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DETERMINATION OF THE EXPRESSION PATTERNS OF BOVINE NON-CLASSICAL
MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) CLASS I PROTEINS

by

Parveen Parasar

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Animal, Dairy and Veterinary Sciences

Approved:

Dr. Christopher J Davies
Major Professor

Dr. Gregory J Podgorski
Committee Member

Dr. Kenneth L White
Committee Member

Dr. Lee F Rickords
Committee Member

Dr. Thomas D Bunch
Committee Member

Dr. Dirk K Vanderwall
ADVS Department Head

Dr. Mark R McLellan
Vice President for Research and
Dean of the School of Graduate Studies

UTAH STATE UNIVERSITY
Logan, Utah

2013

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ABSTRACT

Determination of the Expression Patterns of Bovine Non-Classical Major Histocompatibility Complex (MHC) Class I Proteins

by

Parveen Parasar, Doctor of Philosophy

Utah State University, 2013

Major Professor: Dr. Christopher J. Davies
Department: Animal, Dairy and Veterinary Sciences

My dissertation hypothesis is that bovine trophoblast cells express cell-surface and secreted non-classical major histocompatibility complex class I (MHC-Ib) proteins which inhibit NK cells and other leukocytes by binding to inhibitory receptors (e.g., LILRB1, LILRB2, KIR2DL4, and/or CD94/NKG2A).

Extremely polymorphic and ubiquitously expressed classical MHC class I (MHC-Ia) proteins, which present foreign antigenic peptides to CD8⁺ T lymphocytes, are involved in acceptance or rejection of tissue grafts. Non-classical MHC class I (MHC-Ib) glycoproteins, such as Human Leukocyte Antigen-G (HLA-G) and murine Qa-2, are important modulators of the maternal immune system during pregnancy. MHC-Ib proteins are: (a) oligomorph or monomorph, (b) expressed in specific tissues under specific conditions, and (c) produced as surface and/or soluble isoforms due to alternative splicing.

Third trimester-bovine trophoblast cells express both MHC-Ia and MHC-Ib proteins. The MHC-Ib proteins expressed by trophoblast cells during the third trimester of pregnancy are encoded by four bovine leukocyte antigen (BoLA) loci: BoLA-NC1, BoLA-NC2, BoLA-NC3, and BoLA-NC4.

Two MHC-Ia (N*01701 and N*01802) and three MHC-Ib (NC1*00501, NC3*00101 and NC4*00201) proteins showed cell-surface expression in transfection studies performed in murine P815 and human K562 cells. Two additional isoforms, NC1*00401 and NC2*00102, were not detected on the surface of these cells. Nevertheless, both class Ia proteins, N*01701 and N*01802, and five class Ib proteins, NC1*00401, NC1*00501, NC2*00102, NC3*00101, and NC4*00201, were detected in crude cell lysates on Western blots. Precipitation of proteins from culture supernatants showed that cell-surface MHC-Ia (N*01701 and N*01802) and MHC-Ib proteins (NC1*00501, NC3*00101, and NC4*00201) are shed from the surface of these cells into the media. The mechanism of shedding of these proteins is, however, not known.

Monoclonal antibodies W6/32, IL-A88, H1A, H6A, H11A, H58A, and PT-85A recognized surface MHC-I isoforms with varying affinity. We were able to develop a sandwich enzyme-linked immunosorbent assay (ELISA) using either H1A or IL-A88 antibody as the capture antibody and the W6/32 antibody for detection. We produced monoclonal antibodies against cattle NC1*00501 and NC3*00101 proteins. One monoclonal antibody generated against BoLA-NC3*00101 was highly specific. Unfortunately, due to failure to clone the NC3*00101-hybridoma, we no longer have an infinite source of this monoclonal antibody for NC3*00101. We eluted peptides from NC3*00101-transfected MHC-null K562 cells and identified peptides using liquid chromatography-mass spectrum (LC-MS) analysis. Analysis of peptide binding data using the SAS Proc mixed statistical program, suggested that the peptide EVTNQLVVL is a potential peptide ligand, which can be used to make tetramers for enumeration of antigen-specific leukocytes.

PUBLIC ABSTRACT

Determination of the Expression Patterns of Bovine Non-Classical Major Histocompatibility Complex (MHC) Class I Proteins

Parveen Parasar

This project was funded by the United States of Department of Agriculture (USDA), which funds research aimed at improving production and animal health. The aim of this study was to advance knowledge of maternal immune tolerance to the fetus and mechanisms bovine non-classical MHC class I proteins employ to interact with immune cells and render them inert towards the fetus.

A fetus is a tissue graft inside the mother's uterus yet must be accepted by the mother to maintain a successful pregnancy. Reproductive insufficiency and pregnancy failure are major causes of production loss in cattle, especially in cloned animals. Knowledge of the receptors that non-classical MHC class I proteins interact with may make it possible to improve reproductive efficiency in cattle. We have shown that three non-classical MHC class I proteins expressed during the third trimester, NC1*00501, NC3*00101, and NC4*00201, are expressed as cell-surface isoforms. The other non-classical class I proteins, that we studied, NC1*00401 and NC2*00102, were not expressed on the surface of our cell lines and may be secreted or soluble proteins. The non-classical class I proteins expressed and/or secreted by embryos at different gestational stages can be detected using enzyme-linked immunosorbent assays (ELISA). We were able to produce a NC3*00101-specific monoclonal antibody for use in ELISA; however, we lost the cell line when we attempted to clone it.

Understanding the patterns of expression of these proteins during different stages of pregnancy will help elucidate the association of these proteins with pregnancy success in normal and cloned cattle and will provide insights into mechanisms that prevent rejection of the fetus by the potentially hostile maternal immune system. To elucidate how non-classical class I proteins interact with white blood cells in the uterus, we initiated studies to identify NC3*00101-specific peptide ligands needed to make NC3*00101 tetramers, which can be used to understand interactions between MHC molecules and white blood cells. Understanding the interactions of non-classical class I proteins with maternal immune cells will reveal the mechanisms of inhibitory action that are used by class Ib proteins to suppress the maternal immune system and protect the fetus.

This study helped to elucidate patterns of expression of cattle non-classical MHC class I proteins, which are important to maintain a favorable environment within uterus during pregnancy.

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Parveen Parasar

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LIST OF ABBREVIATIONS

- AP – Alkaline Phosphatase
- APC – Antigen Presenting Cells
- BNC – Binucleate Cells
- BoLA – Bovine Leukocyte Antigen
- BoLA-I – Bovine Leukocyte Antigen class I
- BoLA-Ia – Classical BoLA class I
- BoLA-Ib – Non-classical BoLA class I
- BoLA-II – BoLA class II
- BoLA-IIa – BoLA class IIa genetic subregion
- BoLA-IIb – BoLA class IIb genetic subregion
- BoLA-III – BoLA-III genetic region
- CD4 – Cluster of Differentiation 4 antigen
- CD8 – Cluster of Differentiation 8 antigen
- CHCA - cyano-4-hydroxy cinnamic acid
- DC – Dendritic Cell
- DMEM – Dulbecco's Modified Eagle Medium
- DMSO – Dimethyl Sulfoxide
- DNA – Deoxyribonucleic Acid
- EDTA – Ethylene Diamine Tetraacetic Acid
- FACS – Fluorescence Activated Cell Sorter
- FB – Fluorescence Buffer
- FITC – Fluorescein Isothiocyanate

HLA-Human Leukocyte Antigen

IL – Interleukin

IMDM – Iscove’s Modified Dulbecco’s Medium

ITIM - Immunoreceptor Tyrosine-based Inhibitory Motifs

ITAM - Immunoreceptor Tyrosine-based Activating Motifs

kD – Kilo Dalton

KIR – Killer Immunoglobulin-Like Receptor

LC/MS – Liquid Chromatography Mass-Spectra

LILR – Leukocyte Immunoglobulin-Like Receptor

LRC – Leukocyte Receptor Complex

MALDI – Matrix-Assisted Laser Desorption Ionization

MW – Molecular Weight

NC –Non-classical

NK - Natural Killer

NKC – Natural Killer Complex

MS – Mass Spectra

PAG – Pregnancy Associated Glycoprotein

PBS – Phosphate Buffered Saline

RBC-Red Blood Cells

RPM – Revolutions Per Minute

RPMI - Roswell Park Memorial Institute Medium

RP-HPLC – Reverse Phase Liquid Chromatography

SCNT – Somatic Cell Nuclear Transfer

TGF- β – Transforming zgrowth Factor- β

Th – T Helper

Treg – Regulatory T cells

TNF- α – Tumor Necrosis Factor- α

CHAPTER 1

LITERATURE REVIEW

Pregnancy includes the complex steps of fertilization, cleavage of newly formed zygote by mitotic divisions to form the embryo (morula), blastocyst (blastula), and gastrula, and organogenesis resulting in a fully developed fetus. The average gestation time in cattle is 285 days. The blastocyst develops at ~7th day and hatches 8-9 days post-conception. After gastrula formation (16-18th day post-conception) and elongation, embryo implantation begins at ~22 days and completes in ~40 days post-conception. Despite its allogeneic nature and its antigenicity, a fetus is carried to term with successful completion of each of these complex steps of pregnancy without being rejected by the maternal immune system.

The Major Histocompatibility Complex (MHC) is the genetic region that encodes the transplantation antigens or MHC class I (MHC-I) and MHC class II (MHC-II) molecules, which are primarily involved in protecting the body against pathogens. Immunological recognition of pathogens includes proteolysis of foreign proteins into peptides, assembly of peptides on MHC-I and -II glycoproteins specialized for presenting the peptides on the cell-surface, and interaction of the MHC-I and MHC-II-peptide complexes with CD8 and CD4 T cell-receptors, respectively, leading to effector functions including subsequent killing or removal of the infected cells to clear the infection.

Because MHC glycoproteins present peptides, they play an important role in the acceptance or rejection of the tissue grafts. A successful pregnancy requires that the fetal allograft remains unharmed by the mother's potentially hostile immune system throughout pregnancy. In humans and mice, studies indicate that another class of

transplantation antigens, referred to as “non-classical MHC class I (MHC-Ib) proteins,” are expressed as alternatively spliced isoforms in fetal trophoblast cells and interact with inhibitory receptors on natural killer cells and other maternal leukocytes resulting in the inhibition of these cells. Examples of class Ib proteins are human leukocyte antigens (HLA)-E, -F and -G, *Qa-2* in mice, *Mamu-AG* in Rhesus Macaques, and *Paan-AG* in olive baboons.

After years of research on immune tolerance to the fetus during pregnancy, novel mechanisms of interaction of MHC-Ib proteins with receptors on maternal leukocytes have been identified in humans and mice. In cattle, identification of bovine leukocyte antigen (BoLA) class Ib proteins begs further research to investigate the mechanisms of bovine maternal-fetal immune-regulation during pregnancy and achievement of an immunologically tolerant state in the uterus.

The Structure of MHC Glycoproteins

Histocompatibility genes, which produce transplantation or histocompatibility antigens, were first discovered in mice [58]. This finding led to the identification of loci encoding human and mouse [59, 60, 110]. MHC class I antigens and successful organ transplantation in patients. The genes that encode MHC proteins and several other proteins with related functions are tightly linked in the MHC genetic region. The human MHC, human leukocyte antigen (HLA) complex, and mouse MHC, the H-2 complex, are located on chromosomes 6 and 17, respectively. The bovine MHC, which is known as the bovine leukocyte antigen “(BoLA) complex,” is located on bovine autosome 23. The genetic structure of BoLA was first described by Amerona and Stone (1978) and Spooner et al. [105, 106].

There are two classes of MHC glycoproteins: MHC-I and MHC-II. Class I proteins comprise an alpha (α) polypeptide chain which is a large subunit of 44 kilodaltons (kD) molecular weight (MW) that is non-covalently linked with a 12 kD-light chain called β_2 microglobulin (β_2m) to form a stable cell surface protein. The gene for the β_2m is not located in the MHC region. MHC-II proteins are heterodimers composed of an alpha (α) and a beta (β) polypeptide chain. Class II molecules are expressed on professional antigen presenting cells (APCs) such as dendritic cells (DCs), macrophages and B cells. Class II molecules are not expressed on placental trophoblast cells and, therefore, will not be described in detail here.

The general structure of the MHC in mammalian species is relatively conserved. The BoLA complex is divided into three regions - BoLA-I, BoLA-II and BoLA-III with different functions and roles. There are at least six classical MHC-I genes in the BoLA-I region which are expressed in a number of different combinations [11]. In addition, the BoLA-I region contains four non-classical BoLA-class I loci, NC1-NC4 [25]. BoLA-II was subdivided into BoLA-IIa and -IIb based on genetic mapping [5, 119]. The BoLA-IIa subregion contains the DR and DQ cluster of genes [4, 98]. The BoLA-IIb and BoLA-IIa subregions are about 15cM apart. The BoLA-IIb subregion includes the DMA, DMB, LMP2, LMP7 and TAP genes whose products are involved in antigen processing and transport [22, 66, 92]. The class IIb region also carries some other genes of unknown function such as DOA, DOB, DYA and DYB [66].

The BoLA-III region contains genes such as CYP21, BF, HSP70 and C4 which are not considered part of the MHC proper [6, 66]. Figure 1-1 shows the linkage map of bovine MHC genetic regions as reported earlier [3].

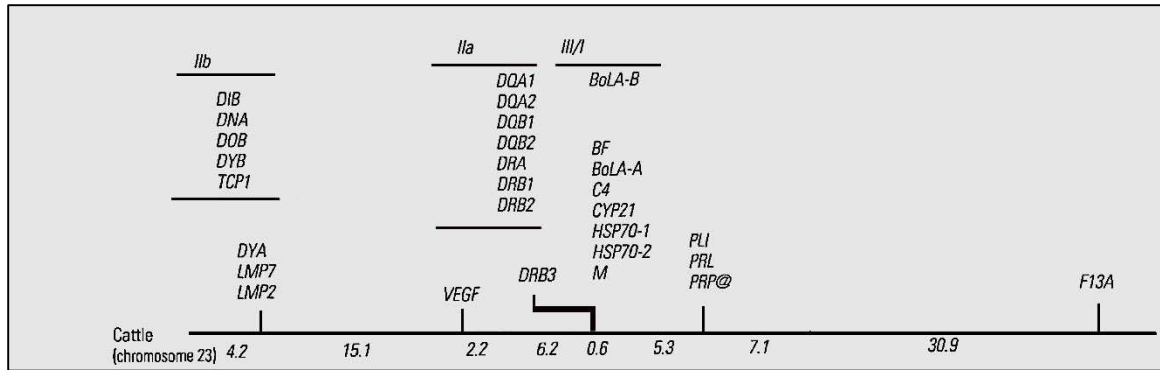


Fig 1-1 Genetic linkage map of the major histocompatibility complex (MHC) region in cattle

Classical (MHC-Ia) and Non-classical (MHC-Ib) MHC-I Proteins

MHC-Ia proteins show ubiquitous expression. They are absent from mature erythrocytes of larger mammals such as humans and pigs but in rodents they are present on erythrocytes at low density. The MHC-Ia molecules are extremely polymorphic with a large number of alleles present in the population. These proteins are transmembrane glycoproteins and play an important role in immune regulation.

MHC-Ib genes are monomorphic or oligomorphic and often possess premature stop codons, and/or putative non-classical amino acid motifs (IPI and VPI) in the transmembrane domain. Similar to class Ia genes, most class Ib genes have eight exons which encode the heavy chain. Exon 1 encodes the signal sequence that is cleaved after the newly synthesized protein is targeted into the endoplasmic reticulum (ER). Exons 2, 3 and 4 encode the $\alpha 1$, $\alpha 2$ and $\alpha 3$ domains, which form the extracellular portion of the protein. The transmembrane domain is encoded by exon 5. Exons 6, 7, and sometimes part of exon 8 encode the cytoplasmic domain. In contrast to MHC-Ia proteins, non-classical class I proteins (MHC-Ib) are expressed in specific tissues and under specific conditions. These proteins often have a truncated cytoplasmic domain. As a result of

alternative splicing, these proteins are produced as transmembrane and soluble isoforms [49]. The process of alternative splicing determines the secreted or lipid-linked nature of some class Ib molecules such as HLA-G (HLA-G2) and Qa-2 [50]. Alternative splicing of HLA-G or Qa-2 transcripts that eliminates or splices out exon 5, results in only secreted isoforms. In contrast to class Ia molecules which require binding with a light chain or β 2-microglobulin (β 2m) for their cell-surface expression [51, 95, 115, 116], MHC-Ib proteins do not always require a light chain for their expression. Membrane-HLA-G1 and soluble HLA-G5 associate with a light chain whereas membrane HLA-G2 and -G3 and soluble HLA-G6 do not associate with a light chain or β 2m [49].

Peptide Binding to MHC Glycoproteins

MHC class I proteins bind peptides from intracellular pathogens and the animal's own protein-derived peptides. The protein complexes are digested in the cytosol by proteasomes into 8-10 amino acid long peptides [71]. These peptides are accommodated in the peptide-binding cleft formed by the α 1 and α 2 domains of MHC-I proteins. The N-termini of freshly synthesized MHC-I glycoproteins contain N-linked glycan. N-linked glycans are trimmed by Glucosidases I and II (GlsI/II) to a single terminal glucose residue, which allows the MHC-I protein to interact with chaperon proteins. After this, the first interaction is with calnexin (CNX), which allows β 2m to bind with the MHC-I heavy chain. Calreticulin (CRT) then recruits the MHC- protein to the peptide loading complex (PLC) [94]. The PLC is formed by the transporter associated with antigen processing (TAP) heterodimer together with other proteins and chaperon molecules. Tapasin, Erp 57, and CRT in association with other chaperons in the PLC help to locate the MHC-I protein to the PLC. Peptides longer than 8-10 amino acids are trimmed by ER

aminopeptidases known as “ERAAP/ERAP1 and ERAP2.” Finally, tapasin-mediated editing results in preferential binding of approximately sized peptides in the peptide-binding groove [28, 79, 80, 112]. The MHC-I-peptide complexes then move to the cell surface for recognition by T cell-receptors on CD8+ T lymphocytes.

MHC-II glycoproteins bind peptides derived from endocytosed extracellular proteins. The α and β chains of class II proteins associate with the invariant chain (Ii chain) and the complex moves to a mature endosome via the trans-Golgi network (TGN). In mature endosomes, the invariant chain is cleaved by proteolysis to form the class II associated invariant chain peptide (CLIP) [18, 37, 55, 63, 78, 83, 109, 127]. Endocytosed proteins internalized by a cell from exogenous sources are degraded in increasingly acidic and proteolytic endosomal compartments of early and late endosomes, and lysosomes by acidic lysosomal proteases called cathepsins into peptides [123]. The MHC-II-related chaperons such as HLA-DM and HLA-DO are expressed in the ER and form complex. DM-DO complexes co-transport to late endosomes, where DM catalyzes the replacement of the CLIP fragment in the MHC-II binding groove by exogenous peptides [26, 70, 73, 74]. The DM protein regulates the binding of high-affinity peptides to MHC-II [100]. The MHC-II-peptide complexes move to the cell surface for recognition by the CD4+ T cells.

MHC Glycoproteins and Transplantation

The process of recognition of self and foreign peptides by T lymphocytes was first studied in mice [128]. The findings demonstrated that cytotoxic T lymphocytes lyse virus-infected cells under specific immunological conditions. Virus-infected target cells are killed only when they carry the same MHC-I antigens as the immune T cells, which explains MHC-mediated restriction of T lymphocyte function. The researchers reported

that virus-infected cellular targets of cytotoxic T lymphocytes contain two parts, the viral antigen and the MHC-I antigen. Similar studies revealed that MHC-II antigens on APCs must be the same as the MHC-II proteins that primed the T lymphocyte in order for the T cells to recognize the antigens. They found that T helper cells only recognize antigens on the surface of APCs and the class II molecule is part of the antigenic complex recognized by the T helper cells [101, 113]. Therefore, the MHC molecules, both class I and II, govern the effector functions of cytotoxic and helper T cells, respectively. MHC-I and MHC-II polymorphism have been associated with susceptibility and resistance to many pathological conditions [21, 27]. Certain alleles are more frequent in one population than in another. One consequence of the MHC-I and MHC-II polymorphism is that when a diseased organ is replaced by transplantation of a healthy organ, the new organ may be accepted or rejected based on the degree of similarity between the class I and class II antigens on the cells of the donor and recipient tissues.

It is helpful to understand the following terms that describe transplantation between animals:

Autograft - A tissue graft where the tissue is transplanted back onto the original donor.

Isograft - A graft between individuals of identical genetic composition (i.e. syngeneic individuals) such as identical twins or mice of the same inbred mouse line.

Allograft - A graft between individuals of the same species but of different genetic compositions (i.e., allogeneic individuals) such as human to human and from one strain of mouse to another.

Xenograft - A graft between members of different species (i.e., xenogeneic individuals) such as a graft from a monkey to human.

The greatest concern is with allografts because allografting is commonly practiced for treatment of many human disorders. Blood transfusions as well as skin, kidney, liver, and other organ transplants are all examples of allografts. An allogeneic skin graft is sloughed within 10 days due to the infiltration of the graft bed with lymphocytes, monocytes and a few plasma cells. The second time, a graft from the same donor is rejected much faster than the first time and there is more infiltration with polymorphonuclear leukocytes, lymphoid cells, and plasma cells, including thrombosis and acute cell destruction. Therefore, the second-set rejection is more severe than the first-set rejection. Grafts from new donors are rejected at the same rate as the original first-set rejection. The presence of immunological memory suggests that graft rejection is mediated by lymphocytes. Transplantation rejection can be prevented or delayed by matching the MHC class I and class II antigens of the donor and recipient. Another method of preventing graft rejection is to use immunosuppressive drugs that inhibit T lymphocyte activation.

Fetal Allograft and Placentation in Cattle

Despite its allogeneic nature, the fetal allograft is not normally rejected by the potentially hostile maternal immune system. The early embryo is made of blastomeres which are undifferentiated cells resulting from cleavage of the fertilized egg (zygote). After a series of divisions and compaction, the embryo forms a compact mass of 16-32 blastomeres known as the morula. The morula develops into a fluid-filled hollow ball of over a hundred cells known as the blastocyst. A blastocyst has two distinctive tissues, the outer single-cell layer of trophoderm or trophoblasts surrounding the fluid-filled cavity (blastocoel) which gives rise to extra-embryonic tissues and the inner cell mass gives rise

to the embryonic disc and eventually the embryo proper. Together with the somatic mesoderm, the trophoblast layer is referred to as the “chorion.”

In cows, the gestation length is 285 days and the chorioallantois starts attaching to the gravid uterus at about 4 weeks of gestation. With the growth of extraembryonic membranes inside the uterine lumen chorioallantois starts to form “cotyledons” over specialized areas of the endometrium known as “caruncles.” The caruncular surface develops crypts and the apposing chorioallantois forms finger-like projections, which enter into the crypts, greatly enhancing the contact surface area between the endometrium and the trophoblast [97]. The combination of cotyledonary and caruncular tissues forms the “placentomes.” Placentomes are 10-12 cm long and 2-3 cm thick. The chorioallantois that develops and apposes with endometrium between placentomes is the interplacentomal chorioallantois. The chorioallantois, both cotyledonary and intercotyledonary, is responsible for nutrient uptake that provides for the fetal metabolic demands and fetal tissue growth. This type of placenta is known as a “cotyledonary placenta” and is found in ruminants. The majority of ruminant trophoblast cells is uninucleate and functions in nutrient exchange and metabolism. An important feature of the ruminant-placenta is the presence of binucleate cells (BNC) or giant trophoblast cells, which develop by acytokinetic mitosis of uninucleate trophoblast cells. Binucleate cells constitute 15-20% of the bovine trophoblast cells. Binucleate trophoblast cells fuse with uterine epithelial cells both in the placentomes and the interplacentomal region to form trinucleate cells, which are feto-maternal hybrids, throughout pregnancy [61, 124]. BNC secrete bovine pregnancy-associated glycoproteins (PAGs) [125], progesterone, transforming growth factor- β (TGF- β), and placental lactogen which act as local

immunomodulators to maintain pregnancy as well as help in the metabolism and development of placentomes [34, 75, 89, 90].

Ruminant placenta is often referred to as “synepitheliochorial”. The bovine chorion (trophoblast) layer is in intimate contact with the maternal epithelium. The binucleate cells migrate toward the uterine epithelium and fuse with the epithelial cells, secreting their granules to the basal side of the uterus. These secretory granules provide a mechanism for transfer of MHC-I proteins to the maternal side of the placenta. The $\gamma\delta$ -T cells also have been identified in the uterine epithelium during pregnancy in ruminants [64, 68, 85]. These cells may play a role in the development of the conceptus, immunosuppression, and placental detachment during parturition.

The Role of MHC Class I Proteins in Reproduction

In most species, mature trophoblast cells do not express highly polymorphic classical class I protein [33, 40, 48]. Fetal allografts expressing classical class I proteins during the first trimester of pregnancy do not survive in the uterus [9]. In ewes, a study showed that intrauterine skin autografts were immunologically accepted, whereas the allografts were rejected [88].

In cattle, trophoblast cells in interplacentomal, arcade, and villous/crypt regions possess unique MHC-I expression patterns. In normal pregnancy, cattle trophoblasts do not show MHC-I expression before 120 days of pregnancy. However, MHC-I expression by interplacentomal and arcade trophoblast cells during the last trimester in cattle is normal [23]. Cows carrying MHC-compatible pregnancies at term have reduced levels of immunoreactive interleukin-2 (IL-2), less apoptosis, less tumor necrosis factor-alpha (TNF- α) in macrophages, and reduced degranulation of binucleate trophoblast cells,

which is similar to the conditions seen with retained placenta [24]. In the third trimester of pregnancy, MHC class Ia and class Ib proteins are expressed in the interplacentomal and arcade regions [25]. The ratio of class Ia to class Ib gene expression varies extensively among pregnancies. About 34-79% of transcripts from interplacentomal trophoblast cells are encoded by class Ib genes. Gene sequence analysis led to the discovery of four bovine non-classical loci: BoLA-NC1, BoLA-NC2, BoLA-NC3, and BoLA-NC4.

Normally, cattle have a greater number of lymphocytes in the non-gravid uterine horn than in the gravid horn. In early pregnancy, the endometrial lymphocytes decrease in number in sheep, pigs, and cattle [12, 57, 64, 122]. In 34-63 day old SCNT pregnancies, trophoblast MHC-I expression was widespread and accompanied by endometrial infiltration of CD3⁺ T lymphocyte that formed aggregates as compared to normal pregnancies [45]. This maternal lymphocytic response involved 80% CD4⁺ helper T cells, with the remaining cells comprised of equal numbers of CD8⁺ cytotoxic T cells and B cells with a minimal number of γ/δ -T lymphocytes (Davies unpublished). The large number of CD4⁺ T helper cells suggests that allogeneic trophoblast MHC-I proteins are processed by maternal antigen presenting cells (APCs) and that MHC-I derived fetal peptides are presented on maternal MHC class II proteins. This is an indirect recognition pathway, where recognition is restricted by the host MHC class II molecules that have bound peptides derived from an allogeneic MHC molecule [43]. Fetal antigens are probably carried towards the uterine endometrial epithelium by binucleate cells and released with the secretory granules after the BNC fuse with the uterine epithelial cells. Presentation of peptides derived from fetal MHC-Ia antigens triggers recruitment of Th1

cells and initiation of an inflammatory response involving release of tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-12, and IFN- γ , which interferes with placental attachment and causes embryonic death. The success rate of nuclear transfer in cattle ranges from 0% to 10% [114].

These findings suggest that abnormal trophoblast MHC-I expression accompanied by lymphocytic infiltration of the endometrium causes early embryonic mortality of cloned fetuses. Therefore, appropriate expression of fetal MHC-I antigens at the bovine fetal-maternal interface is critically important for immunological acceptance of the allogeneic fetus by its mother.

Non-classical MHC-I (MHC-Ib) Proteins and Tolerance to the Fetal-allograft

The mystery of immunological acceptance of the fetal allograft has been the focus of many studies. There are various mechanisms that induce maternal tolerance. These include production of TGF- β 1 and interleukin-10 (IL-10) by the T_{reg} subset of CD4⁺/CD25 cells, secretion of prolactin, gonadotropin and progesterone by both fetal and endometrial cells, and expression of high levels of complement regulatory proteins by fetal cells and expression of inhibitory members of the B7 and the TNF family of ligands [19, 49, 95]. Fetal cells also produce immunosuppressive cytokines, chemokines, and prostaglandins, which dampen T lymphocyte proliferation, and secrete the immunosuppressive hormone progesterone. Importantly, trophoblast cells regulate the expression of MHC-Ia and MHC-Ib proteins [23, 24, 25]. As mentioned earlier, villous trophoblast cells do not express class Ia and class II molecules. However, in cattle interplacentomal and arcade trophoblast cells express class I proteins during the second

and third trimesters of pregnancy. MHC-Ib proteins are uniquely expressed in human, mouse, rhesus macaques, baboons, and cattle trophoblast cells.

HLA-E, HLA-F, and HLA-G are human class Ib proteins and Qa-1 and Qa-2 are murine class Ib proteins. Similar class Ib proteins studied in non-human primates include Mamu-AG in *Macaca mulata* and Paan-AG in olive baboons. HLA-G and Qa-2 proteins have been studied most extensively for their role in maternal tolerance to fetal allografts [20, 35]. HLA-G is predominantly expressed on fetal extravillous trophoblasts [35, 62]. HLA-G provides inhibitory signals to NK cells, macrophages, monocytes, and lymphocytes by interacting with various inhibitory receptors expressed by leukocytes to induce immunosuppression and fetal survival [49]. These receptors are inhibitory leukocyte immunoglobulin-like receptors 1 and 2 (LILRB1/LIR-1/ILT-2 and LILRB2/LIR-2/ILT-4), CD94/NKG2A, and killer-immunoglobulin-like receptor (KIR) 2DL4 (KIR2DL4) [2, 7, 38, 65, 84, 86, 102, 104].

Murine Q9 antigen (referred to as Qa-2) of the Qa-2 family of proteins is encoded at the *Ped* (preimplantation embryonic development) gene locus. It was recognized because of its role in embryonic development and reproductive tolerance. The murine Q9 antigen is a functional homologue of HLA-G [20]. Both HLA-G and Qa-2 play a role in tumor surveillance via peptide presentation to CD8⁺ T cells [41]. HLA-G and Qa-2 can present a larger array of peptides than HLA-E and Qa-1 [54, 91, 111]. HLA-G can display restricted but still a diverse set of peptides [29, 52]. HLA-G has two free cysteine residues (Cys⁴² and Cys¹⁴²) unlike most of other MHC-I alleles. Both soluble and cell-surface HLA-G isoforms can exist as disulfide-linked dimer with intermolecular Cys⁴²-Cys⁴² disulfide bond. The HLA-G dimer has oblique orientation which exposes the

receptor-binding sites upward and more accessible. HLA-G dimer has much higher overall affinity toward LILRBs than the monomer because it has increased avidity for its ligand [15, 103]. The HLA-G dimer on the cell surface inhibits NK cells through LILRB1 binding [42]. The peptide binding surfaces of each HLA-G monomer is too small to be accessible by T cell receptor; therefore, it cannot work as antigen presenting molecule for the T cell response. However, it can present LILR- and CD8- binding sites, which suggests that HLA-G dimers are important in immunosuppression.

HLA-E and Qa-1 are orthologues and present peptides derived from the leader sequences of other class I molecules [16, 53]. HLA-E and Qa-1 play a role in the innate immune response as ligands for the receptors of the CD94/NKG2 family expressed by NK and T cells [17, 120]. In some bacterial and viral infections, class Ia proteins are downregulated. Due to non-availability of leader sequence, HLA-E and Qa-1 are not expressed properly. NK cells can detect and eliminate the compromised MHC deficient host cells [1].

