Chapter 2
Comparative View of Lung Vascular Endothelium of Cattle, Horses, and Water Buffalo

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Abstract Endothelium plays an important role in maintaining the vascular barrier and physiological homeostasis. Endothelium also is fundamental to the initiation and regulation of inflammation. Endothelium demonstrates phenotypic and functional heterogeneity not only among various organs but also within an organ. One of the striking examples would be the pulmonary endothelium that participates in creating blood–air barrier. Endothelium in large pulmonary blood vessels is distinct in structure and function from that lining of the pulmonary capillaries. This chapter focuses on the comparative aspects of pulmonary endothelium and highlight unique differences such as the presence of pulmonary intravascular macrophages among select species.

2.1 Introduction

The pulmonary endothelium is a dynamic and metabolically active layer of squamous endothelial cells that is ideally placed to mediate lung homoeostasis (Millar et al. 2016). The pulmonary capillary is made up of a few (usually 2–3) thin, squamous endothelial cells (Dornan and Meban 1985; King et al. 2004), and capillary endothelial cells constitute 30–50% and 46–50% of the total cell population in the alveolar septal wall

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in human (Crapo et al. 1982) and nonhuman primate (Crapo et al. 1983), respectively. Horse lung showed an average of 252.4 capillaries per centimeter of lung alveolar wall with endothelial cells constituting 49.5% of total tissue cells in the alveolar septal wall (Gillespie and Tyler 1967). Endothelium is the gatekeeper of the tissues and regulates traffic of circulating cells and molecules into the tissues (Cotran 1989).

Endothelial cells of the lung can be easily split into two subgroups, one comprising the lining of larger blood vessels such as arteries and veins and another population that constitutes capillary endothelial cells of the microvasculature, each with distinct functions. Both subsets of endothelial cells contain rough endoplasmic reticulum, numerous vesicles such as caveolae, a low number of mitochondria, and filaments around the basal cell surface amongst other common cellular features (King et al. 2004). Filaments are more common in arterial cells which also exhibit projections from the apical surface, particularly near cellular junctions (King et al. 2004). As there is transition from arterial to microvascular endothelial cells, there is a flattening and elongation of the cells, with a greater restriction of cytoplasm near the flattened periphery. Many of the organelles are excluded from the flattened parts and concentrated around the nucleus. While apical projections were present, they were not as pronounced in this population (King et al. 2004; Ochoa et al. 2010).

A key intracellular feature of endothelial cells are dark bodies in the cytoplasm referred to as Weibel–Palade bodies (WPb) which contain a variety of secretable products such as von Willebrand Factor (vWF), interleukin (IL)-8, factor XIIIa, and P-selectin. While these bodies are present in arterial endothelial cells they are not present in the capillary endothelial cells, though these factors are still secreted by these cells as well (Fuchs and Weibel 1966; Lowenstein et al. 2005). Upon injury or another activating stimulus, these bodies fuse with the cellular membrane and degranulate, releasing their contents into circulation and on the surface of the endothelial cells. vWF, a substrate of XIIIa, plays a role in adhesion of platelets to the endothelium and also serves as a carrier for coagulation factor VIII (functions reviewed in Wang and Eikenboom 2010). IL-8 on the other hand is a strong neutrophil chemoattractant and activator, which is likely to cause damage to the endothelium (discussed later; Fig. 2.1). vWF can bind to platelets and initiate clot formation (Ochoa et al. 2010). While this is one of the most obvious and recognizable difference between both endothelial cell populations, a host of other differences exist.

Another function of the endothelium is the active regulation of vascular permeability and relaxation of arterial vessels. Relaxation is controlled by nitric oxide (NO) release from arterial endothelial cells (Furchgott and Vanhoutte 1989; Furchgott and Zawadzki 1980) which in turn prompts smooth muscle to release guanylate cyclase, increasing cGMP and vasodilation (Furchgott and Vanhoutte 1989). This mechanism is observed during exercise (Pelletier and Leith 1993), even at rest (Rees et al. 1989), and can be prompted by shear force of circulating blood (Kaiser and Sparks 1986; Koller et al. 1994). In hypoxia, removal of endothelial cells from vessels significantly reduces arterial NO production and the resulting vasoconstriction (around 65–70%), indicating a role for these cells in this phenomenon as well (MacEachern et al. 2004). However, such removal in human and sheep completely abrogated this response (Demiryurek et al. 1991, 1993), suggesting that this function is somewhat less

