



Alternaria aerosol during a bovine respiratory syncytial virus infection alters the severity of subsequent re-infection and enhances IgE production

W.V. Kalina¹, M.L. Anderson, L.J. Gershwin^{*}

Department of Pathology, Microbiology, and Immunology, School of Veterinary Medicine,
University of California, Davis, CA 95616, USA

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Abstract

Background: Previous studies with cattle and rodent models have shown that bovine and human RSV infections influence the immune response to inhaled allergen. In the present study, we extended these observations to examine the effect of fungal allergen *Alternaria alternata* aerosol exposure (prior to and during BRSV infection) on the immune response and clinical outcome of a secondary BRSV infection.

Methods: Calves were either *Alternaria* (Alt)/mock Alt (mAlt) and BRSV/mBRSV exposed. Exposures began on day –6 and continued every other day until day 6 post infection. A second set of aerosols/infection began on day 103 and continued as before. Clinical outcome during infections was measured in each group. IgG1, IgA, and IgE responses to *Alternaria* were measured in serum or bronchiolar alveolar lavage fluid (BALF). Cytokine responses, including IL-4, were also measured.

Results: *Alternaria* did not influence primary infection; however, the Alt/BRSV group had less disease than mAlt/BRSV group (median clinical score 8 vs 476.5; $p \leq 0.01$) after secondary infection. Exposure to *Alternaria* facilitated IgE antibody production in BRSV infected calves. IgE responses to *Alternaria* were higher in Alt/BRSV than Alt/mBRSV animals on day 10 (mean baseline fold increase 1.97 vs 1.06; $p = 0.013$) and 109 (1.40 vs 0.810; $p = 0.008$). Comparatively, Alt/BRSV calves had less *Alternaria* specific IgG1 than Alt/mBRSV calves on days 0, 107, 109, 113, 115, and 120 ($p \leq 0.05$) with more lung eosinophils and IL-4 secreting PBMCs.

^{*} Corresponding author. Tel.: +1 530 752 6643; fax: +1 530 752 3349.

E-mail address: ljgershwin@ucdavis.edu (L.J. Gershwin).

¹ Present address: Virology Division, United States Army Medical Research Institute of Infectious Disease, Fort Detrick, MD 21702, USA

Conclusion: *Alternaria* aerosols during primary and secondary BRSV infections decreased disease in secondary infections; however, BRSV infection enhanced Th2 responses against inhaled *Alternaria*.
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Keywords: *Alternaria alternata*; IgE; BRSV

Résumé

Contexte: Les études précédentes sur des modèles bovin et rongeur ont montré que les infections RSV bovine et humaine influence la réponse immunitaire aux allergènes aériens. Dans cette étude nous avons élargi ces observations pour examiner les effets d'une exposition aérienne de l'allergène fongique *Alternaria alternaria* (avant et pendant une infection B-RSV) sur la réponse immunitaire et sur les résultats cliniques d'une infection BRSV secondaire.

Méthodes: Les veaux étaient exposés à soit *Aternaria* (Alt)/mock Alt (malt) et B-RSV/mB-RSV. L'exposition commença au jour moins 6 et continua un jour sur deux jusqu'au jour 6 après l'infection. Une deuxième vague d'infection aérienne commença au jour 103 et continua comme la précédente. Les résultats cliniques pendant l'infection ont été mesurés sur chaque groupe. La réponse des IgG1, IgA, et IgE a *Alternaria* ont été mesurés dans le sérum ou le fluide alvéolaire bronchial (BALF). La réponse des cytokines, comprenant IL-4, ont aussi été mesurés.

Résultats: *Alternaria* n'a pas d'influence sur l'infection primaire; par contre, le groupe Alt/BRSV a eu moins de maladie que le groupe malt/BRSV (médiane du score clinique 8 vs 476.5; $p < 0.01$) après l'infection secondaire. L'exposition par *Alternaria* a facilité la production des anticorps IgE des veaux infectés par B-RSV. La réponse des IgE a *Alternaria* était plus haute dans les groupes Alt/B-RSV que Alt/mB-RSV au jour 10 (augmentation moyenne de la ligne de base par 1.97 vs 1.06; $p = 0.013$) et jour 109 (1.40 vs 0.810; $p = 0.008$). Comparativement, les veaux du groupe Alt/B-RSV ont eu moins de IgG1 spécifique par *Alternaria* que le groupe Alt/mB-RSV au jour 0, 107, 109, 113, 115, et 120 ($p < 0.05$) avec plus d'éosinophiles pulmonaires et de PBMC sécrétant IL-4.

Conclusion: *Alternaria* aérien pendant les infections B-RSV primaire et secondaire ont diminué les maladies dans les infections secondaires; par contre, les infections B-RSV ont augmenté la réponse Th2 contre *Alternaria* aérien.

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Mots clés: *Alternaria alternaria*; IgE; B-RSV

1. Introduction

Alternaria alternata is a common fungal allergen associated with rhinoconjunctivitis among atopic children [1]. There have been multiple reports, throughout the world, in which allergies to *Alternaria* have been associated with high *Alternaria* specific IgE [2,3]. The small spore size allows for easier access deep into the lower airways than other larger fungal spores and pollen [1]. Spore germination results in the release of several identified allergens that allergic individuals frequently respond to [4,5]. Warm and moist environments are required for spore germination, which are common within areas of the respiratory tract where nasal clearance is less efficient [4,6]. With respect to nasal clearance, respiratory viruses such as respiratory syncytial virus (RSV) can reduce mucociliary action by infecting bronchial epithelial cells. Under these circumstances, the

upper and lower respiratory tract are less efficiently cleared of particulate matter—some of which are undoubtedly allergens.

In addition to creating an environment where allergens accumulate, RSV infection has been hypothesized to modulate other immune responses towards a Th2 phenotype [7–12]. Studies have shown that allergies and asthma tend to follow severe RSV infections at a young age [13]. Various experimental animal models, primarily the BALB/c mouse, have been used to explain this aberrant phenomenon. Mice aerosolized with ovalbumin and then infected with RSV display more airway hyperresponsiveness than those infected with RSV alone [8,9]. Furthermore, when mice are challenged with *Dermatophagoides farinae* and infected repeatedly, the overall immune response to RSV is consistently Th2-like [11]. However, current research is lacking data on RSV/allergen immune responses in outbred animal populations.

