



## Ex-vivo delivery of monoclonal antibody (Rituximab) to treat human donor lungs prior to transplantation

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### ABSTRACT

**Background:** Ex-vivo lung perfusion (EVLP) is an innovative platform for assessing donor lungs in the pre-transplant window. In this study, we demonstrate an extension of its utility by administering the anti-CD20 monoclonal antibody, Rituximab, during EVLP. We hypothesized that this would lead to targeted depletion of allograft B-cells which may provide significant clinical benefit, including the potential to reduce latent Epstein-Barr virus (EBV) and decrease the incidence of post-transplant lymphoproliferative malignancies.

**Methods:** Twenty human donor lungs rejected for transplantation were placed on EVLP with ( $n = 10$ ) or without ( $n = 10$ ) 500 mg of Rituximab. Safety parameters such as lung physiology and inflammatory cytokines were evaluated. We measured the delivery efficacy through flow cytometry, immunohistochemistry and ELISA. An *in-vitro* culture assay, in the presence of complement, was further conducted to monitor whether B-cell depletion would occur in Rituximab-perfused samples.

**Findings:** Rituximab was successfully delivered to human lungs during EVLP as evidenced by flow cytometric binding assays where lung tissue and lymph node biopsies demonstrated occupied CD20 epitopes after perfusion with the antibody. Lymph nodes from Rituximab perfusions demonstrated a 10.9 fold-reduction in CD20+ staining compared to controls ( $p = 0.0003$ ). In lung tissue, Rituximab resulted in an 8.75 fold-reduction in CD20+ staining relative to controls ( $p = 0.0002$ ). This decrease in CD20+ binding illustrates the successful delivery and occupation of epitopes after perfusion with the Rituximab. No apparent safety concerns were seen as exhibited by markers associated with acute cell injury (e.g., proinflammatory cytokines), cell death (e.g., TUNEL staining), or pulmonary physiology. In a post-perfusion tissue culture model, the addition of complement (human serum) resulted in evidence of B-cell depletion consistent with what would be expected with posttransplant activation of bound Rituximab.

**Interpretation:** Our experiments illustrate the potential of EVLP as a platform to deliver monoclonal antibody therapies to treat donor lungs pretransplant with the goal of eliminating a latent virus responsible for considerable morbidity after lung transplantation.

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## 1. Introduction

Normothermic *ex vivo* lung perfusion (EVLP) is an emerging technology that serves as a platform for evaluation, preservation and conditioning of donor lung grafts prior to transplantation [1]. In contrast to cold static preservation, EVLP maintains organ metabolism active during preservation, hence allowing for continuous evaluation of organ function and treatment. Transplantation of extended-criteria

lungs that have undergone EVLP have demonstrated equivalent success compared to standard-criteria lungs that have undergone cold preservation [2,3]. In addition, we have recently demonstrated the efficacy of EVLP as a stable platform to treat donor lung grafts prior to transplant, including the use of gene therapy and anti-viral strategies [4,5]. The improvement of EVLP as a platform for treatment will serve to provide a larger and safer donor pool and address the growing demand of patients in need of lung transplantation [6].

Rituximab (RTX) is a chimeric human-mouse monoclonal antibody targeting CD20, a surface marker present on B-cells [7]. RTX has a long history as it was one of the first monoclonal antibody therapies to be approved for clinical use in humans [8]. RTX has been effective

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## Research in Context

### Evidence before this study

*Ex-vivo* lung perfusion (EVLP) is a state-of-the-art platform that has allowed us to assess and condition donor lungs prior to transplantation. EVLP also offers an invaluable opportunity for grafts to be treated, allowing for the potential to create a larger and safer donor pool. Recent studies have shown the utility of EVLP in treating viral and microbial infections such as the use of the platform to target hepatitis C virus. Another possible intervention could be the administration of the monoclonal antibody Rituximab. Rituximab has been used as induction therapy as well as a mode of prophylaxis for posttransplant lymphoproliferative disorder (PTLD). PTLD is especially a risk in Epstein Barr-virus (EBV) donor seropositive, recipient seronegative (D+/R-) transplant scenarios. This is because EBV is transmitted to the naïve recipient via donor B-cells, the latent reservoir of EBV. Therefore, administration of Rituximab through EVLP could help remove EBV and reduce transmission.

### Added value of this study

This study demonstrates that Rituximab can be safely and effectively delivered through the EVLP platform. The majority of B-cell targets in both lymph nodes and lung tissue were successfully bound following perfusion. No adverse effects were shown after perfusion with Rituximab compared to normal perfusion. Bound Rituximab was also shown to deplete B-cells post-perfusion in an *in vitro* culture.

