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Gene Expression Analysis of Bovine Peripheral Blood Mononuclear Cells in Response to Adenovirus-Vectored Foot-and-Mouth Disease Vaccine

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Gene Expression Analysis in Bovine Peripheral Blood Mononuclear Cells in Response to Adenovirus-Vectored Foot-and-Mouth Disease Vaccine

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Abstract

Foot-and-mouth disease virus (FMDV) gives rise to a highly contagious and economically important disease of cloven-hooved animals. Vaccination reduces the economic impact by inducing serotype-specific protection. Recently, a replication-defective adenovirus-vectored foot-and-mouth disease (FMD) subunit vaccine was developed and licensed. Serum virus neutralization (SVN) titer ≥1.5 to FMDV is the best predictor of vaccine-induced protection. However, protection does not always correlate with the presence of neutralizing antibodies. For example, some animals with high SVN titer develop signs of disease, and conversely, some animals with negligible SVN titer are protected. Categorizing cattle on the parameters of seroconversion and protection status yields four groups of cattle. Two of these groups are the expected outcome, protected with SVN titer ≥1.2 and unprotected with SVN titer <1.2. The other two groups are the rare but unexpected outcome, unprotected with SVN titer ≥1.2 and protected with SVN titer <1.2. The current study examines these rare but unexpected outcomes. Studying these unexpected outcomes may enhance our understanding of vaccine-induced protection in cattle administered the replication-defective Ad-5 vectored FMDV subunit vaccine (Ad-5 FMDV). Additionally, an alternative indicator of protection may provide additional clues as to the mechanism of immune protection that affords protection in animals with low SVN titers, and ultimately may lead to improvements in vaccine design. I hypothesized that gene expression analysis of bovine peripheral blood cells would provide a genomic tool for predicting Ad5-FMDV vaccine efficacy for foot-and-mouth disease. Thus, I sought to identify genes associated with protection after vaccination with Ad-5 FMDV.

Microarray-based analysis of mRNA transcripts from peripheral blood leukocytes of vaccinated cattle (n=21), drawn on 0, 1, 14 and 15 days post-vaccination, revealed that in response to Ad-5 FMDV, protected cattle (n=5) demonstrated a rapid but short-lived induction of stress-related genes. Vaccination with the adenovirus vaccine vector alone, ad-null, (n=4) resulted in a similar, but less robust, expression pattern, whereas unprotected cattle (n=4) exhibited an initially mild, but increasing expression profile of stress-related genes. A subset of 12 immune response-related genes was verified by RT-qPCR. A separate list of genes that correlated with protection was identified. One gene in particular, CCL8, an inflammatory mediator, resulted in a transient up-regulation that was associated with protection, whereas vaccinated, but unprotected animals, exhibited a pattern of prolonged expression that did not return to baseline levels. Rapid but transient induction of the CCL8 gene in Ad5-FMDV vaccinated cattle correlated with protection, irrespective of SVN titer. These microarray-based results were verified with RT-qPCR using a *TaqMan*™ probe. Capture ELISA was unable to detect the CCL8 protein in plasma. This small study was expanded to a larger cohort of cattle (n=39) utilizing RT-qPCR. From the 32 protected cattle in this expanded study, I was unable to conclude that transient up-regulation of CCL8 mRNA following vaccination correlated with protection. Thus, although CCL8 transient up-regulation could not be confirmed as a sole correlate of immune protection, it may be possible that transient up-regulation of CCL8 mRNA contributes to protection with other mechanistic immune functions that supplement or co-correlate with protection. Perhaps transient up-regulation of CCL8 is one of multiple biomarkers that contribute to protection. Once the biological underpinnings of protection

are better understood perhaps these complex correlates of vaccine-induced protection will be readily identified.

Introduction

Foot-and-Mouth Disease

Foot-and-mouth disease (FMD) is highly contagious and economically devastating, affecting wild and domestic cloven-hoofed animal species [1]. The acute viral-borne disease is characterized by vesicular lesions on the tongue, snout, buccal cavity, feet and teats [2]. The incubation period in individual animals is highly variable ranging from 1-14 days and is dependent on the strain and dose of virus, the route of transmission, the animal species and the husbandry conditions [3]. FMD generally resolves within 7-10 days without treatment. Adult convalescent animals may serve as carriers of FMDV and potentially initiate new outbreaks [4-7]. Carrier animals for FMD are animals, which have a persistent unobvious infection, from which it is possible to isolate infectious FMDV intermittently 28 days or more after infection. Fatal cases are rare in adult animals but may occur in young animals due to myocarditis [8]. Thus, the morbidity, high transmissibility, wide dissemination and significant economic impact makes FMD among the most feared livestock diseases and a major research focus for more than a century.

FMD is the first disease for which the *world organization for animal health* (OIE) (formerly known as the Office International des Epizooties) established an official list of FMDV-free countries and zones [9]. It was recently estimated that the visible production losses and vaccination costs in endemic countries alone is between US$6.5 and 21 billion. Additionally, outbreaks in FMD free countries may cause losses of US$1.5 billion per year [10]. The economic importance of being officially recognized by the OIE as a member country to the *free of FMD without using vaccination* list allows these

countries to freely export animals and animal products. The use of vaccination during an outbreak in a previously FMD free zone in order to regain disease-free status requires at least three more months when a vaccinate-to-live policy is enacted compared to stamping out or slaughter of vaccinated animals [11]. The reason for this delay might result from difficulty distinguishing infected from vaccinated animals (DIVA) and that vaccinated animals exposed to the virus may remain asymptomatic but become virus carriers potentially initiating new outbreaks [4] further complicating the process of regaining FMD-free status.

The causative agent, foot-and-mouth disease virus (FMDV) is the type species of the genus *Apthovirus* in the family Picornaviridae [2]. FMDV consists of seven serotypes (A, O, C, SAT 1-3 and Asia1) and multiple strains within each serotype, which differ antigenically. Infection with one serotype usually results in protection from subsequent exposure to the same serotype but affords little, if any, cross-protection with other serotypes. Its genome is comprised of a single-stranded positive-sense RNA of about 8.5 kb encoding a single long open reading frame (ORF). The polyprotein is processed post-translationally to yield intermediate and mature structural and nonstructural (NS) proteins. The icosahedral protein capsid contains 60 copies each of 4 structural proteins (VP1–4), whose assembly is dependent on NS viral protein $3C^{pro}$ cleavage of the structural protein precursor, P1-2A [12].

The innate immune system, the first line of immune defense, is comprised of cells and mechanisms that defend the host from infection by FMDV in a non-specific manner through interactions of conserved viral motif with pattern recognition receptors. The innate immune responses are responsible for recruiting cells to the site of infection

through cytokines. These recruited cells are then responsible for activating the adaptive immune response, discuss below, through the process of antigen presentation. Disease occurs when FMDV succeeds in overwhelming innate host responses to establish a local site of infection, and then replicates there to allow its further transmission within the host. Infection of cattle generally occurs via the respiratory route by aerosolized virus but can also occur through abrasions on the skin or mucous membranes [13]. FMDV infects and replicates efficiently in epithelial cells, which contribute to the innate immune response through the production of interferons, chemokines and other cytokines. Recruitment of immune cells to the site of infection is mediated through chemokines [14]. In the current work, FMDV appears to induce expression of the CCL8 gene, an inflammatory mediator. The gene product of CCL8 is a chemokine that attracts and activates leukocytes. It is possible that the early induction of CCL8 in the protected cattle results in the recruitment of phagocytic antigen presenting cells to the site of infection where they encounter FMDV antigens. The reduction in gene expression of CCL8 at later time points might correlate with recruitment of these phagocytic antigen presenting cells to secondary lymphoid tissues, such as lymph nodes. These cells may then encounter the cognate receptor on a specific B or T lymphocyte for the antigen they are presenting. This antigen presentation can initiate the adaptive immune response which may result in protection from subsequent exposure. Genome replication and virion assembly occurs in the infected cell cytoplasm [14]. FMDV replication outpaces the host innate immune response because FMDV is able to shut down host de novo protein synthesis [14]. Epithelial cells contribute to the innate immune response to FMDV through the release of cytokines, including interferons. FMDV is highly susceptible to the action of interferon

type I in vitro [15] and in vivo [16]. Innate immune defenses can control FMDV, but it is still unclear to what extent such responses are induced during natural infection. Rapid control of viremia and early induction of adaptive immune responses would indicate innate immune responses are active. However, the characterization of such responses are not yet comprehensive.

Virus clearance is dependent on specific neutralizing antibodies [17, 18], which are directed towards epitopes on the three external structural proteins [2]. Vaccine-induced antibodies confer protection by neutralization and/or opsonization. In so doing, preventing entry into target cells and mediating degradation of virus by phagocytic cells through Fc receptor mediated uptake respectively [19, 20]. Vaccine-induced serotype-specific protection is generally measured as a function of FMDV-specific neutralizing antibody titer [21, 22]. However, protection from disease has been reported in the absence of serum virus neutralization (SVN) titer [19, 21, 23], and vaccinated animals with medium to high neutralizing antibody titers may not always be protected [21]. Although neutralizing antibodies are an important component of vaccine-induced protection [24, 25]; other mechanisms of antibody-dependent protection likely exist that may inhibit viral maturation or release without affecting viral entry; thus, do not neutralize in vitro [25-28] but contribute to protection from reinfection or infection in the case of vaccination.

The implication of cell-mediated immune responses in terms of protective immunity to FMDV remains unclear, though it has been suggested that secretion of IFN-γ, a marker for development of cellular immunity which also has antiviral properties, may be important in controlling the virus in at least some animals [29, 30]. Additionally, the

enhanced efficacy of an Ad5-vectored FMD subunit vaccine correlated with an increase in IFN-γ producing T-cells following challenge when compared to challenged controls [31]. Although an MHC class I-restricted $CD8^+$ T-cell response is induced by FMDV and inactivated FMDV [32], the correlation of circulating effector and memory $CD8^+$ T-cells specific for FMDV and protection from disease remains to be well described [32]. The role of vaccine-induced antibodies in preventing infection for this intracellular pathogen may be more important than cellular immune functions. Thus, the immune response to FMDV and vaccination is likely shifted towards a Th2 response. Accordingly, a gap in our understanding of vaccine-induced protection following viral challenge exists and further understanding of the host's immune response to vaccination and/or viral challenge may enhance progress towards development of effective tools and countermeasures for FMD.

Traditional Chemically-inactivated FMD Vaccines

Licensed traditional vaccines that induce immunity to FMD are chemically inactivated cell-culture-derived whole virus preparations emulsified with adjuvant [2, 33]. These vaccines are most commonly used in enzootic areas and have been successful in reducing outbreaks worldwide [2, 33, 34]. Standard potency commercial vaccines are formulated with enough antigen and adjuvant to provide a minimum potency level of 3 PD_{50} (50% protective dose) and provide six months of immunity when two initial doses are administered a month apart. Higher potency emergency vaccines are recommended for vaccination in naïve populations for wider range of immunity along with rapid induction of protection. These emergency vaccines are formulated to have a minimum potency level of 6 PD_{50}.

The limitations of these vaccines include difficulty distinguishing infected and vaccinated animals, the need for high-containment manufacturing facilities to produce vaccine and inability to induce rapid protection [2, 34, 35].

Adenovirus

Adenoviruses are DNA viruses generally causing mild infection in the upper or lower respiratory tract, gastrointestinal tract, or conjunctiva [36]. Its double-stranded DNA genome is approximately 34-43 kb surrounded by a non-enveloped icosahedral protein capsid of approximately 90 nm. The tropism of adenoviruses is determined by their ability to associate with host cell receptors. Most human adenoviruses, including serotype 5, initially bind to the cocksackie adenovirus receptor (CAR) [37], which is expressed on many cell types, although lymphoid cells do not express CAR. Adenoviruses are highly immunogenic activating the innate immune system by expressing pathogen-associated molecular patterns (PAMPs). These PAMPs bind pattern recognition receptors (PRRs) on host cells, which includes receptors of the innate immune system, inducing proinflammatory cytokines and differentiation of immature dendritic cells into professional antigen presenting cells (APCs) [38].

Adenovirus-vectored Vaccines

Adenoviruses have an efficient cell entry mechanism, are able to propagate to high titer and are capable of eliciting T and B cell responses to antigens encoded as a transgene product and the adenovirus vector itself [39-46]. Adenoviruses have demonstrated high efficiency as vehicles for introducing foreign DNA into target cells. Taken together, the intrinsic immunogenicity and ease of transducing foreign DNA, these features accompanied by extensive knowledge of adenovirus molecular biology and

methods for manipulating the viral genome that are now available, make adenoviruses appealing candidates for vaccine vector development [46].

Human Adenovirus-Vectored FMD Vaccines

Traditional approaches to vaccine development for FMD is problematic for a number of reasons. Manufacturing these vaccines in countries where FMD is eradicated presents a moderate risk due to the possibility of incomplete inactivation or virus escape from high-containment vaccine-producing facilities. Traditional vaccines are generally incompatible with DIVA assays. Additionally, since FMDV is an RNA virus, repeated serial passage in culture would lead to antigenic variation making serial passage attenuation unappealing for vaccine development. The use of adenovirus vectors continues to eliminate the limitations of traditional approaches to FMD vaccine. The advantage of adenoviral-vectors is its ability to bind to and internalize in many cell types. This feature guarantees rapid uptake and expression of desired genes. Thus, acting as an efficient vehicle for transducing genetic material.