Unlike mouse RSV infections, bovine RSV infections more closely mimic their human counterpart in young children, including natural infection and similar immunologic responses to the infection. However, the effects of recurring RSV infections on the outcome of immunological responses to allergens have not been extensively studied. Cattle, like humans, are susceptible to re-infection throughout life; however, the influence of subsequent infections and the incidences of atopy and chronic allergies have not been thoroughly studied. Previous BRSV studies involving the fungal allergen *Saccharopolyspora rectivirgula* (formerly known as *Micropolyspora faeni*) have shown that allergen exposure in previously sensitized and concurrently BRSV infected calves results in viral and allergen specific IgE production [19]. The same group also showed that timing of allergen exposure with respect to BRSV infection could enhance disease in a secondary infection. In the present study we show that, a similar fungal species *A. alternata*, when administered as an allergen in aerosol triggers a Th2 allergen specific response that is enhanced in calves infected with BRSV. However, repeated allergen exposures of *Alternaria* 6 days prior to infection and 6 days following infection, completely ameliorate the onset of clinical signs following a secondary infection despite inducing humoral and cellular responses that mimic the Th2 phenotype.

2. Materials and methods

2.1. Animals

Eight to ten weeks old conventionally raised Holstein calves were castrated prior to beginning the study. All calves were negative at a 1:10 dilution in for BRSV specific antibodies as determined by IFA. They were maintained in stalls at the Animal Resource Service of UC Davis under ALAC approved conditions. Calves were fed calf starter and alfalfa hay.

2.2. BRSV infection of calves

Calves were infected with a clinical isolate of BRSV CA-1 [14]. This virus was grown on bovine turbinate (BT) cells. Prior to in vitro infection, BT cell monolayers were washed

with Hank's balanced salt solution (HBSS) (Sigma H2387) and incubated with 2 ml of previously frozen virus. Cells were incubated with virus for 7 days and then removed by freeze fracture. Cells and media were centrifuged at 1732g for 10 min at 4 °C. Virus inoculum was given to steers by aerosol using a facemask as previously described and attached to a nebulizer (DeVilbiss) [15].

2.3. BRSV titer determination by TCID₅₀

BRSV titer, of virus infected BT cell lysate, was determined by the TCID₅₀ method. BT cells were grown in a 24 well plate in EMEM (Sigma M-0268) with 10% fetal calf serum (FCS). After 1 day of growth and immediately prior to aerosol, cell cultured BRSV lysate was titered by adding 200 µl to three wells seeded with BT cells. Successive serial dilutions were performed up to 10⁻⁷. Results were expressed as viral titer at which 50% cytopathic effect was observed. Viral titers used to infect calves ranged from 2 × 10⁴ to 1 × 10⁵ TCID₅₀/ml.

2.4. Treatment groups and aerosol exposures

Steers were divided into three groups: *Alternaria* exposed/BRSV infected (Alt/BRSV)(n=5), *Alternaria* exposed/mock infected (Alt/mBRSV)(n=4), and mock *Alternaria* exposed/BRSV infected (mAlt/BRSV)(n=5).

For mock aerosols, allergen was substituted with phosphate buffered saline (PBS) and mock virus aerosols contained spent tissue culture media. The volume used in each aerosol was 5 ml. *A. alternata* cellular antigen grown on enriched trypticase media (Greer XPM1-D20) (1.5 mg/ml concentration) or mock extract was given on the following days: -6, -4, -2, 2, 4, 6 and 103, 105, 107, 111, 113, 115. BRSV or mock infection was given on day 0 and 109.

2.5. Viral shedding

On days 0–10 after BRSV infection posterior nasal swabs were obtained using cotton tipped applicators. Swabs were immersed in HBSS, which were then centrifuged at 1732g for 5 min. The cell pellet was placed onto microscope slides, fixed for 5 min in acetone, and examined for immunofluorescence after staining with anti-RSV using the Merifluor RSV detection kit (Meridian Bioscience 500112).

2.6. Clinical score evaluation

Clinical scores were measured daily from day 0 to 10 using a method developed by Collie et al. and modified by us to put less emphasis on temperature [14,16]. Parameters such as respiratory rate, amount and characteristics of nasal secretions, cough, lung sounds, attitude, breathing pattern, and temperature were recorded by a veterinarian. Clinical scores from day 5 to 10, from both infection periods, were summed and median totals from each group of calves were compared.

2.7. Indirect immunofluorescence assay (IFA)

Serum samples from pre-infection day 0, day 20, 109, and 120 were evaluated using a BRSV IFA titer test performed by the California Veterinary Diagnostic Laboratory System (CVDLS) at the University of California, Davis.

2.8. Necropsy of animals

Calves were euthanized, on day 120 (10 days post second BRSV infection), with pentobarbital sodium (Schering-Plough Animal Health, Kenilworth, NJ), weighed and a necropsy performed. The entire respiratory tract was removed, photographed, and lesions diagramed on a lung drawing. Areas of atelectasis and consolidation were estimated as a percentage for each lobe and for the entire lung. The left lung was removed intact from the left mainstem bronchus and infused via an intrabronchial catheter with 10% neutral buffered formalin until fully expanded. Samples from the right lung were obtained for aerobic and Mycoplasma cultures, and fluorescent antibody examination for BRSV. Sampling of tissues for histologic examination was performed on sections of the trachea, right mainstem bronchus and from lung samples from the right and left apical, middle and caudal lobes. Lung samples included fourteen standard sample sites and additional lesion sites when gross lesions were present and not included in the standard sample site. All histologic samples were labeled as to site, fixed in 10% neutral buffered formalin and routinely processed for histologic slides (sections cut at 3–4 μ m) and stained with haemotoxylin and eosin.

2.9. IgG1 and IgE ELISA

Alternaria or BRSV specific ELISAs were performed in 96 well polystyrene plates where 1 μ g protein of *Alternaria* extract or BRSV was added to each well in 0.1 M carbonate/bicarbonate buffer pH 9.6 and incubated overnight. Each plate was blocked with 1% rabbit serum albumin (Sigma A0639-10G) for 1 h and subsequently washed with phosphate buffered saline (PBS) containing 0.1% tween-20 (Fischer BP337-500). Serum was added at a 1:25 dilution for IgG1 analysis and undiluted for IgE analysis. When probing for IgG1 a 1:10,000 dilution of sheep anti-bovine IgG1-HRP was used (Bethyl A10-116P). For IgE detection, a 1:750 dilution of anti-bovine IgE monoclonal was added followed by a goat anti-mouse IgG(h+l)-HRP (Zymed 81-6520) diluted 1:2500 [17]. Each antibody was incubated for 1 h. Plates were developed with *o*-phenylenediamine dihydrochloride (Sigma P1526-506) in citrate buffer pH 3.0 and read on a UVmax spectrophotometer in dual wavelength mode 450/650 nm. Results were expressed as fold increase over baseline (serum prior to commencement of *Alternaria* aerosols) measurement.

