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# Increased Concentration of Iodide in Airway Secretions is Associated with Reduced RSV Disease Severity

## Abstract

Recent studies have revealed that the human and nonrodent mammalian airway mucosa contains an oxidative host defense system. This three-component system consists of the hydrogen peroxide ( $\text{H}_2\text{O}_2$ )-producing enzymes dual oxidase (Duox)1 and Duox2, thiocyanate ( $\text{SCN}^-$ ), and secreted lactoperoxidase (LPO). The LPO-catalyzed reaction between  $\text{H}_2\text{O}_2$  and  $\text{SCN}^-$  yields the bactericidal hypothiocyanite ( $\text{OSCN}^-$ ) in airway surface liquid (ASL). Although  $\text{SCN}^-$  is the physiological substrate of LPO, the Duox/LPO/halide system can generate hypoiodous acid when the iodide ( $\text{I}^-$ ) concentration is elevated in ASL. Because hypoiodous acid, but not  $\text{OSCN}^-$ , inactivates respiratory syncytial virus (RSV) in cell culture, we used a lamb model of RSV to test whether potassium iodide (KI) could enhance this system *in vivo*. Newborn lambs received KI by intragastric gavage or were left untreated before intratracheal inoculation of RSV. KI treatment led to a 10-fold increase in ASL  $\text{I}^-$  concentration, and this  $\text{I}^-$  concentration was approximately 30-fold higher than that measured in the serum. Also, expiratory effort, gross lung lesions, and pulmonary expression of an RSV antigen and IL-8 were reduced in the KI-treated lambs as compared with nontreated control lambs. Inhibition of LPO activity significantly increased lesions, RSV mRNA, and antigen. Similar experiments in 3-week-old lambs demonstrated that KI administration was associated with reduced gross lesions, decreased RSV titers in bronchoalveolar lavage fluid, and reduced RSV antigen expression. Overall, these data indicate that high-dose KI supplementation can be used *in vivo* to lessen the severity of RSV infections, potentially through the augmentation of mucosal oxidative defenses.

## Keywords

mucosal immunity, oxidative defense system, respiratory syncytial virus, potassium iodide, ovine model

## Disciplines

Veterinary Microbiology and Immunobiology | Veterinary Pathology and Pathobiology

## Comments

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## **Increased concentration of iodide in airway secretions is associated with reduced RSV disease severity**

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**Running title:** Oral KI reduces RSV by enhancing oxidative defense

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**ABSTRACT**

Recent studies have revealed that the human and non-rodent mammalian airway mucosa contains an oxidative host defense system. This three-component system consists of hydrogen peroxide ( $H_2O_2$ )-producing enzymes dual oxidase (Duox) 1 and 2, thiocyanate ( $SCN^-$ ), and secreted lactoperoxidase (LPO). The LPO-catalyzed reaction between  $H_2O_2$  and  $SCN^-$  yields the bactericidal hypothiocyanite ( $OSCN^-$ ) in airway surface liquid (ASL). Although  $SCN^-$  is the physiological substrate of LPO, the Duox/LPO/halide system can also generate hypiodous acid (HOI) when the iodide ( $I^-$ ) concentration is elevated in ASL. Since HOI, but not  $OSCN^-$ , inactivates respiratory syncytial virus (RSV) in cell culture, we used a lamb model of RSV to test whether potassium iodide (KI) could enhance this system *in vivo*. Newborn lambs received KI by intragastric gavage or were left untreated prior to the intratracheal inoculation of RSV. The KI treatment led to a 10-fold increase in ASL  $I^-$  concentration, and this  $I^-$  concentration was ~30-fold higher than that measured in the serum. Also, expiratory effort, gross lung lesions, and pulmonary expression of an RSV antigen and interleukin-8 were reduced in the KI-treated lambs as compared to non-treated controls. Inhibition of LPO significantly increased lesions, and RSV mRNA and antigen. Similar experiments in 3-week-old lambs also demonstrated that KI administration was associated with reduced gross lesions, decreased RSV titers in bronchoalveolar lavage fluid, and reduced RSV antigen expression. Overall these data indicate that high-dose KI supplementation can be used *in vivo* to lessen the severity of RSV infections, potentially through the augmentation of mucosal oxidative defenses.

**Keywords:** mucosal immunity; oxidative defense system; Duox; lactoperoxidase; thiocyanate; airway surface liquid; respiratory syncytial virus; potassium iodide; ovine model

## INTRODUCTION

Respiratory syncytial virus (RSV) is a major cause of acute lower respiratory infection in infants and young children and is a leading cause of infantile bronchiolitis worldwide (1, 2). In industrialized countries, hRSV accounts for up to 70% of hospitalized bronchiolitis cases (1-3). Although the antiviral drugs Palivizumab and Ribavirin have virucidal activity against RSV *in vivo*, there are no fully satisfactory therapeutic regimens or vaccines (4-6). It is estimated that 3% of RSV cases in the United States and 10% of cases worldwide result in hospitalization; the reported number of new cases of lower respiratory infection due to RSV in children under 5 in 2005 was 33.8 million (7, 8). Thus, there is a need for new therapies for RSV infection.

The host defense activity of the airway epithelium is critical for the continuous inactivation and removal of inhaled microbes from the respiratory tract. While the role of mucociliary clearance and epithelial host defense proteins and peptides in lung immunity is well established, the recognition that airway epithelial cells express an oxidative microbicidal system is recent (9-13; reviewed in 11, 12). This three-component oxidative system consists of two H<sub>2</sub>O<sub>2</sub>-generating enzymes of airway epithelia, dual oxidase (Duox) 1 and 2, along with a pseudohalide anion (thiocyanate, SCN<sup>-</sup>), and the enzyme lactoperoxidase (LPO). The Duox enzymes generate H<sub>2</sub>O<sub>2</sub> into the apical extracellular space where H<sub>2</sub>O<sub>2</sub> reacts with secreted SCN<sup>-</sup> in a LPO-catalyzed reaction to form the antibacterial hypothiocyanite (OSCN<sup>-</sup>) molecule; (H<sub>2</sub>O<sub>2</sub> + SCN<sup>-</sup> → OSCN<sup>-</sup>) (10, 13-21). Both LPO and SCN<sup>-</sup> are highly concentrated in the airway surface liquid (ASL). The Duox/LPO/SCN<sup>-</sup> system generates sufficient OSCN<sup>-</sup> to eliminate bacteria *in vitro* and *in vivo* (13, 22, 23). *In vitro*, the substitution of iodide (I<sup>-</sup>) for SCN<sup>-</sup> in this system yields hypoiodous acid (H<sub>2</sub>O<sub>2</sub> + I<sup>-</sup> → HOI) instead of the physiological product, OSCN<sup>-</sup> (24). HOI has potent microbicidal activity against bacteria (23) and viruses (26), including activity against RSV, whereas OSCN<sup>-</sup> exhibits little antiviral activity (24). We previously reported that I<sup>-</sup> is concentrated in the nasal ASL of human subjects following oral I<sup>-</sup> supplementation using an FDA-approved formulation (24). However, it was not tested whether I<sup>-</sup> supplementation also led to high I<sup>-</sup> concentration in the ASL of lower airways and whether a high I<sup>-</sup> concentration in the ASL could affect the severity of respiratory viral infections.

The ovine respiratory tract functionally expresses the Duox/LPO/halide system (23), is susceptible to human strains of RSV, and develops microscopic lesions similar to those in infants (for review see 27; 28-30). Some other animal species used to model RSV infection (e.g., mice and rats) lack airway submucosal glands (31) which are the LPO-secreting structures in the airways of larger mammals. Without LPO production in the airways, the Duox/LPO/halide system is not fully functional. Therefore, lambs were used to test the hypothesis that I<sup>-</sup> supplementation can protect the newborn lung against RSV infection. The life cycle of RSV is restricted to the epithelia of the respiratory tract. Because the Duox/LPO/halide system is dependent upon mature epithelia and submucosal glands that can produce adequate levels of enzymes and halide transport proteins, studies were completed in both newborn and 3-week-old lambs.



## MATERIALS AND METHODS

### I<sup>-</sup> concentration measurements in airway secretions and serum.

The concentrations of I<sup>-</sup> and SCN<sup>-</sup> in the nasal secretions and serum of lambs were measured after the administration of NaI (1.7 mg/kg body weight, n = 5) or PBS (control, n = 5) via the external jugular vein. Nasal secretions and blood samples were collected at 4 time points: at 0 hour (just before the injection of NaI), 4 h, 12 h, and 36 h. Blood (8 ml) was drawn from the external jugular vein, and serum was collected. Nasal secretions were collected with microsampling probes (Olympus BC-402C) as previously described (24, 32). Nasal fluid was recovered from the microsampling probes by microcentrifugation. Both serum samples and nasal secretions were diluted in water and analyzed for anion composition using ion-exchange chromatography as described previously (24, 32).

The above described experimental design was slightly modified to determine the effect of intragastric delivery of KI on the concentrations of I<sup>-</sup> and SCN<sup>-</sup> in airway secretions. Briefly, lambs (n = 5) received KI-containing PBS (1.8 mg KI/kg body weight) via an 18 French Foley catheter followed by 6 ml of water flush to clear. Nasal secretions and serum samples were collected at 0 hour (just before the intragastric gavage), 4 h, 12 h, and 36 h. At 4 and 12 hour, one animal/time point was euthanized after the collection of serum and nasal secretions to harvest tracheobronchial secretions as previously described (24, 32). The remaining 3 animals were euthanized at 36 hours for the collection of tracheobronchial secretions.

### *Lamb RSV challenge experiments.*

Lambs were randomly assigned to 3 groups: the first group was inoculated with RSV, the second group was inoculated with RSV and treated with KI, and the third group was neither inoculated with RSV nor treated with KI. Using this experimental design, a pilot experiment was carried out in which the human pathogen RSV strain A2 was delivered through a fiberoptic bronchoscope (10<sup>8</sup> PFU RSV in 10 ml cell culture medium) into the trachea of newborn lambs (2-3 days of age; n = 5). In this pilot experiment, the KI-treated lambs (n = 5) received 1.8 mg KI/kg body weight in PBS by intragastric gavage 4 hours prior to the inoculation of RSV A2 and daily thereafter. Control lambs (n = 3) received I<sup>-</sup> free PBS through via intragastric gavage and sterile cell culture medium (10 ml) through the bronchoscope. In subsequent experiments involving 2-3 day old lambs and 3-week-old lambs, a more pathogenic RSV strain (Memphis 37) (27) was delivered (3.5 x 10<sup>7</sup> PFU) to lambs in 6 ml culture medium using a PARI Sprint™ nebulizer and a cone face mask fitted with a rubber gasket that provided a seal around the mandible and nose. In addition, the dose of KI was increased to 10 mg KI/kg body weight/day. Control lambs received I<sup>-</sup> free PBS intragastrically (6 ml) via nebulization and additional group of lambs received Dapsone which inhibits lactoperoxidase activity. See online data supplement for additional methods.