A recombinant replication-defective human adenovirus serotype 5 vector containing FMDV capsid, P1-2A, and viral 3C protease coding regions was developed [35]. This construct was formulated with *VaxLiant*™ adjuvant [47] and was recently granted a conditional license [48]. This vaccine, Ad5-FMDV, has demonstrated to improve on some of the limitations of traditional FMD vaccines by allowing DIVA and they can be manufactured in the U.S. without the need for a high-containment facility [49].

Research Aim

Categorizing cattle on the parameters of seroconversion and protection status yields four groups of cattle. Two of these groups are the expected outcome, protected with SVN titer ≥1.2 and unprotected with SVN titer <1.2. The other two groups are the rare but unexpected outcome, unprotected with SVN titer ≥1.2 and protected with SVN titer <1.2. The current study examines these rare but unexpected outcomes. I sought to investigate the bovine immune response to Ad5-FMDV. In this study, I used microarray-based analysis to examine the transcriptional response to Ad5-FMDV vaccination, adenovirus vector (ad-null) and FMDV challenge in bovine peripheral blood cells. I hypothesized that this method would provide a genomic tool for predicting vaccine efficacy in FMD vaccination and offer new insight into the immune response to vaccination and viral challenge.

2. Methods

2.1 Animals and Treatment

Holstein steers approximately 6 months old and weighing approximately 150 kg were housed in accordance with BSL3-Ag requirements. Procedures were conducted in accordance with the guidelines of the institutional animal care and use committee (protocol numbers 196-D-11, 232-11-D). Animals were administered a monovalent adenovirus-vectored foot-and-mouth disease (FMD) vaccine[48] with adjuvant (ENABL™, VaxLiant, Lincoln, NE) via IM route. Fourteen days post-vaccination (14dpv); animals were challenged with 10^4 $TCID_{50}$ homologous virus via intradermal lingual (IDL) route [11]. Following viral challenge, animals were observed for signs of disease, defined as presence of pedal lesions within 14 days. Serum virus neutralization (SVN) test was performed according to the OIE standard [11]. Samples were categorized as high titer if the neutralizing titer was ≥1.2 and samples were categorized low titer if SVN titer was <1.2.

2.2 Study Design

I examined transcripts from peripheral blood cells at four sampling time points (fig. 2). Blood was collected on the morning prior to vaccination, 24 hours following vaccination, on the morning just prior to challenge (14 days post-vaccination) and 24 hours following challenge. RNA recovered from PBMCs was assessed for purity and integrity prior to any further downstream analysis. Gene expression analysis from two-color microarray hybridization was performed using an intensity-based approach allowing us to make multiple comparisons [50-52].

2.3 Sample Collection

Blood was drawn during routine husbandry and collected in a vacutainer venous blood collection plasma separating tube with polymer gel separator (Becton, Dickinson and Company, Franklin Lakes, NJ, p/n 367964). Blood samples were spun down at 1,200 x g for 10 min at room temperature. Buffy coats were collected using a transfer pipette and placed in a sterile 2 mL screw cap microcentrifuge tubes containing 1.2 mL RNA*Later* (Life Technologies, Grand Island, NY, p/n AM7021). Microcentrifuge tubes were stored at 4°C overnight to allow *RNA*Later to penetrate cells and then archived at -70°C.

2.4 RNA Recovery

An aliquot of 250 µL was placed in a 1.5 mL microcentrifuge tube containing an equal volume of sterile 1x PBS, mixed by pipetting and spun down at 1,500 x g for 10 minutes at room temperature. The supernatant was removed and RNA recovered utilizing the RNeasy mini kit following the animal cells spin protocol (Qiagen, Valencia, CA, p/n 74106). Samples were further purified and concentrated using the *RNA Clean & Concentrator-5* kit (Zymo Research, Irvine, CA, p/n R1016). The product insert's *general protocol* for total RNA was followed. Samples were then assessed for yield and purity on the NanoDrop ND-1000.

2.5 RNA Integrity Assessment

RNA samples were analyzed on the 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, p/n G2940CA) utilizing the RNA 6000 nano kit (Agilent Technologies, Inc.,

Santa Clara, CA, p/n 5067-1511) according to the manufacturer's instructions. Samples with a minimum RIN of 7.5 were utilized in a microarray-based gene expression study.

2.6 Microarray: RNA Amplification and Labelling

Starting with 50ng total RNA, the *Agilent* two-color microarray-based gene expression analysis version 6.5 (agilent.com) protocol was followed. All incubations were performed utilizing a thermal cycler. The *Agilent* two-color low-input quick amp labeling kit (p/n 5190-2306) was utilized for cDNA synthesis, in-vitro amplification and labelling of nucleic acids. Purification of labelled cRNA was performed with the *Qiagen* RNeasy mini kit (p/n 74104).

2.7 Microarray: Fragmentation and Hybridization

Fragmentation of purified cRNA was performed with the *Agilent* gene expression hybridization kit (p/n 5188-5242). Fragmented cRNA samples were assembled with the Agilent hybridization gasket (p/n G2534-60012), *Agilent* bovine gene expression microarray (p/n G2519F, design id: 023647) and *Agilent* hybridization chamber (p/n G2534A). The assembled hybridization chambers were placed in a rotating hybridization oven set to 65°C with 10 rpm rotation.

2.8 Microarray: Scanning and Feature Extraction

Following washing, the slides were immediately scanned on a GenePix 4000B laser scanner (Molecular Devices). Photo multiplier tube (PMT) gains were adjusted to produce count ratios (cy5/cy3) of approximately 1 (0.9 – 1.1) with no more than $1x10^{-3}$ of

the normalized counts at saturating intensity. Feature extraction was performed with GenePixPro 7 software (Molecular Devices). Microarray images were visually inspected to identify artefacts on features that suggest environmental contaminants and other non-biological fluorescence and excluded them from downstream analysis. Background fluorescence for each feature was determined as the mean of the median values for the five closest negative control features. Intensity values for each feature's channel were calculated as the median fluorescence value minus the calculated background value.

2.9 Microarray-data Analysis

In-silico Experimental Setup:

Each channel's background-corrected median fluorescence value (532 and 635 nm) was used in an intensity-based analysis utilizing *Agilent* GeneSpring software (v.12.5). Feature-level quantile normalization was performed on all the background-corrected intensity values and samples were grouped according to days-post-vaccination, SVN titer and protection status. Features were included in analysis if ≥80% of the features across the replicates were available in all of the time points for a given gene target. Features were excluded if they were at saturating intensity or flagged "bad" in the *GenePix* software. Because some genes were represented by duplicate or triplicate features on the microarray slide, gene-level analysis was performed using mean values of replicate features. Quantile normalization was then performed on the gene-level in-silico experiment.

Identifying Differentially Expressed Genes:

Genes were identified as differentially expressed if there was at least a two-fold change between time points of interest and if those changes were statistically significant (p-value ≤0.05). All grouped samples were representative of four time points (0, 1 days post vaccination and 0, 1 days post challenge). For the purpose of identification of differentially expressed genes, samples were interpreted and grouped according to any of the following: a.) protected/not protected, b.) high SVN/low SVN, c.) protected High SVN/protected low SVN/not protected high SVN/not protected low SVN.

2.10 Real-Time PCR

The primers and probes for *TaqMan* RT-qPCR assay were designed utilizing Beacon Designer 8 (Premier Biosoft International, Palo Alto, CA) with the program's default settings. The internal control genes were selected based on reported stability of transcripts from bovine lymphocytes [53]. PCR reactions were created in triplicate utilizing the *TaqMan* Fast Virus 1-Step Master Mix (Life Technologies, Grand Island, NY, p/n 4444436) at final primer and probe concentrations of 500 nM and 100 nM respectively. Thermal cycling method consisted of the following: 50°C for 20 min, 95°C for 8 min, 50 cycles of [95°C for 15 sec, 60°C for 1 min]. Fold change was calculated via the 2(-delta delta C(T)) method [54].

2.11 Magnetic Activated Cell Sorting

Blood samples from cattle were separated into populations of single cell types according to cell surface markers utilizing the magnetic activated cell sorting system with LS

columns (P/N 130-042-401, Miltenyi Biotec, San Diego, CA) and Goat anti mouse IgG microbeads (p/n 130-048-401, Miltenyi Biotec, San Diego, CA) according to the procedure in the product manual. WC1+ γδ T-cells were positively selected with a mouse anti bovine WC1 monoclonal antibody (p/n IL-A29, VMRD, Pullman, WA). CD14+ cells were then positively selected with a mouse anti bovine CD14 monoclonal antibody (p/n MM61A, VMRD, Pullman, WA). Following these two rounds of cell sorting, T-cells and B-cells were depleted utilizing mouse anti bovine CD3 (p/n MM1A, VMRD, Pullman, WA) and CD21 (p/n GB25A, VMRD, Pullman, WA) antibodies respectively. The remaining population of negatively-selected cells were enriched for natural killer cells.

2.12 Indirect Immunofluorescence Assay

Five hundred μL of diluted cells was applied to a glass slide. Samples were spun down at 800 rpm for 3 minutes in the *cytospin*. Cells were then fixed with ice-cold acetone. Samples were incubated with primary antibody for approximately one hour at 37°C. Primary antibodies were as follows:

- WC1+ γδ T-cells: mouse anti bovine TcR1-N24 (δ chain) (p/n GB21A, VMRD, Pullman, WA)
- CD14+ cells: mouse anti bovine CD172a (p/n MCA2041G, AbD Serotec, Raleigh, NC)
- NK cells: mouse anti bovine CD335 (NKp46) (p/n MCA2365EL, AbD Serotec, Raleigh, NC)

Following washing, samples were incubated with secondary antibodies for one hour at 37°C. Secondary antibody was goat anti mouse conjugated with either Alexa Flour® 594 (p/n A-21145, Life Technologies, Grand Island, NY) or Alexa Flour® 488 (p/n A-11017, Life Technologies, Grand Island, NY). Following washing and air drying, ProLong® Gold antifade reagent with DAPI (p/n P-36931, Life Technologies, Grand Island, NY) was applied.

2.13 ELISA

Capture ELISA for CCL8 (MCP-2) was performed using plasma from cattle to determine if this protein could be detected in vivo. The procedure in the product manual was followed (p/n sE90088Bo, USCN Life Science Inc., Houston, TX).

3. Results

3.1 RNA Assessment

RNA samples from PBMC passing the RNA quality criteria [A260/280 ratio = 2.0 ± 0.2; RNA integrity number (RIN) > 7.5 as assessed using the eukaryotic RNA 6000 kit on the Agilent 2100 Bioanalyzer] were utilized for microarray-based gene expression analysis or RT-qPCR. The average 260/280 ratio was 2.08 and the average RIN was 9.3.

3.2 Indirect Immunofluorescence Assay

Light microscopy visual inspection of slides revealed >90% of the adhered cells were positively-selected WC1+ γδ T-cells. Similar results were observed with positively selected CD14+ cells. Visual inspection of the slides for the negatively-selected cell population revealed approximately 60% of the adhered cells were CD335+ (NKp46) natural killer cells.

3.3 Microarray-based gene expression analysis

Following amplification and labelling, cRNA samples were purified and assessed on the NanoDrop to determine yield and incorporation of cyanine dye (specific activity). Median background-corrected intensity values from microarray features were imported into GeneSpring software (v.12.5) and grouped according to clinical outcome (protected/not protected) with an additional group for cattle receiving the ad-null vaccine vector lacking the transgene. Differential expression was determined between time points representing the innate (0,1 days post vaccination), adaptive (0,14 dpv), immunoregulatory (1,14 dpv) and viral responses (0,1 days post challenge)(fig 2). An

arbitrary group of 12 gene transcripts was validated using RT-qPCR and the expressions patterns were similar to those obtained from microarray-based analysis. Among the protected group, chemokine (C-C motif) ligand 8 (CCL8) was the most up-regulated gene at 1 dpv. Unprotected cattle demonstrated a mild increase of CCL8 following vaccination. Following this increase in protected cattle at 1dpv, CCL8 expression levels returned to baseline prior to viral challenge whereas the converse was observed in unprotected cattle. Thus, I considered the possibility of using this transcript as an indicator of vaccine-induced protection.

The CCL8/MCP-2 gene expression pattern in cattle administered the Ad-null vaccine had a similar pattern as the protected cattle administered the vaccine candidate. These ad-null vaccinated cattle seroconverted to the viral vector and as expected, were not protected from FMD. Whereas 18/21 of cohorts in the same study that were administered the vaccine candidate were protected from FMD (data not shown). The adenovirus vaccine vector induces a primary innate immune response thereby increasing expression of inflammatory mediators setting the stage for an efficacious adaptive immune response to the transgene product. This microarray-based observation suggests that our reported expression profile for CCL8/MCP-2 correlates with protection.

An additional microarray-based gene-expression study was performed on cattle administered a traditional inactivated vaccine for comparison to Ad-5 FMDV. The number of genes induces by vaccination with a traditional inactivated FMD vaccine is much fewer than compared to an adenovirus-vectored FMD vaccine. Additionally, sorted

cells into one of three individual cell types and observed gene expression patterns in WC1+ γδ T-cells, CD14+ cells and CD335+ NK cells that were different than the gene expression pattern in the heterogeneous PBMC population.

3.4 RT-qPCR

Real-time quantitative PCR using a *TaqMan* probe was performed as an independent assay to verify microarray-based gene expression results. Multiple internal control genes were selected based on reported stability of internal control genes from bovine lymphocytes [53]. Twelve immune response genes were chosen because of their robust changes in gene expression in the protected cattle that demonstrated a low SVN titer, suggesting an important role contributing to protection. The mRNA expressions of these 12 chosen immune response genes demonstrated similar behavior as detected expression changes by microarray analyses. Because of limited biological samples available, RT-qPCR verification of microarray data was unable to be performed on some of the samples.