2.10. Brochoalveolar lavage fluid IgA ELISA

BALF was acquired in an unsedated, standing calf using a sterile, lidocaine jelly treated stomach tube with a moveable internal polyethylene tube fitted with a three way stop-cock.

The nasal cavity was treated with 0.5 ml lidocaine prior to insertion of the tube. The tube was gently slipped through the ventral nasal meatus and down to the bifurcation and into a primary bronchus. Hundred and twenty milliliters of sterile phosphate buffered saline (PBS) was inserted and gently withdrawn using the 60 ml syringe. BALF was centrifuged to remove the cell pellet and the supernatant was stored at -20°C until assayed.

Undiluted BALF samples were assayed for BRSV specific IgA in an ELISA format. A rabbit anti-IgA was used to detect BRSV specific IgA and a conjugated secondary goat anti-rabbit (HRP) served as the detection antibody. Relative IgA was determined by dividing the optical density obtained from each sample with the total protein concentration of that particular sample.

2.11. Total lymphocyte and eosinophil counts

Total white blood cell counts were performed by diluting blood 1:10 in lysis buffer (1% acetic acid solution in PBS). White blood cells were counted on a hemocytometer at $40\times$. Differentials were performed on blood smears after addition of Wright stain. Lymphocyte, neutrophil, eosinophil, monocyte, and basophil counts were determined at $100\times$.

2.12. BRSV lymphocyte restimulation assay

PBMCs were harvested from blood collected by jugular venipuncture from the animal of interest. The cells were purified by separation on histopaque (Sigma 1077-1). RBCs were removed by addition of 2 ml of ammonium chloride lysis buffer, resuspended in HBSS (Sigma H2387), and centrifuged at 200g. The pellet was resuspended in DMEM (Sigma D-8900) + 1% FBS and added to a T75 tissue culture flask at a cell density of 2×10^6 cells/ml. This was incubated in a 5% CO₂ incubator for 1 h. Media was then aspirated off and washed two times in with DMEM and 10 ml of cold PBS + 0.02% EDTA (Sigma E-5134) was added and incubated for 10 min on an orbital shaker. The flask was then lightly tapped to remove loosely adhered monocytes. The monocytes were then centrifuged for 5 min at 200g and resuspended in RPMI-1640 (R-6504) + 10% FBS. These monocytes were then added to a U-bottom tissue culture plate at a density of 5×10^4 cells/ml. BRSV isolate ATCC # A5 1908 was added to the culture at an MOIs of 1.0 and incubated for 4 days. Following the fourth day, fresh PBMCs were obtained from the same animal and added to the monocyte culture at an effector to stimulator (E:S) ratio of 10 (5×10^5 PBMCs), unless otherwise noted, and incubated for 16 h. A transport inhibitor, monesien (GolgiStop) (PharMingen 554724), was added to concentrate cytokines within cells.

2.13. FACS analysis

PBMCs were obtained as described above unless taken directly from lymphocyte restimulation cultures. When analyzing markers from fresh PBMCs taken directly from whole blood, 1×10^6 cells were aliquoted into FACS tubes and stained with 1 μg of mouse anti-bovine CD8-FITC (Serotec MCA837F) in FACS buffer (PBS + 1% FBS + 0.1% sodium azide) for 30 min on ice. For IL-4 analysis from restimulated cultures,

lymphocytes were fixed with 1% formalin for 20 min and subsequently washed and incubated with anti-bovine IL-4 (Serotec MCA1820) in permeabilization buffer (PBS + 1% FBS + 0.1% sodium azide + 0.1% saponin). A secondary goat anti-mouse phycoerythrin conjugated antibody (Jackson ImmunoResearch 115-116-075) was added later for detection. All cells were washed in FACS buffer and, resuspended in PBS + 1% formalin prior to analysis on a flow cytometer.

2.14. Statistical analysis

Clinical scores and IFA titers between groups of infected and mock infected animals were analyzed by the Kruskal-Wallis nonparametric ANOVA. When an overall significant difference was observed, pairwise comparisons were made by the Mann-Whitney test. Overall effect between groups on various days, for IgG1 and IgE, was determined by one way ANOVA or, when parametric tests are inappropriate, the Kruskal-Wallis one way ANOVA. When analyzing IgG1 and IgE, group comparisons were performed by the Holm-Sidak procedure or the Mann-Whitney test where noted. All observations were considered significant at the $p < 0.05$ level.

3. Results

3.1. Clinical scores and viral shedding during BRSV infection

Using the Kruskal-Wallis nonparametric ANOVA, there was a significant difference in the median clinical scores for both primary infection ($p < 0.05$) and second infection ($p < 0.05$). When compared to the Alt/mBRSV group (mean = 155), scores for the first infection period were significantly higher in the mAlt/BRSV (mean = 1214) and Alt/BRSV groups (mean = 1071) (see [Table 1](#)). However, there was no significant difference in clinical scores between the mAlt/BRSV and Alt/BRSV groups ($p < 0.05$) (see [Table 1](#)).

Following the second infection, there was a significant difference in clinical scores between the mAlt/BRSV (mean = 749) and Alt/mBRSV (mean = 93) groups ($p < 0.05$). There was no difference in clinical scores between the Alt/BRSV (mean = 45) and Alt/mBRSV groups ($p < 0.05$). However, there was a significant difference in scores of the Alt/BRSV and mAlt/BRSV groups ($p < 0.05$) (see [Table 1](#)).

Viral shedding was documented in experimentally infected calves from the primary and secondary infections ([Table 1](#)). Shedding was most common between days 5 and 8. BRSV was not recovered from mock infected animals.

3.2. Gross macroscopic pathology

All mAlt/BRSV calves had mild to moderate tracheobronchitis, bronchitis, and bronchiolitis. Conditions of bronchiolitis varied but were usually lymphofollicular and/or lymphoplasmacytic or pleocellular. One calf had a focally eosinophilic, chronic obliterating bronchiolitis ([Table 2](#)).

