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Effect of irradiation/bone marrow transplantation on alveolar epithelial type II cells is aggravated in surfactant protein D deficient mice

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Abstract:	Irradiation followed by bone marrow-transplantation (BM-Tx) is a frequent therapeutic intervention causing pathology to the lung. Although alveolar epithelial type II (AE2) cells are essential for lung function and are damaged by irradiation, the long-term consequences of irradiation and BM-Tx are not well characterized. In addition, it is unknown whether surfactant protein D (SP-D) influences the response of AE2 cells to the injurious events. Therefore, wildtype (WT) and SP-D-/- mice were subjected to a myeloablative whole body irradiation dose of 8 Gy and subsequent BM-Tx and compared with age- and sex-matched untreated controls. AE2 cell changes were investigated quantitatively by design-based stereology. Compared with WT, untreated SP-D-/- mice showed a higher number of larger sized AE2 cells and a greater amount of surfactant-storing lamellar bodies. Irradiation and BM-Tx induced hyperplasia and hypertrophy in WT and SP-D-/- mice as well as the formation of giant lamellar bodies. The experimentally induced alterations were more severe in the SP-D-/- than in the WT mice, particularly with respect to the surfactant-storing lamellar bodies which were sometimes extremely enlarged in SP-D-/- mice. In conclusion, irradiation and BM-Tx have profound long-term effects on AE2 cells and their lamellar bodies. These data may explain some of the clinical pulmonary consequences of this procedure. The data should also be taken into account when BM-Tx is used as an experimental procedure to investigate the impact of bone-marrow derived cells for the phenotype of a specific genotype in the mouse.
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Author Comments:	Dear editor, dear Dr. Schrader, we wish to thank you for the opportunity to submit a revised version of our manuscript entitled "Effect of irradiation/bone marrow transplantation on alveolar epithelial type II cells is aggravated in surfactant protein D deficient mice". We also thank the reviewers for critically reviewing the manuscript. Reviewer #1 has raised an abundant number of interesting questions and suggestions for improving the manuscript. With respect to the animal model, we have analyzed the fibrotic response to the irradiation and bone marrow transplantation and have included these data to demonstrate that - besides the AE2 cell alterations - there is also a fibrotic remodeling. Apart from that we feel that it is beyond the scope of the current report to include further animal models (heterozygous SP-D mice and different mouse strains). Several concerns of this reviewer were related to incomplete or not fully understandable description of methods and figures. We have addressed these concerns thoroughly as detailed below. Reviewer #2 has asked for several additional images and further data on the phenotype of the SP-D deficient mice. We have tried to include as many of these suggestions as possible. For example, we have included two new figure panels (low power LM micrographs and EM micrographs), new data on the architecture of the lungs (including volume, surface area and number of alveoli as well as number of alveolar macrophages). The extensive inclusion of these data made it necessary to include a further co-author (Dr. Julia Schipke). We feel that the manuscript has been substantially improved by addressing the reviewer comments and hope that our revised manuscript is now acceptable for publication in Histochemistry and Cell Biology. Yours sincerely, Christian Mühlfeld

Effect of irradiation/bone marrow transplantation on alveolar epithelial type II cells is aggravated in surfactant protein D deficient mice

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Running title: Mouse lung and irradiation/BM-Tx

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Summary statement

This study shows that irradiation and bone marrow transplantation have profound effects on the pulmonary surfactant system – besides clinical implications this is important for conclusions drawn from animal studies.

Abstract

Irradiation followed by bone marrow-transplantation (BM-Tx) is a frequent therapeutic intervention causing pathology to the lung. Although alveolar epithelial type II (AE2) cells are essential for lung function and are damaged by irradiation, the long-term consequences of irradiation and BM-Tx are not well characterized. In addition, it is unknown whether surfactant protein D (SP-D) influences the response of AE2 cells to the injurious events.

Therefore, wildtype (WT) and SP-D-/- mice were subjected to a myeloablative whole body irradiation dose of 8 Gy and subsequent BM-Tx and compared with age- and sex-matched untreated controls. AE2 cell changes were investigated quantitatively by design-based stereology.

Compared with WT, untreated SP-D$^{-/-}$ mice showed a higher number of larger sized AE2 cells and a greater amount of surfactant-storing lamellar bodies. Irradiation and BM-Tx induced hyperplasia and hypertrophy in WT and SP-D$^{-/-}$ mice as well as the formation of giant lamellar bodies. The experimentally induced alterations were more severe in the SP-D$^{-/-}$ than in the WT mice, particularly with respect to the surfactant-storing lamellar bodies which were sometimes extremely enlarged in SP-D$^{-/-}$ mice.

In conclusion, irradiation and BM-Tx have profound long-term effects on AE2 cells and their lamellar bodies. These data may explain some of the clinical pulmonary consequences of this procedure. The data should also be taken into account when BM-Tx is used as an experimental procedure to investigate the impact of bone-marrow derived cells for the phenotype of a specific genotype in the mouse.

Keywords: irradiation, bone marrow transplantation, design-based stereology, alveolar epithelium, surfactant

Introduction

Bone marrow transplantation (BM-Tx) following whole body irradiation is a frequently performed therapeutic intervention in haematological malignancies both in children and adults (Keane et al. 1981; Sampath et al. 2005). Besides irradiation, myeloablation before BM-Tx can be performed by chemotherapeutics such as busulphan or cyclophosphamide (Lund et al. 2004). While this is often the only life-saving option for the patients, its beneficial effects are often counter-balanced by the damage of other organs, in particular the lung. In animal experiments, the sequence of irradiation and BM-Tx is sometimes used to investigate the impact of bone marrow-derived cells for the development of a specific phenotype in genetically altered organisms (Nishinakamura et al. 1996; Cooke et al. 1997). However, the procedure itself may lead to changes in the target organ that are independent of the investigated genotype.

The lung is highly susceptible to irradiation with acute lung injury/pneumonitis and fibrosis leading to functional impairment often after a latent period (Gross 1977). Among the cells damaged by irradiation are alveolar epithelial type II (AE2) cells which serve two major functions in the alveolar region of the lung: First, they produce, store, secrete and recycle pulmonary surfactant which consists of 90 % lipids and 10 % proteins. The protein fraction of surfactant ~~consists~~includes ~~of~~ the four surfactant proteins (SP) SP-A, SP-B, SP-C and SP-D. Second, AE2 cells are progenitor cells for the terminally differentiated alveolar epithelial type I (AE1) cells. Thus, lung function critically depends on the structural and functional preservation of the AE2 cells (Fehrenbach 2001; Ochs 2010).

In fact, AE2 cells are among the first cells that show alterations after irradiation characterized by a loss of lamellar bodies and later degeneration (Penney and Rubin 1977; Penney et al. 1982). The decrease in cellular lamellar bodies was shown to be accompanied by a greater amount of surfactant in the bronchoalveolar lavage (Rubin et al. 1983). At later stages after the radiation insult AE2 cells seem to over compensate the loss of surfactant material suggested by a greater number of lamellar bodies (Penney and Rubin 1977). *In vitro* irradiation of isolated AE2 cells showed a biphasic reaction with a normal secretory activity early after irradiation and an enhanced response to surfactant secretagogues at later time points (Willner et al. 2003). Similar results had already been reported by Shapiro et al. (1984) who concluded that the effect of irradiation on AE2 cells *in vivo* is at least in part direct and not only a response to the damage of AE1 cells. Reports about injury of AE1 cells and endothelial cells are heterogeneous with only mild (Penney et al. 1982; Ward et al. 1993;

Franko et al. 1996) or severe changes (de Saint-Georges et al. 1988; Ward et al. 1993) indicative of acute lung injury. The different observations of the above mentioned studies are related to the dose, the mode (hemi- or bilateral thoracic or whole body irradiation, fractionated or single dose) and the time points of investigation after the injurious event. In addition, great variations exist among the investigated species and even within one species with respect to the strain (Down and Yanch 2010; Jackson et al. 2010). Besides surfactant lipids, surfactant proteins are also influenced by irradiation. For example, in minipigs a single irradiation dose of 15 Gy induced a reduction of SP-A expression (Yue et al. 2015) whereas pro-SP-C was enhanced both by a single 12 Gy irradiation or a fractionated 30 Gy dose in mice (Almeida et al. 2013). Very recently, it was demonstrated that the inflammatory response of SP-D deficient mice to radiation injury is significantly higher than in WT mice (Malaviya et al. 2015).