An additional RT-qPCR study was performed with multiplexed primers for the CCL8 and RPS24 gene to increase the sample size into the investigation of the correlation between protection and relative gene expression patterns of the CCL8 gene. This study included 32 protected animals and 7 unprotected animals which were not previously analyzed by microarray-based analysis. In addition, 4 ad-null control animals which were previously analyzed were also included. I found that the correlation of CCL8 transient up-regulation and protection was not observed in this larger sample set.

3.5 ELISA

The sensitivity of this commercially available kit was 6.4 pg/mL with a detection range of 15.625–1000 pg/mL. Utilizing the standard in the kit, the standard curve had a R^2 value of 0.9859. The plasma from a representative sample all cattle analyzed via microarray was assessed with this kit. There was no signal detected above background from any of the cattle administered the Ad5-FMDV. The protein levels of CCL8 in plasma from cattle administered an ad-null vaccine was decreasing. The protein levels for cattle administered sham vaccination with formulation buffer demonstrated an increase in protein levels.

4. Discussion

FMD is one of the most economically devastating diseases of the livestock industry worldwide. Production losses have a large impact where the poorest are most dependent on livestock. Direct losses limit livestock productivity degrading food security. Countries with ongoing control programs spend a lot of money on FMD control and management. The threat, let alone presence, of FMD prevents access to lucrative international markets [10]. Although the U.S. has been free of FMD since 1929, the presence of FMD in a number of FMD-free countries and the potential as a bio-terrorist threat have significantly increased public awareness of, and interest in this disease [55-58].

Although traditional inactivated FMD vaccines have been efficacious in reducing outbreaks worldwide, the need for high-containment facilities to manufacture, incompatibility with DIVA assays and the need to stockpile large quantities of doses for emergency purposes make traditional vaccine approaches unfavorable. Adenovirus-vectored FMD (Ad-5 FMD) vaccines, improve on some of the short-comings of traditional inactivated FMD vaccines by allowing DIVA and do not require high-containment facilities for manufacturing. Additionally, the adenovirus vaccine vector can be quickly manufactured with the coding region from any serotype, thereby circumventing the need to stockpile many doses.

Vaccines that provide long-lasting immunity stimulate both the innate and adaptive arms of the immune system. The innate immune system is responsible for shaping the nature of the adaptive immune response in terms of its intensity, duration and tailoring of immunological memory. The interplay between antigen presenting cells, B

cells, T helper and killer subtypes, and regulatory T- and B-cells responses is critical for generating a robust immune response and immunological memory against infectious diseases [59-61]. Adjuvants for vaccines should be strategically selected to elicit some of these specific observations that are responsible for shaping the immune response to vaccine + adjuvant that leads to immunity.

The importance of vaccine-induced FMD virus-specific neutralizing antibodies is well known. However, protection from disease does not always correlate with presence of specific neutralizing antibodies [19]. Transcriptomic analysis, with DNA microarray technology, of purified peripheral blood mononuclear cells (PBMCs) reveals that FMD-protected cattle demonstrate a transient induction of genes in response to Ad-5 FMDV vaccination that gene ontology suggests were associated with a stress response. One gene in particular was selected to be more closely studied. Monocyte chemoattractant protein 2 (MPC-2), a chemotactic cytokine that activates many types of immune cells involved in the inflammatory response, was assayed in a larger group of cattle (n=39) not previously studied with DNA microarray.

The reported expression profile of CCL8/MCP-2 appears to correlate with the profile of the immune response to the adenovirus vaccine vector. I expected the adenovirus vector to be immunogenic, as it was, and when the vector was combined with the FMDV transgene product, cattle developed immunological memory that seemed to be denoted at the gene expression level by a more robust immune response compared to ad-null. The gene expression levels of CCL8/MCP-2 correlated with the intensity of the immune response, suggesting that when CCL8/MCP-2 levels do not significantly increase following vaccination, the immune response to the vaccination was insufficient

to confer protection. The use of RT-qPCR to profile gene expression of CCL8/MCP-2 has the potential to enhance the prediction of vaccine-induced protection when used as an adjunctive assay along with SVN.

When this study was expanded to increase the sample size, the correlation was not observed in this larger sample size. Although I also investigated a shorter time point following vaccination (7dpv) in order to determine if the previous correlation in the microarray-based study occurred prior to 14dpv, that data merely added an additional time point.

The significance of this work is that it outlines a systematic process for investigating the immune response to vaccination through a transcriptomics approach. A list of genes that correlated with protection was generated (Table 3) and one gene, CCL8/MCP-2, was chosen for a thorough examination. Although this additional study did not come to fruition, there are other genes on that list that may be of significance. It may be possible that transient up-regulation of CCL8 mRNA contributes to protection with other mechanistic immune functions that supplement or co-correlate with protection. Perhaps transient up-regulation of CCL8 is one of multiple biomarkers that contribute to protection. Once the biological underpinnings of protection are better understood perhaps these complex correlates of vaccine-induced protection will be readily identified.

Although the role of neutralizing antibodies should be considered in the context of factors that correlate with protection in alternative, additive, or synergistic ways rather than the sole correlate of protection [62], at present SVN titer is still the most reliable assay readout to predict vaccine efficacy prior to, or in the absence of, viral challenge.

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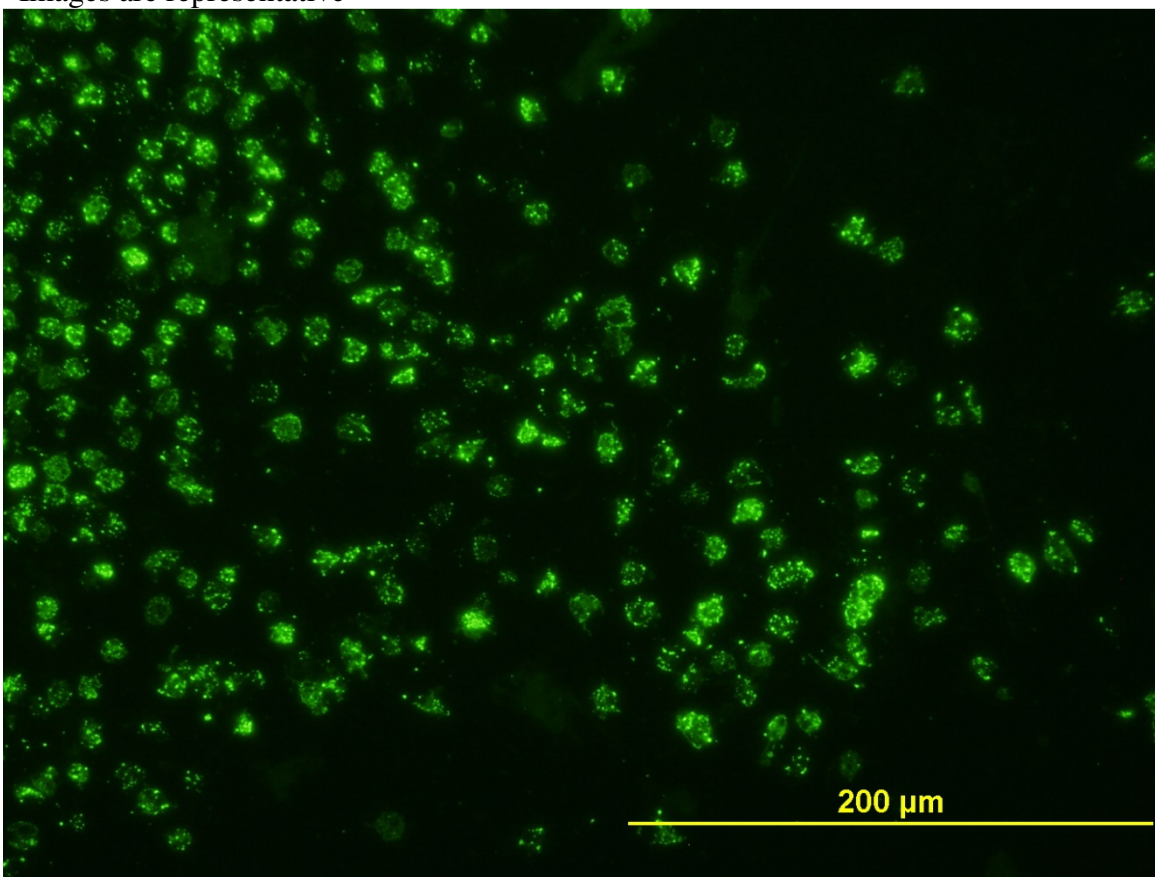
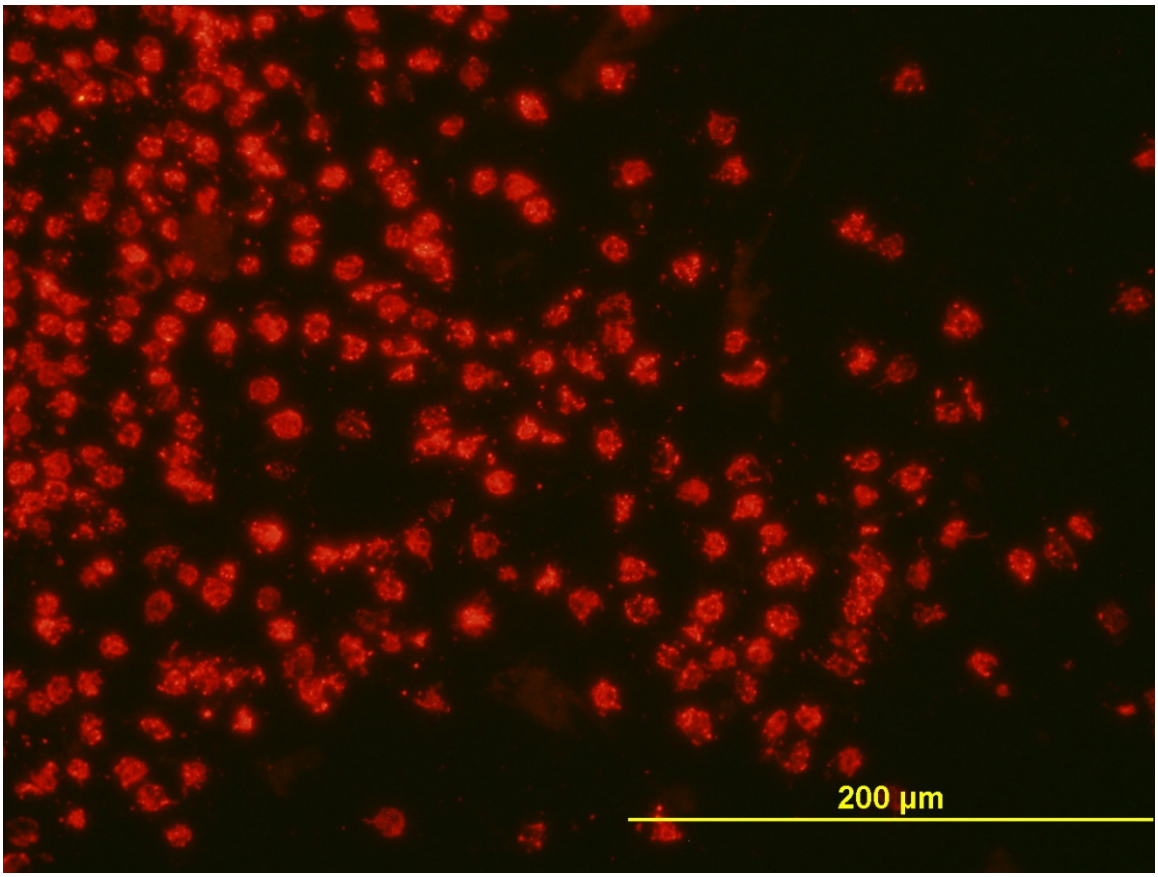
Figures

