Comparative Assessment of Detergent-Based Protocols for Mouse Lung De-Cellularization and Re-Cellularization

John M. Wallis, B.S.,¹ Zachary D. Borg, B.S.,¹ Amanda B. Daly, B.S.,¹ Bin Deng, Ph.D.,²
Bryan A. Ballif, Ph.D.,² Gilman B. Allen, M.D.,¹ Diane M. Jaworski, Ph.D.,³ and Daniel J. Weiss, M.D., Ph.D.¹

Several different detergent-based methods are currently being explored for de-cellularizing whole lungs for subsequent use as three-dimensional scaffolds for *ex vivo* lung tissue generation. However, it is not yet clear which of these methods may provide a scaffold that best supports re-cellularization and generation of functional lung tissue. Notably, the detergents used for de-cellularization activate matrix metalloproteinases that can potentially degrade extracellular matrix (ECM) proteins important for subsequent binding and growth of cells inoculated into the de-cellularized scaffolds. We assessed gelatinase activation and the histologic appearance, protein composition, and lung mechanics of the end product scaffolds produced with three different detergent-based de-cellularization methods utilizing either Triton-X 100/sodium deoxycholate (Triton/SDC), sodium dodecyl sulfate (SDS), or 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). There were significant differences both in gelatinase activation and in the retention of ECM and other intracellular proteins, assessed by immunohistochemistry, mass spectrometry, and western blotting as well as in airways resistance and elastance of lungs de-cellularized with the different methods. However, despite these differences, binding and initial growth following intratracheal inoculation with either bone marrow–derived mesenchymal stromal cells or with C10 mouse lung epithelial cells was similar between lungs de-cellularized with each method. Therefore despite differences in the structural composition of the de-cellularized lungs, initial re-cellularization does not appear significantly different between the three de-cellularization approaches studied.

Introduction

WHILE *ex vivo* engineering of tissues, such as skin, cartilage, and bone, has been successfully used for the regeneration and clinical transplantation,¹ engineering organs with more structural and cellular complexity, such as lung, liver, and heart, is a more challenging endeavor. However, recent advances in regenerative medicine and in tissue engineering techniques have established a foundation upon which the functional replacement of these organs appears possible.^{2–4} One promising approach involves the use of naturally occurring three-dimensional extracellular matrix (ECM) obtained by the de-cellularization of whole organs. The matrix serves as a biologic scaffold for *ex vivo* generation of functional lung tissue with either differentiated adult cells or potentially by stem/progenitor cells.^{5,6} A wide variety of approaches have been used to produce acellular tissues including physical agitation and exposure to chemical and enzymatic agents.^{2,7} Each of these approaches can result in differences in the structure and integrity of the resulting de-cellularized organ scaffold as well as in composition and

amounts of retained ECM and other proteins.^{2,7} Notably, detergents utilized in de-cellularization protocols, including sodium dodecyl sulfate (SDS), sodium deoxycholate (SDC), Triton-X 100, and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), can each activate matrix metalloproteinases (MMPs) and other enzymes that might significantly degrade or alter remaining ECM and other proteins.^{8,9}

Several different detergent-based chemical, enzymatic, and physical methods have recently been utilized to de-cellularize whole lungs obtained from either mice or rats.^{10–14} These different techniques result in de-cellularized lungs that share overall gross and histologic appearances but that differ in ECM content and other features. Importantly, it is unknown whether divergence in the ECM and other protein content and composition of de-cellularized lung scaffolds resulting from the different approaches will affect subsequent re-cellularization. This is a critical consideration for determining the potential clinical utility of de-cellularized human lung scaffolds.

To assess this question, we performed detailed comparative analyses of whole de-cellularized mouse lungs resulting

¹Department of Medicine, University of Vermont College of Medicine, Burlington, Vermont.

²Department of Biology, University of Vermont College of Arts and Sciences, Burlington, Vermont.

³Department of Anatomy and Neurobiology, University of Vermont College of Medicine, Burlington, Vermont.

from three different detergent-based protocols. In parallel, initial binding and short-term growth of two different cell types was assessed following intratracheal administration into the de-cellularized lung scaffolds.

Materials and Methods

Mice

Adult female BALB/c mice (8–24 weeks; Jackson Laboratories) were maintained at UVM in accordance with institutional and American Association for Accreditation of Laboratory Animal Care standards and review.

Lung de-cellularization and preparation of de-cellularized lung slices

Detailed protocols are presented in the Supplementary Methods (Supplementary Data are available online at www.liebertonline.com/tec). In brief, following euthanasia, heart–lung blocs were removed and the lungs were de-cellularized under sterile conditions over 3 days by sequential instillation and rinsing through both trachea and the right ventricle using three different detergent-based protocols based on recently published approaches^{10,12–14}: (i) sequential incubations with distilled water, 0.1% Triton-X 100, 2% SDC, 1 M NaCl, and porcine pancreatic DNase (Sigma)^{10,14}; (ii) sequential incubations with phosphate-buffered saline (PBS), 0.1% SDS, and 0.1% Triton-X 100¹³; or (iii) sequential incubations with PBS, 8 mM CHAPS with 1 M NaCl and 25 mM EDTA, DNase, and fetal bovine serum (FBS).¹² A detailed schematic for each protocol is outlined in Supplementary Figure S1.