### Implications of all of the available evidence

Our study has shown that EVLP is an effective platform to administer monoclonal antibody-based therapies to treat donor lungs. In this respect, the delivery of Rituximab via EVLP may expand treatment options for select transplant recipients and may increase the safety of lung allografts by removing latent infection. Considering the safety of Rituximab within this study, and in the past, clinical trials will begin to evaluate if Rituximab administration through EVLP can reduce EBV transmission as well as assist in reducing inflammatory processes post transplantation.

The risk of PTLD is substantially increased when an organ from an EBV seropositive donor is transplanted into an EBV seronegative recipient (D+/R-) [15]. The EBV D+/R- constellation has consistently been shown to be a major risk factor for PTLD post-solid organ transplant [14,16]. This is because allograft resident donor B-cells transmit EBV virus to a naïve recipient. Depletion of B-cells from the allograft prior to implantation could theoretically mitigate the risk of primary EBV infection.

To establish a safer donor organ pool and investigate the effectiveness of EVLP-mediated delivery of immunotherapies, we assessed the potential efficacy of high-dose localized delivery of RTX via EVLP using clinically rejected human donor lungs. We evaluated the ability of this treatment to reduce B-cells in the graft while evaluating potential lung toxicity and ability to decrease EBV burden.

## 2. Materials and methods

### 2.1. Study protocol

**Donors.** Twenty EBV seropositive human donor lungs, consented by donors' families for research use, were acquired for the study from Canada and the United States. These lungs were deemed unsuitable for transplantation based on standard clinical criteria. Lungs that had pronounced edema, significant physiologic impairment or frank pneumonia were not used for this study. The lungs were randomly allocated to EVLP with RTX (treatment group,  $n = 10$ ) (Roche) or with EVLP alone (control group,  $n = 10$ ) (Supplementary Table 1) without any knowledge of the condition of the lungs. Clinical Rituximab was purchased from the UHN outpatient pharmacy. Within the RTX perfusions, 500 mg doses were administered into the circulating perfusate through the hard-shell reservoir. Lungs were typically placed on EVLP for clinical use intent, and once deemed unacceptable for clinical transplantation they were offered for research except in two RTX cases and 3 control cases where lungs were directly placed on research EVLP (Supplementary Table 2). From this time, lungs were randomized to either control or RTX groups with perfusion durations ranging from 5 to 12 h depending on the level of perfusate consumption. If lungs exceeded 200 ml/h for a double lung EVLP, 100 ml/h for a single lung EVLP or the peak airway pressure reached 25 cmH<sub>2</sub>O the perfusion would be ended [17]. Tissue biopsies were collected before and after experimental perfusion from the periphery of the lung and kept for flow cytometric, histological and cytokine assays. Lymph nodes were collected before and after experimental perfusions from the hilar and interlobar regions of the lungs (Fig. 1b).