~~The protein fraction of surfactant consists of the four surfactant proteins (SP) SP-A, SP-B, SP-C and SP-D.~~ SP-D is a 43 kDa member of the collectin family of collagen and lectin domain-containing proteins which contribute to innate host defense. In mouse and human lungs, SP-D is predominantly expressed by non-ciliated bronchiolar epithelial cells and AE2 cells (Akiyama et al. 2002; Madsen et al. 2000; Stahlman et al. 2002). The pulmonary functions attributed to SP-D include opsonisation and increased phagocytosis of viral, bacterial and fungal pathogens by alveolar macrophages and polymorphonuclear cells (Crouch and Wright 2001; Hawgood and Poulain 2001; Whitsett 2005), modulation of the pulmonary immune response (Madan et al., 2001), regulation of surfactant catabolism by AE2 cells (Ikegami et al. 2005), and homeostasis of AE2 cells and alveolar macrophages (Botas et al. 1998).

The phenotype seen in gene-targeted mice deficient in SP-D (SP-D$^{-/-}$) is associated with surfactant abnormalities including AE2 cell hypertrophy and hyperplasia, the occurrence of giant lamellar bodies in AE2 cells (Botas et al. 1998; Korfhagen et al. 1998), and intra-alveolar accumulation of surfactant phospholipids (Ikegami et al. 2000) as well as the accumulation of foamy AMs in the alveolar airspaces (Botas et al. 1998; Korfhagen et al. 1998; Wert et al. 2000). Other biochemical features of SP-D deficiency include increased expression of matrix metalloproteinases (MMP) and elevated generation of oxidants (Wert et al. 2000), multiple alterations in surfactant metabolism (Ikegami et al. 2000) and nitric oxide metabolism (Atochina et al. 2004). ~~Very recently, it was demonstrated that the inflammatory~~

~~response of SP-D deficient mice to radiation injury is significantly higher than in WT mice (Malaviya et al. 2015).~~

Therefore, the present study had the following objectives: 1) to characterize the effects of the sequence of irradiation and BM-Tx on AE2 cells and their surfactant system by design-based quantitative methods and 2) to test the hypothesis that AE2 cells and surfactant alterations induced by irradiation/BM-Tx are more pronounced in SP-D deficient than in wildtype mice.

Material and Methods

Standard homologous techniques were used to generate mice deficient in SP-D as previously described. The deficient mice were imported from the laboratory of Professor Sam Hawgood, University of California, San Francisco. The SP-D knock-out mice, back-crossed more than 10 generations into a C57BL/6 background were fed ad libitum and housed in isolators in a pathogen free environment in the Biomedical Research Facility at University of Southampton. C57BL/6 wild-type age and sex matched controls were obtained from Harlan-OLAC (Bicester, Oxfordshire, UK). Eight-week old C57BL/6 and SP-$D^{-/-}$ male mice were irradiated at 8 Gy using a 137Cs gamma-ray source and reconstituted with ~~10^7~~ bone marrow cells from age- and sex-matched SP-$D^{-/-}$ and C57BL/6 mice, respectively. Briefly, the bone marrow cells were washed-out from the bones using a 25-gauge needle attached to a 1 ml syringe containing DMEM medium supplemented with 10% FCS. Red cells were lysed by adding 1 ml of RBC lysis buffer. Cells were spun, counted and re-suspended in sterile PBS. A total of 10^7 bone marrow cells in a volume of 150 µl were injected intravenously into the tail vein in each mouse. Mice were kept under sterile conditions during the first two months after the bone marrow transplantation. Successful haemopoietic lineage reconstitution was confirmed by SP-D specific PCR of DNA isolated from peripheral blood. The primers used were: Shared sense: TGG TTT CTG AGA TGG GAG TCG TG; WT antisense: TGG GGC AGT GGA TGG AGT GTG C; ~~DKO~~SP-$D^{-/-}$ (neo) antisense: GTG GAT GTG GAA TGT GTG CGA G.~~.~~ The mice were sacrificed at 5 months and the lungs were fixed as described below. All experimental protocols were approved by appropriate U.K. Home-Office licensing authorities and by the University of Southampton ethical committee.

~~*Genotyping*~~

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~~DNA was extracted from snap frozen blood by using a DNeasy kit from Qiagen (Qiagen, Crawley, UK) according to the manufacturer's instructions. PCR based genotyping was carried out according to the manufacturer's instructions for Platinum Taq DNA polymerase (Invitrogen, Paisley, UK). 1 µL of DNA (approximately 50 ng/µl) was used as template. The reaction contained 1.5 mM MgCl2, 0.2 µM of each shared sense primer, wild-type anti-sense primer and SP-D knock-out anti-sense primer in a volume of 25 µL as described previously (Botas et al., 1998). The primers used were: Shared sense: TGC TTT CTC ACA TGC CAC TGC TG; WT antisense: TGC GGC AGT GGA TGC AGT GTG C; DKO (neo) antisense: GTG GAT GTG GAA TGT GTG CGA G. The PCR reaction was carried out at 94° C for 1 minute then 30 cycles each of 94° C, 57° C, and 72° C, 30 seconds for each temperature followed by a final 72° C step for 10 minutes. The amplified RT-PCR products were analyzed using a 1.5% agarose gel. The amplified products were 377 bp for wild-type mice and 192 bp for SP-D knock-out mice.~~

Fixation, sampling and processing

At the age of 5 months, all animals were sacrificed by schedule 1 method, the thorax was opened and the lungs were instillation-fixed via a tracheal cannula at a hydrostatic pressure of 20 cm H_2O using a mixture containing 1.5% paraformaldehyde/1.5% glutaraldehyde in 0.15 M Hepes buffer (Fehrenbach and Ochs 1998; Mühlfeld et al. 2007). After storage of the lungs in fresh fixative at 4°C, the lungs were sampled for stereological analysis as described previously (Ochs 2006; Mühlfeld et al. 2013). In short, the total volume of the lungs was first measured using Scherle's method which is based on Archimedes' principle (Scherle 1970). The method was applied in water. Afterwards, the lungs were embedded in agar and cut from base to apex into tissue slices of 2 mm average thickness. Starting randomly with the first or second slice, every second slice was sampled for embedding in glycol methacrylate for ~~qualitative~~ light microscopy (Technovit 7100, Heraeus Kulzer, Wehrheim, Germany). The remaining slices were further sampled by projecting a point grid onto the slices and taking a sample from each position hit by one of the points. These samples were further embedded in epoxy resin for light and electron microscopic stereology.

Stereological analysis

In order to ensure that every part of the lungs had an equal chance of being selected and therefore of being represented in the investigation, systematic uniform random sampling was applied at all subsequent stages of microscopic analysis. Investigations were carried out using

a Leica DM6000B light microscope (Leica, Germany) equipped with a computer-assisted stereology system (newCAST, Visiopharm, Denmark) and a Morgagni transmission electron microscope (FEI, Netherlands), respectively.

Three epoxy resin embedded tissue blocks were randomly chosen for sectioning. From each tissue block, the first and fourth section of a consecutive row of 1 µm semithin sections were mounted on one glass slide and stained with methylene blue. These sections were used as physical disector pairs with a disector height of 3 µm. At an oil immersion objective magnification of 63x, the numerical density (N_V(AE2/lung)), the total number (N(AE2,lung)) and the number-weighted mean volume ($\bar{v}_N(AE2)$) of AE2 cells based on nucleoli sampling were estimated (Sterio, 1984; Ochs, 2006). The number-weighted mean volume of AE2 cells was estimated using the rotator for isotropic uniform random sections (Vedel-Jensen and Gundersen 1993). Randomly sampled and corresponding test fields from both sections of the disector pair were analysed by comparing the presence of the nucleolus within the nucleus of the AE2 cells. Whenever the nucleolus was present in one of the sections but not in the other, the cell was counted (cell number) and sampled for the use of the rotator (cell volume) according to the principles of design-based stereology (Ochs and Mühlfeld 2013, Mühlfeld and Ochs 2013).

After cutting of the semithin sections, ultrathin sections (40-60 nm) were prepared for transmission electron microscopy. At a primary magnification of 8900x, systematic uniform random sampling was performed to gather digital images of AE2 cells which were subsequently used to estimate the volume of lamellar bodies per AE2 cell (V(lb,AE2)) by point counting (Weibel 1979) and the volume-weighted mean volume of lamellar bodies $\bar{v}_V(lb)$ by the point-sampled intercepts method as described previously (Jung et al. 2005; Ochs et al. 2004a,b). In short, a point grid was projected onto the test field and the number of points hitting lamellar bodies as well as the number of points hitting the reference volume (i.e. AE2 cell including lamellar bodies) was counted. The volume fraction of lamellar bodies in AE2 cells was calculated by dividing the number of points hitting lamellar bodies by the number of points hitting the reference volume. To obtain the mean total volume per AE2 cell, the volume fraction was multiplied by the mean volume of AE2 cells obtained by the rotator. Whenever a point hit a lamellar body a ruler divided into 15 classes was used to measure the diameter of the lamellar body at that point. From the relative frequency of diameters the volume-weighted mean volume of the lamellar bodies was calculated (Mühlfeld et al. 2013).