To generate de-cellularized lung slices, de-cellularized lungs produced using each protocol were filled with low melting point agarose, sliced with a sterile razor blade to yield transverse sections of ~ 1 mm¹⁴ thickness, covered with sterile PBS, and placed at 37°C until agarose melted out of the tissue.

Lung histology

Lung slices were fixed with 4% paraformaldehyde for 10 min at room temperature, embedded in paraffin, and 5- μ m sections mounted on glass slides. Following de-paraffinization, sections stained with hematoxylin and eosin (H&E), Verhoeff's Van Gieson, Masson's Trichrome, or Alcian blue were assessed by standard light microscopy.¹⁴

To assess the percent parenchyma versus airspace in the native versus de-cellularized lungs, images were taken of six random nonoverlapping parenchymal areas of H&E-stained sections from three naive and three lungs de-cellularized with each protocol (400 $\times$ magnification using Olympus BX50 Light Microscope with QImaging Retiga 2000R digital camera) providing 18 images of the naive and de-cellularized groups, respectively. Percent parenchyma per image was calculated using ImageJ (version 1.43u).¹⁴

Immunohistochemical staining

After de-paraffinization, immunohistochemical staining was performed according to standard protocols with primary antibodies against fibronectin, laminin, smooth muscle myosin heavy chain 2, collagen I, collagen IV, Ki67, cleaved

caspase-3, and smooth muscle actin (see Supplementary Methods for full protocol and reagent details).¹⁴

Mass spectrometry

Six samples (three duplicate pieces, of the same approximate volume and weight, ~ 1 cm³ and 25 μ g, respectively, for each sample, were obtained from similar parenchymal regions of lungs de-cellularized with the Triton-X 100/SDC [Triton/SDC], SDS, and CHAPS protocols, respectively) were processed according to standard protocol (see Supplementary Methods for details¹⁴) and each sample loaded in triplicate onto a fused silica microcapillary LC column packed with C18 reversed-phase resin. Peptides were separated at a flow rate of 250 nL/min for 45 min. Nanospray ESI was used to introduce peptides into a linear ion trap quadrupole (LTQ) Orbitrap mass spectrometer (Thermo Electron). Mass spectrometry data were acquired in a data-dependent acquisition mode, in which a full orbitrap-MS scan (from m/z 400–2000, resolution $r = 30,000$ at m/z 400) was followed by 10 LTQ-MS/MS scans of the most abundant ions.

After an LC-MS run was completed and spectra obtained, the spectra were searched against the IPI Mouse protein sequence databases (V 3.75) using SEQUEST (Bioworks software, version 3.3.1; Thermo Electron), with search parameters detailed in Supplementary Methods. Proteins that were identified by two or more peptides in each of the six samples were regarded as identified. Proteins that were found at least in two out of three LC-MS/MS replicates were compiled and are presented in Supplementary Table S1 and in Figure 2B. Proteins presented in Supplementary Table S1 are broadly categorized and ranked by the average number of peptides identified from the six samples.

Western analyses

Western blot analysis was performed as previously described^{14,15} with antibodies against actin, collagen type I, collagen type IV, fibronectin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), histone H1, laminin, and smooth muscle myosin. After washing, blots were incubated with species-specific horseradish peroxidase-conjugated secondary antibodies and immunoreactive species were identified using enhanced chemiluminescence (PerkinElmer Life Sciences). Densitometry was performed using Quantity One software (Bio-Rad). Full details are in the Supplementary Methods.

Gelatinase assay

Net gelatinolytic activity was determined using the EnzCheck Gelatinase Assay (Molecular Probes) as previously described.¹⁶ Lungs were homogenized in Tris-buffered saline (50 mM Tris-HCl [pH 7.4] and 150 mM NaCl) and gelatinolytic activity (in 80 μ g protein) was reported as the rate of fluorescence increase over 4 h normalized to assay reagent containing DQTM gelatin (Life Technologies) alone. The specificity of the assay was determined by inclusion of protease inhibitors as described.

Lung mechanics

Extracted lungs were intubated with an 18-gauge metal cannula, connected to a flexiVent mechanical ventilator (SCIREQ), and ventilated according to a standard protocol

elaborated in the Supplementary Methods.¹⁷ Respiratory impedance (Z_{rs}) was determined and interpreted by being fit to the constant phase model of the viscoelastic lung, from which were derived values for tissue elastance (H), a parameter reflecting the combined effects of tissue stiffness and surface tension at the air-liquid interface of the lung.¹⁷⁻¹⁹

DNase and RNase assessment

Whole lungs de-cellularized with each protocol were homogenized and the resulting supernatant was mixed with PCR buffer (1×), $MgCl_2$ (1 mM), DNA or RNA ladder, and nuclease-free water. Positive control tubes contained PCR buffer (1×), $MgCl_2$ (1 mM), DNA or RNA ladder, and DNase I or RNase H. Negative control tubes contained nuclease-free water, PCR buffer (1×), $MgCl_2$ (1 mM), and DNA or RNA ladder. All tubes were incubated in C1000 thermocycler (Bio-Rad) at 37°C for 2 h. Tube contents were run on 2% agarose (Invitrogen) gel containing ethidium bromide and visualized under UV conditions. Test was considered valid if positive control tubes showed ladder degradation and negative control tubes showed intact ladder.¹⁴

