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Profiling of local disease-sparing responses to bovine respiratory syncytial virus in intranasally vaccinated and challenged calves

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1. Introduction

Human and bovine respiratory syncytial viruses (HRSV, BRSV) are important paramyxoviral respiratory pathogens in their respective hosts, with very similar pathobiology [1, 2]. Although both viruses can cause disease in all ages of hosts, clinical infections are generally most severe in the young, coincident with the decay of passive immunity, and can be fatal [1,2]. Given its recognized importance in veterinary medicine, a variety of mucosal and parenteral vaccines for BRSV have been commercially available and commonly used since the late 1970's [4]. Many of these vaccines have been shown to confer significant disease-sparing immunity in a challenge model that closely parallels the naturally occurring disease [4]. In contrast, despite years of effort, equivalent immunoprophylactics are still wanting for human pediatric patients [5] due to safety and reactogenicity issues related to

formalin inactivated virus [6,7] and HRSV glycoprotein G vaccinia virus vaccines [8]. As such, human RSV vaccine development is predominately focused on nucleic acid and subunit-based vaccines with several Phase 2 and 3 clinical trials targeting Fusion glycoprotein (gF) as the major neutralizing antigen [7].

In the field of vaccinology, refining vaccine components (in the form of subunit, peptide, virus-like particle or DNA vaccines) is employed under the pretense of supplying the immune system only with the necessary components to generate a measurable (potentially protective) immune response (often assessed by antibody titers) and/or reducing unwanted reactogenic effects. However, it is often seen that such approaches have poor efficacy due to reduced antigenicity from the removal of pathogen associated molecular patterns (PAMPs), requiring adjuvant use to boost suboptimal immunogenicity. Furthermore, multivalent vaccine formulations are further frowned upon as they may dilute the immune response, potentially reducing protection against a single pathogen in favor of protecting against many. The effects of these additional vaccine components, or how such components may alter local immune memory (favorably or unfavorably) to modulate responses to challenge, are often not assessed. This is of particular interest as subunit vaccine formulations often turn to native PAMPs (e.g. TLR agonists) or cytokines to boost efficacy. However, combination vaccine formulations themselves are potentially self-adjuncting, encompassing a more diverse PAMP profile than monovalent vaccine formulations, with the potential to induce trained innate immunity/memory, which being enhanced protects against reinfection and secondary infection through mechanisms independent of T/B cell adaptive responses [9]. Currently, there are few reports that address the effect of vaccine valency on local immune responses or examine the potential to favorably boost or skew subunit vaccine immune responses through combination with safe MLV commercial vaccines.

In the post-genomic era, the rapid growth in 'omic' technologies has led to a greater understanding of physiological processes within the body, allowing researchers to understand how a particular perturbation in a biological system affects up-stream and downstream biological processes

[10,11,12]. Proteomics, in contrast to transcriptomics, has the capacity to highlight actual changes in functional protein levels and provide information not only on proteins produced within tissue of interest but also on those effector proteins originating from the circulatory system. Proteomics is therefore particularly suited to the investigation of immune responses to vaccination which incorporates not only local immune responses but also system responses derived from lymphatic tissue distributed throughout the body. Proteomic marker screening technologies offer the ability to assess potential diagnostic indicators of infection/disease [11] and the application of proteomics to human medicine has resulted in promising prospects for improved vaccinology [13,14]. The pharyngeal tonsil or adenoid, as it is otherwise known in humans, is unique among tonsils, being lined by pseudostratified columnar (respiratory) epithelium [15] in which viruses such as BRSV replicate [16]. This, together with its location between the nasal cavity and the upper airways, makes the pharyngeal tonsil a sentinel site of primary immune responses following natural exposure or mucosal vaccination [17], and therefore a highly relevant tissue for proteomic examination of local responses to vaccination and infection.

Documented disease-sparing has been associated with a variety of mucosal and systemic immune responses in vaccinated cattle including BRSV-specific neutralizing antibody, BRSV-specific total IgG as determined by ELISA, nasal BRSV-specific IgA antibody, and BRSV-specific antibody and cellular immune responses in bronchoalveolar lavage (BAL) fluid [4]. However, the role of the differing viral antigen components within multivalent formulations on disease sparing responses is not clearly understood. A greater understanding of the necessary components required to successfully elicit long-lasting protective immunity could provide significant benefits to design of vaccines for problematic pathogens such as HRSV. Using an established challenge model for BRSV, this study aims to employ proteomic analysis to investigate the effect of additional antigenic material from multivalent mucosal vaccine formulations on local immune responses in the pharyngeal tonsil, and identify potential mechanisms of disease sparing in animals responding favorably to post-vaccination viral challenge.

2. Materials and Methods

2.1 Experimental study

Neonatal Holstein calves were obtained from local dairies and removed from their dams at birth, fed 1.5 L of reconstituted pooled spray-dried bovine colostrum that was previously screened by an ELISA for minimal antibodies to BRSV. Ingestion of this colostrum ensured that calves possessed both low antibody concentrations for BRSV at the time of vaccination (consistent with licensing requirements [17]) and passive immunity to other commonly encountered bovine pathogens. Neonatal calves were considered seronegative if they had virus neutralizing antibody titers of $<1:8$. On arrival at the rearing facility, calves were randomized into individual outdoor pens where they were housed during the vaccination phase, and then housed as a single group during the challenge phase [4]. This husbandry was consistent with the guidelines of the Canadian Council on Animal Care and the local ethical use of animals committee under University of Saskatchewan Animal Use Protocol #19960085.

2.2. Vaccines

Vaccines, a single component modified live (MLV) BRSV and combination containing MLV BRSV, temperature sensitive (TS) BPIV-3, and TS BHV-1 were obtained from the manufacturer (Zoetis, Kalamazoo, MI). All viruses were at commercial release dose (proprietary), and the dose of BRSV was the same in the single component as in combination vaccines. The design of the experiment was a generalized randomized block design with the blocking factor based on order of enrollment (age) and pen where animals were housed during the vaccination phase. At 3-11 days of age calves received: a single 2 ml intranasal (IN) dose of the 3-Way vaccine in one nostril ($n=9$); a single 2 ml IN dose of the BRSV monovalent vaccine in one nostril ($n=9$); or a single 2 ml IN dose of placebo vaccine control

(growth medium and stabilizers) in one nostril (n=9). All calves were challenged as a single cohort, 42 days after vaccination.

2.3. BRSV challenge inoculum

The challenge inoculum consisted of lung wash obtained from a newborn calf infected with BRSV (Asquith strain) as previously described [1]. The lung wash was confirmed negative for bacterial contamination, *Mycoplasma spp*, and BHV-1, BPIV-3, and bovine viral diarrhea viruses by use of standard diagnostic methods.

2.4. Clinical assessment.

Calves were observed for clinical signs post-vaccination, on days -1 and 0 prior to challenge, and on days 1 through 8 after challenge with the BRSV inoculum, at the same time each morning by a veterinarian unaware of treatment status of the calves as previously detailed [1]. Calves were euthanized as previously described [4] by barbiturate overdose via jugular venipuncture 8 days after challenge or earlier if indications of severe respiratory disease onset were observed (clinical score assessment criteria are indicated in S1 Appendix).

2.5. Sample collection

Deep nasal swab specimens were taken from both nares prior to challenge and on day 8 after challenge (or when euthanized due to severe respiratory disease) and placed in transport medium prior to quantification of anti-BRSV IgA. Arterial blood samples were collected from the caudal thoracic aorta [18] and PaO₂ (mm Hg) measurements (corrected for rectal temperature) were performed by use of a gas analyser (model 288, Ciba-Corning, Medfield, MA). Samples of pharyngeal tonsils were collected as soon as possible after euthanasia, snap frozen and maintained at -80 C until analyzed.

2.6 Post mortem analyses

On necropsy the respiratory tract was harvested and percentage of pneumonic lung per lobe visually assessed and scored. Scores were weighted using the following ratios of individual lung lobes to total lung mass: left cranial 5%, left posterior cranial 6%, left caudal 32%, right cranial 6%, right posterior cranial 5%, right middle 7%, right caudal 35% and intermediate 4%. The weighted lung lobe values were summed across lobes to yield the percentage of pneumonic lung for each calf necropsied. Sections of tonsil were collected, fixed with buffered formalin, processed into paraffin blocks and stained with hematoxylin and eosin for routine histological evaluation - selected sections were also immunohistochemically stained for BRSV antigens using routine procedures. ELISAs for BRSV-specific IgG and IgA were performed in serum and nasal swab samples respectively, and analyzed as described previously [19].

