

Dendritic cell recruitment in lesions of human and experimental pulmonary hypertension

Short-title: Dendritic cells in pulmonary hypertension

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Abstract (195 words)

We have tested the hypothesis that dendritic cells (DCs), key players in immunity and tolerance, might be involved in the immunopathology of idiopathic pulmonary arterial hypertension (IPAH).

DCs phenotype and localization were characterized by immunohistochemistry and double-labeling immunofluorescence in lung samples from controls, human IPAH and experimental pulmonary hypertension (monocrotaline-exposed rats).

As compared to controls, morphometric analysis demonstrated increased numbers of DC-SIGN positive cells in muscular pulmonary arteries in IPAH and monocrotaline-induced pulmonary hypertension. In human samples, mean (\pm SEM) number of DC-SIGN positive cells per artery of 100-300 μ m diameter was 1.4 ± 0.4 in controls versus 26.4 ± 2.7 in IPAH ($p < 0.001$). In rats, mean (\pm SEM) number of OX-62 positive cells per artery of 50-150 μ m diameter was 0.5 ± 0.2 in controls, and 0.7 ± 0.5 , 3.1 ± 0.5 , and 8.4 ± 0.6 at day 7, 14 and 28 after monocrotaline exposure, respectively ($p < 0.001$). Human complex lesions of muscular pulmonary arteries showed transmural DC infiltration. Phenotyping revealed an immature DC profile in human and experimental pulmonary hypertension.

Our results support the concept that immature DCs accumulate in remodeled pulmonary vessels and hence could be involved in the immunopathology of pulmonary hypertension.

Key-words

Dendritic cells

Immunopathology

Inflammation

Monocrotaline

Pulmonary arterial hypertension

Introduction

Inflammatory mechanisms play a role in pulmonary arterial hypertension (PAH) [1]. Indeed, severe PAH may complicate the course of systemic inflammatory conditions and treatment with corticosteroids and immunosuppressants has been shown to improve PAH in the setting of connective tissue diseases such as systemic lupus erythematosus [2, 3]. In addition, pathogenic auto-antibodies targeting endothelial cells are capable of inducing vascular endothelial apoptosis and may initiate the development of PAH [4, 5]. A subset of idiopathic PAH (IPAH) patients has been shown to have circulating autoantibodies (antinuclear, anti-endothelial and anti-fibroblast antibodies) [6, 7], as well as elevated circulating levels of the proinflammatory cytokines IL-1 and IL-6 [8]. Lung histology of patients displaying severe IPAH frequently reveals inflammatory cell infiltrates corresponding to macrophages and lymphocytes in the range of plexiform lesions with local expression of chemokines, such as RANTES and fractalkine [9, 10]. Experimental data from monocrotaline-induced pulmonary hypertension in rats further support a link between pulmonary vascular inflammation and remodeling.

Among the different pathways related to the inflammatory process, the role of dendritic cells (DCs) has been increasingly recognized, not only as a simple antigen-presentation cells responsible for the initiation of the inflammatory response but especially as a key modulator of the whole process thus raising its importance in many human disorders, including allergy [11], autoimmunity [12], tumor immunology [13], and allograft rejection [14]. Numerous data clearly demonstrate that pulmonary DCs are at least involved in the pathogenesis of a whole spectrum of highly prevalent respiratory conditions and that for some of those (such as asthma), DCs have been proven to be essential in the development of the disease [15]. In mice, pulmonary DCs have been shown to be the key cells in the pathogenesis of asthma [16], and there is circumstantial

evidence from animal models that pulmonary DCs might contribute to the development of chronic obstructive pulmonary disease in smokers [17]. Furthermore, the number of lung DCs is increased in sarcoidosis [18] and diffuse panbronchiolitis [19]. The ability of DCs to differentiate into other cell phenotypes, including endothelial cells [20] has raised their potential role in vascular disorders. In the present study, we report for the first time data supporting the involvement of DCs in human and experimental pulmonary hypertension.

Methods

Subjects and sample processing

Human PAH pulmonary specimens were obtained at the time of lung transplantation in 8 patients displaying severe IPAH. Right-heart catheterization demonstrated severe precapillary pulmonary hypertension in all cases. Control pulmonary specimens corresponded to 8 lung biopsies performed during surgery for pneumothoraces or at distance from localized pulmonary lesions. Controls had no evidence of pulmonary vascular disease. PAH and control lungs were distended by infusion of ornithyl carbamyl transferase compound (OCT, VWR) diluted in PBS (1:5) into the main bronchi or by needle injection into the parenchyma to preserve lung morphology. Specimens for histology were collected from lung periphery, then snap-frozen in isopentane on dry ice and stored at - 80°C until further analysis. All patients and controls studied were part of the French Network on Pulmonary Hypertension, a programme approved by our Ethics Committee, and gave written informed consent.

Animal model

Adult male Wistar rats (8 to 10 weeks old) were euthanized by overdose of sodium pentobarbital 7, 14 and 28 days after single subcutaneous injection of saline (n=10) or 60 mg/kg monocrotaline (Sigma-Aldrich) (n=30). Lungs from controls (saline) and monocrotaline-exposed rats were distended by infusion of OCT:PBS (1:1) into the trachea, then snap-frozen. Monocrotaline-exposed rats displayed severe pulmonary hypertension at day 28, while mild hemodynamic and histological alterations occurred at day 14. Saline-exposed rats and rats evaluated 7 days after monocrotaline exposure had normal pulmonary hemodynamic values and pulmonary vascular histology. Animal experiments were approved by the administrative panel on

animal care from Centre de Chirurgie Expérimentale Marie Lannelongue, Le Plessis-Robinson, France.

Immunohistochemistry

Immunohistochemistry was performed on 7 µm-thick sections of frozen lung tissue. After routine preparation, human samples were processed with the following antibodies: monoclonal antibodies against DC-SIGN (Pharmingen clone DCN46), CD1a (Pharmingen clone HI149) and CD83 (Immunotech clone HB15A) diluted in PBS containing 1% serum calf fetal serum and 5% human AB serum. Rat DCs were stained with the monoclonal anti-CD103 antibody (Pharmingen clone OX-62) diluted in PBS containing 2 % normal rat serum. After overnight incubation at 4°C, human lung sections were labeled by biotinylated anti-mouse immunoglobulins and peroxidase-labeled streptavidin (Biogenex). Rat OX-62 labeling was revealed with the kit LSAB 2 for use on rat specimens (Dako). Staining was completed after incubation with substrate-chromogen AEC solution (Dako). Slides were counterstained with Mayer's Hematoxyline (RAL) and mounted with aqueous medium (Glycergel, Dako). Controls used for these antibodies included omission of the primary anti-body and substitution of the primary antibody by isotype control. Positive control for CD1a and CD83 was obtained by staining hilar lymph nodes of explanted lungs (data not shown).

Immunofluorescent labeling

For double immunofluorescence, anti human DC-SIGN (Pharmingen clone DCN46) and OX-62 (Pharmingen) antibodies were labeled by Biogenex's or Dako's biotinylated anti-mouse immunoglobulins and streptavidin, Alexa Fluor 594 or 488 conjugate (Molecular Probes) for one hour. Then, the tissues were incubated overnight either with FITC conjugated, anti-smooth

muscle alpha-actin (Sigma-aldrich clone 1A4), anti human CD68 (Dako clone KP1), anti-rat RTIB (HMC class II, Serotec clone OX6) or rhodamine conjugated vimentin (Santa Cruz sc-6260), or Alexa Fluor 488 conjugated anti-rat CD68 (serotec clone ED1) and anti-rat CD4 (serotec clone W3/25). The slides were mounted with Vectashield Mounting Medium (Vector Laboratories).

Immunohistochemical cell quantification

The immunohistochemical cell quantification was carried out using a Nikon 80i microscope with the morphometric analysis software NSI Elements (Nikon). The mean number of pulmonary arterial DCs was calculated by counting DC-SIGN (human DCs) or OX-62 (rat DCs) expressing cells on 3 slides of each sample. For quantification, pulmonary muscular arteries of a cross-sectional diameter of 100 to 300 μm and of 50 to 150 μm , for human and rat lungs respectively, were taken into account.