Table 1
Clinical scores in BRSV infected and mock exposed animals

Calf No.	Group	Clinical scores 1 ^a	Nasal swab days +/– ^b	Clinical scores 2 ^c	Nasal swab days +/–
2233	Alt/BRSV	852	Not done	99	Not done
2226	Alt/BRSV	1654	Not done	8	Not done
2392	Alt/BRSV	1232	6, 7, 8	0	7
2592	Alt/BRSV	493	7, 8	114	7, 8
2508	Alt/BRSV	1124	5, 6, 7, 8	4	7
2321	Alt/mBRSV	364	–	0	–
2324	Alt/mBRSV	178	–	0	–
2305	Alt/mBRSV	80	–	359	–
2306	Alt/mBRSV	0	–	13	–
2513	mAlt/BRSV	894	7, 8, 9	1832	5, 6, 7, 8
2510	mAlt/BRSV	547	7, 8	380	7
2493	mAlt/BRSV	868	7, 8	573	7, 8
5820	mAlt/BRSV	1195	5, 6, 7, 8, 9	212	6, 7, 8
5824	mAlt/BRSV	2564	5, 6, 7, 8	Dead ^d	Dead

^a Clinical scores for the first infection cover the mean sum of days 5–10.

^b A positive shedding result is recorded for days post-infection after day 5. Negative results on days 5–10 are indicated by (–).

^c Clinical scores for the second infection cover the mean sum of days 115–120.

^d Calf 5824 died on day 9.

There were no gross lung lesions in 3 out of 4 Alt/mBRSV calves. One calf did have minimal multifocal atelectasis in the ventral middle left lung lobe with less than 1% involvement (Table 2).

The Alt/BRSV calves did not have any visible lung pathology with the exception of one calf having a mild red lobular atelectasis with 2–3% lung involvement (Table 2).

3.3. Microscopic pathology

Histopathological findings of mAlt/BRSV calves varied but most had infiltrate in sections of the trachea or bronchi (Table 2). The infiltrate consisted of mostly epithelial cells but some calves contained neutrophilic infiltrate and bronchial exudate and another animal contained eosinophilic infiltration in the mucosa from the anteroventral lung bronchi. In one calf there was extensive multifocal to coalescing areas of lobular bronchopneumonia in the cranial portion of the right apical lung lobe and the right middle lung lobe. *Pasteurella multocida* (sulfonamide resistant strain) was isolated from the lung of this animal.

The Alt/mBRSV animals all had some lymphoplasmacytic infiltrate with 2 of 4 having mild lymphoid aggregates. Mild bronchitis or tracheitis was seen in some calves that had lymphoid aggregates and follicles (Table 2). Eosinophils were in the lungs of two calves in this group. One had an eosinophilic interstitial infiltrate with pulmonary vascular muscular hypertrophy, and the other had a mild granulomatous, eosinophilic, focal interstitial pneumonia (Table 2).

Table 2
Pathological findings

Calf No.	Group	Gross pathology	Microscopic pathology	Microbial isolation	BRSV titer ^a	BRSV in Nasal swab ^b
2233	Alt/BRSV	Not done	Not done		1:320	Not done
2226	Alt/BRSV	Not done	Not done	Not done	1:40	Not done
2592	Alt/BRSV	None	Mild diffuse lymphoplasmacytic and focal eosinophilic tracheobronchitis	No growth in 48 h	1:160	Positive
2392	Alt/BRSV	None	Mild diffuse lymphoplasmacytic and focal eosinophilic tracheobronchitis	No growth in 48 h	1:20	Positive
2508	Alt/BRSV	Mild atelectasis (2–3% lung)	Moderate regional lymphofollicular bronchiointerstitial pneumonia with chronic obliterating bronchiolitis; mild focal eosinophilic bronchitis	No growth in 48 h	1:10	Positive
2321	Alt/mBRSV	None	Mild multifocal lymphocytic and eosinophilic interstitial pneumonia	No growth in 48 h	Neg at 1:10	Negative
2324	Alt/mBRSV	None	Mild multifocal histiocytic and eosinophilic interstitial pneumonia; mild lymphofollicular bronchitis and bronchiolitis	No growth in 48 h	Neg at 1:10	Negative
2305	Alt/mBRSV	Minimal focal atelectasis	Mild lymphoplasmacytic, lymphofollicular tracheobronchitis and bronchiolitis	No growth in 48 h	1:40	Negative
2306	Alt/mBRSV	None	None	No growth in 48 h	Neg at 1:10	Negative
2493	mAlt/BRSV	Mild lobular atelectasis, 8% of lung	Mild diffuse lymphoplasmacytic and focal eosinophilic tracheobronchitis and bronchiolitis with chronic obliterating bronchiolitis	Lung—no growth bronchus-mixed flora	> 1:320	Positive
2510	mAlt/BRSV	None	Mild lymphoplasmacytic tracheobronchitis and moderate regional lymphofollicular bronchiolitis	No growth in 48 h	> 1:320	Positive
2513	mAlt/BRSV	Bronchopneumonia, 15% consolidation	Moderate regional neutrophilic bronchopneumonia with chronic obliterating bronchiolitis and pleocellular tracheobronchitis and bronchiolitis	<i>P. multocida</i> in lung and bronchus	> 1:320	Positive
5820	mAlt/BRSV	Not done	Not done	Not done	1:80	Positive

^a BRSV titers are on day 120 (day 10 post second infection).

^b Nasal swabs that detected BRSV antigen on days 5–10 were considered positive.

A lymphoplasmacytic infiltrate was noted in the lungs, trachea, and bronchi of all calves in the Alt/BRSV group. In addition, these calves tended to have focal eosinophilic infiltrates in the bronchi with areas indicating intraepithelial migration to the lumen of the bronchus (see Fig. 1). One calf (2592) had a focal neutrophilic

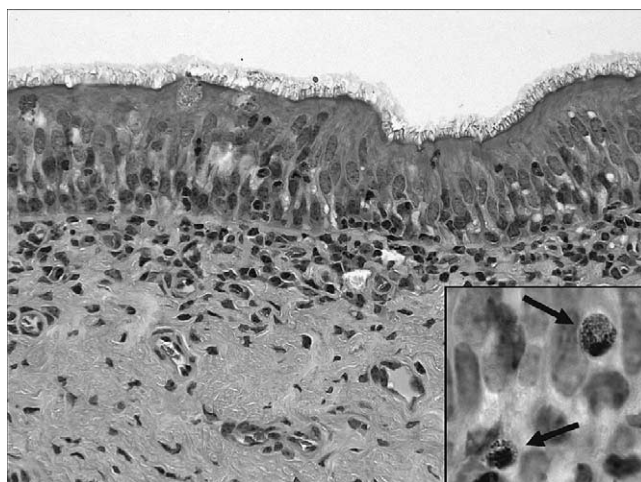


Fig. 1. Mainstem bronchus of calf no. #2392. Bronchial mucosal infiltration by eosinophils, lymphocytes and plasma cells with eosinophil migration into the bronchial epithelium (insert). HE bar = 50 mm.

infiltrate in the trachea and mainstem bronchi. This calf also had a focal interstitial eosinophilic infiltrate in the right apical and accessory lung lobes. Lymphoid aggregates were noted throughout the lung in all Alt/BRSV calves. Widespread bronchus-associated lymphoid tissue (BALT) hyperplasia was noted in one calf (2508). Another calf had less organized BALT (2592).