From the samples embedded in glycol methacrylate, a consecutive row of 1.5 µm thick sections was cut and the first and third section of a row were mounted on a glass slide and stained by orcein and haematoxylin and eosin simultaneously or by toluidine blue. On orcein and haematoxylin/eosin stained sections, using a grid consisting of points and line segments, the volume and surface area of the alveoli was estimated by counting the number of points hitting alveolar lumen and interalveolar septa as well as the number of intersections of the line segments with the alveolar septa. The volume fraction of alveolar lumen and septa was calculated as described above. The surface density of the interalveolar septa was calculated according to $S_V=2*I/L_T$ where I is the number of intersections and L_T is the total length of line segments hitting the reference volume. Volume and surface fractions were multiplied by the reference volume to obtain total data for the lungs. The mean septal thickness of alveolar septa (alvsept) was obtained by τ (alvsept) = 2*V(alvsept,lung)/S(alv,lung). The number of alveoli was estimated by using the sections as a disector pair and by estimating the Euler Poincaré characteristic of the alveolar opening rings. From this the number of alveoli was calculated as described previously (Ochs et al. 2004b, Willführ et al. 2015). On toluidine blue stained sections, the number of alveolar macrophages was estimated using the physical disector (Sterio 1984). The procedure was the same as described for AE2 cells except that the nucleus was used as the counting event.

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Statistics

Stereological data and cell counts are given as "mean (standard deviation)". The data were evaluated by Kruskal-Wallis test, followed by the two-sided nonparametric Mann-Whitney u-test using GraphPad Prism 4.0. Data were regarded statistically significant when $p < 0.05$.

Results

Genotyping

The PCR based genotyping confirmed that BM-Tx WT mice received bone marrow from WT mice giving rise to a single band of 377 bp and BM-Tx DKO mice received bone marrow from DKO mice giving rise to a single band of 192 bp.

Qualitative observations

Fig. 1 provides an overview on the general architecture of the gas-exchange region of the lungs. **Figs. 2~~1~~** and 3 display~~s~~ the characteristic alterations of AE2 cells at light and electron microscopic level and~~.~~ **Fig. 4~~2~~** shows the morphology of alveolar macrophages. The lungs of

WT mice showed a normal appearance with thin alveolar septa and normally sized AE2 cells in the corners of the alveoli. AE2 cell profiles were more frequently seen and appeared to be enlarged in SP-D$^{-/-}$ mice compared with the WT. At EM level, the increased size of AE2 cells and their lamellar bodies after BM-Tx was clearly visible, particularly in the SP-D$^{-/-}$ mice. Alveolar macrophages were present in WT lung sections and were slightly larger than the AE2 cells. They could be detected by their small cytoplasmic protrusions and their localization within the lumen of the alveoli. In SP-D-/- mice, alveolar macrophages were more abundant and enlarged with sometimes foamy appearance. After BM-Tx, the number of alveolar macrophage profiles was strongly enhanced, sometimes forming clusters that filled the alveolar lumen. They were large in size, filled with a great number of vacuoles which gave them a foamy morphology. These alterations were much more pronounced in SP-D$^{-/-}$ than in WT mice.

Stereological data

The stereological data are shown in **Tables 1 and 2** and **Fig. ~~3~~5.** SP-D$^{-/-}$ mice showed the typical AE2 cell phenotype compared with WT mice with a higher number (p=0.0087) of larger AE2 cells (p=0.0043). The larger size was accompanied by a greater volume (p=0.0022) of larger lamellar bodies (p=0.0022). In addition, SP-D$^{-/-}$ mice had a greater number of alveolar macrophages than WT (p=0.041). However, the emphysematous phenotype reported for SP-D$^{-/-}$ mice was not present in the investigated mice as neither volume, surface area nor number of alveoli differed between the untreated groups. By the same token, no sign of fibrosis was present as indicated by the mean septal thickness. Only the number-weighted mean volume of alveoli was higher in SP-D$^{-/-}$ mice. Irradiation and BM-Tx induced hypertrophy and hyperplasia as well as the occurrence of giant lamellar bodies in the lungs of WT mice. The stereological data of this group were similar as the untreated SP-D$^{-/-}$ mice. The same pattern of alterations was observed in the SP-D$^{-/-}$ mice, however, only the mean AE2 cell volume and the surfactant changes reached the level of significance, whereas no significant increase in the number of AE cells was observed in bone marrow transplanted SP-D$^{-/-}$ mice. However, the increase in lamellar body size and size variation after irradiation and BM-Tx was significantly larger in the SP-D$^{-/-}$ mice. WT mice had an increased number of alveolar macrophages after BM-Tx than the untreated WT animals (p=0.0095) whereas no significant increase due to BM-Tx was observed in the SP-D$^{-/-}$ mice. Neither volume, surface area nor total volume of alveoli were affected by BM-Tx. The number-weighted mean volume of alveoli was slightly decreased in WT mice after BM-Tx. However, an equally strong

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increase in the mean septal thickness occurred after BM-Tx in both genotypes indicating a fibrotic response.

Discussion

The present study shows that long-term effects of the sequence of BM-Tx following myeloablative irradiation on AE2 cells of the lung occur in WT and SP-D$^{-/-}$ mice. The effects are characterized by both hypertrophy and hyperplasia of AE2 cells. The hypertrophic response can at least partly be explained by a greater amount of larger sized surfactant-storing lamellar bodies. The effects, especially the alterations of lamellar bodies, were significantly more pronounced in SP-D$^{-/-}$ than in WT mice. In addition, a fibrotic response of equal extent was present in the interalveolar septa of both genotypes. These data are of importance when BM-Tx is used as an experimental intervention, for example to investigate the impact of bone marrow-derived cells (such as alveolar macrophages) for the lung phenotype of a genetically manipulated mouse. In addition, the disturbance of the AE2 cell/surfactant system may help to understand the long-term pulmonary pathology and symptoms observed in patients undergoing irradiation/BM-Tx. As the SP-D$^{-/-}$ mice used here as well as a majority of other genetically altered mice carry the C57BL/6 genetic background the experiments were performed in this mouse strain although it may not be the most appropriate mouse strain to simulate the response of the human lung to irradiation/BM-Tx (Jackson et al. 2012).

Hyperplasia (defined as an increased number) and hypertrophy (defined as an increased size) are frequent and unspecific reactions of AE2 cells in a variety of experimental settings which seem to be related to a persistent pulmonary inflammation (Miller and Hook 1990). We used design-based stereological methods (physical disector in combination with the rotator) to estimate these AE2 cell number and size as these methods provide unbiased estimates which do not rely on any assumptions about shape, size or distribution of the structures of interest (Ochs and Mühlfeld 2013; Mühlfeld and Ochs 2013). In addition, a sample processing and embedding protocol was used that leads to minimal global tissue deformation (Schneider and Ochs 2014), thus adding to the accuracy of the data. For the evaluation of lamellar bodies by transmission electron microscopy, we used an optimized fixation protocol (including prolonged incubation with half-saturated uranyl acetate) which helps to preserve the surfactant lipid lamellae (Fehrenbach and Ochs 1998). Besides total volume of lamellar bodies, the volume-weighted mean volume was estimated which may not be interpreted as the (arithmetic) mean volume of the lamellar bodies because it contains two sources of information: mean volume and volume variation (Ochs 2006). One limitation of the animal

model is that the efficacy of the myeloablation was not investigated which means that a certain degree of chimerism may be present. However, since donor and recipient were always of the same genotype, we consider this to be of minor importance for the conclusions that are drawn from the data.