### 2.2. EVLP system

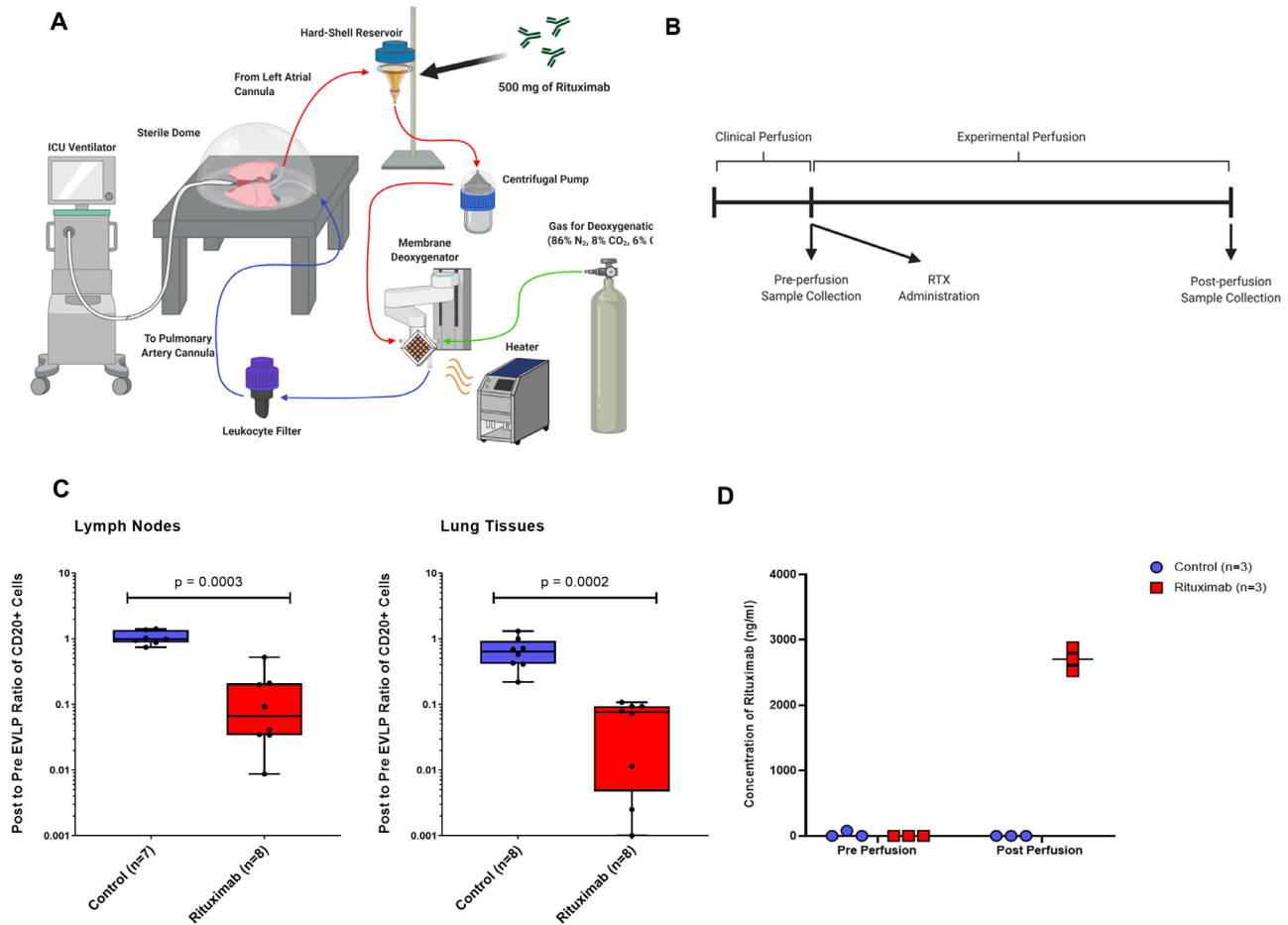
The EVLP system employed in the experiments was the Toronto technique with further details provided elsewhere [18]. The system is primed with 2.0 L of Steen solution (XVIVO): a buffered, low potassium solution with human albumin and dextran 40. This solution is further supplemented with 500 mg of methylprednisolone (Solu-Medrol; Sandoz Canada), 10,000 IU of unfractionated heparin (Leo Pharma) and 500 mg of Imipenem/Cilastatin, (Primaxin; Merck). This solution is circulated by a centrifugal pump into an oxygenator and heat exchanger where it is deoxygenated by a gas mixture (86% N<sub>2</sub>, 8% CO<sub>2</sub> and 6% O<sub>2</sub>) and warmed to normothermia (37 °C). The perfusate then continues past a leukocyte filter before entering back into the lung through the pulmonary artery cannula. Once reoxygenated within the lung, the perfusate returns into a hard-shell reservoir to be re-circulated.

### 2.3. Rituximab ELISA

Frozen samples were mechanically homogenized and incubated in RIPA buffer supplemented with HALT protease inhibitors and

in reducing inflammation as well as treating several disorders such as hematological cancers and rheumatoid arthritis [7]. By reducing B-cells in the graft, EVLP-mediated administration of this drug may serve to reduce inflammatory processes and contribute to better graft outcomes. Delivery through EVLP may avoid the adverse effects of infusion reactions associated with intravenous administration of RTX along with potential prolonged hypogammaglobulinemia that carries an increased risk of opportunistic infection [9,10].

RTX has been used for posttransplant lymphoproliferative disorder (PTLD) treatment by means of targeting Epstein-Barr virus infected B-cells [11]. Epstein-Barr virus (EBV) is a gammaherpesvirus that has a seroprevalence of 90–95% in the general population [12]. After primary infection, EBV establishes latency and remains dormant predominantly in B-cells [13]. EBV reactivates periodically, and under normal conditions, the host immune response, formed primarily of EBV-specific T-cells, is able to efficiently control viral replication and B-cell proliferation. This protection is lost in immunocompromised patients including those that received organ transplantation. This may result in uncontrolled proliferation of EBV-infected B-cells leading to PTLD, a potentially fatal complication of organ transplantation [14].



**Fig. 1.** Flow cytometry and ELISA results indicate that EVLP successfully delivers RTX to both lung tissues and lymph nodes. A) The rejected lungs are placed within a sterile dome and cannulated on the pulmonary artery and the left atrial cuff. Oxygenated perfusate exits the lungs through the atrial cannula into the reservoir. The perfusate is then passaged through a centrifugal pump and propelled into the deoxygenator and heat exchanger. The perfusate is mixed with a gaseous mixture (86% N<sub>2</sub>, 8% CO<sub>2</sub>, and 6% O<sub>2</sub>) and heated to normothermia (37 °C). Lastly, the perfusate will proceed through a leukocyte filter prior to reentering the lung through the pulmonary artery cannula. The 500 mg of RTX is administered into the hard-shell reservoir and circulated. B) The experimental timeline illustrating time of sample collection. C) Lymphocytes were differentiated from total cells based on size and complexity, doublet cells excluded and the proportions of live CD45<sup>+</sup>, CD3<sup>-</sup>, CD20<sup>+</sup> cells were taken. These CD20<sup>+</sup> frequencies were used in ratios of CD20<sup>+</sup> cells post over pre-EVLP observed in lymph nodes ( $n = 8$  with RTX or  $n = 7$  on EVLP alone) and in lung tissue ( $n = 8$  with RTX or  $n = 8$  on EVLP alone). Displayed are the individual ratios for each perfusion with their median and associated maximum and minimum values illustrated by the error bars. Mann–Whitney U statistical analysis was conducted with significance defined at  $p < 0.05$ . D) Tissues that were collected and frozen following perfusions were thawed and dissociated. The resulting lysates ( $n = 3$  with RTX or  $n = 3$  on EVLP alone) were run on a quantitative RTX ELISA. (Abbreviations: Ex-vivo lung perfusion – EVLP, Rituximab – RTX).