Cells and cell inoculation

Mesenchymal stromal cells (MSCs) derived from bone marrow of adult male C57Bl/6 mice were obtained from the NCR/NIH Center for Preparation and Distribution of Adult Stem Cells at Tulane University.²⁰ MSCs were cultured following standard protocol (see Supplementary Methods). Purity was determined by expression of Sca-1, CD106, CD29, absence of CD11b, CD11c, CD34, and CD45 expression, and the ability to differentiate into osteoblasts, chondrocytes, and adipocytes *in vitro*.²⁰ C10 mouse lung epithelial cells were obtained courtesy of Matthew Poynter, Ph.D., University of Vermont and cultured under standard conditions.²¹ To seed each de-cellularized lung, 2×10^6 MSCs or C10 cells were harvested from tissue culture plates, suspended in 3 mL MSCs or C10 basal medium, mixed with low melting point agarose, and inoculated by intratracheal injection into a de-cellularized lung. The lungs were subsequently sliced into pieces of ~1 mm thickness and each slice was placed in a separate well of standard 12-well tissue culture dishes (Corning), incubated at 37°C and 5% CO_2 with lungs immersed in fresh medium every 2 to 3 days.¹⁴ Individual slices were harvested at either 1 or 14 days.

Statistical analyses

Differences between percent parenchyma, western blots, gelatinase activities, and measurements of airways resistance and lung elastance were analyzed by unpaired t-test or by one- or two-way ANOVA with Bonferroni *post hoc* analysis, Tukey's means comparison testing, or posttest Dunnett or Newman-Keuls multiple comparison analyses performed with Prism software as appropriate.^{14-16,18,19,22} Further detailed statistical methods are presented in the Supplementary Methods.

Results

Detailed comparison of de-cellularized mouse lungs

To compare in detail each several detergent-based approaches recently utilized to de-cellularize whole rodent

lungs,^{10-14,23} we first assessed gross appearance and histologic appearance by light microscopy. All lungs looked grossly similar and histologic evaluation with H&E, and of collagen and elastin content demonstrated both absence of intact cells and nuclei throughout the tissues and comparable distribution of elastin and collagen throughout the remaining tissue. In the Triton/SDC and SDS approaches, normal airway, vascular, and alveolar architecture appeared to be well maintained (Fig. 1A). In contrast, mouse lungs de-cellularized using CHAPS tended to have less distinct preservation of normal-appearing structure with thickened-appearing alveolar septa and loss of discrete smaller airways and blood vessels (Fig. 1A). Similar findings were observed in ~1-mm-thick slices produced from the de-cellularized lung scaffolds. Morphometric assessment of native lungs and de-cellularized lungs inflated to equal pressures prior to fixation showed that both the CHAPS and SDS methods resulted in significantly enlarged alveolar septa, and less alveolar space, as compared with native lungs and Triton/SDC de-cellularized lungs (Fig. 1B). Residual RNase activity was present using all methods. However, although DNase was used in the Triton/SDC and CHAPS protocols, no DNase activity was noted in any of the de-cellularized lung scaffolds (Fig. 1C).

Evaluation of the remaining ECM protein composition by Alcian blue staining demonstrated substantial loss of glycosaminoglycan (Alcian blue staining) by visual examination (Fig. 2). In contrast, immunofluorescence demonstrated relatively equivalent preservation of prominent ECM proteins including collagen I, collagen IV, laminin, and fibronectin. The images depicted are reasonable representations for each ECM protein although there can be variability in immunofluorescence staining in different regions of different sections or even on the same section. However, the CHAPS protocol consistently appeared to leave a greater amount of intracellular (smooth muscle actin and smooth muscle myosin) proteins interspersed with the remaining ECM proteins as compared with both SDS and Triton/SDC.

Because immunohistochemical staining is descriptive with variability, as noted previously, quantitative assessments by western blot revealed that detergents differentially affected ECM retention in de-cellularized lungs (Fig. 3A). SDS and CHAPS generally resulted in greater relative enrichment of collagen I, collagen IV, and fibronectin as a proportion of the total protein in the de-cellularized lung scaffolds than did Triton/SDC. SDS and Triton/SDC resulted in comparable relative enrichment of laminin, although each less than that resulting with use of CHAPS (Fig. 3A). However, while Triton/SDC and CHAPS were effective in the removal of nuclear proteins (e.g., histone H1), these detergents were not as effective in the removal of cytosolic proteins (e.g., myosin, GAPDH, and actin) compared with SDS.

As MMPs degrade intracellular as well as ECM substrates,^{9,24} and detergents such as SDS and Triton-X 100 can induce the activation of proMMPs,⁸ we examined net proteolytic activity using a caged substrate (i.e., DQ gelatin) (Fig. 3B). This substrate is cleaved by a broad range of proteases (e.g., all proteases that cleave gelatin), not only the gelatinases MMP-2 and MMP-9. Cleavage yields highly fluorescent peptides, whose fluorescence is proportional to proteolytic activity. To assess differential effects of the different detergents, net proteolytic (gelatinase) activity was assessed following both overnight incubation of whole lungs

