Depletion of pulmonary intravascular macrophages inhibits acute lung inflammation

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Singh, Baljit, Jacqueline W. Pearce, Lakshman N. Gamage, Kyathanahalli Janardhan, and Sarah Caldwell. Depletion of pulmonary intravascular macrophages inhibits acute lung inflammation. Am J Physiol Lung Cell Mol Physiol 286: L363–L372, 2004. First published October 17, 2003; 10.1152/ajplung.00003.2003.—Pulmonary intravascular macrophages (PIMs) are present in ruminants and horses. These species are highly sensitive to acute lung inflammation compared with non-PIM-containing species such as rats and humans. There is evidence that rats and humans may also recruit PIMs under certain conditions. We investigated precise contributions of PIMs to acute lung inflammation in a calf model. First, PIMs were recognized with a combination of in vivo phagocytic tracer Monastral blue and postembedding immunohistology with anti-CD68 monoclonal antibody. Second, gadolinium chloride depleted PIMs within 48 h of treatment (P < 0.05). Finally, PIMs contain TNF-α, and their depletion reduces cells positive for IL-8 (P < 0.05) and TNF-α (P < 0.05) and histopathological signs of acute lung inflammation in calves infected with Mannheimia hemolytica. The majority of IL-8-positive inflammatory cells in lung septa of infected calves were platelets. Platelets from normal cattle contained preformed IL-8 that was released upon in vitro exposure to thrombin (P < 0.05). These novel data show that PIMs, as the source of TNF-α, promote recruitment of inflammatory cells including IL-8-containing platelets to stimulate acute inflammation and pathology in lungs. These data may also be relevant to humans due to our ability to recruit PIMs.

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ACUTE LUNG INFLAMMATION (ALI) accompanies most of the lung diseases that cause significant mortality and morbidity in humans and animals (8). ALI is characterized by the expression of cytokines, adhesion molecules, and recruitment of inflammatory cells. The initial phase of ALI is dominated by neutrophils. Neutrophils promote inflammation by producing molecules such as TNF-α, IL-1β, and oxygen radicals (45). The impact of microvascular recruitment of neutrophils in ALI is much better characterized than the impact of macrophages such as pulmonary intravascular macrophages (PIMs).

PIMs are normally present in domestic animal species such as cattle, horses, and sheep and are recruited in rats that are normally devoid of them (40). PIMs are avidly phagocytic for blood-borne tracer particles and endotoxins and increase the load of inflammatory substances in the pulmonary microvascular bed (2, 15, 35, 38, 47). Physiological data show correlation between recruitment of PIMs in neonatal lambs and increased pulmonary vascular resistance (39). In vivo depletion of PIMs in adult sheep with dichloromethylene-diphosphonate inhibits acute increases in pulmonary arterial pressures in response to endotoxins (39, 41). In vitro data with PIMs isolated from sheep and calves showed secretion of TNF-α, IL-1, and arachidonic acid metabolites (11); however, the identity of these isolated cells has been critically questioned (33). Rogers et al. (33) showed that PIMs are difficult to isolate, even with drastic application of enzymes, due to their intimate attachment to capillary endothelium. The failure to isolate PIMs has restricted investigators to only in vivo studies. In vivo studies have inherent difficulty in attributing a specific contribution to a particular cell since a given function can be the result of contributions by many different cells. Therefore, because of these technical difficulties, very little is known about the specific mechanisms for proposed proinflammatory functions of resident PIMs (14).

We tested a hypothesis that PIMs are proinflammatory and that, therefore, PIM depletion will inhibit ALI. We tested this hypothesis by comparing ALI induced by intratracheal administration of Mannheimia hemolytica in normal and PIM-depleted calves. PIMs were depleted with gadolinium chloride (GC), which is a lanthanide. GC induces apoptosis in alveolar macrophages in vitro, and a single intravenous injection (7 mg/kg) significantly depletes PIMs in sheep in vivo (30, 36). This paper contains in vivo and in vitro data to develop a mechanistic framework for the contributions of resident PIMs in ALI. The data show that PIMs contain TNF-α and that their depletion reduces numbers of cells positive for TNF-α and IL-8 in lung septa and histopathology.