2.7 Proteomic analyses

2.7.1 Sample extraction and DIGE labelling

To minimize bias during DIGE proteomic analysis, pharyngeal tonsil samples (24 in total) were processed individually and at random from 8 animals per group (Figure 1). A section of tonsil tissue (approximately 40 mg) was removed from samples frozen at -80°C using a scalpel, immediately placed into DIGE lysis buffer - 7M Urea, 2M Thiourea, 4% CHAPS, 30mM Tris, pH 8.5 (8:1, w:v), and homogenized using a Precellys tissue lyser (5000rpm, 30sec) – samples were placed on ice between pulses and centrifuged (10,000g, 3min) at 4°C. Samples were similarly processed for two further rounds until completely homogenized, and resulting homogenate centrifuged (16,000g, 15min) to remove cell debris. Protein samples were cleaned up using a 2-D Clean-Up kit (GE Lifesciences) following manufacturer's instructions and resuspended in DIGE lysis buffer (100µl). Protein concentration was determined using a 2-D Quant kit (GE Lifesciences), and reciprocal (dye-swapping) CyDye labelling employed as outlined in Figure 1 to minimize labeling bias. Samples were adjusted to

5mg/ml (pH 8.5), and 50µg protein labelled with 400pmol CyDye (GE Lifesciences) following standard procedures. An equal volume of 7M Urea, 2 M Thiourea, 4 % CHAPS, 130 mM DTT, 4% IPG buffer (pH 3-10) was added and samples combined (as outlined in Figure 1), diluted to a final volume of 450 µl with destreak rehydration (GE Lifesciences) solution and loaded onto 24cm pH 3-10NL immobiline IPG strips (GE Lifesciences) with overnight rehydration. Rehydrated strips were focused using a Multiphor II IEF unit at 20°C with voltage program as follows: 500 V for 1 min, 500 V for 5 h, gradient of 500 V to 3500 V over 1.5 h, 3500 V for 10.5 h - cathode wicks were hydrated with destreak solution to improve resolution in the basic region. As the second dimension was limited to 6 IEF strips per unit the focused strips were frozen at -80 C prior to SDS-PAGE to reduce potential variation from freezing and were all processed over a period of 48 h. Focused strips were equilibrated and loaded onto 10% lab-cast (the gel buffer was degassed prior to casting in low-fluorescent glass plates) polyacrylamide (Readysol IEF 40 % T, 3 % C) gels and second dimension separation performed using a DALT Six electrophoresis unit using a laemelli buffer system (25°C, 10mA/gel for 1h followed by 40mA/gel until complete dye front migration) – 12 ml of 10% SDS was applied to the upper buffer chamber to prevent SDS depletion during protein migration. Gel images were acquired using a Syngene XX9 with DIGE edge lighting unit (no binning, f2.8 with manual focus adjustment, automatic exposure) and saved as 16-bit tiff. Statistical analysis was performed using SameSpots version 5 (TotalLab). Images were quality checked prior to proceeding to alignment and spot picking. A DIGE experimental approach was selected in the software and warping alignment was performed on the Cy2 labeled internal standards only against an automatically selected reference standard. Approximately 30 manual anchors were applied prior to automatic alignment. Quality of automatic alignment was manually assessed and amended where applicable. Spots were automatically detected using a composite image generated from all acquired gel images. Statistical analysis was performed using SameSpots and candidate spots selected for identification based on relative fold-change and significance ($p < 0.05$, ANOVA).

Figure 1: Schematic representation of DIGE experimental setup and CyDye reciprocal labeling approach for proteomic analysis of bovine pharyngeal tonsils.

2.7.2 Mass spectrometry protein identification

Preparative gels were prepared using 800µg of protein from pooled samples, with 2D GE performed as before and stained using with Coomassie labelling [20]. Protein spots of interest were excised manually from preparative gels using a clean scalpel, destained with 50µl of 1:1 acetonitrile:100mM ammonium bicarbonate at 37°C for 1h and dehydrated with addition of 500µl acetonitrile for 15min. Tubes were centrifuged (5000g, 1 min), liquid removed, and gel pieces stored at -80°C. Prior to further use, gel pieces were completely rehydrated with approximately 10µl of 13ng/µl modified trypsin in 10mM ammonium bicarbonate/10% acetonitrile and stored on ice for 2h and incubated overnight at 37°C. Condensate was removed from the Eppendorf lid to desalt the sample, and approximately 2µl of 1% formic acid added to rehydrate gel pieces. Tryptic digests were extracted with 20µl of saturated α-Cyano-4-hydroxycinnamic acid in 0.1% trifluoroacetic acid, 60% acetonitrile with mild sonication in a water bath at 37°C for 15min.

1µl of tryptic digest was spotted onto MTP 384 polished steel and AnchorChip 384 600µm targets (Bruker Daltonics Bellerica, Mass. USA) and air dried. External calibration was performed prior to sample acquisition and every 3 spots using peptide calibration standard II 700-3500Da (Bruker working stock diluted 1:10 with saturated α-Cyano-4-hydroxycinnamic acid in 0.1% trifluoroacetic acid, 60% acetonitrile). Peptide Mass Fingerprinting (PMF) was performed using a Bruker AutoFlex operating in +ve ionization reflective mode with Smartbeam 6 ultra-laser using FlexControl operating software. Operating conditions were as follows: ion source 1, 19.14 kV; ion source 2, 16.79 kV; lens voltage, 8.09 kV, reflector 1, 21.14 kV; reflector 2, 9.67 kV; pulsed ion extraction, 120ns; gain factor,

10x and matrix suppression mass cut-off, 450 Da. Acquisitions were based on an average of 2000 shots at 1000 Hz using random walk sample carrier (800 μ m) and mass range of 700-4000 Da. Smoothing and background subtraction was automatically employed using FlexAnalysis (Bruker) and internal calibration employed using trypsin autodigestion fragments (842.509 Da, 1045.5637 Da and 2211.1040 Da). PMF was performed using the Mascot Server version 2.5 (www.matrixscience.com). Masses with S/N > 10 were searched against SwissProt with 'other mammalian' taxonomy (where applicable contaminant masses were removed via searching against known contaminants in the Mascot database). Search parameters were as follows: trypsin specificity with 1 missed cleavage, monoisotopic mass, carbamidomethyl cysteine fixed modification, oxidized methionine variable modification, peptide tolerance 100 ppm, protein mass at estimated protein molecular weight (MW).

2.8 Data analysis

For lung lesions, arcsine square root transformed total percentages were analyzed using a general linear mixed model with a fixed effect of treatment. Since the overall effect of treatment was significant ($p < 0.05$), pairwise treatment comparisons were made, and treatment least-squared means and standard errors were back-transformed. Clinical and laboratory outcome variables were stratified by study treatment group (i.e. BRSV, PI3V, BHV1 vaccine; BRSV vaccine; or placebo vaccine) and descriptive statistics performed. Prior to investigating any potential differences among groups, data were assessed for normality using the Shapiro-Wilk test. Blood oxygen saturation and IgG data were normally distributed and statistical analysis performed by two-way ANOVA with Bonferroni post-hoc analysis. For IgA, data were not normally distributed and therefore a Kruskal-Wallis test with Dunn's multiple comparison was performed for pairwise comparisons. Normally distributed data results are presented as mean and standard deviation and non-normally distributed data are presented as the median and range of values. Differences were considered statistically significant where $p \leq 0.05$. All statistical analyses

were performed using commercially available software (IBM SPSS Statistics for Windows (V.23 Armonk, NY)). Heteroscedastic t-test was performed to determine the significant differences in protein spot volumes between treatment groups. Protein functional annotation was performed using Reactome with mapping to human homologues.

3. Results

3.1 Clinical and pathological findings.

The mean, standard deviation or median and ranges, where appropriate, for key clinical and pathological findings at day 50 (study completion, or day of euthanization where applicable) are summarized in Table 1 for the 3 study groups. The percentage of pneumonic lung was significantly ($p=0.0001$) reduced in both vaccinated groups as compared to the placebo group, with no significant differences evident between vaccinated cohorts. Although numerically different, there were no statistical differences in blood oxygen saturation between any of the groups at the stringent $p<0.001$ level. Significant ($p<0.001$) differences were noted in serum IgG between the BRSV, PI3V, BHV1 vaccinates and the placebo group, and between the BRSV vaccinates and the placebo - however, no significant differences in anti-BRSV IgG levels were observed between either of the vaccine groups. BRSV, PI3V and BHV-1 3-Way vaccination resulted in a significant ($p<0.001$) elevation in anti-BRSV IgA levels relative to placebo controls, and whilst mean IgA was elevated in the BRSV only vaccine group, this was not significantly different. BRSV challenge also led to mortality in 4 out of 9 calves in the placebo control group, and in 2 out of 9 in the BRSV-vaccinated group, with no mortality occurring within the BRSV, PI3V and BHV-1 vaccinate group. Sections of pharyngeal tonsils from calves in all groups had cortical follicular hyperplasia (Figure 2A) - differences in the extent and magnitude of follicular hyperplasia among groups was not morphometrically determined. Additionally, immunohistochemical staining of tonsillar sections revealed variable amounts of BRSV antigens,

almost exclusively in the respiratory (columnar) epithelial that overlays the lymphoid component of the tonsils (Figure 2B).

Figure 2: (A) Follicular hyperplasia in pharyngeal tonsil from a placebo-vaccinated calf euthanized on day 6 after infection (H and E stain). (B) Growth of BRSV in respiratory epithelium of pharyngeal tonsil from a placebo-vaccinated calf euthanized on day 6 after infection (immunohistochemical stain for BRSV).

Table 1: Clinical outcome of BRSV challenge. Statistical significance of vaccinates was compared to placebo control, *** $p < 0.001$. Values represent mean, standard deviation or median and ranges, where appropriate.