FIG. 1. Histologic assessment of whole mouse lungs de-cellularized using different detergent-based protocols demonstrates significant differences in resulting histologic architecture. **(A)** Hematoxylin and eosin (H&E), Verhoeff's Van Gieson (EVG), and Masson's Trichrome collagen (TRI) staining demonstrate comparable preservation of lung architecture using Triton/sodium deoxycholate (SDC) or sodium dodecyl sulfate (SDS) but loss of detail and parenchymal structure when using 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). a, airway; bv, blood vessel; original magnifications 100 $\times$. **(B)** Mean linear intercept in lungs de-cellularized with Triton/SDC is comparable to that of naive lungs but is significantly increased in lungs de-cellularized with SDS and even more substantially increased in lungs de-cellularized using CHAPS. Values represent means $\pm$ standard error of three lungs per experimental condition. ***Significant difference compared with native lungs ($p < 0.05$). **(C)** Demonstration of residual RNase but not DNase activities in de-cellularized whole lungs. DNA and RNA ladders are shown in the first column (control). Ladders with addition of either DNase or RNase to naive lung homogenates are shown in the second column as positive controls. Two different representative ladders, naive lungs and de-cellularized lungs, are shown for comparison. There is no obvious difference in residual RNase or DNase activities between any of the de-cellularization protocols. Color images available online at www.liebertonline.com/tec

FIG. 2. Immunofluorescent and histologic staining demonstrates reasonably comparable retained extracellular matrix (ECM) proteins in whole lungs de-cellularized using different detergent-based protocols. For each set of IF images, the specific ECM protein is shown in green with a DAPI nuclear co-stain. Glycosaminoglycan (GAG) content is stained blue with Alcian blue stain. Fib, fibronectin; lam, laminin; Col I, type I collagen; Col IV, type IV collagen; SMA, smooth muscle actin; SMM, smooth muscle myosin; a, airway; bv, blood vessel. Representative photomicrographs from lungs de-cellularized with each detergent-based protocol compared with a native lung are shown. Original magnifications are 400 $\times$ for IF photomicrographs and 100 $\times$ for Alcian blue photomicrographs. Color images available online at www.liebertonline.com/tec

with Triton-X 100, SDC, SDS, or CHAPS (Fig. 3A, black bars) and then following the completion of each full de-cellularization protocol (Fig. 3B, gray bars). Proteolytic activity was significantly increased following overnight incubation with Triton-X 100 and even more so with SDC, but not with SDS or CHAPS. Following completion of the full protocol, net proteolytic activity had returned to basal levels found in naive lungs, comparable to observations made by Price *et al.* demonstrating no residual MMP-2 and MMP-9

activities in mouse lungs fully de-cellularized with the Triton/SDC protocol.¹⁰ These results demonstrate that gelatinase and possibly other proteolytic activities are significantly activated early during the course of de-cellularization protocols utilizing Triton or SDC.

To further evaluate the effects of each de-cellularization protocol on residual protein content, de-cellularized lungs underwent mass spectrometric analyses (Fig. 3C and Supplementary Table S1). ECM proteins detected included

collagens, fibronectin, and laminin but did not include elastin. We and others have previously found that elastin is particularly labile and does not survive well with Triton/SDC-based or other methods of de-cellularization,^{10–14} in our studies falling below the limits of detection for mass spectrometry although some elastin can still be detected by histochemical staining.¹⁴ Further, myosin, several isoforms of actin and tubulin, and a number of other intracellular proteins were also detected. While there is no clear pattern in residual intracellular and other proteins, these results highlight that there are substantial differences between the different de-cellularization protocols.

Lung mechanics in de-cellularized whole mouse lungs

As we and others have previously demonstrated,^{10–14} lung de-cellularization affects lung mechanics notably with a significant increase in Newtonian airways resistance (R_N) and in lung stiffness (mean elastance [H]), an increase partially restored by addition of exogenous surfactant.¹⁴ A two-way multiple measures ANOVA was used to evaluate the effects of each de-cellularization method on lung elastance utilizing positive end expiratory pressures (PEEP) of 6, 3, and 1 cm H₂O (Fig. 4A, only PEEP of 3 shown). Significantly higher elastance values were observed in the Triton/SDC group when compared with CHAPS or SDS ($p < 0.05$, Tukey's means comparison testing), but no significant difference was found between the CHAPS and SDS groups. PEEP did not have a significant effect on elastance values. ANOVA testing on values for the coefficient of deviation (COD) at the end of PEEP 6, 3, and 1 cm H₂O yielded similar results (Fig. 4B). PEEP did not have a significant effect on the COD, but the method of de-cellularization did ($p < 0.05$). Means comparison testing demonstrated significantly higher COD values in the Triton/SDC group (better model fit) when compared with CHAPS ($p < 0.05$), and trend toward higher COD values compared with SDS ($p = 0.054$), but no significant difference between SDS and CHAPS. Although Newtonian airways resistance (R_N) values were also higher in the Triton/SDC lungs (Fig. 4C), a much higher variance in R_N values weakened the statistical power of the comparisons.

