Involvement of endothelial progenitor cells in the formation of plexiform lesions in broiler chickens: possible role of local immune/inflammatory response*

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Abstract: Plexiform lesions (PLs), which are often accompanied by perivascular infiltrates of mononuclear cells, represent the hallmark lesions of pulmonary arteries in humans suffering from severe pulmonary arterial hypertension (PAH). Endothelial progenitor cells (EPCs) have been recently implicated in the formation of PLs in human patients. PLs rarely develop in rodent animal models of PAH but can develop spontaneously in broiler chickens. The aim of the present study was to confirm the presence of EPCs in the PLs in broilers. The immune mechanisms involved in EPC dysfunction were also evaluated. Lungs were collected from commercial broilers at 1 to 4 weeks of age. The right/total ventricle ratios indicated normal pulmonary arterial pressures for all sampled birds. Immunohistochemistry was performed to determine the expressions of EPC markers (CD133 and VEGFR-2) and proangiogenic molecule hepatocyte growth factor (HGF) in the lung samples. An EPC/lymphocyte co-culture system was used to investigate the functional changes of EPCs under the challenge of immune cells. PLs with different cellular composition were detected in the lungs of broilers regardless of age, and they were commonly surrounded by moderate to dense perivascular mononuclear cell infiltrates. Immunohistochemical analyses revealed the presence of CD133⁺ and VEGFR-2⁺ cells in PLs. These structures also exhibited a strong expression of HGF. Lymphocyte co-culture enhanced EPC apoptosis and completely blocked HGF-stimulated EPC survival and in vitro tube formation. Taken together, this work provides evidence for the involvement of EPCs in the development of PLs in broilers. It is suggested that the local immune cell infiltrate might serve as a contributor to EPC dysfunction by inducing EPC death and limiting their response to angiogenic stimuli. Broiler chickens may be valuable for investigating reversibility of plexogenic arteriopathy using gene-modified inflammation-resistant EPCs.

Key words: Plexiform lesions; Endothelial progenitor cells; Immune response; Hepatocyte growth factor; Broiler chicken

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1 Introduction

Plexiform lesions (PLs), which are typically located at branching points of small pulmonary arteries and cause vascular lumen obliteration, represent the

hallmark of severe pulmonary arterial hypertension (PAH) in humans, whether idiopathic or secondary (Jonigk *et al.*, 2011; Bockmeyer *et al.*, 2012), and are thought to signify a poor prognosis of PAH (Palevsky *et al.*, 1989). A recent study by Lu *et al.* (2016) demonstrates that severe PAH is present in around 2% of patients suffering from heart diseases. It has long been considered that disordered endothelial cell proliferation gives rise to PLs (Masri *et al.*, 2007; Sakao *et al.*, 2009; Huang *et al.*, 2010), but this remains a

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subject of debate because mature endothelial cells are indeed terminally differentiated with very low proliferative potential.

Recently, the role of progenitor cells in PAH-associated pulmonary vascular remodeling has been a focus of research (Yeager *et al.*, 2011; Schiavon *et al.*, 2012; Dierick *et al.*, 2016). Endothelial progenitor cells (EPCs) are a population of angioblasts which are commonly defined by the co-expression of markers of bone marrow origin such as CD34 or CD133 in addition to the endothelial marker VEGFR-2 (also known as KDR or Flk-1) (Timmermans *et al.*, 2009). Increasing evidence suggests that these cells play a fundamental role in tissue regeneration and vascular repair both by differentiating into mature endothelial cells and by secretion of proangiogenic mediators (George *et al.*, 2011; Zhang *et al.*, 2013). Interestingly, it has been found that PAH patients had increased EPCs (CD133⁺) in remodeled pulmonary arteries, specifically in PLs, leading to the suggestion that the formation of PLs is associated with EPC dysfunction (Toshner *et al.*, 2009). However, the mechanisms underlying EPC dysfunction during the development of PLs remain poorly understood.