3.4. BRSV antibody titer

At necropsy (day 120), there was a significant group effect in serum BRSV antibody titers ($p < 0.05$) (Fig. 2). The mAlt/BRSV group had significantly higher serum BRSV titers than the Alt/mBRSV group but not the Alt/BRSV group. Interestingly, Alt/BRSV calves did not have significantly higher serum titers (total Ig) than their mock infected counterparts (Alt/mBRSV group). There was no significant group effect on pre-second infection (day 109) serum BRSV titers (Fig. 2). However, there was a significant group effect in serum BRSV titer on day 20 ($p < 0.05$) (Fig. 2). The Alt/BRSV group had significantly higher serum antibody titers than the Alt/mBRSV group, while the mAlt/BRSV group was intermediate in titers (not significantly greater than the Alt/mBRSV and not significantly less than the Alt/BRSV group).

3.5. Serum *Alternaria* IgG1 responses among groups

One way ANOVA revealed significant group differences, expressed as fold increase over baseline, in serum *Alternaria* specific IgG1 levels on days 0, 107, 109, 113, 115, and 120 ($p < 0.05$). To convert nonparametric datasets to parametric datasets a $\log_{10}$ transformation was performed on days 107 and 113 for parametric analysis.

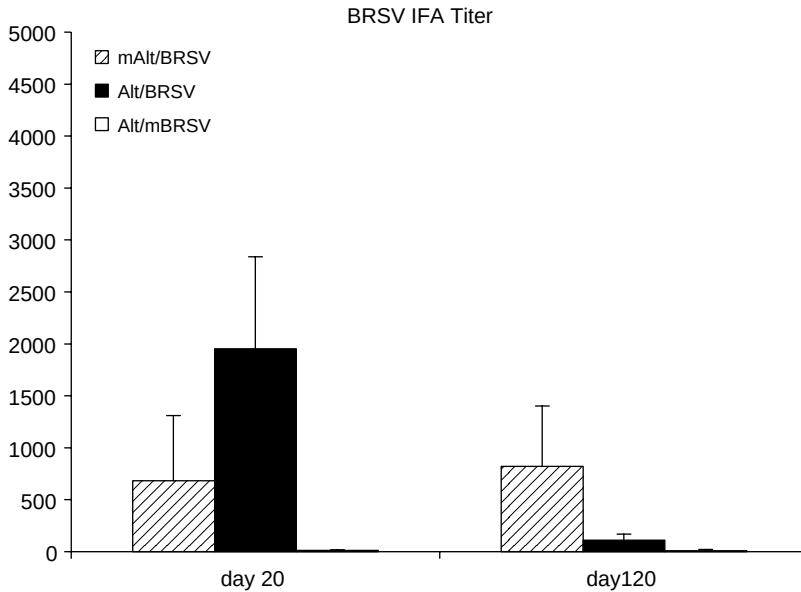


Fig. 2. Days 20 and 120 BRSV antibody titers. Day 120 corresponds to day 10 post-second infection. Bars represent standard error.

The Alt/mBRSV group had higher levels of serum IgG1, except on day 0, than the Alt/BRSV group on days 109 (8.49 vs 4.86), 115 (10.09 vs 5.75), and 120 (12.16 vs 6.64). The Alt/BRSV had higher serum IgG1 levels than the mAlt/BRSV group on days 107 (4.62 vs 1.82), 113 (5.09 vs 1.16), and 115 (5.75 vs 1.87) ($p < 0.05$) (Fig. 3A).

3.6. Serum *Alternaria* specific IgE response

One way ANOVA detected significant group differences in serum *Alternaria* IgE levels on days 10, 103, and 109 ($p < 0.05$). Analysis of day 103 was performed using the Kruskal–Wallis One way ANOVA on ranks. Comparisons between groups on days 10 and 109 were performed using the Holm–Sidak method. For day 10, the Alt/BRSV group had significantly higher serum *Alternaria* specific IgE than Alt/mBRSV (1.97 vs 1.06) group (see Fig. 3B). For day 109, the Alt/BRSV group contained higher serum *Alternaria* specific IgE than the Alt/mBRSV (1.42 vs 0.81) group (see Fig. 3B). Serum from day 103 revealed no significant difference in IgE levels between Alt/BRSV and Alt/mBRSV groups; however, the Alt/BRSV group IgE levels were significantly higher than those of the mAlt/BRSV ($p < 0.05$).

3.7. BALF BRSV specific IgA

Analysis of BALF BRSV specific IgA revealed that exposure to *Alternaria* resulted in higher virus specific IgA that was evident in pre- and post- second infection BALF from the Alt/BRSV group. Of the three animals where BALF was obtained 2/2 (one animal was