Statistical analysis

All values are expressed as mean \pm SEM. In human samples, statistical significance was evaluated with a two-tailed unpaired Student t test for comparisons between control and PAH groups. Analysis of variance (ANOVA) using repeated measures and the Fisher projected least significant difference (PLSD) post test was performed on rat results (4 groups : control, day 7, 14 and 28 after monocrotaline exposure). A p value of less than 0.05 was considered significant.

Results

Detection of dendritic cells in pulmonary arteries from IPAH patients

In all human lung samples from IPAH patients, we identified DC-SIGN expressing DCs presenting network-like organization in adventitial connective tissue of pulmonary vasculature (Figure 1). Affected vessels in PAH lungs displayed DCs infiltration into the adventitial and the medial layer. Transmural infiltration by DCs was frequently observed in plexiform lesions. Morphometric analysis revealed a dramatic increase in the number of DC-SIGN positive cells in muscular pulmonary arteries: the mean (\pm SEM) number of DC-SIGN positive cells per artery of 100-300 μ m diameter was 1.4 ± 0.4 in controls versus 26.4 ± 2.7 in IPAH ($p < 0.001$, two-tailed unpaired Student t test) (Figure 2). Immunohistochemistry indicated that these cells were CD1a and CD83 negative, corresponding to immature DCs (data not shown). Immunofluorescent double labeling with CD68, alpha-actin and vimentin excluded possible cross-reactions with macrophages or fibroblasts (Figure 1). Pulmonary arteries of control lungs showed scattered adventitial DC staining. To a smaller extent, DC-SIGN positive cells were detected in peribronchial lung parenchyma, in lobular septa and visceral pleura of lungs from both IPAH patients and controls. DC-SIGN positive cells were not found in venules or capillaries.

Detection of dendritic cells in pulmonary arteries from experimental pulmonary hypertension

Recruitment of DCs in monocrotaline-induced pulmonary hypertension was studied in Wistar rats (Figure 3). DCs were detected by the CD103 clone OX-62, which has been shown to be an important marker of rat DCs [21]. Increased number of DC was first observed 14 days after monocrotaline exposure. Twenty eight days after monocrotaline injection, pulmonary

hypertensive rats had a marked recruitment of OX-62 positive DCs in the adventitial layer of muscular pulmonary arteries, as well as in the vessel wall of pulmonary veins (Figure 2). The mean (\pm SEM) number of OX-62 positive cells per artery of 50-150 μ m diameter was 0.5 ± 0.2 in controls, and 0.7 ± 0.5 , 3.1 ± 0.5 , and 8.4 ± 0.6 at day 7, 14 and 28 after monocrotaline exposure, respectively ($p < 0.001$, ANOVA) (Figure 2). DCs counts increased with the degree of pulmonary artery medial remodeling and were sparse in control vessels. Immunofluorescence double-labeling revealed an immature pattern of the recruited DCs: CD4 negative, OX-62 positive, CD68 negative mostly MHC class II negative phenotype (Figure 3). OX-62 positive scattered DCs were present in bronchial-associated lymphatic tissue, as well as in the bronchial wall of PAH-rats and controls.

Discussion

The present study reports the first evidence that DCs may contribute to the immunopathology of human and experimental pulmonary hypertension. Our results suggest that in human and experimental pulmonary hypertension, immature DCs accumulate in remodeled pulmonary vessels and hence could be involved in the evolution of the pulmonary vascular lesions. We have shown that mean numbers of arterial DCs increase during the development of vasculopathy in monocrotaline-exposed rats. The arterial DCs accumulation precedes pulmonary arterial thickening and hemodynamic alteration and is constantly present in remodeled vessels, indicating that DC influx is not the mere consequence of increased pulmonary arterial pressure. We have not performed functional testing of DCs recruitment in the present study, such as *in vivo* migration in our animal model. Therefore DCs accumulation in lesions of pulmonary hypertension may be the consequence of recruitment but also local proliferation, decreased efflux from the pulmonary vascular wall or even transdifferentiation from infiltrating macrophages.

The role of inflammatory processes in the natural history of PAH has been discussed since inflammatory infiltrates surrounding remodeled vessels in human PAH have been described by several investigators in the past years. In addition, increased levels and expression of cytokines in blood, lung parenchyma and affected pulmonary arteries, have been recently described. The increased prevalence of PAH in connective tissue disorders and successful treatment with immunosuppressive therapy in some forms of PAH support the concept of inflammatory pathways in the development and modulation of the disease. Therefore a better understanding of the relation between inflammation and pulmonary vascular remodeling is of major importance.

Inflammatory mechanisms and DCs could play a role in the early steps of human PAH development, as demonstrated in the rat inflammatory model of monocrotaline-induced

pulmonary hypertension. All human PAH samples in the present study came from patients suffering from IPAH. This study design was chosen in order to avoid associated inflammatory conditions which can be possibly encountered in PAH related to connective tissue diseases, autoimmune thyroiditis or HIV infection. Our goal was to confront an exaggerated inflammatory animal model of pulmonary hypertension (monocrotaline-induced PH) to “pure” IPAH and to describe common inflammatory mechanisms. Indeed, such mechanisms have not been well defined yet in human IPAH.

As previously shown, human lung DCs lacked CD83 expression [22], corresponding to immature DCs [23]. Also, CD1a, a characteristic cell-surface molecule present on epidermal DCs and blood-derived DC was consistently absent on perivascular lung DCs [22]. CD1a was occasionally seen in bronchial epithelium (data not shown), as confirmed by a recent publication [24]. This observed lack of mature DCs may be explained by the fact that after processing foreign/self antigen, DCs subsequently migrate to lymphoid tissues, where maturation is achieved. Here, DCs initiate the activation of antigen-specific T-cells. Accordingly, rat lung DCs were presenting the immature CD4 negative OX62 positive phenotype [25]. Fibrocytes comprise a subpopulation of circulating mononuclear cells of a monocyte/macrophage CD68 positive lineage that can exhibit fibroblast properties at the site of tissue injury (alpha-actin and vimentin production, differentiation into myofibroblasts) [26, 27], which were shown to participate to the arterial remodelling that occurs in hypoxia-induced PAH in animal models [26]. We checked by immunofluorescent double-labeling that human DC-SIGN and rat OX-62 positive cells did not express vimentin or alpha-actin fibrocytic markers. Hence, we can conclude that a DC subpopulation distinct from fibroblaste/fibrocyte lineages accumulate in human and rat remodeled pulmonary arteries.

Among the different lineage of inflammatory cells, DCs have been recently linked to vascular disorders. In systemic arteries, vascular-associated DCs densely accumulate in arterial regions that are under major hemodynamic stress by turbulent flow conditions [28]. Interestingly, branching points of pulmonary arteries with altered flow conditions are predisposed to the development of plexiform lesions in human PAH [29]. Of note, we observed that transmural infiltration by DCs was most frequently observed in plexiform lesions. Concomitantly to the hemodynamic changes, occurrence of oxidative stress in PAH, could act as a DCs-attracting “danger signal”. Indeed, it has been shown that markers of oxidative stress are constantly present in endothelial and medial layers of pulmonary arteries in PAH [30].

DC-SIGN is a C-type lectin, highly expressed on the surface of immature DCs [31]. DC-SIGN has been shown to allow monocyte-derived DCs to recognize a variety of micro-organisms, including viruses, parasites, and bacteria [32]. DC-SIGN can also bind adhesion molecules on the surface of naive T cells and endothelium suggesting its involvement in T-cell activation and DCs trafficking [31]. Noteworthy, recent findings highlight an involvement of a subset of DCs in tumor angiogenesis by undergoing endothelial-like differentiation *ex vivo* and assembling neovasculature *in vivo* [20].