ethylenediaminetetraacetic acid (ThermoFisher). The resulting lysates were analyzed on an RTX ELISA (MyBioSource). All samples were run in duplicate and the limit of detection of the assay was 1.25 ng/ml. RTX concentration values below the quantification limit were approximated to half of the limit of detection.

#### 2.4. Flow cytometric analysis

**Tissue homogenization.** Collected lung tissue and lymph nodes were incubated in collagenase A at 0.8 mg/mL (Roche) and DNase I at 160 µg/mL (Sigma), homogenized using a GentleMACS Dissociator (MiltenyiBiotec) and filtered through a 40 µm cell strainer (Fisher-Scientific). Following the filtration process, red blood cells were lysed with an ammonium chloride lysis buffer (BD Biosciences) and frozen for 24 h at –80 °C before being stored in liquid nitrogen to be analyzed later via flow cytometry.

**In vitro activity assay.** Samples were acquired before and after perfusion and cultured in RPMI media (ThermoFisher) supplemented with 1% Antibiotic–Antimycotic (ThermoFisher), antibiotic cocktail [19] and gentamicin (ThermoFisher). After an overnight acclimatization period, human AB serum (ValleyBiomedical) was added to induce RTX complement activation. Following an additional 24-h

serum incubation, dissociated cells were collected and stained for flow cytometric analysis.

**Flow cytometry.** CD19<sup>+</sup> and CD20<sup>+</sup> proportions were determined by subjecting homogenized and culture samples to flow cytometric analysis (LSRII-OICR, BD Bioscience) at the UHN-SickKids Flow Cytometry Facility. Samples were blocked with human BD Fc Block (BD Pharmingen) and surface stained with antibodies targeting: CD3 (ThermoFisher), CD19 (Biolegend), CD20 (clone 2H7, BD Pharmingen), CD45 (BD Pharmingen), and the ZombieAqua viability stain (Biolegend). B-cell proportions were determined according to CD19<sup>+</sup> and CD20<sup>+</sup> cells as a percentage of live, CD45<sup>+</sup>, CD3<sup>-</sup> cells.

#### 2.5. Lung inflammation and safety

**Cytokine analysis.** Perfusate was collected before and after either EVLP alone or with RTX. The samples were run on MAGPIX multiplex cytokine assay (Milliplex) by EVE Technologies Corp. The assay assessed concentrations of interferon  $\gamma$  (IFN $\gamma$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), IL-6, IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-2, IL-4, IL-5, IL-12, IL-13, and monocyte chemoattractant protein 1 (MCP-1) with sensitivity ranging from 0.4 to 7.5 pg/ml.

**Physiological parameters.** Six rejected donor lungs were used for assessment of the impact of RTX on the physiological capabilities of the lung. Lungs treated with EVLP + RTX ( $n = 3$ ) or EVLP alone ( $n = 3$ ) were evaluated hourly for six hours to acquire the following parameters: dynamic and static compliance (in ml/cm of water), peak airway pressure (PawP) (in cm of water), pulmonary vascular resistance (PVR) = [(pulmonary artery pressure – left atrial pressure) x 80] / pulmonary artery flow (in dynes•seconds•cm<sup>-5</sup>), and oxygenation in mmHg.

## 2.6. Histological analysis

**Section staining.** Tissue and lymph node samples were formalin-fixed and paraffin-embedded. Blocks were sectioned at 5- $\mu$ m thickness and background stained with hematoxylin. Slides were subsequently stained with antibodies targeting CD19 (Abcam), CD20 (clone EP459Y, Abcam). Sections were also stained with the deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Roche). All histological protocols were conducted by UHN STARR (Spatio-temporal Targeting and Amplification of Radiation Response) Labs. Sections were also stained using *in-situ* hybridization (ISH) RNAScope probes targeting EBV-encoded small RNA 1 (EBER1) (ACDBio). EBV positive control slides used for EBER1 analysis were splenic tissue sections from a patient with Hodgkin's lymphoma (Cell Marque).