MATERIALS AND METHODS

Animals

The experimental protocols were approved by University of Saskatchewan Committee on Animal Care Assurance. Four- to six-week-old (n = 22), male Holstein-Friesian calves were obtained from Saskatoon Auction Mart and physically examined at the auction site. Calves were housed in individual pens, and their temperature and respiration rates were recorded daily for the duration of the experiment. Blood samples were taken for hematology before and during the experiment.

Bacteria Preparation

M. hemolytica-A1 colonies (Dr. Andy Potter, Veterinary Infectious Disease Organization, University of Saskatchewan) were grown in...
brain-heart-infusion broth at 37°C and 7% CO₂ to McFarland’s standard 0.5 (Remel, Lenexa, KS). Serial dilutions, 10⁻¹⁻¹⁰, were prepared in 0.85% normal saline followed by spectrophotometer readings. One hundred microliters of each dilution were inoculated on brain-heart-infusion agar in duplicates and incubated overnight at 37°C with 7% CO₂. Next morning, the colonies were counted, and the experiments were repeated three times to determine the bacterial concentration at McFarland standard 0.5. The concentration of the bacteria in the suspension was adjusted to 2 × 10⁹/ml.

**Experiments**

**Experiment 1.** GC (Sigma-Aldrich) was dissolved in sterile normal saline at a concentration of 100 mg/ml. Five calves were treated with GC at a dose of 10 mg/kg intravenously and euthanized at 48 h. Four other calves given GC were euthanized at 72 and 96 h posttreatment (n = 2 each). Five calves, injected with normal saline, were the controls. All the calves were administered Monastral blue (MB, 0.2 ml/kg intravenous; Sigma-Aldrich) 20 min before euthanasia to label the PIMs (4, 38).

**Experiment 2.** This experiment was designed to compare acute lung inflammation induced by *M. hemolytica* in the presence or absence of PIMs. Eight calves were randomly divided into two groups (n = 4 each). One group was treated with GC as in experiment 1, whereas the second was given normal saline. These calves were anesthetized with xylazine (0.1 mg/kg intramuscular, Bayer) at 36 h after the GC or saline treatments. Ventral surface of the neck was clipped and cleaned with antisepsic solutions. Calves were put in right recumbent position, and a 14-gauge needle was inserted between tracheal rings and directed ventrally. An EZ Cath IV catheter (Desert Pharmaceuticals, Sandy, UT) was run through it past the tracheal bifurcation. Ten milliliters of suspension containing 2 × 10⁹ *M. hemolytica*-A/ml of the inoculum (total dose, 20 × 10⁹ *M. hemolytica*) were deposited through the catheter into the lungs of all the calves. This dosage is higher than the one used in previous studies and induces acute lung inflammation (50).

The calves were monitored every 8 h and did not develop any overt signs of respiratory distress before euthanasia at 24 h after the bacterial challenge, which was 60 h post-GC treatment. This experiment design allowed the inflammation to progress through the window of maximum PIM depletion, which is 48 h post-GC treatment as observed in experiment 1. However, the experiment was terminated before PIM numbers increased, which occurs 72 h post-GC treatment, as was found in calves in experiment 1 and previously in sheep (36).

**Experiment 3: isolation of platelets and thrombin treatment.** Blood was collected from healthy cattle and combined with 0.13 M trisodium citrate. Platelets (1.8 × 10⁹/µl) were suspended in 1 ml of PBS and incubated for 8 min with thrombin (1 unit/µl) at room temperature.

**Tissue Processing**

Lung vasculature was not flushed before systematic sampling of tissues from three different areas. The tissues for histology and immunohistology were fixed in 4% paraformaldehyde for 16 h at 4°C, dehydrated in ascending concentrations of ethanol, and cleared in xylene followed by embedding in paraffin. Sections (5–7 µm) were placed on glass slides coated with Vectabond (Vector Labs) and incubated at 55°C for 30 min to increase adherence of sections. Tissues for immunoelectron microscopy (immuno-EM) were fixed in 6% gluteraldehyde and 2.0% paraformaldehyde for 1 h at 4°C, rinsed in sodium cacodylate buffer, and dehydrated in a graded ethanol series. These pieces were infiltrated with London Resin Co. (LR) white resin and polymerized under ultraviolet (UV) light at –8°C for 48 h. For routine EM, lung pieces were put in 2.5% gluteraldehyde and 2.0% paraformaldehyde for 4 h. These were rinsed in buffer, postfixed with osmium tetroxide, dehydrated, and embedded in Epoxy resin.