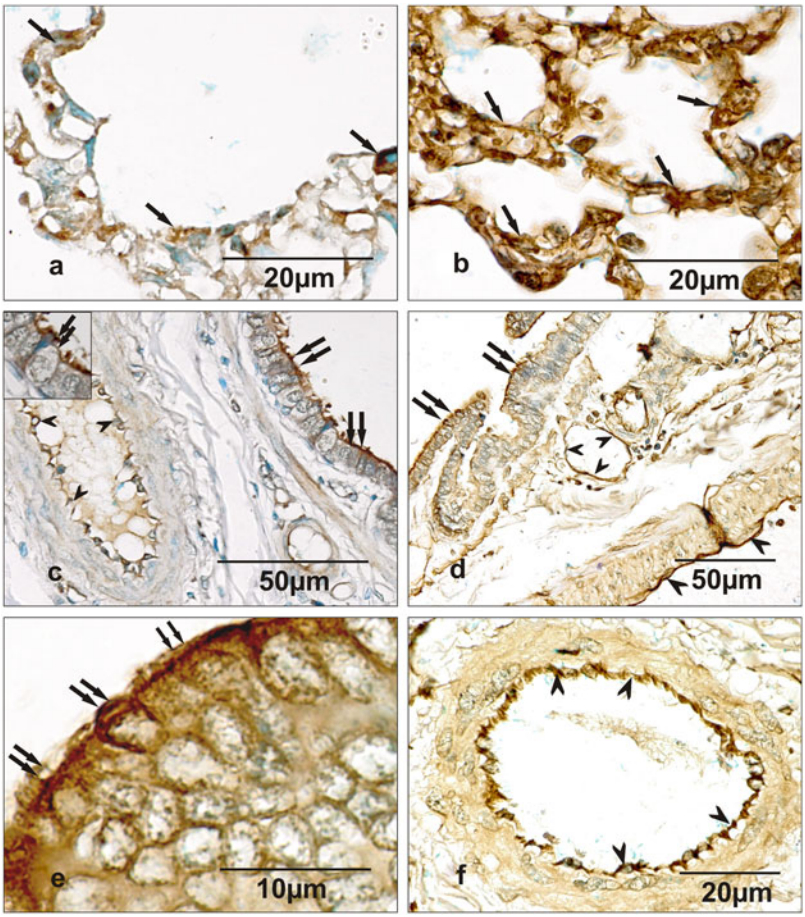

Fig. 2.1 (a) IL-8 expression (arrows) is seen in the alveolar septa of normal and (b) inflamed lungs. (c and e) The airway epithelium (double arrows) and vascular endothelium (arrowheads) of the normal lungs reacts weakly for IL-8 compared with (d) the intense expression in the airway epithelium (double arrows) and (f) the vascular endothelium (arrowheads) of the inflamed lungs. IHC. Reprinted with permission from J Comp Pathol. 2011 Feb–Apr;144(2–3):135–44. doi: https://doi.org/10.1016/j.jcpa.2010.08.003

important in horses and cattle than in other species (MacEachern et al. 2004). The role of NO in this phenomenon may be more complex though as in exercise models of lung injury, inhibition of NO production in horses increased damage seen in the lungs, suggesting mechanisms other than just vessel relaxation (pressure) likely play a role in endothelial damage (Kindig et al. 2000).

2.2 Circulation Constraints in the Lung

Maintenance of vascular integrity is an important function of endothelial cells in the lung. They can however often fail during exertion (Pascoe et al. 1981). Several studies have focused on exercise induced pulmonary hemorrhage in the lungs of race horses, where blood pressure can become very high (Pascoe et al. 1981; West et al. 1993). Arterial pressures can reach up to 120 mmHg in exercising horses (West et al. 1993), and capillary threshold pressure can reach between 75 and 100 mmHg in thoroughbreds (Birks et al. 1997). By comparison, the capillary threshold in rabbits is below 40 mmHg, and 70 mmHg in dogs (Fu et al. 1992; Tsukimoto et al. 1991). While we do not know of any study that examines molecular structural differences between rabbit and horse lung that can account for these differences, it seems likely that such differences must exist to allow for such increased pressures in the capillaries. A study estimating canine and equine capillary strength based on morphology, while accurately predicting increased strength over rabbits, also underestimated actual strength in equines (Birks et al. 1994), suggesting such molecular differences should be present.

Response to pressure in endothelial cells is carried out by the calcium channel TRPV4 (Jian et al. 2008; Yin et al. 2008). Increased pressure elevates cellular calcium, but blockage of TRPV can abrogate most of this change (Jian et al. 2008; Yin et al. 2008). Activation of the channel can also increase lung endothelial permeability (Alvarez et al. 2006). Increase in pressure is also shown to induce exocytosis of endothelial vesicles such as WPb (Kuebler et al. 1999). As these bodies include proteins such as vWF, P-selectin, and IL-8, pressure and calcium control could also be considered mechanisms of inflammation in these cells. Even eNOS production in these cells is controlled partially by calcium and can act as a negative feedback on TRPV4 induced calcium influx (Yin et al. 2008).