## RESULTS

### **Airway surface liquid iodide levels after intravenous and intragastric iodide delivery.**

To evaluate the extent to which systemic I<sup>-</sup> administration to lambs affects the ASL concentration of I<sup>-</sup>, nasal secretions and serum samples were collected from lambs that received sodium iodide (NaI) in PBS intravenously (1.7 mg NaI/kg body weight) or PBS only (negative control). Ion-exchange chromatography analysis of these samples showed that the I<sup>-</sup> concentration increased in the nasal secretions to ~340 μM at the 4-hour time point, whereas the serum I<sup>-</sup> concentration only increased to ~14 μM (Figure 1A and 1B). By the 36-hour time point, the I<sup>-</sup> concentration in the nasal secretions returned to near base-line levels. Interestingly, the SCN<sup>-</sup> concentration decreased in the nasal secretions when the I<sup>-</sup> concentration reached its peak in the serum (supplemental figure E2), suggesting that SCN<sup>-</sup> and I<sup>-</sup> compete for the same transporters in the airway. I<sup>-</sup> administration to humans also leads to a greatly increased [I<sup>-</sup>] in the nasal ASL (24). Thus, our data indicate that the upper airway of lambs recapitulates the halide-transporting activity of the human nasal mucosa.

Next, we examined whether intragastric administration of I<sup>-</sup> also leads to elevated concentration of I<sup>-</sup> in the secretions of the upper and lower airways. We used intragastric gavage to deliver KI (1.8 mg KI/kg body weight) to lambs. Nasal secretions and serum samples were harvested at 4 time points (0, 4, 12 and 36 hours). Tracheobronchial secretions were collected immediately after euthanizing lambs at 4, 12, and 36 hours. Analysis of these samples showed that intragastric KI administration led to a more prolonged increase of I<sup>-</sup> in the serum, nasal secretions, and tracheobronchial secretions than the IV delivery of NaI (Figure 1). Furthermore, the upper and lower airway secretions contained similar levels of I<sup>-</sup> at each time point (Figure 1C). These data indicate that intragastric delivery of I<sup>-</sup> to lambs leads to elevated I<sup>-</sup> concentration in the ASL of both the lower and upper airways.

### **Pilot study: Iodide effects human RSV strain A2 *in vivo*.**

A pilot study was completed in order to establish an initial proof-of-principal that I<sup>-</sup> would have some effect on reducing RSV replication. Lambs received intragastric KI supplementation or control (i.e. I<sup>-</sup> free) solution 4 hours prior to intratracheal inoculation of hRSV A2 and daily thereafter. The severity of hRSV A2 infection was evaluated 6 days later when the RSV-induced lung lesions are maximal in this model (25, 30-33). In the lungs of KI-treated lambs, we found reduced hRSV A2 RNA levels (Figure 2A) and a reduction in gross and histological lesions (consolidation score: 2.22 ± 0.9) compared to untreated lambs infected with hRSV A2 (consolidation score: 4.9 ± 0.8) (p<0.05). After 6 days of I<sup>-</sup> supplementation, treated lambs had significantly increased [I<sup>-</sup>] in tracheal ASL compared to non-supplemented controls (Figure 2B). These findings suggest that I<sup>-</sup> administration is associated with reduced RSV disease severity.

### **Iodide effects on RSV M37 infection in newborn (2-3 day-old) lambs.**



Because the pilot studies demonstrated reductions in both lung lesions and RSV RNA levels, additional studies were completed in 2-3 day-old lambs in order to further investigate the extent to which I<sup>-</sup> reduces RSV severity in newborn lambs. The virus strain RSV Memphis 37 (M37) was used in these studies because M37 is less laboratory-adapted than RSV A2 and the lung disease caused by M37 is very similar in lambs and human infants.

*Effect of KI on clinical parameters, lesions, and bronchoalveolar lavage fluid.* There was a significant reduction in the incidence and severity of enhanced expiratory effort (forced expiration) in lambs receiving KI treatment at days 2, 3, 4, 5 and 6 post-inoculation (Figure 3A) compared to RSV-inoculated lambs without KI prophylaxis. Control lambs showed no increased expiratory effort. Among virus-treated lambs, 2/12 KI-treated RSV-inoculated lambs exhibited mildly increased expiratory effort whereas 9/12 lambs given RSV without KI treatment had increased expiratory effort that was moderate to marked. There were no significant differences between groups of lambs (RSV M37 without KI treatment; RSV M37 with KI treatment; control) in weight gain, temperature, or respiratory rate. Lung lesions were significantly reduced in KI treated lambs compared to untreated RSV-inoculated lambs (Figure 3B). Gross lesions were present in 11/12 of lambs receiving RSV M37 without KI; these were marked in 7/12 and mild in 4/12. In contrast, 2/12 KI-treated lambs receiving RSV M37 had marked gross lesions, 9/12 had mild lesions, and 1/12 had no lesions. To evaluate the effect of KI treatment on the inflammatory cell populations within the lung, BALF differential cell counts were performed. In newborn lambs, no statistically significant differences were detected between groups. However, there was a trend for the RSV-infected lambs that received KI treatment to have an increased proportion of lymphocytes ( $p = 0.096$ ; ANOVA). Consolidation scores determined by histopathology for lambs inoculated with RSV M37 lacking KI treatment ( $0.64 \pm 0.32$ ) were not significantly altered from lambs treated with KI ( $0.70 \pm 0.29$ ); controls (no RSV; no KI) lacked lesions (0.00 consolidation score). Lambs receiving Dapsone, KI and RSV had significantly increased levels of expiratory effort, gross and histologic lesion scores compared to control and KI-treated lambs (no Dapsone) and increased gross and microscopic lesions compared to M37 alone (no KI or Dapsone) (Fig. 3A-D). The findings, demonstrate that KI treatment reduces some, but not all clinical parameters of RSV infection and that inhibition of lactoperoxidase (with Dapsone) eliminates the anti-RSV effects of the Duox/LPO enhanced with KI. Dapsone itself lacks anti-RSV activity (Supplemental Figure E5).

*Effect of KI on RSV replication and cytokines.* By immunohistochemistry, RSV viral antigen abundance was significantly reduced in epithelial cells lining both alveoli (Alv) and airways (bronchioles and bronchi, Br) in KI-treated lambs compared to RSV-inoculated lambs with no KI treatment (Figures 3C, 3D, 3E). Lambs receiving Dapsone, KI and RSV had significantly increased levels of RSV mRNA and antigen compared to control and KI-treated lambs (no Dapsone) and also compared to M37 alone (no KI or Dapsone) (Fig. 3C, 3D). Levels of RSV N gene mRNA, immune and inflammatory gene mRNAs were assessed by RT-qPCR. RSV mRNA levels trended lower in KI-treated lambs but not significantly (supplemental Figure E3). IL-8 mRNA levels were

significantly reduced in KI-treated lambs compared to RSV-inoculated lambs lacking KI (supplemental Figure E3). Also, IFN $\gamma$  mRNA levels were significantly higher in KI-treated lambs compared to lambs receiving RSV M37 without KI treatment (supplemental Figure E3). Control lambs lacked RSV mRNA and increases/alterations in innate and adaptive immunity genes. The levels of IFN $\beta$ , MCP-1 $\alpha$ , MIP1 $\alpha$ , RANTES, SBD-1, SP-A, TGF $\beta$ , and PD-L1 mRNA in KI-treated lambs trended higher compared to lambs inoculated with RSV M37 without KI prophylaxis. In contrast, RNA levels of RSV and mRNA levels of CC10, IL-6, IL-10, IP10, MCP2, MIP1 $\beta$ , SP-D and TNF $\alpha$  trended higher for lambs receiving RSV M37 compared to RSV-inoculated lambs treated with KI. (For more details on the RT-qPCR procedure, see online data supplement). These findings demonstrate that KI treatment is associated with reductions in some parameters of RSV infection.

### **Iodide effects on RSV M37 infection in 3-week-old lambs.**

It is possible that with increased lung maturation, the Duox or LPO enzymes or other components of the Duox/LPO/halide system undergo/exhibit increased expression and function compared to newborn lungs. Therefore, the extent to which KI treatment reduces RSV disease severity in 3-week-old lambs was determined.

*Effect of KI on clinical parameters, lesions, and bronchoalveolar lavage fluid.* Lung lesions were less frequent in 3-week-old lambs with KI treatment ( $8.6 \pm 1.9$ ) compared to RSV inoculated lambs lacking KI ( $4.9 \pm 1.6$ ) but this difference was not significant; control animals lacked lesions. Consolidation scores determined by histopathology for 3-week-old lambs inoculated with RSV M37 and lacking KI treatment ( $1.1 \pm 0.6$ ) trended higher than those from lambs treated with KI ( $0.70 \pm 0.7$ ) but lacked statistical significance; controls (no RSV) lacked lesions (0.00 consolidation score). Clinically, there were no significant differences in expiratory effort among control or treatment groups in 3-week-old lambs. There were no significant differences between groups of 3-week-old lambs (RSV M37 without KI treatment; RSV M37 with KI treatment; control) in weight gain, temperature, heart and respiratory rates, and expiratory effort. To evaluate the effect of KI treatment on inflammatory cell populations within the lung, differential cell counts were performed on BALF. In 3-week-old animals, KI treatment significantly reduced the proportion of macrophages within BALF in RSV-infected lambs. No significant differences were detected in the populations of other cells, although there was a trend for the KI treated lambs to have a higher proportion of lymphocytes ( $p = 0.057$ ) compared with RSV-infected lambs that did not receive treatment (Figure 4A). Comparison between the two RSV-infected groups and uninfected controls were not possible in this age group since too few control lamb samples were present.

*Effect of KI on RSV replication.* RSV titers in BALF were significantly reduced in 3-week-old lambs treated with KI compared to lambs inoculated with RSV lacking KI (Figure 4B). Control lambs (receiving no RSV or KI) lacked viral titers. RSV viral antigen abundance determined by immunohistochemistry was significantly reduced in the epithelial cells lining both the airways (bronchi and bronchioles, Br) and alveoli (Alv) in 3-week-old lambs receiving KI compared to RSV-inoculated lambs lacking KI (Figure 4C). The levels of RSV nucleoprotein mRNA

measured by RT-qPCR were significantly reduced in 3- week-old lambs receiving KI compared to RSV-inoculated lambs lacking KI (Figure 4D).