The data on untreated WT and SP-D$^{-/-}$ mice differ from previous work (e.g. Ochs et al. 2004a) with respect to the emphysema pathology which may be due to a different genetic background. The original genotype was developed in an outbred C57BL6/CD-1 background whereas the SP-D-/- mice used in this study were backcrossed for at least 10 generations in a C57BL/6 background. In contrast, our data confirm the AE2 cell phenotype with increased AE2 cell number and size and increased lamellar body content and size in SP-D$^{-/-}$ (Ochs et al. 2004a, Knudsen et al. 2007). The reasons for the AE2 cell phenotype in SP-D$^{-/-}$ mice are still unclear. Recently, it was tested whether the increased levels of reactive nitrogen species in the SP-D$^{-/-}$ mice were the reason for the pulmonary phenotype in SP-D$^{-/-}$ mice (Knudsen et al. 2014). Although the report showed that a loss of inducible nitric oxide synthase (iNOS) alleviated some of the phenotypic alterations in the SP-D$^{-/-}$ mouse (e.g. emphysema pathology) it failed to affect the AE2 cell changes indicating that these are not caused by reactive nitrogen species. On the contrary, investigations of SP-D and granulocyte/monocyte colony-stimulating factor (GM-CSF) single and double knockout mice have demonstrated that GM-CSF mediates either directly or indirectly the AE2 cell changes in the SP-D$^{-/-}$ mouse but not the emphysema pathology (Ochs et al. 2004a), thus highlighting the role of GM-CSF in surfactant metabolism (Reed and Whitsett 1998). In this regard, it is important to note that alveolar macrophages have been shown to overproduce GM-CSF after bone marrow transplantation in WT mice (Ballinger et al. 2008; Fedorocko et al. 2002), a reaction which is more pronounced in SP-D$^{-/-}$ mice and leads to a more severe inflammation due to BM-Tx in SP-D$^{-/-}$ mice which could be alleviated by exogenous SP-D (Gram et al. 2009).

In the WT mouse, irradiation and subsequent BM-Tx led to hyperplasia and hypertrophy of AE2 cells and an increase in size and amount of lamellar bodies. These observations are in accordance with several previous reports (Penney and Rubin 1977; Penney et al. 1982). Most authors agree that the initial response of the AE2 cell to irradiation is a hypersecretion of surfactant into the alveoli (combined with a loss of intra-cellular material) and a later over-compensation by an increased surfactant production and/or higher number of AE2 cells (Rubin et al. 1983; Penney and Rubin 1977; Willner et al. 2003; Shapiro et al. 1984; Almeida et al. 2013). The stereological analysis in this study was performed at 5 months after irradiation and BM-Tx and the observed effects are similar as the late effects observed after

irradiation alone. BM-Tx has been reported to be accompanied by a nearly 80% replacement of alveolar macrophages from the new bone marrow (Hubbard et al. 2008) and also by a significant replacement of alveolar epithelial cells, however, there is an equal number of studies in favour or against the contribution of bone marrow cells to the lung epithelium (see e.g. Theise et al. 2002; Bernard et al. 2012; Herzog et al. 2006; Chang et al. 2005 and references therein). Thus, some of the effects observed in the present study may either be regarded as a long-term irradiation effect or as a consequence of BM-Tx. It is not possible to discriminate the contribution of each of these factors to the observed pathology as a study group of mice treated by myeloablative irradiation without subsequent BM-Tx would not be viable.

As the lungs of SP-D$^{-/-}$ mice are known to be in a chronic inflammatory state (Wert et al. 2000; Atochina et al. 2004), we had hypothesized that changes observed in the WT after irradiation/BM-Tx would be enhanced in the SP-D deficient mouse. In fact, the stereological data not only support this hypothesis but also provide evidence for an extreme reaction of the intracellular surfactant pool with extraordinarily sized giant lamellar bodies and an overall particularly large size distribution. As mentioned above, the enhanced iNOS levels in SP-D$^{-/-}$ mice do not seem to explain the AE2 cell phenotype (Knudsen et al. 2014), hence it is unlikely that the elevated iNOS levels upon radiation (Giaid et al. 2003) contribute to the observed AE2 cell/lamellar body pathology. Interestingly, a recent study investigated the effects of irradiation on lung injury, oxidative stress and the expression of pro-inflammatory mediators in SP-D$^{-/-}$, iNOS$^{-/-}$ and SP-D$^{-/-}$ iNOS$^{-/-}$ mice (Malaviya et al. 2015). These authors provided evidence that the degree of lung injury and oxidative stress were similar in SP-D$^{-/-}$ and SP-D$^{-/-}$ iNOS$^{-/-}$ mice whereas pro-inflammatory mediator expression was reduced when both SP-D and iNOS were lacking. The study by Malaviya et al. (2015) supports the notion that other mechanisms than iNOS induced inflammation confers the AE2 cell alterations observed in the present study. ~~In contrast, I~~it is tempting to speculate that GM-CSF confers the AE2 cell and surfactant changes after BM-Tx as it is known to be involved in the surfactant alterations in the SP-D$^{-/-}$ mouse (Ochs et al. 2004a) and also to be elevated after irradiation/BM-Tx.

When comparing the degree of change between WT and WT BM-Tx on the one hand, and SP-D-/- and SP-D$^{-/-}$ BM-Tx on the other hand, it becomes apparent that number and mean volume of AE2 cells as well as total volume of lamellar bodies were increased by a similar factor in WT and SP-D$^{-/-}$ after irradiation and BM-Tx (approximately between 1.4 and 2-fold depending on the parameter). The volume-weighted mean volume of lamellar bodies,

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however, showed a 3.5 fold increase in WT mice and a 131 fold increase in SP-D$^{-/-}$ mice. These data not only underline that the procedure of irradiation and BM-Tx leads to profound pulmonary long-term effects but also that the degree of these changes depends on the investigated phenotype. When the effect of bone marrow transplantation on the phenotype of a genetically altered mouse is investigated these results may be of great importance as a possible confounder.

Patients undergoing therapeutic irradiation and bone marrow transplantation often suffer from the pulmonary long-term effects, namely persistent pneumonitis and fibrosis. Recent animal studies (Lutz et al. 2015; Birkelbach et al. 2015) and older pathological reports (Burkhardt and Cottier 1989) support the concept of surfactant dysfunction in the pathogenesis of pulmonary fibrosis. With the observed changes, our study may therefore provide AE2 cells as a possible new therapeutic target in the treatment of irradiation/BM-Tx associated pulmonary side effects.

Acknowledgments

The authors wish to thank Olaf Bahlmann, ~~Julia Schipke,~~ Susanne Fassbender, Susanne Kuhlmann and Christa Lichtenberg for technical help.

Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1. Effects of irradiation/BM-Tx on alveolar architecture in WT and SP-D$^{-/-}$ mice.

Stereological parameter	WT n=6	BM-Tx (WT→WT) n=4	SP-D$^{-/-}$ n=6	BM-Tx (SP-D$^{-/-}$ →SP-D$^{-/-}$ n=4
N(alv,lung) [10^6]	2.32 (0.52)	2.49 (0.38)	2.49 (0.38)	2.54 (0.71)
$\bar{v}_N(alv)$ [µm³]	113 (14) a,b	90 (10)	137 (8)	127 (16)
V(alv,lung) [cm³]	0.266 (0.088)	0.225 (0.050)	0.343 (0.064)	0.329 (0.132)
S(alv,lung) [cm²]	307 (72)	327 (23)	372 (78)	336 (89)
τ (alvsept) [µm]	7.43 (1.79) b	13.94 (1.72)	7.04 (1.45) b	13.87 (1.26)

N(alv,lung), total number of alveoli in the lung, $\bar{v}_N(alv)$, number-weighted mean volume of alveoli, V(alv,lung), total volume of alveoli in the lung, S(alv,lung), total surface area of alveoli in the lung, τ (alvsept), arithmetic mean thickness of interalveolar septa, a, $p<0.05$ vs. SP-D-/-, b, $p<0.05$ vs. BM-Tx group of the same genotype.