Outcome variable	Group		
	Placebo control	BRSV vaccinates	3-Way vaccinates (BRSV, PI3V, BHV-1)
% pneumonic lung	29 (± 6)	7 (± 3) ***	9 (± 4) ***
Arterial PaO ₂ (mmHg)	67.2 (48.9-86.9)	84.6 (47.0-104.5)	77.7 (65.0-97.4)
Mortality (n)	4	2	0
Serum IgG (ELISA units post-challenge)	5.0 (0-10)	50.8 (0-128) ***	59.7 (11-112) ***
Nasal IgA (ELISA units post-challenge)	6.9 (0-21)	19.6 (0-51)	19 (20-75) ***

3.2 DIGE analysis of pharyngeal tonsils of differentially vaccinated calves following BRSV challenge

Proteomic analysis of pharyngeal tonsils (n=8 per group) from vaccinated (BRSV and 3-Way (BRSV, PI3 and BHV-1)) and placebo control study groups was performed using 2D-DIGE methodology -

Figure 3 illustrates a representative 2D-DIGE gel image of bovine pharyngeal tonsils. 2D gels revealed

excellent protein separation and resolution by pI and MW, with an average of 867 spots detected within pharyngeal tonsil lysates across all gels. Spot volumes were normalized automatically via SameSpots using Cy2 internal pools, and unsupervised multivariate analysis was performed using principal component analysis (PCA) of all protein spot volumes without data pre-filtering. The scores plot in Figure 4 illustrates a clustering of animals into their respective treatment groups based on components 1 ($R^2X = 14.0\%$) and 2 ($R^2X = 8.65\%$). However, there was an overlap, particularly with animals from the 3-Way vaccine group with placebo controls (animals 521 and 527). Upon further inspection it can be seen that these specific animals from vaccine treatment groups responded poorly to BRSV challenge, as indicated by early euthanization due to poor clinical score (calves 521 and 527) and low D50 IgG ELISA titre (calve 522) which was comparable to non-vaccinated calves. Due to the clustering and separation of calves by vaccine treatment and mortality, downstream analysis focused on identification and functional analysis of proteins associated with (1) differential response to BRSV challenge in differing vaccine formulations, and (2) investigation of the function of proteins differentially regulated in calves which succumbed early to BRSV challenge as potential correlates of BRSV mortality in pharyngeal tonsil.

Figure 3: Representative 2D-DIGE gel of bovine pharyngeal tonsil analysed on 24cm pH 3-10NL IPG strips and 10% SDS-PAGE. Cy3 and Cy5 are representative of 3-Way vaccinated and placebo control animals respectively.

Figure 4: Unsupervised principal component analysis (PCA) scores plot of pharyngeal tonsil proteome in individual calves (labelled by identifier number) – coloured by (A) vaccine group and (B) day of euthanization post-BRSV challenge.

3.2.1: *Differential response in pharyngeal tonsils proteome in response to vaccination.*

Statistical analysis was performed to select proteins exhibiting significantly different levels within pharyngeal tonsil of animals from different vaccine study groups for downstream identification. Seventy-three protein spots were found to be significantly altered in BRSV and 3-Way vaccinated animals relative to placebo control - 29 of which were specific to the 3-Way vaccine and 3 to BRSV vaccinates. A further 3 protein spots were found to be significantly altered in BRSV vaccinated animals relative to 3-Way vaccinated animals. Figure 5A illustrates the 76 gel spots selected for downstream analysis from which 62 proteins were successfully identified, corresponding to 39 unique proteins (Table 2). Spot quantification within individual samples is presented in Supplementary Table S1 - complete annotation of protein identifications including number of searched peptides, matched peptides and mass deviation is presented in Supplementary Table S2.

Figure 5: Gel location of differentially altered proteins in pharyngeal tonsil in animals differing in (A) pre-challenge vaccine treatment and (B) post-BRSV challenge mortality.

3.2.2: *Functional attribution of proteins differentially altered in pharyngeal tonsil in response to vaccination*

Table 3 illustrates the functional attribution of differentially altered proteins performed using the Reactome pathway database with mapping to human homologues. Significantly regulated proteins were primarily associated with immune response processes including MHC Class II antigen presentation and neutrophil degranulation from proteins differentially regulated by both vaccine treatments relative to placebo controls, with interleukin-12 (IL12) signaling and downstream JAK-STAT signaling only

associated with the 3-Way vaccine formulation. BRSV challenge resulted in a net increase in proteins associated with platelet degranulation in placebo control relative to both vaccine formulations. Other specific pathways only associated with the 3-Way vaccine formulation included chaperonin-mediated protein folding, activation of BAD, and translocation of GLUT4 to the plasma membrane. Whilst mapping could not be achieved for plastin-3 and chloride intracellular channel protein 5, plastin-3 gene ontology revealed association with cellular component biogenesis and cytoskeleton organization with actin binding function - gene ontology annotation was not available for chloride intracellular channel protein 5.

Table 2: Detected proteins in pharyngeal tonsil post-BRSV challenge within differing vaccine treatment groups. Relative protein spot profiles are indicated versus PBS control group animals.

Protein name	Spot no.	Spot normalized volume [mean $\pm$ SEM]			Relative spot volume versus PBS group				Protein spot MASCOT identification						
		PBS	BRS V	3-Way	BRSV vaccine s		3-Way vaccine s		Accession	Score	Expect	Matches	Coverage	MW	pI
Galectin-9	800	0.965 $\pm$ 0.017	1.039 $\pm$ 0.054	1.358 $\pm$ 0.03	Fold - change	p-value	Fold - change	p-value	LEG9_BOVIN	88	2.2E-05	11	32	39309	943
Chloride intracellular channel protein 5	794	0.76 $\pm$ 0.017	0.913 $\pm$ 0.044	1.024 $\pm$ 0.038	Fold - change	p-value	Fold - change	p-value	CLIC5_BOVIN	62	7.4E-03	7	25	49100	447
Annexin A2	746	0.797 $\pm$ 0.029	0.872 $\pm$ 0.036	1.07 $\pm$ 0.037	Fold - change	p-value	Fold - change	p-value	ANXA2_BOVIN	110	1.3E-07	13	41	38873	692
	736	0.834 $\pm$ 0.036	0.946 $\pm$ 0.058	1.128 $\pm$ 0.046	Fold - change	p-value	Fold - change	p-value	ANXA2_BOVIN	90	1.4E-05	10	32	38873	692
	10	0.74	0.86	1.03	Fold - change	p-value	Fold - change	p-value	ANXA	1	3.	11	34	38	6
					Fold - change	p-value	Fold - change	p-value	ANXA	1	3.	11	34	38	6

	37	8 ± 0.03 8	± 0.06 5	2 ± 0.05 5		8E -01		7E -03	2_BO VIN	1 6	3E - 08			87 3	. 9 2
	10 36	0.80 9 ± 0.04 1	0.85 2 ± 0.05 6	1.02 9 ± 0.04 7	1.05	5.6 8E -01	1.27	5.0 5E -03	ANXA 2_BO VIN	1 5 5	3. 3E - 12	12	41	38 87 3	6 .9 2
Peroxiredoxin-6	85 0	0.85 3 ± 0.03 5	1.15 8 ± 0.08 1	1.31 ± 0.08 9	1.36	9.6 0E -03	1.54	1.5 0E -03	PRDX 6_BO VIN	1 1 4	5. 2E - 08	10	37	25 10 8	6
	85 5	0.93 2 ± 0.04 6	1.14 4 ± 0.10 5	1.31 8 ± 0.06 1	1.23	1.1 3E -01	1.42	3.5 0E -04	PRDX 6_BO VIN	1 4 2	8. 3E - 11	13	52	25 10 8	6
	85 9	0.91 7 ± 0.06 4	1.22 1 ± 0.07 6	1.36 5 ± 0.06 7	1.33	1.2 3E -02	1.49	4.4 6E -04	PRDX 6_BO VIN	2 1 6	3. 3E - 18	20	68	25 10 8	6
	87 1	0.96 8 ± 0.04	1.16 6 ± 0.05 6	1.18 9 ± 0.03 9	1.20	1.8 1E -02	1.23	2.1 9E -03	PRDX 6_BO VIN	5 6	3. 1E - 02	5	25	25 10 8	6
	86 2	0.84 8 ± 0.06 7	0.88 5 ± 0.06 1	1.24 ± 0.08 6	1.04	7.0 2E -01	1.46	4.6 3E -03	GSTA 2_BO VIN	1 3 2	8. 3E - 10	15	47	25 81 4	8 .6 6
Aldehyde dehydrogenase, cytosolic	47 1	0.95 6 ± 0.04 4	1.06 7 ± 0.04 9	1.24 6 ± 0.04 5	1.12	1.3 6E -01	1.30	6.8 5E -04	AL1A 1_BO VIN	8 9	1. 5E - 05	14	41	55 39 8	6 .2 4
14-3-3 protein epsilon	81 3	0.85 6 ± 0.04 1	0.88 5 ± 0.03 2	1.07 2 ± 0.04 7	1.03	5.9 6E -01	1.25	5.7 6E -03	1433 E_BO VIN	8 2	8. 5E - 04	11	34	29 32 6	4 .6 3
Purine nucleoside phosphorylase	82 1	0.84 1 ± 0.04 3	1.05 7 ± 0.06 7	1.14 4 ± 0.04 5	1.26	2.5 0E -02	1.36	3.9 5E -04	PNPH _BOV IN	8 3	7. 2E - 05	12	28	32 24 4	5 .9 2
Glutathione peroxidase 1	90 3	0.92 3 ± 0.03 2	0.99 3 ± 0.01 7	1.07 6 ± 0.03 1	1.08	9.0 6E -02	1.17	5.2 0E -03	GPX1 _BOV IN	1 2 1	1. 0E - 08	13	61	22 93 0	6 .7 4
Vimentin	10 08	0.88 2 ± 0.04 8	0.83 ± 0.05 7	1.14 4 ± 0.07 2	0.94	5.2 5E -01	1.30	1.4 1E -02	VIME _BOV IN	9 0	1. 4E - 05	12	40	53 75 2	5 .0 6
	10 09	0.93 7 ± 0.03 8	0.95 ± 0.03 2	1.09 ± 0.04 4	1.01	8.1 5E -01	1.16	2.7 1E -02	VIME _BOV IN	9 1	1. 0E - 05	16	34	53 75 2	5 .0 6
Actin, cytoplasmic 1	58 2	0.85 6 ± 0.04 4	1.01 3 ± 0.06 4	1.10 1 ± 0.05 4	1.18	7.9 9E -02	1.29	5.2 2E -03	ACTB _BOV IN	1 2 2	8. 3E - 09	15	58	42 05 2	5 .2 9
	10 20	0.76 4 ±	0.85 7 ±	0.92 9 ±	1.12	8.9 2E	1.22	8.3 1E	ACTB _BOV	1 0	1. 7E	12	42	42 05	5 . .