Finally, endothelial cells are constrained by the competing requirements of gas diffusion and blood pressure. Thus, it is no surprise that their capillary cell morphology takes on a more flattened structure compared to arterial endothelial cells to improve gas exchange. Increased ability to handle pressure will come at a cost of increased thickness, which will also trade off gas exchange efficiency. Thoroughbred horses in particular fall into this conundrum. While bred for work and speed, cardiac output has been increased, but the lungs often cannot handle this output pressure, such that alveolar bleeding is a common occurrence (West 2000). In the end, selection pressure may remain on improving endothelial cell thinness to maintain gas diffusion efficiency, and increase of extracelluar matrix toughness to improve strength of the alveoli (West 2000). While improvement of endothelial cell junctions would be expected, disruptions when they happen are observed frequently in the cells and not at junctions (Costello et al. 1992), and often seal quickly upon relief of pressure (Elliott et al. 1992).

Capillary endothelium is surrounded by mainly alveolar type I epithelial cells and occasional type II cell (Townsley 2012). Further, capillary endothelium is connected to other cellular constituents of the alveolar septal wall via fibroblasts

by intercellular junctions (Sirianni et al. 2003) and pericytes by myoendothelial junctions (Michel et al. 1995). Most of these cell junctions are not random but strategically placed at the interface between the thick and the thin sides of the septal wall to provide structural stability and minimize the distensibility, especially during the increase in the mass of surrounding tissue (Walker et al. 1995).

Capillary endothelium lacks large gap junctions unlike endothelial junctions in extra-alveolar arteries and veins, but contains occasional small gaps in capillary inter-endothelial cell junctional complexes (Schneeberger 1982). Further, a third adjoining endothelial cell forms a flap overlaying the borders of these cells and this flap has specific organization of junctional strands (Walker et al. 1994). These strands run parallel instead of perpendicular to the plane of the overlying cell to provide some protection against transendothelial fluid movement during increased intravascular pressure as the increased hydrostatic force results in "sealing" of this junctional complex (Walker et al. 1994).

Endothelial cells are capable of allowing diffusion of a number of molecules across the cellular barrier, but studies in several species have shown a general resistance to diffusion, particularly in lung capillary endothelial cells. Capillary endothelial cells of the lung show lower permeability to water and solutes compared to vascular endothelial cells (Kelly et al. 1998; Parker and Yoshikawa 2002; Parker et al. 2006). Studies of hydraulic conductance also show values much lower for microvascular endothelial cells suggesting tighter junctions and lower diffusion (Kelly et al. 1998; Parker et al. 2006; Ofori-Acquah et al. 2008). This makes sense when considering the need to maximize diffusion of oxygen across the cell membrane and minimize solute and fluid transport, the last of which could cause tissue or alveolar edema problems if not closely controlled. Both arterial and microvascular endothelial cells have about the same amount of intracellular actin. However, the chemical disruption of actin wtih cytochalasin D is much harder in microvascular endothelial cells (Ofori-Acquah et al. 2008).

A reason for this more restrictive permeability is alterations in localized cAMP expression in endothelial cells. High levels of cAMP are generated at the membrane of microvascular endothelial cells by adenylate cyclase 6 which enhances barrier function (Ludwig and Seuwen 2002). This adenylate cyclase is calcium regulated and thus permeability can be increased by increased intracellular (cytosolic) calcium (Cioffi et al. 2002; Stevens et al. 1995; Sayner and Stevens 2006; Sayner et al. 2006), though a specific cAMP effector of barrier strength is not fully resolved (Prasain and Stevens 2009). One such example is endothelial response to thrombin. Thrombin is a pro-inflammatory molecule that binds to PAR receptors (PAR-1 and PAR-3) and activates endothelial cells (reviewed in Minami et al. 2004). Amongst these cellular changes is Gq activation, which leads to calcium release from the endoplasmic reticulum resulting in calcium depletion which is replenished by opening calcium channels (TRPC1 and TRPC4 subunits). This increase in free cytosolic calcium thus disrupts the endothelial cell barrier (Cioffi et al. 2009). Another ion channel, TVRP4, is selectively expressed in lung microvasculature but not the arterial endothelial cells (Alvarez et al. 2006). Activation of this channel

leads to leakage into the alveoli, but in this case leakage is due to sloughing of cells and/or loss of attachment to cell matrix (Alvarez et al. 2006).

Neuropeptides play important roles in the regulation of respiratory function and topographical inflammation and are localized in both motor and sensory neurons in the mammalian respiratory tract (Lundberg et al. 1988). Substance P (SP) and calcitonin gene-related peptide (CGRP)-immunoreactive nerve fibers are present around pulmonary blood vessels throughout the respiratory tract of calves and cows (Nishi et al. 2000). These fibers are few in number in the lung compared to nasal and laryngeal mucosae and tracheal bronchus, and are more numerous in calves than in cows (Nishi et al. 2000). Co-localization of SP and CGRP in most of the nerve fibers suggests that these nerve fibers are involved in the regulation of the bovine respiratory tract.