Figure 1 – Indirect Immunofluorescence Assay

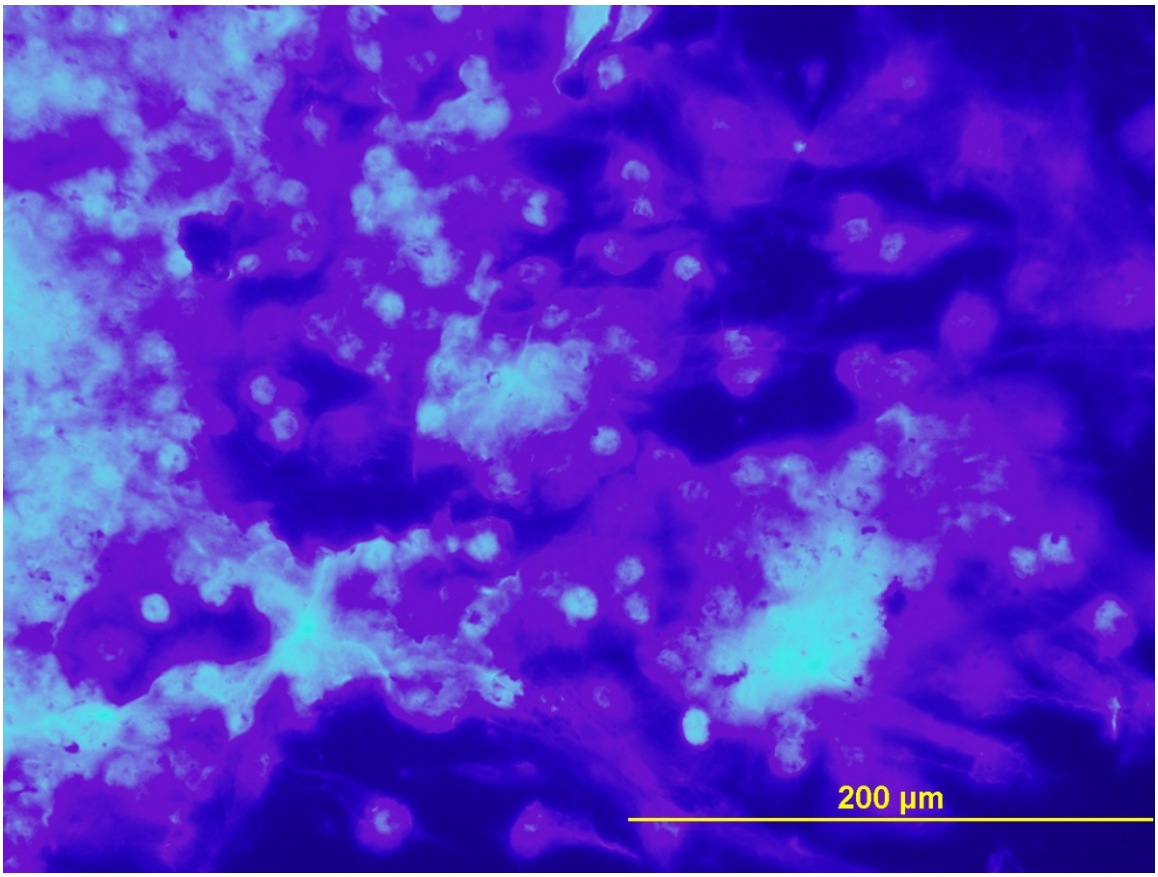
*Images are representative



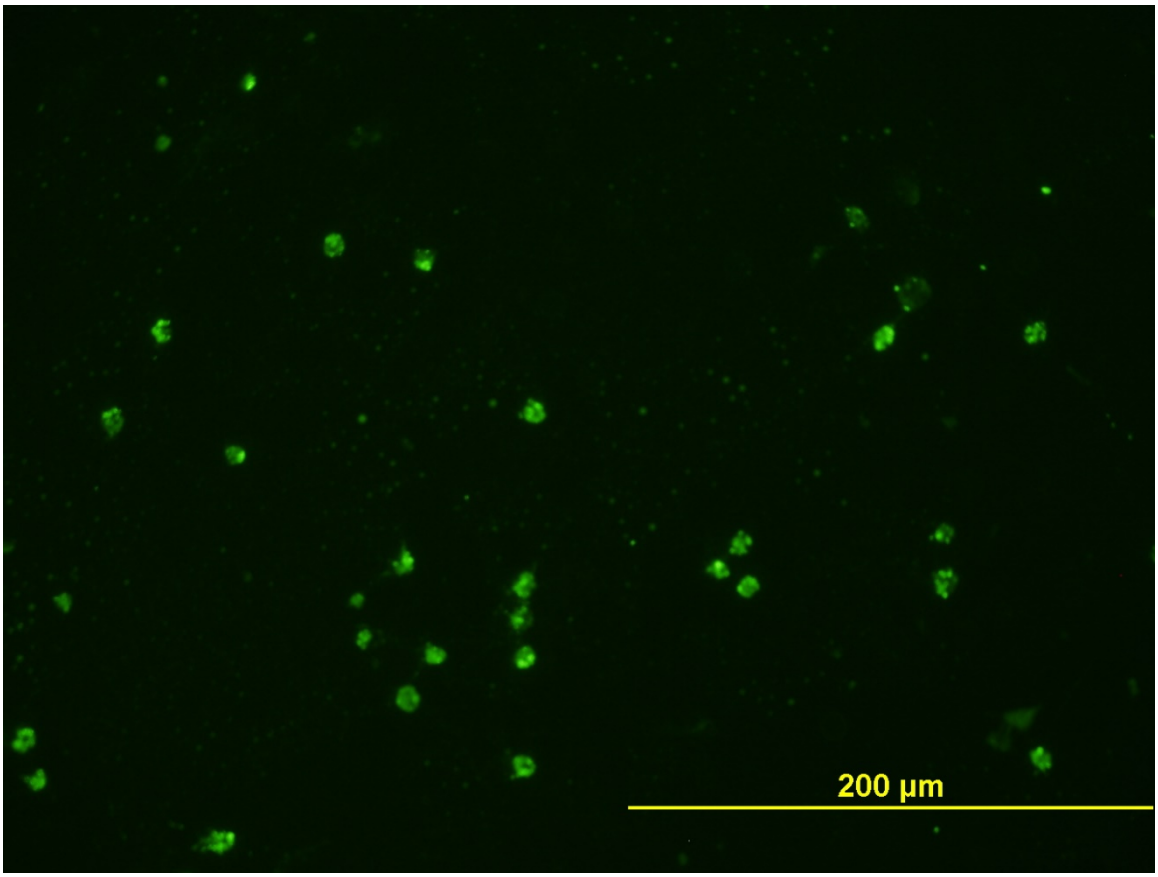
WC1 Positively selected γδ T-cells. Primary antibody, mouse anti-bovine WC1. Secondary goat anti-mouse Alexa 488 conjugated.



WC1 Positively selected γδ T-cells. Primary antibody, mouse anti-bovine δ chain. Secondary antibody, goat anti-mouse Alexa 594 conjugated.

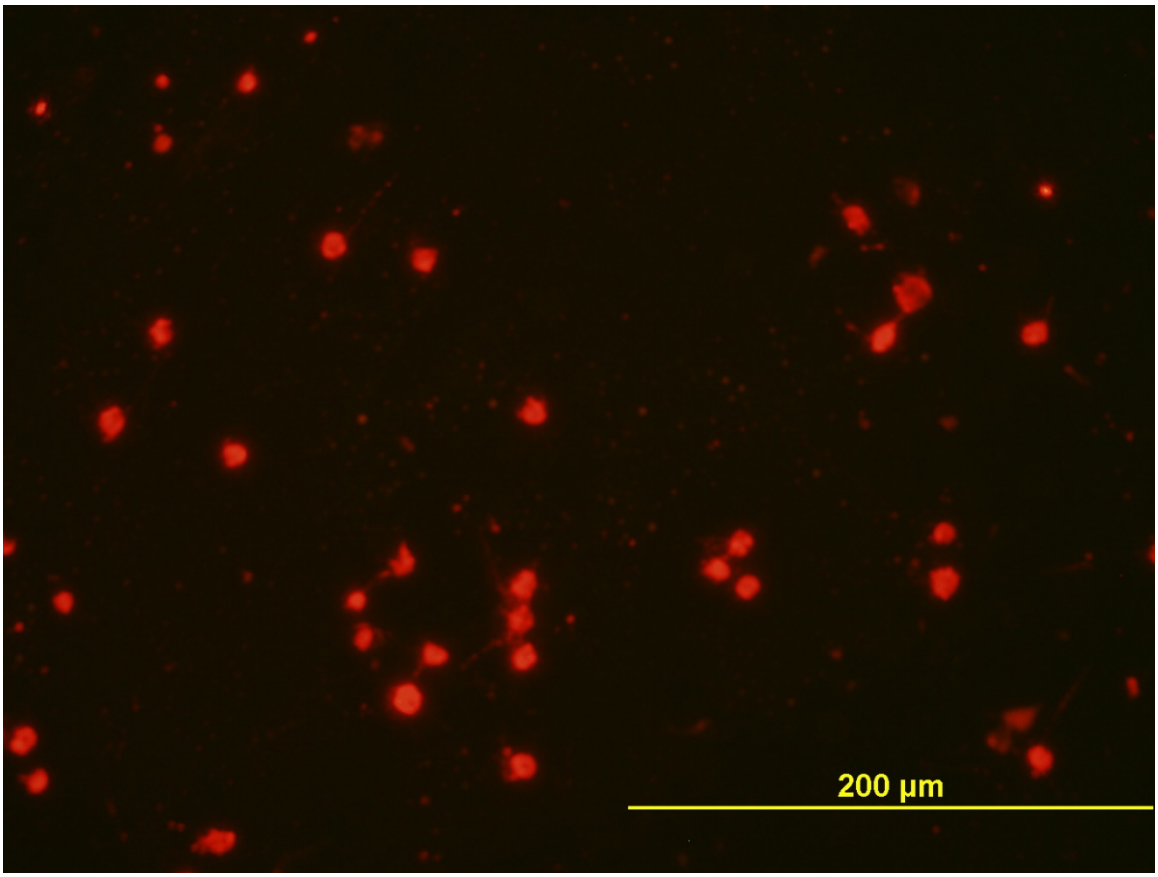


WC1 Positively selected γδ T-cells, DAPI stained

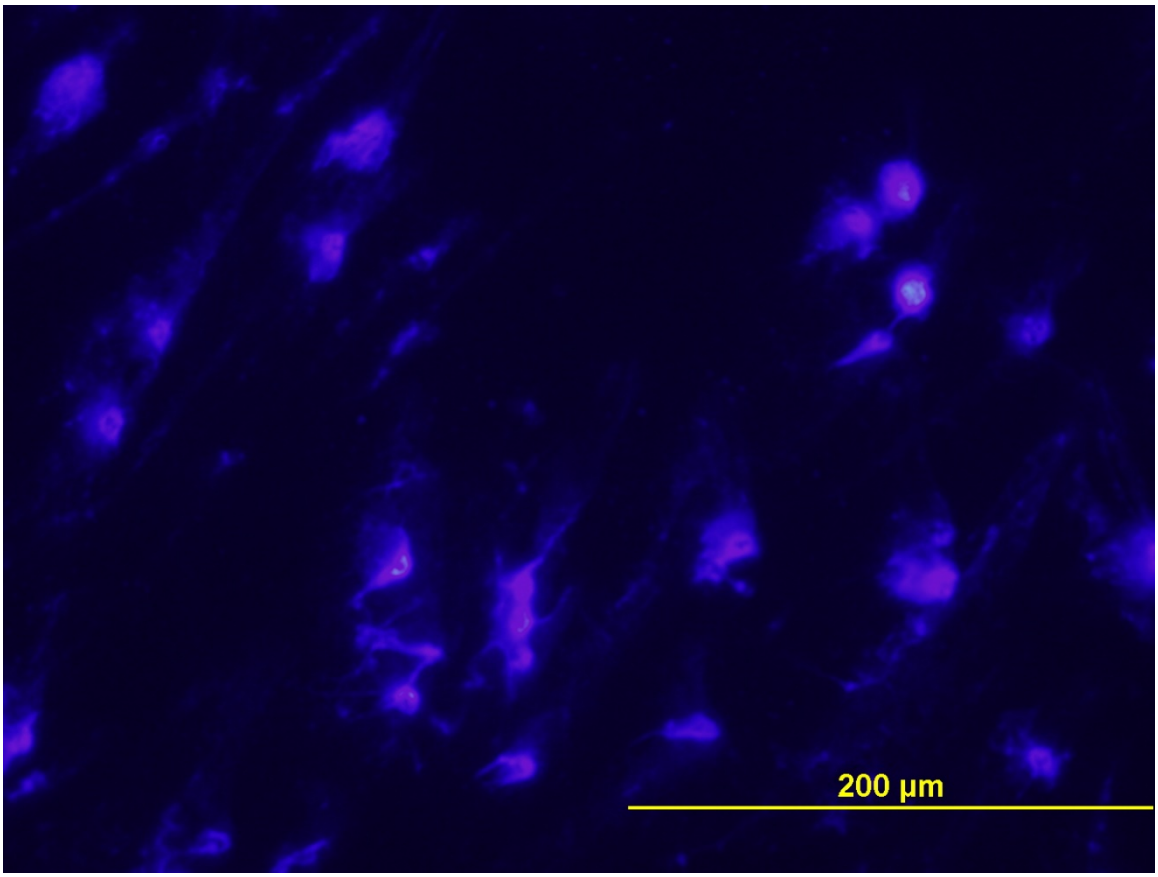


Monocytes: staining for CD14 @ 488nm

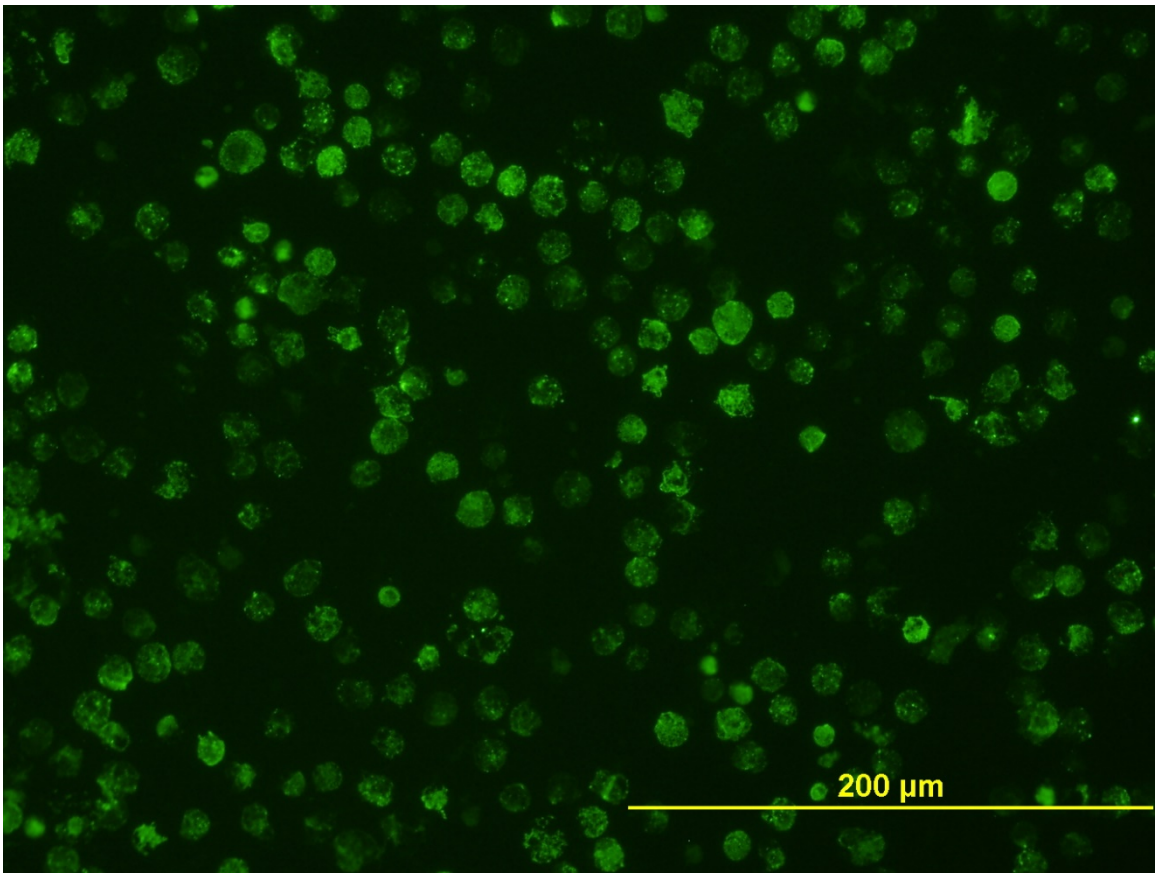
CD14 positively selected monocytes. Primary antibody mouse anti-bovine CD14. Secondary antibody, goat anti-mouse Alexa 488 conjugated.