Multiple angiogenic molecules have been identified in PLs. Some of these molecules, e.g. vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) (Tuder *et al.*, 2001; Jonigk *et al.*, 2011), have indeed been repeatedly demonstrated to enhance EPC-dependent vascular repair in various animal models (Sanada *et al.*, 2009; Song *et al.*, 2009; Yu *et al.*, 2010; 2014). In particular, HGF has been found to play a critical role in the angiogenic process in experimentally induced lung injury (Ishizawa *et al.*, 2004). Given that the increased expressions of angiogenic molecules (e.g. HGF) in diseased vessels represent a counter-system against endothelial dysfunction (Morishita *et al.*, 1998), some other factors contributing to the formation of PLs might have a detrimental effect on EPC-dependent angiogenesis. It is known that the formation of PLs is usually accompanied by a moderate to intense mononuclear infiltrate present around or within the affected arteries (Tuder *et al.*, 1994; Cool *et al.*, 1997). Therefore, the local immune/inflammatory environment might be involved in this process. However, the experimental evidence supportive of the hypothesis still needs to be established.

Plexogenic arteriopathy rarely develops in existing experimental animals (Firth *et al.*, 2010; Colvin and Yeager, 2014), but can develop spontaneously in broiler chickens. Previous studies showed that PLs in broilers had histological and molecular features closely resembling human PLs (Wideman and Hamal, 2011; Wideman *et al.*, 2011; Hamal *et al.*, 2012), rendering the broilers particularly suitable for studying the molecular and cellular basis of the formation of PLs. The present work was conducted to confirm the presence of EPCs in the PLs in this avian model. We also examined the effect of lymphocyte co-culture on the function of EPCs, with an attempt to understand whether immune cell infiltrate contributes to the formation of PLs by impairing the angiogenic activity of EPCs.

2 Materials and methods

2.1 Lung tissue preparation

Sixty one-day-old broiler chickens (Cobb 500) were purchased from a commercial hatchery and reared under normoxic and thermoneutral conditions. The birds were fed a commercial maize-soybean pelleted feed formulated to meet or exceed minimum National Research Council (NRC, 1994) standards for all ingredients (21% crude protein, 3100 kcal (1 kcal=4.1868 kJ) metabolizable energy (ME)/kg). Feed and water were provided *ad libitum*. The lighting schedule remained at continuous lighting (24 h light:0 h dark) during the experiment. At 1, 2, 3, and 4 weeks of age, a minimum of 8 birds were randomly selected and killed by cervical dislocation. Hearts were dissected and weighed to calculate the right/total ventricular weight (RV/TV) ratios. Birds with RV/TV>0.299 were considered to suffer from PAH (Walton *et al.*, 2001). The whole left lung was collected and cut in the transverse plane at the major rib indentations (costal sulci). One interrib division from the middle of each lung was fixed in 4% (0.04 g/ml) paraformaldehyde.

2.2 Histological and immunohistochemical staining

Lung tissues were embedded in paraffin and serially sectioned in the transverse plane at 5 μ m thickness. One slide of each lung was stained with haematoxylin and eosin to ascertain the presence of PLs. The remaining sections were used for immunochemical

staining of CD133, VEGFR-2, and HGF. Briefly, sections were deparaffinized and treated with 3% (v/v) hydrogen peroxide to block endogenous peroxidase activity. Heat-induced antigen retrieval was performed in Tris/ethylene diamine tetraacetic acid (EDTA) (pH 9.0) in a pressure cooker. After blocking with 5% (0.05 g/ml) bovine serum albumin, the slides were incubated with the following antibodies: polyclone rabbit anti-rat CD133 (1:50 (v/v), Bostar Biological Technology, China), VEGFR-2 (1:50 (v/v), Boster Biotechnology Technology, China), and HGF (1:50 (v/v), Bostar Biological Technology, China). The primary antibody detection was performed using a standard labeled streptavidin-biotinylated antibody method and visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB), followed by counter-staining with haematoxylin. Omitting primary antibodies served as negative controls.