Novel therapeutic strategies may target pulmonary artery remodeling and its inflammatory component in PAH. Schermuly et al. have reported that the PDGF receptor antagonist STI571 (imatinib mesylate) reverses pulmonary vascular remodeling in two different animal models of pulmonary hypertension including monocrotaline-exposed rats [33]. Moreover preliminary case reports plead in favor of imatinib as an antiremodeling agent in severe human IPAH [34-36]. Although, curative properties of imatinib in experimental and human PAH are supposed to rely on *in vitro*-demonstrated antiproliferative, antimigratory and proapoptotic effects on pulmonary artery smooth muscle cells [33], recent publications highlight the DC-modulatory effects of

imatinib *in vitro* and *in vivo* [37-40]. Indeed, Mohty et al. have shown that disease response to imatinib in patients with chronic myeloid leukemia is accompanied by restoration of plasmacytoid DCs function *in vivo*. These findings provide evidence that imatinib is capable to restore some DC-related immune functions in CML [39]. In addition, Smyth et al. state that imatinib can activate a cell type named “interferon-producing killer DCs”, which is considered to be a cross between DCs and natural killer cells [40]. Moreover, a recent report indicates that imatinib activates interferon-producing killer DCs in a mouse model of cancer, explaining the therapeutic effect of this drug in gastrointestinal stromal tumors that are resistant to its antiproliferative effect [41]. Thus, imatinib effects in IPAH could be linked at least in part to the modulation of DC function.

The possible contribution of DCs to the immunopathology of both human and experimental pulmonary hypertension is of major interest. Further phenotyping of the immature DC-SIGN⁺ subsets in lungs of human PAH should help to better characterize DCs in PAH. The possible involvement of immature DCs in a well established animal model of inflammatory pulmonary hypertension will help analyzing the effects of specific therapeutic interventions to modulate the role of these cells *in vivo*. Last, evaluation of the cross-talk between immature DCs and endothelial and smooth muscle cells should be evaluated *in vivo* and *in vitro*. These data will allow us to better understand the links between immune cells, abnormal angiogenesis and pulmonary artery remodeling in pulmonary hypertension.