Cattle have two MHC clusters that are equivalent to the human MHC-I beta and kappa blocks. The orthologous MHC-I region in cattle carries the heavy chain genes for BoLA-NC2, -NC3, and -NC4. The only expressed MHC-I gene in the orthologous human kappa block is HLA-E. In addition, this block contains six to eight MHC-Ia genes and expresses two to three MHC-Ia proteins. The cattle orthologous kappa block has one expressed MHC-Ib gene, BoLA-NC1 (Davies unpublished), that maps very close to HLA-E, which suggests that these genes can be true orthologs.

In Rhesus monkeys, an ortholog of HLA-G named “Mamu-G” was identified in trophoblast cells from a day-36 post-conception placenta [13]. This class Ib protein is

non-functional as it contained premature stop codons and frameshift mutations. Therefore, it is encoded by a pseudogene. Another non-classical molecule expressed exclusively in rhesus monkey placenta, Mamu-AG, shares functional similarities with HLA-G [14]. It has both membrane and soluble isoforms as a result of alternative splicing [93].

Peptide Presentation by Non-classical MHC-I Proteins

Human and murine class Ib proteins have been extensively studied for their peptide-presenting characteristics. Generally, class Ib molecules have peptide-presenting capacity, which varies among all the expressed class Ib molecules [29, 99]. Compared to class Ia molecules, class Ib molecules present a far narrower range of peptides, which correlates well with their narrow tissue distribution. HLA-G molecules present nonamer peptides, which are similar to those of class Ia molecules. The anchor residues for HLA-G are isoleucine (I) or leucine (L) at position 2, proline (P) at position 3, and leucine at position 9. HLA-G has a preference for non-anchor residues. For instance, position 1 is generally positively charged and position 7 is a hydrophobic amino acid residue in most of the ligand motifs [87]. Therefore, HLA-G glycoproteins which present the specific sequence motif with the consensus sequence XI/LPXXXXXL resemble classical MHC class I proteins [96]. Because HLA-G interacts with NK cell inhibitory receptors and provides a signal peptide for increased expression of HLA-E, it inhibits the NK cells that express C-type lectin like inhibitory receptors [76, 77].

The murine class Ib molecule Qa-2 binds with specifically histidine-containing nonapeptides (9-amino acids long) with two anchor residues, Histidine (H) at position 7 and Leucine, Isoleucine (I) or Phenylalanine (F) at position 9 [91]. Positions 2, 3, 5, and 6

are auxiliary anchors occupied by aliphatic residues. This suggests that a relatively small number of nonapeptides fit the Qa-2 motif compared to the large number of peptides that bind to other class I proteins; however, Qa-2 binds with a large array of endogenous peptides [54, 91].

Peptide binding information is helpful to determine the physiological and immunological role of class Ib proteins, which are expressed in tissues under specific conditions. The peptide motif for BoLA class Ia protein, BoLA-N*01701, has been identified [8, 39], but BoLA-Ib peptide motifs have not yet been identified.

Bovine MHC-Ib (BoLA-Ib) Proteins

Transcripts from four bovine non-classical class I loci, BoLA-NC1, BoLA-NC2, BoLA-NC3 and BoLA-NC4 were identified [25] in third trimester-interplacentomal and arcade region trophoblast cells. Amino acid alignment of class Ia and class Ib alleles at the four BoLA-NC loci revealed that all of the BoLA-Ib alleles possess characteristic features such as limited or no polymorphism, putative non-classical amino acid motifs (IPI, VPI or VLI) in the transmembrane domain, differential splicing in the transmembrane domain, and/or premature stop codons.

The BoLA-NC1 locus encodes seven alleles. The NC1*00101 allele has already been described [46]. NC1*00201 (AH19), NC1*00301(AH12), and NC1*00401(AH11) were identified by Davies et al. (2006) [25]. Most recently NC1*00501, NC1*00601, and NC1*00701 alleles have been identified (Davies et al. unpublished). Multiple NC1 splice variants with partial or complete deletion of exon 5, which encodes the transmembrane domain, have been identified. Examples include NC1*00101 SV, NC1*00201, NC1*00202 SV, and NC1*00401

(<http://www.ebi.ac.uk/ipd/mhc/bola/nomenclature.html>). These splice variants suggest that both membrane-bound and soluble isoforms are encoded at this locus.

The NC2 locus exhibits minimal polymorphism with only three closely related alleles identified: NC2*00101 [36], NC2*00102 [25], and NC2*00103 (Davies et al. unpublished).

NC3 and NC4 were new loci identified by Davies et al. (2006) [25]. The NC3 locus appears to be monomorphic and encodes an allele NC3*00101 that has an early stop codon resulting in a truncated cytoplasmic domain. Four alleles have been identified at the NC4 locus: NC4*00101 (AH12), NC4*00201 (AH11), NC4*00202 and NC4*00301. The NC4 locus may not be expressed in all haplotypes. The variation in the ratio of expression of classical to non-classical gene expression in trophoblast cells appears to be influenced by the haplotype composition of the fetus. Haplotypes that have a higher ratio of non-classical class I expression may be associated with higher immunological acceptance of the fetal allograft.

Receptors for MHC-Ib Ligands

MHC-Ib proteins interact with inhibitory and activating receptors expressed on maternal leukocytes and direct the actions of the leukocytes. NK cell receptors are encoded at two genetic regions, the natural killer complex (NKC) and the leukocyte receptor complex (LRC). The NKC on bovine Chr5, murine Chr6, and human Chr12 encodes killer cell lectin-like receptors such as inhibitory and activating members of the CD94/NKG2 receptor family. These are expressed as heterodimers; CD94 combines with various NKG2 isoforms to form functional receptors. The NKG2A isoforms have immunoreceptor tyrosine-based inhibitory motifs (ITIM), whereas NKG2B and NKG2C

isoforms have immunoreceptor tyrosine-based activating motifs (ITAM). Human HLA-E interacts with CD94/NKG2A, B, and C receptors [17, 49, 50, 82]. Similarly, murine Qa-1 binds with CD94/NKG2C and CD94/NKG2E activating receptors on NK cells [67, 121]. There are two CD94 genes, seven NKG2A genes, and one NKG2C gene expressed in cattle [10]. In rodents, the NKC also encodes the Ly49 family of receptors [126]. The Ly49 receptors are homodimeric type-II C type lectin-like molecules [30]. In cattle, a single Ly49 locus has been identified that is polymorphic and produces three alternatively spliced Ly49 receptors. These receptors were reported to be inhibitory as reported earlier. They therefore may act as inhibitory receptors for NK cells [32, 72].

The other genetic region, the LRC present on bovine Chr18, human Chr19, and murine Chr7, encodes three types of receptors: the killer immunoglobulin-like receptors (KIR), the leukocyte immunoglobulin-like receptors (LILR), and the leukocyte-associated immunoglobulin-like receptors (LAIR). The KIR in primates are functional homologues of the Ly49 receptors in rodents [56, 81, 115]. Both KIR in primates and Ly49 receptors in rodents are highly polymorphic with unique combinations of alleles expressed on NK cells in different individuals [117, 118]. Killer Immunoglobulin-like receptors recognize mostly MHC-Ia proteins [72]. In cattle, multiple KIR members have been demonstrated [107, 108]. Soluble HLA-G binds with KIR2DL4 present on NK cells and activates the NK cells. KIR2DL4 is predominantly localized in endosomes. Soluble HLA-G is thus endocytosed to interact with KIR2DL4. Interaction between sHLA-G and KIR2DL4 activates a proangiogenic response, which supports a role for soluble HLA-G in augmenting vascularization early in pregnancy. KIR2DL4 possesses structural elements associated with both the activation and inhibition of NK cells [81]. In addition

to HLA-G, human trophoblasts express HLA-C, but not HLA-A or -B [69]. HLA-G and HLA-C binding with KIR on human NK cells promotes conversion of maternal spiral arteries into blood and nutrient-supplying channels to the placenta [44].

Summary

There is extensive literature on the role of MHC-Ib proteins in inducing maternal immune tolerance to the fetus. This literature is the basis for my research to better understand the mechanism of immune regulation at the maternal-fetal interface during bovine pregnancy. Fetal-allografts are immunologically accepted in normal pregnancies. Evidence of expression of class Ib molecules by human and murine trophoblast cells and their role in inducing immune tolerance by reacting with the inhibitory receptors expressed on different leukocytes have been reported. Therefore, cattle class Ib proteins are potentially key players in immunomodulation at the maternal-fetal interface. Identification of the expression patterns of the bovine trophoblast class Ib proteins and their peptide binding motifs will help identify pathways these proteins use to maintain maternal immunological tolerance to fetus.

Research Impact and Applications

This research project is based on the hypothesis that cattle MHC class Ib proteins are produced as surface and/or secreted isoforms and they interact with maternal NK cells and other leukocytes to provide an immunological state within the uterus favorable for maintenance of the fetus.

The research addressed the following specific aims:

- 1) Determine which bovine class Ib proteins are produced as transmembrane proteins and/or secreted isoforms.
- 2) Develop or identify monoclonal antibodies specific for BoLA-Ib proteins
- 3) Determine peptide motifs recognized by BoLA-Ib proteins and to make BoLA-Ib tetramers to identify the maternal leukocytes that express receptors that bind BoLA-Ib proteins.

Development of an enzyme-linked immunosorbent assay (ELISA) to measure secreted MHC-Ib proteins in embryo and trophoblast culture supernatants and cattle serum is important because secreted MHC-Ib proteins may be a biomarker for fetal health. Once the importance of class Ib proteins in maintaining pregnancy has been established, these proteins may offer a tool for preventing immune-mediated abortions in naturally bred and cloned cattle.

Identification of the BoLA-Ib peptide motifs will facilitate BoLA-Ib tetramer production. The BoLA-Ib tetramers can be used to identify and sort lymphocytes that react with cattle MHC-Ib proteins so that their inhibitory and activating receptors can be characterized.

References

1. Adams EJ, Luoma AM. The adaptable Major Histocompatibility Complex (MHC) fold: Structure and function of nonclassical and MHC class I-like molecules. *Annu Rev Immunol* 2013; 31:529-561.
2. Allan DS, Colonna M, Lanier LL, Churakova TD, Abrams JS. Tetrameric complexes of human histocompatibility leukocyte antigen (HLA)-G bind to peripheral blood myelomonocytic cells. *J Exp Med* 1999; 189:1149-1156.

- 3.** Amills M, Ramiya V, Norimine J, Lewin HA. The major histocompatibility complex of ruminants. *Rev Sci Tech* 1998; April 17(1):108-120.
- 4.** Andersson L, Bohme J, Peterson PA, Rask L. Genomic hybridization of bovine class II major histocompatibility genes: polymorphism of DR genes and linkage disequilibrium in the DQ-DR region. *Anim Genetics* 1986a; 17:295-304.
- 5.** Andersson L, Lunden A, Sigurdardottir S, Davies CJ, Rask L. Linkage relationships in the bovine MHC region. High recombination frequency between class II subregions. *Immunogenetics* 1988; 27:273-280.
- 6.** Antczak DF. Structure and function of the major histocompatibility complex in domestic animals. *JAVMA* 1982; 181 (10): 1030-1036.
- 7.** Bainbridge DR, Ellis SA, Sargent IL. HLA-G suppresses proliferation of CD4(+) T lymphocytes. *J Reprod Immunol* 2000; 48:17-26.
- 8.** Bamford AI, Douglas A, Friede T, Stevanovic S, Rammensee HG, Adair BM. Peptide motif of a cattle MHC class I molecule. *Immunol Letters* 1995; 45(1-2):129-136.
- 9.** Beer AE, Billingham RE. Host responses to intra-uterine tissue, cellular and fetal allografts. *J Reprod Fertil Suppl* 1974; 21:59-88.
- 10.** Birch J, Ellis SA. Complexity in the cattle CD94/NKG2 gene families. *Immunogenetics* 2007; 59(4):273-280.
- 11.** Birch J, Codner G, Guzman E, Ellis SA. Genomic location and characterization of non-classical MHC class I genes in cattle. *Immunogenetics* 2008; 60(5):267-273.
- 12.** Bischof RJ, Brandon MR, Lee CS. Cellular immune responses in the pig uterus during pregnancy. *J Reprod Immunol* 1995; 29:161-178.

- 13.** Boyson JE, Iwanaga KK, Golos TG, Watkins DI. Identification of the rhesus monkey HLA-G ortholog. Mamu-G is a pseudogene. *J Immunol* 1996; 157:5428-5437.
- 14.** Boyson JE, Iwanaga KK, Golos TG, Watkins DI. Identification of a novel MHC class I gene, Mamu-AG, expressed in the placenta of a primate with an inactivated G locus. *J Immunol* 1997; 159:3311-3321.
- 15.** Boyson JE, Erskine R, Whitman MC, Chiu M, Lau JM. Disulfide bond-mediated dimerization of HLA-G on the cell surface. *Proc Natl Acad Sci USA* 2002; 99:16180-16185.
- 16.** Braud V, Jones EY, McMichael A. The human major histocompatibility complex class-Ib molecule HLA-E binds signal sequence-derived peptides with primary anchor residues at positions 2 and 9. *Eur J Immunol* 1997; 27:1164-1169.
- 17.** Braud VM, Allan DS, O'Callaghan CA, Soderstrom K, D'Andrea A. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature* 1998; 391:795-799.
- 18.** Ceman S, Sant AJ. The function of invariant chain in class II-restricted antigen presentation. *Semin Immunol* 1995; 7:373-387.
- 19.** Chen HL, Yang Y, Hu XL. Tumor necrosis factor alpha mRNA and protein are present in human placental and uterine cells at early and late stages of gestation. *Am J Pathol* 1991; 139:327-335.
- 20.** Comiskey M, Goldstein CY, De Fazio SR, Mammolenti M, Newmark JA, Warner CM. Evidence that HLA-G is the functional homolog of mouse Qa-2, the Ped gene product. *Hum Immunol* 2003; 64:999-1004.
- 21.** Dausset J, Svejgaard A. (eds). *HLA and Disease*. Copenhagen, Munksgaard: 1977.

- 22.** Davies CJ, Andersson L, Ellis SA, Hensen EJ, Lewin HA, Mikko S, Muggli-Cockett NE, Van der Poel JJ, Russell GC. Nomenclature for factors of the BoLA system, 1996: report of the ISAG BoLA Nomenclature Committee. *Anim Genetics* 1997; 28:159-168.
- 23.** Davies CJ, Fisher PJ, Schlafer DH. Temporal and regional regulation of major histocompatibility complex class I expression at the bovine uterine/placental interface. *Placenta* 2000; 21:194-202.
- 24.** Davies CJ, Hill JR, Edwards JL, Schrick FN, Fisher PJ, Eldridge JA, Schlafer DH. Major histocompatibility antigen expression on the bovine placenta: its relationship to abnormal pregnancies and retained placenta. *Anim Reprod Sci* 2004; 82-83:267-280.
- 25.** Davies CJ, Eldridge JA, Fisher PJ, Schlafer DH. Evidence for expression of both classical and non-classical major histocompatibility complex class I genes in bovine trophoblast cells. *Am J Reprod Immunol* 2006; 55:188-200.
- 26.** Denzin LK, Cresswell P. HLA-DM induces CLIP dissociation from MHC class II $\alpha\beta$ dimers and facilitates peptide loading. *Cell* 1995; 82:155-165.
- 27.** Dick HM. HLA and disease. An introductory review. *Br Med Bull* 1978; 34(3):271-274.
- 28.** Dick TP, Bangia N, Peaper DR, Cresswell P. Disulfide bond isomerization and the assembly of MHC class I-peptide complexes. *Immunity* 2002; 16:87-98.
- 29.** Diehl M, Münz C, Keilholz W, Stevanović S, Holmes N, Loke YW, Rammensee HG. Non-classical HLA-G molecules are classical peptide presenters. *Curr Biol* 1996; 6(3):305-314.
- 30.** Dimasi N, Biassoni R. Structural and functional aspects of the Ly49 natural killer cell receptors. *Immunol Cell Biol* 2005; 83:1-8.

- 31.** Dobromylskyj MJ, Ellis S. Complexity in cattle KIR genes: transcription and genome analysis. *Immunogenetics* 2007; 59(6):463-472.
- 32.** Dobromylskyj MJ, Connelley T, Hammond JA, Ellis SA. Cattle Ly49 is polymorphic. *Immunogenetics* 2009; 61(11-12):789-795.
- 33.** Donaldson WL, Zhang CH, Oriol JG, Antczak DF. Invasive equine trophoblast expresses conventional class I major histocompatibility complex antigens. *Development* 1990; 110:63-71.
- 34.** Duello TM, Byatt JC, Bremel RD. Immunohistochemical localization of placental lactogen in binucleate cells of bovine placentomes. *Endocrinology* 1986; 119:1351-1355.
- 35.** Ellis SA, Sargent IL, Redman CW, McMichael AJ. Evidence for a novel HLA antigen found on human extravillous trophoblast and a choriocarcinoma cell line. *Immunology* 1986; 59:595-601.
- 36.** Ellis SA, Staines KA, Morrison WI. cDNA sequence of cattle MHC class I genes transcribed in serologically defined haplotypes A18 and A31. *Immunogenetics* 1996; 43(3):156-159.
- 37.** Fineschi B, Sakaguchi K, Appella E, Miller J. The proteolytic environment involved in MHC class II-restricted antigen presentation can be modulated by the p41 form of invariant chain. *J Immunol* 1996; 157:3211-3215.
- 38.** Fournel S, Aguerre-Girr M, Huc X, Lenfant F, Alam A, Toubert A, Bensussan A, Le Bouteiller P. Cutting edge: soluble HLA-G1 triggers CD95/CD95 ligand-mediated apoptosis in activated CD8⁺ cells by interacting with CD8. *J Immunol* 2000; 164:6100-6104.

39. Gaddum RM, Willis AC, Ellis SA. Peptide motifs from three cattle MHC (BoLA) class I antigens. *Immunogenetics* 1996; 43(4):238-239.
40. Gogolin-Ewens KJ, Lee CS, Mercer WR, Brandon MR. Site-directed differences in the immune response to the fetus. *Immunology* 1989; 66:312-317.
41. Gomes AQ, Correia DV, Silva-Santos B. Non-classical major histocompatibility complex proteins as determinants of tumor immunosurveillance. *EMBO Rep* 2007; 8:1024-1030.
42. Gonen-Gross T, Achdout H, Gazit R, Hanna J, Mizrahi S. Complexes of HLA-G protein on the cell surface are important for leukocyte Ig-like receptor-1 function. *J Immunol* 2003; 171:1343-1351.
43. Gould DS, Auchincloss H Jr. Direct and indirect recognition: the role of MHC antigens in graft rejection. *Immunol Today* 1999; 20:77-82.
44. Hiby SE, Apps R, Sharkey AM, Farrell LE, Gardner L, Mulder A, Claas FH, Walker JJ, Redman CW, Morgan L, Tower C, Regan L, Moore GE, Carrington M, Moffett A. Maternal activating KIRs protect against human reproductive failure mediated by fetal HLA-C2. *J Clin Invest* 2010; 120(11):4102-4110.
45. Hill JR, Schlafer DH, Fisher PJ, Davies CJ. Abnormal expression of trophoblast major histocompatibility complex class I antigens in cloned bovine pregnancies is associated with a pronounced endometrial lymphocytic response. *Biol Reprod* 2002; 67:55-63.
46. Holmes EC, Roberts AF, Staines KA, Ellis SA. Evolution of major histocompatibility complex class I genes in Cetartiodactyls. *Immunogenetics* 2003; 55(4):193-202.
47. Huotari J, Helenius A. Endosome maturation. *EMBO J* 2011; 30:3481-3500.

- 48.** Hunt JS, Andrews GU, Wood GW. Normal trophoblasts resist induction of class I HLA. *J Immunol* 1987; 138:2481-2487.
- 49.** Hunt JS, Petroff MG, McIntire RH, Ober C. HLA-G and immune tolerance in pregnancy. *FASEB J* 2005; 19:681-693.
- 50.** Hunt JS, Langat DL. HLA-G: a human pregnancy-related immunomodulators. *Curr Opin Pharmacol* 2009; 9(4):462-469.
- 51.** Ishitani A, Geraghty DE. Alternative splicing of HLA-G transcripts yields proteins with primary structures resembling both class I and class II antigens. *Proc Natl Acad Sci USA* 1992; 89:3947-3951.
- 52.** Ishitani A, Sageshima N, Lee N, Dorofeeva N, Hatake K. Protein expression and peptide binding suggest unique and interacting functional roles for HLA-E, F, and G in maternal-placental immune recognition. *J Immunol* 2003; 171:1376-1384.
- 53.** Joly E, Rouillon V. The orthology of HLA-E and H2-Qa1 is hidden by their concerted evolution with other MHC class I molecules. *Biol Direct* 2006; 1:2.
- 54.** Joyce S, Tabaczewski P, Angeletti RH, Nathenson SG, Stroynowski I. A nonpolymorphic major histocompatibility complex class Ib molecule binds a large array of diverse self-peptides. *J Exp Med* 1994; 179:579-588.
- 55.** Kang SJ, Cresswell P. Regulation of intracellular trafficking of human CD1d by association with MHC class II molecules. *EMBO J* 2002; 21:1650-1660.
- 56.** Kelley J, Walter L, Trowsdale J. Comparative genomics of natural killer cell receptor gene clusters. *PLoS Genet* 2005; 1:129-139.
- 57.** King GJ. Reduction in uterine intra-epithelial lymphocytes during early gestation in pigs. *J Reprod Immunol* 1988; 14:41-46.

- 58.** Klein J. *Biology of the Mouse Histocompatibility-2 Complex: principles of immunogenetics applied to a single system*, Berlin and New York: Springer-Verlag; 1975.
- 59.** Klein J. H-2 mutations: their genetics and effect on immune functions. *Adv Immunol* 1978; 26:55-146.
- 60.** Klein J. The major histocompatibility complex of the mouse. *Science* 1979; 203(4380):516-521.
- 61.** Klisch K, Wooding FB, Jones CJ. The glycosylation pattern of secretory granules in binucleate trophoblast cells is highly conserved in ruminants. *Placenta* 2010; 31:11-17.
- 62.** Kovats S, Main EK, Librach C, Stubblebine M, Fisher SJ, DeMars R. A class I antigen, HLA-G expressed in human trophoblasts. *Science* 1990; 248:220-223.
- 63.** Landsverk OJ, Bakke O, Gregers TF. MHC II and the endocytic pathway: regulation by invariant chain. *Scand J Immunol* 2009; 70:184-193.
- 64.** Lee CS, Meeusen E, Gogolin-Ewens K, Brandon MR. Quantitative and qualitative changes in the intraepithelial lymphocyte population in the uterus of nonpregnant and pregnant sheep. *Am. J Reprod Immunol* 1992; 28:90-96.
- 65.** LeMaout J, Krawice-Radanne I, Dausset J, Carosella ED. HLA-G1-expressing antigen-presenting cells induce immunosuppressive CD4+ T cells. *Proc Natl Acad Sci USA* 2004; 101:7064-7069.
- 66.** Lewin HA. Genetic organization, polymorphism, and function of the bovine major histocompatibility complex. In: Schook LB, Lamont SJ (eds.), *The Major Histocompatibility Complex Region of Domestic Animal Species*. Florida: CRC Press; 1996: 65-98.

- 67.** Lohwasser S, Kubota A, Salcedo M, Lian RH, Takei F. The non-classical MHC class I molecule Qa-1(b) inhibits classical MHC class I-restricted cytotoxicity of cytotoxic T lymphocytes. *Int Immunol* 2001; 13(3):321-327.
- 68.** Majewski AC, Hansen PJ. Progesterone promotes survival of xenogeneic transplants in the sheep uterus. *Horm Res.* 2002; 58:128-135.
- 69.** Male V, Trundley A, Gardner L, Northfield J, Chang C, Apps R, Moffett A. Natural killer cells in human pregnancy. *Methods Mol Biol* 2010; 612:447-463.
- 70.** Marks MS, Roche PA, van Donselaar E, Woodruff L, Peters PJ, Bonifacino JS. A lysosomal targeting signal in the cytoplasmic tail of the β chain directs HLA-DM to MHC class II compartments. *J Cell Biol* 1995; 131:351-369.
- 71.** Maupin-Furlow J. Proteasomes and protein conjugation across domains of life. *Nat Rev Microbiol* 2012; 10:100-111.
- 72.** McQueen KL, Wilhelm BT, Harden KD, Mager DL. Evolution of NK receptors: a single Ly49 and multiple KIR genes in the cow. *Eur J Immunol* 2002; 32:810-817.
- 73.** Morris P, Shaman J, Attaya M, Amaya M, Goodman S. An essential role for HLA-DM in antigen presentation by class II major histocompatibility molecules. *Nature* 1994; 368:551-554.
- 74.** Mosyak L, Zaller DM, Wiley DC. The structure of HLA-DM, the peptide exchange catalyst that loads antigen onto class II MHC molecules during antigen presentation. *Immunity* 1998; 9:377-383.
- 75.** Munson L, Wilhite A, Boltz VF, Wilkinson JE. Transforming growth factor beta in bovine placentas. *Biol Reprod* 1996; 55:748-755.

- 76.** Münz C, Stevanović S, Rammensee HG. Peptide presentation and NK inhibition by HLA-G. *J Reprod Immunol* 1999; 43(2):139-155.
- 77.** Münz C, Nickolaus P, Lammert E, Pascolo S, Stevanović S, Rammensee HG. The role of peptide presentation in the physiological function of HLA-G. *Semin Cancer Biol* 1999; 9(1):47-54.
- 78.** Naujokas MF, Morin M, Anderson MS, Peterson M, Miller J. The chondroitin sulfate form of invariant chain can enhance stimulation of T cell responses through interaction with CD44. *Cell* 1993; 74:257-268.
- 79.** Oancea G, O'Mara ML, Bennett WF, Tieleman DP, Abele R, Tampe R. Structural arrangement of the transmission interface in the antigen ABC transport complex TAP. *Proc Natl Acad Sci USA* 2009; 106:5551-5555.
- 80.** Ortmann B, Copeman J, Lehner PJ, Sadasivan B, Herberg JA. A critical role for tapasin in the assembly and function of multimeric MHC class I-TAP complexes. *Science* 1997; 277:1306-1309.
- 81.** Parham P. MHC class I molecules and KIR in human history, health and survival. *Nat Rev Immunol* 2005; 5(3):201-214.
- 82.** Petrie EJ, Clements CS, Lin J, Sullivan LC, Johnson D, Huyton T, Heroux A, Hoare HL, Beddoe T, Reid HH, Wilce MC, Brooks AG, Rossjohn J. CD94-NKG2A recognition of human leukocyte antigen (HLA)-E bound to an HLA class I leader sequence. *J Exp Med* 2008; 17; 205(3):725-735.
- 83.** Pond L, Kuhn LA, Teyton L, Schutze MP, Tainer JA. A role for acidic residues in di-leucine motif-based targeting to the endocytic pathway. *J Biol Chem* 1995; 270:19989-19997.

- 84.** Ponte M, Cantoni C, Biassoni R, Tradori-Cappai A, Bentivoglio G. Inhibitory receptors sensing HLA-G1 molecules in pregnancy: decidua-associated natural killer cells express LIR-1 and CD94/NKG2A and acquire p49, an HLA-G1-specific receptor. *Proc. Natl Acad Sci USA* 1999; 96:5674-5679.
- 85.** Rahman ANMA. Hormonal changes in the uterus during pregnancy- lessons from the ewe: a review. *J Agric Rural Dev* 2006; 4(1&2):1-7.
- 86.** Rajagopalan S, Long EO. A human histocompatibility leukocyte antigen (HLA)-G-specific receptor expressed on all natural killer cells. *J Exp Med* 1999; 189:1093-1100.
- 87.** Rammensee HG. Chemistry of peptides associated with MHC class I and class II molecules. *Curr Opin Immunol* 1995; 7:85-96.
- 88.** Reimers TJ, Dziuk PJ. The survival of intrauterine skin autografts and allografts in sheep. *J Reprod Fertil* 1974; 38:465-467.
- 89.** Reimers TJ, Ullman MB, Hansel W. Progesterone and prostanoid production by bovine binucleate trophoblast cells. *Biol Reprod* 1985; 33:1227-1236.
- 90.** Roberts RM, Xie S, Nagel RJ, Low B, Green J, Beckers JF. Glycoproteins of the aspartyl proteinase gene family secreted by the developing placenta. *Adv Exp Med Biol* 1995; 362:231-240.
- 91.** Rotzschke O, Falk K, Stevanovic S, Grahovac B, Soloski MJ. Qa-2 molecules are peptide receptors of higher stringency than ordinary class I molecules. *Nature* 1993; 361:642-644.
- 92.** Russell GC, Davies CJ, Andersson L, Ellis SA, Hensen EJ, Lewin HA, Mikko S, Muggli-Cockett NE, Van der Poel JJ. BoLA class II nucleotide sequences, 1996: report of ISAG BoLA Nomenclature Committee. *Anim Genetics* 1997; 28:169-180.

- 93.** Ryan AF, Grendell RL, Geraghty DE, Golos TG. A soluble isoform of the rhesus monkey nonclassical MHC class I molecule Mamu-AG is expressed in the placenta and the testis. *J Immunol* 2002; 169:673-683.
- 94.** Sadasivan B, Lehner PJ, Ortmann B, Spies T, Cresswell P. Roles for calreticulin and a novel glycoprotein, tapasin, in the interaction of MHC class I molecules with TAP. *Immunity* 1996; 5:103-114.
- 95.** Sakaguchi S. Regulatory T cells: Key controllers of immunologic self-tolerance. *Cell* 2000; 101: 455-458.
- 96.** Saper MA, Bjorkman PJ, Wiley DC. Refined structure of the human histocompatibility antigen HLA-A2 at 2.6 AA resolution. *J Mol Biol* 1991; 219(2):277-319.
- 97.** Schlafer DH, Fisher PJ, Davies CJ. The bovine placenta before and after birth: placental development and function in health and disease. *Anim Reprod Sci* 2000; 60-61:145-160.
- 98.** Scott PC, Choi CL, Brandon MR. Genetic organization of the ovine MHC class II region. *Immunogenetics* 1987; 25:116-122.
- 99.** Shawar SM, Vyas JM, Rodgers JR, Rich RR. Antigen presentation by major histocompatibility complex class I-B molecules. *Annu Rev Immunol* 1994; 12:839-880.
- 100.** Sherman MA, Weber DA, Jensen PE. DM enhances peptide binding to class II MHC by release of invariant chain-derived peptide. *Immunity* 1995; 3:197-205.
- 101.** Shevach EM. The guinea pig i-region. A functional analysis of Ia-Ir associations. *Springer Semin. Immunopathol* 1978; 1(2):207-234.

- 102.** Shiroishi M, Tsumoto K, Amano K, Shirakihara Y, Colonna M, Braud VM, Allan DS, Makadzange A, Rowland-Jones S, Willcox B, Jones EY, van der Merwe PA, Kumagai I, Maenaka K. Human inhibitory receptors Ig-like transcript 2 (ILT2) and ILT4 compete with CD8 for MHC class I binding and bind preferentially to HLA-G. *Proc Natl Acad Sci USA* 2003; 100:8856-8861.
- 103.** Shiroishi M, Kuroki K, Ose T, Rasubala L, Shiratori I. Efficient leukocyte Ig-like receptor signaling and crystal structure of disulfide-linked HLA-G dimer. *J Biol Chem* 2006; 281:10439-10447.
- 104.** Solier C, Aguerre-Girr M, Lenfant F, Campan A, Berrebi A, Rebmann V, Grosse-Wilde H, Le Bouteiller P. Secretion of pro-apoptotic intron 4-retaining soluble HLA-G1 by human villous trophoblast. *Eur J Immunol* 2002; 32:3576-3586.
- 105.** Spooner RL, Leveziel H, Grosclaude F, Oliver RA, Vaiman M. Evidence for a possible major histocompatibility complex (BLA) in cattle. *J Immunogenetics* 1978; 325-346.
- 106.** Spooner RL, Millar P, Oliver RA. The production and analysis of antilymphocyte sera following pregnancy and skin grafting of cattle. *Anim Blood Groups and Biochem Genetics* 1979; 10:99-105.
- 107.** Storset AK, Slettedal IO, Williams JL, Law A, Dissen E. Natural killer cell receptors in cattle: a bovine killer cell immunoglobulin-like receptor multigene family contains members with divergent signaling motifs. *Eur J Immunol* 2003; 33:980-990.
- 108.** Storset AK, Kulberg S, Berg I, Boysen P, Hope JC, Dissen E. NKp46 defines a subset of bovine leukocytes with natural killer cell characteristics. *Eur J Immunol* 2004; 34:669-676.