2.3 Ex vivo expansion of EPCs from peripheral blood

Isolation and ex vivo expansion of EPCs were carried out as previously described (Bi *et al.*, 2014; Shah *et al.*, 2014). Whole blood was collected from clinically healthy broilers at 4 weeks of age. Mononuclear cell fraction was obtained by density gradient centrifugation on a Ficoll-Paque (Tianjin Haoyang Biological Manufacture Co., Ltd., China). Cells were seeded on rat tail type 1 collagen-coated 6-well plates at a density of 1×10^7 cells/well, and cultured in Endothelial cell growth medium (EGM)-2 (Lonza, Walkersvil, MD, USA) containing 2% (v/v) fetal bovine serum (FBS; Gibco, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin. After 24 h, the nonadherent cells were removed and the attached cells were cultured further in fresh growth medium. The culture medium was replaced every 3 d. Cell morphology was constantly monitored using phase contrast microscopy.

On Day 7 after plating, cells were co-stained with Dil-labeled acetylated low-density lipoprotein (Dil-ac-LDL, Invitrogen, Carlsbad, CA, USA) and fluorescein isothiocyanate (FITC)-labeled ulex europaeus agglutinin 1 (UEA-1) lectin (Sigma-Aldrich, St. Louis, MO, USA) as previously described (Bi *et al.*, 2014; Shah *et al.*, 2014). Cells presenting double-positive fluorescence were considered to be EPCs. For the determination of the progenitor and endothelial markers, cells were labeled with a phycoerythrin (PE)-conjugated monoclonal antibody against mouse CD133

(eBioscience, San Diego, CA, USA), or an allophycocyanin (APC)-conjugated anti-human VEGFR-2 (Miltenyi Biotec, Germany), at 4 °C for 20 min. After two steps of washing, the cells were subjected to flow cytometry using a fluorescence-activated cell sorter (FACS) Calibur flow cytometry (Beckman Coulter, CA, USA).

2.4 EPC/lymphocyte co-culture system

Peripheral blood mononuclear cell fraction was prepared from 6-week-old healthy birds as described above. The cells were incubated in Dulbecco's modified Eagle's medium (Gibco) at 39 °C in 5% CO₂ for 2 h to allow the monocytes to adhere to the bottom of the culture plates. The lymphocytes floating in the medium were pelleted by centrifugation at 400g for 10 min. The viable cells were counted and resuspended in DMEM.

EPCs were trypsinized and replated onto 24-well plates at 1×10^5 cells/well. Cells were allowed to adhere for 12 h and then co-cultured with lymphocytes in DMEM containing 2% (v/v) FBS with or without human recombinant HGF (20 ng/ml; Sino Biological Inc., Beijing, China) for 24 h at 39 °C in 5% CO₂. Results from our preliminary studies showed that EPCs treated with 20 ng/ml HGF for 24 h had increased proliferation and enhanced in vitro tube formation (data not shown). The ratio of lymphocytes to EPCs in co-cultures was 1:1. This ratio was chosen because our in vivo findings showed that the number of infiltrating mononuclear cells around most of the PLs was almost equal to that of the putative EPCs.

2.5 Methyl thiazolyl tetrazolium assay

The viability of EPCs in the co-culture system was determined using the methyl thiazolyl tetrazolium (MTT) assay. In the EPC/lymphocyte co-culture system, EPCs were adherent to the bottom of the culture plates and the lymphocytes were suspended in the culture medium, ensuring the removal of lymphocytes by washing. Briefly, cells were washed with 0.1 mol/L phosphate buffered saline (PBS) to remove lymphocytes and cultured in 200 µl fresh DMEM containing 10 mg/ml MTT (Sigma-Aldrich) for a further 4 h. At the end of incubation, the supernatant was carefully removed and 200 µl dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals for 15 min in the dark. The absorbance of the solubilized product at 570 nm (A_{570})

was measured. Cell densities were plotted as the mean A_{570} value of five replicates.