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Table 2. Effects of irradiation/BM-Tx on ~~**Stereological data on**~~ **AE2 cells and their lamellar bodies as well as the number of alveolar macrophages in WT and SP-D$^{-/-}$ mice.**

Stereological parameter	WT n=6	BM-Tx (WT→WT) n=4	SP-D$^{-/-}$ n=6	BM-Tx (SP-D$^{-/-}$ →SP-D$^{-/-}$ n=4
$\bar{v}_N(AE2)$ [µm³]	487 (42) a,b	705 (90)	612 (92) b	1084 (122)
V_V(lb/AE2) [%]	15.3 (2.0) a,b	22.0 (2.9)	22.5 (0.7) b	32.0 (3.3)
V(lb,AE2) [µm³]	74.8 (15.5) a,b	156 (32.2)	137 (19.2) b	345 (38.3)
$\bar{v}_V(lb)$ [µm³]	0.53 (0.14) a,b	1.92 (0.44)	1.15 (0.27) b	150.4 (151.5)
N(AE2,lung) [*10^6]	2.68 (0.53) a,b	5.45 (1.32)	5.17 (1.24) b	6.73 (1.04)
N(mac,lung) [*10^6]	1.58 (0.34) a,b	2.25 (0.29)	2.07 (0.29)	2.63 (0.61)

$\bar{v}_N(AE2)$, number-weighted mean volume of AE2 cells, V_V(lb/AE2), volume fraction of lamellar bodies related to AE2 cells as reference volume, V(lb,AE2), total volume of lamellar bodies in AE2 cells, $\bar{v}_V(lb)$, volume-weighted mean volume of lamellar bodies, N(AE2, lung), total number of AE2 cells in the lung, N(mac,lung), total number of alveolar macrophages in the lung, a, $p<0.05$ vs. SP-D-/-, b, $p<0.05$ vs. BM-Tx group of the same genotype.~~.~~

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Figure legends.

Fig. 1. Effects of irradiation/BM-Tx on alveolar architecture in WT and SP-D$^{-/-}$ mice. Micrographs of each study group at 20x lens magnification. Haematoxylin and eosin staining, scale bar 200 µm. A: WT. B: WT→WT BM-Tx. C: SP-D$^{-/-}$. D: SP-D$^{-/-}$→ SP-D$^{-/-}$ BM-Tx. bro: bronchus, art: artery. Images were acquired using a Zeiss Axioscan.Z1 slide scanner.

Fig. 2~~1~~

Effects of irradiation/BM-Tx on alveolar type II cells and their microenvironment in WT and SP-D$^{-/-}$ mice.~~Morphology of AE2 cells~~. Micrographs of each study group at 40x lens magnification. Toluidine blue staining, scale bar ~~10~~ 20 µm. Some AE2 cells are indicated by arrows. A: WT. B: WT→WT BM-Tx. C: SP-D$^{-/-}$. D: SP-D$^{-/-}$→ SP-D$^{-/-}$ BM-Tx. Some AE2 cells were extremely enlarged with giant lamellar bodies in SP-D$^{-/-}$ mice after BM-Tx. Images were acquired using a Zeiss Axioscan.Z1 slide scanner.

Fig. 3

Effects of irradiation/BM-Tx on the ultrastructure of alveolar type II cells in WT and SP-D$^{-/-}$ mice. Micrographs of each study group at 4,400x primary magnification. Scale bar 5 µm. Some lamellar bodies are indicated by arrows. A: WT. B: WT→WT BM-Tx. C: SP-D$^{-/-}$. D: SP-D$^{-/-}$→ SP-D$^{-/-}$ BM-Tx. glb: giant lamellar body. Images were acquired using a FEI Morgani transmission electron microscope.

Fig. 4~~2~~

Effects of irradiation/BM-Tx on alveolar macrophages and their microenvironment in WT and SP-D$^{-/-}$ mice.~~Morphology of alveolar macrophages.~~
Micrographs of each study group at 20x lens magnification. Toluidine blue staining, scale bar ~~20~~ 50 µm. Some alveolar macrophages are indicated by arrows. A: WT. B: WT→WT BM-Tx. C: SP-D$^{-/-}$. D: SP-D$^{-/-}$→ SP-D$^{-/-}$ BM-Tx. Note the abundant and enlarged alveolar macrophages in untreated and transplanted SP-D$^{-/-}$ mice. Images were acquired using a Zeiss Axioscan.Z1 slide scanner.

Fig. 5~~3~~

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Scatterplots of the stereological data. A: Number of AE2 cells. B: Number-weighted mean volume of AE2 cells. C: Total volume of lamellar bodies. D: Volume-weighted mean volume of lamellar bodies. Note the logarithmic scale of the y axis in D. **, $p<0.01$. Circles and squares represent the data of the individual animals, horizontal bars show the group means.

Figure 1

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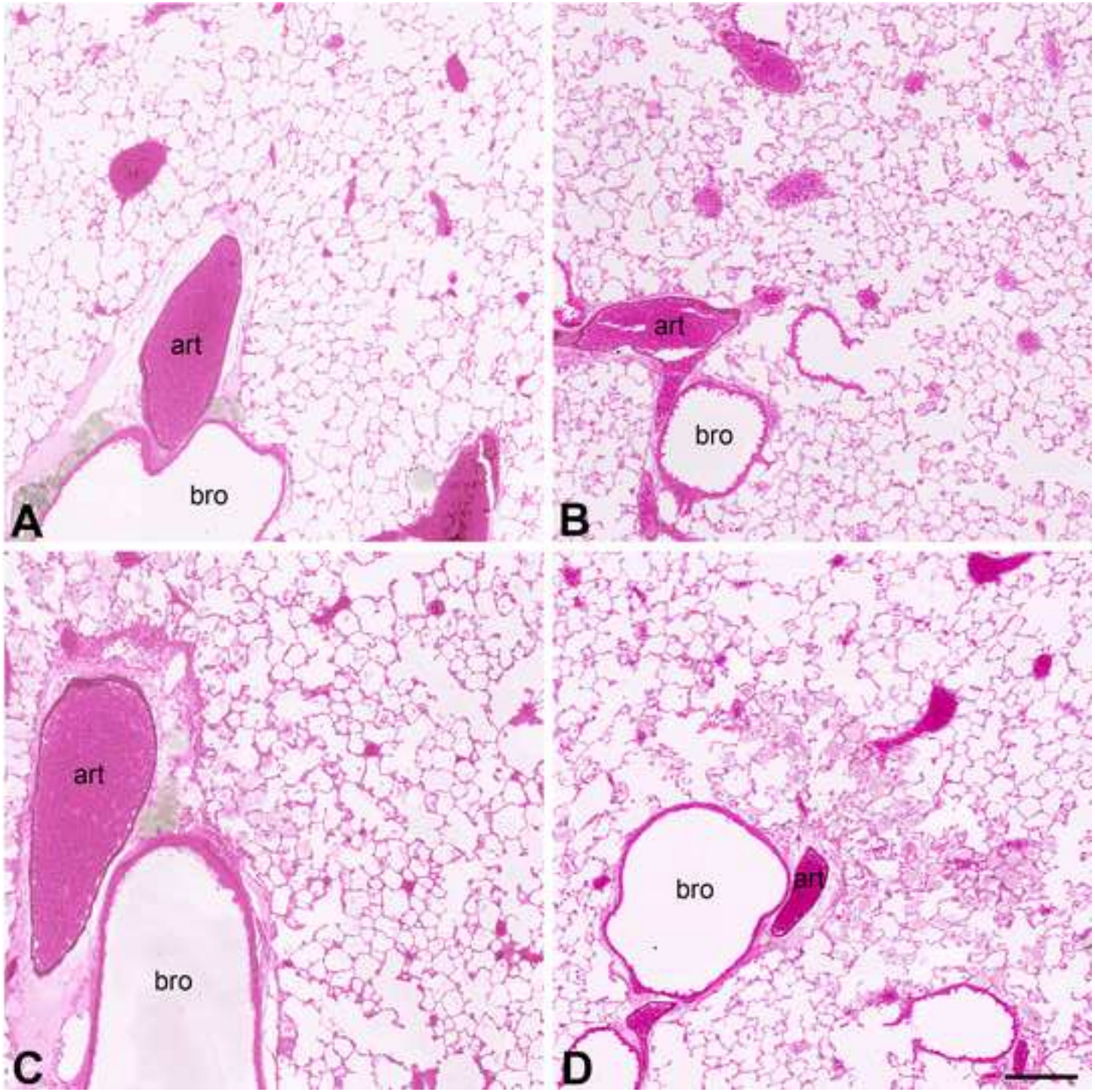