#### **Ontologic expression of Duox 1, 2 and Lactoperoxidase genes in lamb lung.**

Because the lung of newborns continues to develop and mature after birth and also because expression of Duox1, Duox2, and LPO is essential for converting I<sup>-</sup> into hypoiodous acid, the extent of expression of these genes during ontogeny was measured by RT-qPCR using total RNA isolated from lung tissues collected from lambs at various time points during gestation and compared to adult expression levels as described previously (41, 42). Expression of lactoperoxidase and Duox1 was low preterm, at gestational days 115 and 130, and at birth, while expression was markedly increased in adults (Figure 5A and 5B). Expression of Duox2 was increased at day 115 of gestation, decreased progressively with gestational age, and was also markedly increased in adults (Figure 5C). These data indicate that Duox1, Duox2, and LPO are expressed in the airways of newborn lambs albeit at lower levels than in adult sheep.

## DISCUSSION

Viral respiratory tract infections are common and can be life threatening and there are no fully effective therapies or approved vaccines. Overall our findings indicate that the use of high-dose KI supplementation *in vivo* lessens the severity of RSV infections in newborn and 3-week-old lambs, through the augmentation of mucosal oxidative defenses.

There are several animal models of RSV infection and each has unique features. Lambs, like infants, have submucosal glands which produce LPO and also express sufficient levels of Dual oxidases and halide transport systems (23, 24) to support formation of the pseudohypohalide  $\text{OSCN}^-$  or, in the presence of sufficient  $\text{I}^-$ ,  $\text{HOI}^-$ . Rodents lack significant submucosal gland formation in the intrapulmonary airways and thereby also lack sufficient LPO production for a fully functional oxidative defense system within the airways (31). In addition, lambs are susceptible to infection by several strains of RSV, including hRSV M37 used in this study (27, 28-30, 33-36, 41). Gerson et al. demonstrated that the ovine airway secretions contain LPO and that a reduction of LPO function by dapsone administration reduces antibacterial activity (23), thus demonstrating that the ovine airway has a functional LPO-dependent antibacterial system. The present study further demonstrates that the ovine ASL contains  $\text{SCN}^-$  and for the first time shows that intravenous (i.v.) or intragastric delivery of iodide increases ovine ASL  $[\text{I}^-]$ . The results also suggest that the upper airway of lambs recapitulates the halide-transporting activity of the human nasal mucosa. Furthermore, the study is the first to show reductions in RSV disease severity following KI supplementation. In addition to lacking significant submucosal glands, mice do not have NIS (sodium/iodide symporter) in the trachea (Banfi, unpublished observations) and thus would require enormous amounts of KI in order to reach the air surface liquid  $\text{I}^-$  concentrations attained in lambs and humans receiving much less.

Newborn lambs receiving KI exhibited reduced levels of expiratory effort, gross lesions, RSV antigen distribution, and a trend of reduced RSV N gene mRNA levels. KI treatment in the older, 3-week-old lambs was associated with reduced RSV titers in BALF along with reduced RSV antigen load by immunohistochemistry, and reduced RSV nucleoprotein mRNA levels by RT-qPCR. Microscopic lesions (consolidation scores) were not significantly reduced with KI treatment in either newborn or 3-week-old lambs, although there was a trend towards reduced lesions in the 3-week-old lambs. Thus, while not all measured parameters of RSV infection were altered by KI treatment, reductions in viral parameters (e.g., viral titer, antigen, and RNA levels) and clinical features (reduced expiratory effort) are of particular significance in lessening RSV disease severity. Mechanistically, administration of Dapsone with RSV and KI in newborn lambs reversed the effects of the KI treated and resulted in disease severity equal to and often significantly greater than M37 alone (no KI or Dapsone).  $\text{HOI}^-$  and/or hypoiodite ( $\text{OI}^-$ ) production is very difficult to measure *in vivo* because such reactive oxygen species have very short molecular half-lives in general (12, 14). Thus, the extent to which  $\text{HOI}^-$  was formed in the ASL of the KI-treated lambs in this study is not known. However, because Dapsone inhibits lactoperoxidase (LPO) and LPO is essential for

catalyzing the reaction of KI and H<sub>2</sub>O<sub>2</sub> to HOI, these findings suggest that: 1) the Duox/LPO system is essential to the anti-RSV activity seen in these studies and 2) KI itself is not sufficient for the anti-RSV activity. These findings are consistent with *in vivo* studies that demonstrated significant anti-RSV activity by HOI and a lack of direct RSV killing by KI, LPO, or H<sub>2</sub>O<sub>2</sub> individually (24). Dapsone has been used previously in sheep to inhibit LPO activity which allowed increased colonization by a Gram-negative bacterial pathogen in the respiratory tract (23). Also, lambs in this study demonstrated high ASL [I<sup>-</sup>] levels when receiving daily KI. It is also possible that there are other non-oxidative properties of KI that result in anti-RSV activity, such as its efficacy as an expectorant agent (43) but expectorants are generally not effective in reducing RSV replication.

KI treatment reduced disease severity to a greater degree in 3-week-old lambs than in newborn lambs as reflected by the large reductions in titer, antigen, and viral mRNA levels (viral mRNA levels trended lower in KI-treated 2-3 day old lambs were not significantly reduced despite a larger group number). While the reason for this is not fully understood, we speculate that the respiratory tracts of 3-week-old lambs are more mature and have more fully differentiated epithelia than newborns and, therefore, have more available Duox/LPO expression and activity to convert KI to HOI than do newborn lambs. Alternatively, we have shown previously that younger lambs (especially preterm) exhibit increased disease severity compared to older lambs. Therefore, it could be that RSV disease severity was slightly less in the 3-week-old lambs as reflected by their lack of increased expiratory effort compared to the markedly enhanced expiratory efforts observed in the younger lambs. Thus, if RSV disease severity is less in older lambs than newborn, the effects of KI treatment may appear more pronounced in older lambs. It is also possible that older lambs may have enhanced innate and adaptive immune responses that synergize with KI treatment.

KI was delivered prophylactically in this study and thus, iodide levels in the ASL were high at the time of RSV nebulization. Because of this, it was suspected that nebulized particles would come into contact with HOI present on the ASL - inhibiting initial RSV attachment and infection. However, while prophylactic administration of KI reduced many parameters of RSV infection and RSV disease severity, it did not completely prevent RSV infection; since KI-treated lambs developed some gross and histologic lesions, and viral RNA and antigen were present (albeit at significantly reduced levels) compared to untreated animals. It is possible that not all RSV virions were inactivated upon initial contact with the respiratory mucosa during nebulization. More specifically, it is likely that HOI levels are higher in the nasal mucosa, trachea and bronchi, all three of which are anatomical locations where submucosal glands (and LPO) are present, than in the distal bronchioles and alveoli (both lacking in submucosal glands). Thus, nebulized virions deposited onto the upper airways may be damaged/destroyed by HOI whereas those virions deposited onto bronchioles or alveoli may have evaded HOI. Deposition and infection by RSV at these distal locations may allow some level of viral infection since the distal bronchioles and alveoli are important sites of RSV replication. In other words, there may be a mismatch between sites of HOI production and some areas of the airways and alveoli that are susceptible to RSV infection.

Because KI was delivered daily after initial infection, it is also possible that HOI has effects on RSV virions produced in the lung after RSV nebulization. KI treatment may impair/damage virus released from respiratory mucosa during replication cycles several days after inoculation of RSV. Prophylactic protection by KI, as demonstrated in this study, might be effective in preventing spread of RSV to non-infected individuals treated with KI. It is also possible that KI treatment might have some beneficial effect in individuals already infected with RSV. Further studies are needed to optimize KI as a therapeutic, including determining its effectiveness with different routes of infection (intratracheal, intrabronchial, or aerosolized RSV), activity against different viral and bacterial pathogens, optimizing the dose and timing of KI administration, and the effects of inhibition of various components of the Duox/LPO/halide system.

In a previous study, we showed that the  $[I^-]$  increased in the upper airway secretions of human subjects following oral intake of KI (24). In the current study, through the use of the lamb model, we determined that  $I^-$  was secreted not only in the upper airways, but also in the trachea following  $I^-$  supplementation. In both humans and lambs, the  $[I^-]$  was found to be more than 20-fold higher in ASL than in serum. The mechanism responsible for secretion of  $I^-$  in the airways is not known. Frago and colleagues have reported that the sodium-iodide symporter (NIS) is expressed in the submucosal glands of airways where it is localized to the basolateral membrane of epithelial cells (16). On the apical side of the epithelium,  $I^-$  may be exported through  $I^-$  permeable ion channels or anion transporters. Among the apical anion channels of airway epithelia,  $Ca^{2+}$ -activated  $Cl^-$  channels and the cystic fibrosis transmembrane conductance regulator (CFTR) are known to be  $I^-$  permeable (44). Furthermore, the anion transporter, pendrin, has been detected in the apical membrane of airway epithelial cells (20). NIS, CFTR,  $Ca^{2+}$ -activated  $Cl^-$  channels, and pendrin have also been implicated in the secretion of  $SCN^-$ . The fact that the increased ASL  $[I^-]$  is accompanied by reduced  $SCN^-$  levels supports the notion that the same ion transporters are involved in both  $SCN^-$  and  $I^-$  secretion in the airways.

Previous studies have shown that lambs express all components of this mucosal oxidative defense system at levels sufficient for activity in the airways (23), and our results indicate that prophylactic administration of KI reduces RSV disease severity. These findings have implications for use of KI in infants against RSV or other respiratory pathogens since KI is relatively inexpensive, stable, can be readily transported, and does not require refrigeration or specialized storage. The extent to which KI treatment may reduce RSV disease severity in preterm and premature lambs and in lambs was not assessed in this study. RSV disease in humans often occurs in infants born prematurely and therapies are needed, but additional studies will be required in order to better understand the ontogeny and activity of the components of the Duox/LPO/halide host defense system in the preterm lung. Overall our findings indicate that the use of high-dose KI supplementation *in vivo* lessens the severity of RSV infections, potentially through the augmentation of mucosal oxidative defenses.



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## Figure legends

**Figure 1.** I<sup>-</sup> concentrations in the airway secretions and serum of lambs following systemic administration of I<sup>-</sup>. **(A)** I<sup>-</sup> concentration in nasal secretions of lambs at the indicated time points following intravenous (i.v.) injection of NaI (filled circles, n = 5) or PBS (open circles, n = 5) at 0 hour. **(B)** I<sup>-</sup> concentrations in the serum of lambs at the indicated time points following i.v. administration of NaI (filled triangles, n = 5) or PBS (open triangles, n = 5) at 0 hour. **(C)** I<sup>-</sup> concentration in the nasal and tracheobronchial secretions of lambs (filled circles and squares, respectively) at the indicated time points following intragastric (i.g.) KI administration at 0 hour. The number of animals used for the collection of nasal secretions was 5 (0 h and 4 h), 4 (12 h), and 3 (36 h). The number of animals used for the collection of tracheal secretions was 1 (4 h), 1 (12 h), and 3 (36 h). **(D)** I<sup>-</sup> concentration in the serum of lambs (n ≥ 3) at the indicated time points following i.g. delivery of KI. Error bars = SEM.