2.6 Flow cytometric analysis of cell apoptosis

The EPC apoptosis in the co-culture system was assessed using an annexin V-FITC kit (Beyotime Institute of Biotechnology, Nanjing, China) following the manufacturer's protocol. Briefly, EPCs were detached with 0.25% trypsin/EDTA (Gibco) and then re-suspended in 195 μ l annexin V binding buffer. Cells were incubated with 5 μ l of annexin V-FITC at room temperature for 10 min, followed by incubation with 10 μ l propidium iodide (PI) in 190 μ l annexin V-binding buffer on ice. Annexin V-FITC⁺/PI⁻ and annexin V-FITC⁺/PI⁺ cells were considered as apoptotic cells and quantified by flow cytometry (FACScan, Beckman Coulter).

2.7 Tubular formation assay

Matrigel matrix (BD Biosciences, San Jose, CA, USA), which was used to promote the differentiation of EPCs into capillary tube-like structures, was added into 96-well plates and allowed to polymerize by incubating at 39 °C for 30 min. Following treatment, EPCs were plated onto the surface of the solidified gel at 1×10^4 cells/well and incubated at 39 °C for 4 h. Network formation was observed with a phase-contrast microscope (Nikon, Japan) and representative fields ($\times 20$ magnification) were photographed. The number of master segments formed by cells was automatically counted in five random fields per well using the angiogenesis macro for ImageJ (Version 1.50b, NIH, USA).

2.8 Statistical analyses

Data are presented as mean $\pm$ standard error of the mean (SEM). Data were analyzed using SPSS software (Version 22, IBM Corp., Armonk, NY, USA). Comparisons were made using one-way analysis of variance (ANOVA) as appropriate. The observed significance levels were adjusted with the Bonferroni test for multiple comparisons. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Histological findings

The RV/TV ratios of all sampled birds were indicative of normal pulmonary arterial pressures, alt-

hough the birds at 1-week-old had a higher mean RV/TV ratio than those at ages of 2, 3, and 4 weeks (Table 1). PLs with different cellular compositions, presumed to be at different stages of maturation or differentiation, were determined in the lungs of birds at all ages sampled. Representative photomicrographs of PLs are shown in Fig. 1. The "immature" lesions displayed the cluster of epithelia-like cells with hyperchromatic nuclei and scanty cytoplasm; no elastic tissue formed the wall of the plexogenic vessels (Fig. 1a). These lesions closely resembled the PLs in human PAH described by Tuder *et al.* (1994). There were frequent observations of lesions consisting of macrophage-like cells (Fig. 1b). During the maturing process of the lesions, myofibroblasts and connective tissue matrix appeared and the macrophages became foam-like cells, with the formation of multiple slit-like vascular channels lined by flat and mildly atypical endothelial cells (Fig. 1c). More mature lesions exhibited a typical glomeruloid-like appearance, which had fewer cellular components in the matrix with multiple foam-like cells aligning around the periphery of the main body (Figs. 1d and 1e). Moderate to dense mononuclear cell infiltrates were often found around the affected arterioles (Figs. 1d and 1e). Lesions with multinucleated giant cell response were also identified (Fig. 1f); this type of plexogenic pulmonary arteriopathy was occasionally identified in human PAH (Heath and Smith, 1982).

Table 1 Right/total ventricle weight ratios of broilers at 1 to 4 weeks of age

Week	Number	RV/TV ratio			
		Mean	SE	Median	Range
1	9	0.216 ^a	0.08	0.209	0.183–0.251
2	8	0.183 ^b	0.04	0.182	0.169–0.200
3	11	0.171 ^b	0.04	0.169	0.148–0.194
4	10	0.186 ^b	0.06	0.183	0.154–0.220

Mean values of right/total ventricle weight (RV/TV) ratio were analyzed using the one-way ANOVA procedure. Different superscript letters indicate significant difference ($P < 0.01$). SE: standard error

3.2 Expressions of CD133, VEGFR-2, and HGF in PLs

We observed widespread vascular endothelial expression of CD133 in normal arterioles (Fig. 2a). On adjacent sections, these vessels were found also to express VEGFR-2 (Fig. 2b). Of particular note was the strong immunoreactivity of epithelial-like cells