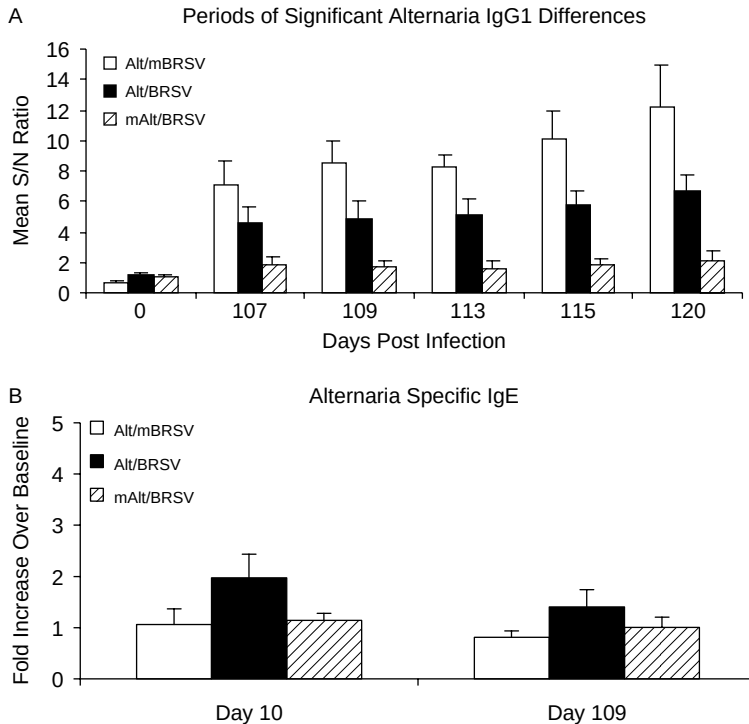


Fig. 3. (A) Periods of significant *Alternaria* IgG1 group differences using one way ANOVA. The figure clearly demonstrates the significant ($p < 0.05$) phenomenon of enhanced Th2 phenotype in allergen aerosolized animals. (B). *Alternaria* specific IgE measured in serum 10 days after BRSV infection, which represents 16 days after the first aerosol. Collectively, a depreciated IgG1 response to *Alternaria* in the Alt/BRSV group is oppositely flanked by a high IgE response ($p < 0.05$) in the same group. Bars represent standard error for the Alt/BRSV ($n = 5$), Alt/mBRSV ($n = 4$), and mAlt/BRSV ($n = 4$) groups. Day 109 corresponds to day 0 of the second infection and day 6 post *Alternaria* challenge. Asterisk represents a significant difference among other groups ($p < 0.05$). See results section for specific group comparisons.

not tested) had high levels of BRSV specific IgA pre-second infection, whereas, only 1/3 in the mAlt/BRSV group had high BRSV specific IgA levels (see Table 3). After 10 days following the second BRSV infection did not result in any noteworthy increase in BRSV specific IgA in either infected group (see Table 3).

4. IL-4 expression in BRSV stimulated effector cells

IL-4 secretion in BRSV stimulated effector cells was typically small by inference of the relatively minor shifts in mean fluorescent intensity between stimulated and non-stimulated effectors (data not shown). In the latter case, monocytes were infected with BRSV and served as viable antigen presenting cells. Monocytes were infected with live BRSV for 4 days and then co-cultured with PBMCs from the same calf at the various time points following an in vivo BRSV infection. When looking at individual calves, BRSV stimulated, IL-4 secreting cells are commonly present on

Table 3
BRSV specific IgA in BALF

Calf no. ^a	Group	Pre ^b	Post ^c
2592	Alt/BRSV	0.340	0.355
2392	Alt/BRSV	Not done	0.264
2508	Alt/BRSV	0.207	0.043
2321	Alt/mBRSV	0.128	0.124
2324	Alt/mBRSV	0.137	0.127
2305	Alt/mBRSV	0.077	0.096
2306	Alt/mBRSV	0.120	0.109
2493	mAlt/BRSV	0.282	Not done
2510	mAlt/BRSV	0.053	0.068
2513	mAlt/BRSV	0.053	0.081

^a Only calves with data from BALF are shown.

^b Pre refers to pre-second infection ranging from days 103 to 106.

^c Post refers to day 10 following second infection (day 120).

day 4 post infection in the Alt/BRSV group but not commonly seen in the others. IL-4 levels in BRSV stimulated PBMCs also became elevated 4 days after the second infection (day 113) in the mAlt/BRSV and Alt/BRSV group (Fig. 4). Some calves either at baseline or in the mock infected group contained PBMCs that secreted IL-4 in response to BRSV. Almost always lower, this may either be background associated with the assay or non-specific bystander stimulation of other Th2 cells by the manner in which BRSV infects and interacts with pattern recognition receptors on monocytes.

As a control fresh PBMCs collected at the same time intervals as outlined for stimulation were added to non-infected monocytes. Any IL-4 release observed for a particular calf, under these conditions, was considered background and subtracted from the observed IL-4 release from PBMCs cultured with BRSV infected monocytes. A graphical display of the data corrected for background is shown in Fig. 4.

4.1. FACS analysis of CD8+PBMCs

When comparing CD8+T cell number in peripheral blood, there were significant differences on days 4, 6, and 10 but not day 0 post infection. *Alternaria* aerosol alone did not affect the number of circulating CD8+T cells over time; however, BRSV infection (mAlt/BRSV group) significantly decreased the number of circulating CD8+T cells over time (Fig. 5B). This was evident on days 4, 6, and 10 when compared to counts obtained in non-infected animals (Alt/mBRSV group) ($p < 0.05$). There was no significant drop in CD8+cell count in the Alt/BRSV group when compared to the Alt/mBRSV group; however, the Alt/BRSV group contained significantly higher numbers of circulating CD8+cells than the mAlt/BRSV group on days 6 and 10 ($p < 0.05$) (see Fig. 5B). Total lymphocyte counts were lower in the Alt/BRSV group but similar to those in the mAlt/BRSV group on days 4 and 6 (see Fig. 5A).