Growth of MSCs and C10 cells in de-cellularized whole lungs

To assess the ability of mouse lung epithelial C10 cells and mouse bone marrow-derived MSCs to bind to and subsequently proliferate in the de-cellularized lungs, each cell type was inoculated by intratracheal administration (2×10^6 cells/lung) into different lungs and cultured for either 1 or 14 days

in their respective media. We have previously demonstrated that MSCs and C10 cells have different initial distributions following intratracheal administration into Triton/SDC de-cellularized lungs.¹⁴ MSCs initially localized to regions enriched in fibronectin, collagens I and IV, and laminin whereas C10 cells did not localize in fibronectin-enriched regions rather localized to areas enriched in laminin and collagens I and IV.¹⁴ These patterns appeared to be maintained following inoculation into SDS or CHAPS de-cellularized lungs (Fig. 5A, B). Both inoculated MSCs and C10 cells appeared to thrive predominantly in parenchymal regions (Fig. 5A, B) but also along both large and small airways 1 day after intratracheal administration. There was no obvious difference in the number of cells that initially remained in the lungs 1 day after inoculation or in the initial distribution of the inoculated cells relative to different retained ECM proteins (Fig. 5B).

Fourteen days after inoculation, MSCs tended to remain in parenchymal regions while C10 cells lined many of the large and small airways located in the inoculated lungs with a smaller number located in parenchymal regions (Fig. 6A, B). These patterns were similar regardless of the lung de-cellularization approach utilized. In each case, several different morphologies were observed for both cell types growing in parenchymal regions including both flattened elongated and rounded cells. Cells growing along airways, predominantly the C10 cells, remained flattened and squamous in appearance and no cells of pseudostratified non-ciliated or polarized ciliated or epithelial appearance were observed. Although the number of cells on histologic sections was not quantified due to the stochastic nature of the inoculation process, the total number of both MSCs and C10 cells appeared to proliferate over the 14-day incubation period as assessed by Ki67 staining (Fig. 7). Caspase-3 staining to assess apoptosis remained consistently low in both MSCs and C10 cells throughout the 14-day culture period (Fig. 7). No obvious difference in either Ki67 or caspase-3 staining was observed between cells inoculated into lungs de-cellularized with the different detergents.

Discussion

Although 1000–1500 lung transplants per year are performed in the United States, a significant shortage of suitable donor lungs and the drawbacks of lung transplantation, including an approximate 50% 5-year mortality and need for lifelong immunosuppression, demonstrate a critical need for new approaches.²⁵ Unlike cadaveric organs such as kidneys, which have been successfully utilized for many years,

FIG. 3. Western blot, mass spectrometric, and gelatinase analyses demonstrate significant difference in content of ECM and intracellular proteins and also in activation of matrix metalloproteinases (gelatinase) between whole mouse lungs de-cellularized using the different protocols. **(A)** Representative western blots are shown, respectively, for naive lungs and lungs undergoing each full de-cellularization protocol. Quantitative data are presented as mean $\pm$ SEM. Significant interactions were identified by one-way analysis of variance with Bonferroni *post hoc* analysis using Prism software. * means < 0.05 , ** means < 0.01 and # means < 0.001 relative to naive. **(B)** Proteolytic (net gelatinolytic) activity was significantly increased in lungs de-cellularized with Triton and SDC, but not SDS or CHAPS. Two-way ANOVA revealed a significant detergent effect ($p = 0.0073$) and time effect ($p = 0.0014$), but a time by detergent interaction ($p = 0.0038$). ** is < 0.01 while # is < 0.001 relative to naive. **(C)** Comparative mass spectrometric analyses demonstrating average values of duplicate assessments of de-cellularized lungs resulting from each detergent-based protocol. The key to identification of each peptide and the corresponding International Protein Index (IPI) reference is listed in Supplementary Table S1. Color images available online at www.liebertonline.com/tec

transplantation of cadaveric lungs has not yet been feasible. *Ex vivo* re-cellularization of de-cellularized three-dimensional whole lung scaffolds to produce functional lung tissue suitable for transplantation has recently been investigated as an alternative approach that would allow use of cadaveric lungs.^{10–14} Initial promising results have been obtained in rodent models and de-cellularized rat lungs, re-cellularized with a mixture of endothelial cells, fetal lung homogenates, and tumorigenic lung epithelial cells, have been implanted and maintained for up to 2 weeks.^{12,13,26}

However, different techniques have been utilized to de-cellularize whole lungs including physical methods (multiple freeze thaws) as well as use of different detergents.^{10–14} We have previously provided detailed characterization of de-cellularized mouse lungs¹⁴ utilizing a Triton/SDC-based method originally described by Lwebuga-Mukasa *et al.*²³ and modified by Price *et al.*¹⁰ Other groups have described SDS or CHAPS-based protocols.^{11–13} It is not clear which, if any, of these might produce an optimal de-cellularized lung scaffold. Further it is not clear what an “optimal” de-cellularized lung scaffold will be.² Considerations include appropriate maintenance of gross and microscopic structure, maintenance of key ECM proteins, and the ability to support appropriate binding and growth of a range of cells that might potentially be utilized for re-cellularization.^{2,5–7} Whether different residual contents of intracellular and other proteins or whether differences in mechanical properties of the de-cellularized lungs will affect re-cellularization and generation of functional lung tissue has not yet been clarified. Further, the potential immunogenicity of the de-cellularized scaffolds is a critical consideration for clinical transplantation of re-cellularized human lung scaffolds.