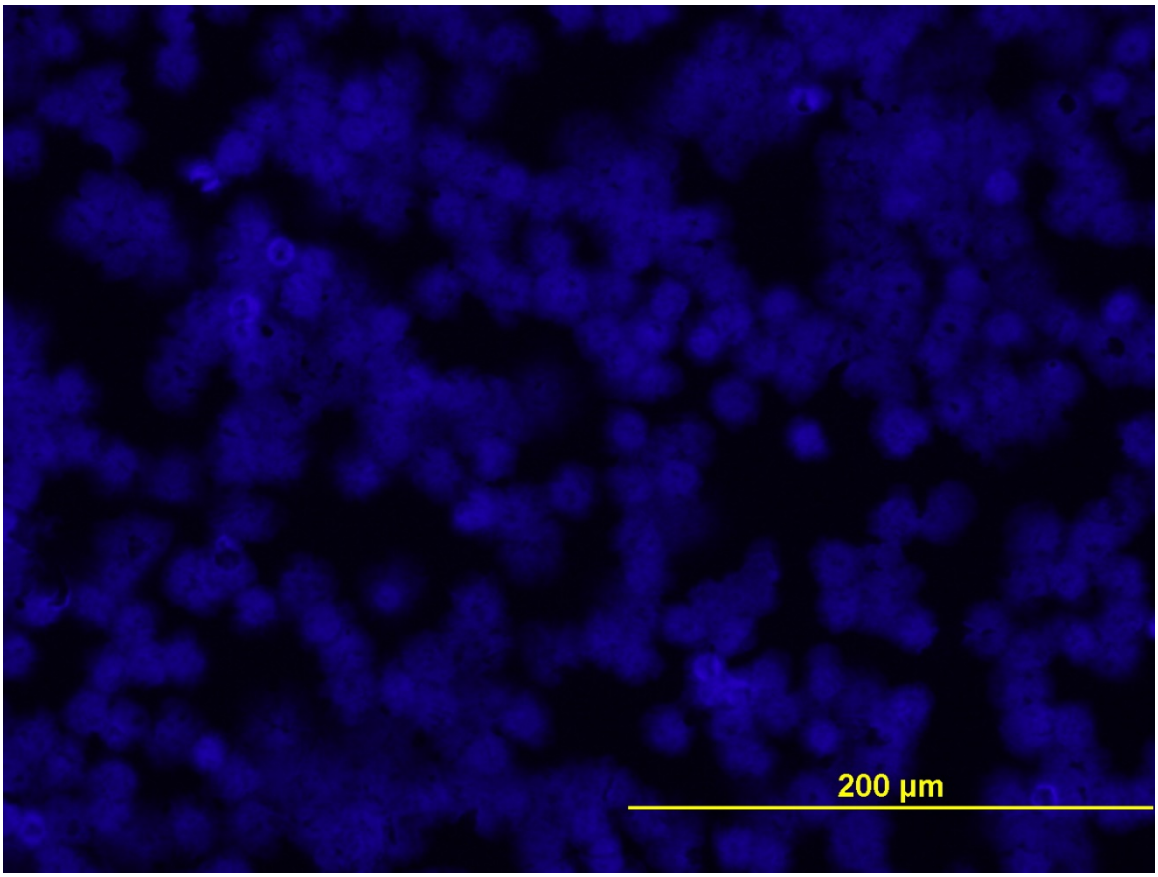
CD14 positively selected monocytes. Primary antibody mouse anti-bovine CD172. Secondary antibody, goat anti-mouse Alexa 594 conjugated.



CD14 positively selected monocytes, DAPI stained



WC1, CD14, CD3, CD21 depleted, negatively selected natural killer cells. Primary antibody, mouse anti-bovine CD335 (NKp46). Secondary antibody, goat anti-mouse Alexa 488 conjugated.

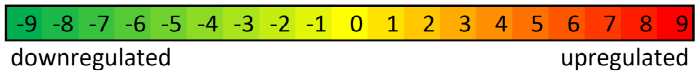


WC1, CD14, CD3, CD21 depleted, negatively selected natural killer cells, DAPI stained.

Figure 2 – CCL8/MCP-2 Expression Profiles

CCL8/MCP-2 log(2) fold change

Microarray	1	2	3
High SVN, Protected	3.66	-4.49	2.69
Low SVN, Protected	2.36	-3.96	6.51
High SVN, Unprotected	0.36	1.41	3.5
Low SVN, Unprotected	0.5	0.42	4.09
Ad-Null, Unprotected	0.37	-1.63	6.88
RT-qPCR			
High SVN, Protected	4.55	-3.68	4.79
Low SVN, Protected	5.95	-7.13	8.05
High SVN, Unprotected	-0.09	1.1	6.82
Ad-Null, Unprotected	2.63	-2.73	7.02



(1) log2 fold change at 1dpv compared to 0dpv
(2) log2 fold change at 14dpv compared to 1dpv
(3) fold change at 1dpc compared to 0dpc

Normalized intensity values from microarray over time course of vaccine efficacy study. Protected (n=5), not protected (n=4), ad-null (n=4)

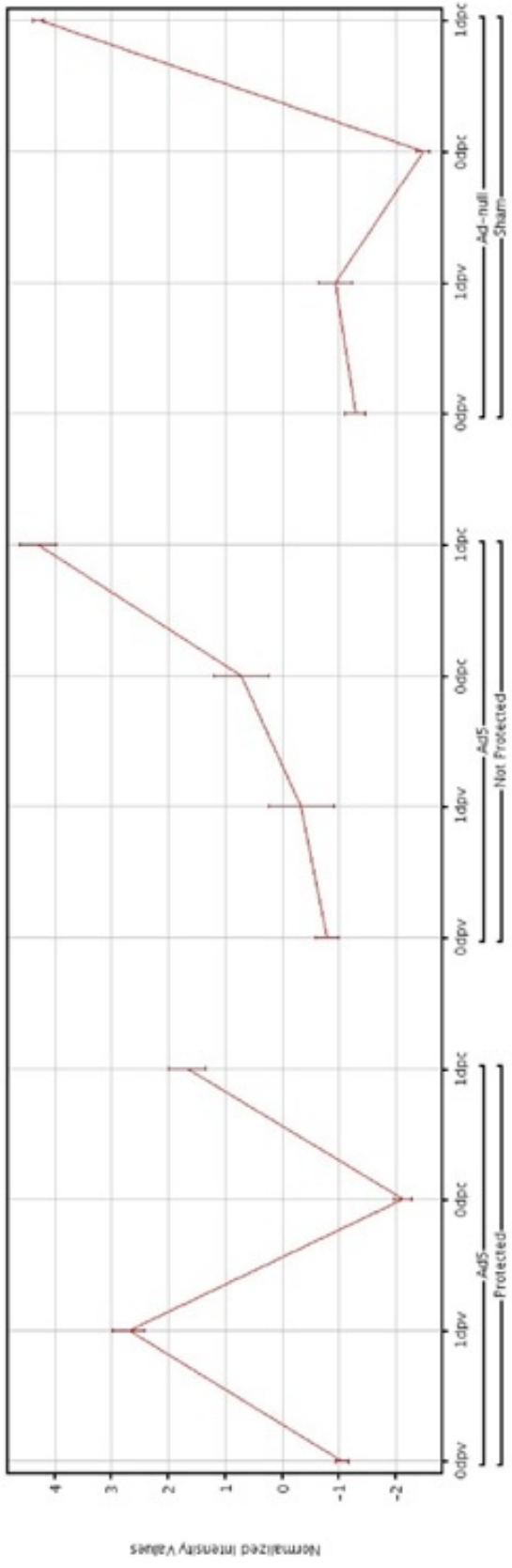


Figure 3 – Microarray-based Differential Expression

Microarray-based log(2) fold change results

High SVN, Protected				**Low SVN, Protected**			
interval:	1	2	3	interval:	1	2	3
APOBEC3A	1.26	-0.94	2.03	APOBEC3A	1.67	-1.79	4.34
CCL8	3.66	-4.49	2.69	CCL8	2.36	-3.96	6.51
GBP5	-0.14	-0.24	-0.25	GBP5	1.01	-2.00	3.48
IL8	0.53	-1.39	2.97	IL8	0.08	2.62	-0.48
IL10	0.60	-0.30	0.07	IL10	-0.44	0.16	0.96
IL16	-0.34	0.49	-0.24	IL16	0.48	-0.77	0.28
ISG15	1.53	-1.91	1.60	ISG15	1.53	-1.40	4.04
MX2	0.81	-1.35	0.58	MX2	1.00	-1.41	2.43
OAS2	0.53	-0.73	0.18	OAS2	2.33	-1.61	2.72
TLR3	-0.38	0.16	-0.06	TLR3	0.11	-0.94	1.04
TLR4	0.88	-1.21	1.36	TLR4	0.45	0.51	1.30
TLR10	-0.48	1.04	-0.62	TLR10	1.00	-1.41	0.95
High SVN, Unprotected				**Low SVN, Unprotected**			
APOBEC3A	0.61	-0.46	1.63	APOBEC3A	-0.69	2.15	0.29
CCL8	0.36	1.41	3.50	CCL8	0.50	0.42	4.09
GBP5	-0.06	-0.38	2.70	GBP5	-1.28	0.43	0.54
IL8	0.76	0.26	0.15	IL8	0.29	1.20	0.82
IL10	0.55	0.57	-0.04	IL10	-1.64	0.95	1.52
IL16	0.03	-0.07	-0.23	IL16	0.45	-0.77	0.23
ISG15	0.18	-0.04	2.36	ISG15	0.26	0.56	1.21
MX2	-0.03	-0.32	2.67	MX2	0.19	0.26	0.20
OAS2	-0.50	-0.26	2.93	OAS2	-0.67	1.04	-0.34
TLR3	0.03	-0.20	0.29	TLR3	-0.14	-0.58	0.24
TLR4	0.84	0.44	-1.24	TLR4	-0.53	1.75	0.75
TLR10	-0.14	-0.44	0.59	TLR10	1.03	-0.95	-0.45

(1) log2 fold change at 1dpv compared to 0dpv
(2) log2 fold change at 14dpv compared to 1dpv
(3) fold change at 1dpc compared to 0dpc

Microarray-based log(2) fold change results

interval:	1	2	3
Ad-Null			
APOBEC3A	0.7	-0.48	4
CCL8	0.37	-1.63	6.88
GBP5	0.29	-0.4	1.7
IL8	-2.42	0.63	0.14
IL10	-0.7	-0.24	0.25
IL16	0.26	0.18	-0.58
ISG15	0.41	-1.22	4.19
MX2	0.74	-1.04	3.69
OAS2	1.25	-0.96	3.88
TLR3	0.31	0	0.11
TLR4	-0.95	-0.08	1.81
TLR10	0.72	-0.32	0.01

*intervals 1,2 & 3: 0/1dpv, 1/14dpv & 0/1dpc respectively

downregulated upregulated

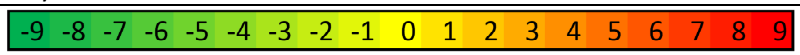
(1) log2 fold change at 1dpv compared to 0dpv
(2) log2 fold change at 14dpv compared to 1dpv
(3) fold change at 1dpc compared to 0dpc