Fig. 1 Representative images showing plexiform lesions (PLs) in the lungs

It is noted that we examined the lung sections from 1- to 4-week-old broilers and that the maturing process of PLs was not related to the ages. (a) Photomicrographs of a lesion in the lung of a 1-week-old broiler photographed in the same section at 200 $\times$ (left panel) and 400 $\times$ (right panel) magnifications. Note the epithelial-like cells in the body of the lesion. (b) A PL in the lung of a 1-week-old broiler. The lesion contains mainly macrophage-like cells (M ϕ). (c) A view of a small pulmonary arteriole in the lung of a 2-week-old broiler completely occluded by the complex lesion, showing foam-like macrophages (M ϕ), myofibroblasts (arrow), and spindle-like cells (arrowhead) embedded in the matrix. Multiple slit-like vascular channels (C) are found in the body. (d, e) More mature glomeruloid-like PLs in the lung of a 4-week-old broiler. These “mature” lesions are indicated by the multiple vascular channels (C) and foam-like cells (M ϕ) aligning around the periphery of the main body. The perivascular connective tissue displays mononuclear cell infiltrate (arrow). (f) A cross-sectional view of a lesion containing multinucleated giant cell (arrow) in a bird at 3 weeks of age. Scale bar=50 μ m

with CD133 (Fig. 2d) and VEGFR-2 (Fig. 2e) within the immature PLs, indicating a progenitor phenotype. In the glomeruloid-like PLs, the foam-like macrophages also showed strong expression in CD133 (Fig. 2g) and VEGFR-2 (Fig. 2h). HGF was also determined in normal vessels and PLs (Figs. 2c, 2f, and 2i).

3.3 Characterization of cultured EPCs

On Day 7 of plating, peripheral mononuclear cells displayed typical EPC characteristics such as spindle-shaped appearance (early EPCs) (Fig. 3a), Dil-ac-LDL/UEA-1-lectin double-positive (Fig. 3c), and the expressions of CD133 and VEGFR-2 (Fig. 3d). Nearly 97% of the cells were Dil-ac-LDL/lectin dual-

positive. These cells grew and differentiated into cobblestone-like late EPCs around Day 14 after plating (Fig. 3b) and were used for experiments.

3.4 Effect of lymphocyte challenge on EPC function in the presence or absence of HGF

Lymphocyte exposure did not alter the proliferative capacity of EPCs stimulated either with or without HGF (Fig. 4). Nevertheless, EPCs showed increased apoptosis in the presence of lymphocytes and the pro-survival effect of HGF on EPCs was completely inhibited by the lymphocyte challenge (Fig. 5).

We next used a Matrigel model to assess the in vitro capacity of EPCs to form capillary-like

Fig. 2 Representative photomicrographs of immunohistochemical staining

Adjacent sections of lung tissues showing the expressions of CD133, VEGFR-2 as well as HGF in a normal pulmonary arteriole (a–c), an immature plexiform lesion (PL) (d–f), and a mature lesion (g–i). The lung sections presented here are from 2-week-old broilers. Mφ: foam-like macrophage. Scale bar=25 μ m

structures after they were exposed to lymphocytes for 24 h. As shown in Fig. 6, the total number as well as the total length of the master segments formed by EPCs on Matrigel matrix surface was not affected by lymphocyte challenge. HGF stimulation augmented the ability of EPCs to form tube-like structures; however, this effect was significantly inhibited by lymphocyte challenge.

4 Discussion

PLs in the lungs of broilers have been documented previously in both PAH-susceptible and PAH-resistant broiler lines (Hamal *et al.*, 2012; Wideman *et al.*, 2015). In line with these findings, the present study demonstrated the presence of PLs in the lungs of commercial broilers. PLs were constantly

detected in birds with normal pulmonary arterial pressures, supporting the notion that the development of PLs does not require pre-existing pulmonary hypertension (Wideman *et al.*, 2015). In addition to the typical glomeruloid-like lesions described in previous studies (Wideman *et al.*, 2015), we detected PLs composed mainly of epithelial-like cells, which appeared remarkably similar to those from humans with PAH (Tuder *et al.*, 1994). In particular, an unusual form of plexogenic arteriopathy in human PAH, i.e. lesions accompanied by multinucleated giant cell response (Heath and Smith, 1982), was also detected in our commercial birds. Taken together, findings reported here demonstrate that PLs developed in broilers share all the reported morphological features of PLs from human PAH, validating the broiler chickens as a particularly useful model of spontaneous plexogenic arteriopathy.