2.3 Inflammation and Immunity

Endothelial cells are located at the interface between blood and tissues not only to gate the traffic of molecules and cells across the vessel wall but also contribute to hemostasis, inflammatory reactions, and immunity (Mantovani et al. 1992). When not activated, endothelial cells can secrete a variety of products that can aid in inhibiting clotting and inappropriate activation of innate immune functions (reviewed in Pober and Sessa 2007). Amongst these is NO, which can inhibit platelet aggregation and adhesion in addition to being a vasorelaxant as mentioned earlier (Sessa 2004). In this state, P-selectin is sequestered within the cell (Bonfanti et al. 1989), and other leukocytes adherence molecules such as E-selectin, VCAM, and ICAM-1 are suppressed to a greater degree (Pober and Sessa 2007).

During inflammation, there is binding to cell-surface receptors by one of a number of potential molecules (e.g., histamine, thrombin, LPS, etc.). For those that bind to G-protein receptors, there is a release of cytosolic Ca^{2+} that will result in increased endothelial permeability as discussed earlier (Pober and Cotran 1990; Pober and Sessa 2007) causing release of WPb (Birch et al. 1992, 1994). Calcium also activates phospholipase A2 which catalyzes arachidonic acid formation and subsequently its conversion to COX1 resulting in increased blood flow (Egan and FitzGerald 2006). A by-product of arachidonic acid formation, lysophosphatidylcholine, at the same time acts as a platelet activating factor. When this is combined with the released P-selectin it leads to neutrophil attachment, integrin activation, and the extravasation of said neutrophils across the endothelial barrier (Prescott et al. 1984; Lorant et al. 1991; Pober and Sessa 2007).

Another activated protein, RHO, also aids in contraction of cellular actin filaments, resulting in creation of gaps between endothelial cells, especially post-capillary venules (Heltianu et al. 1982). Passage of most cells through the endothelial barrier appears to be between such endothelial cells and supported by increased expression in these intercellular gaps of PECAM1 and CD99 (Marchesi 1961; Schenkel et al. 2002). Cytokines such as TNF-α and IL-1 can also induce a similar response

(reviewed in Pober and Sessa 2007), but leading to transcriptional activation of NF-kB and AP-1, which activates a number of the same effector pathways already mentioned (Pober and Sessa 2007). Secretion of IL-8 will lead to activation, firm attachment, and diapedesis of neutrophils to the endothelial cells, but still requires E-selectin to tether such cells (Pan et al. 1998).

Specific to pulmonary microvascular capillary endothelial cells is a 1G T-type calcium channel. Activated by thrombin, the channel causes membrane depolarization, but in this case does not increase permeability but instead promotes P-selectin expression for neutrophil recruitment (Wu et al. 2003, 2009). Thus, calcium again appears to play a very important role in induction of inflammatory responses in endothelial cells, particularly with regard to P-selectin expression (Kuebler et al. 1999; Parthasarathi et al. 2006).

2.4 Toll-Like Receptors

Toll-like receptors (TLRs), the mammalian homologues of the Drosophila Toll family, are critical for recognition of conserved pathogen-associated molecular patterns in bacteria and viruses by immune cells (Aderem 2001). TLRs are expressed in the endothelium to activate the immune system when encountering specific antigen (Xu et al. 2011). TLR4 recognizes lipopolysaccharides (LPS) and plays important roles in host defense against bacterial infections (Lymboussaki et al. 1998; Takeda et al. 2003). We reported the presence of weak TLR4 immunoreactivity in the vascular endothelium in normal lung of cattle (Wassef et al. 2004) and buffalo (Sethi et al. 2011), which is consistent to what is observed in humans (Faure et al. 2000). Some TLR4 staining was reported in peribronchiolar blood vessels and alveolar septal endothelial cells in horse lungs (Singh Suri et al. 2006). TLR4 activates endothelium and induces recruitment of leukocytes in lung along with expression of adhesion molecules such as P-selectin and vascular cell adhesion molecule-1 (Andonegui et al. 2002, 2003). Interestingly, TLR4 appeared unchanged or reduced in the vascular endothelium of inflamed lungs from calves and Water buffalo infected with *Mannheimia haemolytica* and *Pasteurella multocida*, respectively (Wassef et al. 2004; Sethi et al. 2011). Lack of TLR4 in vascular endothelium during *Mannheimia haemolytica* and *Pasteurella multocida* infection may be a protective mechanism against unwanted inflammation.

Similarly, early studies with LPS treatment of horse lungs showed endothelial cells to be fairly unaffected by the exposure. This was, however, in the absence of immune cells such as neutrophils. While this study primarily looked at electron microscopic examination of endothelial cells, it does suggest that endothelial cells maintain morphology through exposure. By contrast, cell death was common in the presence of neutrophils, suggesting that when other immune cells are present endothelial cells are sensitive to products of these cells in the horse (Turek et al. 1987). Later experiments have also suggested TNF-α and IL-1b secretion by these cells to LPS in a whole animal exposure system (Parbhakar et al. 2005).