**Image quantification.** Lung tissue and lymph node slides were scanned and digitized using the ScanScope AT2 scanner (Leica Biosystems) at 20X magnification. Image analysis algorithms were developed using HALO<sup>®</sup> image analysis software (Indica Labs). Different algorithms and parameters were established to measure CD19, CD20, EBER1 and TUNEL cell staining within each slide (Supplementary Fig. 1). The proportion of CD19 and CD20 positive cells was determined by taking the fraction of medium and strong positive cells from the total number of cells read by the software.

## 2.7. Statistical analysis

To adjust for the baseline variability across all metrics of the rejected human donor lungs, measurements were expressed as the proportional changes after perfusion. The Mann–Whitney U test was used to compare the ratios of post-to-pre perfusion measurements created in the flow cytometric and histological analyses. The creation of all figures and statistical analyses conducted were done using GraphPad Prism 8.0 software (GraphPad Software). Error bars within the figures delineate the range between the maximum value and the minimum value. Statistical significance was defined at  $p < 0.05$ .

## 2.8. Ethical approval

All donor families provided written informed consent for lungs to be used for research if deemed unsuitable for transplantation by standard criteria. This protocol was approved by the University Health Network's Research Ethics Board (Coordinated Approval Process for Clinical Research (CAPCR) #06-0283) and Trillium Gift of Life (Ontario organ donation organization). The research is performed in accordance with the ethical principles of research involving human participants as outlined in the Declaration of Helsinki.

## 2.9. Role of funding source

This study was funded by the University Health Network Transplant Care Center. The funders had no role in data collection, data analysis, data interpretation, or writing of the manuscript. TK, RR, VH, MC, and AH had full access to all study data and were responsible for the decision to submit for publication.

## 3. Results

### 3.1. RTX is efficiently delivered to the lungs via EVLP

To assess whether we could deliver RTX via EVLP, twenty human deceased donor lungs (Supplementary Table 2) from EBV seropositive donors that had been rejected for transplant underwent either EVLP alone or EVLP with administration of 500 mg of RTX (Fig. 1a) (Supplementary Table 1). This drug dose was selected so that we could administer a clinically used systemic dose but deliver it specifically to the lungs [20]. Lung tissue and lymph nodes were collected before and after experimental EVLP and analyzed with flow cytometry (Fig. 1b). The flow cytometry antibody, clone 2H7, targeted CD20 and has overlapping specificity to the epitope targeted by RTX. However, RTX binds with a higher affinity, preventing 2H7 from binding [21]. As such, we assessed RTX binding efficiency by comparing 2H7-associated targeting of CD20 in RTX perfused tissues and controls (Supplementary Fig. 2). To account for the heterogeneity in our samples, we used post-to-pre EVLP ratios to analyze the proportional change of each measurement from baseline. Lymph nodes collected from lungs administered with RTX demonstrated a 10.9 fold-reduction in CD20+ staining compared to controls ( $p = 0.0003$ ) (Fig. 1c). In lung tissue, RTX perfusion resulted in an 8.75 fold-reduction in CD20+ staining relative to controls ( $p = 0.0002$ ). This suggests that RTX was successfully delivered during EVLP and efficiently bound to B-cells in both lung tissue as well as hilar and interlobar lymph nodes (Fig. 1c). RTX delivery was further confirmed by measuring rituximab concentration in homogenized lung tissue (Fig. 1d). Samples perfused with RTX demonstrated a median concentration of 2705.16 ng/ml while RTX levels in controls were undetectable.