- 109.** Sugita M, Brenner MB. Association of the invariant chain with major histocompatibility complex class I molecules directs trafficking to endocytic compartments. *J Biol Chem* 1995; 270:1443-1448.
- 110.** Svejgaard A, Hauge M, Jersild C, Platz P, Ryder LP, Nielsen LS, Thomsen M. The HLA system. An introductory survey. *Monogr Hum Genet* 1975; 7:1-100.
- 111.** Tabaczewski P, Chiang E, Henson M, Stroynowski I. Alternative peptide binding motifs of Qa-2 class Ib molecules define rules for binding of self and nonself peptides. *J Immunol* 1997; 159:2771-2781.
- 112.** Tan P, Kropshofer H, Mandelboim O, Bulbuc N, Hammerling GJ, Momburg F. Recruitment of MHC class I molecules by tapasin into the transporter associated with antigen processing-associated complex is essential for optimal peptide loading. *J Immunol* 2002; 168:1950-1960.
- 113.** Thomas DW, Yamashita U, Shevach EM. The role of Ia antigens in T cell activation. *Immunol Rev* 1979; 35(1): 97-120.
- 114.** Tian XC, Kubota C, Enright B, Yang X. Cloning animals by somatic cell nuclear transfer-biological factors. *Reprod Biol Endocrinol* 2003; 1:98.
- 115.** Trowsdale J. Genetic and functional relationships between MHC and NK receptor genes. *Immunity* 2001; 15(3):363-374.
- 116.** Tysoe-Calnon VA, Grundy JE, Perkins SJ. Molecular comparisons of the β 2-microglobulin-binding site in class I major-histocompatibility-complex alpha-chains and proteins of related sequences. *Biochem J* 1991; 277:359-369.

- 117.** Uhrberg M, Valiante NM, Shum BP, Shilling HG, Lienert-Weidenbach K, Corliss B, Tyan D, Lanier LL, Parham P. Human diversity in killer cell inhibitory receptor genes. *Immunity* 1997; 7(6):753-763.
- 118.** Valiante NM, Uhrberg M, Shilling HG, Lienert-Weidenbach K, Arnett KL, D'Andrea A, Phillips JH, Lanier LL, Parham P. Functionally and structurally distinct NK cell receptor repertoires in the peripheral blood of two human donors. *Immunity* 1997; 7(6):739-751.
- 119.** van Eijk MJ, Russ I, Lewin HA. Order of bovine DRB3, DYA, and PRL determined by sperm typing. *Mammalian Genome* 1993; 4(2):113-118.
- 120.** Vance RE, Kraft JR, Altman JD, Jensen PE, Raulet DH. Mouse CD94/NKG2A is a natural killer cell receptor for the non-classical major histocompatibility complex (MHC) class I molecule Qa-1(b). *J Exp Med* 1998; 188:1841-1848.
- 121.** Vance RE, Jamieson AM, Raulet DH. Recognition of the class Ib molecule Qa-1(b) by putative activating receptors CD94/NKG2C and CD94/NKG2E on mouse natural killer cells. *J Exp Med* 1999; 190:1801-1812.
- 122.** Vander Wielen AL, King GJ. Intraepithelial lymphocytes in the bovine uterus during the oestrous cycle and early gestation. *J Reprod Fertil* 1984; 70:457-462.
- 123.** Watts C. The exogenous pathway for antigen presentation on major histocompatibility complex class II and CD1 molecules. *Nat Immunol* 2004; 5:685-692.
- 124.** Wooding FBP. Current topic: the synepitheliochorial placenta of ruminants: binucleate cell fusions and hormone production. *Placenta* 1992; 13:101-113.
- 125.** Wooding FBP, Roberts RM, Green JA. Light and electron microscope immunocytochemical studies of the distribution of pregnancy associated glycoproteins

(PAGs) throughout pregnancy in the cow: possible functional implications. *Placenta* 2005; 26:807-827.

126. Yokoyama WM, Plougastel BF. Immune functions encoded by the natural killer gene complex. *Nat Rev Immunol* 2003; 3(4):304-316.

127. Zhong G, Romagnoli P, Germain RN. Related leucine-based cytoplasmic targeting signals in invariant chain and major histocompatibility complex class II molecules control endocytic presentation of distinct determinants in a single protein. *J Exp Med* 1997; 185:429-438.

128. Zinkernagel RM, Doherty PC. MHC-restricted cytotoxic T cells: studies on the biological role of polymorphic major transplantation antigens determining T-cell restriction-specificity, function, and responsiveness. *Adv Immunol* 1979; 27:51-177.

CHAPTER 2

EXPRESSION OF BOVINE CLASSICAL (BoLA-Ia) AND NON-CLASSICAL (BoLA-Ib) CLASS I PROTEINS IN MOUSE P815 AND HUMAN K562 CELLS

Abstract

During the third trimester of pregnancy in cattle, interplacentomal trophoblast cells express both classical (BoLA-Ia) and non-classical (BoLA-Ib) MHC class I proteins. To investigate whether these proteins are expressed as surface or secreted proteins, we cloned protein-coding DNAs in the pcDNA 3.1 mammalian expression vector and sequenced the inserts to identify plasmids to transfect murine P815 and human K562 (MHC null) cultured cells. Stably transfected P815 and K562 cell lines were stained with H1A and W6/32 antibodies, respectively, to detect expression of the proteins by flow cytometry. We identified two bovine classical (N*01701 and N*01802) and three non-classical (NC1*00501, NC3*00101, and NC4*00201) proteins on the cell surface. Other non-classical proteins (NC1*00401 and NC2*00102), which may be released or secreted by cells into their surrounding extracellular spaces (soluble or secreted proteins), do not exhibit cell surface expression. The surface expressing cells were sorted and enriched. Both classical and non-classical proteins were precipitated from the cell culture supernatant using ammonium sulfate. The cell lines with surface expressing BoLA-Ia and BoLA-Ib proteins shed and/or released more MHC-I proteins into the culture supernatants than unsorted non-surface expressing cell lines. Transfected cells were lysed and histidine tagged proteins were purified using nickel affinity column chromatography. Western blot analysis with an anti-V5 antibody demonstrated the presence of MHC-I

heavy chains in all of the transfected cell lines. These data confirm that bovine class Ib molecules NC1*00501, NC3*00101, and NC4*00201 are expressed as surface isoforms.

Introduction

The MHC, the genetic region that encodes the proteins responsible for tissue graft compatibility [21], encodes MHC class I (MHC-I) and MHC class II (MHC-II) glycoproteins. There are two subclasses of MHC-I proteins. MHC-Ia proteins are membrane-bound isoforms that are expressed in all nucleated cells of the body and present intracellular pathogen-derived peptides or the animal's own peptides on the cell surface for immune recognition by CD8 T cells. With the discovery of HLA-G [12], another category of MHC-I proteins referred, non-classical class I (MHC-Ib), was recognized. MHC-Ib molecules are less polymorphic, possess specific molecular motifs in their transmembrane domains and contain premature stop codons. These features make MHC-Ib proteins important immunomodulatory molecules which may induce immunotolerance during pregnancy. MHC-II proteins are expressed only on professional antigen presenting cells (APCs) which present extracellular pathogen-derived peptides on the cell surface for recognition by CD4 T cells.

An allogeneic transplant is a tissue graft from a genetically distinct member of the same species. A fetus carrying half of its genome from the father is a semi-allogeneic tissue inside the uterus, yet it circumvents the maternal immune system. Many studies performed on humans, mice, and non-human primates reveal that both fetal and maternal mechanisms contribute to the immunological tolerance of the mother to the fetus. Placental lactogen, progesterone, prostaglandins and other immunomodulatory hormones as well as chemokines synthesized by uterine cells, including T-regulatory cells and

trophoblast cells, contribute to tolerance [17, 19, 38]. Non-classical class I molecules produced by fetal trophoblast cells interact with inhibitory receptors expressed by T-lymphocytes and natural killer (NK) cells to inhibit these immune cells, thus protecting the conceptus from maternal immune attack [1, 3, 11, 12, 17, 19, 23, 32]. The human class Ib molecule HLA-G interacts with leukocyte inhibitory receptors such as leukocyte immunoglobulin-like receptors 1 (LILR1) and LILR2 to induce immunosuppression [6, 19, 36]. HLA-G also upregulates expression of ILT2, ILT3, ILT4 and KIR2DL4 in APCs, NK cells and T cells, which protects the HLA-G expressing tissues from immune cell attack [26]. Qa-2, a mouse class Ib molecule and functional homolog of HLA-G, controls the rate of cleavage and survival of mouse preimplantation embryos [6].

Alternative splicing is a mechanism for generating protein diversity in non-classical major histocompatibility complex class I (MHC-Ib) molecules. Alternative splicing produces membrane and soluble isoforms. In humans, differential mRNA splicing of HLA-G results in synthesis of membrane isoforms, HLA-G1, -G2, and -G3 and soluble isoforms, HLA-G5 and -G6 [12, 19]. Similarly, murine Qa-2 encodes two soluble isoforms, S1 Qa-2 and S2 Qa-2 [6], *mamu-AG* in rhesus monkeys encodes membrane-bound isoforms *Mamu-AG1*, *Mamu-AG2* and *Mamu-AG3* [4], and *Paan-AG* in baboons encodes four membrane isoforms, *Paan-AG1*, *-AG2*, *-AG3* and *-AG4*, and a soluble isoform, *sPaan-AG1* [22]. Alternatively spliced variants of bovine class Ib protein, BoLA-NC1 have also been found (Davies unpublished). However, splice variants of BoLA-NC2, -NC3, and -NC4 have yet to be positively confirmed. This is the first study to investigate the surface expression and/or secretion of BoLA-Ib proteins. Here we report that the bovine class Ib molecules NC1*00501, NC3*00101, and

NC4*00201 are expressed as cell surface proteins. However, membrane expression of the bovine class Ib proteins NC1*00401 and NC2*00102 was not detected.

Most mammalian trophoblastic cells do not express polymorphic, classical, MHC-I molecules [10, 18]. However, cattle interplacentomal and arcade trophoblast cells normally express BoLA-Ia molecules during the third trimester of pregnancy [9]. MHC-Ia expression during the last trimester triggers the release of the placenta during parturition [8, 16]. Interplacentomal trophoblast cells from late pregnancy expressed classical (BoLA-Ia) and non-classical (BoLA-Ib) genes at varying levels depending on the specific MHC class I haplotypes carried by the conceptus [7]. Comparatively, trophoblast cells expressed more MHC class I transcripts encoded at non-classical loci than peripheral blood mononuclear cells (PBMC). However, all of the classical genes expressed in PBMC were also expressed in trophoblast cells.

We propose that bovine trophoblast cells express cell surface and secreted non-classical MHC-I proteins. To investigate this hypothesis, we used the murine mastocytoma cell line P815 (ATCC TIB-64) and the human MHC-null cell line K562 (ATCC CCL-243) to express cattle class I proteins. Cells were transfected with bovine transgenes encoding classical and non-classical class I proteins. Post-transfection analysis of cell surface expression was performed by flow cytometry. Secreted proteins in culture supernatants were precipitated by ammonium sulfate precipitation and then detected using Western blots.

Materials and Methods

Samples

Full-length MHC-I cDNA was reverse transcribed from interplacentomal trophoblast-RNA, cloned in the pCR II TOPO vector (Invitrogen), and stored at -80°C as part of a previous study [7]. These were the initial samples used in the current study.

Subcloning of Classical and Non-Classical MHC Class I Genes

We amplified classical (N*01802 and N*01701) and non-classical (NC1*00401, NC1*00501, NC2*00102, NC3*00101 and NC4*00201) class I alleles from the AH11 haplotype using *Platinum Pfx* DNA polymerase (Invitrogen) and subcloned them into the pcDNA3.1 One Directional V5-His mammalian expression vector (Invitrogen) to express the protein with a 3' 6x His tag and a V5 epitope. Fifty µL PCR reactions were prepared by adding 1 U *Taq DNA Polymerase* (proofreading), 0.8 µM of each primer (listed below), 2.0 mM MgSO₄, 0.2 mM dNTPs, 1X optimized PCR buffer, and 2 µL of the diluted (1:1000) cDNA from the pCR II TOPO clone. PCR amplification was carried out using an Eppendorf Master cycler (Brinkmann) with the following parameters: 1 min 30 sec at 94°C; 25 cycles of 30 sec at 94°C, 15 sec at 60°C and 90 sec 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 pcDNA 3.1 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. There was no blue/white screening. Consequently, 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. One forward and two reverse amplification primers were used:

Forward primer (BoC1FP-E1B)	ACCATGGGGCCGCGAACCCCTC
Reverse primer (BoC1RP-3'A)	GATGAAGCATCACTCAGTCCCC
Reverse primer (BoC1RP-E7A)	TTTAGGAACCGTGAGAGACACATC

By using two different reverse primers, clones with the normal stop codon (reverse primer BoC1RP-3'A) and clones expressing a 3' 6x histidine tag and a V5 epitope (reverse primer BoC1RP-E7A) were produced.

Sequencing of Subclones

Correct size subclones were sequenced using T-7 forward and BGH reverse sequencing primers and subclones with full-size insert were selected for expression. For all the alleles, clones from multiple cell preparations were sequenced. 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 software (DNASTAR, Inc.) to trim vector sequences and generate MHC class I consensus sequences. The ClustalW method of Lasergene MegAlign™ software (DNASTAR, Inc.) was used to align sequences.

Cell Lines and Transfection

P815 cells are mast cells derived from a DBA/2 strain-mouse (*Mus musculus*) with a mastocytoma. The majority of these cells grow in suspension with some (<5%)

adherent cells. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Caisson Laboratories) with 10% bovine calf serum (Hyclone), 2mM L-glutamine (Hyclone) and penicillin 100 units/ml and streptomycin 100 µg/ml (Hyclone) at 37°C, 5% CO₂.

The K562 cell line (CCL-243) is an MHC null cell line originated 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% CO₂. Both cell lines were obtained from American Type Culture Collection (ATCC, USA).

Cells were transfected with subclones of either a classical or non-classical MHC class I gene in the pcDNA3.1 vector using Lipofectamine 2000 (Invitrogen) transfection reagent. Untransfected cells were used as a negative control. Beta-galactosidase supplied with the vector kit was expressed in P815 cells as a transfection positive control. Briefly, plasmids from the correct subclones were isolated using a QIA plasmid Mini Prep kit (Qiagen). Plasmids carrying cDNA for N*01802, N*01701, NC1*00401, NC1*00501, NC2*00102, NC3*00101 and NC4*00201 were used. Cells were washed 1x with DMEM or IMDM (without serum or antibiotics) and resuspended at 1×10^6 cells/ml. For the transfections, 2 ml of cells (2×10^6 cells) were plated per well in a 6-well tissue culture plate and incubated at 37°C, 5% CO₂. Four micrograms of plasmid DNA was diluted to 250 µl in DMEM and mixed gently. Lipofectamine was mixed gently before use, and then 10 µl was diluted in 250 µl of DMEM or IMDM and incubated for 5 min at room temperature. The diluted DNA was combined with diluted Lipofectamine (total volume =

500 μ l) and mixed gently. The mixture was incubated for 20 min at room temperature. After the 20 min incubation, 500 μ l of the complex (plasmid + Lipofectamine) was added to each well containing cells and mixed gently. Cells were incubated at 37°C, 5% CO₂ for 3 hours and then 5 ml of DMEM or IMDM with 10% bovine calf serum was added to each well. Transfected cells were incubated for 24 to 48 hours prior to selection of stable transfectants by addition of G418 (*Invivogen*) antibiotic (500 μ g/ml). After two weeks in selective media the transfectants were screened by flow cytometry.

Flow Cytometry

The following monoclonal antibodies were used in the analysis. The anti-human MHC-I monoclonal antibody W6/32, which recognizes MHC class I heavy chains associated with human or bovine beta-2-microglobulin (β 2m), was used as a negative control for MHC-null K562 cells and as a positive control for mouse P815 cells because they express mouse class I proteins that bind bovine β 2m present in the culture medium. ColiS205D1 (IgG2a), which is specific for *Escherichia coli* antigen, was used as an isotype negative control. The anti-bovine MHC class I monoclonal antibody H1A was used to detect bovine MHC class I heavy chains. Other anti-bovine MHC class I antibodies that were also used included: H6A, H11A, H58A, PT85-A, and IL-A88. All of the monoclonal antibodies were obtained from the Monoclonal Antibody Center at Washington State University, Pullman, WA. Fluorescein isothiocyanate (FITC) conjugated anti-mouse IgG antibody (KPL) was used as a secondary antibody. For staining, cells were resuspended in fluorescence buffer (FB; PBS with 0.1% Sodium Azide, 1% bovine serum albumin) and incubated with primary antibody (15 μ g/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% formaldehyde or paraformaldehyde. One million cells were stained for each sample. Cells were analyzed using a Becton-Dickinson FACS Aria II fluorescence activated cell sorter (FACS) equipped with FACS Diva software. The FACS Aria II was also used to sort the transfected cells to enrich the high expressing cells.

Western Blot

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

Purification of Recombinant Histidine Tagged Proteins

Transfected P815 and K562 cells were grown in T75 cell culture flasks (Corning) to harvest $\sim 100 \times 10^6$ cells. Cells were harvested by centrifuging at 1500 RPM for 10 min at RT. 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). Lysis was performed by incubating the cells for 1 hour at 4°C. One micromole protease inhibitor cocktail (Sigma) for mammalian cell extracts and 1mM PMSF (Phenyl Methane Sulfonyl Fluoride) were also added to the suspension. Cells were centrifuged at 10,000 RPM for 10 min at 4°C and the supernatant was filtered using 0.2 μ M filters and stored in sterile tubes at -20°C until used for purification.

HisGraviTrap Columns (GE Healthcare) were used to purify histidine-tagged proteins. The manufacturer's recommended purification procedure was followed. After loading lysate to the column, it was washed 4 times with wash buffer with 40 mM imidazole. Elution was performed with elution buffer containing 500 mM imidazole. Purified eluates were stored at -20°C. Eluates with specific strong bands 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 [15]. 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 (VIVASPIN). Concentrations of dialysates were measured using a BCA protein assay kit (Thermo Scientific).

Ammonium Sulfate Precipitation

Thirty percent ammonium sulfate (AS) was used to precipitate proteins in the culture supernatants (exhausted media) from the cultured transfected cells. 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 hour. The suspension was centrifuged at 10,000 x g for 10 min at 4°C to pellet the insoluble 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.

Results

Subcloning and Sequencing Analysis

Most of the subclones had inserts with the correct sequences in the correct orientation and contained the C-terminal 6x Histidine tag and V5 epitope in-frame. The following alleles from the AH11 haplotype were expressed in the two cell lines: N*01701, N*01802, N*01701, NC1*00401, NC1*00501, NC2*00102, NC3*00101 and NC4*00201.

Evaluation of Cell Surface Expression by Flow Cytometry

In order to determine which bovine class Ib proteins were expressed on the cell surface, selected and stably transfected cell lines were stained and analyzed using flow cytometry. W6/32, which reacts with the MHC class I heavy chains associated with β 2m, was positive on untransfected P815 cells as these cells express mouse class I proteins. On the other hand, W6/32 antibody was negative on untransfected K562 cells because this

cell line does not express MHC antigens. ColiS205D1 showed no reactivity with any of the cell lines and served as a negative control antibody for both the P815 and K562 cells. Monoclonal antibody H1A recognizes bovine class I heavy chains. H1A antibody reacted with P815 cells expressing N*01701, N*01802, NC1*00501 or NC3*00101 proteins, whereas it did not recognize P815 cells expressing the other bovine non-classical class I proteins (Figure 2-1). The same pattern was seen with the H6A, H11A, H58A, PT-85A and IL-A88 antibodies.

K562 cells expressing N*01701, N*01802, NC1*00501, NC3*00101 and NC4*00201 were positive with W6/32 (Figure 2-2). H1A, H6A, H11A, H58A, PT-85A, IL-A88 were also positive on K562 cells transfected with these proteins. Twenty-four hour post-transfection expression was <20% compared to untransfected cells which increased to ~40% after selection with G418 for 2 weeks. Therefore, we used FACS to sort the positive stably transfected cells to enrich transgene expression. Sorting of transfected P815 cells was done using cells stained with the H1A antibody. Transfected K562 cells were sorted on the basis of staining with W6/32. Sorted cells were cultured in appropriate media for one week and rechecked on the flow cytometer to determine the post-sort MHC-I expression level. N*01701, N*01802, NC1*00501, NC3*00101, and NC4*00201 positive cell lines were enriched to more than 90% expression of the proteins after sorting. Due to the lack of surface expression of the NC1*00401 and NC2*00102 non-classical class I proteins, we were not able to sort the cell lines expressing these transgenes (Figures 2-1 and 2-2). The results of the class I monoclonal antibody screening of the transfected cell lines using flow cytometry are summarized in Tables 2-1 and 2-2.

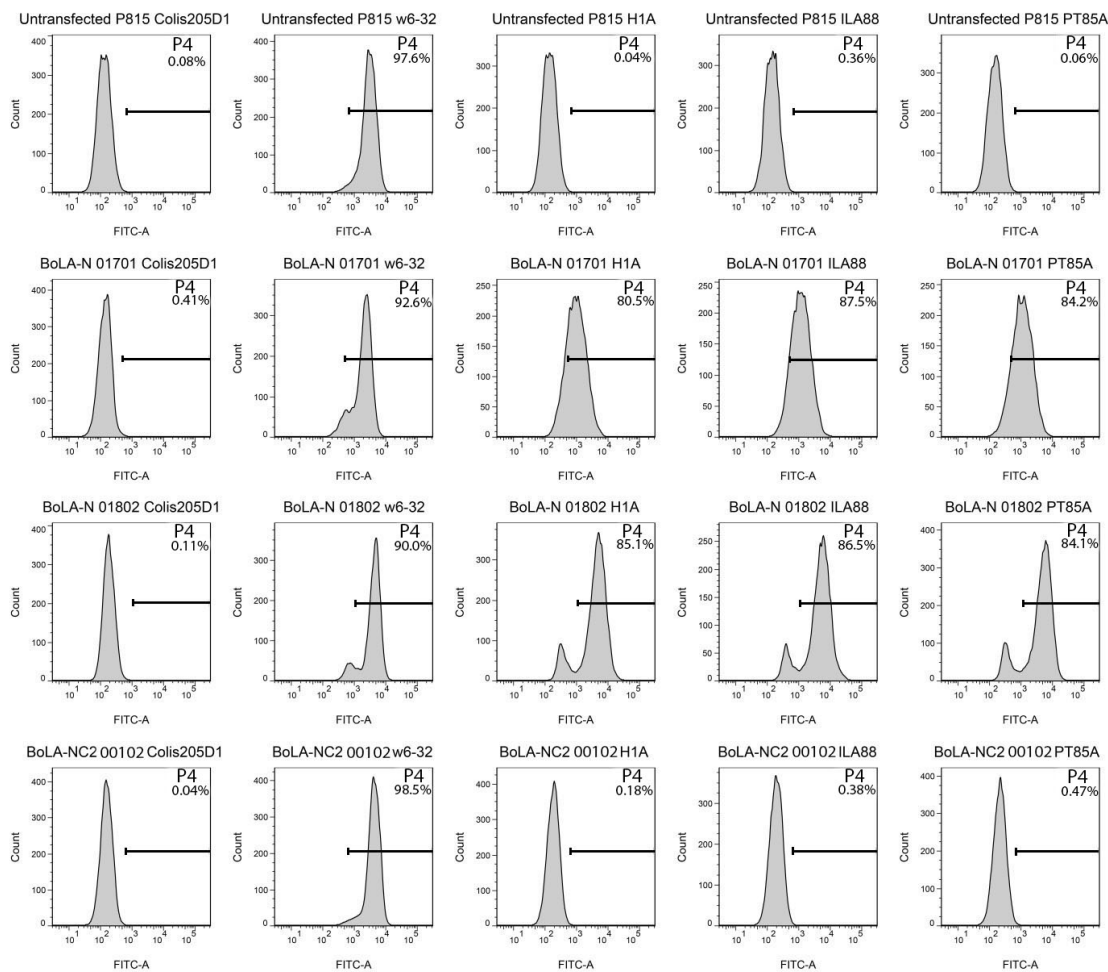
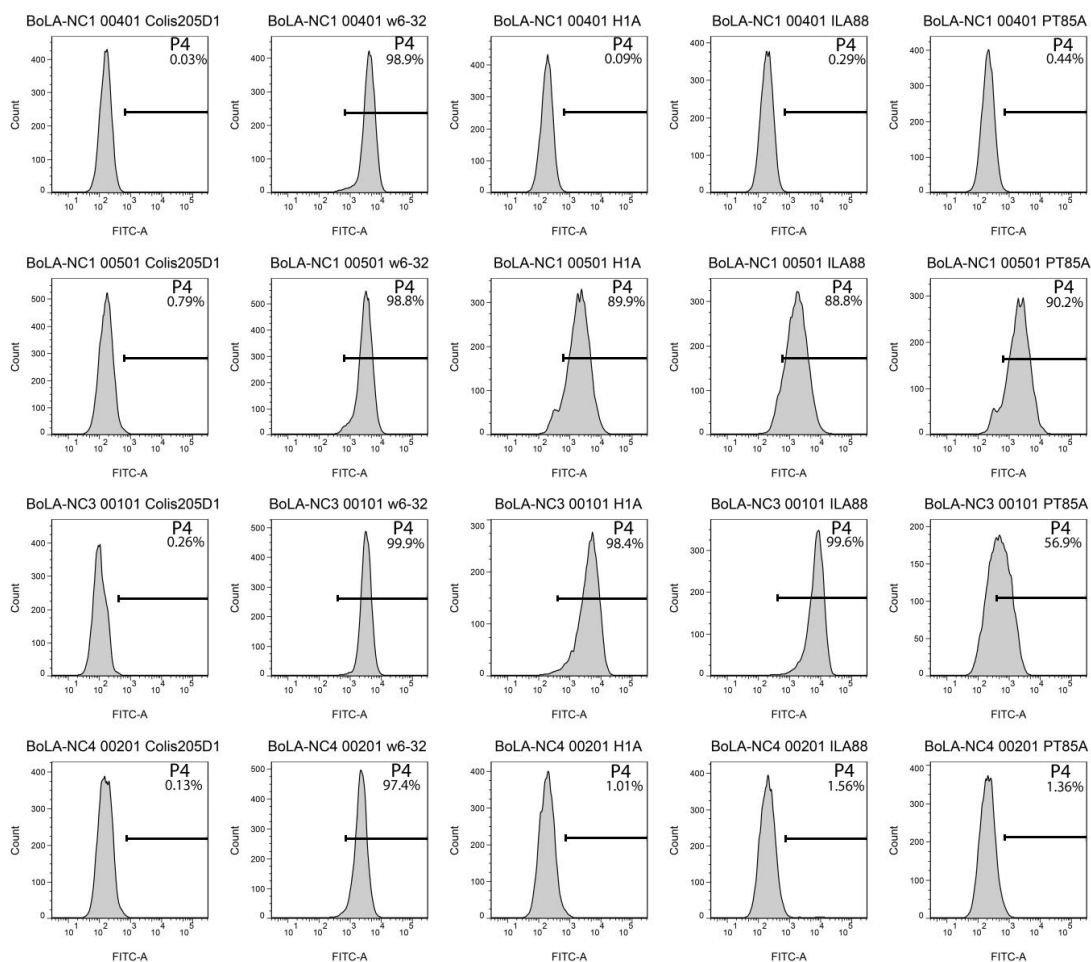


Fig. 2-1: Flow cytometric analysis of murine P815 cells transfected with cattle MHC class Ia and class Ib transgenes. Colis205D1 and W6/32 were used as negative and positive controls, respectively. Three anti-bovine MHC-I monoclonal antibodies were used: H1A, IL-A88, and PT-85A. As shown, the BoLA-NC1*00401, BoLA-NC2*00102, and BoLA-NC4*00201 class Ib proteins did not exhibit surface expression on P815 cells. On the other hand, BoLA-Ia proteins, N*01701 and N*01802, and BoLA-Ib proteins NC1*00501 and NC3*00101 proteins were expressed on the cell membrane of transfected P815 cells.



We noticed that the antibodies tested varied in their affinity for different proteins. In addition, there was considerable variation in the level of expression of the bovine MHC-I proteins. The classical N*01701 protein was expressed at the lowest level and the non-classical NC*00101 protein was expressed at highest level in both P815 and K562 cells.