Figure 2

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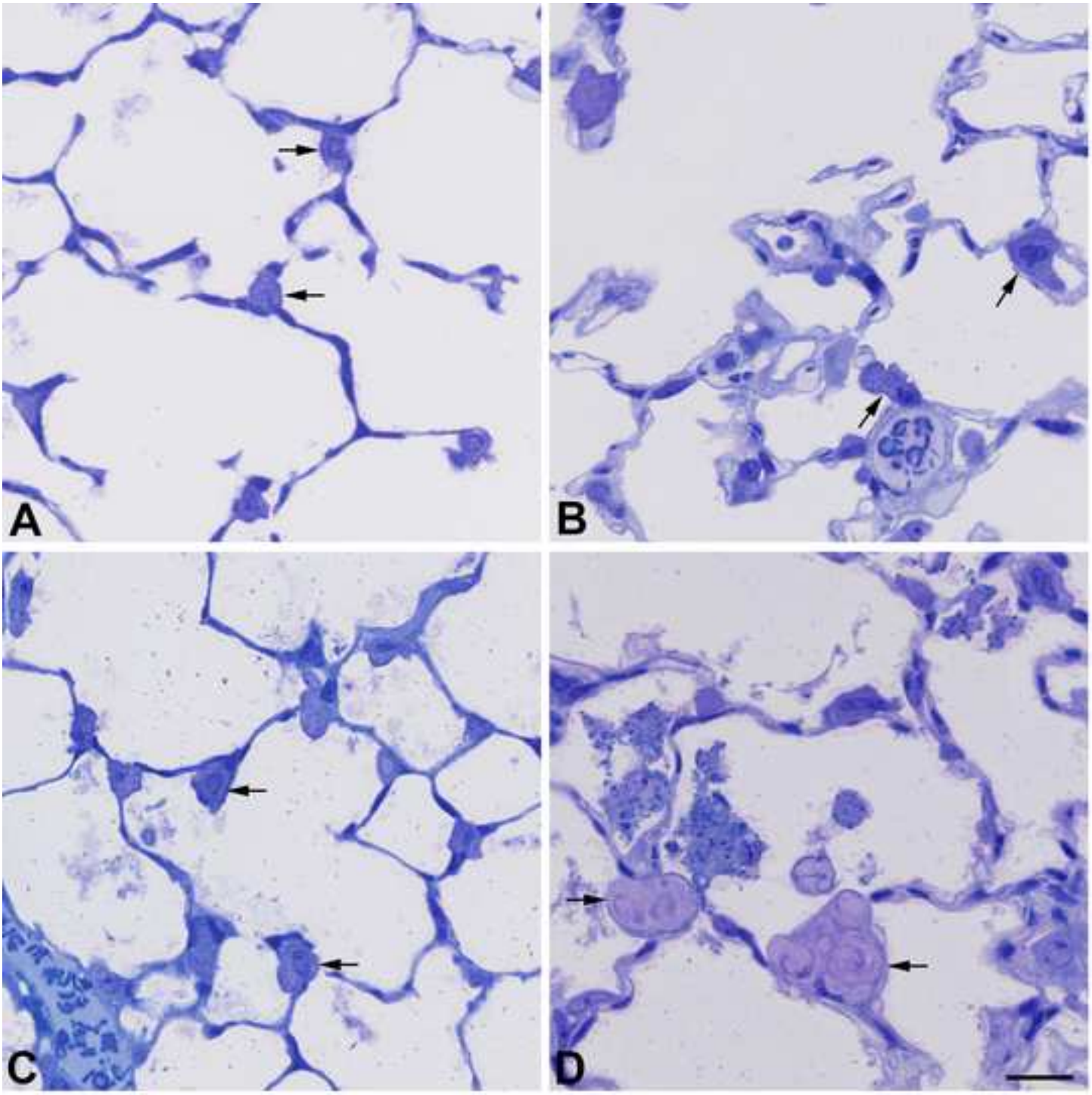

Figure 3

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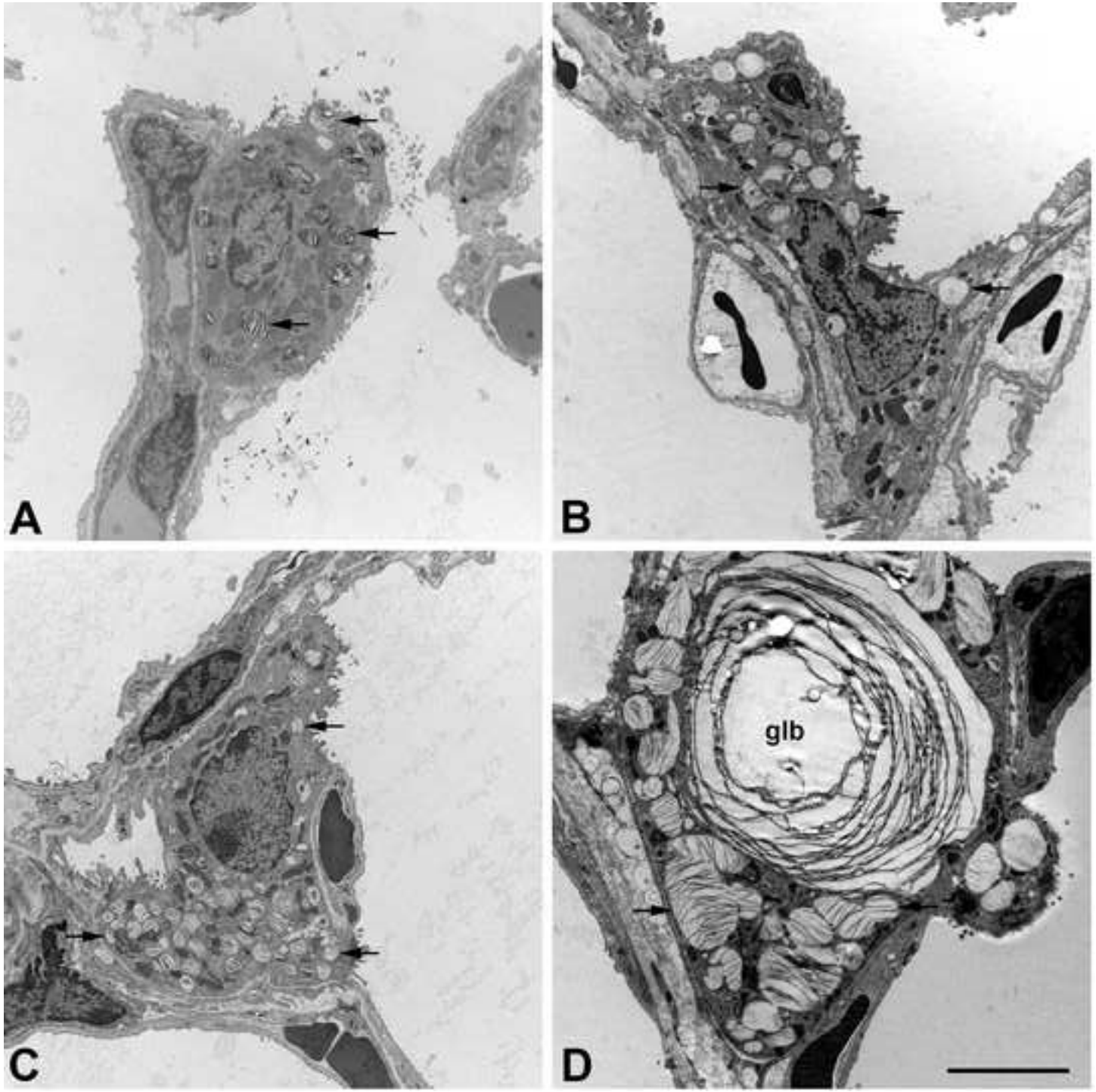