TLR9 identifies bacterial DNA and is a key player in bacterial DNA induced cell signaling (Bauer and Wagner 2002). TLR9 is not only activated by unmethylated CpG motifs within ssDNA, but also by CpG motifs in nucleic acids released during vascular apoptosis and necrosis (Krogmann et al. 2016). We observed that pulmonary vascular endothelium of water buffalo did not show any TLR9 immunopositive reactivity which is similar to expression of TLR4 (Sethi et al. 2011) (Fig. 2.2). However, unlike TLR4, TLR9 expression was increased in the vascular endothelium during *P. multocida* induced lung inflammation in water buffalo (Sethi et al. 2011). The data suggest that increased TLR9 expression may handle the DNA released either from the phagocytosed bacteria or from the dying extracellular bacteria at the later stages of infection. However, the contradictory finding of TLR9 agonism in vascular biology remains the potential area of research. In horse and cattle, expression of TLR9 was clearly shown in vascular endothelial cells (Fig. 2.3), with some possible capillary staining, though if present it was much lighter in comparison (Schneberger et al. 2009, 2011).

Recently, TLR10 mRNA upregulation in THP-1 cell line has been linked with reactive oxygen species induced during hypoxia (Kim et al. 2010). TLR10 is expressed in lungs of human although at very low levels and immune cells viz neutrophils, macrophages, and dendritic cells (Chuang and Ulevitch 2001). We recently reported immuno-histochemical and immuno-electron microscopic data indicating expression of TLR10 in the pulmonary vascular endothelium of cattle along with other veterinary species (Balachandran et al. 2015). However, TLR10 immunohistological expression was much reduced in the *M. haemolytica* infected animals compared to the controls (Balachandran et al. 2015). The data suggest implications of TLR10 during *M. haemolytica* infection in terms of entry of bacteria to establish infection in cattle lung.

2.5 Pulmonary Intravascular Macrophages

One of the most striking differences between species in the lung capillaries is the presence of pulmonary intravascular macrophages (PIMs) attached to the microvascular endothelial cells of the lung. This population of macrophages colonizes the lung shortly after birth and are maintained through life in animals such as in horses, Artiodactyla, odontoceti, and cats, while in other species they may be induced under certain circumstances (reviewed in Schneberger et al. 2012). They are attached strongly to the endothelial cell layer through a number of darkly staining structures, which may complicate creating primary cell cultures. These attachments to the endothelial cells are unknown but are thought to be a glycosyl-phosphatidyl inositol anchor (Atwal et al. 1992; Singh et al. 1995; Singh and Atwal 1997). Attachment is seen on the thicker side of the endothelial cell, possibly to reduce interference with gas exchange, (Winkler 1988) and the cells range from 20 to 80 μM in size, with horse PIMs being the largest. While similar to other macrophages, PIMs possess a unique decoration of lipid/lipoprotein vesicles at or near the