**Immunohistology of Lung Sections**

Paraffin-embedded sections were dewaxed, rehydrated, and incubated with 0.5% hydrogen peroxide in methanol to quench endogenous tissue peroxidase. Sections were incubated with pepsin (2 mg/ml 0.01 N hydrochloric acid) for 45 min for antigen retrieval. After blocking nonspecific sites with 1% BSA in PBS, we treated sections with primary [CD68 (DAKO), IL-8, and TNF-α (Santa Cruz Biotechnology)] and with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 90 and 45 min, respectively. Reaction was visualized with use of a color development kit (Vector Laboratories). Some of the slides were counterstained with methyl green (Vector Laboratories). Immunohistology controls included staining of lung sections without primary antibodies or without both primary and secondary antibodies or staining with isotype-matched goat, rabbit, and mouse immunoglobulins. Some of the lung sections were also stained with von Willebrand factor antibody to delineate lung vasculature that also served as a control.

**Staining of Lung Sections for Apoptosis**

Lung sections were stained with a terminal deoxynucleotidyl transferase-uridine nucleotide end-labeling (TUNEL) kit (Roche). Sections were stained according to the manufacturer’s instructions followed by color development with diaminobenzidine substrate.

**Numerical Counts**

Sections obtained from three different tissue blocks from each of the animals were stained. The cells were counted in a random and blind manner in the tissue areas free of large blood vessels, larger airways, and bronchioles. The numbers of intravascular cells labeled for CD68, TUNEL reaction, TNF-α, or IL-8 were counted in 10 high-power fields (×100) in each of the sections (tissue area: 0.025 mm²/field, 0.25 mm²/section, 0.75 mm²/lung). Alveolar septal cells that contained both MB and CD68 were counted to obtain PIM numbers.

**Immunohistology of Platelets**

Cytospins of the control (untreated) and thrombin-treated platelets were fixed in absolute methanol and blocked with 10% BSA in PBS for 30 min at room temperature. Slides were incubated overnight with rabbit polyclonal IL-8 antibodies (50 µg/ml) at 4°C, rinsed thrice with cold PBS for 5 min each, and exposed to HRP-conjugated goat anti-rabbit antibody (1:400) in 5% BSA for 30 min at room temperature. After three washes in cold PBS, antigen-antibody complex was visualized using a color development kit (Vector Labs). Controls were maintained by omitting the primary antibody.

**Immunoelectron of Lung Sections**

Sections were placed on nickel grids and floated on a blocking buffer (1% BSA and 0.1% Tween 20 in Tris-buffered saline, pH 7.9) for 30 min followed by incubation with primary antibody (CD68, IL-8, or TNF-α) for 1 h. Tissue sections were rinsed three times for 5 min each in Tris-buffered saline and incubated with appropriate gold-conjugated secondary antibodies for 1 h followed by staining with 2% aqueous uranyl acetate and then lead citrate. Controls included omission of primary antibody or staining with anti-von Willebrand factor antibody (DAKO).

**Immunoelectron of Platelets**

Thrombin-treated and control platelets were fixed in 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M sodium cacodylate
buffer for 1 h and suspended in 1% molten agarose. The solidified agarose that contained platelets was cut into small pieces, suspended in 0.1 M sodium cacodylate buffer, and stored at 4°C. The specimens were dehydrated in graded ethanol series and infiltrated with LR white resin (medium grade) and polymerized under UV light for 2 days. Ultra-thin sections (90 nm) prepared from the specimens were stained with anti-IL-8 antibody and gold-conjugated secondary antibody. Gold particles were counted in normal and thrombin-treated platelets (n = 25 each).