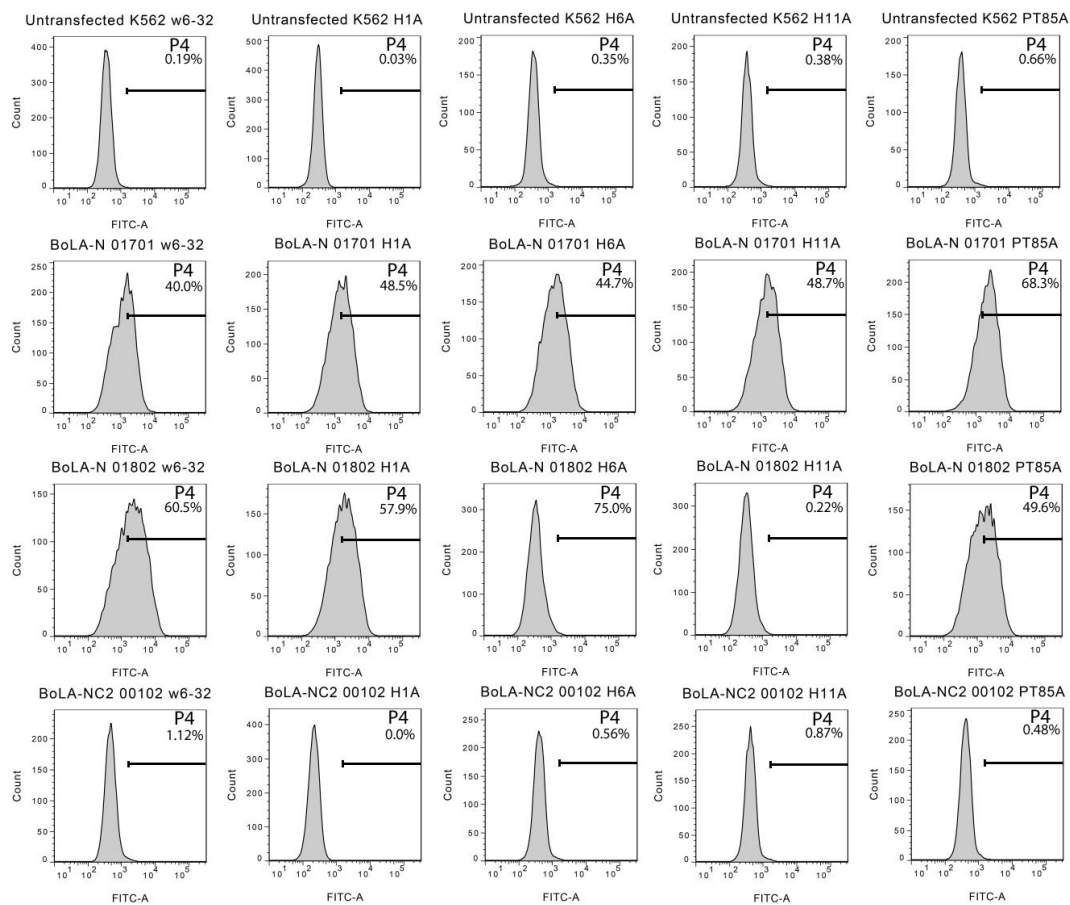
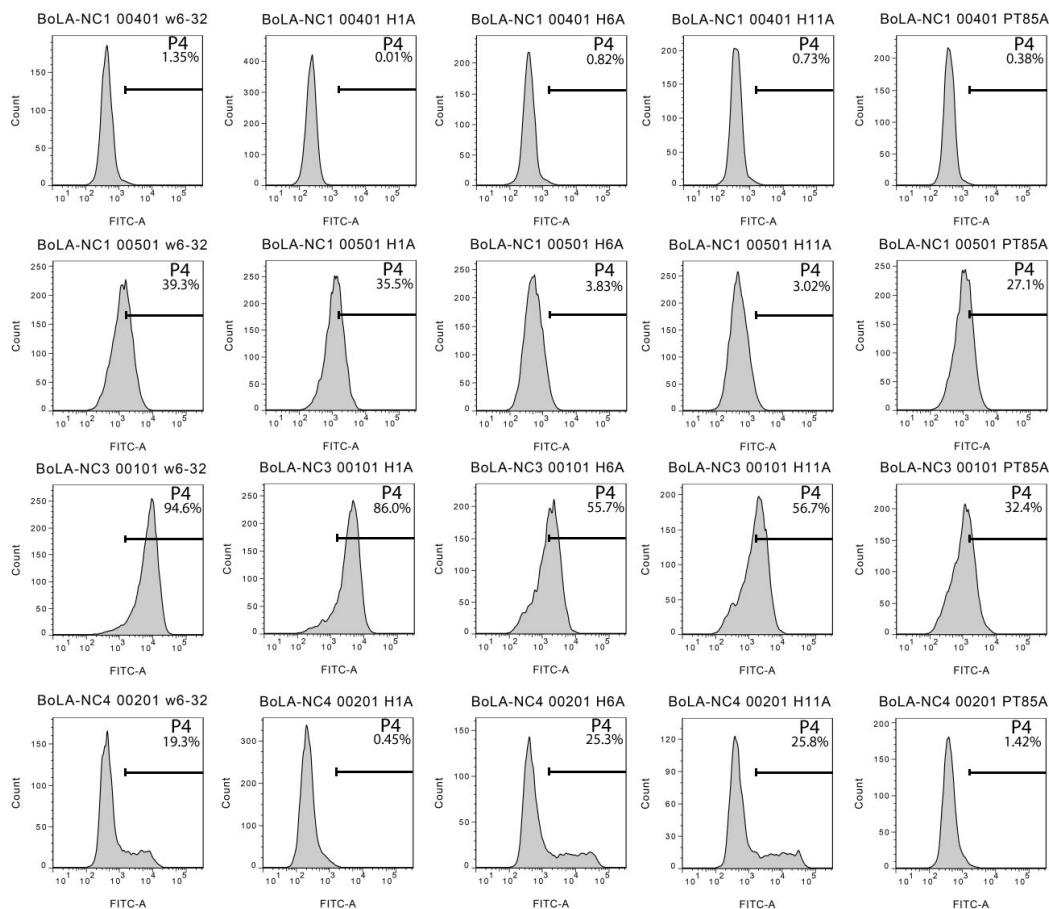


Fig. 2-2: Flow cytometric analysis of BoLA-Ia and -Ib expression on transfected human MHC-null K562 cells. Untransfected cells served as a negative control. W6/32, H1A, H6A, H11A, and PT-85A antibodies were used to detect bovine class I proteins. As shown, BoLA-NC1*00401 and BoLA-NC2*00102 class Ib proteins did not exhibit surface expression on K562 cells. BoLA-Ia proteins, N*01701 and N*01802, and BoLA-Ib proteins NC1*00501, NC3*00101, and NC4*00201 proteins were expressed on the surface of transfected K562 cells.



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. We also tested an antibody directed against the C-terminal His tag (Invitrogen) but this antibody did not work. A cell line transfected with β -galactosidase was used as a positive control and had a 120 kD band as expected. A lysate from untransfected cells was used as a negative control. All of the bovine class Ia and class Ib proteins were identified in lysates from transfected cells with the anti-V5 antibody. The bands were all approximately 45 kD in size as determined with the Magic

Table 2-1 Reactivity pattern of monoclonal antibodies on P815 cells transfected with cattle MHC-I genes

Antibody	Untransfected P815 Cells	Transfected P815 Cells						
		N*01701	N*01802	NC1*00401	NC1*00501	NC2*00102	NC3*00101	NC4*00201
ColIS205D1	-	-	-	-	-	-	-	-
W6/32	++++	++++	++++	++++	++++	++++	++++	++++
IL-A88	-	+++	++++	-	++++	-	++++	-
H1A	-	+++	++++	-	++++	-	++++	-
H6A	-	++	++++	-	+++	-	++++	-
H11A	-	++	++++	-	+++	-	++++	-
H58A	-	+++	++++	-	+++	-	++++	-
PT-85A	-	+++	++++	-	++++	-	++	-

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

Antibody	Untransfected K562 Cells	Transfected K562 Cells						
		N*01701	N*01802	NC1*00401	NC1*00501	NC2*00102	NC3*00101	NC4*00201
W6/32	-	++++	++++	-	++++	-	++++	++
H1A	-	+++	++++	-	+++	-	++++	++
H6A	-	+++	++	-	++	-	++++	++
H11A	-	+++	++++	-	++	-	++++	++
H58A	-	+++	++++	-	++	-	++++	++
PT-85A	-	+++	++++	-	+++	-	++	+

Mark (Invitrogen) ladder. The NC1*00401 protein, which has a complete deletion of the transmembrane domain, was slightly smaller than the other MHC class I proteins.

Sometimes there were non-specific protein bands seen in the crude lysates of the transfected cells, but these bands disappeared after purification of the proteins with His GraviTrap Columns (GE Healthcare; Figures 2-3 and 2-4).

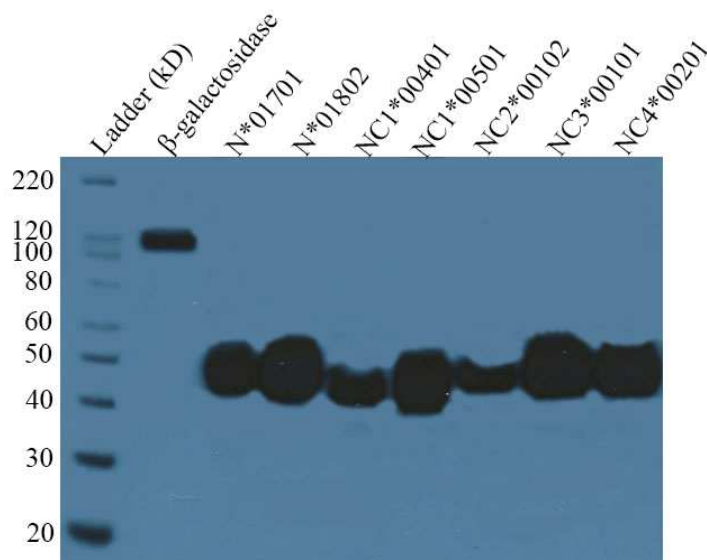


Fig 2-3: Purified proteins from transfected murine P815 cells

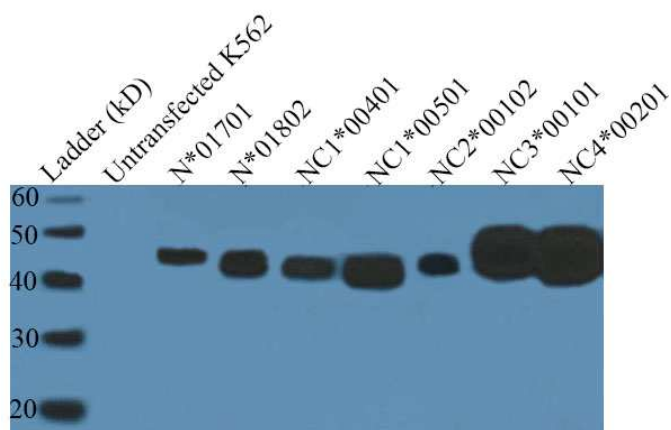


Fig 2-4: Purified proteins from transfected human MHC null K562 cells

Assessment of Protein Secretion by Ammonium Sulfate Precipitation

Ammonium sulfate precipitation was performed on exhausted media from all of the cultured cell lines. Precipitated protein pellets were dissolved in 50% phosphate buffer with L-Arg and L-Glu amino acids and 50% DMSO and processed to run on Western blots. MHC class I proteins in the culture supernatants were detected by Western blotting with anti-V5 antibody (Figures 2-5 and 2-6). Strong bands were present for all of the surface expressed MHC class I proteins: N*01701, N*01802, NC3*00101, and NC4*00201 (only in K562 cells). This suggests that the class I proteins are being shed from the cell membrane at a significant rate. There was a clear but fairly weak band for NC1*00401, which lacks the transmembrane domain, in the supernatant from K562 cells, suggesting that this may be a secreted isoform. The NC2*00102 protein apparently remained trapped inside the cells as it was not detected in the culture supernatants.

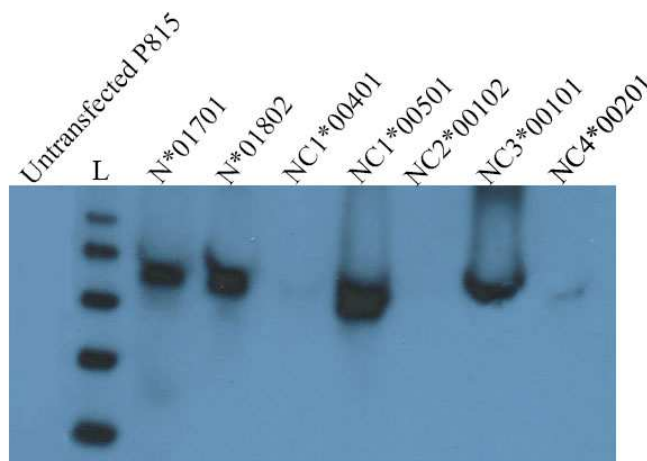


Fig 2-5: Ammonium sulfate-precipitated proteins from transfected murine P815 cell-culture supernatants

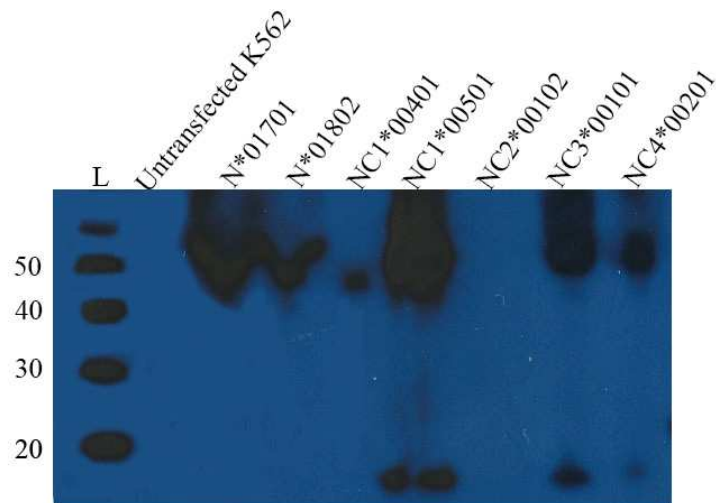


Fig 2-6: Precipitated proteins from transfected human K562 cell-culture supernatants

Discussion

We performed transfection experiments to identify proteins encoded by two classical and four non-classical genes. Our experiments showed that the two classical isoforms, N*01701 and N*01802, and the non-classical isoforms, NC1*00501, NC3*00101, and NC4*00201, are surface or membrane proteins expressed on the cell surface. Immunoblotting experiments with proteins precipitated from culture supernatants showed that these proteins are also shed or released from the cell membrane. NC1*00401, which lacks a transmembrane domain, was secreted at a noticeable but low level by transfected K562. The NC2*00102 protein was not expressed on the cell membrane or secreted by either of the transfected cell lines that were tested. Detection of the MHC class I proteins by flow cytometry and Western blots provides clear evidence that the classical and non-classical MHC class I transcripts isolated from third-trimester interplacental trophoblast cells are translated into proteins.

We do not have monoclonal antibodies that are specific for the bovine non-classical class I proteins. We are currently in progress to produce monoclonal antibodies to NC3*00101 protein by direct immunization of mice with transfected cell lines. We could not detect surface expression of NC1*00401 and NC2*00102 with W6/32, H1A or the other antibodies that were tested. A previous transfection study done with NC2*00101 showed no surface expression with ILA-88 [13]. NC1*00401 does not have a transmembrane domain [7], hence we did not expect this protein to be expressed on the cell surface. NC4*00201, which was not expressed on the surface of P815 cells, showed surface expression on K562 cells. It is likely that human β 2m can associate with this protein thereby allowing peptide binding and cell surface expression, while murine β 2m does not form a functional heterodimer with this bovine MHC class I heavy chain.

Expression of membrane bound and secreted forms of BoLA-Ib proteins is important because these proteins may inhibit maternal leukocytes by interacting with inhibitory receptors expressed by the leukocytes. Membrane bound and secreted class Ib proteins, such as HLA-E, -F, and -G in humans, Qa-2 in mice, and Mamu-E in rhesus monkeys, interact with receptors such as LILRB1, LILRB2, KIR2DL4 and CD94/NKG2A expressed by maternal leukocytes and inhibit their cell-lysis properties [1, 2, 6, 14, 17, 19, 20]. Membrane bound HLA-G isoforms as well as soluble isoforms play an immunosuppressive role in human pregnancy. Membrane isoforms induce suppression of CD4⁺ T cells and NK cells [25, 31]. It also has been reported that T cells and NK cells are rendered immunosuppressive by the transfer of membrane patches containing HLA-G from APCs or tumor cells [5, 31]. The soluble HLA-G1 isoform induces maternal fetal tolerance by inducing apoptosis of activated CD8 T cells [24, 37] and down-regulating

CD4 T cell proliferation [29]. It has been reported that sHLA-G inhibits NK cell-mediated cytotoxicity [30, 32, 33, 35]. It is possible, that secreted or shed cattle class Ib molecules act as soluble immunosuppressive factors during pregnancy.

There is nothing known about the role of class Ib proteins in cattle. The surface expressed BoLA-Ib proteins, secreted NC1*00401, and even NC2*00102 may interact with inhibitory receptors expressed by maternal leukocytes and provide inhibitory signals. Consequently, it is important to conduct studies to identify the leukocytes and inhibitory receptors that interact with BoLA-Ib proteins. To identify bovine class Ib glycoproteins in trophoblast cell culture supernatants, there is a need for monoclonal antibodies to these proteins.

We were not able to see NC2*00102 protein on the cell surface. In humans, HLA-E does not bind with the peptides derived from intracellular proteins. Instead they bind with peptides derived from other HLA class I signal sequences as required for cell surface expression [3, 27, 28, 34]. It is possible that NC2*00101 is a homologue of human HLA-E and requires a specific peptide or leader peptide for its cell surface expression.

In summary, we have identified that cattle class Ia proteins, N*01701 and N801802, and class Ib proteins, NC1*00501, NC3*00101, and NC4*00201, are the surface expressing proteins whereas, NC1*00401 and NC2*00102 proteins, are not expressed on the cell-surface.

References

1. Bainbridge DRJ, Ellis SA, Sargent IL. The short forms of HLA-G are unlikely to play a role in pregnancy because they are not expressed at the cell surface. *J Reprod Immunol* 2000; 47:1-16.
2. Bainbridge DRJ, Ellis SA, Le Bouteiller P, Sargent I. HLA-G remains a mystery. *Trends Immunol* 2001; 22:548-552.
3. Braud VM, Allan DS, O'Callaghan CA, Soderstrom K, D'Andrea A, Ogg GS, Lazetic S, Young NT, Bell JI, Phillips JH, Lanier LL, McMichael AJ. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature* 1998; 391:795-799.
4. Boyson JE, Iwanaga KK, Golos TG, Watkins DI. Identification of a novel MHC class I gene, Mamu-AG, expressed in the placenta of a primate with an inactivated G locus. *J Immunol* 1997; 159:3311-3321.
5. Caumartin J, Favier B, Daouya M, Guillard C, Moreau P, Carosella ED, LeMaoult J. Trogocytosis-based generation of suppressive NK cells. *EMBO J* 2007; 26(5):1423-1433.
6. Comiskey M, Goldstein CY, De Fazio SR, Mammolenti M, Newmark JA, Warner CM. Evidence that HLA-G is the functional homolog of mouse Qa-2, the Ped gene product. *Hum Immunol* 2003; 64:999-1004.
7. Davies CJ, Eldridge JA, Fisher PJ, Schlafer DH. Evidence for expression of both classical and non-classical major histocompatibility complex class I genes in bovine trophoblast cells. *Am J Reprod Immunol* 2006; 55:188-200.
8. Davies CJ, Hill JR, Edwards JL, Schrick FN, Fisher PJ, Eldridge JA, Schlafer DH. Major histocompatibility antigen expression on the bovine placenta: Its relationship to abnormal pregnancies and retained placenta. *Anim Reprod Sc* 2004; 82-83:267-280.

- 9.** Davies CJ, Fisher PJ, Schlafer DH. Temporal and regional regulation of major histocompatibility complex class I expression at the bovine uterine/placental interface. *Placenta* 2000; 21:194-202.
- 10.** Donaldson WL, Zhang CH, Oriol JG, Antczak DF. Invasive equine trophoblast expresses conventional class I major histocompatibility complex antigens. *Development* 1990; 110:63-71.
- 11.** Ellis SA, Palmer MS, McMichael AJ. Human trophoblast and the choriocarcinoma cell line BeWo express a truncated HLA class I molecule. *J Immunol* 1990; 144:731-735.
- 12.** Ellis SA, Sargent IL, Redman CW, McMichael AJ. Evidence for a novel HLA antigen found on human extravillous trophoblast and a choriocarcinoma cell line. *Immunology* 1986; 59:595-601.
- 13.** Ellis SA, Staines KA, Morrison WI. cDNA sequence of cattle MHC class I genes transcribed in serologically defined haplotypes A18 and A31. *Immunogenetics* 1996; 43:156-159.
- 14.** Fournel S, Aguerre-Girr M, Huc X, Lenfant F, Alam A, Toubert A, Bensussan A, Le Bouteiller P. Cutting edge: soluble HLA-G1 triggers CD95/CD95 ligand-mediated apoptosis in activated CD8⁺ cells by interacting with CD8. *J Immunol* 2000; 164:6100-6104.
- 15.** Golovanov AP, Hautbergue GM, Wilson SA, Lian Lu-Yun. A simple method for improving protein solubility and long-term stability. *J Am Chem Soc* 2004; 126(29):8933-8939.
- 16.** Hill JR, Schlafer DH, Fisher PJ, Davies CJ. Abnormal expression of trophoblast major histocompatibility complex class I antigens in cloned bovine pregnancies is

associated with a pronounced endometrial lymphocytic response. *Biol Reprod* 2002; 67:55-63.

17. Hunt JS. Stranger in a strange land. *Immunological Reviews* 2006; 213:36-47.

18. Hunt JS, Andrews GU, Wood GW. Normal trophoblasts resist induction of class I HLA. *J Immunol* 1987; 138:2481-2487.

19. Hunt JS, Petroff MG, McIntire RH, Ober C. HLAG and immune tolerance in pregnancy. *FASEB J* 2005; 19:681-693.

20. Ishitani A, Sageshima N, Lee N, Dorofeeva N, Hatake K, Marquardt H, Geraghty DE. Protein expression and peptide binding suggest unique and interacting functional roles for HLA-E, F, and G in maternal placental immune recognition. *J Immunol* 2003; 171:1376-1384.

21. Klein J. *Biology of the Mouse Histocompatibility-2 Complex: principles of immunogenetics applied to a single system*, Berlin and New York: Springer-Verlag; 1975.

22. Langat DK, Morales PJ, Fazleabas AT, Mwenda JM, Hunt JS. Baboon placentas express soluble and membrane-bound Paan-AG proteins encoded by alternatively spliced transcripts of the class Ib major histocompatibility complex gene, Paan-AG. *Immunogenetics* 2002; 54:164-173.

Immunogenetics 2002; 54:164-173.

23. Le Bouteiller P. HLA-G in the human placenta: expression and potential functions. *Biochem Soc Trans* 2000; 8:208-212.

24. Le Bouteiller P, Legrand-Abrevanel F, Solier C. Soluble HLA-G1 at the materno-foetal interface-a review. *Placenta* 2003; 24 Supplement A Trophoblast Res Vol 17, S10-S15.

- 25.** LeMaoult J, Krawice-Radanne I, Dausset J, Carosella ED. HLA-G1-expressing antigen-presenting cells induce immunosuppressive CD4⁺ T cells. *Proc Natl Acad Sci USA* 2004; 101(18):7064-7069.
- 26.** LeMaoult J, Zafaranloo K, Danff CL, Carosella E D. HLA-G up-regulates ILT2, ILT3, ILT4, and KIR2DL4 in antigen presenting cells, NK cells, and T cells. *FASEB J* 2005; 19:662-664.
- 27.** Lee N, Geraghty DE. Genetic diversity of HLA: functional and medical implications. Proceedings of the 12th International Histocompatibility Conference, Paris, France, June 9–12. *Hum Immunol Abstracts* 1996; 47:1-184.
- 28.** Lee N, Goodlett DR, Ishitani A, Marquardt H, Geraghty DE. HLA-E surface expression depends on binding of TAP-dependent peptides derived from certain HLA class I signal sequences. *J Immunol* 1998a; 160:4951-4960.
- 29.** Lila N, Rouass-Freiss N, Dausset J, Carpentier A, Carosella ED. Soluble HLA-G protein secreted by allo-specific CD4⁺ T cells suppresses the alloproliferative response: a CD4⁺ T cell regulatory mechanism. *Proc Natl Acad Sci USA* 2001; 98:2150-2155.
- 30.** Lindaman A, Dowden A, Zavazava N. Soluble HLA-G molecules induce apoptosis in natural killer cells. *Am J Reprod Immunol* 2006; 56:68-76.
- 31.** Naji A, Le Rond S, Durrbach A, Krawice-Radanne I, Creput C, Daouya M, Caumartin J, LeMaoult J, Carosella ED, Rouas-Freiss N. CD3⁺CD4^{low} and CD3⁺CD8^{low} are induced by HLA-G: novel human peripheral blood suppressor T-cell subsets involved in transplant acceptance. *Blood* 2007; 110(12):3936-3948.

- 32.** Park GM, Lee S, Park B, Kim E, Shin J, Cho K, Ahn K. Soluble HLA-G generated by proteolytic shedding inhibits NK-mediated cell lysis. *Biochem Biophys Res Commun* 2004; 313:606-611.
- 33.** Poehlmann TG, Schaumann A, Busch S, Fitzgerald JS, Aguerre-Girr M, Le Bouteiller P, Schleussner E, Markert UR. Inhibition of term decidua NK cell cytotoxicity by soluble HLAG1. *Am J Reprod Immunol* 2006; 56(5-6):275-285.
- 34.** Sala FG, Moral PD, Pizzato N, Legrand-Abravanel F, Le Bouteiller P, Lenfant F. The HLA-G*0105N null allele induces cell surface expression of HLA-E molecule and promotes CD94/NKG2A-mediated recognition in JAR choriocarcinoma cell line. *Immunogenetics* 2004; 56:617-624.
- 35.** Selmani Z, Naji A, Zidi I, Favier B, Gaiffe E, Obert L. Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce CD4⁺CD25^{high}FOXP3⁺ regulatory T cells. *Stem Cells* Jan; 2008; 26(1):212-222.
- 36.** Shiroishi M, Tsumoto M, Amano K, Shirakihara Y, Colonna M, Braud VM, Allan DS, Makadzange A, Rowland-Jones S, Willcox B, Jones EY, van der Merwe PA, Kumagai I, Maenaka I. Human inhibitory receptors Ig-like transcript 2 (ILT2) and ILT4 compete with CD8 for MHC class I binding and bind preferentially to HLA-G. *Proc Natl Acad Sci USA* 2003; 100:8856-8861.
- 37.** Solier C, Aguerre-Girr M, Lenfant F, Campan A, Berrebi A, Rebmann V, Grosse-Wilde H, Le Bouteiller P. Secretion of pro-apoptotic soluble HLA-G1 by human villous trophoblast. *Eur J Immunol* 2002; 32:3576-3586.

38. Wu X, Jin LP, Yuan MM, Zhu Y, Wang MY, Li DJ. Human first-trimester trophoblast cells recruit CD56brightCD16-NK cells into decidua by way of expressing and secreting of CXCL12/stromal cell-derived factor 1. *J Immunol* 2005; 175:61-68.

CHAPTER 3

SCALE-UP OF BoLA-NC3*00101 PROTEIN EXPRESSION IN MOUSE P815 CELLS USING A PITCHED-BLADE

Abstract

In an attempt to express and isolate BoLA-NC3*00101, a bovine non-classical MHC (Major Histocompatibility Complex) class I protein (class Ib), the mouse mastocytoma cell line P815 was transfected with the NC3*00101 transgene. The transfected cells were checked for expression by flow cytometry and positive stable transfectants were sorted using a fluorescence activated cell sorter (FACS). A large amount of purified protein is required to immunize and boost the host for several months to produce monoclonal antibodies. To avoid the use of a large number of cell culture flasks and shorten the time to obtain a sufficient quantity of protein, stably transfected, BoLA-NC3*00101 expressing cells were grown in a pitch-blade bioreactor. One week of culturing in a pitched-blade bioreactor yielded a large cell mass, which was used to isolate and purify the protein.

Introduction

Transplantation antigens were discovered as molecules responsible for acceptance or rejection of tissue grafts in mice [1]. The acceptance or rejection depends on the degree of similarity among these antigens on the cells of the donor and recipient animals. The genetic region encoding the most important proteins for tissue graft compatibility is called the major histocompatibility complex (MHC). The MHC encodes two types of highly polymorphic cell surface glycoproteins, the MHC class I (MHC-I) and MHC class

II (MHC-II) proteins, which present peptide antigens to T lymphocytes. MHC-I proteins have two subsets, classical (MHC-Ia) and non-classical (MHC-Ib). MHC-Ia proteins are expressed on all nucleated cells in the mammalian body. Consequently, any tissue graft will express class Ia proteins and thus the recipient will react to the graft. In addition, class Ia proteins are extremely polymorphic or highly variable in the population, therefore tissue graft donor-recipient pairs are rarely MHC-matched [2]. MHC-Ia proteins are usually expressed as membrane bound isoforms and interact directly with the T cell receptor. In contrast, class Ib proteins are expressed in specific tissues or organs and their expression may or may not be conditional. They are less polymorphic and have few variants; therefore they do not induce a transplant rejection. MHC-Ib proteins are expressed as membrane-bound and soluble isoforms.

A tissue graft from another member of the same species is known as an allogeneic transplant. A fetus is an allogeneic tissue that resides inside the maternal uterus during pregnancy. The MHC genetic region of cattle is known as the bovine leukocyte antigen (BoLA) complex. Bovine trophoblast cells (the cells that form the outer membrane of the placenta and attach the embryo to the uterine wall) express four non-classical MHC class I genes, BoLA-NC1, -NC2, -NC3 and -NC4 [3]. These class Ib proteins are believed to play an important role in protecting the fetal tissue graft from hostile maternal immune attack throughout pregnancy. Both class Ia and Ib proteins help protect cells against attack by natural killer (NK) cells. MHC class I deficient cells are usually susceptible to NK cell-attack and macrophage killing [4].

To study whether the bovine non-classical class I proteins are expressed as cell surface and/or soluble isoforms, P815 (mouse mastocytoma) cells were transfected with

the transgenes encoding these proteins. The mouse P815 cell line is a transfection competent cell line that has been used for expressing a variety of MHC class I proteins, including HLA-B27 [5]. Transfected P815 cell lines have been used as immunogens to immunize mice to produce monoclonal antibodies against HLA-B27 and other HLA-B antigens [5]. In other studies P815 cells have been transfected with equine [6, 7] and bovine MHC class I protein-encoding transgenes [3, 11].

Transfection studies revealed that BoLA-NC3*00101, was expressed on the cell surface whereas the other three bovine MHC-Ib proteins (BoLA-NC1*00401, NC2*00102 and NC4*00201) were not expressed as cell-surface isoforms as determined with flow-cytometric analysis of transfected P815 cells (Parasar *et al.* in preparation). The other three MHC-Ib proteins are probably expressed as secreted isoforms. Our objective was to produce monoclonal antibodies against bovine MHC-Ib proteins and utilize the antibodies to detect soluble MHC-Ib proteins in trophoblast culture supernatants and serum from pregnant cows. Since NC3*00101 protein is expressed on the cell surface as a membrane bound protein, it was possible to sort the positive and stable NC3*00101 transfectants using FACS. Scale-up in a bioreactor was used to achieve higher cell mass and isolate more protein in shorter time than in tissue culture flasks.

To date there is no report of any monoclonal antibody that can differentiate the bovine NC3*00101 protein from other bovine MHC-I proteins. Availability of an NC3*00101 specific monoclonal antibody will greatly assist in determining the level of NC3*00101 protein expressed on the surface of trophoblast cells at different stages of pregnancy and the amount of protein secreted in trophoblast cultures. In order to isolate a

sufficient amount of NC3*00101 protein to immunize mice for antibody production, a high number of P815 cells expressing bovine NC3*00101 protein was required. A bioreactor is an efficient, well-established, specialized, simulated biologically active environment. Growing cells in a bioreactor yields a far greater cell mass in a shorter time without affecting the integrity and quality of the cells or their products. Pitched-blade impellers have flat blades that are set at a 45° angle so that they provide simultaneous radial and axial flow. Combined radial and axial flow produces better mixing and promotes a high oxygen transfer rate. Animal and plant cells often have low resistance to shear [9]. Pitched-blade impellers are low shear blades that cause less cell damage and provide gentle and smooth mixing of the cells in culture. This type of impeller is widely used with mammalian, insect and other shear sensitive cell-lines growing in suspension [10]. Because of these characteristics, we decided to grow transfected P815 cells expressing NC3*00101 in a pitched-blade bioreactor.

Materials and Methods

Cell Line

The P815 cell line is a mast cell line derived from a DBA/2 strain-mouse (*Mus musculus*) with a mastocytoma. The majority of these cells grow in suspension with some (<5%) adherent cells. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% bovine calf serum at 37°C and 5% CO₂. The cell line was obtained from American Type Culture Collection (ATCC, USA).

Transfection and FACS Sorting of Stable NC3*00101 Transfectants

The BoLA-NC3*00101 coding sequence was amplified from complementary DNA (cDNA) using specific primers (Forward primer CACCATGGGGCCGCGAACCCCTC and reverse primer GATGAAGCATCACTCAGTCCCC). The pcDNA3.1 directional TOPO expression vector (Invitrogen) was used for cloning the cDNA and expressing the protein in mammalian P815 cells. Positive clones were sequenced and full-length NC3*00101 clone was selected for transfection. Plasmid was purified using a QIA plasmid Mini Prep kit (Qiagen). Lipofectamine 2000 (Invitrogen) was used to transfect P815 cells with the pcDNA3.1 plasmid. This vector allows for expression of a specific protein with a V5 epitope and a C-terminal polyhistidine (His-His-His-His-His-His) fusion tag. The V5 epitope permits easy detection of recombinant protein by Western blot with anti-V5 antibody and the polyhistidine tag allows rapid purification on nickel-chelating resin, which binds His-tagged proteins.