Figure 4 – RT-qPCR verification of microarray-based data

RT-qPCR log(2) fold change results

High SVN, Protected				**Low SVN, Protected**			
interval:	**1**	**2**	**3**	interval:	**1**	**2**	**3**
APOBEC3A	1.66	-1.72	1.93	APOBEC3A	4.06	-2.95	5.04
CCL8	4.55	-3.68	4.79	CCL8	5.95	-7.13	8.05
GBP5	0.14	0.15	-0.01	GBP5	2.82	-3.40	2.83
IL8	0.54	-2.33	3.20	IL8	1.51	1.01	0.95
IL10	-0.04	-1.29	2.54	IL10	0.94	0.83	-0.11
IL16	-0.33	-0.23	-0.41	IL16	1.40	-2.17	0.31
ISG15	0.98	-1.96	1.82	ISG15	3.26	-2.46	3.82
MX2	0.83	-2.31	0.65	MX2	3.38	-4.27	3.43
OAS2	0.70	-1.88	1.07	OAS2	3.80	-4.35	4.57
TLR3	-0.04	-0.22	-0.27	TLR3	2.00	-2.49	0.77
TLR4	0.82	-1.92	1.20	TLR4	1.89	-1.41	1.59
TLR10	-0.49	-0.03	-0.73	TLR10	2.69	-2.31	0.44
High SVN, Unprotected	**1**	**2**	**3**	**Ad-Null**	**1**	**2**	**3**
APOBEC3A	0.28	-1.61	3.36	APOBEC3A	1.86	-2.12	4.25
CCL8	-0.09	1.10	6.82	CCL8	2.63	-2.73	7.02
GBP5	-0.36	-1.19	2.84	GBP5	1.63	-1.67	2.92
IL8	0.27	-0.06	0.37	IL8	0.11	-1.53	0.91
IL10	1.20	0.97	-0.46	IL10	-3.98	0.86	0.64
IL16	0.17	-0.54	0.11	IL16	0.78	-1.78	0.14
ISG15	-0.87	-1.47	5.42	ISG15	1.38	-2.29	5.08
MX2	-0.58	-1.33	5.07	MX2	1.92	-3.74	4.64
OAS2	-0.74	-2.76	5.44	OAS2	1.88	-3.06	4.37
TLR3	-0.18	-1.10	0.70	TLR3	1.20	-1.60	0.81
TLR4	0.22	-0.33	1.49	TLR4	-0.23	-1.96	2.30
TLR10	-0.11	-1.33	0.84	TLR10	0.99	-0.66	0.50

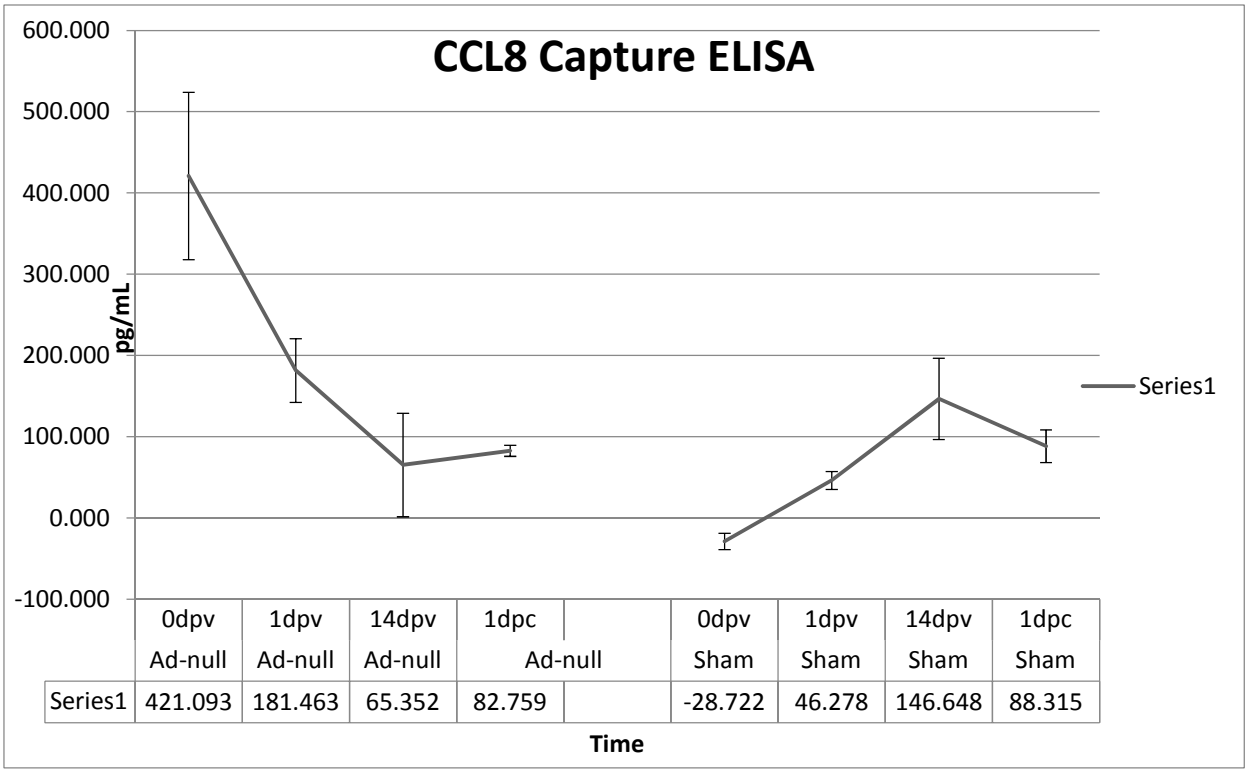
*intervals 1,2 & 3: 0/1dpv, 1/14dpv & 0/1dpc respectively



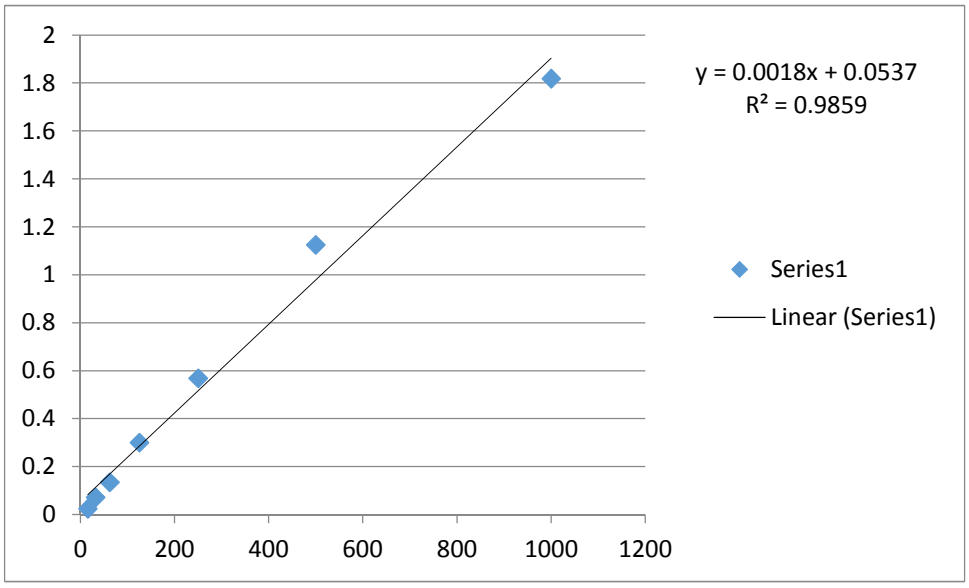
downregulated upregulated

(1) log2 fold change at 1dpv compared to 0dpv
(2) log2 fold change at 14dpv compared to 1dpv
(3) fold change at 1dpc compared to 0dpc

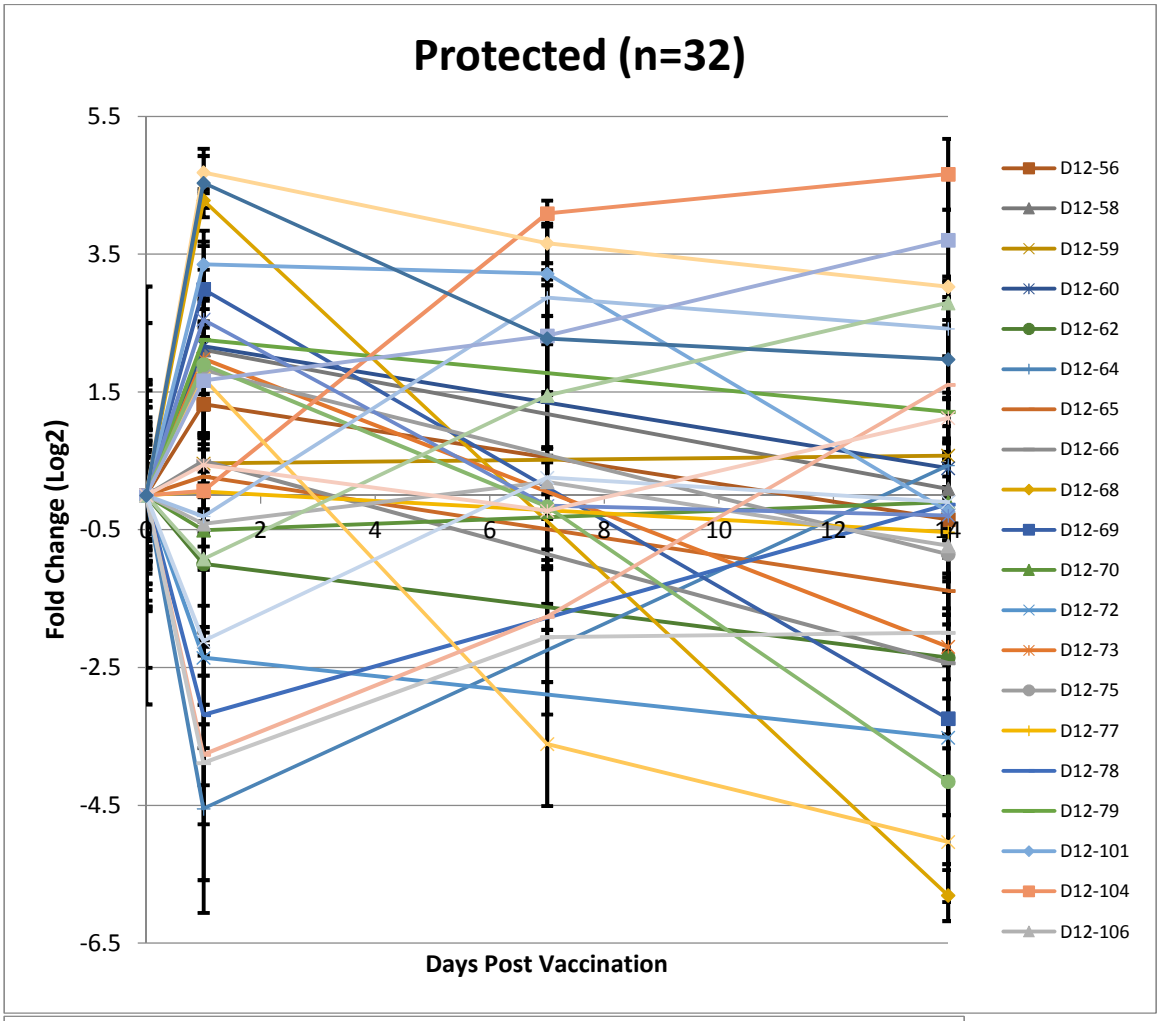
Figure 5 – CCL8 ELISA from Plasma & Standard Curve

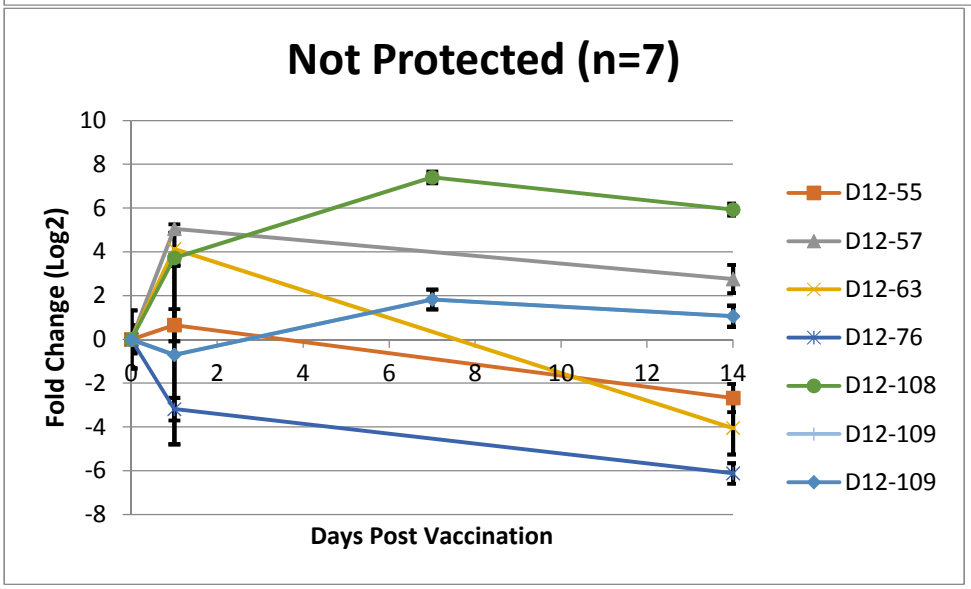


	0dpv Ad-null	1dpv Ad-null	14dpv Ad-null	1dpc Ad-null	0dpv Sham	1dpv Sham	14dpv Sham	1dpc Sham
Series1	421.093	181.463	65.352	82.759	-28.722	46.278	146.648	88.315