Figure 4

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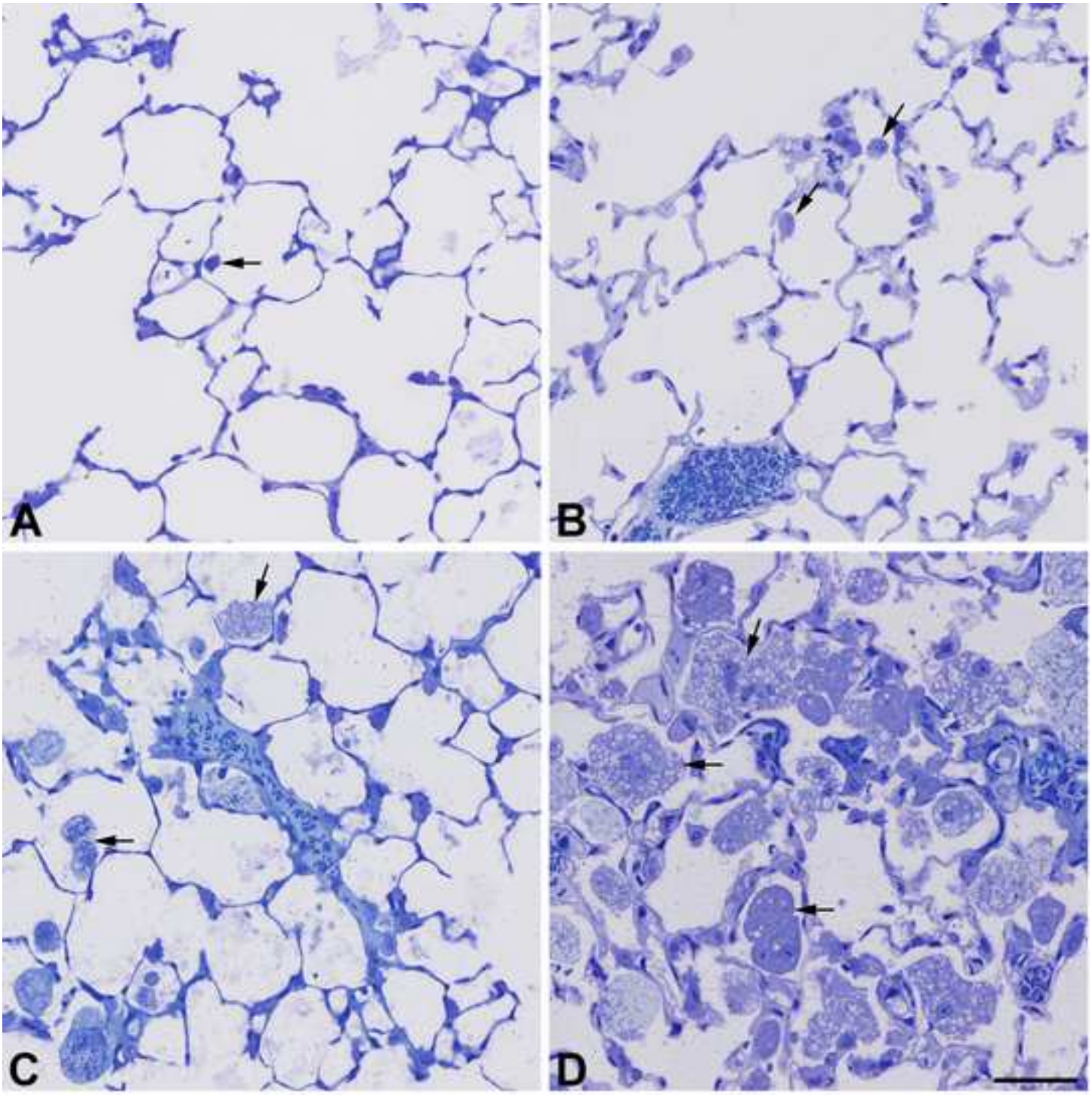

Figure 5

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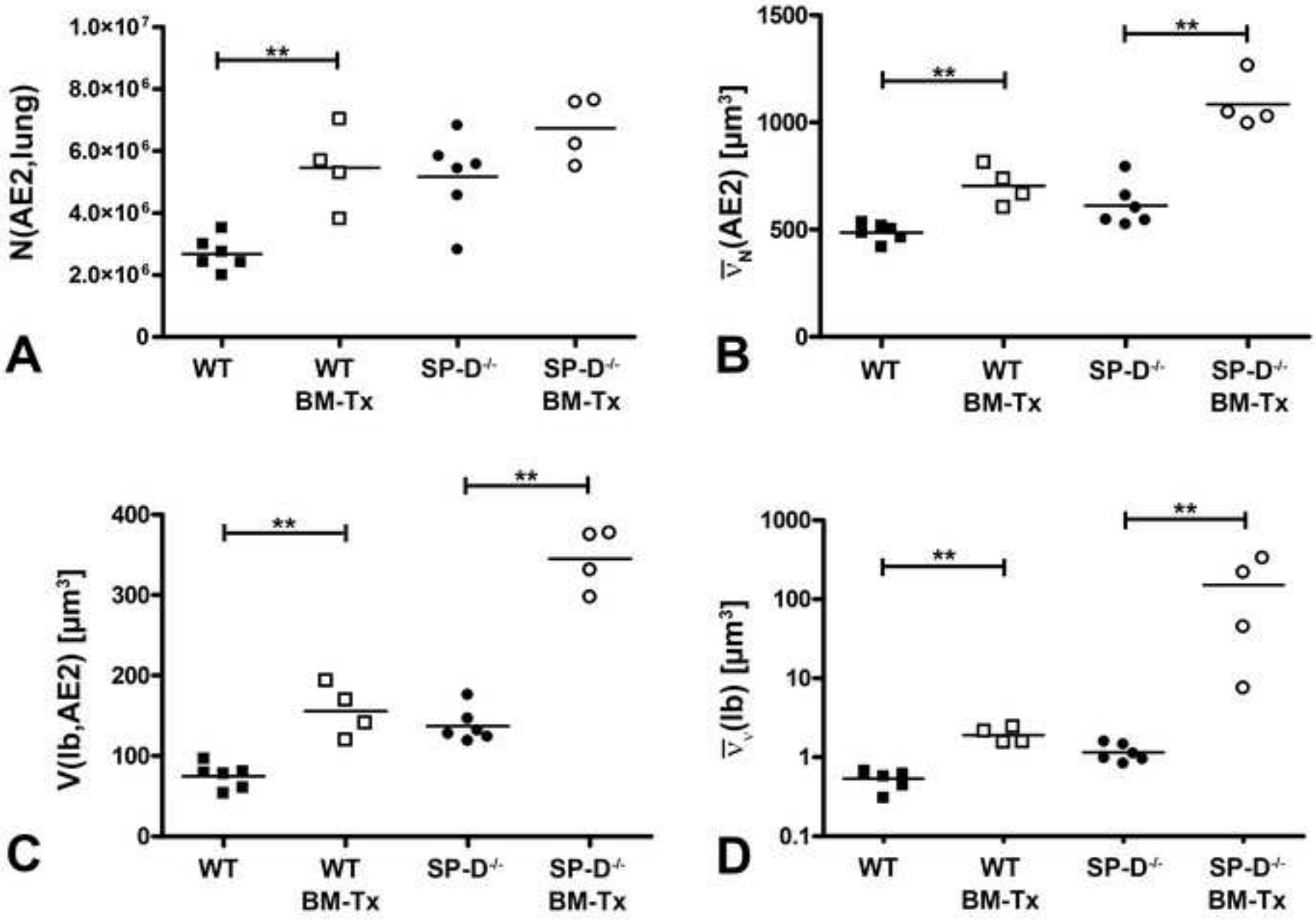

Point-to-point response to reviewers

Reviewer #1

Reviewer comment:

Introduction:
The authors should separate bone marrow transplantation, which is often carried out with Busulfan and Cytoxan or other chemotherapy drugs rather than irradiation, from irradiation. They should also separate the effect of total body irradiation from single fraction total lung irradiation and fractionated irradiation all of which can damage alveolar type II cells. The authors focus on a specific surfactant, which has not been studied extensively in the pathophysiology of lung irradiation damage. There is much interest in the interaction of bone marrow transplanted cells (donor cells) in the interaction with the irradiated lungs, and the topic is timely and interesting. The introduction should focus more on the role of what is known about surfactant components in irradiation lung damage and also in the biology of transplanted lung cells on irradiated lung with respect to alveolar type II cells.

Author response:

According to the reviewer suggestion we have mentioned the use of chemotherapeutics in the context of bone marrow transplantation and have further addressed what is known about surfactant proteins in irradiation and bone marrow transplantation.

Reviewer comment:

Materials and Methods:

The authors should explain why they have chosen C57BL/6 mice, which develop a late lung fibrosis. Why did they not use Balb/c or C3H/HEJ mice, which develop an acute radiation pneumonitis? Another mouse strain should be studied to see if these findings apply to both those mice that are susceptible to acute compared to those that are susceptible to late lung irradiation damage.

There is a section on genotyping, which is not clear with respect to its use in this manuscript.

The stereological analysis is also not clear.

The results section focuses on one table and several figures.

Table 1 is improperly titled. A table should not be titled "Stereological data", but rather the table should focus on what is shown in the table.

The column on the left is not titled. What are the authors measuring? Abbreviations are not clear in the horizontal axis. There is no statistical analysis and no p value.

Figures have morphology of AE2 cells, but it is not clear what is being studied here. What radiation doses were given? What marrow was transplanted, and there should be arrows placed on what is being described.

Figure 2 has morphology of alveolar macrophages. The macrophages are not labeled, but there are some arrows in panel D. Are these the macrophages? Is there a specific histochemical staining? Are these of donor or recipient origin?

Figure 3 shows scatterplots of stereological data. What is stereological data? This is not explained. Stereo suggests 3-dimensional, none of which is shown.