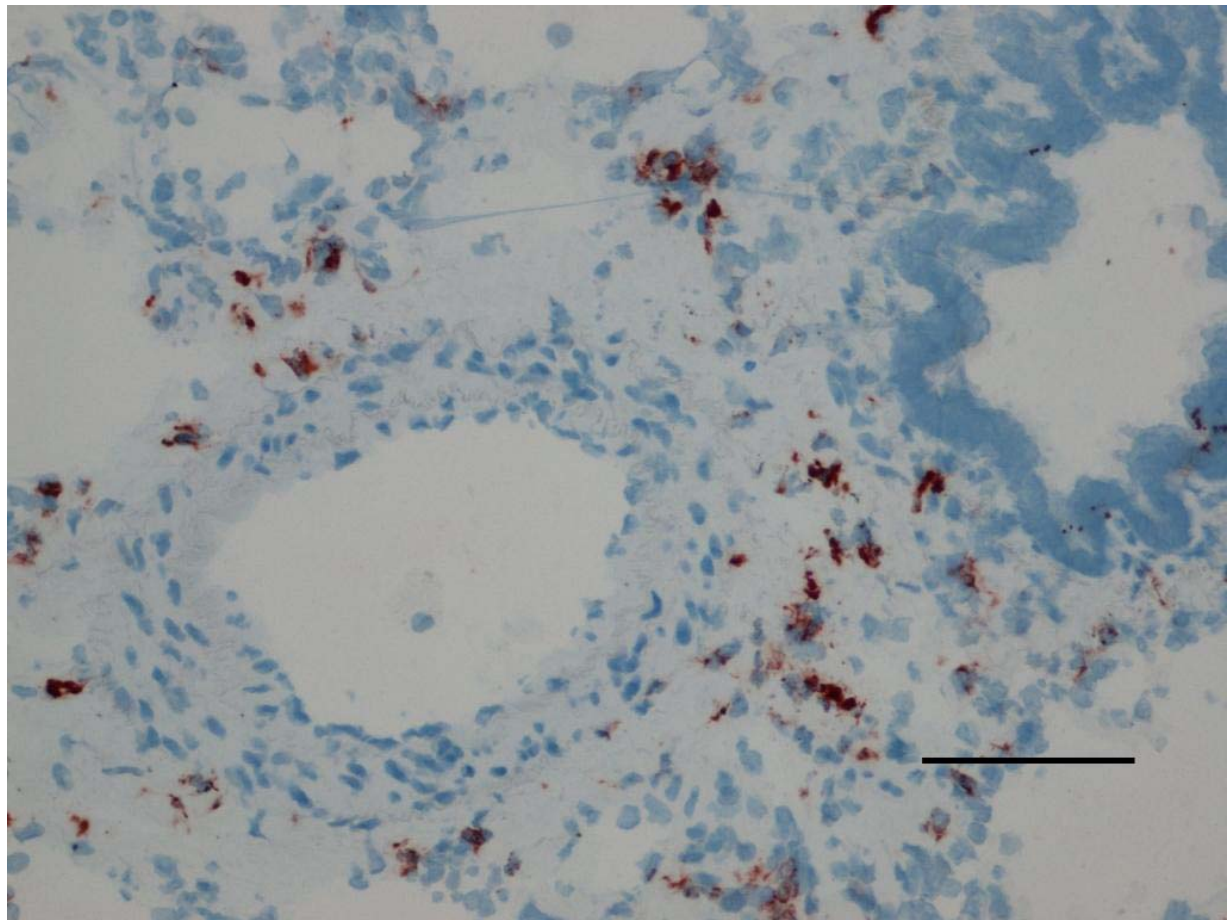
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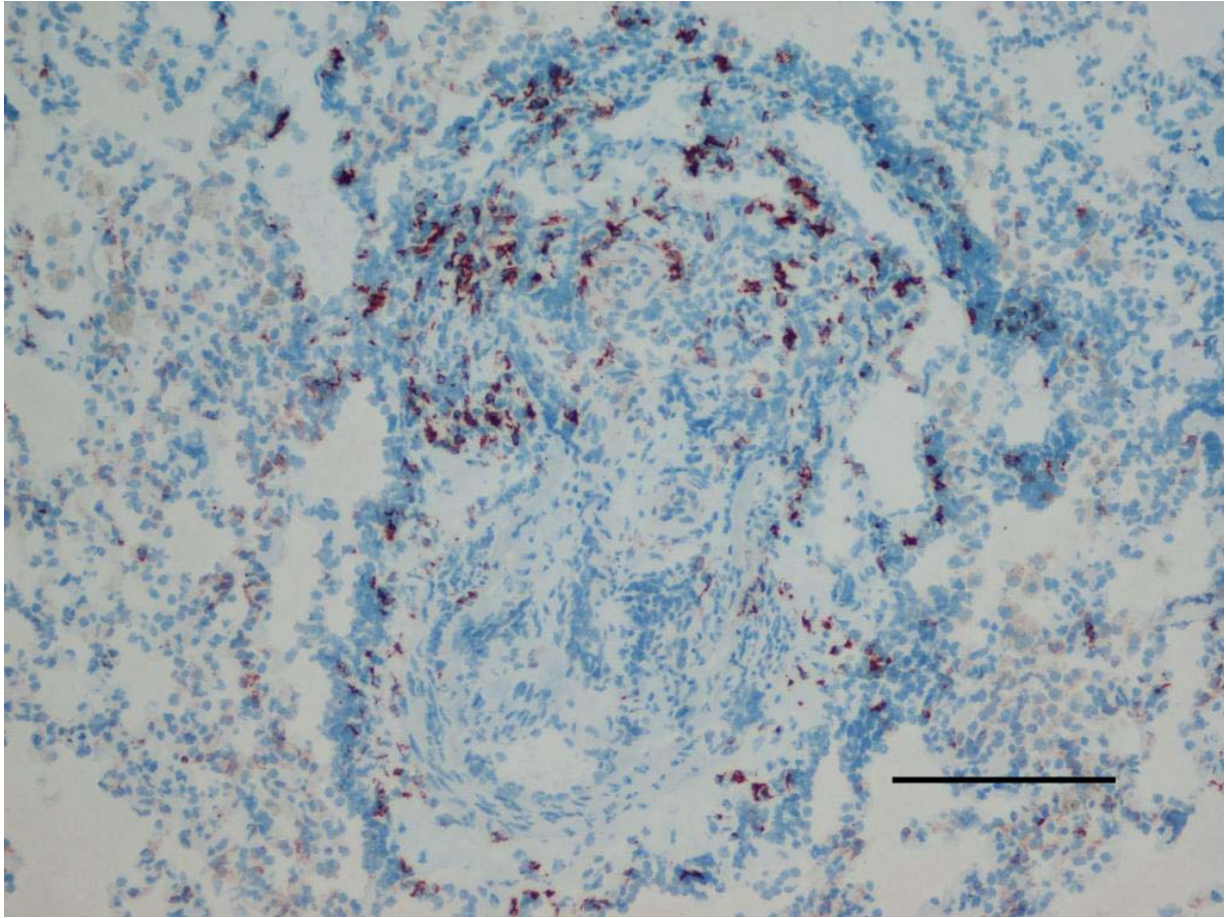
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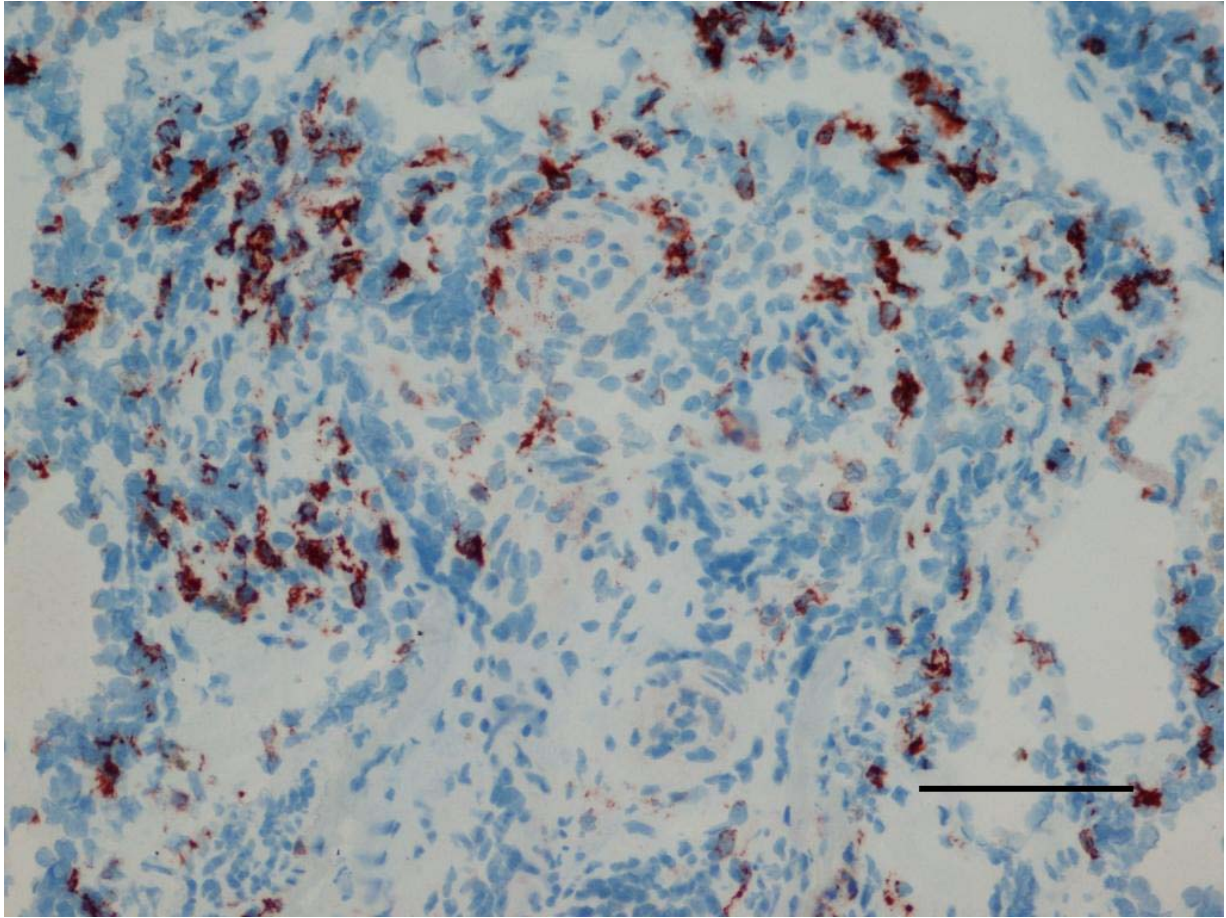
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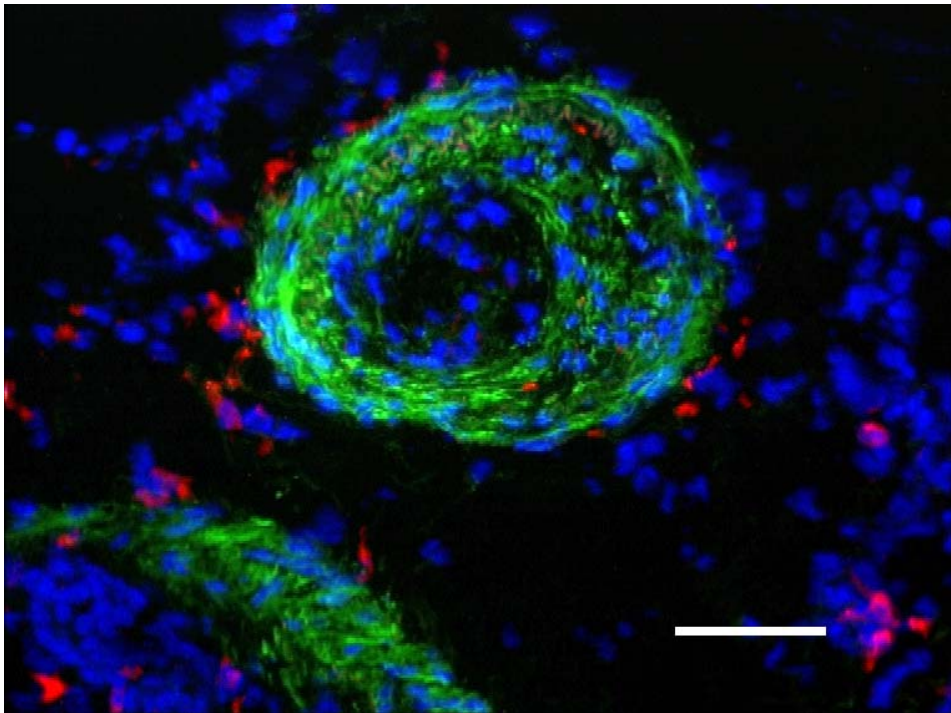
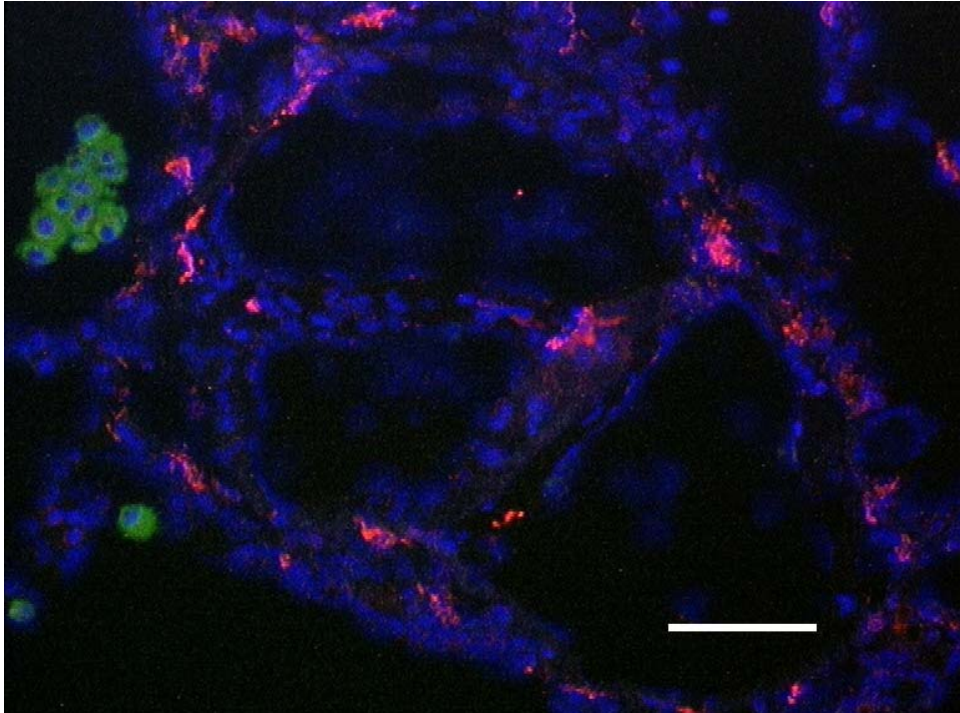
Figure 1