F igure 6 – RT-qPCR-based Differential Expression of CCL8 mRNA





Tables

Table 1 – Primers and *TaqMan* Probes

Gene	**APOBEC3A**	Gene	**ISG15**
Accession	NM_001163936	Accession	NM_174366
Forward	CTGACTGAGCATACCATC	Forward	GAGCTGAAGCAGTTCATC
Reverse	CCTTGATTGTTGAAGTTCTC	Reverse	GATGGAGATGCAGTTCTG
Probe	TATTCGTCCATCAGGTGTCGTCC	Probe	CCAGAAGATCAATGTGCCTGCTT
Gene	**CCL8**	Gene	**MX2**
Accession	NM_174007	Accession	NM_173941
Forward	GGTCCAGACTTCCATAAG	Forward	GCCTTTGAAGTACAAGAG
Reverse	GGCCATTATAATACAATAATACAC	Reverse	AGCTGATTGAAGTTGTTG
Probe	CGAACACCGAAGCCTTGAACC	Probe	CTCAGACTTCCACTCAGCACCA
Gene	**GBP5**	Gene	**OAS2**
Accession	NM_001075746	Accession	NM_001024557
Forward	TCTGGAGAATTCACTGAG	Forward	CTCACCTCTTGTCTCTTC
Reverse	CCAGATCATCATTATGTAGTG	Reverse	CTGCTGATGTCCATCTAA
Probe	AGGCACCAACCAGCATCTTCA	Probe	TATCTCAACAGTCACAATCCAAGCCT
Gene	**IL8**	Gene	**TLR3**
Accession	NM_173925	Accession	NM_001008664
Forward	CCAGAAGAAACCTGACAA	Forward	CTGGAGTATAATAACATAGAGC
Reverse	CGAAGTTCTGTACTCATTC	Reverse	CAGTGAAATGCTTTGTCTA
Probe	AAGCCTCTTGTTCAATATGACTTCCAA	Probe	TCAAGTCCAGGCGTCTCAAGTT
Gene	**IL10**	Gene	**TLR4**
Accession	NM_174088	Accession	NM_174198
Forward	CGGAAATGATCCAGTTTTAC	Forward	CAGAGCCTTTAGATATGGA
Reverse	AGCTCACTGAAGACTCTC	Reverse	GGTGGAAATAGAACTTGTAG
Probe	TTCTCCACCGCCTTGCTCTT	Probe	TACCACAGACACCAGGAGCAC
Gene	**IL16**	Gene	**TLR10**
Accession	NM_001075253	Accession	NM_001076918
Forward	CAGAGCTAAGAGAATACAC	Forward	GCATCAATGGACTCAGAC
Reverse	CAAGGAGATAACCGATTG	Reverse	CAGGCAGAATCATGTTCA
Probe	CGGAAGCCAACGACTGTGAC	Probe	AGGAAGACAACTCAGGAACAACTCA

Gene	**YWHAZ**	Gene	**PPIA**
Accession	NM_174814	Accession	NM_178320
Forward	CTGGTGATGACAAGAAAG	Forward	GTCCATCTATGGCGAGAA
Reverse	GGAGTTCAGAATCTCATAATAG	Reverse	ATGCCCTCTTTCACCTTG
Probe	CCAGTCACAGCAAGCATACCAAG	Probe	TGCCATCCAACCACTCAGTCTT
Gene	**RPS24**		
Accession	NM_001025339		
Forward	GAACGCAAGAACAGAATG		
Reverse	GCACAATCACCACAGATA		
Probe	ACTCCTTCTGTTGTCCAATCTCCA		

Multiplexed CCL8

Gene	**CCL8**	Gene	**RPS24**
Accession	NM_174007	Accession	NM_001025339
Forward	GGTCCAGACTTCCATAAG	Forward	GAACGCAAGAACAGAATG
Reverse	GGCCATTATAATACAATAATACAC	Reverse	GCACAATCACCACAGATA
Probe	CCGAACACCGAAGCCTTGAAC	Probe	ACTCCTTCTGTTGTCCAATCTCCA

Table 2 – Gene List & Internal Controls

Gene List

1. **Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3A**
2. **Chemokine (C-C motif) ligand 8**, also known as monocyte chemoattractant protein 2 (MCP-2)
3. **Guanylate binding protein 5**
4. **Interleukin 8** (**IL-8**), also known as neutrophil chemotactic factor
5. **Interleukin-10** (**IL-10**), also known as cytokine synthesis inhibitory factor (CSIF)
6. **Interleukin-16** (**IL-16**), previously known as lymphocyte chemoattractant factor (LCF)
7. **Interferon-induced 17 kDa protein**
8. **Interferon-induced GTP-binding protein Mx2**
9. **2'-5'-oligoadenylate synthetase 2**
10. **Toll-like receptor 3** (TLR3) also known as CD283
11. **Toll-like receptor 4** (TLR4)
12. **Toll-like receptor 10,** also designated as CD290

Internal Control Genes

1. **Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide**
2. **Peptidylprolyl isomerase A** also known as cyclophilin A or rotamase A
3. **40S ribosomal protein S24**

Table 3 – Candidate Surrogate Markers for Protection

Description	Symbol	Entrez Gene	Fold Change 1dpv	Accession No.
Chemokine (C-C motif) ligand 8	CCL8	788169	4.6000476	NM_174007
Collagen, type I, alpha 1	COL1A1	282187	2.0105424	NM_001034039
Drebrin 1	DBN1	505406	8.14972	NM_001045917
PREDICTED: Exonuclease 3'-5' domain containing 2	EXD2	539532	7.7476177	XM_587937
Hyaluronan and proteoglycan link protein 3	HAPLN3	515224	7.0904818	NM_001098981
5-hydroxytryptamine (serotonin) receptor 4	HTR4	317708	3.5694902	NM_001040485
Katanin p60 subunit A-like 1, isoform 2	KATNAL1	537739	7.1488013	NM_001192918
Meprin A, beta	MEP1B	540701	3.7190018	NM_001144098
N-acetyl-glucosaminyltransferase, isozyme B	*MGAT5B*	*785213*	5.833619	NM_001102337
Nudix (nucleoside diphosphate linked moiety X)-type motif 13	NUDT13	504993	6.997903	NM_001045905
Ribosomal protein S6 kinase, 90kDa, polypeptide 2	RPS6KA2	517953	4.665269	NM_001192478

Fold change at 1dpv compared to 0dpv in protected cattle with low SVN titer at time of challenge.

Table 4 – Serum Virus Neutralization Titer

a.)	SVN FMDV				
ET#	**0 dpv**	**7 dpv**	**14 dpv**	**7 dpc**	**14 dpc**
D10-100	0.6	1.8	**2.1**	2.4	2.7
D10-109	0.6	0.9	**1.8**	1.8	2.1
D10-121	0.6	1.2	**2.1**	2.4	3
D10-132	0.6	0.9	**1.8**	1.2	2.4
D10-115	0.6	0.6	**0.6**	2.4	2.7
D10-173	0.6	1.2	**1.2**	3	2.4
D10-184	0.6	1.2	**1.8**	3	3.3
D10-186	0.6	1.2	**1.5**	3.3	3
D10-101	0.6	0.6	**0.6**	2.1	3
D10-113	0.6	0.6	**0.6**	2.1	3
D10-118	0.6	0.6	**0.6**	2.1	2.4
D10-131	0.6	0.6	**0.6**	2.1	3
D10-133	0.6	0.6	**0.6**	2.1	3
D12-55	0.6	1.2	**1.5**	3.0	2.4
D12-56	0.6	1.8	**2.7**	3.0	3.3
D12-57	0.6	1.5	**2.1**	3.3	3.0
D12-58	0.6	2.4	**2.4**	2.7	3.3
D12-59	0.6	2.1	**2.7**	2.7	3.3
D12-60	0.6	0.6	**1.8**	3.0	3.3
D12-62	0.6	1.5	**2.1**	2.7	2.7
D12-63	0.6	1.5	**2.1**	3.0	3.3
D12-64	0.6	1.5	**2.4**	2.7	3.0
D12-65	0.6	1.8	**3.0**	3.3	3.6
D12-66	0.6	1.8	**2.1**	3.0	3.3
D12-68	0.6	0.6	**0.9**	2.1	3.6
D12-69	0.6	1.5	**2.1**	3.0	3.6
D12-70	0.6	2.4	**2.7**	3.0	3.6
D12-72	0.6	0.9	**1.8**	3.6	3.0
D12-73	0.6	1.5	**2.7**	3.0	3.3
D12-75	0.6	0.9	**2.4**	2.4	3.3
D12-76	0.6	0.6	**0.6**	3.0	2.7
D12-77	0.6	1.8	**2.7**	3.3	3.3
D12-78	0.6	1.2	**2.4**	2.4	3.6
D12-79	0.6	2.4	**3**	3	3.3

Log_{10} of inverse two-fold dilution of inactivated sera capable of 50% reduction of cytopathic effect on BHK cells

D12-97	0.6	0.6	**0.6**	2.1	3.3
D12-98	0.6	0.6	**0.6**	2.4	3
D12-99	0.6	0.6	**0.6**	2.4	3
D12-100	0.6	0.6	**0.6**	2.4	3
D12-101	0.6	1.2	**1.8**	2.7	3.3
D12-104	0.6	1.8	**1.5**	3	3
D12-106	0.6	1.2	**1.8**	3	3
D12-107	0.6	0.6	**0.6**	3	2.7
D12-108	0.6	1.2	**1.8**	3	3
D12-109	0.6	0.6	**1.2**	3	3
D12-110	0.6	0.6	**1.2**	3	3
D12-111	0.6	0.6	**1.2**	3	3
D12-112	0.6	1.2	**1.5**	3	3
D12-113	0.6	0.6	**1.2**	2.4	3
D12-114	0.6	1.8	**1.8**	3	3
D12-115	0.6	1.2	**1.8**	3	3
D12-116	0.6	1.2	**1.5**	3	3
D12-117	0.6	1.5	**1.2**	3	3.6
D12-118	0.6	1.8	**1.8**	2.7	3.3
D12-119	0.6	0.6	**1.5**	3	3.3
D12-120	0.6	1.8	**1.8**	2.4	3
D12-121	0.6	1.2	**1.8**	3	3

b.)

	SVN Adenovirus-5				
ET#	**0 dpv**	**7 dpv**	**14 dpv**	**7 dpc**	**14 dpc**
D10-100	0.6	1.5	**2.1**	2.4	1.8
D10-109	0.6	0.9	**3**	2.7	2.7
D10-121	0.6	1.2	**3**	2.4	2.7
D10-132	0.6	1.5	**3**	2.7	2.4
D10-115	0.6	1.2	**1.8**	2.7	2.4
D10-173	0.6	1.8	**3**	3	2.1
D10-184	0.6	0.6	**2.7**	3	3
D10-186	0.6	1.8	**2.7**	2.4	2.4
D10-101	0.6	1.5	**2.4**	2.4	2.4
D10-113	0.6	0.6	**0.6**	0.6	0.6
D10-118	0.6	0.6	**0.6**	0.6	0.6
D10-131	0.6	0.6	**0.6**	0.6	0.6
D10-133	0.6	0.6	**0.6**	0.6	0.6

D12-55	0.6	0.6	**0.6**	0.6	0.6
D12-56	0.6	2.1	**2.4**	2.4	2.1
D12-57	0.6	1.5	**2.4**	3.3	2.1
D12-58	0.6	1.8	**2.4**	3.0	2.7
D12-59	0.6	1.5	**2.4**	1.8	2.1
D12-60	0.6	1.5	**2.4**	1.5	2.1
D12-62	0.6	1.5	**2.4**	2.4	2.4
D12-63	0.6	0.6	**0.6**	0.6	0.6
D12-64	0.6	0.6	**0.6**	0.6	0.6
D12-65	0.6	2.1	**2.1**	2.1	2.7
D12-66	0.6	1.2	**2.7**	2.4	2.4
D12-68	0.6	0.6	**0.6**	0.6	0.6
D12-69	0.6	0.6	**0.6**	0.6	0.6
D12-70	0.6	0.6	**0.6**	0.6	0.6
D12-72	0.6	0.6	**0.6**	0.6	0.6
D12-73	0.6	0.6	**1.8**	1.8	1.8
D12-75	0.6	0.6	**0.6**	0.6	0.6
D12-76	0.6	0.6	**0.6**	0.6	0.6
D12-77	0.6	2.4	**2.1**	2.7	2.7
D12-78	0.6	1.2	**2.4**	2.4	1.8
D12-79	0.6	0.6	**0.6**	0.6	0.6
D12-97	0.6	1.2	**3**	2.7	3
D12-98	0.6	0.6	**3**	3.6	3.3
D12-99	0.6	1.2	**3**	3.3	3
D12-100	0.6	1.8	**3**	3.6	3
D12-101	0.6	1.8	**3**	3.6	3.3
D12-104	0.6	1.2	**2.7**	2.7	2.4
D12-106	0.6	1.2	**2.1**	3	3.3
D12-107	0.6	0.9	**2.7**	2.1	1.8
D12-108	0.6	2.1	**3**	2.7	3
D12-109	0.6	2.4	**2.4**	2.4	2.1
D12-110	0.6	2.7	**3**	3	3
D12-111	0.6	2.4	**3**	3	3
D12-112	0.6	1.5	**3**	2.7	2.7
D12-113	0.6	2.4	**2.4**	2.4	2.7
D12-114	0.6	2.1	**2.7**	2.7	2.4
D12-115	0.6	2.4	**3.6**	3.6	3

D12-116	0.6	1.8	**3**	2.7	2.7
D12-117	0.6	2.4	**3**	2.7	2.4
D12-118	0.6	2.1	**3**	3	2.4
D12-119	0.6	2.1	**3.3**	3.3	2.4
D12-120	0.6	1.8	**3**	2.7	3
D12-121	0.6	2.4	**3.3**	3.6	3

Additional files

Supplemental Table 1 – RNA Assessment (NanoDrop, Bioanalyzer)