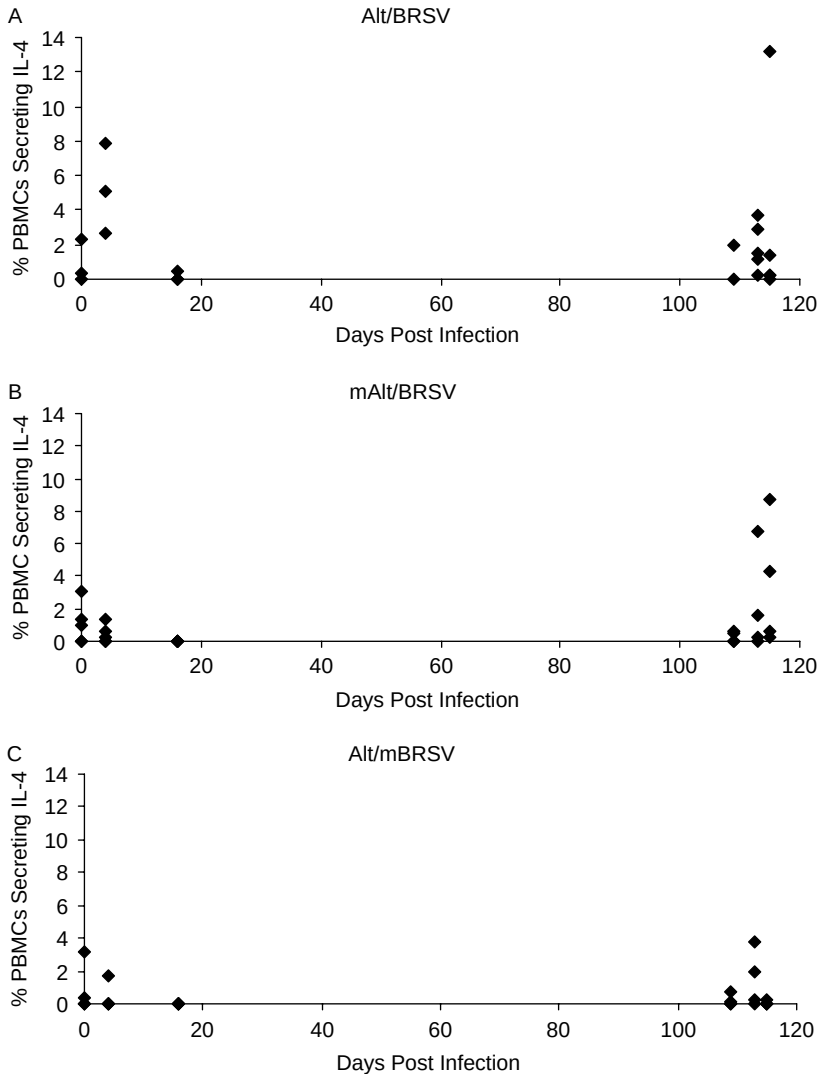


Fig. 4. BRSV responsive IL-4 secreting PBMCs from individual calves are shown for the Alt/BRSV (A), mAlt/BRSV (B), and Alt/mBRSV (C) groups. Each point represents the percentage of PBMCs secreting IL-4 when added to 4 day cultures of BRSV infected monocytes at the designated times post infection. Time periods of stimulation shown are days 0, 4, 16, 109 (pre-second infection), 113, and 115.

5. Discussion

We hypothesized that BRSV infection would augment a generalized Th2 immune response and inadvertently boost allergen specific IgE production. The results from this study show that BRSV infection depresses serum *Alternaria* specific IgG1 while

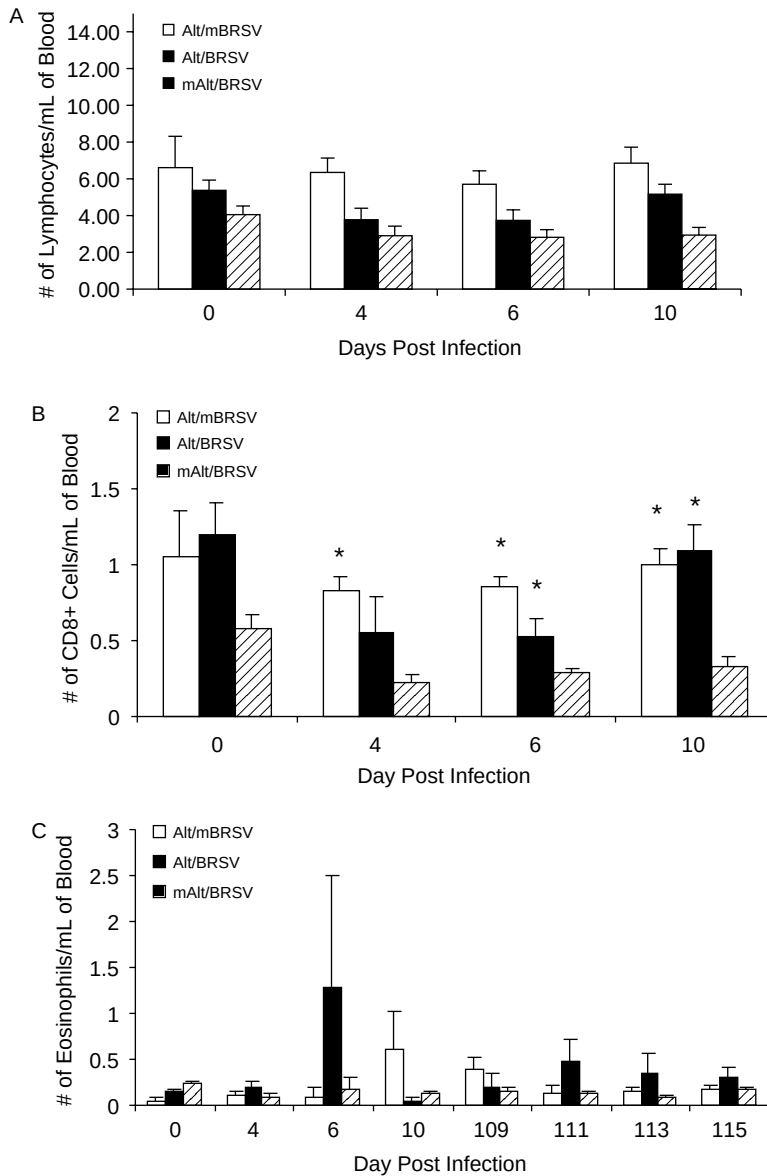


Fig. 5. (A) Mean total lymphocyte counts in peripheral blood for each group are represented pre and post infection out to day 10. (B) Mean total CD8+T cell counts in peripheral blood. Graph shows results from Alt/mBRSV group ($n=6$), Alt/BRSV group ($n=5$), and mAlt/BRSV group ($n=5$) on days 0, 4, 6, and 10. Where noted, asterisks indicate where groups have significantly higher ($p<0.05$) mean CD8+cells than animals in the mAlt/BRSV group. (C) Mean total eosinophils from peripheral blood of calf groups. Days 0–10 represent an interval during the primary BRSV infection, and days 109–115 represent days 0–6 of the second infection period. ANOVA did not find a significant difference in eosinophil numbers throughout the study. In all cases, bars represent standard error.

enhancing serum *Alternaria* specific IgE. This is also corroborated by the ambiguity in BRSV responding IL-4 producing PBMCs disseminated throughout the bloodstream in allergen treated (Alt) and BRSV infected animals. RSV infected animals commonly hyper-respond to allergens and this widely accepted observation was reproduced in our dual RSV and *Alternaria* exposed animals (Alt/BRSV) [8,13,15,18–20]. However, allergen aerosols did not enhance disease in a primary or secondary infection and despite evidence of a mounting allergen specific Th2 response, calves exposed to *Alternaria* and BRSV infected were highly resistant to a secondary BRSV infection. The pioneering results suggest that fungal allergens such as *Alternaria* may have utility in boosting a forthcoming immature and mature RSV specific immune response. Although the mechanisms of such a conundrum are obscure, the results obtained and documented herein suggest that *Alternaria* exposure leads to the formation of a Th1 disease known as hypersensitivity pneumonitis (HP). HP is manifested as a respiratory disease and usually involves a both antibody and cellular responses directed against a persistently inhaled environmental antigen. Typically, CD8+ T cells and macrophages infiltrate into the lung during chronic exposure and contribute to granuloma formation in areas of high antigen concentration; antibodies have also been shown to induce type III hypersensitivity responses in humans and cattle commonly exposed to *M. faeni* (currently *S. rectivirgula*) [19,21]. In addition, the pathological circumstances that arise from acute HP have been hypothesized, by others, to offer enhanced cellular and humoral responses.