**Figure 2.** Effect of oral KI supplementation on hRSV A2 mRNA levels and ASL [I<sup>-</sup>]. **(A)** RSV replication and **(B)** [I<sup>-</sup>] in tracheal ASL were evaluated 6 days after inoculating lambs with hRSV A2; the indicated groups of animals also received daily doses of KI (1.8 mg/kg BW). **(A)** RSV mRNA signal normalized to total RNA loaded per RT-qPCR: Control n = 5; RSV+KI n = 5; RSV n = 5. **(B)** No KI n = 5; KI n = 5. Error bars = SEM, \*p<0.05.

**Figure 3.** The effect of KI on RSV M37 infection newborn (2-3 day old) lambs. **(A)** Enhanced expiratory effort (forced expiration). Lambs inoculated with RSV M37 and receiving KI had significantly reduced expiratory efforts at days 3, 4 and 5 post-inoculation compared to RSV-inoculated lambs lacking KI and control lambs. Control group n = 6; M37 group n = 11; M37+KI group n = 10. Error bars = SEM, \*p<0.05, \*\*\*p<0.001. **(B)** Lambs receiving KI had significantly reduced gross lesions compared to RSV-inoculated lambs lacking KI and control lambs. RSV M37 infected lambs receiving KI and Dapsone had significantly increased gross lesions compared to control and RSV-infected (no KI). Control group n = 14; M37 group n = 11; M37+KI group n = 10; M37 + KI + Dapsone (n = 5). Error bars = SEM, \*p<0.05, \*\*\*p<0.001. The minor control group lesions were found (by immunohistochemistry) to not be RSV. **(C)** Lambs receiving KI had a trend of reduced RSV M37 mRNA compared to lambs lacking KI whereas lambs receiving RSV M37, KI and Dapsone had significantly increased RSV M37 mRNA levels compared to M37 alone and M37 + KI. Error bars = SEM, \*p<0.05, \*\*\*p<0.001. **(D)** Newborn lambs inoculated with RSV M37 and receiving KI had significantly reduced levels of viral antigen in alveolar regions compared to RSV-inoculated lambs lacking KI. In bronchioles, RSV M37 antigen was not significantly

altered; control lambs lacked RSV antigen. RSV M37-infected lambs receiving KI and Dapsone had significantly increased RSV antigen compared to control and RSV-infected (no KI). Control group n = 14; M37 group n = 11; M37+KI group n = 10; M37 + KI + Dapsone (n = 5). Error bars = SEM, \*p<0.05, \*\*\*p<0.001 (E, F). Lung, newborn lambs infected with RSV M37 and stained for RSV antigen. (E) Lung of lamb lacking KI administration in which there is abundant RSV antigen (brown) in most airway epithelial cells. (F) Lung of lamb that received KI; a few cells contain RSV antigen.

**Figure 4.** The effect of KI on RSV M37 infection in 3-week old lambs. (A) Effect of KI treatment on the cellular composition of BALF in RSV-infected 3-week-old lambs. Cytospin preparations of BALF were stained with modified Wright's, and 300 cell differentials were performed to assess relative differences in populations of inflammatory cells. Values are expressed as mean  $\pm$  SD, \*p<0.01 based on ANOVA followed by Tukey-Kramer multiple comparisons test. (B) Bronchoalveolar lavage fluid (BALF) titers of RSV in 3-week-old lambs. Lambs receiving KI had significantly less viable RSV than lambs lacking KI. Control lambs lacked RSV titers. Control group n = 4; M37 group n = 5; M37+KI group n = 5. Error bars = SEM, \*p<0.05. (C) RSV M37 mRNA levels in lungs of lambs receiving KI trended toward significant reductions compared to RSV-inoculated lambs lacking KI; controls lack RSV mRNA. Control group n = 8; M37 group n = 10; M37+KI group n = 10. Error bars = SEM, \*p<0.05, \*\*p<0.001. (D) 3-week-old lambs receiving KI had significantly reduced levels of RSV antigen in both bronchiolar and alveolar regions compared to RSV-inoculated lambs lacking KI; controls lacked RSV antigen. Control Br n = 8; Control Alv n = 8; M37 Br n = 30; M37 Alv n = 30; M37+KI Br n = 30; M37+KI Alv n = 30. Error bars = SEM, \*p<0.05, \*\*p<0.01.

**Figure 5.** Ontogeny of lung expression of lactoperoxidase (LPO) and dual functioning oxidases 1, 2 (Duox1 and Duox2) in lambs as assessed by RT-qPCR. (A) Expression of LPO mRNA at various stages throughout lamb development. 115 day (of gestation) group n = 5; 130 day (of gestation) group n = 4; Term lamb group n = 4; Adult lamb group n = 4. Error bars = SEM, \*p < 0.05. (B) Expression of Duox1 mRNA at various stages throughout lamb development. (C) Expression of Duox2 mRNA at various stages throughout lamb development. 115 day group n = 3; 130 day group n = 3; Term lamb group n = 4; Adult lamb group n = 4. Error bars = SEM, \*p < 0.05.

**Fig. 1.**

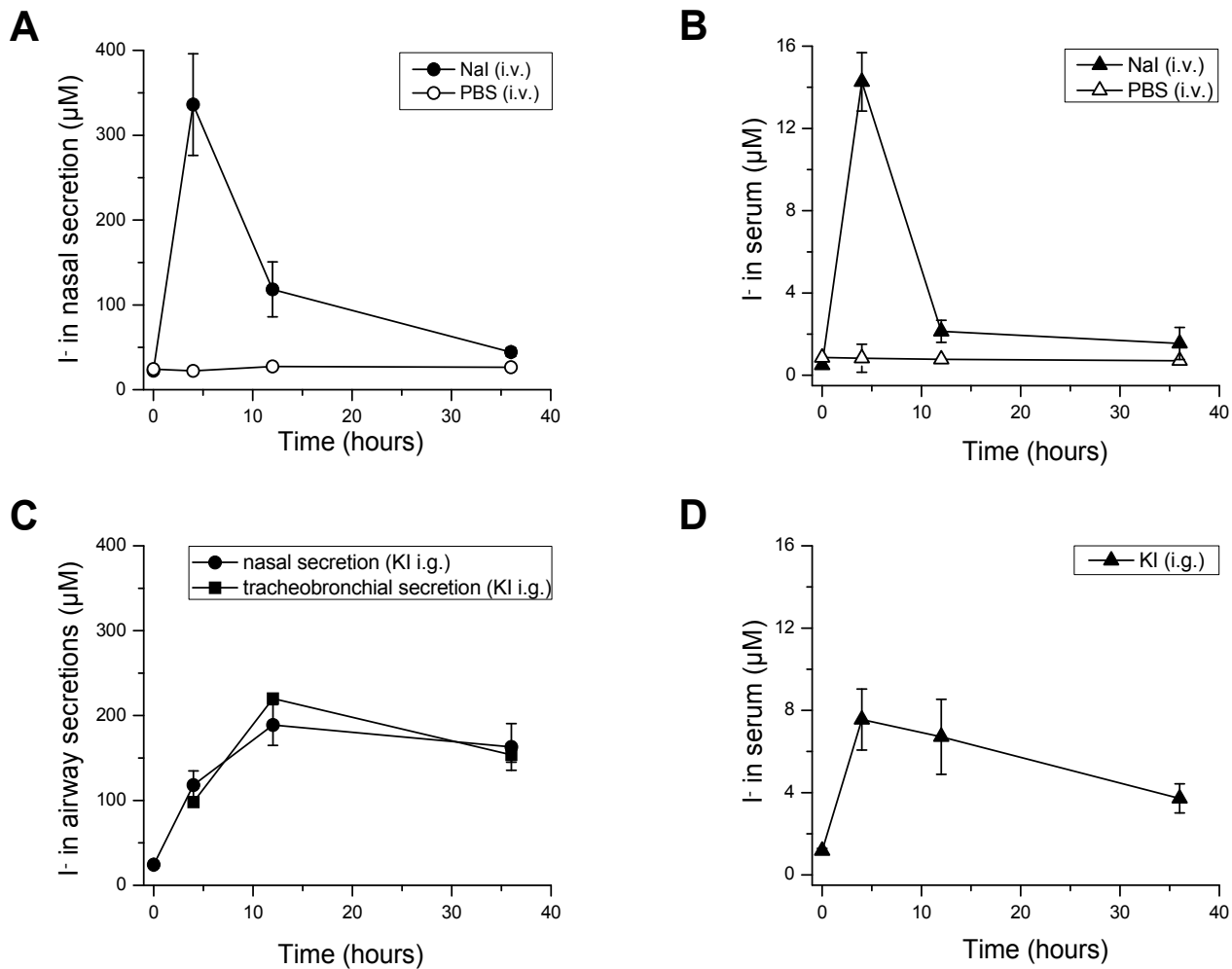
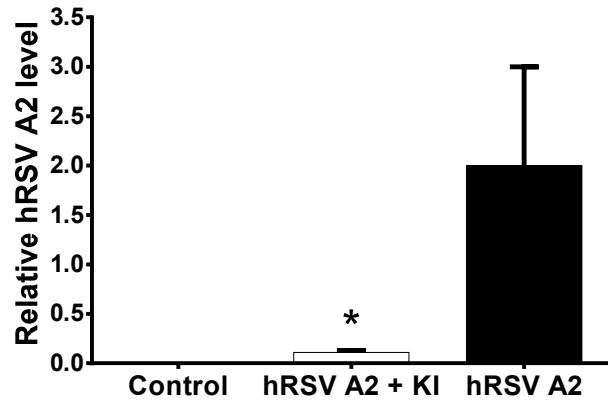




Fig. 2.

A



B

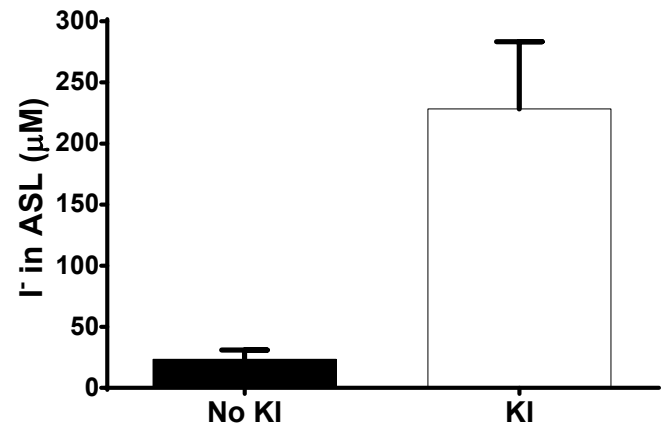


Fig. 3.