		0.02 6	0.04	0.04 2		-02		-03	IN	9	- 07			2	2
	10 22	0.89 7 ± 0.04 3	1.04 2 ± 0.05 3	1.14 7 ± 0.04 1		6.2 3E -02		1.3 4E -03	ACTB _BOV IN	1 0 5	4. 2E -			42 05 2	5 . 2 9
	10 24	0.93 7 ± 0.05	1.04 9 ± 0.04 1	1.17 6 ± 0.03 7		1.2 4E -01		3.1 1E -03	ACTB _BOV IN	1 2 4	5. 2E -			42 05 2	5 . 2 9
	10 25	0.81 9 ± 0.05 3	0.96 8 ± 0.05 9	1.04 8 ± 0.06 4		9.7 9E -02		2.2 0E -02	ACTB _BOV IN	1 2 9	1. 7E -			42 05 2	5 . 2 9
	10 26	0.89 2 ± 0.04 9	1.00 6 ± 0.03 6	1.15 1 ± 0.05 8		1.0 0E -01		6.1 7E -03	ACTB _BOV IN	9 8	2. 0E -			42 05 2	5 . 2 9
14-3-3 protein theta	10 39	0.85 8 ± 0.03 4	0.92 6 ± 0.04 2	1.08 2 ± 0.06 1		2.5 4E -01		1.1 7E -02	1433T _BOV IN	7 8	1. 9E -			28 03 2	4 . 6 8
Carbonic anhydrase 3	83 8	0.84 5 ± 0.03 9	0.94 ± 0.03 9	1.04 2 ± 0.03 6		1.2 4E -01		3.3 1E -03	CAH3 _BOV IN	1 0 4	5. 2E -			29 63 7	7 . 1
Triosephosphate isomerase	86 9	0.86 8 ± 0.03 1	0.86 ± 0.02 5	1.02 1 ± 0.04 5		8.4 9E -01		2.0 8E -02	TPIS_ BOVI N	1 9 1	1. 0E -			26 90 1	6 . 4 5
Vinculin	87	0.93 4 ± 0.04 7	1.06 8 ± 0.05 8	1.22 8 ± 0.06 3		1.1 6E -01		3.8 1E -03	XP_0 05226 540.1	2 5 2	1. 3E -			11 72 13	5 . 8 3
	95	0.98 9 ± 0.04 6	1.13 9 ± 0.04 8	1.21 4 ± 0.06 4		5.1 5E -02		1.8 7E -02	XP_0 05226 540.1	1 7 2	1. 3E -			11 72 13	5 . 8 3
Tubulin alpha- 1B chain	10 06	0.89 8 ± 0.05 4	0.85 4 ± 0.05 6	1.14 1 ± 0.06 5		6.0 7E -01		1.7 4E -02	TBA1 B_BO VIN	1 5 0	1. 3E -			50 80 4	4 . 8 4
	10 07	0.91 6 ± 0.05	1.00 7 ± 0.04 4	1.16 2 ± 0.05 5		2.1 8E -01		7.3 3E -03	TBA1 B_BO VIN	1 2 6	3. 3E -			50 80 4	4 . 9 4
Tropomyosin alpha-3 chain	79 3	0.94 8 ± 0.04 2	0.91 6 ± 0.03 2	1.09 7 ± 0.04 4		5.7 4E -01		3.5 3E -02	TPM3 _BOV IN	4 4	5. 5E -			32 85 6	4 . 6 8
Plastin-3	32 2	0.88 3 ± 0.03 9	0.99 1 ± 0.02 2	1.09 ± 0.06 4		4.4 8E -02		2.4 0E -02	PLST _BOV IN	8 2	7. 4E -			71 26 6	5 . 4 1
Phosphoglycerat e kinase 1	60 6	0.87 5 ± 0.04	0.89 2 ± 0.02	1.02 9 ± 0.03		7.4 4E -01		1.6 7E -02	PGK1 _BOV IN	1 2 8	2. 1E -			44 90 8	8 . 5

			5	6							12				
Desmin	45 1	0.87 4 ± 0.05	1.01 2 ± 0.05 1	1.09 6 ± 0.04 8	1.16	9.1 7E -02	1.25	9.3 7E -03	DES M_BO VIN	7 2	8. 9E - 04	10	33	53 55 6	5 . 2 1
Moesin	28 2	0.99 9 ± 0.03 7	1.13 1 ± 0.06 3	1.22 8 ± 0.05 8	1.13	1.1 5E -01	1.23	8.5 7E -03	MOE S_BO VIN	1 9 7	2. 6E - 16	28	34	68 04 7	5 . 9
	28 6	0.99 4 ± 0.03 9	1.10 1 ± 0.05 2	1.21 ± 0.04 9	1.11	1.4 5E -01	1.22	5.7 9E -03	MOE S_BO VIN	5 8	2. 1E - 02	8	17	68 04 7	5 . 9
	28 8	1.03 9 ± 0.03 9	1.08 6 ± 0.03 2	1.21 9 ± 0.04 9	1.04	4.0 3E -01	1.17	1.6 9E -02	MOE S_BO VIN	2 4 8	2. 1E - 21	34	51	68 04 7	5 . 9
Heat shock protein beta-1	87 8	1.08 ± 0.09 1	0.99 1 ± 0.04 8	1.28 7 ± 0.07 5	0.92	4.3 1E -01	1.19	1.2 2E -01	HSPB 1_BO VIN	1 8 2	8. 3E - 15	15	78	22 43 6	5 . 8
Isocitrate dehydrogenase [NADP] cytoplasmic	61 1	1.04 6 ± 0.06 3	1.07 1 ± 0.05 6	1.28 4 ± 0.06 1	1.02	7.7 9E -01	1.23	2.2 6E -02	IDHC _BOV IN	2 5 9	1. 7E - 22	34	67	47 09 8	6 . 1 3
Sulfotransferase 1A1	80 9	0.77 3 ± 0.03 9	0.84 1 ± 0.03 8	0.96 7 ± 0.05 6	1.09	2.5 1E -01	1.25	1.9 4E -02	ST1A 1_BO VIN	1 0 6	3. 3E - 07	10	44	34 33 3	6 . 5 2
Guanine nucleotide- binding protein G	74 4	0.81 8 ± 0.02 8	0.94 7 ± 0.05 1	1 ± 0.05 1	1.16	5.7 2E -02	1.22	1.3 1E -02	GBB1 _BOV IN	1 0 0	1. 4E - 06	11	51	38 15 1	5 . 6
Carbonic anhydrase 2	84 5	0.87 7 ± 0.06 5	1.54 9 ± 0.27 2	1.17 5 ± 0.13 1	1.77	5.5 3E -02	1.34	8.3 5E -02	CAH2 _BOV IN	6 4	5. 0E - 03	6	35	29 09 6	6 . 4 1
F-actin capping protein subunit beta	78 8	0.95 8 ± 0.03 1	1.10 8 ± 0.04 9	1.06 1 ± 0.02 4	1.16	3.0 7E -02	1.11	2.7 7E -02	CAPZ B_BO VIN	1 8 0	1. 3E - 14	18	49	34 17 6	6 . 0 2
Cathepsin D	61 5	0.99 6 ± 0.06 9	1.24 1 ± 0.06 2	1.24 8 ± 0.07 6	1.25	2.5 8E -02	1.25	3.6 8E -02	CATD _BOV IN	3 3	7. 1E +0	5	15	42 92 0	6 . 6 6
	62 5	1.04 4 ± 0.04 5	1.26 4 ± 0.08 2	1.27 4 ± 0.03 7	1.21	4.8 4E -02	1.22	2.3 6E -03	CATD _BOV IN	5 4	4. 9E - 02	6	15	42 92 0	6 . 6 6
Peroxiredoxin-2	90 4	0.86 5 ± 0.07 5	1.25 4 ± 0.14 3	1.15 ± 0.07 9	1.45	4.6 0E -02	1.33	2.7 6E -02	PRDX 2_BO VIN	9 6	3. 0E - 06	8	41	21 93 2	5 . 3 7
Serpin A3-5	10 00	1.21 6 ± 0.06	0.88 4 ± 0.06 4	0.91 ± 0.02 4	0.73	2.9 8E -03	0.75	1.4 4E -03	SPA3 5_BO VIN	1 0 8	2. 8E - 06	13	25	46 48 2	6 . 4 8