DC-SIGN⁺ DCs surrounding the adventitia of a normal pulmonary artery (A) (x20) and located inside a complex lesion of a PAH-lung (B) (x10) and (C) (x20). The DC-SIGN positive cells (red) were distinct from the macrophagic CD68⁺ population (green) (D) (x20). DCs (red) neither co-localized with alpha-actin positive cells (green) excluding possible cross-reactions with smooth muscle cells and/or myofibroblasts (E) (x20). At last DCs (green) did not express vimentin (red), a mesenchymal marker, eliminating any confusion with fibroblastic/fibrocytic elements which can be involved in fibrotic PAH-lesions (F) (x20). In A, C, D and E, internal scale bar=100 μ m, in B internal scale bar = 200 μ m.









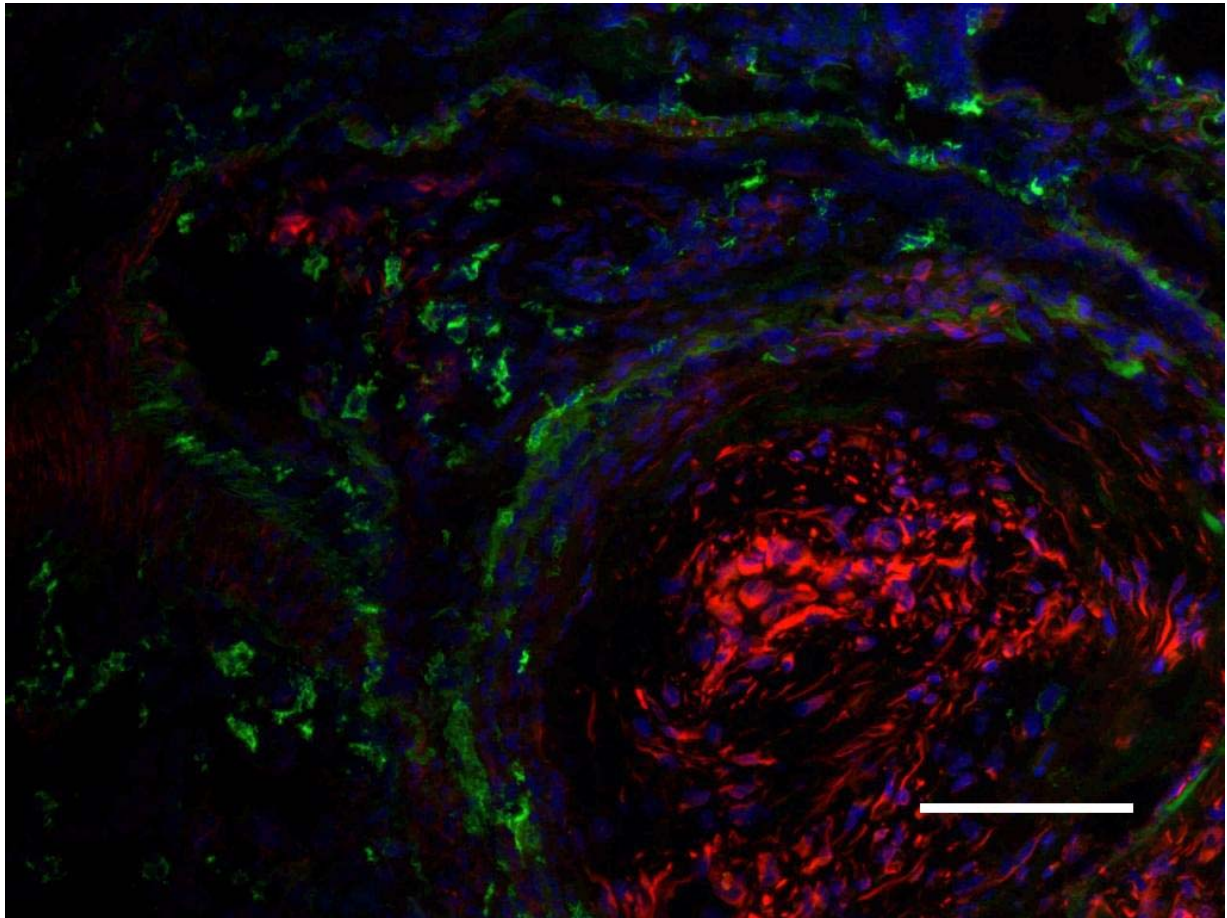