To transfect, the cells, 2×10^6 cells (1×10^6 cells/ml of DMEM) were plated per well in a 6-well tissue culture plate and incubated at 37°C with 5% CO₂. Four µg of plasmid DNA was diluted to 250 µl in DMEM and mixed gently. Lipofectamine was mixed gently before use, and then 10 µl was diluted in 250 µl of DMEM and incubated for 5 min at room temperature. The diluted plasmid DNA was combined with diluted Lipofectamine (total volume = 500 µl), mixed gently and incubated for 20 min at room temperature. An aliquot of 500 µl of plasmid and Lipofectamine was added to each well containing cells and medium and mixed gently. Cells were incubated at 37°C, 5% CO₂ for 3 hours, then 5 ml of DMEM with 10% bovine calf serum was added to each well.

Transfected cells were incubated for 24 to 48 hours prior to addition of G418 antibiotic (500 μ g/ml; Invivogen) to select for stable transfectants. After two weeks in selective media the transfectants were screened using flow cytometry.

The flow cytometry staining procedure was as follows. One million cells were resuspended in fluorescence buffer (FB) [Phosphate Buffered Saline (PBS) with 0.1% sodium azide, 1% bovine serum albumin] and incubated with primary antibody for 15 min. The H1A monoclonal antibody was used for labeling the NC3*00101 protein and an irrelevant antibody, ColiS169A, which does not recognize the mouse P815 cells or the bovine class I protein, was used as a negative control. Both monoclonal antibodies were obtained from the Monoclonal Antibody Center at Washington State University, Pullman, WA. Cells were washed twice with FB and then incubated with a fluorescein labeled anti-mouse IgG (Heavy+Light) secondary antibody for 15 min. All incubations were performed at 4°C. Cells were washed twice and fixed in PBS with 1% formaldehyde. Cells were analyzed using a BD Biosciences FACSAria II flow cytometer equipped with Diva software for data acquisition and analysis.

Prior to culturing the cells in the bioreactor, Fluorescence Activated Cell Sorting (FACS-sorting) was performed with the FACSAria II to isolate a subpopulation of cells expressing a high level of NC3*00101 protein. The cells were stained with the H1A monoclonal antibody as described above but were not fixed in fluorescence fixative. The sorted high-expressing cells were grown in T75 flasks until they reached a sufficient number to seed the bioreactor.

Culturing of Sorted NC3*00101-Transfectants in the Pitch-Blade Bioreactor

Sorted NC3*00101 cells were grown in T-75 flasks at a seeding concentration of 1×10^6 cells/ml until a sufficient number of cells were obtained. Cells were harvested from eight T-75 flasks by centrifugation at 1000 RPM (500 g) for 10 min at room temperature. Cells were stained with Trypan Blue (0.4% solution) and enumerated with a Countess Automated Cell Counter (Invitrogen). The cells used to seed the bioreactor had a viability of $\geq 95\%$.

A New Brunswick Scientific Celligen@310 stirred tank bioreactor vessel with 5 liter capacity (3.5 L working volume) was used for culturing the cells. Because of the shear sensitivity of the mouse P815 cells, a pitched-blade impeller was selected for this experiment (Figure 3-1). The bioreactor vessel was installed on the control station. The vessel was filled with PBS before autoclaving and the pH probe was calibrated before vessel sterilization. The sterile assembly was taken to a laminar flow hood and the PBS was replaced with 3.5 liters of sterile prewarmed (37°C) DMEM supplemented with 10% bovine calf serum, 2 mM L-glutamine and 2 mM penicillin-streptomycin using a peristaltic pump. The reactor was seeded with 2×10^5 cells/ml and the dissolved oxygen (DO) probe was calibrated. Cell viability was monitored by counting sample of cells daily using a Countess Automated Cell Counter (Invitrogen). The BioFlo 310 cascades were used to control DO and pH levels. After three days of cell growth the culture was collected into sterile bottles and cells were harvested by centrifugation at 2000 g for 10 min at 4°C . The process parameters and cascade loops are shown below.

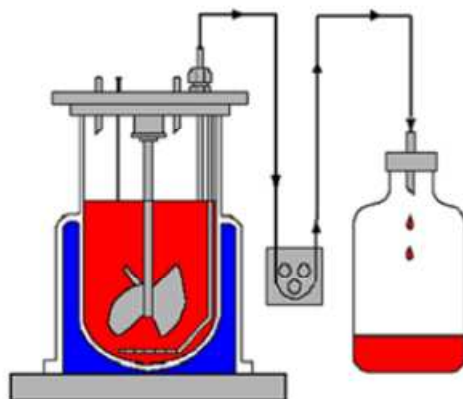


Fig 3-1 Pitched-Blade Bioreactor

Process Parameters

Air	4-Gas mixture (Air, O ₂ , CO ₂ , N ₂)
Temperature`	37°C
Gas Flo	0.5 slpm
Agitation	35 rpm
pH	7.2
Dissolved Oxygen (DO)	50%

Cascade Loops

From	To	Start Set Point	DO Start Output %	End Set Point	DO End Output%
DO	DO	0.0	50	100%	100
pH	pH	0	25	100%	100

*Purification of NC3*00101 Protein*

Cells were kept on ice during lysis. One ml of 1X lysis buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl with 1 mM mammalian protease inhibitor cocktail (Sigma), 1 mM PMSF) was added per 1×10^7 cells. Cells were incubated at 4°C for 1 hour with rotation. The suspension was then centrifuged at 10,000 g for 15 min at 4°C to remove insoluble

material and nuclei. The supernatant containing protein was collected in fresh tubes and stored at -20°C until processed for protein purification.

His-GraviTrap Columns (GE Healthcare) were used to purify the histidine tagged protein. After loading lysate on the column, it was washed 4 times with wash buffer with 40 mM imidazole. Elution was performed with elution buffer containing 500 mM imidazole. Purified eluates were stored at -20°C. Eluates with specific bands on Western blots were pooled and dialyzed against phosphate buffer solution (20 mM sodium phosphate, 500 mM NaCl, pH 7.4) using 20 kilodalton (kD) MWCO Slide-A-Dialysis cassettes (Pierce). To prevent precipitation and to maximize the stability of proteins 50 mM charged amino acids, L-arginine and L-glutamate were added to all the purification buffers and dialysis buffers [8]. 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 Vivaspın-20 concentrators with 30 KD MWCO membranes (Vivaproducts). The concentration of each fraction was measured using a BCA protein assay kit (Thermo Scientific).

Western Blotting

Western blots were performed with a highly sensitive Western Breeze Chemilluminescence Kit (Invitrogen). Ten µl of NC3*00101 protein was mixed with 5 µl LDS sample buffer (Invitrogen) and 5 µl deionized water and heated at 70°C for 10 min. Fifteen µl of denatured protein was loaded in each lane of 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 volts. Membranes were blocked with blocking buffer provided with the Western Breeze Kit and stained with AP conjugated anti-V5 antibody (Invitrogen) as per the instructions recommended by the manufacturer. Blots were evaluated by exposure of Blue X-ray film (*ISC Bioexpress*) for different exposure times.

Results

Flow Cytometric Analysis of Transfected Cells and Sorting

Less than 20% of newly transfected P815 cells expressed BoLA-NC3*00101 protein that could be detected by immunostaining and flow cytometry. Growth in G418 antibiotic was used to select stable transfectants. Following two weeks of selection approximately 50% of the cells expressed immunoreactive NC3*00101 protein. Following FACS sorting with an anti-bovine MHC class I monoclonal antibody (H1A), 98.4% of the cells expressed the bovine MHC class I protein. NC3*00101-transfected cells after sorting with H1A antibody had an expression of 98.4%. The pre-sorting and post-sorting data are presented in Figure 3-2.

Growth of Cells in Pitched-Blade Bioreactor

NC3*00101-transfected cells grew with a doubling time of 24 hours. The reported doubling time for this cell line is 18-22 hours. pH was maintained by utilizing CO₂ from the pH cascade. The total number of cells harvested was 3×10^9 cells after 3 days of cell growth. This was an adequate number for isolation of enough purified NC3*00101 protein for immunization of 10 mice and subsequent screening of the hybridoma-supernatants by enzyme-linked immunosorbent assay (ELISA).

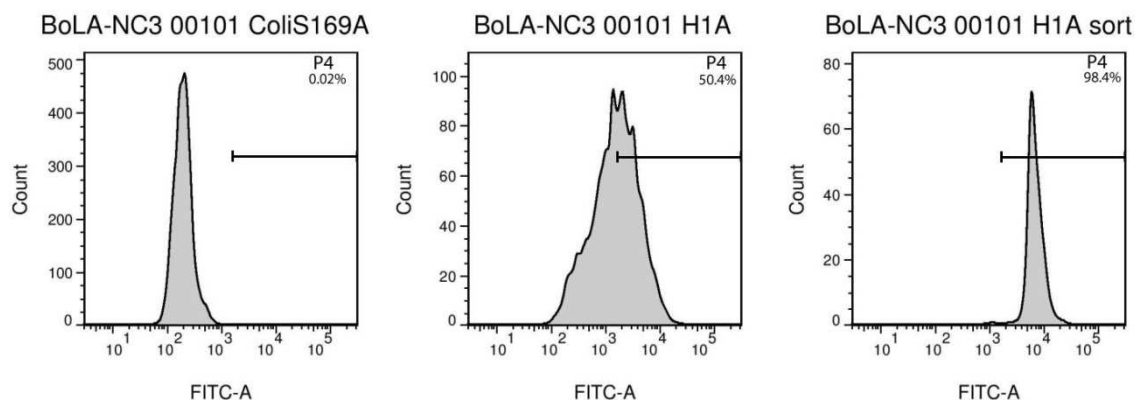


Fig 3-2: Flow cytometric analysis of murine P815 cells transfected with an expression vector encoding the bovine NC3*00101 protein. Positive, stably transfected cells were sorted by FACS on the basis of H1A monoclonal antibody staining.

Isolation of BoLA-NC3*00101 Protein

The concentrations of BoLA-NC3*00101-crude cell lysate and purified concentrated protein sample were 1.9 mg/ml and 0.5 mg/ml, respectively. This was adequate for the downstream immunization and screening of the hybridoma supernatants using ELISA.

Western Blot

A Western blot was performed to detect the specific protein in the crude cell lysate and the purified protein sample. The blot was probed with an antibody that recognizes the V5 epitope at the C-terminal end of the protein. Untransfected cell lysate was used as a negative control. Beta-galactosidase protein (120 kD) isolated from cells transfected with the beta-galactosidase control plasmid was used as a positive control.

The bovine NC3*00101 class I protein was detected as a 45 kDa band in the crude lysate and the purified concentrated sample (Figure 3-3).

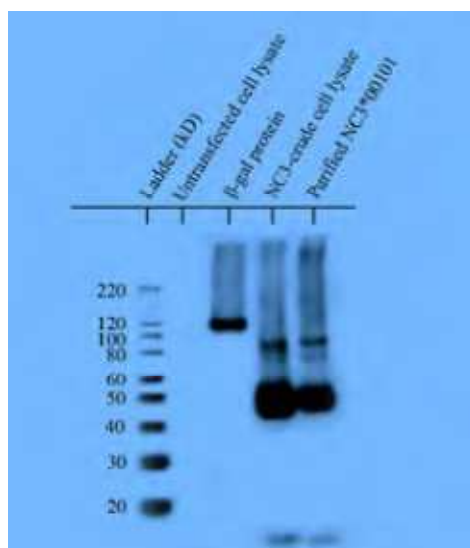


Fig 3-3 BoLA-NC3*00101 protein (45kD) specifically recognized by anti-V5 antibody

Discussion

The pitched-blade bioreactor was useful in the scale-up of P815 cell growth and protein production. The product quality did not change as measured by SDS-PAGE and subsequent detection with Western blotting. It is evident from the trends in DO and pH values that P815 cells, like CHO cells [12], utilize large amounts of oxygen for respiration in their early exponential growth phase and thus, acidify the media. Later during the proliferation phase the pH establishes equilibrium, followed by an elevation in pH indicating a stationary and death phase. A high pH value at 72 hours indicates high alkalinity, which possibly is marker of lack of nutrients and cell death.

Stirred tank bioreactors have been used for culturing hybridoma cells in suspension for monoclonal antibody production [13]. Mouse P815 cells grow well in

vessels with pitched-blade impellers at moderate agitation rates. Shear sensitivity of mammalian cells has been an important issue for scaling up production but advances in technology have helped overcome this limitation.

This study resulted in successful scale-up in production of murine P815 cells expressing BoLA-NC3*00101 protein using sophisticated, automated bioreactor process control machinery in batch-culture. This study demonstrated the feasibility of growing FACS sorted murine P815 cells in a large vessel without loss of protein quality or cell viability. Bioreactors are an efficient means by which difficult cells can be cultured under controlled conditions to maintaining cell health and increase productivity.

The non-classical MHC class I proteins are important immunoregulatory molecules at the maternal-fetal interface. With the generation of antibodies against these glycoproteins it will be possible to quantify the level of secretion of these proteins at different stages of pregnancy. With the protein produced in the bioreactor we were able to produce hybridomas that secrete monoclonal antibodies to the NC3*00101 protein. Therefore, our findings show that pitched-blade bioreactor is a useful tool to scale-up the expression of BoLA-NC3*00101 protein by culturing the transfected cells on a large scale.

References

1. Bainbridge DRJ. Evolution of mammalian pregnancy in the presence of the maternal immune system. *Rev Reprod* 2000; 5(2):67-74.
2. Bertschmann M, Käsermann D, Keller R. MHC class-I antigen deficiency, malignancy and susceptibility of P815 mastocytoma to NK and macrophage killing. *Int J Cancer* 1990; 46(4):739-744.

- 3.** Chishti Y. Animal cell cultures in stirred bioreactors: observations on scale-up. *Process Biochem* 1993; 28:511-517.
- 4.** Davies CJ, Eldridge JA, Fisher PJ, Schlafer DH. Evidence for expression of both classical and non-classical major histocompatibility complex class I genes in bovine trophoblast cells. *Am J Reprod Immunol* 2006; 55:188-200.
- 5.** Golovanov AP, Hautbergue GM, Wilson SA, Lian LY. A simple method for improving protein solubility and long-term stability. *J Am Chem Soc* 2004; 126(29):8933-8939.
- 6.** Guzman E, Taylor G, Charleston B, Ellis SA. Induction of a cross-reactive CD8-T cell response following foot-and-mouth disease virus vaccination. *J Virol* 2010; 84(23):12375-12384.
- 7.** Klein J. *Biology of the Mouse Histocompatibility-2 Complex*. Springer-Verlag, Berlin, 1975.
- 8.** Kydd JH, Davis-Poynter NJ, Birch J, Hannant D, Minke J, Audonnet JC, Antczak DF, Ellis SA. A molecular approach to the identification of cytotoxic T-lymphocyte epitopes within equine herpes virus 1. *J Gen Virol* 2006; 87:2507-2515.
- 9.** Mirro R, Voll K. Which impeller is right for your cell line? *BioProcess International* 2009; 7(1):52-57.
- 10.** Naciri M, Kuystermans D, Al-Rubeai M. Monitoring pH and dissolved oxygen in mammalian cell culture using optical sensors. *Cytotechnology* 2008; 57:245-250
- 11.** Rappocciolo G, Birch J, Ellis SA. Down-regulation of MHC class I expression by equine herpesvirus-1. *J Gen Virol* 2003; 84:293-300.

12. Reisman HB. Problems in scale-up of biotechnology production processes. *Crit Rev Biotechnol* 1993; 13(3):195-253.

13. Thureau SR, Wildner G, Kuon W, Weiss EH, Riethmüller G. Expression and immunogenicity of HLA-B27 in high transfection recipient P815: a new method to induce monoclonal antibodies directed against HLA-B27. *Tissue antigens* 1989; 33:511-519.

CHAPTER 4
PRODUCTION OF MONOCLONAL ANTIBODIES FOR BoLA-NC3*00101 AND
NC1*00501 PROTEINS

Abstract

We previously identified that cattle classical (MHC-Ia) proteins, N*01701 and N*01802, and three non-classical (MHC-Ib) proteins, NC1*00501, NC3*00101, and NC4*00201, are expressed on the cell surface. Other cattle non-classical proteins, NC1*00401 and NC2*00102, are not expressed on the cell surface and may be secreted or soluble in nature. To detect secreted or soluble class Ib isoforms produced by bovine embryos and trophoblast cells and to determine the level of expression of class Ib immunoregulatory molecules during pregnancy, we immunized mice and developed monoclonal antibodies for two class Ib proteins, BoLA-NC1*00501 and NC3*00101. Large numbers of P815 cells expressing NC1*00501 and NC3*00101 proteins were grown in multiple T75 flasks and/or a pitched blade bioreactor. The polyhistidine or 6X-histidine-tagged proteins were isolated using His-Gravi trap nickel affinity column chromatography and were used as immunogens in mice. Monoclonal antibodies were produced at Washington State University (WSU) Monoclonal Antibody Center. Screening of sera from immunized mice and culture supernatants from hybridomas was done by flow cytometry and enzyme linked immunosorbent assay (ELISA). Our objectives are to use the monoclonal antibodies to develop an ELISA that can be used to detect soluble MHC-Ib proteins from embryos and trophoblast cells and to use the monoclonal antibodies in immunohistochemistry to examine the regulation of MHC-Ib expression at the maternal-fetal interface.

Introduction

An antibody recognizes a specific linear or conformational “epitope” on the antigen. While conformational epitopes are dependent on the tertiary structure of the protein, linear epitope remains intact when the protein is denatured. Polyclonal antibodies or antisera collected from an animal immunized with a specific antigen contain antibodies with different specificities and epitope affinities. Monoclonal antibodies are produced by progeny from a single ancestral B cell from an immunized animal and recognize a single epitope. B cells from the spleen or a lymph node of an immunized animal are harvested and cultured *in vitro*. However, antibody producing B cells have limited life span. B cells are, therefore, immortalized by fusing them with myeloma cells. Kohler and Milstein [9] were the first investigators to synthesize “hybridomas” by fusion of mouse myeloma and mouse spleen cells from an immunized donor mouse. Monoclonal antibodies are purified from hybridoma culture supernatants or ascitic fluid. Once a hybridoma cell line is established via single cell cloning, it can be frozen and stored in liquid nitrogen for an indefinite period. Monoclonal antibody (mAb or MoAb) production technology not only enables researchers to isolate a specific antibody from an immunized animal but also allows scientists to produce specific antibodies in large quantity *in vitro*.

By virtue of their single epitope specificity, monoclonal antibodies decrease background noise and cross-reactivity with non-specific antigens and give reproducible results. Use of a mAb allows for efficient affinity purification of the target antigens. Monoclonal antibodies are used in various diagnostic tests to detect minute quantity of antigens such as hormones, enzymes, drugs, toxins etc. For instance, in human medicine detection of human chorionic gonadotropin (hCG) in urine or serum is used for

pregnancy diagnosis and an ELISA test is used to diagnose AIDS. Rodents are routinely used to produce monoclonal antibodies. Other animals are not commonly used because of difficulties in establishing immortalized cell lines by hybridoma formation, viral transformation or reprogramming [10, 11, 15].

Our objective is to use the mAb against bovine MHC class Ib proteins to develop ELISA that can be used to detect secreted or soluble BoLA-Ib proteins in: serum from pregnant cows, culture supernatants from *in vitro* derived embryos, and culture supernatants from cultured trophoblast cells from different terms of pregnancy. Surface expressed bovine MHC class Ib NC1*00501 and NC3*00101 purified proteins were used to immunize mice. Sera and hybridoma secreting mAbs were tested by ELISA and flow cytometry to check their specificity.

Materials and Methods

Cell Lines and Transfection

The murine mastocytoma P815 cell line was obtained from American Type Culture Collection (ATCC, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% bovine calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin (Hyclone) at 37°C and 5% CO₂. Cells were transfected with bovine MHC class I proteins. Coding sequences for BoLA-NC1*00501 and NC3*00101 were amplified from complementary DNA (cDNA) using the specific primers shown below:

Forward Primer CACCATGGGGCCGCGAACCCTC

Reverse Primer for NC1*00501 GATGAAGCATCACTCAGTCCCC

Reverse Primer for NC3*00101 GGCAGTGTCACTGCTTGCAGTCTG

The pcDNA3.1 directional TOPO expression vector (Invitrogen), which expresses the protein with a C-terminal polyhistidine tag and a V5 epitope, was used for cloning the cDNA. Subclones were sequenced and NC1*00501 and NC3*00101 subclones were selected to transfect P815 cells. Cells were transfected using our previously described method [13]. Transfected cells were fluorescently labeled using specific mAb and analyzed by flow cytometry to check initial expression. Transfectants were selected with G418 antibiotic (InvivoGen) and stably transfected cells were sorted using a BD FACSAria II fluorescence activated cell sorter (FACS). Sorted, high expressing NC1*00501 and NC3*00101 cells were cultured in multiple T150 flasks for antigen production. For large-scale antigen production, cells were sometimes grown in a pitched-blade bioreactor. Sorted NC1*00501 cells were cultured in multiple T150 flasks.

Purification of NC1*00501 and NC3*00101 Proteins

Cells were lysed in lysis buffer (50 mM Tris-HCl, 150 mM NaCl with 1 mM mammalian protease inhibitor cocktail (Sigma), 1 mM PMSF; pH 7.4) with 1×10^7 cells/ml. After an hour-incubation at 4°C, the supernatant was collected by centrifugation at 10,000 g for 15 min at 4°C.

Specific histidine-tagged NC1*00501 and NC3*00101 proteins were purified using His-GraviTrap Columns (GE Healthcare) as previously described [13]. Eluates which had specific protein bands on Western blots were pooled and dialyzed against phosphate buffer solution (20 mM Sodium Phosphate, 500 mM NaCl, pH 7.4) using 20 kilodalton (kD) molecular weight cut-off (MWCO) Slide-A-Dialysis cassettes (Pierce). To prevent precipitation and to maximize the stability of proteins, 50 mM charged amino acids, L-arginine and L-glutamate, were added to all the purification buffers and dialysis

buffers [8]. Dialysates were centrifuged at 12100 RPM for 10 min at 4°C and the supernatant was transferred to a sterile tube. Dialysates were concentrated using Vivaspin-20 concentrators with 30 KD MWCO membranes (Vivaproducts). The concentration of each fraction was measured using a BCA protein assay kit (Thermo Scientific).

Immunization

Antibodies to BoLA-NC1*00501 and NC3*00101 proteins were produced at the monoclonal antibody center at WSU, Pullman using the protocol described by Hamilton and Davis [6]. Three mice per antigen were hyperimmunized with purified antigen in Ribi's Adjuvant (Sigma-Aldrich). Each mouse had four immunizations each with 50 ug antigen/mouse. Because titer was low with initial immunizations with purified antigens, subsequent immunizations were done with P815 cells transfected with NC1*00501 and NC3*00101 cells and mice were boosted with the purified antigens. A final booster dose of antigen was given intravenously through the tail vein three days before the fusion of spleen and myeloma cells.

Fusion

For each antigen spleen cells were collected and pooled from three mice. Spleen cells ($\sim 1 \times 10^8$) were fused with 4×10^7 X63 Ag8.653 myeloma cells [8]. Fused cells were resuspended in growth medium containing hypoxanthine aminopterin and thymidine (HAT) medium and cultured in ten 96-well culture plates. We received three live cultures (A, B, and C) of each of the NC1-19, NC1-21, and NC1-40 hybridomas

which were grown and supernatants were collected and screened by flow cytometry and ELISA.

Flow Cytometry

Flow cytometry was used to test hybridoma supernatants for the presence of specific antibodies by indirect ELISA and flow cytometry [1]. For screening by flow cytometry, NC1*00501 and NC3*00101-transfected cells were stained using culture supernatants and a fluorescein conjugated anti-mouse IgG (H+L) secondary reagent. Staining was evaluated using a FACSAria II flow cytometer. Untransfected P815 cells were used as a negative control. Positive hybridomas were expanded in 12-well culture plates. Two ampoules of each positive hybridoma line were cryopreserved. Positive cultures were cloned, and the clones were expanded and cryopreserved. Supernatants from the clones were collected at the time of cryopreservation and characterized by ELISA, flow cytometry, and Western blotting.

ELISA

ELISA plates (CoStar Inc.) were coated with antigen at a concentration of 16 µg/ml in ELISA binding buffer (0.05M Tris, pH 9.5) and incubated overnight at 4°C. Plates were washed with 1 X ELISA wash buffer (10X Wash Buffer: PBS with 1.4 M Sodium Chloride, 0.5% Tween 20, 1% of 20% Sodium Azide) and blocked for 2 hours at 37°C using blocking buffer (Binding buffer with 0.3% Bovine serum albumin, BSA). Plates were washed four times prior to addition of antibodies. Monoclonal antibodies H1A, W6/32, H6A, and H11A were used at 1 µg/ml with 100 µl used per well. The NC1*00501 and NC3*00101 hybridoma supernatants were tested at 1:1 and 1:100

dilutions of NC1*00501- and NC3*00101-hybridoma culture supernatants in dilution buffer I (PBS with 0.3% BSA and 0.05% Tween-20). The secondary antibody goat anti-mouse-biotin was used at 1:10000 dilution in dilution buffer I with 100 μ l used per well. Plates were incubated at 37°C for 1 hour. After washing the plate four times, the streptavidin-AP diluted 1:1000 in dilution buffer II (PBS with 0.3% BSA) was added and incubated for 1 hour at 37°C. Finally, freshly made pNPP solution in substrate buffer (50 mM Potassium carbonate; 2 mM Magnesium Chloride) was added to the wells and incubated at 37°C. The optical density (OD) at 405 nm was measured at 15 min intervals from 15 min up to an hour. Data were plotted with time on the X axis and OD on the Y axis.

For Sandwich ELISA, plates were coated with antibodies or hybridoma culture-supernatants overnight at 4°C. After blocking the plate, purified cattle class I antigens were added. A biotinylated W6/32 antibody was used as detection antibody. In other studies capture (sandwich) ELISA are routinely used to detect the soluble class I proteins [3, 4, 5, 7, 14].

Production of Exhausted Hybridoma Culture Supernatants

Exhausted culture supernatants for the NC3*00101 antigen were prepared at Washington State University while exhausted culture supernatants for the NC1*00501 antigen were prepared at Utah State University. Cells were thawed, enumerated and cultured in a 6-well plate at a seeding concentration of 1×10^6 cells/ml in RPMI with 1% Zap Hybridoma supplement (InVitria). After 1 week of culture, cells were transferred to a T25 flask. When the cells were ~90% confluent, they were transferred to T75 flasks. Multiple T75 flasks were inoculated with 5 million cells in 50 ml of culture medium and

cultured at 37°C at 5% CO₂ for a week by which time the media was exhausted and cells had died. The exhausted supernatants containing antibodies were collected by centrifugation at 1500 RPM for 10 min.

Antibody Isotyping

The NC3*00101- and NC1*00501-hybridoma culture supernatants were screened using an ELISA murine antibody isotyping kit (Pierce) to determine the isotypes of the antibodies. H1A, W6/32 and IL-A88 were used as positive controls.

Results

Establishment of High-Expressing Transfected Cell Lines

Less than 20% of newly transfected P815 cells expressed BoLA-NC1*00501 or NC3*00101 proteins that could be detected by flow cytometry. Growth in G418 antibiotic was used to select stable transfectants. Following two weeks of selection approximately 25-50% of the cells expressed immunoreactive NC1*00501 or NC3*00101 proteins. Following FACS sorting with an anti-bovine MHC class I monoclonal antibody (H1A), 92.3% of NC1*00501 and 98.4% of NC3*00101 cells expressed the bovine MHC class I protein. Pre-sort and post-sort data are presented in Figure 4-1.

ELISA Screening

Antibodies against NC1*00501

First Fusion: Supernatants NC1-19, NC1-21, and NC1-40 had positive reactivity in the initial screening ELISA (data not shown). The NC1-19 supernatant had stronger reactivity than other two supernatants. Exhausted supernatants were produced and used to

test the monoclonal antibodies against all of our cloned MHC class I proteins. These supernatants reacted with all of the cattle MHC class I proteins but had higher affinity for NC1*00501 protein (Figure 4-2). NC1-19, which appeared to be strong in ELISA, did not appear to be a true clone in isotyping assay (Figure 4-2).

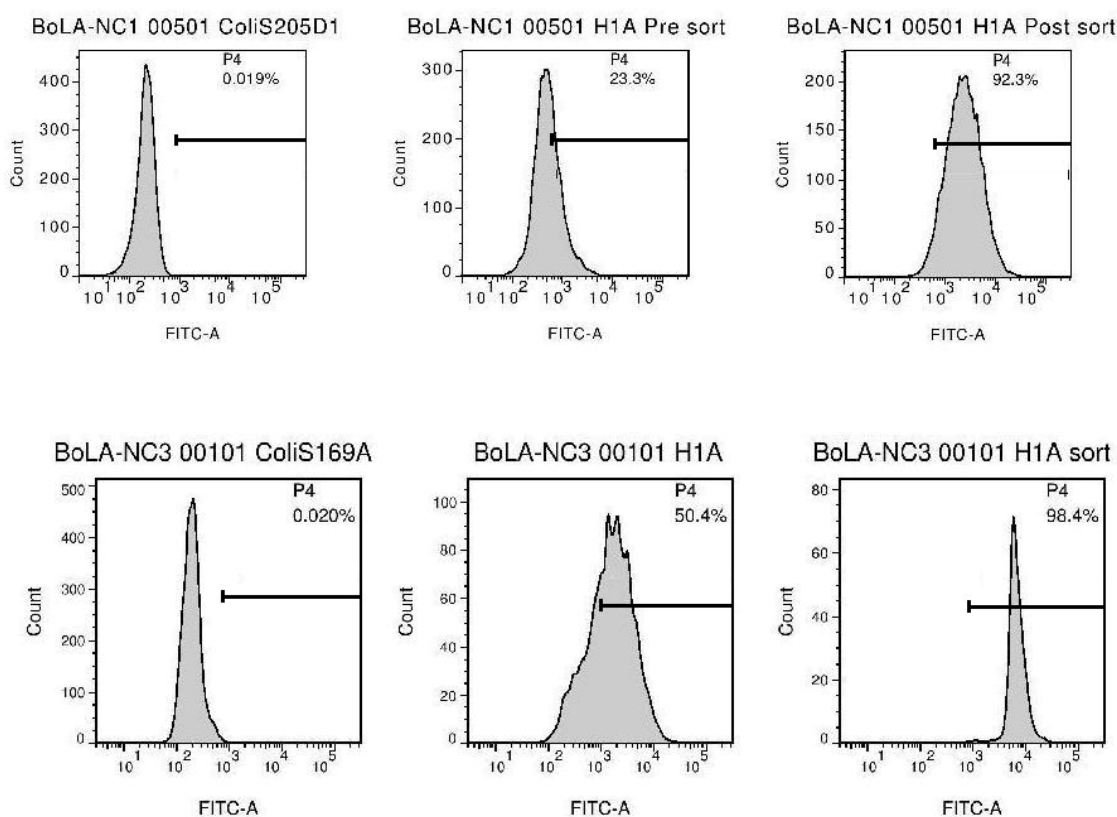


Fig 4-1: Presort and post sort analysis of NC1*00501 and NC3*00101 transfectants

NC3*00101

First Fusion: NC3*00101-specific antibody secreting hybridomas were screened by ELISA and flow cytometry at WSU. Twelve positive NC3 hybridoma colonies were found positive from the first fusion. NC3-4 and NC3-9 were slightly positive in ELISA.

Second Fusion: From the second fusion of spleen cells with myeloma cells, one positive hybridoma (NC3 supernatant 2) clone was selected and the supernatant was sent to us for secondary screening. NC3-02 clone had a specific and positive reactivity with NC3*00101 protein as determined by ELISA (Figure 4-3).