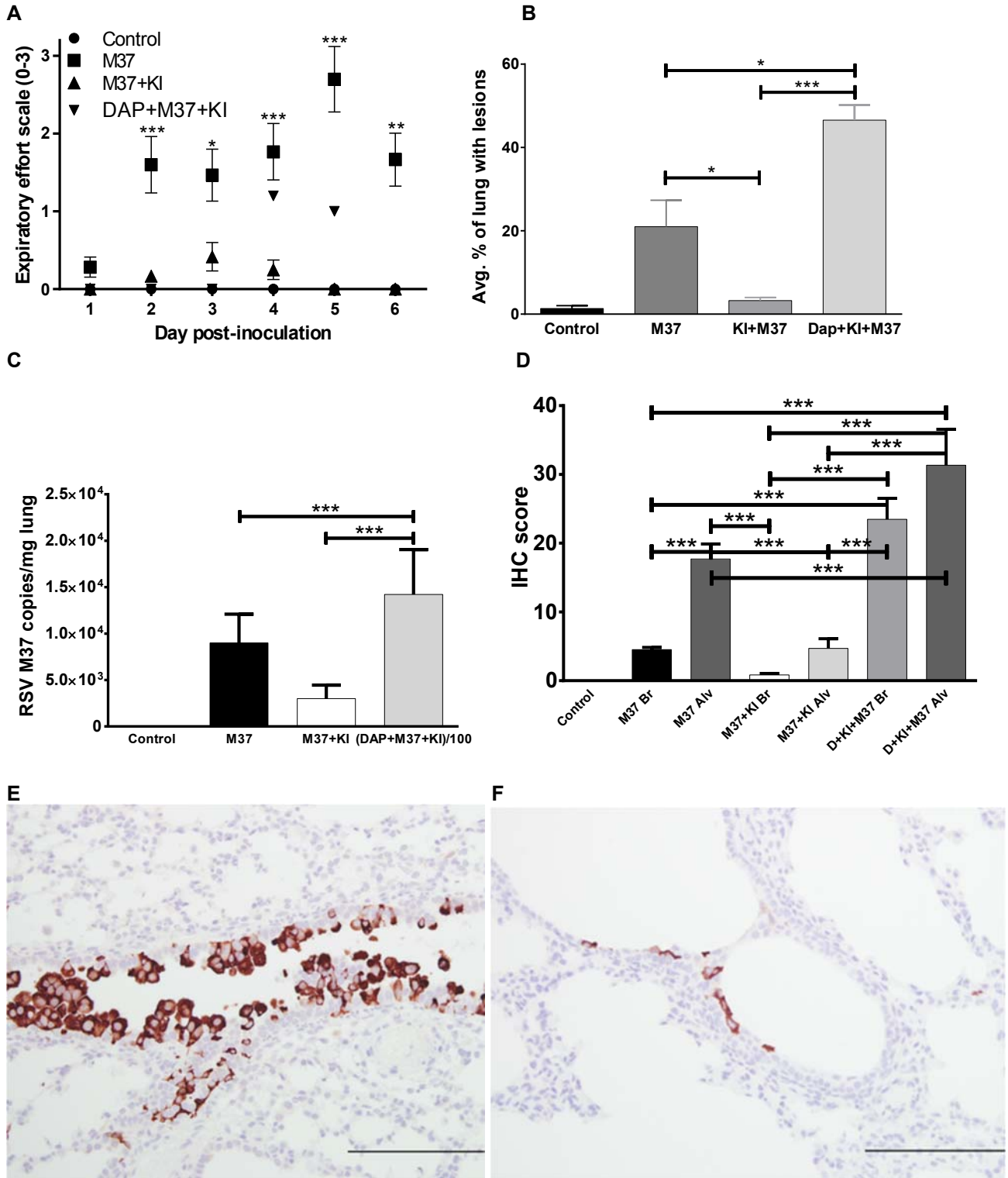


Fig. 4.

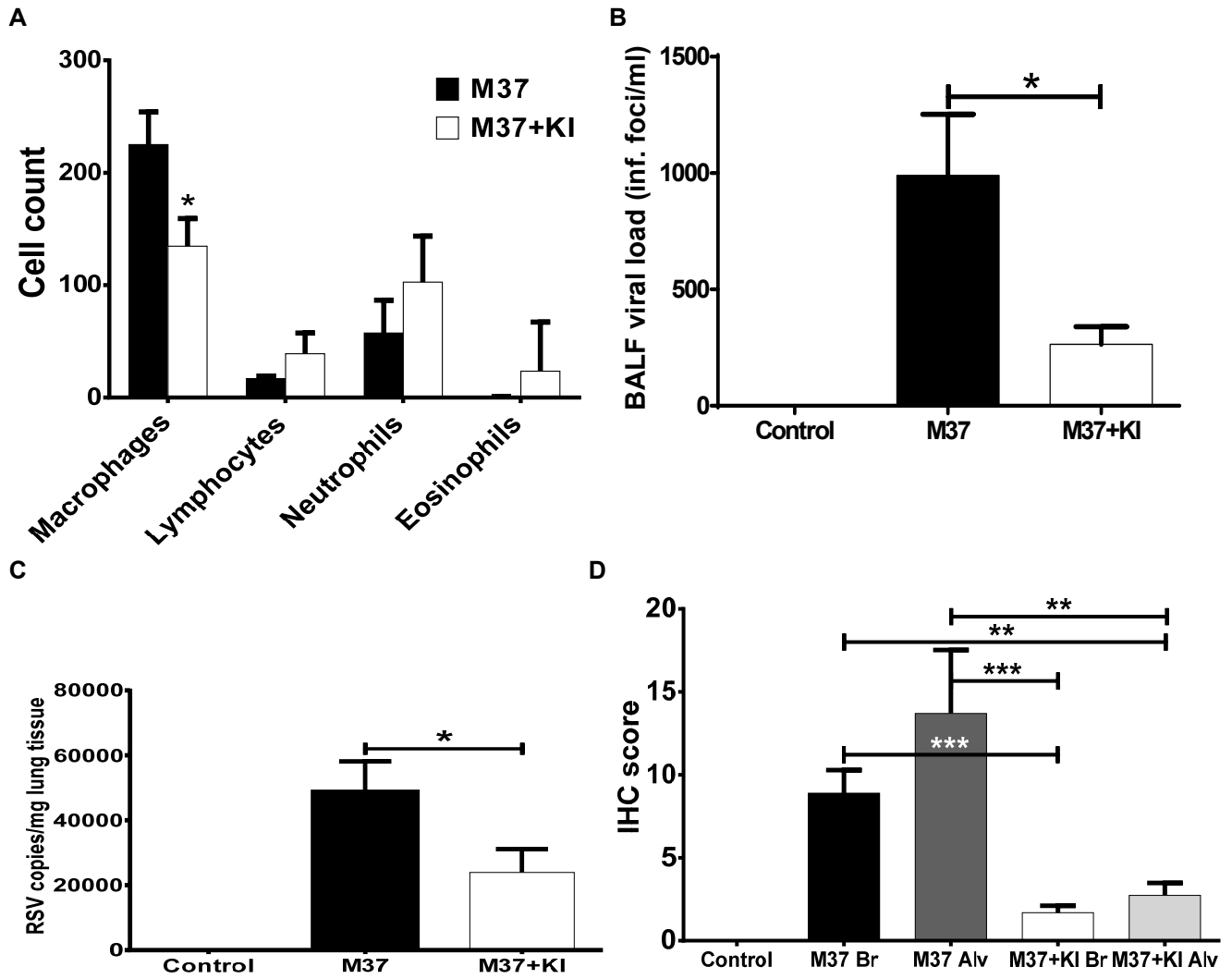
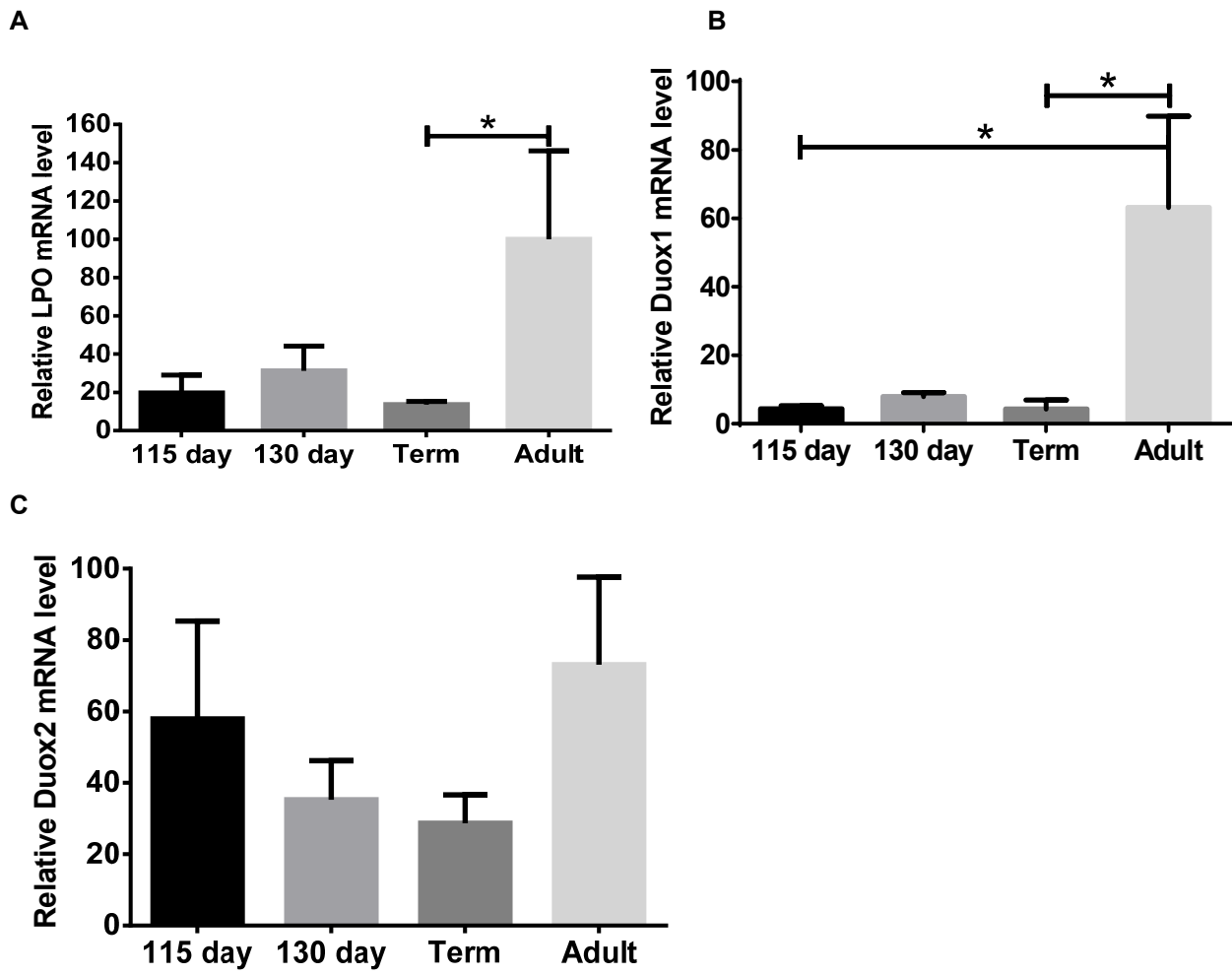


Fig. 5.