To address whether re-cellularization would be affected by different de-cellularization approaches, we compared three different detergent-based lung de-cellularization protocols and found significant differences in histologic appearance, gelatinase activation, content and distribution of ECM proteins, and a range of intracellular proteins. To best assess the specific effects of the different detergents utilized, the SDS- and CHAPS-based de-cellularization protocols were otherwise standardized to the timeline of the Triton/SDC approach we have previously utilized based on the method of Price *et al.*^{10,14} Additional steps in the de-cellularization

approaches, including use of hypertonic saline, EDTA, DNase, and FBS, were utilized as had been done in previous reports of lung de-cellularization.^{10–14} Notably, 0.1% Triton alone resulted in increased proteolytic activity at a much lower concentration than what is normally used to activate MMPs for gelatin zymography (e.g., 2.5% Triton-X 100 at 37°C).⁸ These results highlight that the different protocols will result in different de-cellularized lungs and notably that detergents will differentially activate proteolytic activities early

FIG. 4. Significant differences in lung mechanics in lungs de-cellularized with the different detergent-based protocols. Panel (A) plots elastance (H) values over time at positive end expiratory pressure (PEEP) 3 $\text{cm H}_2\text{O}$ for individual lungs de-cellularized by CHAPS, SDS, or Triton/SDC. Mean values are exhibited by the superimposed horizontal bars (gray or black). * signifies a significant difference between Triton/SDC and CHAPS (ANOVA, $p < 0.05$). # signifies a significant difference between Triton/SDC and SDS (ANOVA, $p < 0.05$). Panel (B) plots mean coefficient of deviation (COD) $\pm$ SEM over time for Triton/SDC (gray circles), SDS (black triangles), and CHAPS (upside down gray triangles) at PEEP 6, 3, and 1 $\text{cm H}_2\text{O}$. * signifies a significant difference between Triton and CHAPS (ANOVA, $p < 0.05$). Panel (C) plots Newtonian airways resistance (R_N) values over time at PEEP 3 $\text{cm H}_2\text{O}$ for individual lungs de-cellularized by CHAPS, SDS, or Triton/SDC. Mean values are exhibited by the superimposed horizontal bars (gray or black).

FIG. 5. No significant difference in initial binding is observed following intratracheal inoculation of either mesenchymal stromal cells (MSCs) or C10 epithelial cells into whole mouse lungs de-cellularized with different detergent-based protocols. Representative photomicrographs obtained 1 day after intratracheal inoculation of bone marrow-derived mouse MSCs (**A**) or C10 mouse lung epithelial cell (**B**) into de-cellularized whole mouse lungs are shown. For each panel, specific ECM protein immunofluorescence is depicted in green with DAPI nuclear co-staining. a, airways; bv, blood vessels; Fib, fibronectin; Lam, laminin; Col I, type I collagen; Col IV, type IV collagen; SMA, smooth muscle actin. Original magnifications 100 $\times$, 400 $\times$. Color images available online at www.liebertonline.com/tec

FIG. 6. No significant difference in short-term (14 day) growth is observed following intratracheal inoculation of either MSCs or C10 epithelial cells into whole mouse lungs de-cellularized with different detergent-based protocols. Representative photomicrographs obtained 14 days after intratracheal inoculation of bone marrow-derived mouse MSCs (**A**) or C10 mouse lung epithelial cells (**B**) into de-cellularized whole mouse lungs are shown. For each panel, specific ECM protein immunofluorescence is depicted in green with DAPI nuclear co-staining. Higher magnification images of the regions indicated by the * in Col I-stained sections detail that C10 cells robustly re-epithelialize medium- and small-sized airways following 14 days in culture with no obvious difference between de-cellularization conditions. a, airways; bv, blood vessels; Fib, fibronectin; Lam, laminin; Col I, type I collagen; Col IV, type IV collagen; SMA, smooth muscle actin. Original magnifications 100 $\times$, 400 $\times$. Color images available online at www.liebertonline.com/tec

FIG. 7. No significant differences in proliferation or early apoptosis are observed following intratracheal inoculation of either MSCs (**A**) or C10 epithelial (**B**) cells into whole mouse lungs de-cellularized with different detergent-based protocols. Representative photomicrographs obtained 1 and 14 days after intratracheal inoculation of bone marrow-derived mouse MSCs or C10 mouse lung epithelial cells into de-cellularized whole mouse lungs. For each panel, Ki67 or caspase-3 immunofluorescence is depicted in red with DAPI nuclear co-staining. Original magnification 400 $\times$. Color images available online at www.liebertonline.com/tec

during the de-cellularization process that may critically affect the remaining ECM matrix. A recent comparison of SDS-versus CHAPS-based lung de-cellularization protocols similarly found differential retention of ECM proteins (collagen, elastin, and glycosaminoglycans) with more marked loss of collagen and elastin in SDS de-cellularized lungs.²⁷ Notably, mechanical testing of lung strips demonstrated better retention of tensile strength in lungs de-cellularized with CHAPS as compared with SDS, possibly related to better retention of collagen and elastin. In contrast, we found that airways resistance and whole lung elastance were relatively similar with the use of CHAPS and SDS although elastance was decreased with use of Triton/SDC. The consistently lower than physiologic levels of elastance (Fig. 4A) and negative values for resistance (Fig. 4C) in the CHAPS and SDS lungs were likely due to a significantly greater degree of leak during the delivery of oscillatory perturbations rather than being obviously attributable to differences in any given ECM proteins. In contrast, despite a few lungs in the Triton/SDC group exhibiting abnormally low elastance and resistance values, the majority of the Triton/SDC lungs exhibited elevated elastance values and above-zero resistance values that were reproducible over time, likely representing a loss of surfactant function in the absence of significant leak. Likewise, values for the COD ("goodness of

fit"), derived when fitting the Zrs raw data to the viscoelastic lung model, were significantly higher in the Triton lungs (Fig. 4B), suggesting much more linear pressure response and significantly less leak in these lungs. Taken together, these data suggest that use of Triton/SDC as utilized is significantly less disruptive to the integrity of the de-cellularized matrix, and thus more conducive to following lung mechanical changes over time, both during de-cellularization and re-cellularization.