Fig. 3 Characterization of endothelial progenitor cells (EPCs)

EPCs displayed a spindle-shape appearance on Day 7 of plating (a) and differentiated into monolayer of cobblestone-like cells on Day 14 (b). The ability of EPCs for DiI-ac-LDL uptake (red color) and the binding of FITC-UEA-1 lectin (green color) are shown in (c), while cells carrying both characteristics appear in orange. (d) Expressions of CD133 and VEGFR-2 were assessed by flow cytometry (gray line indicates the isotype control; black line indicates the antibody staining). Representative results are shown. Scale bar=100 μ m (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

Fig. 4 Proliferation of endothelial progenitor cells (EPCs) determined by MTT assay at 570 nm

Cells were exposed to lymphocytes in the presence or absence of hepatocyte growth factor (HGF) for 24 h. The values are expressed as mean $\pm$ SEM of five replicates. * $P<0.05$ vs. control; ## $P<0.01$ vs. lymphocyte treatment alone

One of the major findings in this work is that the epithelial-like cells accumulated in the immature PLs exhibited an EPC phenotype (CD133⁺ and VEGFR-2⁺). This finding from our avian model provides the first supportive evidence for the claim that EPCs contribute to the formation of PLs in the human (Toshner *et al.*, 2009). Interestingly, in mature PLs with glomeruloid-like appearance, the foam-like macrophages also expressed both CD133 and VEGFR-2. These results raise a possibility that there is an intrinsic relationship between the two morphologically different cell types in the PLs. Indeed, it has been reported that CD133⁺ progenitors can differentiate into both fibroblasts and monocytes/macrophages in inflamed tissue (Kania *et al.*, 2009). Therefore, it is very likely that there is a process of EPC-to-macrophage transition during the maturing of PLs. In context, our results suggest that

Fig. 6 In vitro tube morphogenesis of endothelial progenitor cells (EPCs)

Cells were seeded on a Matrigel surface after treatment with lymphocytes in the presence or absence of hepatocyte growth factor (HGF). A chart bar shows the number of master segments (a) and the total length of master segments relative to control (b), respectively. The values are expressed as mean±SEM of three replicates. *** $P<0.001$ vs. control; ## $P<0.01$, ### $P<0.001$. (c) Representative images of segments formed by EPCs on Matrigel surface

EPCs may contribute to the initiation and perpetuation of PLs in the lungs of broilers.

Numerous angiogenic molecules have been previously identified in PLs of broilers (Hamal *et al.*, 2012). In the present study, we found a strong expression of HGF in PLs, consistent with the studies on human PAH (Farha *et al.*, 2011). As an important factor responsible for vascular repair, HGF has been shown to enhance EPC function and improve EPC transplant efficiency in experimentally induced arterial injury (Song *et al.*, 2009; Yu *et al.*, 2015). Therefore, the strong expression of HGF in PLs might be explained as an unsuccessful attempt of this molecule to stimulate EPC-dependent vascular repair or angiogenesis.