Hi Pro	ng/ul	260/280
D10-100 8-3	193.27	2.15
D10-100 8-4	183.77	2.15
D10-100 8-17	100.58	2.13
D10-100 8-18	35.45	2.05
D10-109 8-3	665.35	2.15
D10-109 8-4	416.97	2.14
D10-109 8-17	87.64	2.16
D10-109 8-18	550.41	2.14
D10-121 8-3	317.19	2.14
D10-121 8-4	357.77	2.15
D10-121 8-17	213.93	2.17
D10-121 8-18	255.03	2.14
D10-132 8-3	329.39	2.13
D10-132 8-4	419.73	2.11
D10-132 8-17	480.74	2.13
D10-132 8-18	319.09	2.15

Ad Null		
12-97 6/12	244.76	2.07
12-97 6/13	124.47	2.02
12-97 6/26	89.82	1.98
12-97 6/27	51.95	1.95
12-98 6/12	264.52	2.04
12-98 6/13	130.03	1.98
12-98 6/26	112.41	2.02
12-98 6/27	86.8	1.98
12-99 6/12	297.47	2.05
12-99 6/13	103.85	1.95
12-99 6/26	39.92	1.79
12-99 6/27	27.91	1.52
12-100 6/12	254.07	2.02
12-100 6/13	128.45	1.99
12-100 6/26	44.57	1.82
12-100 6/27	57.24	1.89

Hi No Pro	ng/ul	260/280
D10-173 11-15	47.33	2.07
D10-173 11-16	114.97	2.13
D10-173 11-29	96.82	2.14
D10-173 11-30	69.33	2.18
D10-184 11-15	81.29	2.15
D10-184 11-16	125.66	2.12
D10-184 11-29	96.83	2.15
D10-184 11-30	86.27	2.12
D10-186 11-15	63.59	1.99
D10-186 11-16	27.77	2.15
D10-186 11-29	179.2	2.15
D10-186 11-30	60.01	2.16

Lo No Pro		
D10-101 8-3	413.59	2.06
D10-101 8-4	342.51	2.11
D10-101 8-17	125.22	2.13
D10-101 8-18	207.13	2.09

Sham	ng/ul	260/280
D10-113 8/17	316.56	2.15
D10-113 8/18	351.92	2.14
D10-118 8/17	267.88	2.16
D10-118 8/18	138.35	2.17
D10-131 8/17	324.01	2.16
D10-131 8/18	293.5	2.15
D10-133 8/17	80.12	2.15
D10-133 8/18	266.38	2.17

Lo Pro		
D10-115 8-3	345.94	2.14
D10-115 8-4	270.05	2.15
D10-115 8-17	85.18	2.17
D10-115 8-18	166.04	2.12

Hi Pro

D10-100 8-3	RIN: 9.10
D10-100 8-4	RIN: 8.80
D10-100 8-17	RIN: 9.30
D10-100 8-18	RIN: 9.30
D10-109 8-3	RIN: 8.90
D10-109 8-4	RIN: 9.40
D10-109 8-17	RIN: 9.60
D10-109 8-18	RIN: 9.20
D10-121 8-3	RIN: 8.70
D10-121 8-4	RIN: 9.50
D10-121 8-17	RIN: 9.60
D10-121 8-18	RIN: 9.60
D10-132 8-3	RIN: 9.70
D10-132 8-4	RIN: 9.30
D10-132 8-17	RIN: 8.90
D10-132 8-18	RIN: 9.50

Sham

D10-113 8/17	RIN: 9.10
D10-113 8/18	RIN: 9.30
D10-118 8/17	RIN: 9.50
D10-118 8/18	RIN: 9.60
D10-131 8/17	RIN: 9.60
D10-131 8/18	RIN: 9.50
D10-133 8/17	RIN: 8.80
D10-133 8/18	RIN: 9.70

Hi No Pro

D10-173 11-15	RIN: 9.40
D10-173 11-16	RIN: 9.30
D10-173 11-29	RIN: 9.30
D10-173 11-30	RIN: 9.60
D10-184 11-15	RIN: 9.50
D10-184 11-16	RIN: 8.60
D10-184 11-29	RIN: 9.30
D10-184 11-30	RIN: 9.60
D10-186 11-15	RIN: 8.90
D10-186 11-16	RIN: 9.20
D10-186 11-29	RIN: 9.30
D10-186 11-30	RIN: 9.60

Lo Pro

D10-115 8-3	RIN: 9.30
D10-115 8-4	RIN: 9.70
D10-115 8-17	RIN: 9.20
D10-115 8-18	RIN: 9.60

Lo No Pro

D10-101 8-3	RIN: 9.30
D10-101 8-4	RIN: 9.80
D10-101 8-17	RIN: 8.80
D10-101 8-18	RIN: 9.50

RNA Integrity Number (RIN) ranges from 0 to 10 where 10 is greatest integrity

Supplemental Table 2 – Cyanine Dye Incorporation

Sample ID **Hi Pro**	Cy-3 pmol/µl	Cy-5 pmol/µl	ng/µl	activity
D10-100 8-3 Cy-3	1.08	-0.11	97.86	11.04
D10-100 8-4 Cy-3	0.81	-0.07	90.2	8.98
D10-100 8-17 Cy-3	0.63	-0.19	88.52	7.12
D10-100 8-18 Cy-3	0.62	-0.05	54.81	11.31
D10-109 8-3 Cy-3	1.39	-0.05	99.66	13.95
D10-109 8-4 Cy-3	1.11	-0.15	109.15	10.17
D10-109 8-17 Cy-3	0.84	-0.2	109.46	7.67
D10-109 8-18 Cy-3	1.02	-0.16	91.96	11.09
D10-121 8-3 Cy-3	1.64	0.06	109.79	14.94
D10-121 8-4 Cy-3	0.99	-0.03	73.98	13.38
D10-121 8-17 Cy-3	1	0.01	71.11	14.06
D10-121 8-18 Cy-3	1.29	-0.04	96.43	13.38
D10-132 8-3 Cy-3	1.29	0	89.94	14.34
D10-132 8-4 Cy-3	0.93	0.02	71.29	13.05
D10-132 8-17 Cy-3	1.14	-0.07	89.86	12.69
D10-132 8-18 Cy-3	1.13	-0.13	100.36	11.26
D10-100 8-3 Cy-5	-0.03	1.5	88.23	17
D10-100 8-4 Cy-5	-0.27	0.94	75.97	12.37
D10-100 8-17 Cy-5	-0.2	1.23	95.64	12.86
D10-100 8-18 Cy-5	0.08	0.78	55.59	14.03
D10-109 8-3 Cy-5	-0.17	1.47	96.63	15.21
D10-109 8-4 Cy-5	-0.06	1.4	87.32	16.03
D10-109 8-17 Cy-5	0.21	1.61	95.9	16.79
D10-109 8-18 Cy-5	0.13	1.51	96.62	15.63
D10-121 8-3 Cy-5	-0.13	1.86	124.53	14.94
D10-121 8-4 Cy-5	-0.06	1.24	81.72	15.17
D10-121 8-17 Cy-5	0.05	1.49	97.82	15.23
D10-121 8-18 Cy-5	0.07	1.3	81.32	15.99
D10-132 8-3 Cy-5	-0.22	1.2	85.72	14
D10-132 8-4 Cy-5	-0.23	1.21	84.26	14.36
D10-132 8-17 Cy-5	0	1.19	82.04	14.51
D10-132 8-18 Cy-5	0.13	1.27	78.37	16.21

Hi No Pro

173 11-15 Cy-3	0.91	-0.01	78.99	11.52
173 11-16 Cy-3	1.2	-0.01	101.79	11.79
173 11-29 Cy-3	0.84	0	82.34	10.2
173 11-30 Cy-3	0.86	0.05	78.47	10.96
184 11-15 Cy-3	1.48	-0.02	117.48	12.6
184 11-16 Cy-3	1.26	0.08	101.57	12.41
184 11-29 Cy-3	1.41	0.13	107.64	13.1
184 11-30 Cy-3	0.92	0.11	76.11	12.09
186 11-15 Cy-3	0.74	0.04	74.21	9.97
186 11-16 Cy-3	0.66	0.05	67.51	9.78
186 11-29 Cy-3	0.92	0.05	86.9	10.59
186 11-30 Cy-3	0.73	0.11	70.95	10.29
173 11-15 Cy-5	-0.08	1.23	79.19	15.53
173 11-16 Cy-5	-0.02	1.24	74.72	16.6
173 11-29 Cy-5	-0.09	1.3	75.32	17.26
173 11-30 Cy-5	0.46	1.07	65.07	16.44
184 11-15 Cy-5	0	1.58	98.93	15.97
184 11-16 Cy-5	-0.02	1.57	90.6	17.33
184 11-29 Cy-5	-0.09	1.55	93.72	16.54
184 11-30 Cy-5	-0.16	1.01	62.5	16.16
186 11-15 Cy-5	-0.12	1.11	72.37	15.34
186 11-16 Cy-5	-0.1	0.96	58.02	16.55
186 11-29 Cy-5	-0.11	1.31	75.28	17.4
186 11-30 Cy-5	-0.2	0.91	50.96	17.86

Lo Pro

D10-115 8-3 Cy-3	1.41	0	100.05	14.09
D10-115 8-4 Cy-3	1.11	-0.02	93.04	11.93
D10-115 8-17 Cy-3	1.25	-0.05	98.39	12.7
D10-115 8-18 Cy-3	1.26	-0.1	97.1	12.98
D10-115 8-3 Cy-5	-0.51	1.14	99	11.52
D10-115 8-4 Cy-5	0.08	1.26	76.36	16.5
D10-115 8-17 Cy-5	0.16	1.46	89.82	16.25
D10-115 8-18 Cy-5	0.11	1.39	86.49	16.07

Lo No Pro

D10-101 8-3 Cy-3	1.29	-0.05	89.04	14.49
D10-101 8-4 Cy-3	1.11	-0.14	108.14	10.26
D10-101 8-17 Cy-3	1.25	-0.08	100.06	12.49
D10-101 8-18 Cy-3	1.07	-0.13	87.47	12.23
D10-101 8-3 Cy-5	-0.06	1.48	90.43	16.37
D10-101 8-4 Cy-5	-0.08	1.44	85.77	16.79
D10-101 8-17 Cy-5	0.23	1.76	104.28	16.88
D10-101 8-18 Cy-5	0.22	1.67	96.22	17.36

Sham

113 8/17 Cy3	0.95	-0.07	83.69	11.35
113 8/18 Cy3	0.65	-0.33	64.66	10.05
118 8/17 Cy3	0.9	-0.1	77.93	11.55
118 8/18 Cy3	1.12	-0.34	96.13	11.65
131 8/17 Cy3	1.24	-0.08	94.53	13.12
131 8/18 Cy3	1.02	-0.11	83.9	12.16
133 8/17 Cy3	0.87	-0.17	97.78	8.9
133 8/18 Cy3	1.07	-0.09	82.37	12.99
113 8/17 Cy5	-0.21	1.2	96.95	12.38
113 8/18 Cy5	0.09	1.25	78.54	15.92
118 8/17 Cy5	-0.78	0.83	104.49	7.94
118 8/18 Cy5	0.12	1.2	76.77	15.63
131 8/17 Cy5	0.13	1.22	76.59	15.93
131 8/18 Cy5	0.15	1.25	80.54	15.52
133 8/17 Cy5	-0.34	1.13	100.33	11.26
133 8/18 Cy5	-0.23	0.97	85	11.41

Ad Null

12-97 6/12 Cy3	1.38	0.05	120.45	11.46
12-97 6/13 Cy3	1.29	-0.08	125.63	10.27
12-97 6/26 Cy3	1.42	-0.09	126.86	11.19
12-97 6/27 Cy3	1.26	0	118.3	10.65
12-98 6/12 Cy3	1.04	-0.03	104.4	9.96
12-98 6/13 Cy3	1.38	0.05	118.88	11.61
12-98 6/26 Cy3	1.33	-0.11	130.67	10.18
12-98 6/27 Cy3	1.44	0.01	133.06	10.82
12-99 6/12 Cy3	1.05	-0.03	105.75	9.93
12-99 6/13 Cy3	0.98	-0.01	91.44	10.72
12-99 6/26 Cy3	1.15	-0.07	110.01	10.45
12-99 6/27 Cy3	0.97	-0.02	87.87	11.04
12-100 6/12 Cy3	0.85	-0.05	91.35	9.3
12-100 6/13 Cy3	1.12	-0.14	116.47	9.62
12-100 6/26 Cy3	1.22	0.03	107.66	11.33
12-100 6/27 Cy3	1.17	-0.13	114.41	10.23
12-97 6/12 Cy5	0.21	2.04	108.74	18.76
12-97 6/13 Cy5	0.17	2.2	121.29	18.14
12-97 6/26 Cy5	0.06	2.21	128.19	17.24
12-97 6/27 Cy5	0.1	2.25	124.89	18.02
12-98 6/12 Cy5	0.04	1.81	107.08	16.9
12-98 6/13 Cy5	0.13	1.79	102.95	17.39
12-98 6/26 Cy5	0.23	2.07	112.99	18.32
12-98 6/27 Cy5	0.31	2.48	135.87	18.25
12-99 6/12 Cy5	0.23	1.67	85.09	19.63
12-99 6/13 Cy5	0.08	1.53	90.53	16.9
12-99 6/26 Cy5	0.31	2.01	110.63	18.17
12-99 6/27 Cy5	0.05	1.55	95.57	16.22
12-100 6/12 Cy5	0.12	1.86	104.94	17.72
12-100 6/13 Cy5	0.1	1.83	108.38	16.89
12-100 6/26 Cy5	0.24	1.86	105.79	17.58
12-100 6/27 Cy5	0.31	2.15	114.41	18.79

Specific activity is defined as 1,000 multiplied by the ratio of dye concentration over cRNA concentration. Values ≥ 7 are appropriate for hybridization.