However, despite differences in ECM and other protein content and differences in whole lung elastance, initial binding and short-term (2 weeks) proliferation of two different cell types, a stromal progenitor cell (MSC) and a mouse lung epithelial cell line (C10), appeared comparable when cells were intratracheally inoculated into lungs de-cellularized with the different protocols and lung slices grown at normoxia under static (i.e., nonventilated or perfused) conditions. We have previously found that MSCs inoculated into and similarly cultured in Triton/SDC-derived de-cellularized lungs thrived for at least up to 1 month.¹⁴ These results suggest that despite significant differences in the composition of lungs produced with the different detergent-based protocols, inoculated cells are able to recognize appropriate ligands that will allow binding and initial proliferation. Notably the C10 cells appear to

comparably re-epithelialize medium-sized and small airways in each of the protocols utilized.

One additional point of comparison is that use of allogeneic re-cellularization with MSCs (C57Bl/6 MSCs inoculated into de-cellularized BALB/c mouse lungs) in the current studies resulted in similar initial patterns of cell localization as compared with the syngeneic re-cellularization (C57Bl/6 MSCs inoculated into de-cellularized C57Bl/6 mouse lungs) utilized in our previous studies.¹⁴ C10 cells are of BALB/c origin²¹ and we have not yet compared syngeneic versus allogeneic inoculation of C10 cells into de-cellularized C57Bl/6 lungs. Further comparison of syngeneic versus allogeneic re-cellularization approaches may provide clues about the critical factors in the de-cellularized lungs that direct re-cellularization as well as the degree of acceptable genetic mismatch compatibility.

These results suggest that development of techniques for production of an "optimum" de-cellularized scaffold is more dependent on re-cellularization potential rather than de-cellularization methodology. However, despite similarities in initial re-cellularization between the three detergent-based protocols assessed, there are still many questions to be answered, including long-term growth and differentiation of inoculated cells, reconstitution of lung functions including gas exchange and appropriate lung mechanics, and also appropriate antiinflammatory and immune functions of the lung.²⁸ Further, there are other permutations yet to be clarified for de-cellularization approaches as some published protocols have utilized only vascular perfusion with de-cellularizing agents and also shorter incubation times with the different detergents.^{12,13,26,27} As such, the optimal de-cellularization protocol is still to be determined.

Conclusion

These studies demonstrate that different detergent-based de-cellularization protocols result in significant differences in histologic appearance, gelatinase activation, content and distribution of ECM and intracellular proteins, and lung mechanics, and in each case they result in lungs that are immunogenic. Nonetheless, initial binding and proliferation of intratracheally inoculated cells appears to be comparable between the protocols. This suggests that a range of approaches may be feasible for producing de-cellularized scaffolds that might be suitable for clinical transplantation. However, there remain many questions about what constitutes an optimally de-cellularized lung scaffold and what the criteria for optimization should be.

Acknowledgments

The authors gratefully acknowledge the staffs Bruce Bunnell, Christine Finck, and Andrew Hoffman of the Offices of Animal Care Management at the University of Vermont for critical reads of the manuscript and Katie Polakowski and Kevin Weiss for valuable contributions to the experimental studies. Studies were supported by NIH ARRA RC4HL106625 (D.J.W.) and NHLBI R21HL094611 (D.J.W.). Facilities and equipment were supported by the UVM Lung Biology COBRE (NIH NCRR P20 RR-155557), NIH Neuroscience COBRE (NIH NCRR P20 RR016435), and the Vermont Cancer Center DNA Analysis facility (NIH P30 CA22435).

Disclosure Statement

No competing financial interests exist.