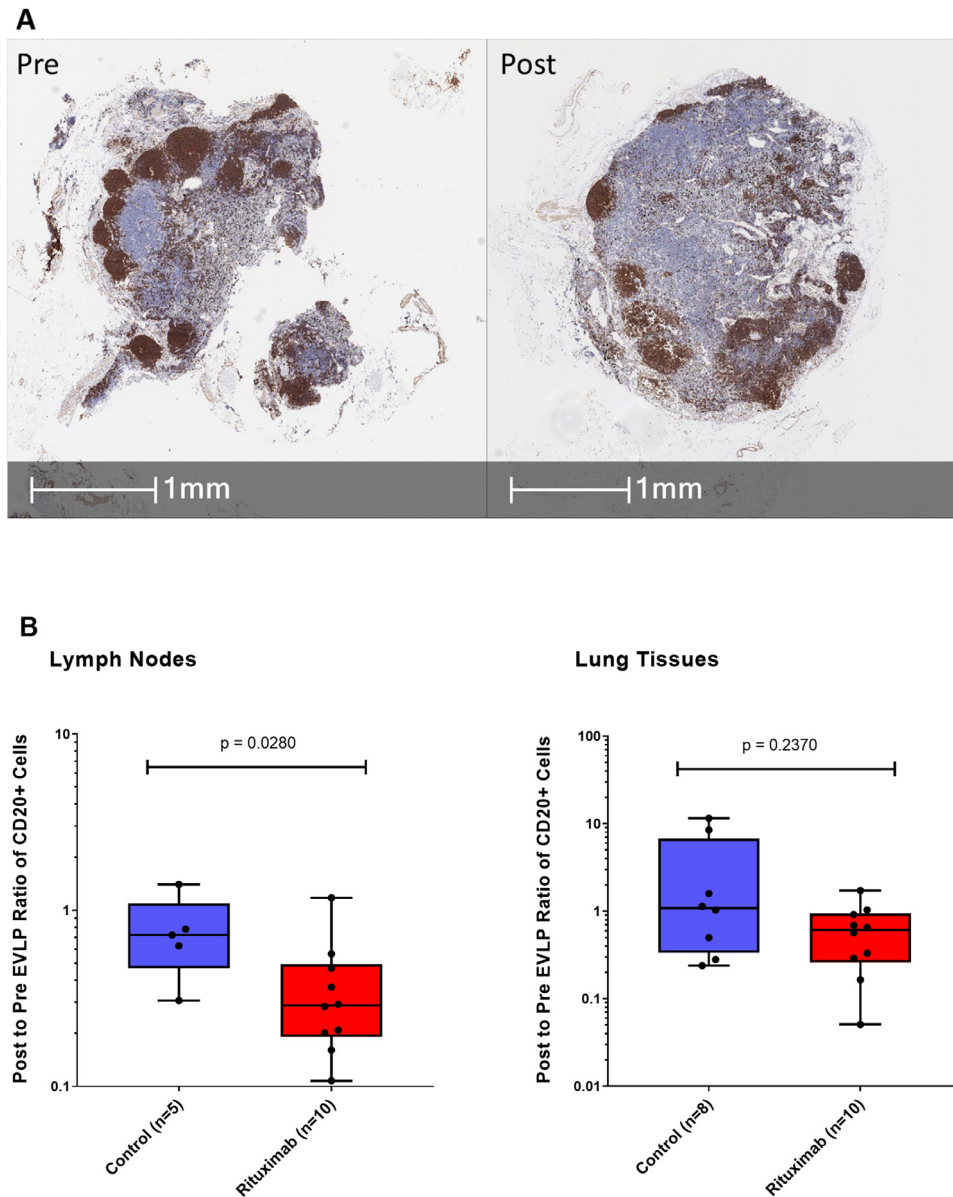
Lung tissues and lymph nodes were further analyzed using immunohistochemistry with the CD20 antibody clone, EP459Y. This different CD20 antibody clone was used because it binds to a sequence between amino acids 270 and 297 of CD20 whereas RTX binds amino acids 171–174 [22]. The EP459Y epitope also lies intracellularly and distal from the RTX epitope, decreasing the likelihood of it being occluded by bound RTX. The proportion of CD20 positive cells was found to be significantly reduced in RTX perfused lymph nodes, but not significant in perfused lung tissue (Fig. 2a and b).

### 3.2. RTX delivery through EVLP does not increase inflammation

Safety was assessed through several parameters. Cytokines associated with lung and RTX injury including IL-6, IL-8, IL-1 $\beta$ , IFN $\gamma$ , TNF $\alpha$  and IL-10 were assessed in the perfusate before and after EVLP with or without RTX [23,24] (Supplementary Table 3). No significant difference was observed in any of the cytokines assessed within the panel in RTX EVLP perfusate as compared to controls (Fig. 3a). An extended panel of cytokines (GM-CSF, IL-2, IL-4, IL-5, IL-12, IL-13 and MCP-1) was also measured in perfusate due to their involvement in EVLP cytokine profiles [25]. A significant reduction in IL-13 and MCP-1 was observed within RTX EVLP perfusate (Supplementary Fig. 3).

To further assess the potential toxicity related to EVLP-mediated RTX administration, the TUNEL assay was used to quantify cell death. There was no significant increase in TUNEL staining following RTX perfusion, suggesting that RTX perfusion did not increase non-specific cell death in lung tissue (Fig. 3b).

Importantly, parameters of lung function such as graft oxygenation, static and dynamic compliance, peak and plateau airway pressure as well as pulmonary vascular resistance were recorded over the course of six hours within donor lungs in controls and RTX groups ( $n = 3$  per group) (Supplementary Table 4). No differences were observed in *ex vivo* lung function between the two groups (Fig. 4).