**SUPPLEMENTAL INFORMATION**

**Oral potassium iodide reduces RSV disease severity in lambs by augmenting mucosal oxidative defenses**

Rachel J. Derscheid, Albert van Geelen, Jack M. Gallup, Shannon J. Hostetter, Botond Banfi, Paul B. McCray Jr., Mark R. Ackermann



Table 1. RT-qPCR primers and hydrolysis probes used in this study.

Primer name	Sequence (5'→3')	Accession # or source
RSV M37 Forward RSV M37 Reverse RSV M37 Probe	GCTCTTAGCAAAGTCAAGTTGAA[C]GA TGCTCCGTTGGATGGTGTATT 6FAM-ACACTCAACAAAGATCAACTTCTGTCATCCAGC	M11486 and Meridian LifeScience literature; nucleoprotein sequence. [ ] = T for hRSV A2 forward primer
CC-10 Forward CC-10 Reverse CC-10 Probe	GCTGCCCTGGAACCTTCA CCTTCAGCTGTCTTGTGCTTCT 6FAM-CCCTGACGAAGACATG-MGBNFQ	Cloned and sequenced in our lab by Dr. Albert van Geelen, 2008
IFN $\beta$ Forward IFN $\beta$ Reverse IFN $\beta$ Probe	TGGTTCTCCTGCTGTGTTTCTC CGTTGTTGGAATCGAAGCAA 6FAM-ACCACAGCTCTTCCAGGAGCTACA-TAMRA	NM_174350
IFN $\gamma$ Forward IFN $\gamma$ Reverse IFN $\gamma$ Probe	TGGAGGACTTCAAAGGCTGAT GATGGCTTTGCGCTGGAT 6FAM-CAAATTCCGGTGGATGATCTGC-TAMRA	NM_001009803
IL-6 Forward IL-6 Reverse IL-6 Probe	GCTGCTCCTGGTGATGACTTC GGTGGTGCATTTTTGAAATCTTCT 6FAM-CTTTCCTACCCCGGGTCCCCTG-MBGNFQ	NM_001009392
IL-8 Forward IL-8 Reverse IL-8 Probe	TTCCAAGCTGGCTGTTGCT TTGACAGAACTGCAGCTTCACA 6FAM-CCGCTTCTGCTCTCTGCAGCTC-TAMRA	X78306
IL-10 Forward IL-10 Reverse IL-10 Probe	GTCGGAAATGATCCAGTTTTACCT GTCAGGCCCATGGTTCTCA 6FAM-AGGAGGTGATGCCACAGG-MBGNFQ	Z29362
IP10 Forward IP10 Reverse IP10 Probe	CACCCTGAGCTGTTGAGTAGTGA GCGCCCCGGTATAGCTTAC 6FAM-CCCTGGCACTGTGAC-MBGNFQ	NM_001009191
MCP-1 $\alpha$ Forward MCP-1 $\alpha$ Reverse MCP-1 $\alpha$ Probe	GCTGTGATTTCAAGACCATCCT GGCGTCTGGACCCATTT 6FAM-AAAGAGTTTTGTGCAGACCCCAACC-TAMRA	<sup>†</sup> Vet Immunol Immunopathol 2001;82:153–64.
MCP2 Forward MCP2 Reverse MCP2 Probe	GCTCGCTCAGCCAGATTCAG CTTCCCATTGGTCACACTAAAGC 6FAM-TTCTATCCCAATCACCTGC-MBGNFQ	<sup>†</sup> Vet Immunol Immunopathol 2001;82:153–64.
MIP1 $\alpha$ Forward MIP1 $\alpha$ Reverse MIP1 $\alpha$ Probe	GGCCTGCTGCTTCTCCTATG TGGTCTCAAATAGTCAGCTACGATT 6FAM-CGCCCCGGCAGCTTCTCGC-TAMRA	NM_174511
MIP1 $\beta$ Forward MIP1 $\beta$ Reverse MIP1 $\beta$ Probe	GCGCCAACCCAGTGA CAGCTCAGTTCAATTCAGGTCAT 6FAM-TGGGTCCAGGAGTACG-MBGNFQ	EU366479 and NM_001075147 XM_582039
RANTES Forward RANTES Reverse RANTES Probe	TGCTTCTGCCTCCCCATATG GGGCGGGAGATATAGGCAA 6FAM-CACCACGCCCTGCT-MBGNFQ	Based on alignment of human (NM_002985) and bovine (NM_175827) sequences
SBD-1 Forward SBD-1 Reverse SBD-1 Probe	CCATAGGAATAAAGGCGTCTGTGT CGCGACAGGTGCCAATCT 6FAM-CCGAGCAGGTGCCCTAGACACATGA-TAMRA	U75250
SP-A Forward SP-A Reverse SP-A Probe	TGACCCTTATGCTCCTCTGGAT GGGCTTCCAAGACAACTTCTCT 6FAM-TGGCTTCTGGCCTCGAGTGC-TAMRA	AF076633
SP-D Forward SP-D Reverse SP-D Probe	ACGTTCTGCAGCTGAGAAT TCGGTCATGCTCAGGAAAGC 6FAM-TTGACTCAGCTGGCCACAGCCAGAACA- TAMRA	AJ133002
TGF $\beta$ Forward TGF $\beta$ Reverse TGF $\beta$ Probe	TGTGTTGCTCAGCTCTACATTGAC TAGCCCTTGGTTCGTGAAT 6FAM-TCCAGCCCAGGTCTTCCGGA-TAMRA	NM_001009400
TNF $\alpha$ Forward TNF $\alpha$ Reverse TNF $\alpha$ Probe	CAACCTGGGACACCCAGAAT TCTCAAGGAACGTTGCGAAGT 6FAM-CAAGGGCCAGGGTCTTACCGGAA-TAMRA	NM_001024860
PD-L1 Forward PD-L1 Reverse PD-L1 Probe	TGGTCATCCCAGAACCATATCTAG CCCAGAGTCACCAAGTGATTCC 6FAM-TCCAGCAAAAAAG-MBGNFQ	XM_613366

**Table 1 Footnotes:** †For MCP-1 $\alpha$  and MCP2 sequences. Dunphy J, Horvath A, Barcham G, Balic A, Bischof R, Meeusen E. Isolation, characterization and expression of mRNAs encoding the ovine CC chemokines, monocyte chemoattractant protein (MCP)-1alpha and -2. *Vet Immunol Immunopathol* 2001;82:153-164.

## **MATERIALS AND METHODS**

*Lamb RSV challenge experiments (methods continued from text).*

In the 2-3 day old lambs and 3-week-old lambs, each of the 3 groups of lambs contained 6 animals, and the experiment was carried out twice in 2-3 day-old (newborn) lambs (n = 12 in each treatment group) and once in an additional group (n = 5 lambs) that received daily administration of 2.014 mM Dapsone (Sigma-Aldrich; 4-aminophenyl sulfone in 3 mL sterile 0.9% saline containing 1 % w/v ethanol and 0.1% w/v DMSO nebulized to completion with a PARI nebulizer (13 minutes) along with RSV and KI. Dapsone inhibits lactoperoxidase and thereby reduces conversion of H<sub>2</sub>O<sub>2</sub> and I<sup>-</sup> to OI<sup>-</sup>. Also, the design was used once in 3-week-old lambs (n = 6 in each treatment group). Both RSV strains were grown in HEp-2 cells. After inoculation of RSV, lambs in all groups received daily antibiotics (Ceftiofur, 1-2 mg/kg body weight, intramuscular) to prevent secondary bacterial infections. The animals' temperature, weight, and respiratory rates, and clinical severity of RSV disease for each lamb was assessed daily and scored as described (28, 33-36); increased expiratory effort (also termed forced expiration) was scored blindly daily as: 0 = no increased effort; 1 = earliest detectable; 2 = moderate (>1 sec) with some abdominal effort; 3 = marked (>3 sec) with hard abdominal effort. Lambs were euthanized by sodium pentobarbital overdose on day 6 post-inoculation when RSV-induced lesions are maximal in this model (28, 33-36). During the RSV challenge experiments, lambs received a custom diet (Milk Products Inc., Chilton, WI, USA) that lacked supplemental I<sup>-</sup> and the lack of I<sup>-</sup> in this diet was verified in our laboratory using anion-exchange chromatography as previously described (24, 32). All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Iowa State University.

*Post-mortem analysis of RSV disease severity.*

The thoraxes of euthanized lambs were opened, lungs photographed *in situ* and *ex vivo*, and gross lesions were scored as described previously (28, 33-36). Multiple samples from each lung lobe were collected and processed for RNA isolation as described previously (28, 33-41). The isolated RNA was assessed for quality (supplemental figure E1) and used for RT-qPCR analysis of the expressions of RSV nucleoprotein (N gene) mRNA, immune and inflammatory gene



mRNAs according to the PREXCEL-Q Method (30-33, see protocol, below). Primers and probes were designed using ABI Primer Express 2.0 (see oligo sequences in supplemental Table 1). Two additional samples from each lobe were fixed in 10% neutral-buffered formalin, embedded into paraffin, sectioned, stained with H & E, and assessed by light microscopy for lung lesions as described previously (28, 33-36). Briefly, a 4-point scale was used to describe the percentage involvement of lung parenchyma: 0% consolidation = 0, 1-9% consolidation = 1, 10-39% consolidation = 2, 40-69% consolidation = 3, 70-100% consolidation = 4. Other formalin-fixed paraffin-embedded (FFPE) sections of the lung lobes were used for the immunohistochemical detection of RSV antigen expression, and the expression of RSV antigen was scored as described previously (25, 30-33, see more details below).

In addition to the lung collected for RNA isolation and histological samples, bronchoalveolar lavage fluid (BALF) was also collected from each 3-week-old lamb and used for the determination of viral titers and cytology (see details in the online data supplement). Briefly, the right caudal lung lobe was flushed through a major bronchus with 5 ml of cold modified Iscove's media (containing 42.5% Iscove's modified Dulbecco's medium, 7.5% glycerol, 1% heat-inactivated FBS, 49% DMEM, and 5 µg/ml kanamycin sulfate), and 1 ml of the collected BALF was used for infectious focus assay (see detailed protocol in the online data supplement). BALF was also collected from the accessory lobe by flushing the lobe through its major bronchus with 1 ml sterile PBS. These BALF samples were analyzed for total nucleated cell count and differential cell count by a board certified veterinary clinical pathologist blinded to the identity of individual samples (see details of the cytology method in the online data supplement). In some BALF samples, too few intact nucleated cells were present to perform a differential cell count; these samples were excluded from the statistical analysis.