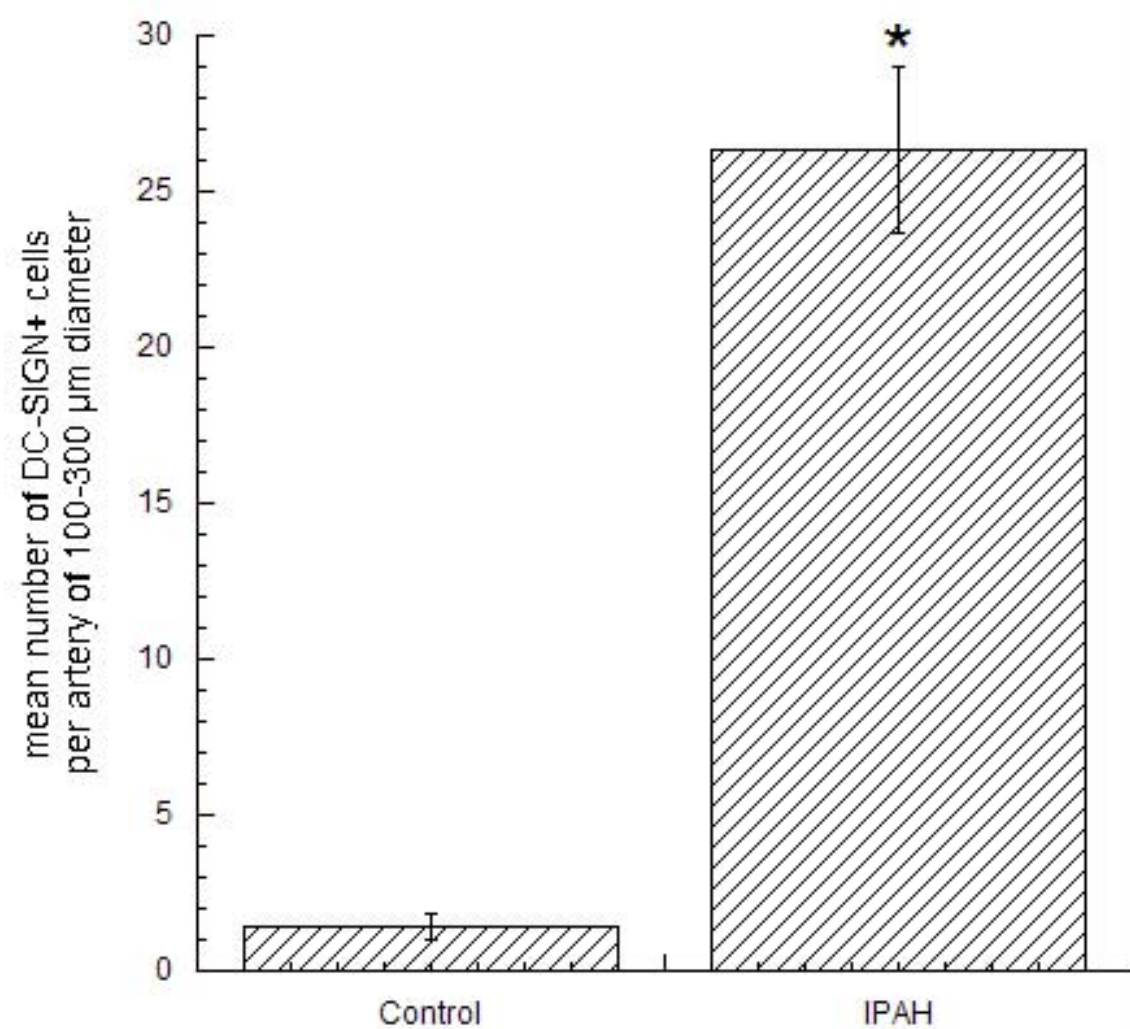
Figure 2

Quantification of DCs in human and rat pulmonary arteries. In IPAH patients, an increase of DC-SIGN positive cells in the pulmonary arteries of 100 to 300 μm diameter is observed (A). Monocrotaline-exposed rats show a significant increase in the mean number of OX-62 positive cells in pulmonary arteries of 50 to 150 μm diameter, in a time-related fashion (B).

*: $p < 0.001$, two-tailed unpaired Student t test

#: $p < 0.001$, analysis of variance (ANOVA)

Abbreviations: D: day; IPAH: idiopathic pulmonary arterial hypertension



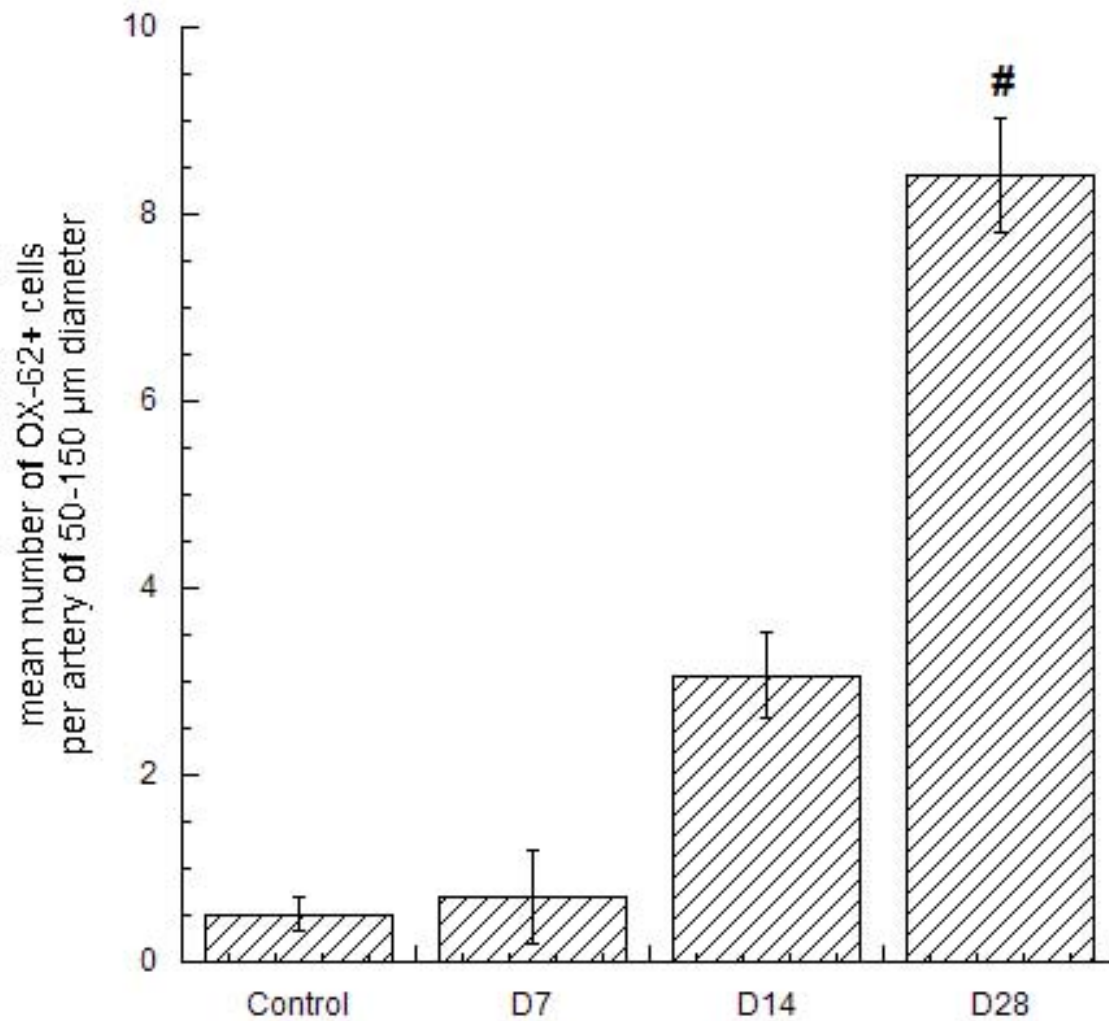
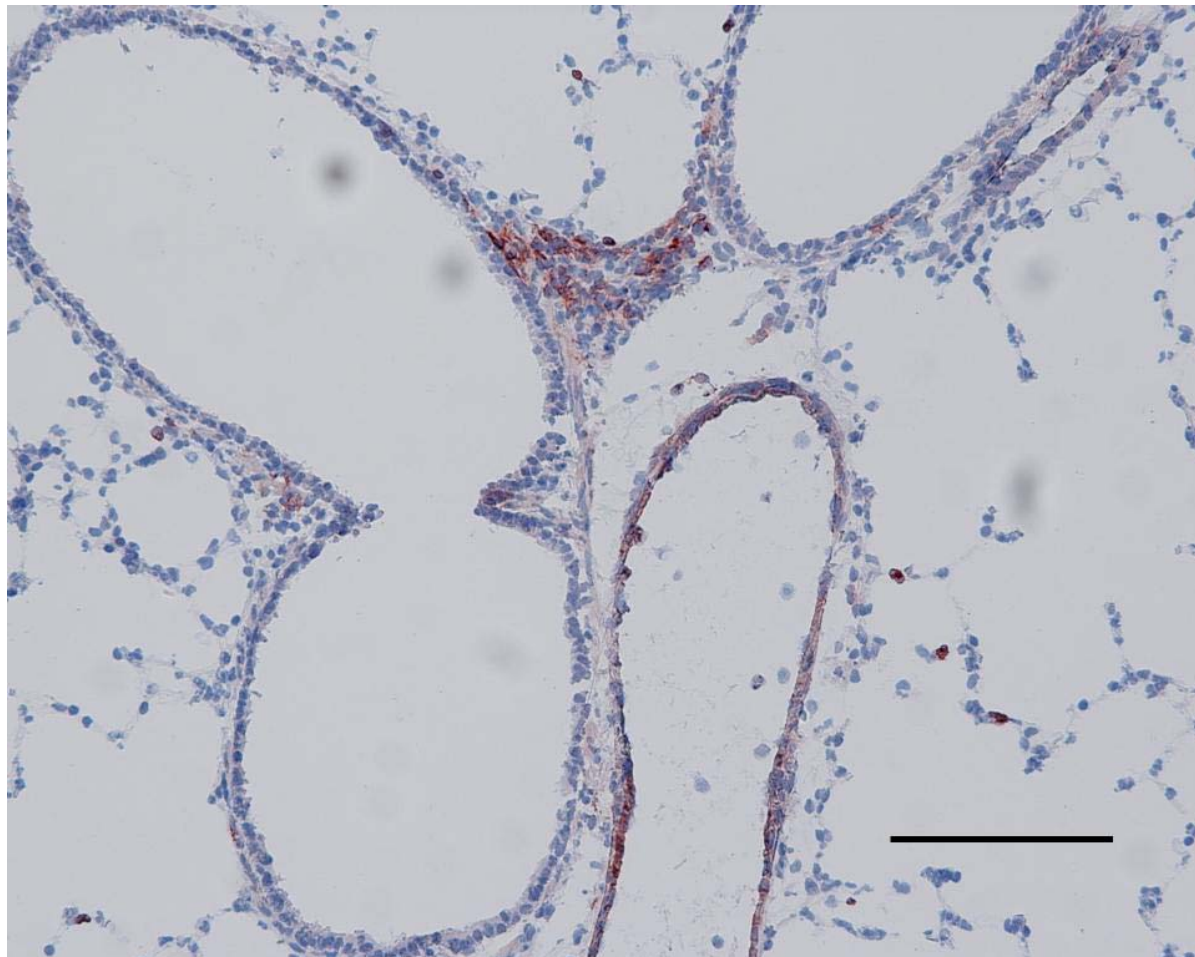
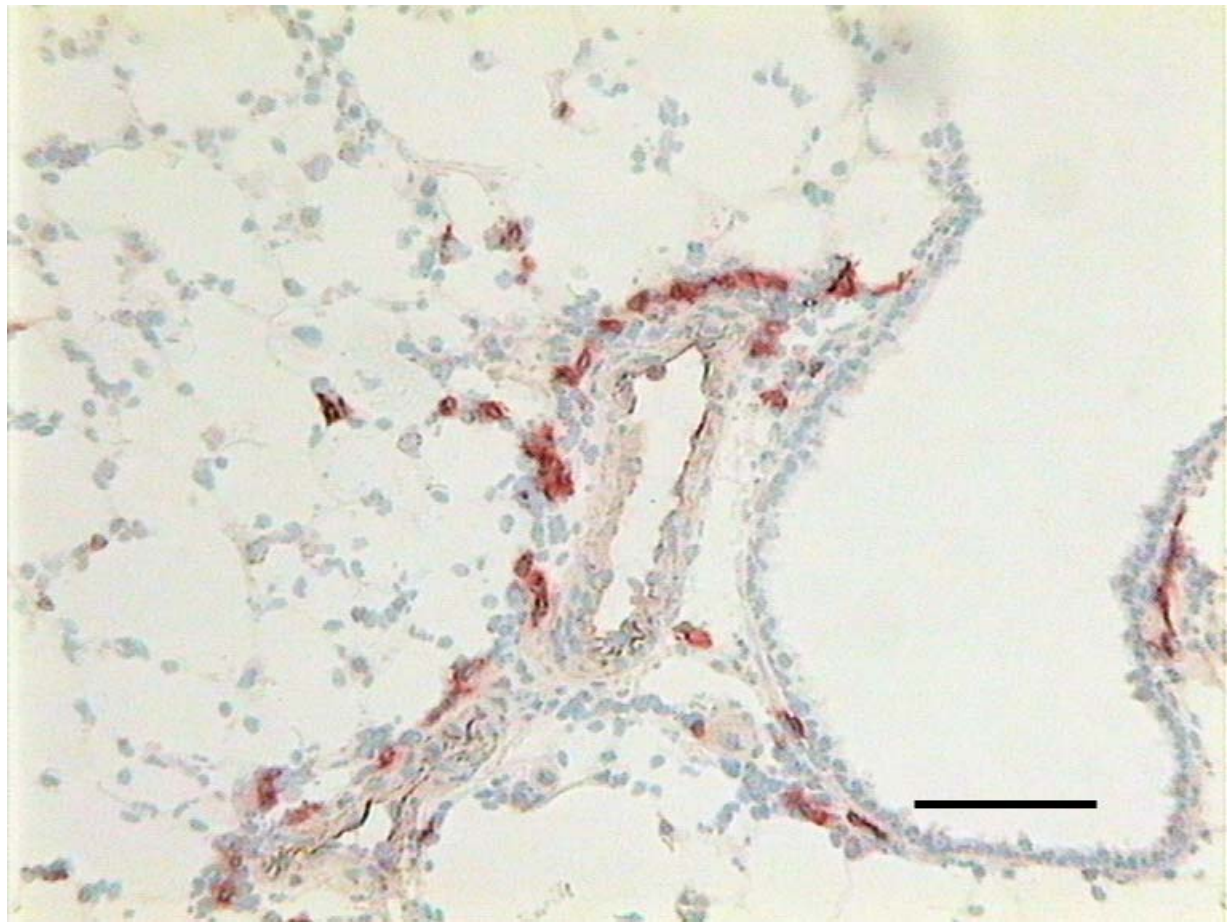