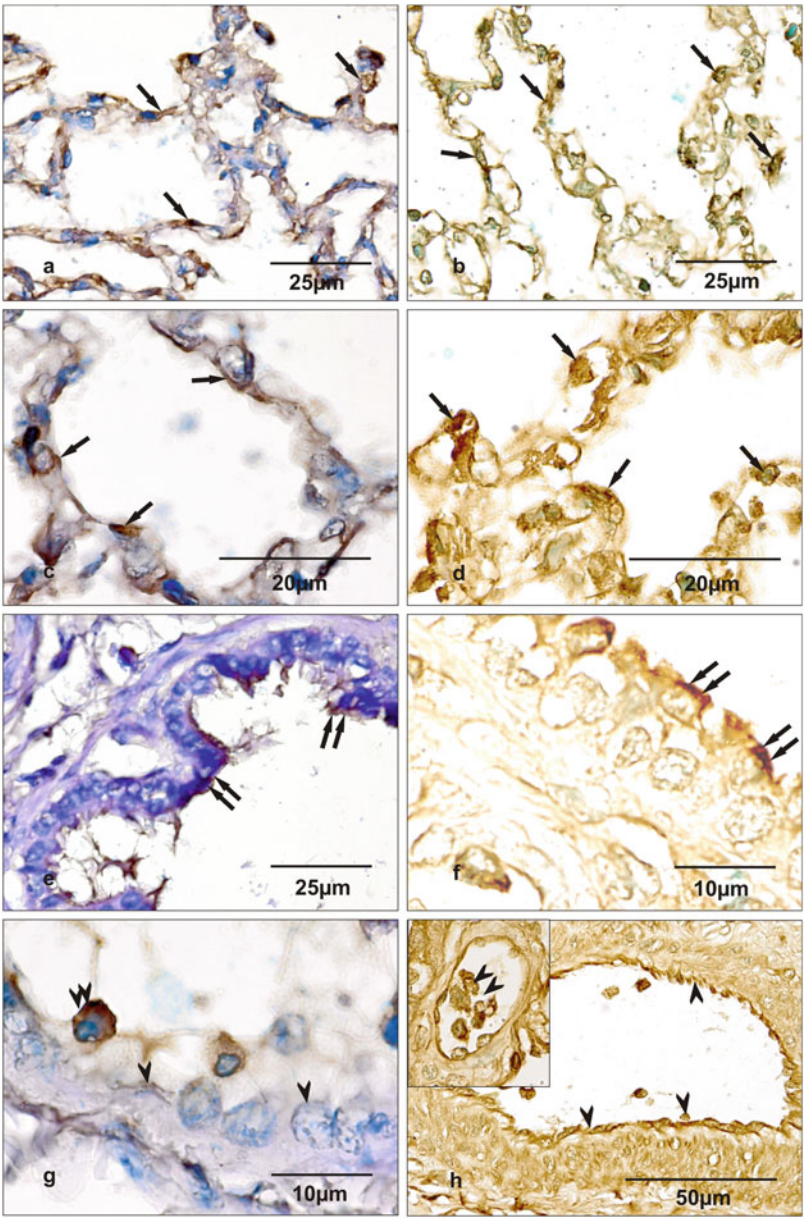

Fig. 2.2 (a) Normal lung section shows weak expression of TLR9 in alveolar septa (arrows) and (b) airway epithelium (double arrows), (c) but not in the endothelium of large blood vessels (arrowheads). (d) TLR9 expression was increased in the alveolar septa (arrows), (e) airway epithelium (double arrows) and (f) the endothelium of large blood vessels (arrowheads) in inflamed lungs. The blood cells (double arrowheads) in the control (c) and the infected lung (f) were also positive for TLR9 expression. IHC. Reprinted with permission from J Comp Pathol. 2011 Feb–Apr;144(2–3):135-44. doi: https://doi.org/10.1016/j.jcpa.2010.08.003

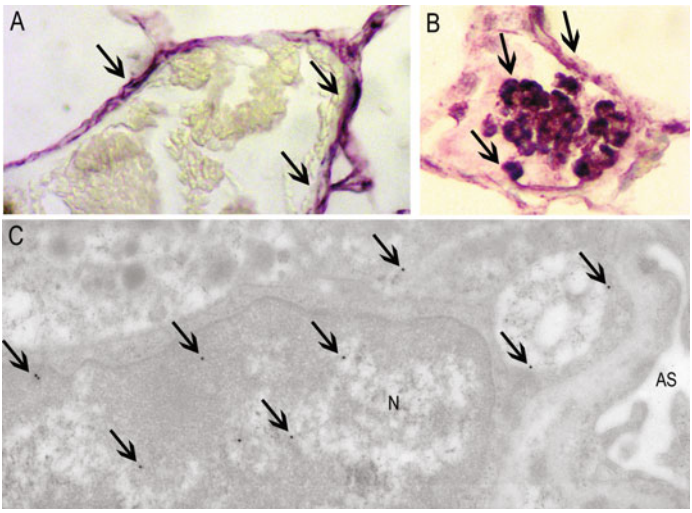

Fig. 2.3 TLR9 immunostaining of vascular endothelium in lungs of control (A) and LPS-treated horses (B) (noted by arrows). Immuno-electron microscopy (C) showed TLR9 staining in nucleus (N) of capillary endothelial cells in the lung (arrows). Original magnification A-B: ×400, C: ×10,000. Reprinted with permission from Anat Rec (Hoboken). 2009 Jul;292(7):1068–77. doi: https://doi.org/10.1002/ar.20927

cell surface membrane which are believed to help in phagocytosis (Atwal et al. 1992; Singh and Atwal 1997). Recently, PIMs were also identified in Water buffalo (Sethi et al. 2011; Fig. 2.4). Like other macrophage populations, PIMs are decorated with a number of TLR receptors (Wassef et al. 2004; Singh Suri et al. 2006; Schneberger et al. 2009, 2011; Sethi et al. 2011) (Fig. 2.5) and play a role in lung inflammation (Singh and de la Concha-Bermejillo 1998; Singh et al. 2004; Parbhakar et al. 2005), and phagocytosis (Atwal and Saldanha 1985). In disease, abrogation of PIMs can greatly reduce symptoms in recurrent airway obstruction in horses (Aharonson-Raz et al. 2012). Recently, we have reported the recruitment of PIMs in dogs that died due to acute necrotizing pancreatitis (Vrolyk et al. 2017). These data raise the intriguing possibility of PIM recruitment in domestic animal species that may not have constitutive PIMs and that the recruited PIMs may predispose these to higher susceptibility for lung inflammation and disease.