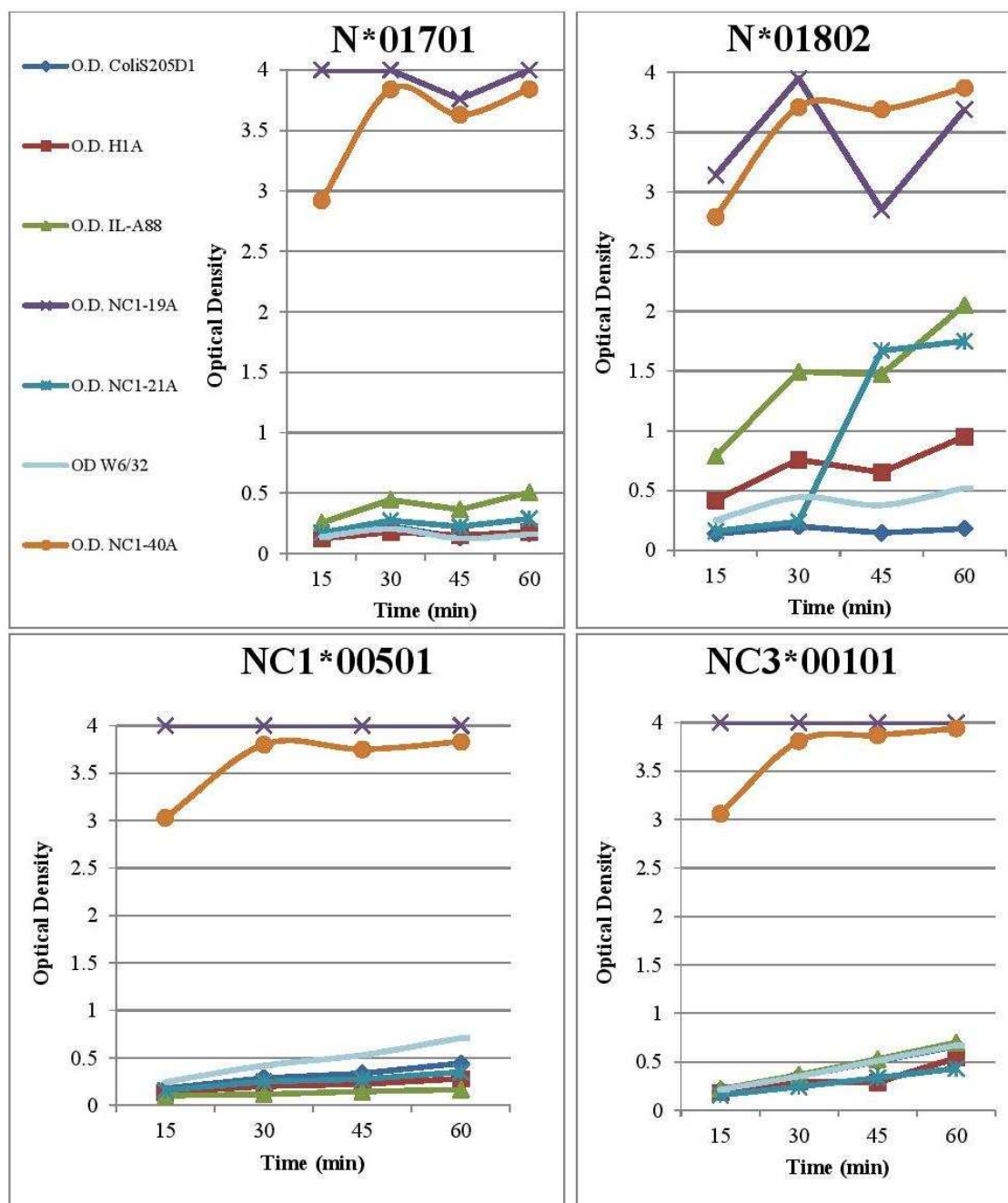


Fig 4-2: ELISA screening of anti-NC1*00501 exhausted supernatants

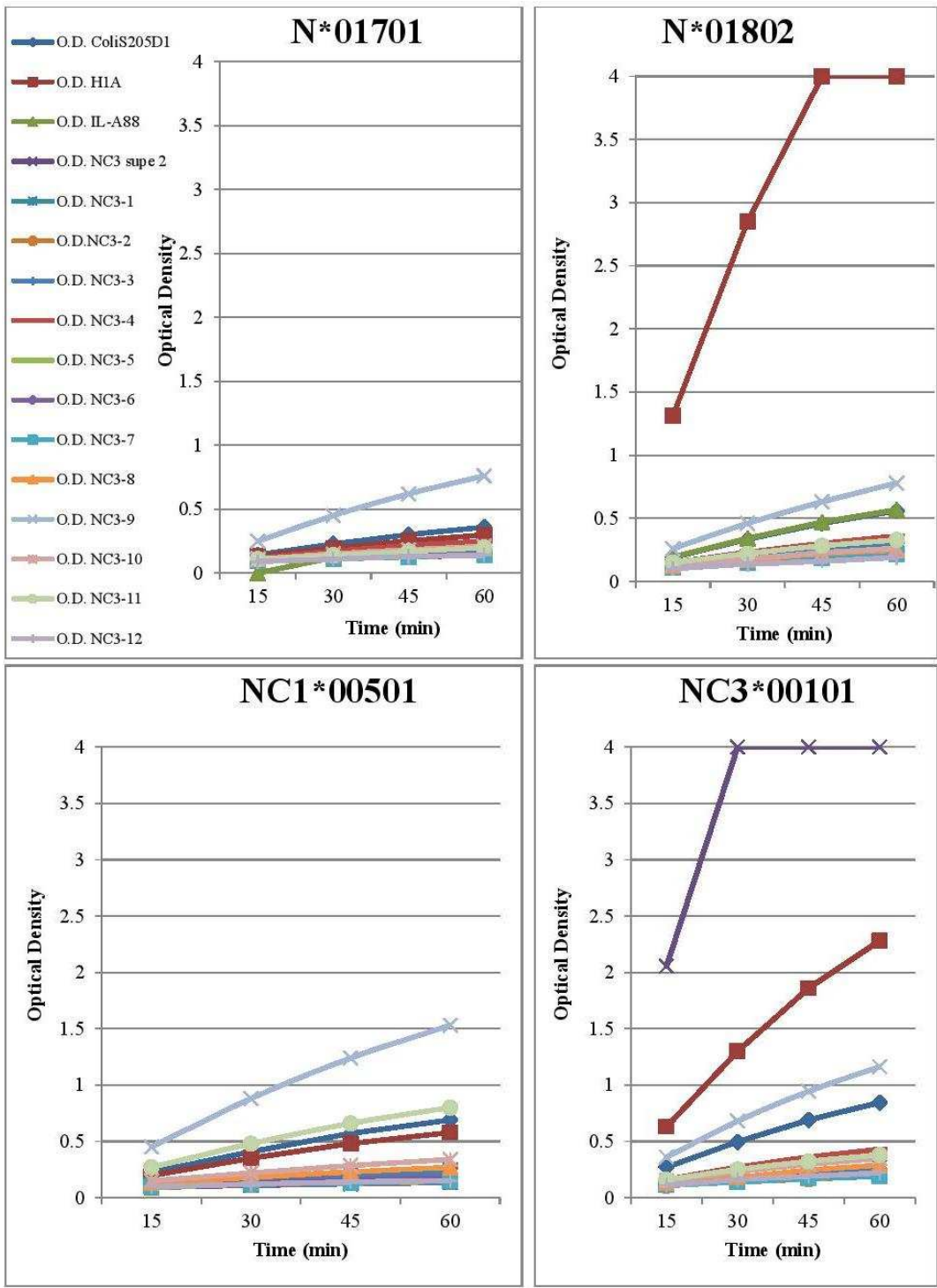


Fig 4-3: ELISA screening of NC3*00101 supernatants. Note that NC3 supe 2 has a specific reaction with NC3*00101 protein as shown in the last line graph with the crossed purple line of NC3 supe 2.

Third Fusion: From the third fusion, 7A and 7B supernatants were positive in initial screening but they were non-specific and reacted with all other class I proteins (Table 4-1).

Table 4-1: The summary of ELISA results of NC1 and NC3 supernatants

mAb	N*01701	N*01802	NC1*00501	NC3*00101
H1A	-	++++	++	+++
W6/32	-	+++	++++	-
IL-A88 neat	++++	++++	-	++++
IL-A88 1:10	++++	++++	-	++++
NC1-19A 1:10	+++	++	++++	+++
NC1-19A 1:100	-	-	-	-
NC1-21A 1:10	-	-	-	-
NC1-21A 1:100	-	-	-	-
NC1-40A 1:10	++	+	+++	+
NC1-40A 1:100	+	-	++	+/-
NC3-supe 2	-	-	-	++++
NC3-1	-	-	-	-
NC3-2	-	-	-	-
NC3-3	-	-	-	-
NC3-4	+	+	+	+
NC3-5	-	-	-	-
NC3-6	-	-	-	-
NC3-7	-	-	-	-
NC3-8	-	-	-	-
NC3-9	++++	++	++	++++
NC3-10	-	-	-	-
NC3-11	-	-	-	-
NC3-12	-	-	-	-

Flow Cytometry

*NC1*00501*

In the initial screening of NC1 serum from immunized mice, we saw a strong NC1-specific response. However, it also produced a weak response to untransfected P815 cells. In the first fusion one NC1 hybridoma was positive; however, there was no outgrowth after transfer to the 12-well plate. The second fusion yielded positive hybridomas whose expanded supernatants were tested with flow cytometry.

First Fusion: One supernatant NC1-19 had a detectable activity but it reacted with other cell lines including untransfected P815 cells. As shown in Table 4-2, 11 of the supernatants (#24, #25, #27, #28, #30, #33, #34, #36, #38, #40 and #41) appeared to have a low titer against NC1*00501 protein. They all seemed to be the mixed population. We rescreened all supernatants with 1:1 and 1:10 dilutions of supernatants. The titer of antibody in all anti-NC1 supernatants was likely low. NC1-19A and 40A reacted more strongly with untransfected P815 cells than with NC1*00501-transfected cells (Figure 4-4).

*NC3*00101*

First Fusion: None of the twelve sera was positive.

Second Fusion: Supernatants of NC3-1 and NC-9 hybridomas reacted with all cattle MHC-I proteins with a weak reactivity to untransfected P815 cells which suggests that these supernatants may have reacted with unknown cell-cycle antigen on mouse P815 cells as mice were immunized with transfected P815 cells. We did not see any

specific reactivity in the NC3-9 supernatant. Consequently, we did not clone NC3-1 and NC3-9 hybridomas.

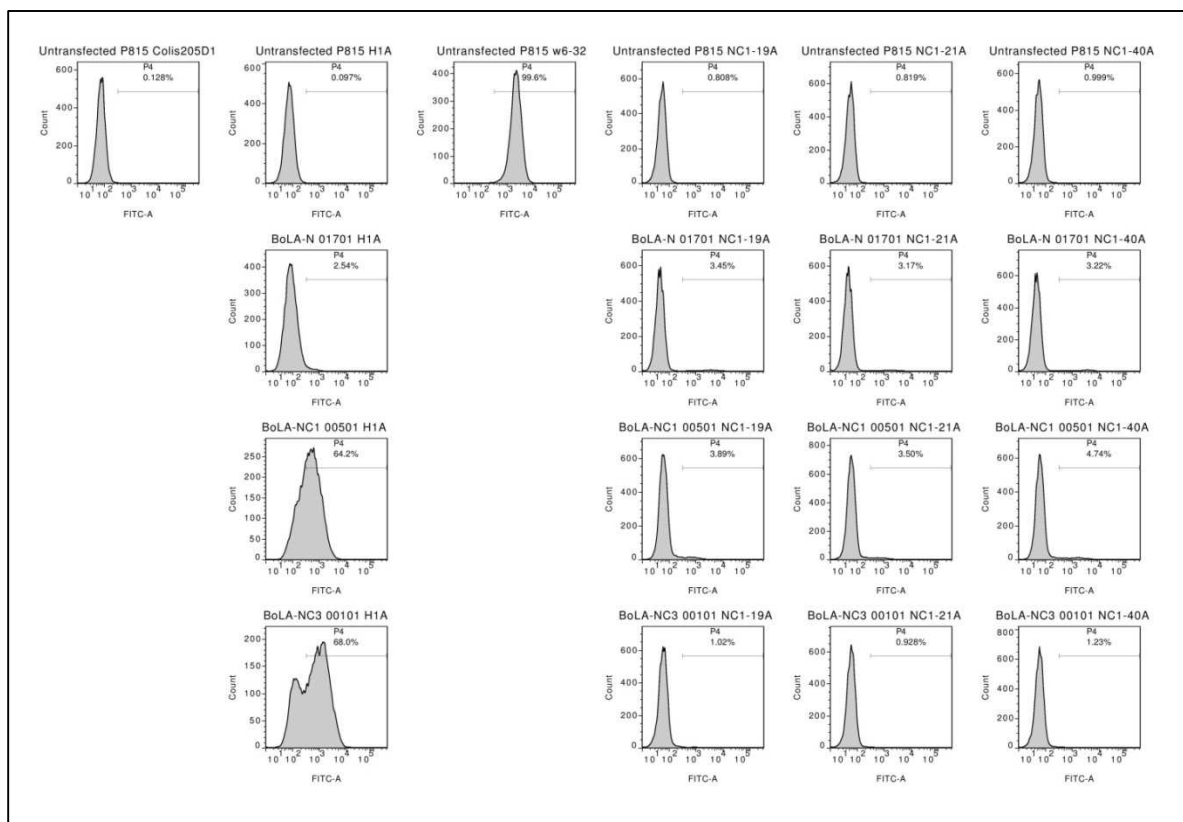


Fig 4-4: Flow-cytometry staining of culture supernatants of NC1*00501-hybridomas.

Third Fusion: From the third fusion of spleen cells, initial screening produced ten NC3 positive clones. However, only two of them expanded. These were frozen. We received supernatants of NC3-1, NC3-2 and NC3-3 from the third fusion. The NC3-2 supernatant looked very promising and positive on NC3*00101-transfected cells even at 1:100 dilution (Figure 4-5). We decided to clone the NC3-02 clone from the third fusion. Cloning of the NC3-02 hybridoma cell line was attempted twice at WSU, but the cell line was not cloned or rescued. More fusions were attempted but all the clones had non-

specific reactivities. From the third fusion we cultured NC3-2 (A, B, C, D, E, F), NC3-4 (A, B, C, D, E, F), NC3-7 (A, B), and NC3-8 (A, B, C, D, E, F) cell lines and tested them by flow cytometry. 7A and 7B supernatants reacted with the NC3*00101-transfected cell line but they also reacted with untransfected P815 cells with the same intensity.

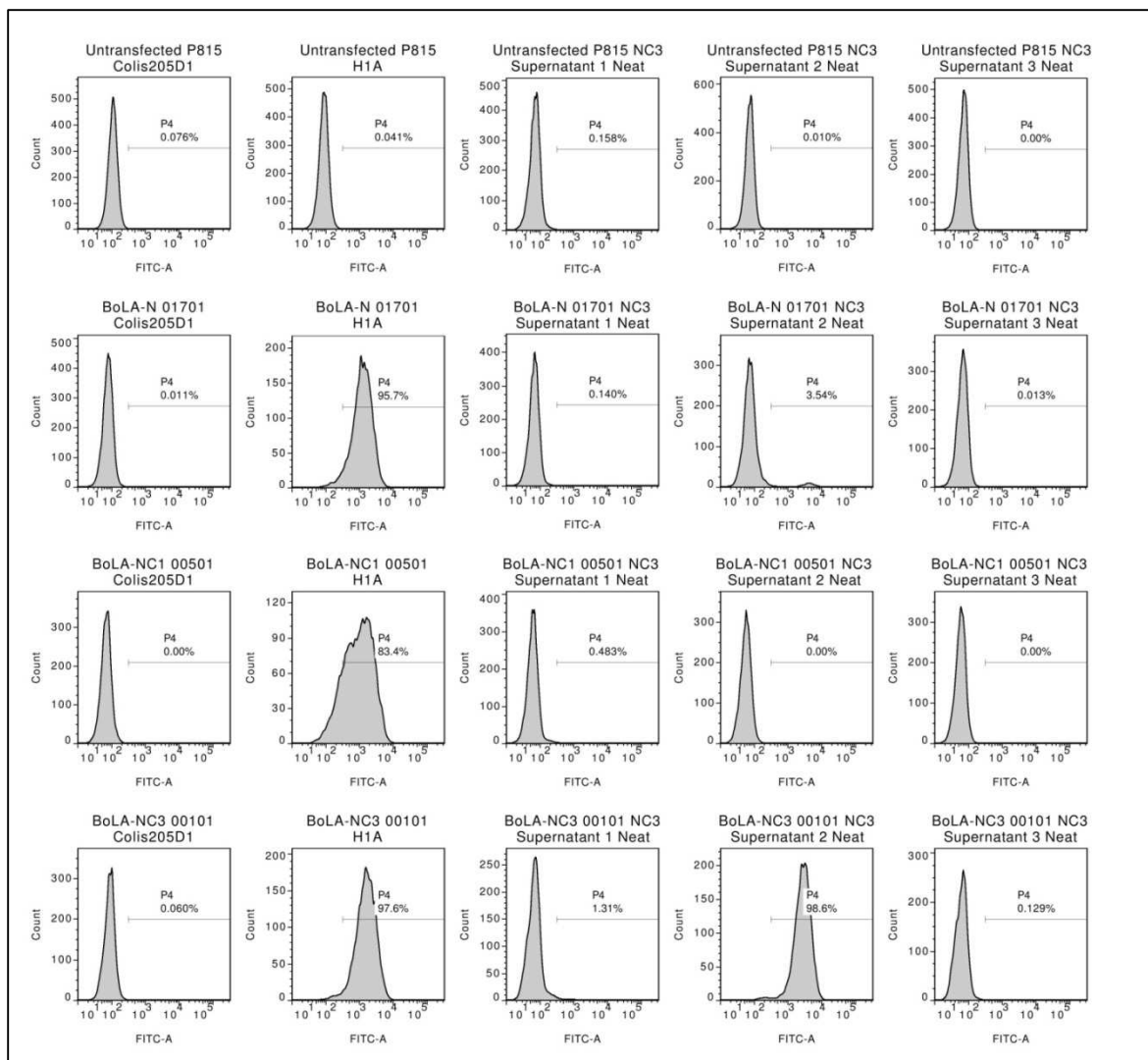


Fig 4-5: Flow-cytometry staining of culture supernatants of NC3*00101-hybridomas.

Note that BoLA-NC3*00101-NC3-Supernatant 2 neat has a specific positive response

Antibody Isotyping

All NC3*00101 and NC1*00501-hybridoma culture supernatants secreted IgG1 isotypes of immunoglobulins. As expected H1A, W6/32, and IL-A88 antibodies were of IgG2a isotypes. All samples tested were found to have kappa light chains (Table 4-2).

Table 4-2: Summary of Isotyping of NC1*00501 and NC3*00101 supernatants

mAb	Isotype	Light Chain
H1A	IgG2a	Kappa
IL-A88	IgG2a	Kappa
W6/32	IgG2a	Kappa
NC1-19A	IgG1	Kappa
NC1-21A	IgG1	Kappa
NC1-40A	IgG1	Kappa
NC3-2 original	IgG1	Kappa
NC3-5	IgG1	Kappa
NC3-6	IgG1	Kappa
NC3-9	IgG1	Kappa
NC3-10	IgG1	Kappa
NC3-11	IgG1	Kappa

Discussion

The report of bovine monoclonal antibody to red blood cell antigen was published by Tucker et al. 1987 [16]. Monoclonal antibodies against cattle MHC-I, IL-A88 and IL-A19, were produced at International Laboratory for Research on Animal Diseases (ILRAD), Kenya. In one study, researchers noticed that the MHC-I specific antibodies recognized MHC class I heavy chain on Western blot [2]. We tested H1A, H6A, H11A, PT-85A antibodies but they did not work on Western blot. It is reported that in non-reducing Western blotting conditions, they may all react with the light chain (Beta-2 microglobulin) [12].

To develop ELISA to detect secreted proteins from trophoblast cell culture supernatants, we attempted to develop monoclonal antibodies against two bovine class Ib molecules, NC1*00501 and NC3*00101. We were able to produce a hybridoma cell line which secreted NC3-specific antibodies. Unfortunately, the NC3-2 hybridoma, which produced specific and high-titer anti-NC3*00101 antibody, was not recovered and thus we failed to retain the positive hybridoma. We were able to use the NC3-2 original supernatant in ELISA as a capture antibody for NC3*00101 protein.

In the future, we need to perform more fusions and screenings of hybridomas to produce a positive hybridoma that is more stable and able to secrete specific antibodies in the supernatant. The use of transfected murine P815 cells as immunogens may also be one of the considering factors as the hybridoma supernatants reacted with untransfected cells when tested on flow cytometry. Using an MHC-null cell line such as human K562 and/or another host animal for immunization are parameters to consider for future projects to produce cattle class Ib specific antibodies.

References

1. Davis WC, Davis JE, Hamilton MJ. Use of monoclonal antibodies and flow cytometry to cluster and analyze leukocyte differentiation molecules. In: Davis WC (eds.), *Monoclonal Antibody Protocols, Methods in Molecular Biology*, vol. 45. Totowa, NJ: The Humana Press; 1995: 149-167.
2. Davis WC, Marusic S, Lewin HA, Splitter GA, Perryman LE, McGuire TC, Gorham JR. The development and analysis of species specific and cross reactive monoclonal antibodies to leukocyte differentiation antigens and antigens of the major histocompatibility complex for use in the study of the immune system in cattle and other species. *Vet Immunol Immunopathol* 1987; 15:337-376.
3. Desai N, Filipovits J, Goldfarb J. Secretion of soluble HLA-G by day 3 human embryos associated with higher pregnancy and implantation rates: assay of culture media using a new ELISA kit. *Reprod Biomed Online* 2006; 13:272-277.
4. Fournel S, Huc X, Aguerre-Girr M, Solier C, Legros M, Praud-Brethenou C, Moussa M, Chaouat G, Berrebi A, Bensussan A, Lenfant F, Le Bouteiller P. Comparative reactivity of different HLA-G monoclonal antibodies to soluble HLA-G molecules. *Tissue Antigens* 2000; 55:510-518.
5. Fuzzi B, Rizzo R, Criscuoli L, Noci I, Melchiorri L, Scarselli B, Bencini E, Menicucci A, Baricordi OR. HLA-G expression in early embryos is a fundamental prerequisite for the obtainment of pregnancy. *Eur J Immunol* 2002; 32:311-315.
6. Hamilton MJ, Davis WC. Culture conditions that optimize outgrowth of hybridomas, 1995. p. 17-28, *Monoclonal Antibody Protocols, Methods in Molecular Biology*, vol. 45. The Humana Press Inc., Totowa, N.J.

- 7.** Hviid TV, Rizzo R, Christiansen OB, Melchiorri L, Lindhard A, Baricordi OR. HLA-G and IL-10 in serum in relation to HLA-G genotype and polymorphisms. *Immunogenetics* 2004; 56:135-141.
- 8.** Kearney JF, Radbruch A, Liesegang B, Rajewsky K. A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines. *J Immunol* 1995; 123:1548-1550.
- 9.** Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 1975; 256:495-497.
- 10.** Kurosawa N, Yoshioka M, Fujimoto R, Yamagishi F, Isobe M. Rapid production of antigen-specific monoclonal antibodies from a variety of animals. *BMC Biol.* 2012; 10:80:1-14.
- 11.** Kwakkenbos MJ, Diehl SA, Yasuda E, Bakker AQ, van Geelen CM, Lukens MV, van Bleek GM, Widjojoatmodjo MN, Bogers WM, Mei H, Radbruch A, Scheeren FA, Spits H, Beaumont T. Generation of stable monoclonal antibody-producing B cell receptor-positive human memory B cells by genetic programming. *Nat Med* 2010; 16:123-128.
- 12.** Li W, O'Reilly KL, Davis WC, Splitter GA. Bovine major histocompatibility complex class I specific monoclonal antibodies characterized by flow cytometry, one- and two-dimensional electrophoresis, Western blot and inhibition of cytotoxic T lymphocyte function. *Vet Immunol Immunopath* 1992; 33:309-320.
- 13.** Parasar P, Barnett S, Wilhelm A, Rashid K, Davies CJ. Large-scale growth of mouse P815 cells expressing a bovine non-classical major histocompatibility complex class I protein utilizing a pitched-blade bioreactor, *Bio Process J*, 2012; 11(3): 27-34.

- 14.** Sargent I, Swales A, Ledee N, Kozma N, Tabiasco J, Le Bouteiller P. sHLA-G production by human IVF embryos: Can it be measured reliably? *J Reprod Immunol* 2007; 75(2):128-132.
- 15.** Traggiai E, Becker S, Subbarao K, Kolesnikova L, Uematsu Y, Gismondo MR, Murphy BR, Rappuoli R, Lanzavecchia A. An efficient method to make human monoclonal antibodies from memory B cells: potent neutralization of SARS coronavirus. *Nat Med* 2004; 10:871-875.
- 16.** Tucker EM, Clarke SW, Metenier L. Murine/bovine hybridomas producing monoclonal alloantibodies to bovine red cell antigens. *Anim Genetics* 1987; 18, 29-39.

CHAPTER 5

IDENTIFICATION OF THE PEPTIDE MOTIFS OF CATTLE NON-CLASSICAL MHC-I (BoLA-NC3*00101) PROTEIN

Abstract

We eluted and identified peptides which bind to the peptide binding groove of the NC3*00101 protein. We used a MHC-null K562 cell line, which only expresses the transgene. K562 cells were transfected with the bovine transgene coding for NC3*00101 protein and grown on a large scale. Peptides were released and eluted using mild acid treatment. After purification with reverse phase high pressure column chromatography (RP-HPLC) and mass-spectroscopy (MS) the peptide sequences were identified. Sequences were matched with the mammalian database of National Center for Biotechnology Information (NCBI). Candidate peptide-sequences were identified by creating sequence logos (<http://weblogo.berkeley.edu/logo.cgi>) and comparing with human non-classical class I specific peptide ligands. Candidate peptides were tested for their specificity in refolding assays and peptide binding assays. Our results indicate that peptide EVTNQLVVL is potential peptide ligand of NC3*00101 protein as identified by peptide binding assay and statistical analysis using mixed procedure of statistical analysis software (SAS).

Introduction

There are two types of major histocompatibility complex class I (MHC) proteins, classical (MHC-Ia) and non-classical (MHC-Ib) class I glycoproteins. Class I proteins bind with intracellular pathogen-derived peptides or the animal's own peptides and

present these peptides to the cell surface for cytotoxic T cell-receptor interaction. Self-peptides normally do not elicit a cytotoxic T-response owing to their tolerance to self-antigens. In bacterial, viral, and other infections, peptides produced from the foreign pathogen are subsequently loaded onto peptide binding grooves of MHC-I glycoproteins for presentation on the cell surface and immunological recognition by class I restricted cytotoxic T cells. Thus, an infected cell with a foreign peptide in the MHC-I peptide-binding groove is recognized and destroyed by cytotoxic T cells.

Binding of peptides to the peptide binding groove is critically important for the expression of MHC-I proteins on the cell-surface. Class I specific peptides are 8-10 amino acid long. These peptides tightly bind in the groove with their N- and C- termini buried interacting with MHC residues. Side chains of peptide amino acid residues interact with corresponding MHC residues to form pockets that vary in shape and location depending on the allelic forms of the MHC molecules [31]. MHC-Ia proteins have been studied to a significant extent in terms of their peptide presentation characteristics. Extremely polymorphic MHC-Ia proteins present a vast array of peptides to T cells. On the other hand, MHC-Ib proteins bind with a narrow range of peptides. Human MHC-Ib proteins, human leukocyte antigen (HLA)-E and HLA-G display more restricted sets of peptides than classical HLA-A, -B, and -C antigens. HLA-E binds peptides derived from leader sequences from certain HLA class I leader sequences [3, 4, 21, 22]. HLA-G binds naturally processed endogenous peptides [6, 23]. HLA-F is uniquely expressed as empty MHC-I protein [10]. The murine class Ib molecule Qa-2 binds nonapeptide which has a histidine as an anchor residue at P7 [18].

MHC-Ib proteins are important immunomodulatory proteins which interact with immunoglobulin-like receptors, LILRB1 and LILRB2, and inhibit immune cells [1, 2, 9, 13, 14, 15, 24, 28, 35]. Soluble HLA-G isoform, HLA-G1 induces maternal fetal tolerance uterus by inducing apoptosis of activated CD8 T cells [9]. It also inhibits NK cell-mediated cytotoxicity [25, 29, 30, 34].

The MHC of cattle, bovine leukocyte antigen (BoLA), encodes class Ib proteins at four loci, BoLA-NC1, -NC2, -NC3, and -NC4. It is known that class Ib proteins undergo alternative splicing so as to produce membrane and secreted isoforms. During the third trimester of pregnancy, cattle trophoblast cells express both class Ia and class Ib protein. The characteristic features of BoLA-Ib proteins are less polymorphism (monomorphic or oligomorphic), putative class Ib amino acid motifs (IPI, VPI or VLI) in their transmembrane domains and/or premature stop codons, and surface and/or soluble isoforms due to alternative splicing in the transmembrane domain [5]. Transfection studies revealed that BoLA-Ia proteins, N*01701 and N*01802, and BoLA-Ib proteins, NC1*00501, NC3*00101, and NC4*00201, are expressed on the cell-surface. NC3*00101 protein is expressed at a high level compared to other BoLA-Ib proteins, and is an important immunoregulatory molecule. It is important to investigate peptide motifs that bind with NC3*00101 protein to understand the mechanism of immune tolerance of this protein during pregnancy.

Endogenous peptides associated with MHC-I proteins can be identified using reported methods [33]. MHC-I bound peptides can be acid extracted from whole cells [7, 8, 31, 32, 33, 36, 37] or they can be acid-eluted from purified MHC-I molecules [6, 7, 8]. Although the elution of peptides from purified MHC-I proteins gives better results, citrate

shock or mild acid-elution can also be a method of choice when a MHC-I specific antibody capable of working in immunoprecipitation is not available.

We transfected a K562 cell line with a cattle NC3*00101 transgene and used the citrate shock method to elute peptides after mild acid treatment of transfected cells. Peptides were purified using chromatography and analyzed by liquid chromatography mass-spectroscopy (LC-MS) to identify sequences of peptides extracted from NC3*00101-transfected K562 cells.

Materials and Methods

Cell Lines and Culture Conditions

The MHC-null cell line K562 (ATCC CCL243) is a lymphoblastic cell line derived from a person with chronic myelogenous leukemia (CML). Cells were cultured and maintained at 37°C and 5% CO₂ in Iscove's Modified Dulbecco's Medium (IMDM) (Fisher Scientific) with 10% heat-inactivated bovine calf serum (Hyclone), 100 units/ml penicillin and 100 µg/ml streptomycin (Hyclone) in a humidified incubator.

Flow Cytometry

The BoLA-NC3*00101 gene was amplified (Forward primer CACCATGGGGCCGCGAACCCTC and reverse primer GGCAGTCTGACTGCTTGCAGTCTG) and cloned in the pcDNA3.1 mammalian expression vector. Subclones were sequenced to confirm the size of insert and full length subclones were used to isolate the plasmid using a Qia-prep kit (Qiagen). Cells were transfected with the subclones of the NC3*00101 gene in the pcDNA3.1 vector using Lipofectamine 2000 (Invitrogen) transfection reagent. Untransfected cells were used as a

negative control. Four micrograms plasmid DNA was used to transfect 2×10^6 cells using the method described in the chapter 3. For staining, cells were resuspended in fluorescence buffer (FB; PBS with 0.1% sodium azide, 1% bovine serum albumin) and incubated with anti-MHC class I monoclonal antibody W6/32 (15 μ g/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% formaldehyde or paraformaldehyde. One million cells were stained for each sample. Cells were analyzed using a Becton-Dickinson FACS Aria II fluorescence activated cell sorter (FACS) equipped with FACS Diva software. The FACS Aria II was also used to sort the transfected cells to enrich the high expressing cells.