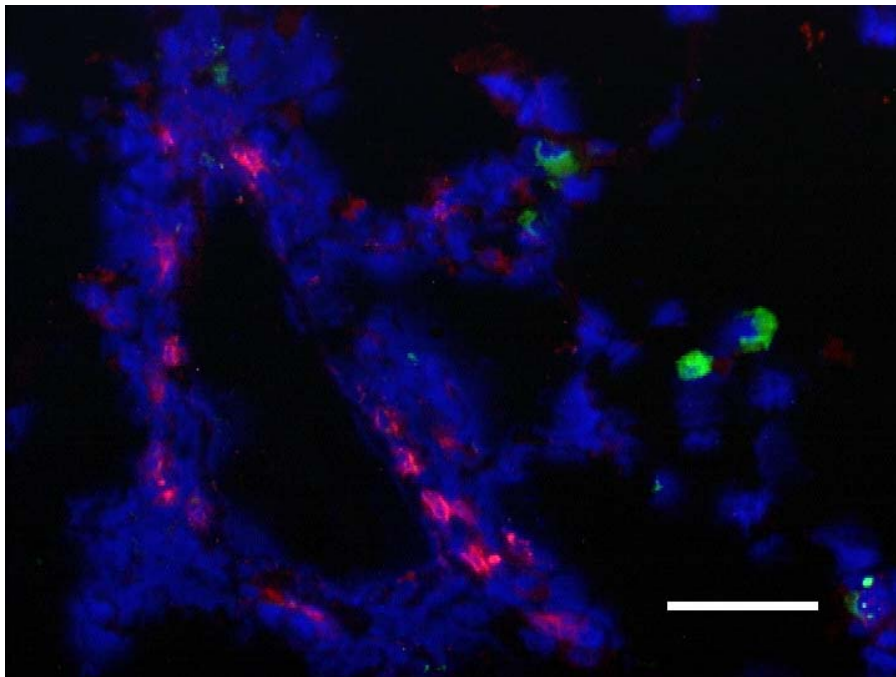
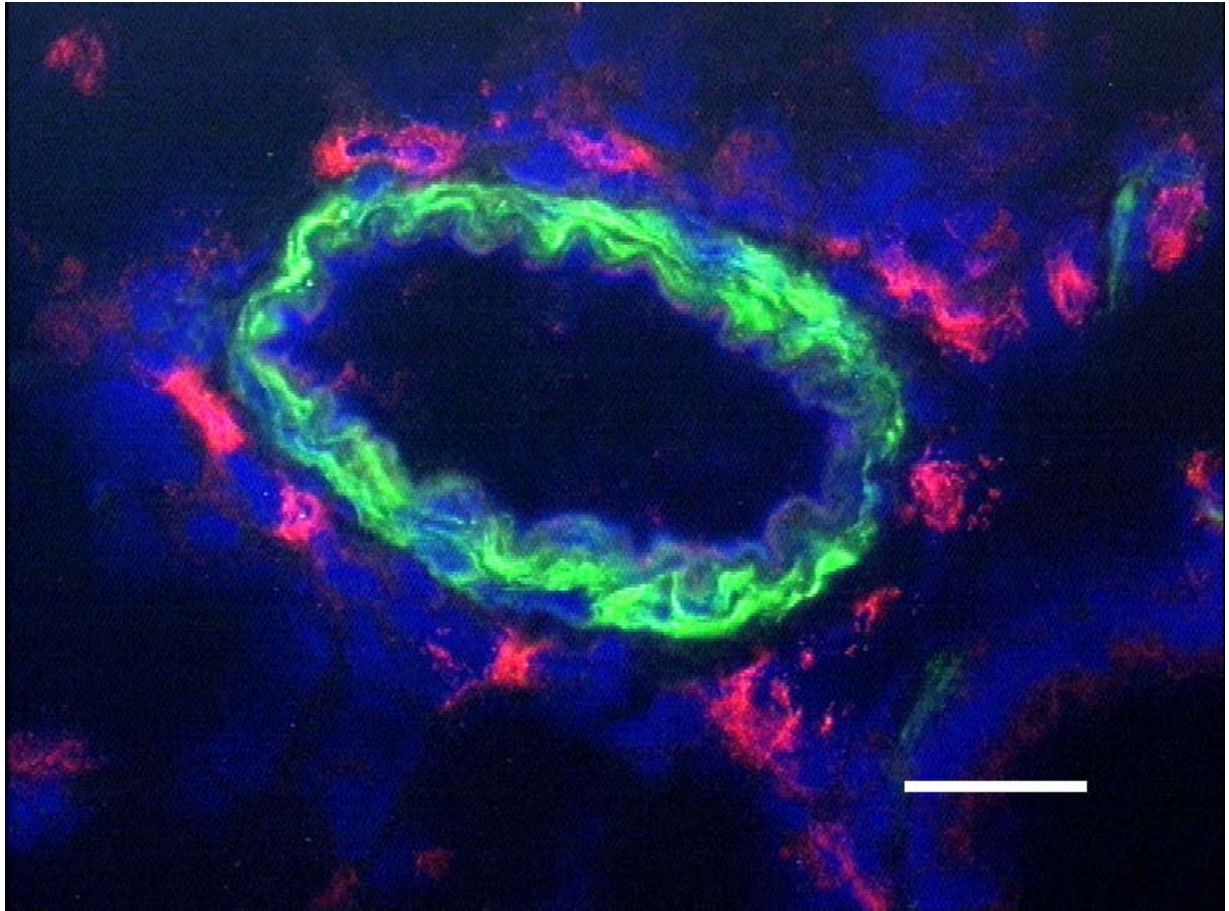
Figure 3