Western Blots for IL-8 in Platelet Lysates

Platelets were lysed by adding three volumes of lysis buffer (10 mM Tris-hydrochloric acid, pH 8.0, with 0.5% Triton X-100 and 100 μl of protease inhibitor cocktail; Sigma-Aldrich). Supernatants were electrophoresed on 15% SDS-PAGE under reducing conditions. The proteins were transferred electrophoretically onto a nitrocellulose membrane. The membrane was blocked overnight using 5% nonfat dried milk in Tris-buffered saline with 0.1% Tween 20 (TBST), washed, and incubated with mouse anti-bovine IL-8 antibody (1:200 in blocking buffer, Serotec) for 1 h at room temperature. Recombinant bovine IL-8 was used as a positive control. The membrane was washed with TBST and exposed to HRP-conjugated goat anti-mouse antibody (1:2,000 in TBST) for 1 h at room temperature. The reaction was visualized with a peroxidase substrate kit (Vector Labs).

Statistical Analyses

All values are presented as means ± SE. Analyses were performed using a statistical package (SPSS, release 10.05). Differences between two groups were tested by independent-samples t-test, and more than two groups were compared by parametric, one-way analysis of variance. Statistical significance was accepted at P < 0.05.

RESULTS

Clinical Symptoms

Calves showed transient dyspnea following injection of GC and MB. These symptoms have been noticed previously (36).

Hematology

No differences were detected in cell counts in blood samples within and between the groups (data not shown).

Characterization of PIMs

Lungs appeared normal on gross examination except for a bluish color in calves injected with MB. The intensity of the color appeared to be less in calves euthanized at 48 h post-GC treatment. Light microscopy revealed pulmonary septal cells that contained MB (Fig. 1A). Postembedding immunohistology showed that CD68 antibody stained pulmonary septal cells that contained MB (Fig. 1A). Lung sections stained with isotype-matched antibodies lacked any reaction (Fig. 1B). Separate counts of septal cells labeled with MB or CD68 antibody or both were made. The data showed that >90% of MB-containing pulmonary septal cells also reacted with CD68. Immunohistochemistry identified PIMs in situ and a method to deplete them was compared with the control (Fig. 2, A, B, and C; P < 0.05). EM showed classical signs of apoptosis such as nuclear fragments and membrane-bound apoptotic bodies in PIMs of GC-treated calves (Fig. 2D). Lung microvascular endothelium, alveolar epithelium, and other vascular cells appeared to be normal (Fig. 2D).

In Vivo Effects of PIM Depletion on Lung Inflammation

We tested the hypothesis that PIMs are proinflammatory. To test this hypothesis, it was essential to compare ALI in the presence and absence of PIMs. Therefore, calves were challenged with M. hemolytica at 36 h after the saline or GC treatments, and ALI was compared at 24 h postbacterial inoculations. Because GC causes maximum depletion of PIMs at 48 h posttreatment, ALI in GC-treated calves was allowed to progress for 24 h to include this time point (36–60 h post-GC treatment).

Lung sections from control calves appeared normal. However, M. hemolytica infection induced ALI, as indicated by microvascular congestion and accumulation of inflammatory cells, a feature that was markedly reduced in GC-treated infected calves (data not shown). Immunohistology detected TNF-α staining in MB-containing PIMs of infected calves, whereas sections stained with normal goat IgG did not show any reaction (Fig. 3, A and B). Immuno-EM confirmed TNF-α staining in PIMs that were adherent to capillary endothelium (Fig. 3C). We counted all the cells in lung sections labeled with TNF-α antibody. The numbers of TNF-α-positive cells declined in GC-treated M. hemolytica-infected calves compared with saline-treated calves infected with bacteria (P < 0.05, Fig. 3D); a part of this decline may be a direct result of depletion of TNF-α-containing PIMs. Staining with IL-8 antibody detected positive cells in alveolar septa of calves (Fig. 4, A and B), whereas normal rabbit IgG caused only minimal background staining (Fig. 4C). Numerous IL-8-positive cells were detected in the septa, especially in the vicinity of MB-containing PIMs, of M. hemolytica-infected control calves (Fig. 4A and inset). Immuno-EM confirmed that the majority of the cells accumulated around the PIMs were platelets, which reacted with anti-IL-8 antibody (Fig. 4D). Treatment of calves with GC before infection with M. hemolytica reduced numbers of cells positive for IL-8 (P < 0.05; Fig. 4, B and E).