The pathology seen in the Alt/mBRSV group is similar to that commonly observed in cases of hypersensitivity pneumonitis. Lung lymphocytic infiltrates and scattered granulomas were commonly seen in all calves of this group; in addition, Alt/mBRSV calves had the highest levels of *Alternaria* specific IgG1. In addition to having high *Alternaria* specific IgG1, post mortem findings revealed that several Alt/mBRSV calves had bronchitis and vascular inflammation accompanied by lymphoplasmacytic, neutrophilic, or eosinophilic infiltrates (see Table 2), which is consistent with HP. High levels of *Alternaria* specific IgG1 are commonly detected in humans suffering from Woodworker's lung, a condition in which types III and IV hypersensitivity reactions usually account for the pathological changes observed. HP is instigated by cellular and humoral responses to inhaled, primarily fungal, antigens [22–25]. The characterization of a Th1 disease is based on the fact that Th2 prone mice strains, such as DBA/2 and BALB/c, develop less severe HP than Th1 prone strains such as C57Bl. In line with our data, calves with evidence of Th2 responses, such as those observed in the Alt/BRSV group, may have indirectly suppressed the development of clinical and pathological characteristics of HP. Lung and lumen eosinophils were more commonly seen in the dual exposure group, which suggests a dominant Th2 predisposition (see Fig. 1). Th2 cytokines have generally been considered suppressive to the progression of HP and, therefore, may explain the lack of any related inflammatory condition in the Alt/BRSV group.

Bronchitis and CD8+ T cell migration to the lung, likely due to CXCR3 Th1 chemokines, are commonly seen in chronic forms of the HP [26]. Interestingly, increased numbers of lung CD8+ T cells, which are elevated in cases of HP may indirectly help combat respiratory viral infections. In this study, we did not note a depressed CD8+ population in peripheral blood of the Alt/BRSV group, which is normally seen in

BRSV infections. CD8 + T cell number actually increased on day 6, despite lymphopenia (Fig. 5A and B). The observed lymphopenia in the infected groups was due to depressed peripheral blood CD4, CD21, and $\gamma\delta$ T cell populations, which did not significantly differ between infected groups (data not shown). Recruitment of CD8 + T cells into the lung is commonly observed in cases of HP. However, we did not measure lung CD8 + T cells in this study so we can only suggest that increasing blood CD8 + T cell numbers may have resulted in increased lung CD8 + T cells within the lymphocytic infiltrate observed in most cases of *Alternaria* exposure.

Not all CD8 + T cell populations follow strict Th1 profiles. Tc2 cells secrete IL-5, which can promote recruitment of eosinophils to the lung. Post mortem analysis of Alt/BRSV calves showed areas of focal eosinophilic lung infiltrate, which may have been recruited by increasing numbers of lung Tc2 cells, but we did not find a direct effect of BRSV or *Alternaria* on eosinophil numbers in peripheral blood (Fig. 5C). However, we noted that eosinophils were more common in the airways of Alt/BRSV calves (Fig. 1). This disposition in eosinophil location between treatment groups is likely the result of locally produced chemokines following a BRSV infection. The source and nature of these chemokines was not explored in this study, but one could speculate their source to be either of lymphocyte or epithelial origin.

Alt/BRSV calves did not become ill following *Alternaria* and BRSV challenge; whereas, calves not exposed to *Alternaria* but BRSV infected did become ill following viral challenge. Some memory, possibly cytotoxic (Tc1), CD8 + T cells may have been recruited to the lung by secondary pre infection *Alternaria* aerosols (days 103, 105, and 107), which may have been responsible for protection from a second BRSV infection. This speculation is based on the fact the Tc2 cells do not provide adequate anti-viral defense [27]. This is a feasible conclusion, because cytotoxic T cells have been shown to be important in controlling RSV infections [28]. Furthermore, it has been shown, in humans, that low nasal RSV specific IgA significantly increases the risk of RSV infection [30]. IgA is well noted for its mucosal proximity and for its ability to provide a first line of defense against viruses. Table 3 shows that BALF BRSV specific IgA was present in all of the calves from the dual exposed group (Alt/BRSV); whereas, it was absent in mock infected animals and only present in one out of three animals tested in the mAlt/BRSV group.

The effect of IgE production to *Alternaria* on pathogenesis of disease in primary and secondary infections is uncertain. Cattle that inhale *Alternaria* do not develop asthma so the effect of higher *Alternaria* specific IgE levels in the calf is unknown. Several calves from the Alt/BRSV group produced IgE to *Alternaria* proteins but not Alt a 1, which is considered the most potent human allergen of *Alternaria* [29,31] (data not shown). Spores, the primary source of Alt a 1 and other allergens, are commonly found in barns and are produced year-round but are most common in late summer and fall [32]. It is well known that BRSV infection facilitates a Th2-type cytokine response [15,33,34]. Cytokine responses to *Alternaria* have not been reported in cattle, but it is assumed that, since *Alternaria* antigens induce IgE production in humans, they have a similar effect in cattle. Since, some *Alternaria* aerosols took place before infection, a premature *Alternaria* immune response may have been influenced by an early immune response to BRSV. All calves exposed to *Alternaria*, especially those infected with BRSV, produced a positive type 1 hypersensitivity skin reaction when injected intradermally with *Alternaria*

(data not shown). In addition, Gershwin et al. showed that BRSV infection enhances IgE responses in *M. faeni* sensitized and challenged cattle and is associated with greater clinical disease [15]. Early BRSV responses involve IL-2 and IL-4, which may influence *Alternaria* specific T cell and or B cell responses [33]. Thus, while the effect of *Alternaria* specific IgE on development of bovine respiratory disease is unknown, it is likely that sensitization of mast cells with *Alternaria* specific IgE may predispose cattle to airway hyper-responsiveness during periods of chronic exposure to dust borne *Alternaria*.

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