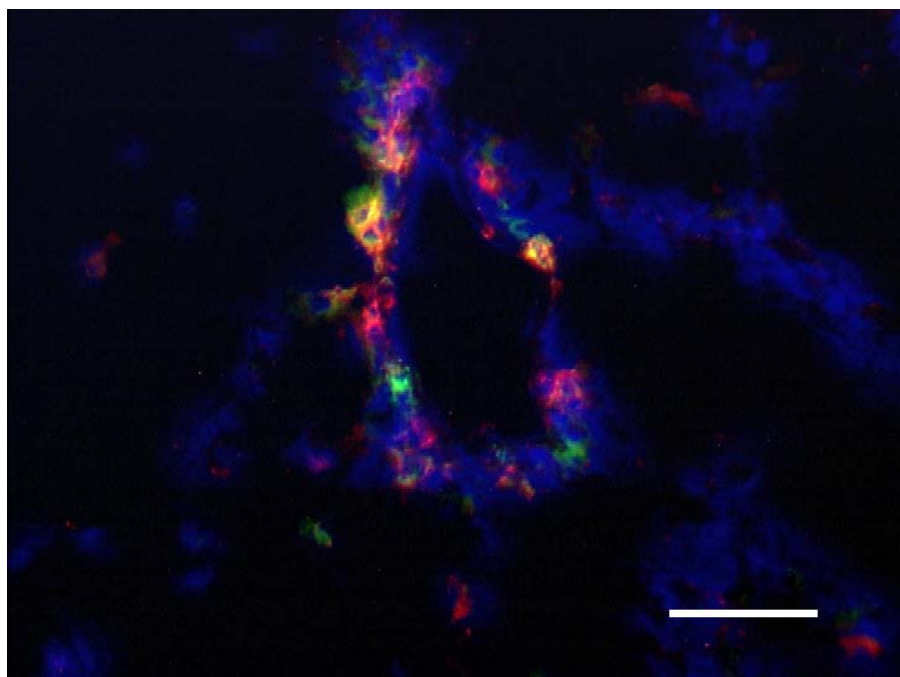
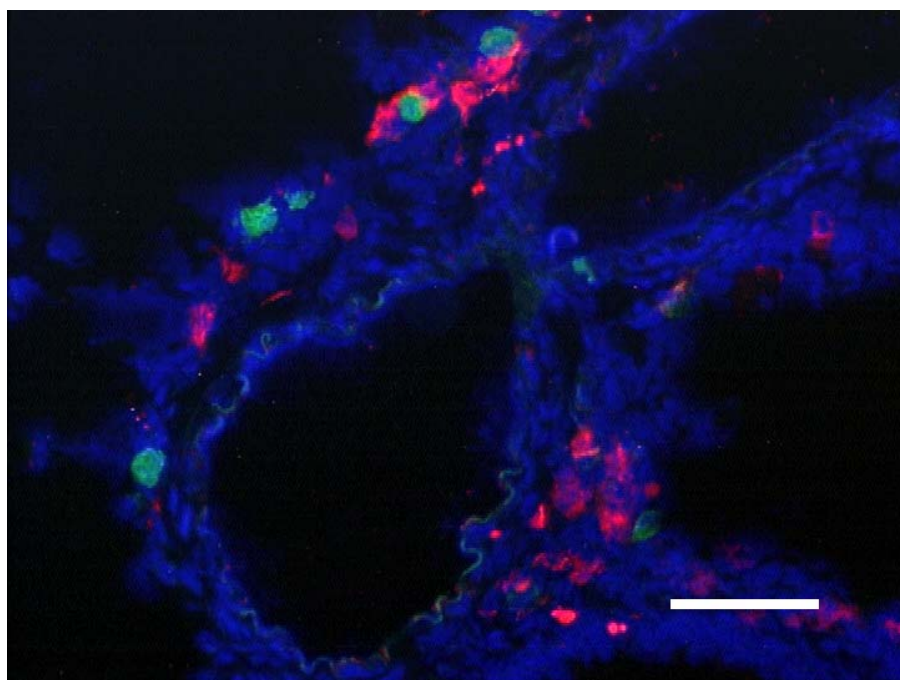
OX-62+ rat DCs were only detected in bronchus-associated lymphatic tissue of control lungs (A) (x10), while presenting a network-like organization in the adventitia of affected arteries in monocrotaline-exposed rats (B) (x20). Recruited OX-62+ DC (red) were distinct from the medial smooth muscle cells (green) (C) (x40), but also from macrophagic populations (green) (D) (x40), rat monocytic/lymphocytic cells and DCs CD4+ subsets (green) (E) (x40). OX-62 (red) and MHC class II (green) were occasionally coexpressed (yellow staining) highlighting different

maturation stages or different DC subpopulations (F) (x40). In A, internal scale bar=200 μm , in B internal scale bar = 100 μm , in C, D, E and F, internal scale bar=25 μm .









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