	36 3	1.32 7 ± 0.06 6	0.85 ± 0.08 1	0.91 5 ± 0.06	0.64	8.0 7E -04	0.69	7.0 2E -04	SPA3 5_BO VIN	1 0 8	2. 8E - 06	13	25	46 48 2	6 .4 8
	99 9	1.24 2 ± 0.04 9	0.87 2 ± 0.06 2	0.91 1 ± 0.03 8	0.70	6.6 2E -04	0.73	2.2 7E -04	SPA3 5_BO VIN	1 0 8	2. 8E - 06	13	25	46 48 2	6 .4 8
Proteasome subunit alpha type-4	84 0	1.22 1 ± 0.06	1.12 4 ± 0.04 1	0.92 3 ± 0.06 8	0.92	2.3 4E -01	0.76	8.0 8E -03	PSA4 _BOV IN	7 6	3. 2E - 04	9	48	29 75 0	7 .5 7
Threonine-- tRNA ligase, cytoplasmic	24 1	1.15 4 ± 0.06 3	0.91 2 ± 0.08	0.88 6 ± 0.03 1	0.79	4.2 8E -02	0.77	4.6 1E -03	SYTC _BOV IN	6 4	4. 8E - 03	6	9	84 35 1	6 .3 4
Serotransferrin	27 6	1.29 ± 0.10 7	0.95 5 ± 0.04 9	0.92 8 ± 0.06 4	0.74	2.3 8E -02	0.72	1.8 9E -02	TRFE _BOV IN	1 7 6	3. 3E - 14	17	28	79 87 0	6 .7 5
	27 7	1.35 7 ± 0.11 9	0.78 8 ± 0.08 4	0.90 4 ± 0.11 7	0.58	2.9 1E -03	0.67	2.3 0E -02	TRFE _BOV IN	2 0 3	6. 6E - 17	23	37	79 87 0	6 .7 5
	30 2	1.34 5 ± 0.12	1.05 ± 0.06 8	0.98 9 ± 0.09	0.78	7.0 0E -02	0.74	4.4 3E -02	TRFE _BOV IN	1 6 3	6. 6E - 13	19	34	79 87 0	6 .7 5
Rab GDP dissociation inhibitor alpha	38 1	1.07 5 ± 0.05	0.88 3 ± 0.08 3	0.83 8 ± 0.04 3	0.82	8.5 8E -02	0.78	4.3 3E -03	GDIA _BOV IN	8 8	2. 2E - 05	13	32	51 10 3	5
	38 8	1.16 6 ± 0.07 3	0.90 8 ± 0.04 2	0.90 7 ± 0.06 5	0.78	1.4 2E -02	0.78	2.5 9E -02	GDIA _BOV IN	9 1	1. 1E - 05	15	33	51 10 3	5
Fibrinogen beta	10 43	1.07 6 ± 0.05 6	0.89 8 ± 0.05	0.91 2 ± 0.02 5	0.84	4.3 3E -02	0.85	3.1 4E -02	FIBB_ BOVI N	5 7	2. 4E - 02	9	15	53 93 3	8 .4 5
Vitamin D- binding protein	38 9	1.13 5 ± 0.08 7	0.96 6 ± 0.02 8	0.87 2 ± 0.06 2	0.85	1.1 7E -01	0.77	3.7 2E -02	VTDB _BOV IN	6 7	2. 4E - 03	10	28	54 90 4	5 .3 6
WD repeat- containing protein 1	35 2	1.14 3 ± 0.11 2	0.90 1 ± 0.08	0.77 8 ± 0.04 8	0.79	1.2 3E -01	0.68	1.9 4E -02	WDR 1_BO VIN	7 3	7. 2E - 04	6	10	66 21 6	6 .2 4
Serum albumin	10 33	1.35 6 ± 0.09	1.10 3 ± 0.07 3	1.03 3 ± 0.07 4	0.81	6.0 8E -02	0.76	2.1 3E -02	ALBU _BOV IN	1 4 6	3. 3E - 11	19	35	71 24 4	5 .8 2
	10 45	1.15 4 ± 0.08 3	0.92 ± 0.06 7	0.87 1 ± 0.06 2	0.80	5.8 2E -02	0.75	2.2 8E -02	ALBU _BOV IN	1 2 4	5. 2E - 10	16	31	71 24 4	5 .8 2
2-oxoglutarate	14	1.28	1.11	0.99	0.87	2.0	0.78	3.2	ODO1	6	5.	12	11	11	6

dehydrogenase, mitochondrial	6	6 ± 0.09 7	7 ± 0.07	9 ± 0.05 2		7E -01		7E -02	_BOV IN	4	7E - 03			68 75	. 2 8
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Table 3: Reactome analysis of differentially altered proteins in pharyngeal tonsils of vaccinated calves following BRSV-challenge.

Pathway name	No. Entities found	No. Entities total	Entities p-value	Entities FDR	Submitted entities found (3-way only: bold , <u>BRSV only</u> : underlined)
Immune System	14	2229	7.7E-03	5.3E-02	PNP, VIM, CTSD, CAPZB, FGB, PRDX6, SERPINA3-5, VCL, MSN, ACTB, TBA1B, ANXA2, LGALS9, IDH1
Adaptive Immune System	3	947	6.1E-01	6.1E-01	CTSD, CAPZB, TBA1B
MHC class II antigen presentation	3	141	1.1E-02	6.3E-02	CTSD, CAPZB, TBA1B
Innate Immune System	9	1182	1.2E-02	6.3E-02	PRDX6, PNP, CTSD, FGB, SERPINA3-5, ACTB, IDH1, ANXA2, VCL
Neutrophil degranulation	7	480	8.1E-04	1.4E-02	PRDX6, PNP, CTSD, SERPINA3-5, IDH1, ANXA2, VCL
Cytokine Signaling in Immune system	4	772	2.4E-01	2.4E-01	MSN, LGALS9, VIM, ANXA2
Interleukin-12 signaling	2	47	1.0E-02	6.2E-02	MSN, ANXA2
Gene and protein expression by JAK-STAT signaling after Interleukin-12 stimulation	2	38	6.9E-03	4.8E-02	MSN, ANXA2
Hemostasis	11	743	1.6E-05	1.6E-03	TF, SERPINA3-5, CAPZB, FGB, VCL, GNB1, WDR1, ACTB, TBA1B, ALB, ANXA2
Platelet activation, signaling and aggregation	7	279	2.9E-05	2.1E-03	VCL, TF, SERPINA3-5, FGB, ALB, GNB1, WDR1
Response to elevated platelet cytosolic Ca2+	6	133	4.4E-06	6.4E-04	VCL, TF, SERPINA3-5, FGB, ALB, WDR1
Platelet degranulation	6	128	3.5E-06	6.4E-04	VCL, TF, SERPINA3-5, FGB, ALB, WDR1
Signal Transduction	9	2675	5.2E-01	5.2E-01	GDI1, FGB, VCL, ALDH1A1, GNB1, YWHAE, ACTB, YWHAQ, TBA1B, HSPB1
MAP2K and MAPK activation	3	40	3.1E-04	7.5E-03	FGB, VCL, ACTB,
MAPK6/MAPK4 signaling	1	89	8.1E-03	1.6E-02	<u>HSPB1</u>
RHO GTPases activate IQGAPs	2	31	4.7E-03	3.7E-02	ACTB, TBA1B
Signaling by VEGF	1	106	9.6E-03	1.9E-02	<u>HSPB1</u>
VEGFA-VEGFR2 Pathway	1	97	8.8E-03	1.8E-02	<u>HSPB1</u>
Metabolism of proteins	8	2010	3.3E-01	3.3E-01	CTSD, TF, CAPZB, GNB1, ACTB, TBA1B, ALB, TARS
Protein folding	3	101	4.4E-03	3.5E-02	GNB1, ACTB, TBA1B
Chaperonin-mediated protein folding	3	95	3.7E-03	3.5E-02	GNB1, ACTB, TBA1B
Cooperation of Prefoldin and TriC/CCT in actin and tubulin folding	2	32	5.0E-03	4.0E-02	ACTB, TBA1B
Vesicle-mediated transport	8	765	2.8E-03	3.1E-02	TF, CAPZB, GDI1, YWHAE, ACTB, YWHAQ, TBA1B, ALB
Membrane Trafficking	7	639	4.1E-03	3.5E-02	TF, CAPZB, GDI1, YWHAE, ACTB, YWHAQ, TBA1B
Translocation of GLUT4 to the plasma membrane	4	80	1.4E-04	7.5E-03	YWHAE, ACTB, YWHAQ, TBA1B
Cellular responses to external stimuli	6	482	4.4E-03	3.5E-02	PRDX6, CAPZB, PRDX2, TBA1B, YWHAE, GPX1
Cellular responses to stress	6	405	1.8E-03	2.6E-02	PRDX6, CAPZB, PRDX2, TBA1B, YWHAE, GPX1
Detoxification of Reactive Oxygen Species	3	39	2.9E-04	7.5E-03	PRDX6, PRDX2, GPX1
HSP90 chaperone cycle for steroid hormone	2	56	1.4E-02	7.2E-02	TBA1B, CAPZB