**Fig. 2.** RTX administration and subsequent binding downregulates CD20 expression. A) Lymph nodes ( $n = 10$  with RTX or  $n = 5$  on EVLP alone) and lung tissue biopsies ( $n = 10$  with RTX or  $n = 8$  on EVLP alone) were fixed in formalin and embedded into paraffin blocks. Sections were taken from the blocks, placed on to slides and stained with an antibody against CD20 (brown). Illustrated is a lymph node taken pre and post RTX perfusion. B) Slides were electronically scanned and analyzed with HALO image quantification software. Displayed are the ratios of medium and strong CD20 positively stained cells post- to pre-EVLP, their medians and associated ranges from the maximum to minimum as noted by the error bars. Mann–Whitney U statistical analysis was conducted with significance defined at  $p < 0.05$ . (Abbreviations: Ex-vivo lung perfusion – EVLP, Rituximab – RTX).

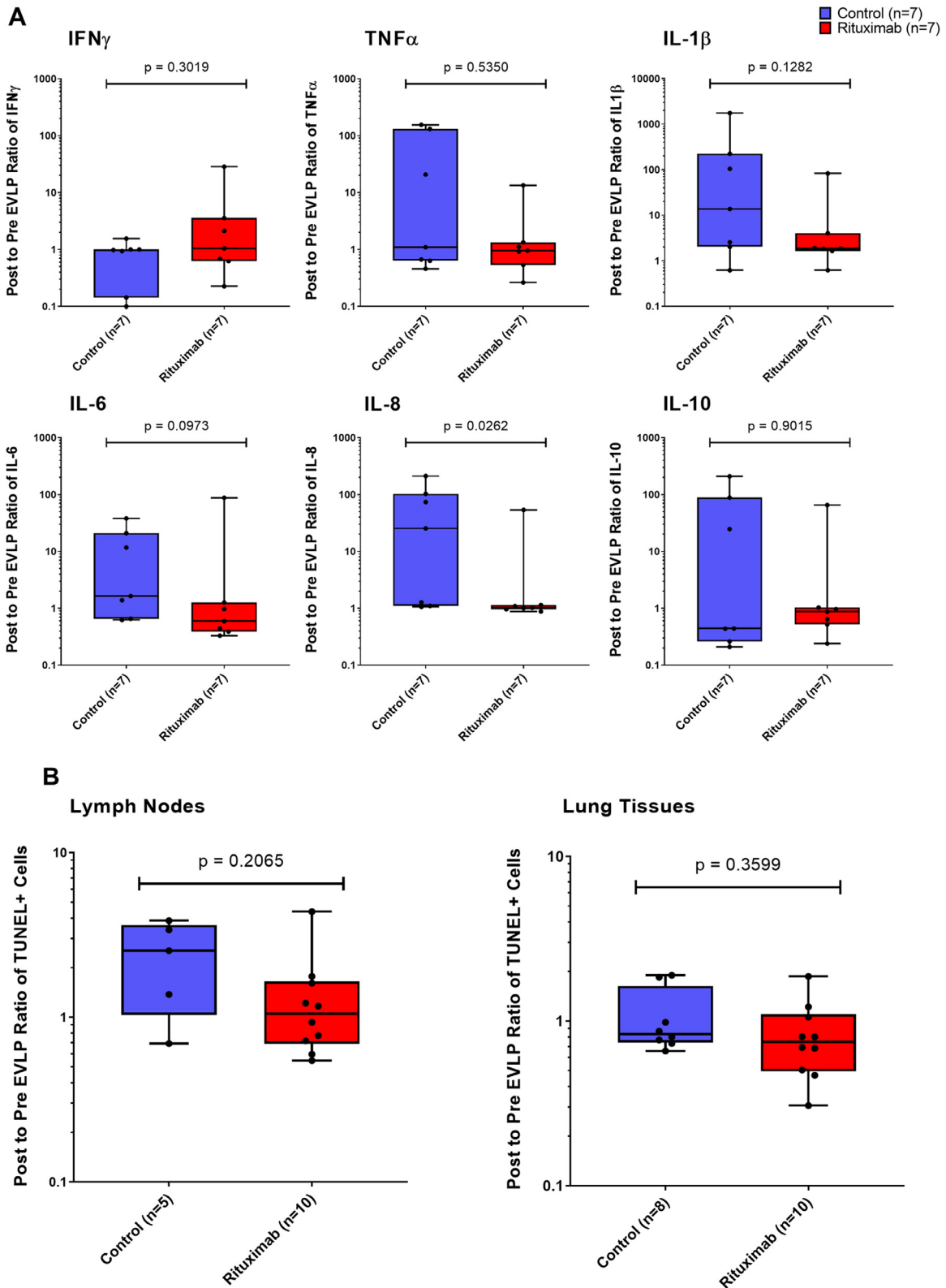
### 3.3. B-Cell depletion occurs following RTX perfusion in the presence of human serum