#### *Effect of Dapsone on RSV in vitro*

The extent to which dapsone may have anti-RSV activity was assessed *in vitro*. HEP-2 cells were grown to 90% confluence in 12-well culture plates in MEM media supplemented with 10% FBS and 1% pen/strep.  $5 \times 10^3$  FFU of RSV-GFP was mixed with increasing concentrations of Dapsone solution (Sigma-Aldrich; 4-aminophenyl sulfone in sterile 0.9% saline containing 1% w/v ethanol and 0.1% w/v DMSO) with a final volume of 300 µl. For controls, RSV-GFP (recombinant replication competent RSV-A2 strain expressing GFP) (E8) was added to either the diluent alone or serum free MEM. The final concentration of ethanol and DMSO for the Dapsone treated virus and diluent control was 0.97% and 0.097% respectively. The samples were incubated together

at 37°C and 5% CO<sub>2</sub> for 2 hours. The RSV-Dapsone mixture was then added to HEp-2 cells and incubated for 2 hours at 37°C and 5% CO<sub>2</sub>. The remaining virus was then washed off with 1X PBS and fresh media applied. Following overnight incubation, green fluorescent foci were counted for 5 random fields per well using the GFP filter on an inverted stage fluorescence microscope (Olympus IX70, Center Valley, PA, USA).

*Ontologic expression of Duox1, Duox2, and LPO genes in lamb lung.*

The extent to which Duox1, Duox2, and LPO mRNA are expressed in the lung during development was assessed by RT-qPCR using lung RNA from lambs at various developmental time points: 115 days gestation (n = 5), 130 days gestation (n = 4), at birth (n = 4) and adults (n = 4) as described previously (41, 42).

*Infectious focus assay used on collected BALF samples.*

BALF was collected as described above, and 1 ml samples from each lamb were tested for the presence of live virus by an infectious focus assay. Briefly, HEp-2 cells were grown to 70% confluence in 12-well culture plates (Fisher Scientific, Hanover, IL, USA) in DMEM media (Mediatech, Inc., Manassas, VA, USA) supplemented to 10% with heat-inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA, USA) and 50 µg/ml kanamycin sulfate (Invitrogen). Each sample was analyzed at full-strength and at four additional serial-dilutions of 10-fold, 100-fold, 1,000-fold and 10,000-fold. 200 µl of each BALF sample dilution was added in duplicate to wells of a 12-well plate (e.g., each BALF sample used an entire 12-well culture plate); two control wells on each plate received virus-free cell culture medium. Plates were incubated for 80 minutes in a CO<sub>2</sub> incubator at 37°C and 5% CO<sub>2</sub> with manual rocking every 20 minutes. 1 ml of culture medium was added to each well and plates were allowed to incubate for 48 hours after which medium was removed from all wells and cells in all wells were immediately fixed with 60% acetone/40% methanol solution for 1 minute. The acetone/methanol was removed and plates were allowed to air dry for about 2 minutes after which each well was rehydrated with 1 ml TBS-0.05% Tween 20, pH 7.4-7.6 (TBS-tw) for 1 minute with mild rotation. To block non-specific binding, 1 ml of 3% BSA (Fisher Scientific) in TBS-tw was added to all wells at room temperature for 30 minutes with gentle rocking. Primary antibody (MAb to RSV Fusion Protein, Clone: RSV 3216 (B016), Meridian Bioscience, Cincinnati, OH, USA) was diluted 1:800 in TBS-tw containing 3% BSA; 325 µl of this was added to each well and plates were allowed to incubate overnight at 4°C with gentle rocking. In the morning, plates were washed gently three times for 5 minutes each with TBS-tw, then 325 µl secondary antibody (Goat anti-Mouse Fab' conjugated to

AlexaFluor 488, Invitrogen) diluted 1:800 in TBS-tw containing 3% BSA was added to each well and allowed to incubate at room temperature for 30 minutes with gentle orbital rotation. Plates were rinsed two times for 5 minutes each with TBS-tw and 1 ml of TBS-tw was added back to each well prior to microscopic inspection. Plates were inspected for the presence of fluorescent foci of infection using the FITC/GFP filter on an inverted stage fluorescence microscope (Olympus CKX41, Center Valley, PA, USA). Clusters of 5 or more fluorescing cells were counted as single infectious focal events. Results were calculated according to the following equation: an average of 100 counts in a 1:10-diluted (duplicate) sample would indicate that the original BAL sample had a “titer” of 5000 since  $[100 \text{ counts} \times \text{dilution of } 10 \times 1000 \mu\text{l/ml}]/200 \mu\text{l assessed} = 5000 \text{ infectious foci/ml}$ .

*Reverse transcription polymerase chain reaction (RT-qPCR) measurement of levels of: RSV and immune and inflammatory gene mRNA.*

Tissues from the right and left cranial, right and left middle, and right and left caudal lung lobes (0.3 - 0.4 g of each) were homogenized in TRIzol (Invitrogen, Carlsbad, CA, USA), pooled in equal portions to create a composite slurry for each animal, then adjusted to 0.091 g/ml with additional TRIzol. RNA isolation continued as per manufacturer’s instructions (Invitrogen), followed by DNase treatment (Ambion, TURBO DNase, Austin, TX, USA) and 10-fold dilution with a combination of RNaseOUT (Invitrogen) and ultrapure water (Millipore). A NanoDrop (NanoDrop-1000, Thermo Scientific, Waltham, MA, USA) was used to assess each sample for simple RNA purity and total RNA quantity ( $A_{260\text{nm}}/A_{280\text{nm}}$  all >1.95). Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) analysis of total RNA prior to DNase treatment and dilution routinely showed RIN values of 8.0 or higher (Figure E1). RT-qPCR was carried out using One-Step Fast qRT-PCR Kit master mix (Quanta, BioScience, Gaithersburg, MD, USA) in a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA) employing PREXCEL-Q for all set up calculations (E1-E5). The following targets were assessed: RSV M37 nucleoprotein mRNA (M37), Clara cell secretory protein (CC10), surfactant protein A (SP-A), interleukin 8 (IL-8), interleukin 10 (IL-10), macrophage inflammatory protein (MIP1 $\alpha$ ), monocyte chemotactic protein (MCP1 $\alpha$ ), tumor necrosis factor alpha (TNF $\alpha$ ), interferon beta (IFN $\beta$ ), interferon gamma (IFN $\gamma$ ), and regulated on activation normal T-cell expressed and secreted (RANTES). Thermocycling conditions were 3 minutes at 50°C, 30 seconds at 95°C followed by 45 cycles of: 3 seconds 95°C and 30 seconds at 60°C. Each 10-fold diluted total RNA sample was further diluted so that each final RT-qPCR contained 0.784 ng RNA/ $\mu\text{l}$  (determined to be optimal by PREXCEL-Q). Each sample and standard was assessed in duplicate and each target

gene quantification cycle (Cq) value converted to a relative quantity (rQ) based on the corresponding standard curve using the following equation:  $rQ = 10^{((Cq-b)/m)}$ , where b and m are the y-intercept and slope, respectively, from the Stock I-derived standard curve for each target (E1, E2). The efficiency-corrected delta Cq ( $E_{AMP}^{\Delta Cq}$ ) method was employed for RT-qPCR quantification analysis. Results were normalized to total lung RNA loaded per RT-qPCR; which was identical for all reactions. No-RT control qPCR reactions (NRC) consistently gave either no signal, or generated Cq values >13 cycles away from the RT-qPCR target reactions. Inhibition was absent from all reactions as determined by the PREXCEL-Q method.

#### *Immunohistochemistry:*

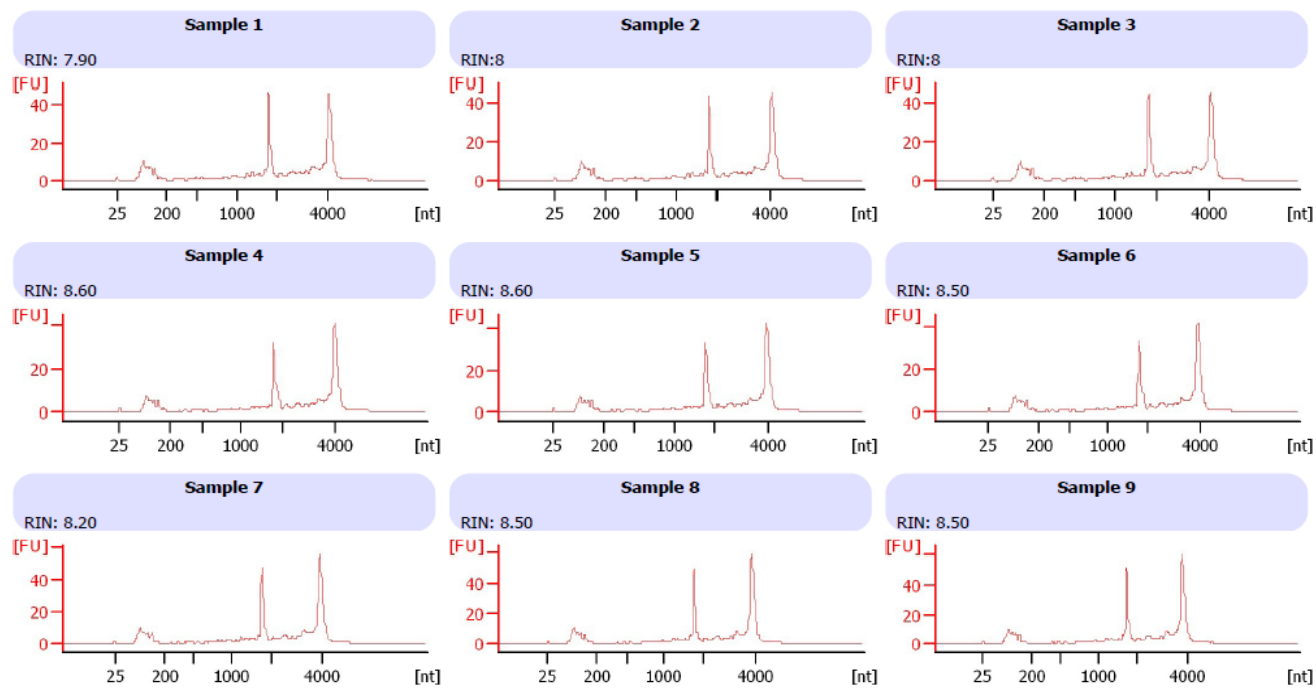
Immunohistochemistry for detection, localization, and quantification of RSV antigen was performed on paraffin-embedded tissue as described previously (E5, E6), with the following variations: instead of Pronase E antigen retrieval, heated buffer antigen retrieval was performed in TE-0.05% Tween 20, pH 9.0 in a pressure cooking device (Decloaking Chamber™ Plus, Biocare Medical, Concord, CA) using the factory default program which lasted about 40 minutes, during which time the peak temperature of 125°C was reached in about 18 minutes, and after which the system cooled down to 80°C in another 22 minutes. Blocking for 15 minutes with 3% bovine serum albumin (BSA) (Fisher Scientific) in TBS-0.05% Tween 20, pH 7.4 (TBS-tw) was followed by blocking with 20% normal swine serum (NSS) (Gibco/Invitrogen) in TBS-tw for 15 minutes. Primary polyclonal goat anti-RSV (all antigens) antibody (EMD/Millipore/Chemicon, Billerica, MA) was applied for 1.5 hours at room temperature (~22°C) at a dilution of 1:500 (~8 µg/ml) in TBS-tw containing 10% NSS and 3% BSA. After rinsing with TBS-tw, 1:300-diluted biotinylated rabbit anti-goat secondary antibody (Kirkegaard-Perry Labs, Gaithersburg, MD) in TBS-tw containing 10% NSS and 3% BSA was applied for 45 minutes, slides were rinsed, blocked with 3% peroxide in TBS-tw for 25 minutes, and streptavidin-conjugated HRP (Invitrogen) diluted 1:200 in TBS-tw was applied for 30 minutes. After rinsing the slides, color was developed using Nova Red (Vector, Burlingame, CA, USA) for about 1.5 minutes followed by water rinses, counterstaining with Harris' hematoxylin, bluing with alkaline Scott's water, dehydration and cover-slipping. Slides (containing two pieces of lung tissue each) were scored by the following method: 20 unique 10X fields on each slide were assessed for antigen staining and immunoreactive cells were counted within bronchioles and alveoli. The number of cells immunoreactive for RSV per field was then given a score according to the following scale: 0 = 0, 1 = 1-10 immunoreactive cells, 2 = 11-39 immunoreactive cells, 3 = 40-99 immunoreactive cells, 4 = >100 immunoreactive cells.