In Vitro Characterization of Bovine Platelets

Platelets isolated from normal calves were positive for IL-8 (Fig. 5A). However, thrombin-treated platelets were mostly negative for IL-8 (Fig. 5B). Western blots prepared from lysates of normal platelets showed IL-8 (Fig. 5C). Immuno-EM confirmed reduced IL-8 staining in thrombin-treated platelets compared with the untreated platelets from normal calves (P < 0.05; Fig. 5, D–F).

DISCUSSION

This paper contains data on specific proinflammatory contributions of PIMs in ALI. We have characterized a phenotypic marker to identify PIMs in situ and a method to deplete them in vivo to compare lung inflammation in their absence. Traditionally, PIMs have been recognized by intravenous injections of vascular tracers and EM due to a lack of a phenotypic

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marker (3, 29). CD68 recognizes differentiated macrophages, and recently an anti-CD68 monoclonal antibody was used to identify pulmonary alveolar macrophages in calves (1). We combined in vivo tracer MB, which has been extensively used to label PIMs, with subsequent postembedding CD68 immunohistology (4, 5, 38). More than 90% of MB-positive PIMs reacted with anti-human CD68 antibody, thus validating both markers. Therefore, CD68 antibody and MB provide phenotypic and phagocytic markers, respectively, to precisely recognize PIMs in situ.

It is a technical challenge to delineate specific in vivo contributions of inflammatory cells since many cells contribute to an observed pathophysiological outcome. Because PIMs are difficult to isolate (34), we depleted them with GC to investigate ALI in their absence and to compare with hosts with intact PIMs. Many investigators have used GC to selectively remove alveolar and liver macrophages to investigate their functions (7, 32, 34, 43). Because phagocytosis of GC is a prerequisite for its efficacy, macrophages become its prime targets (19, 20, 43). PIMs are strategically placed to interact with and remove
Such preferential removal of GC by PIMs will make them a premier target for GC administered in jugular vein and spare vascular macrophages in other organs such as liver and spleen. Our data show that calves treated with GC underwent a decline in CD68/MB-positive PIMs.

In vitro studies showed that GC activates apoptosis in alveolar macrophages (30). We observed TUNEL-positive apoptotic cells in alveolar septa under light microscopy and ultrastructural signs of apoptosis such as nuclear fragments and apoptotic bodies in PIMs but not in endothelial or other vascular cells (22). Because we did not perform dual labeling on lung sections with TUNEL and CD68, which is a highly sensitive method for identification of apoptotic macrophages, we cannot term TUNEL cells in alveolar septa as apoptotic PIMs (44). Despite this weakness in the study to determine in vivo mechanisms of action of GC, our data obtained with CD68 and MB dual labeling accomplish our central objective to deplete PIMs with GC to develop a model by which to investigate ALI in their absence.

ALI is characterized by inflammatory cell recruitment that is facilitated by cytokine-induced expression of adhesion molecules on endothelium and inflammatory cells (6). Therefore, secretion of cytokines such as TNF-α following interaction...
with microbes or their products such as endotoxins is important for recruitment of inflammatory cells. It is known that PIMs rapidly remove a majority of the vascular endotoxins in sheep (15, 35, 47). Even intratracheally instilled *M. hemolytica* leukotoxin was detected in PIMs to suggest leukotoxin’s transfer across the blood-air barrier (48). Although it has been suspected that endotoxins and bacteria activate PIMs to secrete inflammatory mediators such as thromboxane-2 (12), our data are the first to directly localize TNF-α in these cells. It appears that not all the PIMs stained for TNF-α in these cells. It appears that not all the PIMs stained for TNF-α, which may partly be due to technical reasons or to the absence of TNF-α in some of the PIMs. TNF-α is a potent stimulator of expression of other cytokines and adhesion molecules and subsequent recruitment of inflammatory cells (6, 13). Therefore, PIMs’ ability to produce TNF-α in conjunction with their strategic vascular location makes them central to initiation of ALI.