References

- Bhatia, S.K. Tissue engineering for clinical applications. *Biotech J* 5, 1309, 2010.
- Badylak, S.F., Taylor, D., and Uygun, K. Whole-organ tissue engineering: decellularization and recellularization of three-dimensional matrix scaffolds. *Ann Rev Biomed Eng* 13, 27, 2011.
- Ott, H.C., Matthiesen, T.S., Goh, S.K., Black, L.D., Kren, S.M., Netoff, T.I., and Taylor, D.A. Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart. *Nat Med* 14, 213, 2008.
- Uygun, B.E., Soto-Gutierrez, A., Yagi, H., Izamis, M.L., Guzzardi, M.A., Shulman, C., Milwid, J., Kobayashi, N., Tilles, A., Berthiaume, F., Hertl, M., Nahmias, Y., Yarmush, M.L., and Uygun, K. Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix. *Nat Med* 16, 814, 2010.
- Badylak, S.F. The extracellular matrix as a biologic scaffold material. *Biomaterials* 28, 3587, 2007.
- Badylak, S.F., Freytes, D.O., and Gilbert, T.W. Extracellular matrix as a biological scaffold material: structure and function. *Acta Biomater* 5, 1, 2009.
- Crapo, P.M., Gilbert, T.W., and Badylak, S.F. An overview of tissue and whole organ decellularization processes. *Biomaterials* 32, 3233, 2011.
- Nagase, H. Activation mechanisms of matrix metalloproteinases. *Biol Chem* 378, 151, 1997.
- Cauwe, B., and Opdenakker, G. Intracellular substrate cleavage: a novel dimension in the biochemistry, biology and pathology of matrix metalloproteinases. *Crit Rev Biochem Mol Biol* 45, 351, 2010.
- Price, A.P., et al. Development of a decellularized lung bio-reactor system for bioengineering the lung: the matrix re-loaded. *Tissue Eng Part A* 16, 2581, 2010.
- Cortiella, J., et al. Influence of acellular natural lung matrix on murine embryonic stem cell differentiation and tissue formation. *Tissue Eng Part A* 16, 2565, 2010.
- Petersen, T.H., et al. Tissue-engineered lungs for *in vivo* implantation. *Science* 329, 538, 2010.
- Ott, H.C., Clippinger, B., Conrad, C., Schuetz, C., Pomerantseva, I., Ikononou, L., Kotton, D., and Vacanti, J.P. Regeneration and orthotopic transplantation of a bioartificial lung. *Nat Med* 16, 927, 2010.
- Daly, A.B., Wallis, J.M., Borg, Z.D., Bonvillain, R.W., Deng, B., Baliff, B.A., Jaworski, D.M., Allen, G.B., and Weiss, D.J. Initial binding and re-cellularization of de-cellularized mouse lung scaffolds with bone marrow-derived mesenchymal stromal cells. *Tissue Eng Part A* 2011 [Epub ahead of print]; DOI:10.1089/ten.TEA.2011.0301.
- Lluri, G., Langlois, G.D., Soloway, P.D., and Jaworski, D.M. Tissue inhibitor of metalloproteinase-2 (TIMP-2) regulates myogenesis and beta1 integrin expression *in vitro*. *Exp Cell Res* 314, 11, 2008.
- Lluri, G., Langlois, G.D., McClellan, B., Soloway, P.D., and Jaworski, D.M. Tissue inhibitor of metalloproteinase-2 (TIMP-2) regulates neuromuscular junction development via a β 1 integrin-mediated mechanism. *J Neurobiol* 66, 1365, 2006.
- Gomes, R.F., Shardonofsky, F., Eidelman, D.H., and Bates, J.H.T. Respiratory mechanics and lung development in the rat from early age to adulthood. *J Appl Physiol* 90, 1631, 2001.

18. Allen, G.B., Suratt, B.T., Rinaldi, L., Petty, J.M., and Bates, J.H. Choosing the frequency of deep inflation in mice: balancing recruitment against ventilator-induced lung injury. *Am J Physiol Lung Cell Mol Physiol* **291**, L710, 2006.
19. Allen, G.B., Pavone, L.A., DiRocco, J.D., Bates, J.H.T., and Nieman, G.F. Pulmonary impedance and alveolar instability during injurious ventilation in rats. *J Appl Physiol* **99**, 723, 2005.
20. Sekiya, I., Larson, B.L., Smith, J.R., Pochampally, R., Cui, J.G., and Prockop, D.J. Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. *Stem Cells* **20**, 530, 2002.
21. Malkinson, A.M., Dwyer-Nield, L.D., Rice, P.L., and Dinsdale, D. Mouse lung epithelial cell lines-tools for the study of differentiation and the neoplastic phenotype. *Toxicology* **123**, 53, 1997.
22. Zar, J. *Biostatistical Analysis*. Englewood Cliffs, NJ: Prentice-Hall, Inc., 1974.
23. Lwebuga-Mukasa, J.S., Ingbar, D.H., and Madri, J.A. Repopulation of a human alveolar matrix by adult rat type II pneumocytes *in vitro*. *Exp Lung Res* **162**, 423, 1986.
24. McCawley, L.J., and Matrisian, L.M. Matrix metalloproteinases: they're not just for matrix anymore! *Curr Opin Cell Biol* **13**, 534, 2001.
25. Orens, J.B., and Garrity, E.R. General overview of lung transplantation and review of organ allocation. *Proc Am Thorac Soc* **6**, 13, 2009.
26. Song, J.J., Kim, S.S., Liu, Z., Madsen, J.C., Mathisen, D.J., Vacanti, J.P., and Ott, H.C. Enhanced *in vivo* function of bioartificial lungs in rats. *Ann Thorac Surg* **92**, 998, 2011.
27. Petersen, T.H., Calle, E.A., Colehour, M.B., and Niklason, L.E. Matric composition and mechanics of decellularized lung scaffolds. *Cells Tissues Organs* 2011 [Epub ahead of print]; DOI: 10.1159/000324896.
28. Panoskaltis-Mortari, A., and Weiss, D.J. Breathing new life into lung transplantation therapy. *Mol Ther* **18**, 1581, 2010.

Address correspondence to:

Daniel J. Weiss, M.D., Ph.D.

Department of Medicine

University of Vermont College of Medicine

226 Health Science Research Facility

Burlington, VT 05405

E-mail: dweiss@uvm.edu

Received: October 12, 2011

Accepted: December 13, 2011

Online Publication Date: January 26, 2012