For FACS sorting, cells were stained in IMDM medium using the method described above. Stable NC3*00101 transfectants of K562 cells were cultured in T150 flasks and monitored by counting the cells and visualizing daily under a microscope.

Mild-Acid Elution of NC3*00101-Peptides

Stably transfected and highly expressing NC3*00101-transfectants were harvested ($\sim 1 \times 10^9$ cells) by centrifuging cells at 2000 xg at 4°C for 10 min. Cells were washed twice with phosphate buffered saline (PBS) by spinning each wash at 2000 xg for 5 min. Cells were incubated on ice for 5 min. Ice-cold citrate shock buffer (0.131 M citric acid, 0.066 M Na₂HPO₄, and 150 mM NaCl, pH 3.3) was added to cells at 1 ml/ 10^8 cells. Cells were resuspended and incubated on ice for 5 min. Cells were spun down to remove the cell debris and supernatant was collected in a sterile 15 ml centrifuge tube (Corning). Cells were centrifuged at 15000 RPM for 30 min to clear the supernatant of the precipitated material to avoid the blockade of centrifugal filters in the subsequent

procedure. In the meantime, 3 kD MWCO centrifugal filters (Amicon) were washed with 50% methanol and liquid chromatography-mass spectrometry (LC-MS) grade-water (Optima) by centrifuging filters at 4000 RPM for 30 min. Clear supernatant from citrate-treated cells was added to the filter and centrifuged at 4000 RPM for 30 min. Flow through containing peptides lower than 3 kD size was collected and pooled. The pooled flow through was vacuum concentrated to 500 μ l and purified using reverse-phase-high pressure liquid chromatography (RP-HPLC) C18 Zip Tip (Millipore). Peptide extractions were performed in three replicates each of $\sim 1 \times 10^9$ cells and analyzed by mass-assisted laser desorption ionization (MALDI) mass spectroscopy for initial analysis of mass-spectra to identify the presence of peptides and potential m/z peaks of interest. Synthetic peptide (YPAIPVLQI), which is the reported peptide motif of cattle class I antigen BoLA-A11 or N*01701 protein (<http://www.ebi.ac.uk/ipd/mhc/bola/index.html>) [11, 12] was ordered from Genscript and used as a positive control reference peptide for the MALDI protocol.

MALDI and LC-FT MS/MS

MALDI and liquid chromatography Fourier Transform (LC-FT) MSMS analyses were carried out at the mass spectrometry and proteomics core facility, University of Utah, Salt Lake City. Briefly, peptide samples were spotted using the dried-droplet method. The matrix, α -cyano-4-hydroxy cinnamic acid (CHCA), was prepared in water/acetonitrile [50:50] with 0.1 % trifluoroacetate (TFA) by thoroughly mixing the matrix powder with 0.5 mL of solvent in a 1.7 mL eppendorf tube, and centrifuging to pellet the undissolved matrix. The supernatant was used for sample preparation for MALDI analysis. Peptide samples (0.5 μ l of 1 pmol/ μ l) were loaded onto a stainless

steel target plate and mixed on the target with 0.5 μL of supernatant of saturated matrix solution. After the sample spot was air-dried and the mixture of matrix supernatant and peptide co-crystallized, the spot was ablated with a 1 kHz smartbeam-IITM laser from the plate while the sample was simultaneously desorbed, ionized, and then accelerated into a flight tube. The MALDI spectrum was acquired in reflector mode, which was operated at around 30,000 resolutions over a mass range from 500 to 5000 Da.

LC/MS Analysis of Peptides

LC-MS/MS analysis was performed using a LTQ-FT hybrid mass spectrometer (ThermoElectron Corp) equipped with Ion Trap Fourier Transform Ion-Cyclotron Resonance (FT-ICR) technologies. Primary mass spectra were acquired with the (FT-ICR) part of the instrument. MS/MS fragmentation spectra (i.e. peptide sequence information) were acquired in the Ion Trap part of the instrument. Peptide molecular masses were measured by FT-ICR, yielding primary mass spectra of peptides with mass errors typically less than 3 ppm. Peptide sequencing was performed by collision-induced dissociation (CID) in the linear ion trap of this hybrid instrument yielding fragment ions with mass errors typically less than 0.3 Da. Peptide samples were introduced by a nanoLC column (2D-Ultra, Eksigent, Inc.) with nano-electrospray ionization spray (ThermoElectron Corp). Typically about 10 to 20 fmoles of peptide samples were injected. NanoLC chromatography was performed using a homemade C18 nanobore column (75 μm ID x 10 cm; Atlantis C18, 3 μm particle-C18 material from Waters Corp) at the University of Utah, Mass Spectrometry and Proteomics -Core Facility. Peptides were eluted during a 78-min linear gradient from 5% acetonitrile (with 0.1% formic acid) to 60% acetonitrile (with 0.1% formic acid) with a flow rate of 350 nl /min.

Peptide ID and Database Searches

All identified peptides were assigned from protein database searches, using in-house processing with the MASCOT search engine (in-house licensed, ver. 2.2.1, Matrix Science, Inc.) at the University of Utah, Mass Spectrometry and Proteomics Core Facility. Mascot searching was performed from an in-house computer using a search of the NCBI protein database. Peptides mass spectra data were searched with non-enzyme-specific cut sites and identified based on the “MS/MS” Mascot search option, with the following criteria:

- 1) Accurate mass measurement of peptide molecular ions by FTMS with search window 5 ppm (peptides typically had less than 3 ppm mass error). Molecular ions with +1, +2, or +3 charge states determined from a FTMS primary mass spectrum (LTQ-FT instrument) were usually considered.
- 2) Peptide sequence information from MS/MS; CID fragmentation of the parent ion of each peptide was obtained in the linear ion trap region of the LTQ-FT instrument. Mass error tolerance of 0.5 Da was allowed for peptide fragment ion masses in the search (MS/MS fragment ions typically had errors less than 0.3 Da).
- 3) Mass spectra peak lists for the Mascot searches were generated using Sequest in QualBrowser software (Excalibur, Thermo Electron Corp.).
- 4) Peptide modification was included in the search (e.g. oxidation on methionine).
- 5) Searches were typically performed for non-specific peptide cleavages. Two missed cleavages were allowed.

6) All identified peptides showed MASCOT scores greater than 20. Mascot threshold cutoffs for acceptable identified peptides had MASCOT scores >20, mass errors <3 ppm, and expected values less than 1.

Purification of NC3*00101-BSP and Bovine β 2m Inclusion Bodies

The NC3*00101 heavy chain was fused with a sequence that can be biotinylated with a biotinylating enzyme (BirA). This sequence tag is therefore known as BirA substrate peptide (BSP*41) tag. The NC3*00101-extracellular domains (alpha 1, 2, and 3 domains) or heavy chain was amplified from genomic DNA and cloned in pTCF33 vector (a generous gift from the NIH Tetramer Facility, Emory University) which expresses the MHC-I heavy chain-BSP (BSP*41) sequence in *E. coli*. The pTCF33 contains a hybrid T7-lac promoter that drives expression of MHC-BSP chain. Bovine β 2m was expressed into pET24a+ vector as per the NIH Tetramer Facility Protocol (<http://tetramer.yerkes.emory.edu/client/protocols#1>). The primers for both the NC3*00101-BSP and bovine β 2m were designed so that the amplicons contain the Bam HI restriction site. Clones were sequenced and checked for the correct insert size. *E. coli* BL21 cells were inoculated with full size subclones and grown as per the NIH protocol. Overinduction of expression was carried out using 1 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG). Cells were harvested by centrifuging at 4000 RPM for 30 min. Bacteria were pooled and resuspended in 50 mM Tris-HCL, 25% (W/V) sucrose, 1 mM EDTA, 0.1% (w/v) NaAzide, and 10 mM freshly added DTT and the suspension was stirred in a 100 ml beaker with a stirring bar. To the stirring mixture was added, 1.2 ml 50 mg/ml lysozyme (final = 1 mg/ml), 300 μ l 1.0 M MgCl₂ (final=5mM), 1.0 ml of 2 mg/ml Dnase I in 50% glycerol containing 75 mM NaCl, 600 μ l Triton-X 100 (final= 1%), and

600 μ l 1M DTT (final=10 mM). The suspension was stirred for 30 min at room temperature and then sonicated for 1.5 min at 0.5 sec alternations at power 4 using sonicator. The suspension was centrifuged in multiple 50 ml centrifuge tubes (Corning) in a Beckman centrifuge at 10000 RPM for 10 min at 4°C. After decanting the supernatant, 1-2 ml of wash buffer, pH 8.0 (50 mM Tris-HCl, 0.5% Triton-X100, 100 mM NaCl, 1 mM EDTA, 0.1% Na-azide, 1 mM DTT added fresh) was added to the pellet. The pellet was resuspended, and centrifuged again. After two washes with wash buffer with Tween-20, cells were washed with wash buffer without Tween-20 and centrifuged. The final white pellet was solubilized in 10 ml urea solution, pH 6.0 (25 mM MES (pH 6.0), 8 M urea, 10 mM EDTA, and 0.1mM DTT added fresh). The protein concentration was measured at A280 measurement on a Nano drop Spectrophotometer. Urea solubilized bodies were frozen at a concentration of 2 mg/ml concentration.

Refolding of Cattle NC3*00101-BSP and β 2m Chains and Biotinylation

Refolding was carried out in 500 ml of folding buffer (400 mM L-arginine, 100 mM Tris, 2 mM EDTA, pH 8.3) chilled at 10°C. To 500 ml refolding buffer, were added 0.76825 g reduced glutathione, 0.15315 g oxidized glutathione, and 0.5 ml 200 mM PMSF. Synthetically ordered peptides (15 mg) were dissolved in 500 μ l DMSO and added to the stirring reaction. Urea solubilized inclusion bodies (500 nmol NC3*00101-BSP and 1000 nmol bovine β 2m) were diluted in injection buffer (3 mM Guanidine HCl, 10 mM Sodium Acetate, 10 mM EDTA; pH 4.2) were loaded into two separate 3 cc syringes using 20 gauge needles and injected forcefully into the stirring reaction on a stir plate. The reaction was incubated at 4°C with stirring overnight. Next morning, 500 nmol of NC3*00101-BSP chain was injected into the reaction followed by 500 nmoles

NC3*00101-BSP in the evening. Folding reaction was incubated for 2 more days at 4°C. After 3 days of incubation, reaction was concentrated using 5 kD cut off filters (Millipore) to 7.5 ml and buffer-exchanged with 10.5 ml biotinylation buffer (100 mM Tris, 200 mM NaCl, 5 mM MgCl₂, pH to 7.5) using PD-10 Sephadex G-25M columns. To 10.5 ml reaction, were added 500 µl ATP (100mM stock), 40 µl Biotin (100mM stock), 10 µl Leupeptin (1000x stock), 10 µl Pepstatin (100x stock), 20 µl PMSF (0.1M stock), and 20 µl BirA enzyme (Avidity) (stock at ~ 1 mg/ml) and the reaction was biotinylated by incubating overnight at RT.

Purification of Biotinylated MHC-Peptide Monomers on the Mono Q Column

Biotinylation reaction was buffer-exchanged with 20 mM Tris, pH 8.0 using Amicon Ultra-15 centrifuge filtering device 10 kD MWCO (Millipore). Final concentrated sample was dissolved in 500 µl Tris buffer. The concentrated sample was transferred to an eppendorf tube and centrifuged at 15300 RPM for 15 min at 4°C to remove any precipitate. The concentrated sample (500 µl) was immediately processed for Mono Q 5/5 purification column (GE Healthcare) using Buffer A (20 mM Tris, pH 8.0) and buffer B (20 mM Tris, pH 8.0 and 500 mM NaCl). Briefly, 2 ml loop was washed two times with 2 ml of 20 mM Tris (pH 8.0). FPLC computer program was opened and set to run the Mono Q column. The parameters were entered manually and the program was run for 2 hours until the MHC peaks showed up. The fractions were collected in 4 ml polypropylene tubes. The specific fractions were pooled and concentrated using 10 KD MWCO Amicon filters. The sample was concentrated <1 ml and the final sample was diluted with ~1 ml PBS. The sample was transferred to an eppendorf tube and stored on ice or 4°C. Protein concentration was measured using A 280 absorbance on a Nano drop

spectrophotometer and the monomer samples were diluted with PBS to achieve the concentration of 2 mg/ml.

Western Blotting Analysis of Biotinylated Refolded MHC-Peptide- β 2m Complex

To 3 μ l of diluted monomer samples from the previous step, was added 12 μ l of PBS (Final concentration 0.4 mg/ml). Five microliters of this monomer sample was added with 5 μ l of 0.8-1 mg/ml streptavidin or water and incubated for 1 hour at room temperature (RT). Samples were run on NuPAGE 4-12 % Bis-Tris gel and Coomassie stained to identify the biotinylated heavy chains to shift when treated with Streptavidin (Biotinylation Shift Assay). For Western blotting, the incubated samples were run on the gel and transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was incubated with anti-mouse β 2m polyclonal (Novus Biologicals) or W6/32 (VMRD Inc.) antibodies for an hour at RT. Anti-mouse AP-conjugated antibody was used as secondary antibody. Both denaturing and native conditions were used to test the antibodies to detect refolded protein complexes.

Peptide Binding Assay

In a 24-well plate, 0.5×10^6 cells (transfected or untransfected) were added. Untransfected K562 cells were used a negative control in each experiment. All cells were treated with or without peptides and incubated at 37°C for 16 hours. Cells treated with DMSO (carrier) were used as reference sample for analysis. Cells were analyzed for NC3*00101 expression using anti-MHC class I antibody, W6/32. Peptides tested were YPAIPVLQI, EVTNQLVVL, LVDGVKRIL, SSKIVGDLA, and GSILSGTAIA. Results were analyzed by creating overlays of flow cytometry data using FlowJo software.

Statistical Analysis

Peptide binding assays were performed and mean fluorescence values were recorded for cell samples. DMSO-treated cells were taken as control and peptides as mentioned above were used as unknown treatment groups. Different days were used as covariables. A mixed procedure using statistical analysis software (SAS) was used to identify significant effects of peptide-treatments on NC3*00101 expression. The least square means and differences between the means were determined for different treatment groups to identify the differences of significance between each peptide. P-value of 0.5 was used to identify the values of significance.

Results

Flow Cytometry

K562 cells transfected with NC3*00101 transgene showed 22.4% expression compared to untransfected cells. We were able to sort highly expressing cells using W6/32 staining. Three sorts were performed which resulted in enrichment of highly expressing cells with an expression level of 90.3% (Figure 5-1).

MALDI and LC-MS/MS Analyses

The mass spectrum of reference peptide YPAIPVLQI yielded a specific m/z peak, which corresponded to a 1013.61 dalton molecular weight (expected MW 1013.65). We had few m/z peaks of interest in the NC3*00101-peptide sample which led us to proceed to subsequent LC-MS/MS analysis on NC3*00101 samples (Figure 5-2).

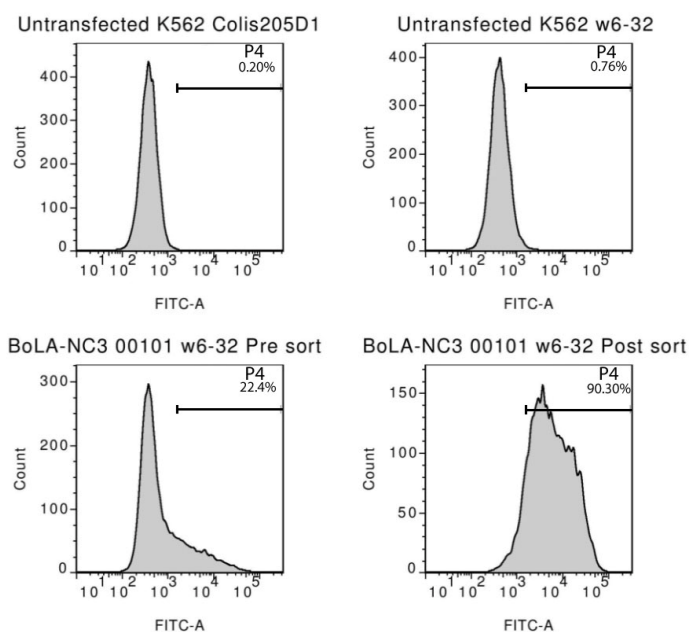


Fig 5-1: Sorting of K562-NC3*00101 transfectants was performed using anti-MHC-I monoclonal antibody W6/32

*LC-MS/MS Analysis of NC3*00101 Extracted Peptides*

Three different analyses were performed using web sequence logo and manual comparisons. Peptide sequences were sorted into 8, 9, and 10-amino acid sequences, which were unique to NC3*00101-transfected cells and different from peptides extracted from untransfected K562 cells. As MHC-I specific peptides are nonamers, we focused on 9-mer peptides. Sequence logos were created and compared with other MHC class I specific peptide residues. Four candidate peptides were selected which had non-polar amino acids at -COOH terminals as reported [31]. Both from sequence logos (<http://weblogo.berkeley.edu/logo.cgi> website, Figure 5-3) and manual sorting methods, candidate peptide motifs of NC3*00101 protein were chosen and ordered from

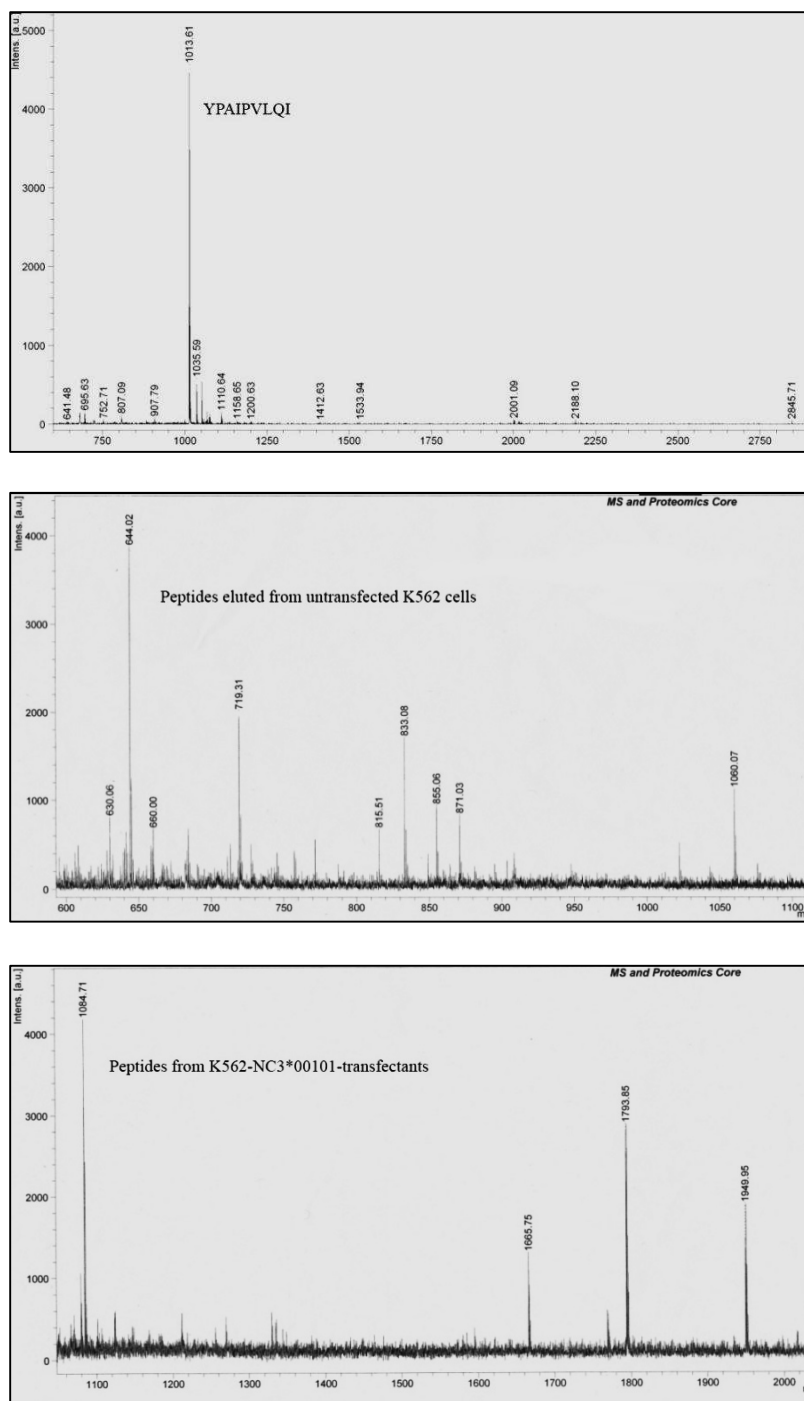


Fig 5-2: MALDI spectra of synthetic (YPAIPVLQI) and acid-eluted peptides from untransfected-K562 cells and transfected NC3*00101-K562 cells

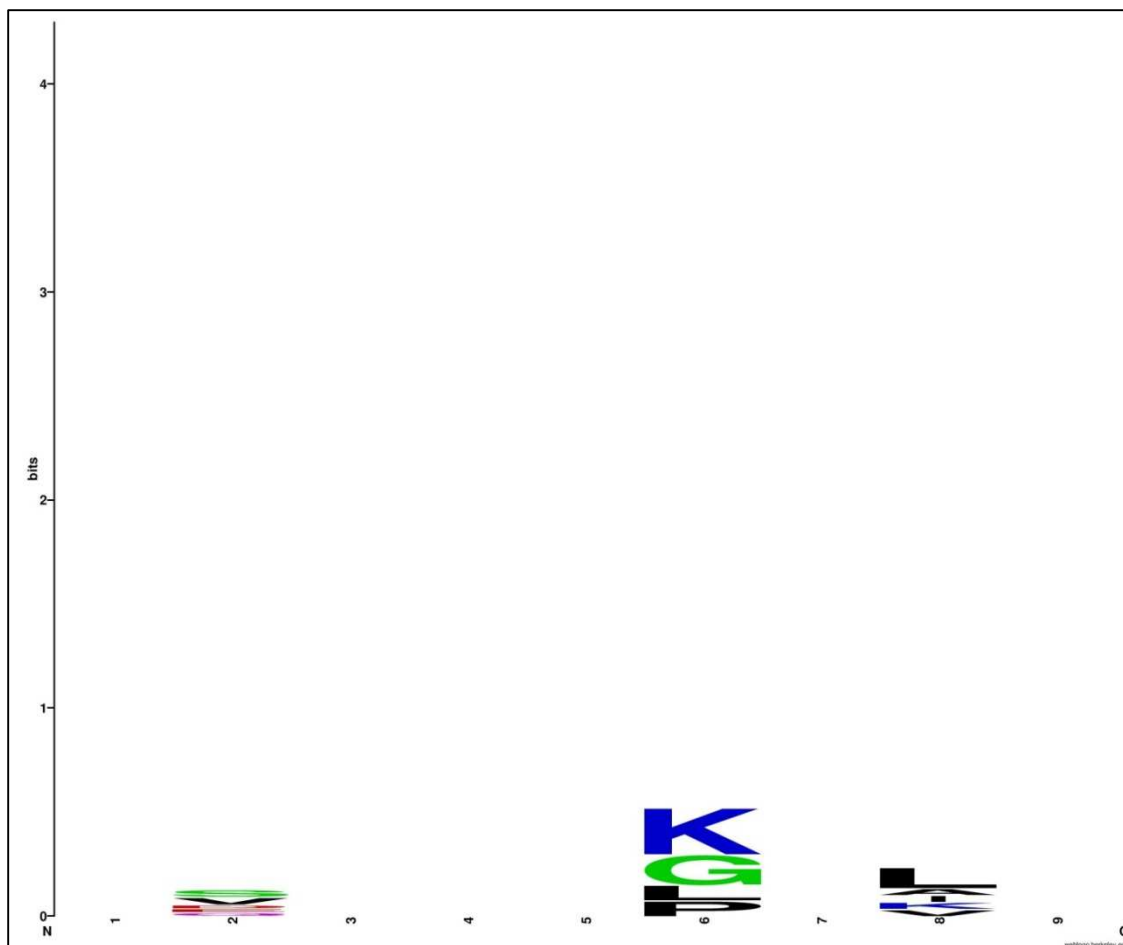


Fig 5-3: Logos created with 9-amino acid long peptides identified with the LC-MS/MS on citrate-acid-eluted peptides

GenScript. These were EVTNQLVVL, LVDGVKRIL, SSKIVGDLA, and GSILSGTAIA.

Refolding of Heavy and Light Chains and Western Blotting

NC3*00101-BSP and bovine β 2m were successfully expressed in bacteria as identified in a Coomassie stained gel (Figure 5-5). Cattle β 2m was detected by Western blotting with anti-mouse β 2m polyclonal antibody (Figure 5-4). We were not able to detect the NC3*00101 heavy chains with the W6/32 antibody in Western blots as W6/32

only recognized N*01701 and NC1*00501 heavy chains and not other cattle class I heavy chains (Figure 5-4). W6/32 and anti-mouse β 2m antibodies did not react with refolded biotinylated MHC-peptide complexes. Refolded complexes were treated with streptavidin and run on the gel followed by Coomassie staining. We noticed the streptavidin band in a biotinylation shift assay but we did not see the expected heavy chains (~45 kD) in streptavidin negative lanes as highlighted with the oval region of Figure 5-5. As the W6/32 antibody did not react with NC3*00101 heavy chain (NC3*00101-HC) on Western blots, it was not a surprise to see that this antibody failed to identify NC3*00101-streptavidin complexes. Native conditions did not produce positive results. Therefore, we proceeded to test our peptides in peptide binding assays.

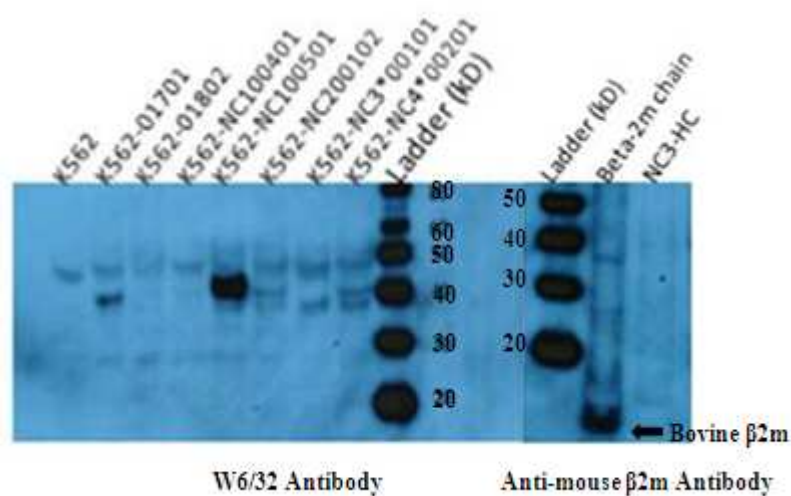


Fig 5-4: Western blotting showing that W6/32 recognizes N*01701 and NC1*00501 proteins in whole cell lysates. Anti-mouse β 2m antibody identifies cattle β 2m inclusion bodies.

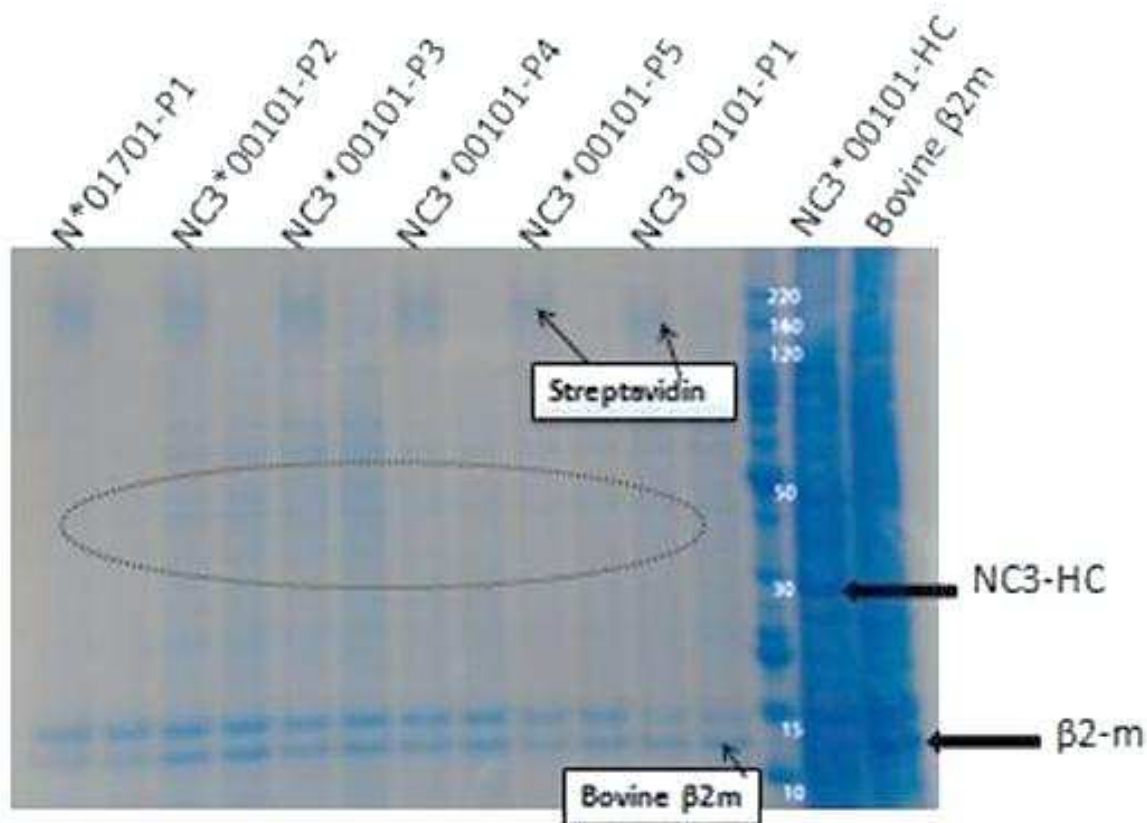


Fig 5-5: Biotinylation shift assay. SDS-PAGE stained with Coomassie stain to test the refolded MHC-peptide- β 2m complexes. As shown NC3*00101 heavy and β 2m light chains were expressed and purified as inclusion bodies.

Peptide Binding Assay

In peptide binding assays, treatment of cells with DMSO produced an increase in the expression of MHC-I protein. As seen in overlays the expression of NC3*00101 was increased by all of our peptides tested (Figure 5-6). However, the increases in the shift varied from batch to batch. An overall comparative analysis of mean fluorescence values, we found that the peptide EVTNQLVVL increased the NC3*00101 expression compared

with other peptides and DMSO treatments. Other peptides, LVDGVKRIL, SSKIVGDLA, and GSILSGTAIA were not consistently effective in increasing the shift as EVTNQLVVL. However, we also noticed increase in expression with YPAIPVLQI treatment, which may be because this peptide fits in the peptide-binding groove stably because of its specificity with MHC-I proteins.

Using Proc mixed program of SAS we identified that the peptide EVTNQLVVL had the highest least square mean among all. We identified that the EVTNQLVVL peptide was significantly different from control DMSO. Other three NC3*00101-cell-eluted peptides were significantly lower than both DMSO and EVTNQLVVL. Therefore, we conclude that EVTNQLVVL is a potential peptide for NC3*00101 protein (see Table 5-1).