Many investigators have previously suggested that PIMs promote ALI (27, 36, 46); however, due to technical difficulties, a direct mechanistic link has been elusive so far. We have attempted to overcome these difficulties with a new experimental design that includes in vivo depletion of PIMs. This design allowed the inflammation to progress for 24 h through a window of time, 36–60 h post-GC treatment, which includes a period of maximum PIM depletion at 48 h of GC treatment. The experiment was terminated before the increase in PIM numbers, which occurs at 72 h post-GC treatment, as we found in calves in experiment 1 and previously observed in sheep (36). The data from experiments reported in this paper show that PIM depletion reduced histopa-
thology and numbers of vascular inflammatory cells that contained IL-8 and TNF-α in *M. hemolytica*-infected calves. Reduction of TNF-α-positive cells in GC-treated infected calves may be due to a direct loss of PIMs. One explanation for this effect could be that PIM depletion removes a source of TNF-α that is critical for the initiation of an inflammatory cascade in lungs by promoting expression of adhesion molecules and recruitment of inflammatory cells.

![Fig. 4. IL-8 immunocytochemistry. Immunocytochemistry with anti-bovine IL-8 antibody showed positive cells in the vicinity of MB-containing PIMs in lung sections from *M. hemolytica*-infected calf (A and inset) and GC-treated *M. hemolytica*-infected calf (B and inset). C: no reaction was observed when the sections were stained with normal rabbit IgG. D: an electron micrograph shows labeling for IL-8 (arrowheads) in platelets. E: numbers of IL-8 positive cells were decreased (*P < 0.05) in *M. hemolytica*- and GC-treated calves compared with *M. hemolytica*-infected control calves. Original magnification, A and B: ×20; insets: ×100; C: ×40; D: ×37,500.](image_url)
Lungs from calves infected with *M. hemolytica* show aggregates of inflammatory cells in microvessels (28, 49). Previously, cell aggregates in lung microvessels of *M. hemolytica*-infected calves that contained IL-8 mRNA were believed to be neutrophils (9). Our data, including immuno-EM, show that platelets contained IL-8 and were the dominant cell in microvascular aggregates around PIMs in the infected animals. Rabbit platelets are known to carry IL-8 in their cytoplasmic granules that can be released following activation (42). Similarly, platelets isolated from normal cattle are enriched in IL-8, which is released upon activation with thrombin. The significance of the accumulation of platelets in lung microvessels may lie in the potency of secreted IL-8 as a chemoattractant for neutrophils and monocytes to propagate lung inflammation (18, 24). Platelets contain P-selectin and are known to promote rolling of neutrophils, which is an initial step in their recruitment (26, 31). Platelets also produce platelet-activating factor, and its inhibition reduced lung inflammation in calves infected with *M. hemolytica* (17). These data show that platelet recruitment mediated by PIMs is a critical component of ALI. Because PIM depletion attenuates microvascular accumulation of inflammatory cells and associated lung pathology, it may be a rational approach to inhibit ALI in host species.

Because PIMs are proinflammatory cells, the relative resistance of humans and rats to ALI may be related to the absence of these cells; conversely, induction of PIMs may predispose them to ALI. One ultrastructural report showed PIMs in biopsies taken from human patients undergoing thoracotomies for excision in noninfectious lung diseases (16). There is some indirect evidence to support accumulation of mononuclear phagocytes in lung vasculature of patients with liver disease (21, 23, 25). These patients were undergoing scans with radioisotopes and showed increased localization of radioactivity in their lungs compared with normal humans (21). Lung samples

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**Fig. 5.** IL-8 immunocytochemistry in isolated calf platelets. IL-8 staining of cytopsins shows reaction in untreated (A) but not in thrombin-treated (B) platelets. C: Western blots with IL-8 antibody show IL-8 in platelet lysates. Immunogold electron microscopy showed IL-8 staining (arrowheads) in normal (D) and thrombin-treated (E) platelets with a decrease in numbers of gold particles (*P < 0.05*) in the latter (F). Original magnification, A and B: ×100; D and E: ×37,500.
from patients who died from sepsis-related lung complications contained large numbers of cells in their alveolar septa that stained with anti-human monocyte/macrophage antibody (B. Singh, K. Janardhan, and R. Kanthan, unpublished observations). However, there is more definitive and direct data on transient recruitment of PIMs in rats infected with *Escherichia coli* or following ligation of the bile duct (10, 37). Although these rats became more susceptible to ALI and mortality, the precise functions of recruited PIMs remain largely unknown (14). Because humans with recruited PIMs, similar to calves with resident PIMs, may be more susceptible to ALI, it is critical to investigate the biology of PIMs further.

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