*Bronchoalveolar lavage fluid (BALF) cytology.*

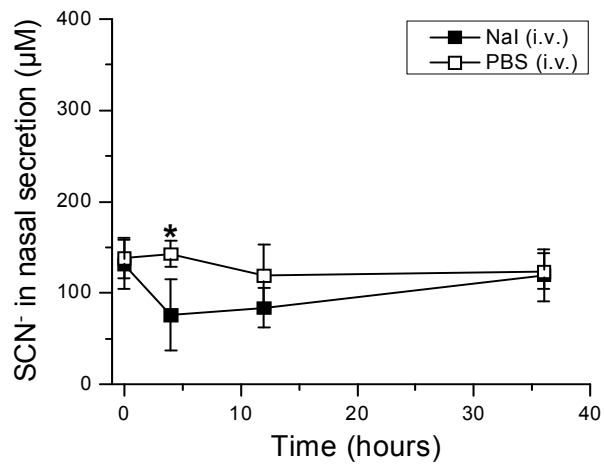
Aliquots of BALF (obtained from each lamb by pipetting 1 ml sterile PBS, pH 7.4 into the major bronchus of the accessory lobe and recovering 500  $\mu$ l of fluid) were submitted to the clinical pathology laboratory (Iowa State University, College of Veterinary Medicine) for measurement of total nucleated cell count and cytology slide preparation. Cell counts were performed on a ADVIA 120™ automated hematology analyzer (samples were diluted 2-fold in isotonic saline prior to cell counts due to low sample volumes). Cytospin preparations of BALF were created using a Shandon Cytospin 3 set at 800 rpm (spun for 10 minutes with low acceleration). Slides were stained with modified Wright's using a Hematek automated stainer. Differential cell counts (based on a 300 cell differential) were performed by a board certified veterinary clinical pathologist blinded to the identity of individual samples. In some BALF samples, too few intact nucleated cells were present on the cytospin samples to perform a 300 cell differential; therefore, these samples were excluded from the final analysis.

*Statistical Analysis.*

All analyses were performed using GraphPad Prism 6 (GraphPad Software Inc, La Jolla, CA). All post-mortem data was assessed by one-way ANOVA followed by Tukey's post-test. All clinical data were assessed by two-way ANOVA, and cumulative weight change was additionally assessed by one-way ANOVA followed by Tukey's post-test.



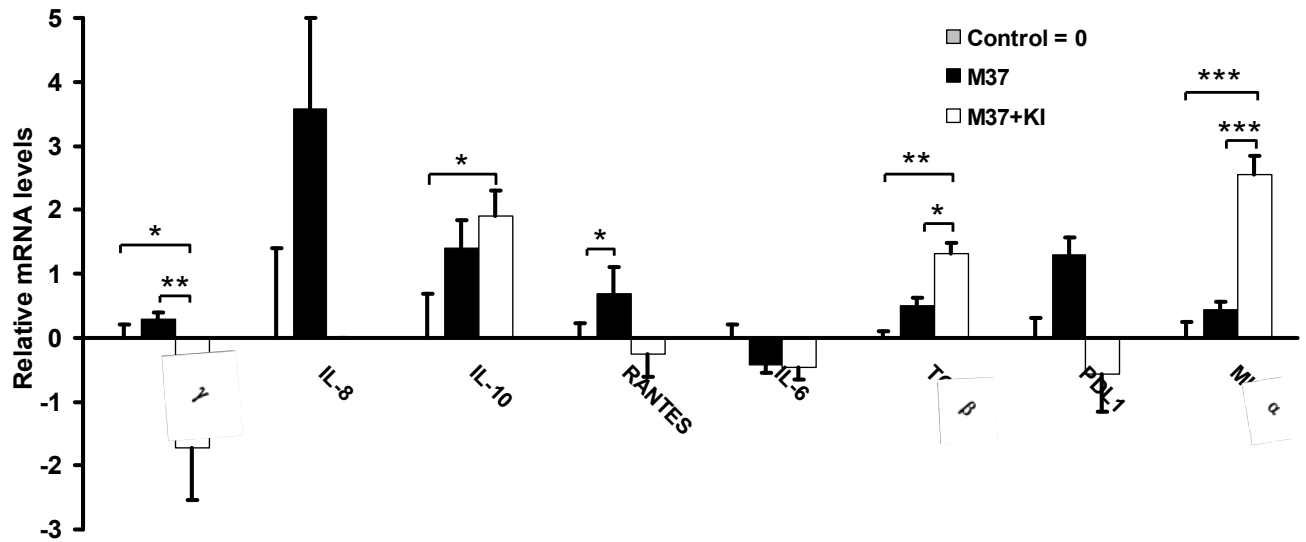
**Figure E1.** Typical RIN values obtained for RNA used for RT-qPCR in this study. Bioanalyzer 2100 assessments for RNA integrity number (RIN) demonstrating high quality TRIZol-isolated total lamb lung RNA samples prior to use in RT-qPCR.



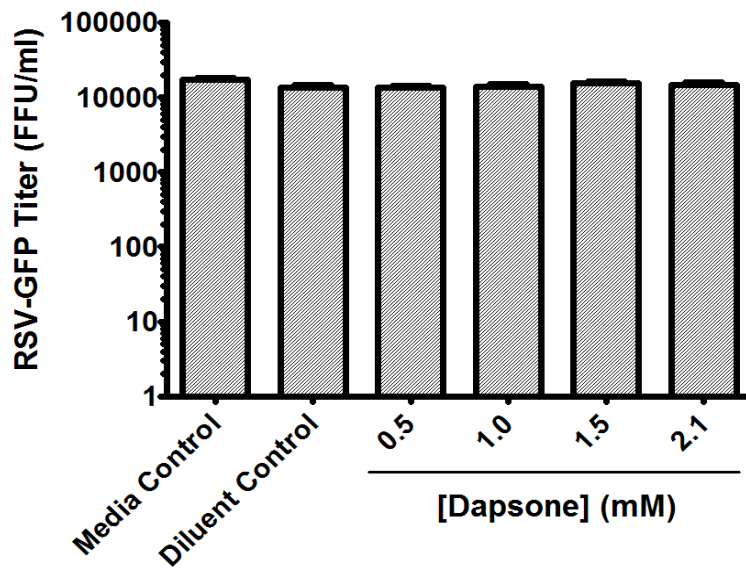
**Figure E2.** SCN<sup>-</sup> concentration in the nasal secretions of lambs declines temporarily following the i.v. injection of Nal (filled squares, n = 5). In contrast, i.v. injection of PBS does not affect the SCN<sup>-</sup> concentration in the nasal secretions (open squares, n = 5). \*p<0.05, unpaired *t* test.

**Figure E3.** Effect of KI on mRNA levels of immune and inflammatory genes six days following initial virus inoculation in 2 to 3-day-old lambs. Messenger RNA levels of interleukin-8 (IL-8) were significantly increased in lambs inoculated with RSV M37 compared to KI-treated lambs; whereas mRNA levels of IFN $\gamma$  were increased significantly in KI-treated lambs compared to lambs receiving RSV M37 without KI treatment. Control lambs lacked increases/alterations in innate and adaptive immune genes. Messenger RNA levels in KI-treated lambs for IFN $\beta$ , MCP-1 $\alpha$ , MIP1 $\alpha$ , RANTES, SBD-1, SP-A, TGF $\beta$ , and PD-L1 trended higher compared to lambs inoculated with RSV M37 but lacking KI. In contrast, levels of CC10, IL-6, IL-10, IP10, MCP2, MIP1 $\beta$ , SP-D, and TNF $\alpha$  mRNA trended higher in lambs receiving RSV M37 compared to RSV-inoculated lambs treated with KI. Control group n = 10; M37 group n = 16; M37+KI group n = 14. Error bars = SEM, \*p<0.05.





**Figure E4.** Effect of KI on mRNA levels of immune and inflammatory genes six days following initial virus inoculation in 3-week-old lambs. Messenger RNA levels of IFN $\gamma$ , IL-8 and RANTES were reduced (IFN $\gamma$  significantly) by KI treatment; whereas IL-10, TGF $\beta$ , and MIP1 $\alpha$  were significantly increased. Control group n = 4; M37 group n = 5; M37+KI group n = 5. Error bars = SEM, \*\*\*p<0.001, \*\*p<0.01, \*p<0.05.



**Figure E5.** Effects of Dapsone or diluent on RSV-A2 infectivity in vitro. Neither Dapsone nor the diluent (1% w/v Ethanol, 0.1% DMSO, in 0.9% sterile saline) affected RSV infectivity of HEp-2 cells (no significant differences). For the media control RSV-A2 was diluted in serum free MEM. The experiment was replicated 4 times. Values shown represent mean  $\pm$  SE.

## References

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