Author response:

Thanks for these comments. It appears that several passages in the Materials and Methods section were not written clearly enough. The following changes have been made:

- With respect to the mouse strain we have included the suggested explanation in the Discussion section.
- We have shortened the passage on genotyping which was mainly used to confirm the genotype of the mice.
- We have expanded the description of the stereological methods to allow the reader a better understanding of what was done.
- Table and figure titles and legends have been revised to provide an immediate and clearer understanding.

Reviewer comment:

Results Section:

In the results section, particularly in Figure 3, need to be amplified by the addition of more controls. Rather than using wild type mice and SP-D-/- mice, were their studies done on SP-D+/- mice? Was there a dose response of genetics? What was the irradiation dose used, and is there a radiation dose response curve?

In the methods section, it suggests that the mice received 8 Gy using a 137Cs gamma-ray source with 107 bone marrow cells transplanted. Why is the 8 Gy dose chosen? This is a low dose for C57BL/6J mice. What is the radiation dose response curve of SP-D-/- compared to wild type mice? This data needs to be shown with respect to lung irradiation compared to total body irradiation.

There is a focus on alveolar type II cells, but no other cells are investigated. What is the radiation survival of purified AE type II cells from wild type compared to knockout mice? What is the effect of the heterozygous genotype on this dose response? Biomarkers for radiation effect need to be demonstrated.

There needs to be a detailed analysis of what cells are coming from donor bone marrow and what cells are coming from the endogenous lung cells. Were these sex-mismatched transplants? Most importantly, other control mouse strains need to be used with respect to the radiation response.

What are the biological damage markers in alveolar type II cells? The only information given appears to be histopathological photographs, and these are not representative of the entire experimental model.

Author response:

Although we appreciate the reviewer's suggestions for including different mouse strains and dose-response-curves, we have the feeling that the issues raised by the reviewer are beyond and aside the actual aim of the current study. It was our aim to analyse if AE2 cells and the surfactant system are altered by a typical model of irradiation and bone marrow transplantation and whether surfactant protein D (or rather its absence) has an influence on the AE2 cell response. Our data clearly answer this question and therefore gain further biological knowledge on the surfactant system. As irradtiation/bone marrow transplantation are frequently used as tools in mouse studies to influence

a certain genotype by bone marrow derived cells from a different genotype, we think that it is important to know that the procedure itself has a great impact on the lung.

Reviewer #2:

Reviewer comment:

Major comments:

The relevance of the genotyping is unclear, since it merely serves to show that mice received the appropriate BM (which is a purely practical consideration). It does not (and cannot) indicate whether the myeloablation was 100% effective, and therefore what degree of chimerism (new BM engraftment on top of any pre-existing remnants) was obtained. Did the authors do a full blood count, to confirm a normal (and comparable) leukocyte repertoire in the mice receiving BM?

Author response:

This is an interesting comment. We agree with the reviewer that the role of the genotyping was not clearly stated and was mainly used as a control of the experiments. It is true that this does not provide information about the degree of chimerism. Although we think that this does not influence the conclusions that can be drawn from the experiments we have now inserted this as a limitation to the discussion.

Reviewer comment:

a) Figs 1 & 2. While the Tol Blue stained histological images give a reasonable idea of the lung morphology, inclusion of additional standard histology would be highly beneficial, particularly some H&E stained sections.

b) In addition, some lower power images would be useful, in order to give an appreciation of the more widespread changes to the lungs following BMT.

c) In a similar vein, did the authors note whether there were any changes evident in the bronchial epithelium, since Clara cell and goblet cells have both been reported to be abnormal in SP-D-/- mice, as well as AEII cells.

d) If feasible, inclusion of immunohistochemistry for SP-C (as a reliable marker for AEII cells) would be informative, to give a better overview of AEII distribution in the lungs.

e) Finally, was any fibrotic change evident in these mice (since irradiation causes fibrosis, and SP-D-/- increases severity of fibrosis in other experimental models)?

Author response:

ad a) and b) We have included further images from H&E stained sections, also at lower magnifications.

ad c) Thank you for this suggestion. Indeed, this is an interesting topic, however, analyzing the bronchiolar/bronchial compartment would be a great deal of work and beyond the scope of the current studies. A quick analysis seemed to us rather superficial, therefore, we have decided to investigate this topic in future studies.

ad d) Unfortunately, the embedding protocols used do not allow immunohistochemistry but we think that the morphological images of semithin sections provide sufficient information on AE2 cell distribution although it doesn't catch the eye as easily as a histochemical stain would do.

ad e) This is an interesting question. We have further analysed the lungs to provide information on the degree of induced fibrosis and have included the mean thickness of interalveolar septa. Interestingly, irradiation/bone marrow transplantation increased the thickness of the septa significantly, however, to a similar degree in both genotypes.

Reviewer comment:

Can the authors indicate on Figure 3 (or in Table 1) the results of the statistical analyses between WT and SP-D-/-, to allow an appreciation of baseline differences (as well as post-BMT).

Author response:

We have integrated the requested information in the new table 1 and table 2 as suggested by the reviewer with respect to the comparison between WT and SP-D-/-. We have not included p values for the comparison between the different genotypes after BM-Tx as we think that this does not provide a suitable statistical comparison because of the dependence on the baseline values.

Reviewer comment:

Since electron microscopy was used as part of the stereological analysis, inclusion of some representative images would be very interesting to the reader.

Author response:

According to the reviewer suggestion we have incorporated a new figure panel showing electron microscopic images of AE2 cells.

Reviewer comment:

Minor comments:

There are a few typographical errors which should be corrected following a re-read of the manuscript, e.g. line 225, '(0.0022)' should read '(p=0.0022) etc.

Author response:

We have thoroughly worked through the manuscript and hope that we have eliminated all errors.

Reviewer comment:

Please include a very brief description of the isolation of bone marrow and preparation for injection (e.g. what volume, and presumably via the tail vein?).

Author response:

We have included the suggested methods description.

Reviewer comment:

Did the authors note a similar range of phenotypic differences in SP-D-/- mice at baseline, as has been reported elsewhere (e.g. emphysematous changes, chronic inflammation etc), to confirm consistency of the phenotype in different animal units? Mention of this would be useful in the results.

Author response:

We have further analysed the lungs with respect to emphysematous changes and added data on the number of alveoli. The originally described loss of alveoli which was also later quantitated by our working group was absent in the current mice indicating either a phenotypic shift or a dependence of this phenotype on the genetic background: In contrast to the original C57BL6/CD-1 genetic background the mice in the current study were backcrossed 10 generations into a C57BL6 background.

Reviewer comment:

The Malaviya paper probably warrants more mention in the Discussion, and further comparison with the current study.

Author response:

We had kept this discussion short because we did not want to be too speculative. However, in line with the reviewer comment we have now extended this discussion.

Reviewer comment:

The authors state that there are differences in the number of alveolar macrophages, but this is not quantified. Do the authors have any bronchoalveolar lavage samples (or a histological analysis) which could provide quantification of this observation?

Author response:

Unfortunately, we do not have BAL samples (as this would have compromised the information from the histological analysis). However, we have performed a further stereological analysis to estimate the number of alveolar macrophages. These data have now been included in the manuscript.

Dear editor, dear Dr. Schrader,

we wish to thank you for the opportunity to submit a revised version of our manuscript entitled "Effect of irradiation/bone marrow transplantation on alveolar epithelial type II cells is aggravated in surfactant protein D deficient mice". We also thank the reviewers for critically reviewing the manuscript. Reviewer #1 has raised an abundant number of interesting questions and suggestions for improving the manuscript. With respect to the animal model, we have analyzed the fibrotic response to the irradiation and bone marrow transplantation and have included these data to demonstrate that – besides the AE2 cell alterations – there is also a fibrotic remodeling. Apart from that we feel that it is beyond the scope of the current report to include further animal models (heterozygous SP-D mice and different mouse strains). Several concerns of this reviewer were related to incomplete or not fully understandable description of methods and figures. We have addressed these concerns thoroughly as detailed below. Reviewer #2 has asked for several additional images and further data on the phenotype of the SP-D deficient mice. We have tried to include as many of these suggestions as possible. For example, we have included two new figure panels (low power LM micrographs and EM micrographs), new data on the architecture of the lungs (including volume, surface area and number of alveoli as well as number of alveolar macrophages). The extensive inclusion of these data made it necessary to include a further co-author (Dr. Julia Schipke).

We feel that the manuscript has been substantially improved by addressing the reviewer comments and hope that our revised manuscript is now acceptable for publication in Histochemistry and Cell Biology.

Yours sincerely,
Christian Mühlfeld