receptors (SHR)					
Programmed Cell Death	3	175	1.9E-02	7.7E-02	YWHAЕ, YWHAQ, VIM
Activation of BH3-only proteins	2	30	4.4E-03	3.5E-02	YWHAЕ, YWHAQ
Activation of BAD and translocation to mitochondria	2	15	1.1E-03	1.7E-02	YWHAЕ, YWHAQ
Transport of small molecules	1	727	6.6E-02	1.3E-01	<u>CA1</u>
O2/CO2 exchange in erythrocytes	1	12	1.1E-03	2.2E-03	<u>CA1</u>
Erythrocytes take up oxygen and release carbon dioxide	1	8	7.2E-04	1.4E-03	<u>CA1</u>
Erythrocytes take up carbon dioxide and release oxygen	1	12	1.1E-03	2.2E-03	<u>CA1</u>

3.2.2: Differential proteome response in post-BRSV challenge pharyngeal tonsil in animals euthanized due to poor clinical score versus animals euthanized at study completion

Animals euthanized early due to severe respiratory distress (n=5) (observed clustering on the PCA scores plot – Figure 4B) were grouped and statistical analysis performed to identify protein spots. This demonstrated levels of protein spots to be significantly different in the early-euthanized cohort relative to that observed in animals surviving to study end (n=19). Through this analysis, Figure 5B illustrates the 65 protein spots that were found to be significantly different of which 35 were identified and found to correspond to 28 unique proteins (Table 4). Table 5 illustrates the Reactome functional analysis of these proteins revealing a significant differential regulation in proteins associated with immune response processes (14 proteins), homeostasis (8 proteins) and cellular responses to external stimuli (detoxification of reactive oxygen species - 3 proteins). Differentially regulated immune response processes included IL-4 and IL-13 signaling (ANXA1, HSP90AA1, VIM and FSCN1), IL-12 signaling (GST, MSN, HNRNPA2B1), TLR regulation by endogenous ligand (FGG and FGB) and neutrophil degranulation (SERPINA3-5, HSP90AA1, GELS and PRDX6). Significant homeostatic alterations were associated with platelet activation, signaling and aggregation, with specific alteration of integrin alpha IIb beta signaling (FGG and FGB) and platelet degranulation (TF, SERPINA3-5, APOA1, ALB, FGG, FGB and ACTN1) and formation of fibrin clots (FGG and FGB). This indication of elevated clotting was also observed in serum samples from calves sacrificed early due to clinical score (data not

shown). Detoxification of reactive oxygen species was associated with decreased PRDX6, GSTA2 and MSN in animals euthanized early.

Table 4: Differentially detected proteins in pharyngeal tonsil within animals euthanized early relative to those surviving to study end

Protein name	Spot no.	Protein spot normalized volume [mean $\pm$ SEM]		Relative spot volume in early euthanised animals		Protein spot MASCOT identification							
		Early euthanised	Day 8 post challenge	Fold - change	p-value	Accession	Gene ID	Score	Expect	Matches	Coverage	MW	pI
Desmin	481	1.48 $\pm$ 0.09	0.88 $\pm$ 0.05	1.68	3.16E-02	DESM_BOVIN	DES	62	7.60E-03	9	18	5356	5.21
	484	1.36 $\pm$ 0.06	0.87 $\pm$ 0.04	1.568	1.60E-02	DESM_BOVIN		16	3.30E-08	16	32	5356	5.21
	502	1.33 $\pm$ 0.08	0.94 $\pm$ 0.05	1.427	6.60E-02	DESM_BOVIN		16	3.30E-11	15	33	5356	5.21
Serotransferrin	301	1.58 $\pm$ 0.09	1.04 $\pm$ 0.07	1.530	4.31E-02	TRFE_BOVIN	TF	19	1.70E-09	15	26	79870	6.75
	302	1.49 $\pm$ 0.07	1.04 $\pm$ 0.05	1.432	3.54E-02	TRFE_BOVIN		13	6.60E-13	19	34	79870	6.75
	271	1.37 $\pm$ 0.08	1.01 $\pm$ 0.06	1.359	9.09E-02	TRFE_BOVIN		10	6.50E-13	18	32	79870	6.75
	27	1.34	1.0	1.2	7.4	TRFE_		1	8.3	20	40	79	6

	0	± 0.06	6 ± 0.05	67	0E- 02	BOVI N		4 2	0E- 11			87 0	. 75
	27 6	1.31 ± 0.08	1.0 0 ± 0.04	1.3 12	1.1 6E- 01	TRFE_ BOVI N		1 7 6	3.3 0E- 14	17	28	79 87 0	. 7 5
Fibrinogen gamma-B chain	50 6	1.34 ± 0.07	0.9 7 ± 0.05	1.3 84	6.1 5E- 02	FIBG_ BOVI N	FIB	1 4 2	8.3 0E- 11	15	31	59 83 8	. 5 4
Apolipoprotein A-I	88 6	1.26 ± 0.06	0.9 4 ± 0.05	1.3 49	6.4 4E- 02	APOA 1_BO VIN	APO	1 3 9	1.7 0E- 10	14	49	20 35 8	. 7 1
Fibrinogen beta chain	10 43	1.19 ± 0.03	0.9 1 ± 0.02	1.3 16	5.2 6E- 03	FIBB_ BOVI N	FIB	5 7	2.4 0E- 02	9	15	53 93 3	. 4 5
Vimentin	41 1	1.21 ± 0.04	0.9 2 ± 0.05	1.3 15	1.3 7E- 02	VIME_ BOVI N	VIM	6 7	2.6 0E- 03	11	26	53 75 2	. 0 6
Tryptophan--tRNA ligase, cytoplasmic	43 5	1.28 ± 0.06	0.9 9 ± 0.08	1.2 98	8.6 2E- 02	SYWC_ BOVI N	WA RS	1 2 8	2.1 0E- 11	15	32	54 17 7	. 4 9
Pyruvate Kinase	41 2	1.15 ± 0.03	0.9 0 ± 0.03	1.2 77	8.2 3E- 03	A5D9 84_B OVIN	PKM 2	7 9	2.2 0E- 03	10	15	58 06 0	. 6 2
T-complex protein 1 subunit gamma	36 7	1.29 ± 0.06	1.0 3 ± 0.03	1.2 54	1.0 9E- 01	TCPG_ BOVI N	CCT 3	7 0	1.2 0E- 03	11	17	61 11 8	. 3 8
Serum Albumin	24 4	1.15 ± 0.02	0.9 3 ± 0.04	1.2 44	2.3 2E- 03	ALBU_ BOVI N	ALB	9 4	5.9 0E- 06	12	22	71 24 4	. 8 2
Serpin A3-5	99 9	1.2 ± 0.04	0.9 6 ± 0.04	1.2 45	3.0 3E- 02	SPA35_ BOVI N	SER PIN A3-5	1 0 8	2.8 0E- 06	13	25	46 48 2	. 4 8
Actin-related protein 3	50 4	1.32 ±	1.0 9 ±	1.2 05	2.3 3E-	ARP3_ BOVI	ARP	7 2	7.6 0E-	7	20	47 79	. 5

		0.04	0.04		02	N			04			7	6
			1.0										1
Heterogeneous nuclear ribonucleoproteins A2/B1	703	1.2 ± 0.04	1.0 ± 0.04	1.186	5.12E-02	ROA2_BOVIN	HNRNPA2B1	124	5.20E-09	13	49	36041	867
Annexin A1	712	1.15 ± 0.05	0.98 ± 0.04	1.175	1.75E-01	ANXA1_BOVIN	ANXA1	71	1.00E-03	9	36	39212	637
Heat shock protein HSP 90-alpha	210	1.02 ± 0.02	0.88 ± 0.03	1.163	2.24E-02	HS90A_BOVIN	HS90	118	2.10E-08	24	35	85077	93
Transketolase	332	1.07 ± 0.04	0.92 ± 0.03	1.158	1.72E-01	TKT_BOVIN	TKT	98	2.00E-06	12	23	68547	56
	335	1.03 ± 0.02	0.92 ± 0.02	1.113	7.04E-02	TKT_BOVIN		109	1.70E-07	16	24	68547	56
Ezrin	269	1.24 ± 0.03	1.07 ± 0.04	1.152	3.84E-02	EZRI_BOVIN	EZR	205	1.00E-16	25	40	68832	06
Fascin	486	1.19 ± 0.04	1.04 ± 0.03	1.146	1.25E-01	NP_001030217.1	FSCN1	173	1.00E-11	19	46	55435	55
T-complex protein 1 subunit gamma	391	1.18 ± 0.03	1.05 ± 0.03	1.125	9.40E-02	TCPG_BOVIN	CCT3	57	2.80E-02	10	21	61118	38
Chloride intracellular channel protein 1	804	1.06 ± 0.01	0.97 ± 0.03	1.099	4.87E-03	CLIC1_BOVIN	CLIC1	105	4.20E-07	10	63	27317	16
Glutathione peroxidase 1	903	0.92 ± 0.02	1.02 ± 0.02	0.898	2.20E-02	GPX1_BOVIN	GPX1	121	1.00E-08	13	61	22930	74
Moesin	282	0.97 ± 0.03	1.16 ± 0.04	0.837	1.30E-02	MOES_BOVIN	MSN	197	2.60E-16	28	34	68047	59