Similar to the findings in previous studies (Hamal *et al.*, 2012; Wideman *et al.*, 2015), we determined constant mononuclear cell infiltrate around PLs, indicating a role of immune/inflammatory cells in the development of PLs in broilers. The initiation of the inflammatory response remains unclear but is presumably related to the formation of disturbed flow in branch points. Disturbed flow naturally occurs in branch points and may lead to endothelial damage and, thereby, local inflammation (Jongstra-Bilen *et al.*, 2006). It has long been considered that immune/inflammatory response contributes to both the physiological and pathological angiogenic process (Naldini and Carraro, 2005). Given that an appropriate inflammatory response contributes to the normal angiogenic process, we posited that the formation of PLs may be associated with abnormal immune/inflammatory response that may lead to EPC dysfunction, either directly or indirectly. We therefore used a cell co-culture system consisting of EPCs and lymphocytes at a ratio of 1:1 to test our hypothesis. We believed that our co-culture system would be more realistic to mimic the *in vivo* events than stimulating the EPC with a single type of proinflammatory cytokine (Balestrieri *et al.*, 2010; Zhao *et al.*, 2012). The EPC population used in this work was composed of so-called “late” EPCs, which have proliferative capacity and are able to differentiate into mature endothelial cells (Hur *et al.*, 2004). Our results showed that lymphocyte exposure alone led to enhanced EPC death; moreover, lymphocyte challenge completely blocked HGF-stimulated EPC survival and *in vitro* tube formation by EPCs. In context, our *in vitro* findings allow us to argue that the formation of PLs

may be associated with increased EPC death and inadequate response of these cells to angiogenic stimulation under the challenge of immune cells.

Some limitations of our *in vitro* study must be taken into consideration for interrupting the *in vivo* events. We acknowledge that our EPC/lymphocyte co-culture system did not adequately model the PL microenvironment in terms of the composition of the infiltrating immune cells, the cell-cell interaction, and the local concentration of HGF. In addition, since we only evaluated the angiogenic response of EPCs to HGF stimulation under lymphocyte challenge, it is not possible to rule out the possibility that EPCs may respond adequately to other proangiogenic factors under the same conditions.

5 Conclusions

The present study provides evidence that EPCs are involved in the formation of PLs in broilers, supporting the findings in human PAH. Our results also suggest that the local immune cell infiltrate may contribute to the development of PLs by inducing EPC death and limiting their response to angiogenic stimuli. Given the therapeutic potential of EPCs in repairing vascular damage (Sukmawati and Tanaka, 2015), transplantation of gene-modified inflammation-resistant EPCs (Liu *et al.*, 2010) may be beneficial for intervention of the formation of PLs. Further studies are warranted to test this hypothesis.

Compliance with ethics guidelines

Xun TAN, Fan-guo JUAN, and Ali Q. SHAH declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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中文概要

题目：免疫炎症反应和内皮祖细胞在肉鸡肺血管丛样病变形成中的作用

目的：肺血管丛样病变是重度肺动脉高压（PAH）病人的特征病变，病变血管周围常伴有单个核细胞浸润。肺血管丛样病变在常用实验动物上难以复制，但可在肉鸡（一种生长快速的肉用型鸡）肺脏中自发形成。内皮祖细胞（EPCs）在组织再生和血管修复过程中发挥重要作用。本研究以肉鸡为模型，探讨 EPCs 与肺血管丛样病变形成之间的关系。

创新点：证实 EPCs 参与了肺血管丛样病变的形成过程，并揭示了导致 EPCs 功能障碍的免疫学机制。

方法：采集 1~4 周龄肉鸡肺组织，常规石蜡切片，观察肺血管丛样病变的形成情况；采用免疫组化法检测 EPCs 表面标志 CD133 和 VEGFR-2 的表达以及肝细胞生长因子（HGF）的表达；分离培养晚期 EPCs，建立 EPCs/淋巴细胞共培养体系，并在共培养体系中添加 20 ng/ml HGF，观察 EPCs 增殖、凋亡和体外管样结构形成的变化。

结论：在不同周龄的肉鸡肺组织中均可观察到处于不同发展阶段的肺血管丛样病变（图 1）。早期的丛样病变主要由 EPCs（CD133⁺和 VEGFR-2⁺细胞）构成，HGF 在病变实体中高表达（图 2）。淋巴细胞共培养显著促进 EPCs 凋亡（图 5），并能阻断 HGF 诱导的 EPCs 存活和体外管样结构形成（图 6）。综上所述，肺血管丛样病变的形成可能与局部免疫炎症反应诱导 EPCs 凋亡并下调 EPCs 对促血管生成因子的反应性有关。

关键词：丛样病变；内皮祖细胞；免疫反应；肝细胞生长因子；肉鸡