2.6 Culture of Endothelial Cells

Isolation and culture of equine endothelial cells has been described in detail (Lamar et al. 1986; MacEachern et al. 1997). Arterial endothelial cells are obtained by surgical removal of vessels and flushing them with PBS supplemented with antibiotics to remove any residual blood and potential bacterial contamination. Warmed DMEM with collagenase added is instilled in vessels which are clamped shut and

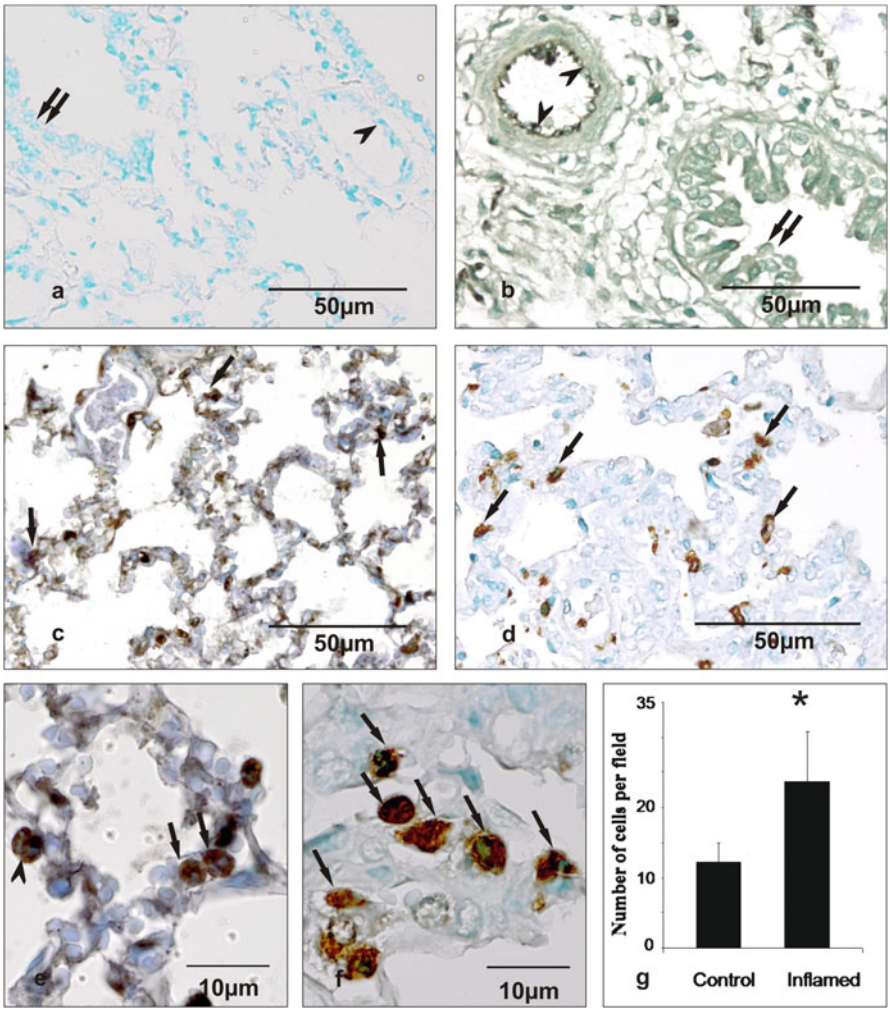

Fig. 2.4 (a) Lung section stained without primary antibody does not show any colour development in airways (double arrows) or bloodvessels (arrowheads). (b) Antibody specific for Factor VIII-related antigen labels vascular endothelium (arrowheads), but not the airways (double arrows). Macrophage antibody reacts with septal cells (arrows) in the normal lungs (c) and the infected lungs (d). (e) High magnification shows intravascular location of labelled macrophages (arrows) in the normal and (f) the infected lungs. An alveolar macrophage (arrowhead) is seen in (e). (g) The number of septal macrophages was significantly increased (P ¼ 0.001) in the inflamed lungs compared with the normal lungs. Reprinted with permission from J Comp Pathol. 2011 Feb–Apr;144(2–3):135–44. doi: https://doi.org/10.1016/j.jcpa.2010.08.003

incubated for 30–40 min at 37 °C. After incubation, vessels are rinsed emptied, media saved, and further washed with PBS and antibiotics. This wash is added to growth media and centrifuged to remove fluid, replacing it with final culture media of DMEM plus fetal calf serum and antibiotics. This is then cultured on 1% gelatin