			4										
Calpain small subunit 1	858	0.86 ± 0.05	1.0 4 ± 0.0 4	0.8 25	1.2 9E- 01	CPNS1 _BOVI N	CPN	1 0 8	2.1 0E- 07	12	55	28 02 8	5 . 0 6
Gelsolin	198	0.77 ± 0.02	1.0 1 ± 0.0 5	0.7 64	1.3 1E- 03	GELS_ BOVI N	GEL	1 2 8	2.1 0E- 09	19	26	80 96 6	5 . 5 4
Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2	750	0.66 ± 0.02	0.9 0 ± 0.0 4	0.7 34	9.4 9E- 04	GBB2 _BOVI N	GBB	7 0	1.4 0E- 03	8	25	38 04 8	5 . 6
Glutathione S-transferase A2	862	0.76 ± 0.02	1.0 6 ± 0.0 7	0.7 19	9.6 2E- 04	GSTA 2_BO VIN	GST	1 3 2	8.3 0E- 10	15	47	25 81 4	8 . 6 6
Alpha-actinin-1	125	0.76 ± 0.06	1.0 6 ± 0.0 5	0.7 13	6.6 8E- 02	ACTN 1_BO VIN	ACT N1	5 5	4.2 0E- 02	6	7	10 34 86	5 . 2 5
Peroxiredoxin-6	868	0.77 ± 0.02	1.1 5 ± 0.0 7	0.6 71	5.5 5E- 05	PRDX 6_BO VIN	PRD X6	7 0	1.4 0E- 03	6	25	25 10 8	6

Table 5: Reactome analysis of differentially detected proteins in pharyngeal tonsils of vaccinated calves following BRSV-challenge.

Pathway identifier	Pathway name	No. entities found	No. entities total	Entities p-value	Entities FDR	Submitted entities found
R-HSA-109582	Hemostasis	8	743	4.07E-04	8.88E-03	TF, SERPINA3-5, APOA1, ALB, FGG, FGB, ACTN1, GBB2
R-HSA-76002	Platelet activation, signaling and aggregation	8	262	2.69E-07	2.29E-05	TF, SERPINA3-5, APOA1, ALB, FGG, FGB, ACTN1, GBB2
R-HSA-76009	Platelet Aggregation (Plug Formation)	2	40	4.96E-03	3.13E-02	FGG, FGB
R-HSA-354192	Integrin alpha(IIb)beta(3) signaling	2	27	2.48E-03	2.73E-02	FGG, FGB
R-HSA-76005	Response to elevated platelet cytosolic Ca ²⁺	7	133	4.45E-08	5.70E-06	TF, SERPINA3-5, APOA1, ALB, FGG, FGB, ACTN1
R-HSA-114608	Platelet degranulation	7	128	3.44E-08	5.70E-06	TF, SERPINA3-5, APOA1, ALB, FGG, FGB, ACTN1
R-HSA-	Formation of Fibrin Clot (Clotting	2	39	4.72E-	3.13E-	FGG, FGB

140877	Cascade)			03	02	
R-HSA-140875	Common Pathway of Fibrin Clot Formation	2	22	1.55E-03	1.85E-02	FGG, FGB
R-HSA-168256	Immune System	14	22 29	6.20E-04	1.02E-02	ACTR3, SERPINA3-5, ANXA1, HSP90AA1, VIM, FGG, PKM, FGB, FSCN1, HNRNPA2B1, PRDX6, MOES, GELS, GSTA2
R-HSA-1280215	Cytokine signaling in Immune system	7	772	3.15E-03	3.13E-02	ANXA1, HSP90AA1, VIM, FSCN1, HNRNPA2B1, GSTA2, MOES
R-HSA-449147	Signaling by Interleukins	7	458	1.42E-04	7.06E-03	ANXA1, HSP90AA1, VIM, FSCN1, HNRNPA2B1, GSTA2, MOES
R-HSA-6785807	Interleukin-4 and 13 signaling	4	111	1.96E-04	7.06E-03	ANXA1, HSP90AA1, VIM, FSCN1
	Interleukin-12 signaling	3	47	2.57E-04	8.22E-03	GSTA2_HUMAN; MOES_HUMAN; ROA2_HUMAN
R-HSA-168249	Innate Immune System	8	1182	9.10E-03	4.55E-02	ACTR3, SERPINA3-5, HSP90AA1, FGG, PKM, FGB, PRDX6, GELS
R-HSA-5686938	Regulation of TLR by endogenous ligand	2	19	1.16E-03	1.51E-02	FGG, FGB
R-HSA-6798695	Neutrophil degranulation	5	480	7.59E-03	4.11E-02	SERPINA3-5, HSP90AA1, PKM, GELS, PRDX6
R-HSA-8953897	Cellular responses to external stimuli	3	48 2	1.3E-01	1.30E-01	PRDX6, GPX1, HS90AA1
R-HSA-3299685	Detoxification of Reactive Oxygen Species	2	39	4.72E-03	3.13E-02	PRDX6, GPX1

4. Discussion

Although the comparative response to similar single component and combination BRSV vaccines has been previously reported [3,4], to our knowledge this is the first study to examine in detail the responses to BRSV (or other viral) challenge in the pharyngeal tonsil of differentially vaccinated animals, an organ critical to the priming of mucosal responses. Upon BRSV challenge, BRSV-only and 3-Way vaccinate groups exhibited an anamnestic response, with significantly elevated systemic anti-BRSV IgG levels relative to non-vaccinated placebo controls (no significant differences observed between differentially vaccinated groups). However, only the 3-Way vaccinates exhibited elevated nasal anti-BRSV IgA and were protected completely from the development of severe respiratory disease after challenge, in contrast to other study groups where early animal euthanization was required (BRSV-vaccine: 2 calves; non-vaccinated placebo: 4 calves). With equivalent BRSV antigen load in

the two vaccine formulations, elevated mucosal priming and protection must be attributed to the inclusion of temperature sensitive BHV-1 and BPI3V, providing additional pattern- or damage-associated molecular patterns (PAMPS or DAMPS respectively) in the form of viral RNA, or tissue damage from vaccine replication in the upper respiratory tract. Difference in Gel Electrophoresis (DIGE) proteomics, employed to investigate proteomic responses in the pharyngeal tonsil to BRSV challenge in differentially vaccinated calves, revealed a differential relative protein profile between the 3 study groups, with the majority of proteins shown to have increased or decreased levels associated with the 3-Way vaccine formulation. In addition, multivariate analysis revealed that the relative levels of proteins within the pharyngeal tonsils correlated with animals that were euthanized early due to severe respiratory disease and resulting poor clinical score.

Functional analysis based on identified altered proteins was performed to elucidate the impact of differing vaccine formulations on local protection to BRSV challenge and to determine potential correlates of mortality or 'failed' vaccine-induced protection. Comparative analysis of proteins with differentially altered tissue levels between groups revealed a number of important biological processes. These processes may be attributed to both the systemic response to infection and the local mucosal immunity associated with elevated anti-BRSV IgA - immune system related biological processes were the primary functional response to BRSV challenge. Both vaccine formulations resulted in elevation of proteins associated with MHC Class II antigen presentation (cathepsin D, F-actin capping protein and tubulin beta chain), neutrophil degranulation, and detoxification of reactive oxygen species, with an increased magnitude of variation in protein levels relative to placebo controls in the 3-Way vaccine group, indicating that shared protective immunity biological responses were enhanced with the incorporation of BHV-1 and BPI3V in that vaccine formulation. Recent proteomic analysis of BAL from BRSV infected calves with differential vaccination status revealed similar functional roles of differentially altered proteins [21] with elevated proteins associated with detoxification of reactive oxygen species in vaccinated animals and a concomitant decrease of neutrophil secreted pro-

inflammatory proteins. In the current study, a significant elevation in neutrophil granule specific and lumen proteins (PRDX6, PNP, Cathepsin D, IDH1) and proteins facilitating degranulation (annexin A2 (ANXA2) and vinculin) was observed in vaccinated calves. Cathepsin D and PRDX6 are associated with anti-inflammatory responses through the activation of neutrophil apoptosis [22] and detoxification of reactive oxygen [22] species respectively. In addition, a net increase in proteins associated with detoxification of reactive oxygen species was observed in vaccinated animals following BRSV challenge relative to non-vaccinated controls (PRDX2, PDRX6 and Glutathione peroxidase 1, glutathione s-transferase A2 [23], aldehyde dehydrogenase [24]). Overall, these findings indicate that the disease-sparing response after BRSV challenge in both sets of vaccinated animals was associated with a diminished inflammatory response in the tonsil relative to non-vaccinates as evidenced by reductions in reactive oxygen species and neutrophil activity.