Discussion

Identification of NC3*00101-specific peptide motifs is the first endeavor to elucidate the amino acid residues binding to NC3*00101 binding groove. It is critically important to understand peptide binding characteristics of cattle NC3*00101 protein to gain more knowledge about mechanisms of interactions of NC3*00101 protein with other leukocytes and the receptors expressed by leukocytes. Here we present a peptide motif that increased the expression of NC3*00101 proteins on transfected K562 cells and was significantly different from other peptides tested. After initial failures of immunoprecipitation method using W6/32 antibody, we switched to citrate shock method to elute NC3*00101-peptides from its transfected cells.

We tested four candidate peptides which were eluted from NC3*00101-expressing K562 cells after citrate-acid treatment. Based on results of refolding and

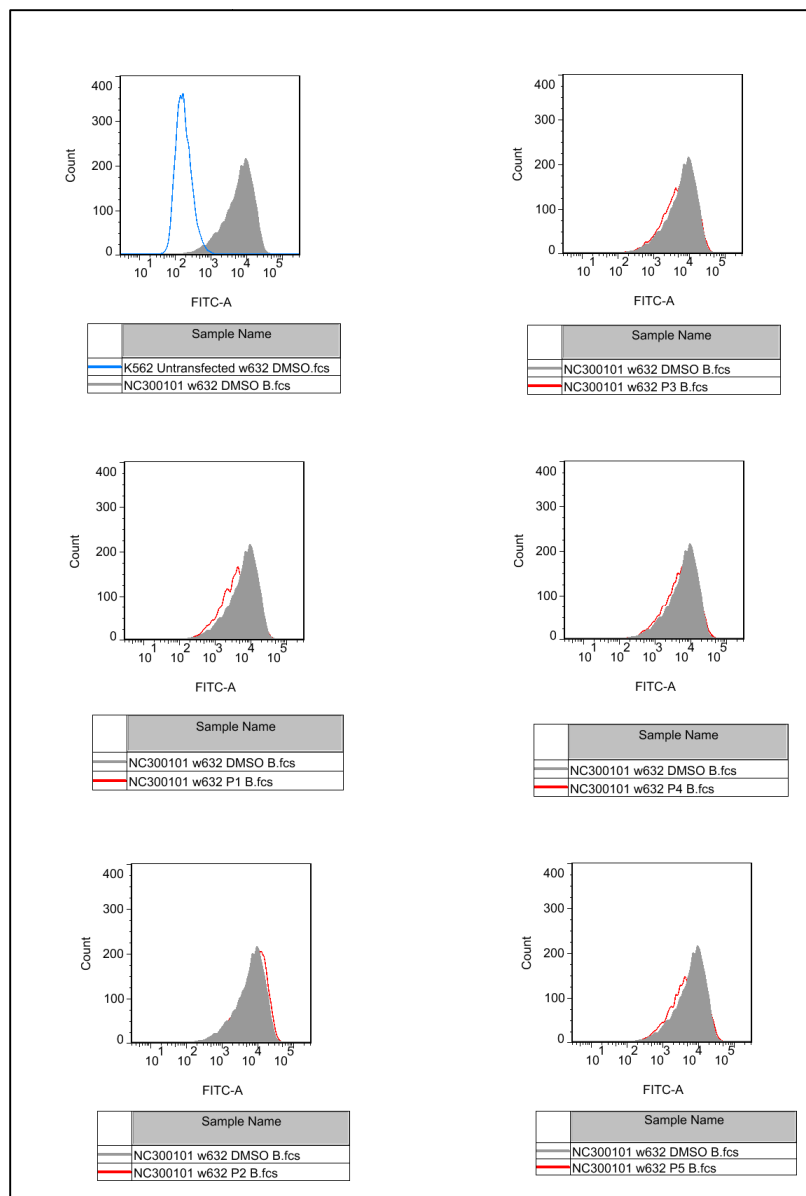


Fig 5-6: Peptide binding assay performed on NC3*00101-transfectants with peptides (P1: YPAIPVLQI; P2: EVTNQLVVL; P3: LVDGVKRIL; P4: SSKIVGDLA; P5: GSILSGTAIA). X axis: Log fluorescence and Y axis: Cell count. Cells were incubated with (red line) and without (black filled histogram) peptides for 15-16 hour at 37°C and checked for the MHC-I (NC3*00101) expression using W6/32 antibody.

Table 5-1: The Mixed Procedure (SAS) output showing the least square means and contrast values for different treatments in peptide binding assay

The SAS System									
The Mixed Procedure									
Type 3 Tests of Fixed Effects									
Effect	Num DF	Den DF	F Value	Pr > F					
trt	5	117	16.71	<.0001					

Contrasts					
Label	Num DF	Den DF	F Value	Pr > F	
DMSO vs P1, P2	1	117	5.74	0.0182	
P1, P2 vs P3, P4, P5	1	117	76.01	<.0001	
P3, P4, P5 vs DMSO	1	117	18.93	<.0001	
P1, P2, P3, P4, P5 vs DMSO	1	117	2.82	0.0956	

Least Squares Means						
Effect	trt	Estimate	Standard Error	DF	t Value	Pr > t
trt	DMSO	7002.85	533.70	117	13.12	<.0001
trt	P1	7181.09	533.70	117	13.46	<.0001
trt	P2	7566.85	533.70	117	14.18	<.0001
trt	P3	6398.71	533.70	117	11.99	<.0001
trt	P4	6364.47	533.70	117	11.93	<.0001
trt	P5	6338.66	533.70	117	11.88	<.0001

Differences of Least Squares Means									
Effect	trt	_trt	Estimate	Standard Error	DF	t Value	Pr > t	Adjustment	Adj P
trt	DMSO	P1	-178.24	178.93	117	-1.00	0.3212	Simulate	0.9146
trt	DMSO	P2	-564.00	178.93	117	-3.15	0.0021	Simulate	0.0231
trt	DMSO	P3	604.14	178.93	117	3.38	0.0010	Simulate	0.0115
trt	DMSO	P4	638.38	178.93	117	3.57	0.0005	Simulate	0.0063
trt	DMSO	P5	664.19	178.93	117	3.71	0.0003	Simulate	0.0040
trt	P1	P2	-385.76	178.93	117	-2.16	0.0331	Simulate	0.2558
trt	P1	P3	782.38	178.93	117	4.37	<.0001	Simulate	0.0002
trt	P1	P4	816.62	178.93	117	4.56	<.0001	Simulate	0.0002
trt	P1	P5	842.43	178.93	117	4.71	<.0001	Simulate	0.0002
trt	P2	P3	1168.14	178.93	117	6.53	<.0001	Simulate	<.0001
trt	P2	P4	1202.38	178.93	117	6.72	<.0001	Simulate	<.0001
trt	P2	P5	1228.19	178.93	117	6.86	<.0001	Simulate	<.0001
trt	P3	P4	34.2381	178.93	117	0.19	0.8486	Simulate	1.0000
trt	P3	P5	60.0476	178.93	117	0.34	0.7378	Simulate	0.9994
trt	P4	P5	25.8095	178.93	117	0.14	0.8856	Simulate	1.0000

biotinylation shift assays, we were not able to detect refolded NC3*00101-peptide- β 2m complexes. We do not have a functional antibody, which recognizes monomeric, dimeric, trimeric and tetrameric complexes of NC3*00101-peptide- β 2m. Natural ligands binding to HLA-E are different than experimentally-derived amino acid residues [27]. It is possible that NC3*00101-specific ligands may bind with endogenous peptides with varying degree of similarity with residues identified in the present study. Nevertheless, treatment of cells with peptides in peptide binding assay shows that the peptide EVTNQLVVL increases the NC3*00101 expression more consistently.

It was not experimentally feasible to screen an entire peptide library based on the LC-MS/MS results. Therefore, it may further need to screen larger number of peptide library to confirm positions of anchor and auxiliary residues of NC3*00101-specific peptides. However, unavailability of antibody to immunoprecipitate native NC3*00101 protein may present a caveat. Reports on HLA-G and other non-classical class I peptides [6] suggest presence of non-polar or neutral amino acid at the carboxyl terminals. Non-polar amino acids are Alanine (A), Isoleucine (I), Leucine (L), Phenylalanine (F), Valine (V), Proline (P), and Glycine (G). By comparing with peptide motifs of human MHC-Ib proteins, HLA-E and HLA-G, we know that experimentally derived amino acid residues may not always be the binding motifs of MHC-I proteins. Therefore, we are confident that NC3*00101-specific peptide motif has a valine as anchor residues at position 2, a Val/Iso/leu at position 8, and Iso/Leu at position 9 as anchor residues (Table 5-2). In order to further confirm that P2 (EVTNQLVVL) is NC3*00101-specific ligand, we identified and statistically tested effects of treatment of each peptide on the NC3*00101 expression with statistical analysis software (SAS) mixed Procedure (Proc Mixed).

Table 5-2: NC3*00101 Peptide Binding Motifs and a comparison with HLA-G and HLA-E peptide motifs

BoLA-NC3*00101	Position								
	1	2	3	4	5	6	7	8	9
INDIVIDUAL LIGANDS	E	V	T	N	Q	L	V	V	L
	L	V	D	G	V	K	R	I	L
	S	S	K	I	V	G	D	L	A
	G	S	I	L	S	G	T	A	I A
POTENTIAL ANCHOR RESIDUES		V						V	I
								I	L
								L	
Possible Preferred Residues					Q			V	
								I	
								L	
								A	
HLA-G	Position								
	1	2	3	4	5	6	7	8	9
	R	I	I	P	R	H	L	Q	L
	R	L	P	K	D	F	R	I	L
	K	L	P	A	Q	F	Y	I	L
Anchor Residues		I	P						L
		L							
Preferred Residues	R					Y	I	Q	
	K						V	M	
							L		
							F		
HLA-E	Positions								
Experimentally derived motifs	1	2	3	4	5	6	7	8	9
		M	K			A	L/I		L
		L	N			T	V		E
		Q	A			V	P		F
			Q						
Residues in endogenous HLA class Ia leader peptides that bind HLA-E	V	M	A	P	R	T	L	L	L
							V	V	
								I	
								F	

The SAS output revealed that the treatment effect was significant between different treatments (Table 5-1). Using contrast method, we identified that treatment effect was divided into two groups, one group containing P1 and P2 which has higher mean fluorescence and the other group containing P3, P4, and P5 which have lower fluorescence as compared to DMSO. The mixed procedure contrast output values reveal that P1 (YPAIPVLQI) and P2 (EVTNQLVVL) as well as P3 (LVDGVKRIL), P4 (SSKIVGDLA), and P5 (GSILSGTAIA) are not significantly different than the control (DMSO). However, it is clear that P1 and P2 are significantly different than P3, P4, and P5. Differences of least square means in the table are in accordance with our hypothesis that P2 is significantly higher than DMSO with an adjusted P value of 0.0231 ($P=0.05$) whereas P1 is not ($P=0.9146$). P3, P4, and P5, on the other hand, are significantly lower than DMSO. This suggests that P2 is the most likely candidate peptide motif of NC3*00101 protein.

In the pursuit of an antibody to detect folded MHC-peptide- β 2m complexes, we tested W6/32 and anti-mouse β 2m antibodies. Monoclonal antibody W6/32 recognizes heavy and light chain conformational epitope under non-reducing conditions. Other reports have been published showing that W6/32 detects certain allelic forms of rabbit, rat, mouse, and guinea pig class I heavy chains only when they are complexed with human or cattle β 2m and not with autologous β 2m [16, 17, 19, 20, 26]. Therefore, it is possible that cattle class I heavy chains which are not detected with W6/32 may require an association with β 2m from other species for their detection with W6/32. We also identified that W6/32 only recognized N*01701 and NC1*00501 free heavy chains under partially denaturing conditions which suggests that all cattle class I proteins do not have

identical association of heavy and light chains which can produce variations in the formation of class I alpha chain loops and normal peptide binding characteristics.

References

1. Bainbridge DRJ, Ellis SA, Sargent IL. The short forms of HLA-G are unlikely to play a role in pregnancy because they are not expressed at the cell surface. *J Reprod Immunol* 2000; 47:1-16.
2. Bainbridge DRJ, Ellis SA, Le Bouteiller P, Sargent I. HLA-G remains a mystery. *Trends Immunol* 2001; 22:548-552.
3. Braud V, Jones EY, McMichael A. The human major histocompatibility complex class Ib molecule HLA-E binds signal sequence-derived peptides with primary anchor residues at positions 2 and 9. *Eur J Immunol* 1997; 27:1164-1169.
4. Braud VM, Allan D. S. O'Callaghan CA, Soderstrom K, D'Andrea A, Ogg GS, Lazetic S, Young NT, Bell JI, Phillips JH, Lanier LL, McMichael AJ. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature* 1998; 391:795-799.
5. Davies CJ, Eldridge JA, Fisher PJ, Schlafer DH. Evidence for expression of both classical and non-classical major histocompatibility complex class I genes in bovine trophoblast cells. *Am J Reprod Immunol* 2006; 55:188-200.
6. Diehl M, Münz C, Keilholz W, Stevanović S, Holmes N, Loke YW, Rammensee HG. Nonclassical HLA-G molecules are classical peptide presenters. *Current Biol* 1996; Mar 1; 6(3):305-314.
7. Falk K, Rötzschke O, Deres K, Metzger J, Jung G, Rammensee HG. Identification of naturally processed viral nonapeptides allows their quantification in infected cells and

suggests an allele-specific T cell epitope forecast. *J Exp Med* 1991; Aug 1; 174(2):425-434.

8. Falk K, Rötzschke O, Stevanović S, Jung G, Rammensee HG. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature* 1991; May 23; 351(6324):290-296.

9. Fournel S, Aguerre-Girr M, Huc X, Lenfant F, Alam A, Toubert A, Bensussan A, Le Bouteiller P. Cutting edge: soluble HLA-G1 triggers CD95/CD95 ligand-mediated apoptosis in activated CD8⁺ cells by interacting with CD8. *J Immunol* 2000; 164:6100-6104.

10. Goodridge JP, Burian A, Lee N, Geraghty DE. HLA-F complex without peptide binds to MHC class I protein in the open conformer form. *J Immunol* 2010; Jun 1; 184(11):6199-6208.

11. Hammond JA, Marsh SG, Robinson J, Davies CJ, Stear MJ, Ellis SA. Cattle MHC nomenclature: is it possible to assign sequences to discrete class I genes? *Immunogenetics* 2012; 64(6):475-480.

12. Hegde NR, Ellis SA, Gaddum RM, Tregaskes CA, Sarath G, Srikumaran S. Peptide motif of the cattle MHC class I antigen BoLA-A11. *Immunogenetics* 1995; 42(4):302-303.

13. Hunt JS. Stranger in a strange land. *Immunological Reviews* 2006; 213:36-47.

14. Hunt JS, Petroff MG, McIntire RH, Ober C. HLAG and immune tolerance in pregnancy. *FASEB J* 2005; 19:681-693.

15. Ishitani A, Sageshima N, Lee N, Dorofeeva N, Hatake K, Marquardt H, Geraghty DE. Protein expression and peptide binding suggest unique and interacting functional

roles for HLA-E, F, and G in maternal placental immune recognition. *J Immunol* 2003; 171:1376-1384.

16. Ivanyi D, van de Meughevel W. A monomorphic HLA-specific monoclonal antibody, W6/32, reacts with the H-2Db molecule of normal mouse lymphocytes. *Immunogenetics* 1984; 20:699–703

17. Jefferies WA, MacPherson GG. Expression of the W6/32 HLA epitope by cells of rat, mouse, human and other species: critical dependence on the interaction of specific MHC heavy chains with human or bovine β 2-microglobulin. *Eur J Immunol* 1987; 17:1257–63

18. Joyce S, Tabaczewski P, Angeletti RH, Nathenson SG, Stroynowski I. A nonpolymorphic major histocompatibility complex class Ib molecule binds a large array of diverse self-peptides. *J Exp Med* 1994; Feb 1; 179(2):579-588.

19. Kahn-Perles B, Boyer C, Arnold B, Sanderson AR, Ferrier P, Lemonnier FA. Acquisition of HLA class I W6/32 defined antigenic determinant by heavy chains from different species following association with bovine β 2-microglobulin *J Immunol* 1987; 138:2190–2196

20. Kievits F, Ivanyi P. Monomorphic anti-HLA monoclonal antibody (W6/32) recognizes polymorphic H-2 heavy chain determinants exposed by association with bovine or human but not murine β 2-microglobulin. *Hum Immunol* 1987; 20:115–126

21. Lee N, Llano M, Carretero M, Ishitani A, Navarro F, Lopez-Botet M, Geraghty DE. HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. *Proc Natl Acad Sci USA* 1998; 95:5199-5204.

- 22.** Lee N, Goodlett DR, Ishitani A, Marquardt H, Geraghty DE. HLA-E surface expression depends on binding of TAP-dependent peptides derived from certain HLA class I signal sequences. *J Immunol* 1998; May 15; 160(10):4951-4960.
- 23.** Lee N, Malacko AR, Ishitani A, Chen MC, Bajorath J, Marquardt H, Geraghty DE. The membrane-bound and soluble forms of HLA-G bind identical sets of endogenous peptides but differ with respect to TAP association. *Immunity* 1995; Nov; 3(5):591-600.
- 24.** LeMaout J, Krawice-Radanne I, Dausset J, Carosella ED. HLA-G1-expressing antigen-presenting cells induce immunosuppressive CD4⁺ T cells. *Proc Natl Acad Sci USA* 2004; 101(18):7064-7069.
- 25.** Lindaman A, Dowden A, Zavazava N. Soluble HLA-G molecules induce apoptosis in natural killer cells. *Am J Reprod Immunol* 2006; 56:68-76.
- 26.** Maziarz RT, Fraser J, Strominger JL, Burakoff SJ. The human HLA-specific monoclonal antibody W6/32 recognizes a discontinuous epitope within the $\alpha 2$ domain of murine H-2Db. *Immunogenetics* 1986; 24:206-208
- 27.** Miller JD, Weber DA, Ibegbu C, Pohl J, Altman JD, Jensen PE. Analysis of HLA-E peptide-binding specificity and contact residues in bound peptide required for recognition by CD94/NKG2. *J Immunol* 2003 Aug 1;171(3):1369-1375.
- 28.** Naji A, Le Rond S, Durrbach A, Krawice-Radanne I, Creput C, Daouya M, Caumartin J, LeMaout J, Carosella ED, Rouas-Freiss N. CD3⁺CD4^{low} and CD3⁺CD8^{low} are induced by HLA-G: novel human peripheral blood suppressor T-cell subsets involved in transplant acceptance. *Blood* 2007; 110(12):3936-3948.

- 29.** Park GM, Lee S, Park B, Kim E, Shin J, Cho K, Ahn K. Soluble HLA-G generated by proteolytic shedding inhibits NK-mediated cell lysis. *Biochem Biophys Res Commun* 2004; 313:606-611.
- 30.** Poehlmann TG, Schaumann A, Busch S, Fitzgerald JS, Aguerre-Girr M, Le Bouteiller P, Schleussner E, Markert UR. Inhibition of term decidua NK cell cytotoxicity by soluble HLAG1. *Am J Reprod Immunol* 2006; 56(5-6):275-285.
- 31.** Rammensee HG, Falk K, Rötzschke O. Peptides naturally presented by MHC class I molecules. *Annu Rev Immunol* 1993; 11:213-44.
- 32.** Rötzschke O, Falk K, Wallny HJ, Faath S, Rammensee HG. Characterization of naturally occurring minor histocompatibility peptides including H-4 and H-Y. *Science* 1990; Jul 20; 249(4966):283-287.
- 33.** Rötzschke O, Falk K, Deres K, Schild H, Norda M, Metzger J, Jung G, Rammensee HG. Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells. *Nature* 1990; Nov 15; 348(6298):252-254.
- 34.** Selmani Z, Naji A, Zidi I, Favier B, Gaiffe E, Obert L. Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce CD4⁺CD25^{high}FOXP3⁺ regulatory T cells. *Stem Cells* Jan; 2008; 26(1):212-222.
- 35.** Shiroishi M, Tsumoto M, Amano K, Shirakihara Y, Colonna M, Braud VM, Allan DS, Makadzange A, Rowland-Jones S, Willcox B, Jones EY, van der Merwe PA, Kumagai I, Maenaka I. Human inhibitory receptors Ig-like transcript 2 (ILT2) and ILT4 compete with CD8 for MHC class I binding and bind preferentially to HLA-G. *Proc Natl Acad Sci USA* 2003; 100:8856-8861.

- 36.** Storkus WJ, Zeh HJ 3rd, Salter RD, Lotze MT. Identification of T-cell epitopes: rapid isolation of class I-presented peptides from viable cells by mild acid elution. *J Immunother Emphasis Tumor Immunol* 1993; Aug; 14(2):94-103.
- 37.** Storkus WJ, Zeh HJ 3rd, Maeurer MJ, Salter RD, Lotze MT. Identification of human melanoma peptides recognized by class I restricted tumor infiltrating T lymphocytes. *J Immunol* 1993; Oct 1; 151(7):3719-3727.

CHAPTER 6

CONCLUSIONS

The first objective of this study was to elucidate and identify whether cattle non-classical major histocompatibility complex class I (MHC-Ib) proteins are expressed as cell-surface and/or secreted isoforms. Transfection studies were conducted to express two classical MHC-I proteins, N*01701 and N*01802, and five non-classical MHC-I proteins, NC1*00401, NC1*00501, NC2*00102, NC3*00101, and NC4*00201, in murine P815 and human K562 cells. We identified that both class Ia and three class Ib proteins, NC1*00501, NC3*00101, and NC4*00201, are expressed on the cell surface. Two additional isoforms, NC1*00401 and NC2*00102, were not detected on the surface of these cells. It is possible that NC1*00401 and NC2*00102 are expressed as secreted isoforms and play important roles in immunosuppression during pregnancy. Nevertheless, all the proteins were detected in crude cell lysates on Western blots. Precipitation of proteins from culture supernatants showed that cell-surface MHC-Ia and MHC-Ib proteins are shed or released from the surface of these cells into the media.

One of the cattle class Ib proteins, NC4*00201, was identified on the cell-surface of human K562 cell line but not on murine P815 cells, which suggests that this protein associates with human beta-2-microglobulin (β 2m) and conforms better with human β 2m than with murine β 2m in P815 cells.

The second objective of the present study was to develop an enzyme-linked immunosorbent assay (ELISA) to quantitate secreted and/or soluble cattle class Ib proteins from trophoblast cell culture supernatants. We collaborated with the monoclonal antibody center at Washington State University, Pullman and produced monoclonal

antibodies for NC1*00501 and NC3*00101 glycoproteins. We were able to produce a positive and NC3*00101-specific hybridoma cell line secreting monoclonal antibodies to NC3*00101 protein as tested with flow cytometry and ELISA. However, the positive NC3*00101-specific hybridoma could not be revived. Therefore, further efforts need to be planned and conducted in order to produce stable clones of hybridomas.

To advance our understanding of functional importance of the cattle class Ib proteins, we identified the peptide motif of the NC3*00101 protein to generate NC3*00101 tetramers. A tetramer is a complex of four identical MHC-peptide- β 2m complexes linked together with streptavidin and a fluorochrome. Leukocytes are stained with tetramers to identify, enumerate, isolate and *in situ* stain antigen-specific T cells. As a prerequisite to constructing NC3*00101-tetramers, we eluted and identified the peptide motif of NC3*00101. First, we isolated NC3*00101 protein in its native conformation with bound peptide by immunoprecipitation and eluted the peptide with acid treatment. However, immunoprecipitation did not work as seen with poor results of liquid chromatography mass-spectrometry (LC-MS). Therefore, we switched to citrate shock method and treated NC3*00101-transfected K562 cells with low pH-citric acid to elute and released peptides from the MHC proteins on the cell-surface. With the help of LC-MS, we identified nonamers which were candidate peptides (EVTNQLVVL, LVDGVKRIL, SSKIVGDLA, and GSILSGTAIA) for the NC3*00101 protein. These peptides were ordered in synthetic forms from GenScript and tested for their specificity to refold NC3*00101-heavy chain with a biotin tag and bovine β 2m. We tested each of the four peptides to refold NC3*00101 heavy and β 2m chains. Western blots with W6/32 and anti-mouse β 2m antibodies failed to detect refolded chains. We were not successful

in detecting refolded proteins with Western blots. We further tested these peptides by peptide binding assays. Transfected NC3*00101 cells were incubated with peptides and analyzed to identify their effects on NC3*00101 expression. Due to inconsistent and varying results of peptide binding assays, we analyzed the effects with a mixed procedure of statistical analysis software (SAS). With the statistical analysis performed on mean fluorescence values of transfected cells incubated with or without peptides, we identified that EVTNQLVVL induces a significantly different response compared with control cells and is a potential peptide motif of NC3*00101 protein. By understanding the peptide ligands of non-classical class I NC3*00101 protein, we will be able to generate the tetramers for this protein. Tetramers are valuable tools to identify the antigen-specific leukocytes that bind to MHC proteins. NC3*00101 tetrameric complexes will be very helpful in identifying the antigen-specific NC3*00101-specific leukocytes which will further give an insight into different receptors expressed by the leukocytes that bind with NC3*00101 and how the NC3*00101-receptor interaction results in immune tolerance during pregnancy.

CURRICULUM VITAE

Parveen Parasar

PhD Candidate
 Department of Animal, Dairy and Veterinary Sciences
 Utah State University
 Logan, UT 84322

Email: suprovvet@gmail.com
 Phone: (435) 797-0995

EDUCATION

Utah State University, Logan, UT **PhD, 2007- Present**
Reproductive Immunology

Washington State University, Pullman, WA **PhD, 08/2005-8/2007**
 Department of Veterinary Microbiology & Pathology

PhD Dissertation: “Determination of the Expression Patterns of Bovine Non-Classical Major Histocompatibility Complex (MHC) Class I Proteins”

Indian Veterinary Research Institute, Izatnagar (India) **MVSc, 2005**
 Division of Animal Genetics and Breeding, **Genetics/Biochemistry**

Thesis: “DQA and DQB polymorphism and their association with mastitis in cattle”

College of Veterinary & Animal Science, Bikaner (India) **BVSc & AH 2003**
 Bachelor of Veterinary Science & Animal Husbandry

AWARDS AND HONORS

- AmeriCorps Segal Education Award 2013
- Recipient Cache Valley Cooperative Dairy Society Scholarship 2012
- Recipient Graduate Research Dissertation Fellowship 2012
- Runners-Up Graduate student research symposium, USU, Logan 2009
- Recipient Junior Research Fellowship, IVRI, Izatnagar, India 2003

RESEARCH EXPERIENCE & LABORATORY SKILLS

Graduate Research Assistant, Utah State University and Washington State University

- **Isolation of peripheral blood mononuclear cells (PBMCs) from blood samples**
- Isolated fetal trophoblast cells from placenta
- Harvested, purified, cryopreserved, processed, cultured, and stored fetal trophoblast cells

- Peptide isolation using HPLC and performed peptide binding assays on transfected cells.
- Purified bovine MHC-I proteins using affinity column-chromatography and purified for generation of monoclonal antibodies at WSU antibody center.
- Experimented on mouse P815 cells, human K562, IL-A88 and IL-A19 hybridomas utilizing mammalian cell culture techniques.
- Investigated and monitored transfection in mouse P815 and human K562 (CCL-243) cells utilizing Lipofectamine and electroporation techniques.
- Conducted and examined DNA cloning and protein expression in eukaryotic and prokaryotic cells.
- Evaluated and interpreted extracellular and intracellular flow cytometry-staining.
- Sorted CD4 and CD8-T cells, dendritic cells, macrophages, natural killer leukocytes with the help of FACS-sorter.
- Identified the specific antibody secreting hybridomas by screening the supernatants by flow cytometry, ELISA (Direct and Indirect), SDS-PAGE, Western blotting.
- Conducted immuno-precipitation with both Protein A and Protein G-Dynabeads and Sepharose beads to purify IL-A88 monoclonal antibody
- Designed primers for genes, performed DNA amplification by PCR and cloned in bacterial and eukaryotic cells.
- Conducted DNA, RNA isolation, Sanger sequencing, PCR, real time RT-PCR, and other molecular biology techniques.
- Identified the peptides which bind bovine MHC class Ib protein with the help of MALDI-tof and LC-MS/MS. Performed peptide-binding assay on P815, K562, and T2 cell lines.
- Experimented to fractionate and purify peptides using C18 RP-HPLC and zip-tip methods.
- Monitored and conducted scale-up of mammalian cells using pitched-blade bioreactor.
- Planned and produced tetrameric complexes (tetramers) for bovine class I proteins and stained the bovine PBMC with the tetramers to identify lymphocytes.
- Acquired experiences of working closely with scientists and staff in cell culture research programs which gave me interaction and discussion skills.

TRAININGS ATTENDED

- Attended the 31st annual meeting (Conference) for American Society for Reproductive Immunology held from May 19-22, 2011 at Salt Lake City, Utah.
- Participated in BD Biosciences' Flow Cytometry Workshop held at University of Utah, Salt Lake City.
- Participated in Animal Cell Culture training held at the Center for Integrated Biosystems (CIB) at USU from November 16-19, 2010 on techniques in animal cell culture and scale-up strategies.
- Participated and assisted in teachers' symposium held at the Center for Integrated Biosystems (CIB), Utah State University.

SEMINAR TALKS

- **Parasar, P.**, and Davies, C.J. Bovine trophoblast non-classical MHC class I proteins and their role in inhibition of natural killer cells and other leukocytes, 2012 & 2009, Annual ADVS graduate Student Symposium Utah State University, Logan (Oral Presentation) (Received second position award for the oral presentation).

PUBLICATIONS

- **Parasar P**, New DD, Suarez C, Wilhelm A, Davies CJ. Bovine Third-Trimester Interplacentomal Trophoblast Cells Express Cell Surface and Secreted Classical and Non Classical MHC Class I Proteins (**In Preparation**).
- **Parasar P**, Wilhelm A, Davies CJ. Identification of peptide motif of non-classical bovine leukocyte antigen (BoLA)-NC3*00101 protein (**In preparation**).
- **Parasar P**, Barnett S, Wilhelm A, Rashid K, Davies CJ. Large-Scale Growth of Mouse P815 Cells Expressing a Bovine Non-Classical Major Histocompatibility Complex Class I Protein Utilizing a Pitched-Blade Bioreactor, *BioProcess J*, **2012**; 11(3): 27-34.
- **Parasar P**, Bhushan B, Yadav JS, Sharma A, Dutt T. DNA polymorphism of major histocompatibility complex Class II DQA and DQB genes in crossbred cattle (*Bos taurus* × *Bos indicus*) *Indian Journal of Animal Sciences*, **2011**; 81 (7): 769–772.
- **Parasar P**, Suarez C, New DD, Davis WC, **Davies CJ**. In vitro expression of bovine classical and non-classical MHC class I proteins. Program and Book of Abstracts 8th International Veterinary Immunology Symposium, Ouro Preto, Brazil. August 15-19, **2007**, pp. 80-81 **Abstract**
- **Davies CJ**, Wilhelm A, Sessions BR, **Parasar P**, Li Y, Schmidt EE, White KL. Expression of non-classical MHC class I genes by cultured bovine embryos. Proceedings of the 28th Annual Meeting of the American Society for Reproductive Immunology, Chicago, Illinois, June 11-14, **2008**, p. 23 **Abstract**
- **Davies CJ**, Wilhelm A, Sessions BR, **Parasar P**, Li Y, Schmidt EE, White KL. Expression of non-classical MHC class I genes by bovine embryos. Proceedings of the XXXI Conference of the International Society for Animal Genetics, Amsterdam, The Netherlands. July 20-24, **2008** **Abstract**
- Bhushan, B., Patra, B. N., **Parasar, P.**, Kumar, P., Bhattacharya T.K., Sharma, Arjava and Dandapat, S. 2005. Characterization of MHC class I haplotypes of river buffalo by PCR based cloning and sequencing. National symposium on “Domestic animal diversity: Status, opportunities and challenges” held at Karnal (India) from February 10 - 11, **2005** **Abstract**