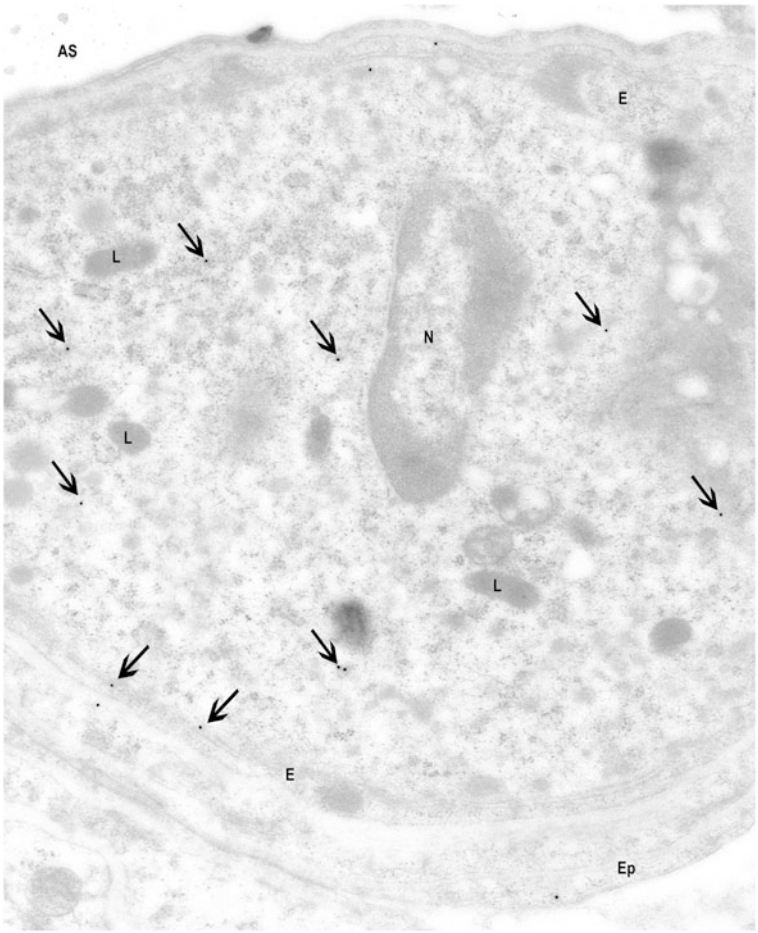

Fig. 2.5 TLR9 staining observed in a PIM and endothelium (E: arrows). L: Lysosomes; Ep: Epithelium; AS: Alveolar Space. Original magnification ×10,000. Anat Rec (Hoboken). 2009 Jul;292(7):1068–77. doi: https://doi.org/10.1002/ar.20927

coated plates, washing cells with PBS and replacing with fresh media until cells reach required density (MacEachern et al. 1997 for more details; Lamar et al. 1986).

This method yields primarily epithelial cells, but some smooth muscle cells will also be harvested in the process. Use of plasma-derived fetal bovine serum in media (10–20%) was able to inhibit growth of these smooth muscle cells, however, as opposed to equine-derived serum which stimulated this population as well as endothelial epithelium. It is believed that a lack of platelet-derived growth factors caused this inhibition of smooth muscle cells (Lamar et al. 1986). Cell type is verified by staining for vWF (Lamar et al. 1986).

Isolation procedures for lung microvascular endothelial cells will follow harvest and isolation techniques seen with capillaries in other tissues. Tissue is aseptically

obtained and minced or otherwise mechanically disrupted in Hank's balanced salt solution, often with addition of antibiotics similar to arterial methods to ensure sterility. This suspension is then treated with collagenase to digest connective tissue and heated for 30 min at 37 °C before passing through a nylon mesh. Filtrate is combined with equal an equal volume of DMEM with 10% fetal bovine serum. After 10 min, cells are centrifuged at low speed, resuspended in DMEM+FBS, and passed through a 30 μM filter. The filter is washed for clumps of endothelial cells which are saved, while flow through is discarded (containing erythrocytes and stromal cells). Harvested cells are washed, re-centrifuged, re-suspended in HBSS and 10 ml gently layered onto 35 ml HBSS + 5% BSA in centrifuged and left stand at room temperature for 15 min. The bottom 20–25 ml containing enriched endothelial cells is saved while the upper layer containing stromal cells is discarded. Cells are centrifuged, resuspended in DMEM + 10% FBS plus antibiotics and endothelial cell growth supplement and plated into dishes coated with fibronectin (Bochsler et al. 1989).

While some microvascular cultures methods will suggest vWF as a method of detection of these cells, expression in microvascular endothelial cells is less robust, and there are no signs of WPb in these cells. Some other suggested markers include uptake of low density lipopeptide and angiotensin converting enzyme in bovine models (Chung-Welch et al. 1988). While not specifically tested in equine, CD34 has also been proven effective at labeling endothelial cells (Muller et al. 2002), and unlike vWF, it appears to be more strongly expressed in microvascular endothelial cells. Similarly, discrimination of different lung endothelial cells has been done using lectins that selectively bind to each endothelial population in several species including cattle (Schnitzer et al. 1994; Magee et al. 1994; Abdi et al. 1995), though these can vary between species, and to our knowledge no screening study of these has yet been done for the horse.

2.7 Conclusions

There are good amounts of data on the morphology and function of lung vascular endothelium in domestic animals. However, there are significant gaps in the molecular phenotyping of lung vascular endothelium in domestic animals. This will always remain the case because of the challenge of creating knock-outs or abilities to functionally block a molecule for precise functional phenotyping. The strength of the data obtained from domestic animal species is their closer physiological relevance to human's pulmonary physiology. There are, however, instances where the data on cells such as PIMs is opening new areas to investigate mechanisms of lung inflammation in rodent models and humans. Therefore, an increased focus on the comparative physiology of domestic animal species and less reliance on rodent models may provide more translational data to understand human lung vascular physiology. The increased use of methods such as electron microscopy especially the correlative light and electron microscopy will yield more precise localization of molecules of functional interest in lung capillary endothelium.

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