Platelet activation and degranulation was significantly higher in non-vaccinated placebo calves compared to both vaccine treated groups, with elevated levels of SEPINA3-5, Vinculin, Transferrin, Albumin, WDR1 and Fibrinogen observed. Pulmonary pathophysiology of severe BRSV induced bovine respiratory disease indicates neutrophil infiltration and fibrin deposition associated with local areas of necrosis [25], and the co-localization of platelets and neutrophils in pulmonary vasculature is a common response to inflammatory stimuli [26]. Neutrophil and platelet degranulation are closely associated with inflammatory responses with platelets promoting neutrophil phagocytosis and neutrophil extracellular traps (NET) through the activation of TLR4 [27] by endogenous fibrinogen released from activated platelets [28]. Activated platelets further enhance oxidative burst and release of various inflammatory cytokines and enzymes of monocytes and neutrophils [29]. Elevated platelet aggregation and degranulation in the pharyngeal tonsil potentially indicates BRSV induced endothelial damage, with accumulation of platelets at sites of necrosis, promoting neutrophil infiltration and inflammation, the primary source of BRSV mortality [30]. Taken together these findings indicate that vaccinated animals have reduced levels of neutrophil-platelet interplay in the pharyngeal tonsil

associated with a reduced BRSV-induced inflammatory response in the respiratory tract (the principal component of mortality).

The induction of Th1 responses by BRSV vaccines with recruitment of CD8⁺ T-cells and interferon γ (IFN- γ) production are thought of as correlates of protective immunity [31,32]. However, the ability to mount a mucosal IgA response has been correlated with reduced viral shedding [3]. Differential comparative analysis of proteins specifically attributable to the 3-Way vaccine formulation, identified significant increases in the levels of proteins associated with IL-12 induced JAK-STAT4 signaling (moesin and ANXA2). IL-12 is a pro-inflammatory cytokine associated with the development of Th1 responses, and IL-12 stimulation of CD4⁺ T cells results in down-regulation of ANXA2 and MSN [33]. Elevated ANXA2 and moesin in 3-way vaccinated calves may potentially indicate a lower level of IL-12 signaling in 3-Way vaccinated calves compared to the other study treatment groups. Relative levels of a number of other proteins (Galectin-9, 14-3-3 proteins beta and epsilon) were significantly higher in animals receiving the 3-Way vaccine formulation as compared to BRSV-vaccine or placebo groups, with proteasome subunit alpha type-4 significantly lower, supporting observed altered Th2 responses. Gal-9 is a binder of the Tim-3 inhibitory receptor involved in suppressing Th1 cells through apoptosis [34], potentially skewing the local T-cell response towards a Th2 phenotype. This switching in local immune tissue may be associated with the elevated IgA observed in 3-Way vaccinated animals. In addition, significantly elevated 14-3-3 proteins are associated with class switching DNA recombination of the immunoglobulin heavy chain [35]. Proteasome subunit alpha type-4, a component of the 20S core proteasome complex involved in the proteolytic degradation of intracellular proteins which is associated with generation of a subset of MHC class I-presented antigenic peptides [36], was also significantly decreased in 3-Way vaccinated animals, further supporting the shift in Th1/Th2 balance in apparent response to challenge to a more effective helper T-cell response via the incorporation of additional viral material in the vaccine formulation. Finally, a significant number of differentially altered proteins specifically associated with

the 3-Way formulation are associated with metabolic processes (GBB1, triosephosphate isomerase, carbonic anhydrase, phosphoglycerate kinase) suggestive of an increased metabolic activity within the pharyngeal tonsil in support of requirements for enhanced local immune responses [37,38].

Comparative analysis of the relative pharyngeal tonsil protein profiles between calves that required euthanasia and those that did not, revealed 28 proteins whose levels were differentially altered (20 significantly elevated and 8 decreased). Functional analysis of these proteins highlighted roles related to platelet homeostasis (platelet activation and degranulation, and integrin signaling), immune responses (neutrophil degranulation, TLR4 activation, and IL-4, IL-13 and IL-12 signaling) and detoxification of reactive oxygen species. As discussed, activation of platelets is associated with bovine respiratory disease-induced tissue necrosis and human RSV infection has been shown to correlate with low mean platelet count [39]. In addition, animals susceptible to BRSV infection had higher levels of proteins associated with Neutrophil Extracellular Traps (NETs) in BAL fluid [21]. NET formation is stimulated by the action of the BRSV fusion glycoprotein on TLR4 [40], resulting in increased viscosity of respiratory mucus. However, elevated levels of platelet degranulation and release of the endogenous TLR4 agonist fibrinogen may assist in exacerbating this effect and lead to elevated morbidity. Significant decreases in antioxidant proteins (PRDX-6, GSTA2) have been shown to correlate with increased levels of neutrophil proteins in the lungs of BRSV infected non-vaccinated calves [21]. This consumption of antioxidants has been previously reported in severe chronic obstructive pulmonary disease (COPD) [41], a disease in which neutrophils play a similar role in inducing respiratory disease as with BRSV. Increased platelet degranulation and activation and oxidative stress within the lung were the most prominent correlates of mortality in calves that succumbed to infection (within both non-vaccinated and vaccinated groups), and indicators of the respiratory tract damage induced by severe BRSV infection. Levels of desmin and serotransferrin displayed the most significant elevation within the pharyngeal tonsil of early euthanized calves. Desmin is a type III intermediate filament and component of the extracellular matrix in lymph node

stromal cells (fibroblastic reticular cells, follicular dendritic cells, and lymph node medullary fibroblasts) [42]. In lambs, ovine RSV infection induces increases in lymph node size and weight, indicating lymph node hyperplasia and extensive expansion of the cortical area, indicating that ovine RSV is capable of inducing lymphoid proliferation resulting in follicle formation [43]. Indications of elevated desmin levels in early euthanized animals potentially suggests a similar virus pathology in BRSV within the pharyngeal tonsil that warrants further investigation. Consistent with this hypothesis, histological examination of tonsils from these calves revealed follicular hyperplasia regardless of the time of euthanasia (Figure 2A). However, determination of whether there were differences in the degree of hyperplasia among groups would require more extensive and detailed morphometric analyses.

5. Conclusion

This study has demonstrated that vaccine valency can alter post-challenge proteome responses within the pharyngeal tonsil that are linked to MHC Class II antigen presentation, neutrophil degranulation, and detoxification of reactive oxygen species. The magnitude of these proteome responses is enhanced through the use of a 3-Way (BRSV, PI3 and BHV-1) vaccine as compared to monovalent (BSRV) formulations. Incorporation of additional live virus material within the 3-Way vaccine resulted in significant induction of anti-BRSV-IgA post-challenge compared to monovalent BRSV vaccine, with observed disease sparing effects related to previously reported pulmonary anti-inflammatory responses. This enhanced proteome response is indicative of a shift in Th1/Th2 balance and immunoglobulin class switching which supports the observation of elevated nasal anti-BRSV IgA levels. In addition, in animals which were euthanized before study completion, the severe respiratory disease induced by

BRSV challenge resulted in elevation of proteins associated with anti-inflammatory responses and lymphoid tissue remodeling. Observed differential immune responses due to distinct vaccine formulations can be attributed to additional antigenic material and viral nucleic acid from multivalent formulations providing additional T-cell epitopes and PAMPS. Whilst the trend in vaccine research tends towards the production of subunit antigens due to safety and reactogenicity concerns, this research indicates that incorporation of subunits within multivalent formulations containing live virus has the potential to induce/skew a favorable immune response, utilising the natural adjuvanting effects of safe and proven live vaccines. Future research will seek to investigate the duration of this enhanced immune response focusing on targeted analysis of specific proteins as potential markers than can be used as direct correlates of vaccine efficiency and improved immune protection. Such work will also examine if this immunomodulation is observed in vaccine and challenge models for other pathogens, helping to determine if the efficacy of subunit vaccines can be enhanced when incorporated within multivalent live vaccines.

The following are the supplementary data related to this article.

S1 Appendix: Clinical scoring criteria.

Table S1: Spot quantification within individual samples.

Table S2: MASCOT search results for protein identification within the study.

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Significance

This study demonstrates that vaccine valency can alter post-challenge proteome responses within the pharyngeal tonsil, a sentinel site of primary immune responses, with the magnitude of response dependent on antigen formulation. Observed differential responses can be attributed to antigenic material and viral nucleic acid from multivalent formulations providing additional T-cell epitopes and PAMPS. These findings indicate that incorporation of subunits proteins within multivalent formulations containing live virus has the potential to induce/skew a favorable immune response, utilising the natural adjuvanting effects of safe proven live vaccines.

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Highlights

- Vaccination alters observed post-challenge proteome responses within the pharyngeal tonsil
- Responses associated with MHC Class II antigen presentation, neutrophil degranulation, and detoxification of reactive oxygen species
- Magnitude of proteome response enhanced by multivalent formulations
- Correlation between pharyngeal tonsil vaccination response and disease-sparing outcomes

Figure 1

A

B

Figure 2

Figure 3

A **B**

Figure 4

Figure 5