We then sought to mimic the organ transplant scenario that occurs after the lung is on EVLP for 4–6 h. Based on the findings that EVLP-delivered RTX successfully binds to B-cells, we hypothesized that intra-graft B-cells would be depleted shortly after transplantation once the graft is exposed to circulating complement, thereby allowing for RTX activation and subsequent B-cell depletion. Samples collected before and after EVLP were cultured in media supplemented with human AB serum (as a source of complement) to stimulate complement-driven B-cell reduction. After a 24-h *in vitro* incubation, B-cell depletion was analyzed using flow cytometry. Tissue and lymph node samples from RTX treated lungs had lower proportions of live CD19+ cells compared to controls (Fig. 5). This effect was more pronounced in lymph nodes (3.226-fold reduction;

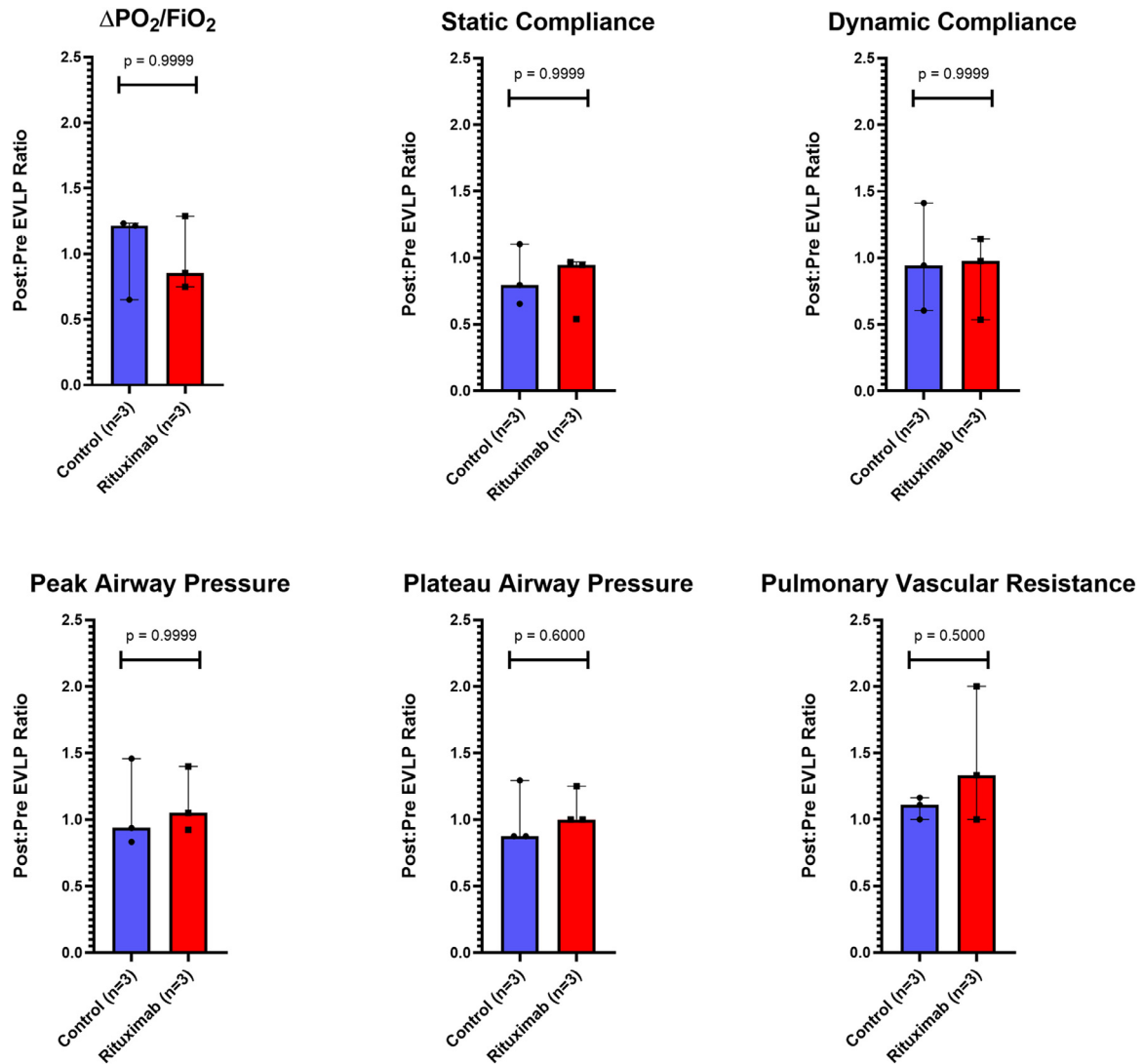
$p = 0.0286$ ) than it was in lung tissue (2.121-fold reduction;  $p = 0.2000$ ). In addition, to confirm that B-cell depletion does not occur within the time-span of EVLP (and in the absence of complement), we also showed that prior to tissue culture, the proportion of CD19+ B-cells is unchanged within lymph nodes (1.06 fold change in RTX vs. 1.06 in controls,  $p = 0.9452$ ) or in lung tissue (1.87 in RTX vs. 1.79 in controls,  $p = 0.5728$ ) (Fig. 6). Similar observations were obtained via CD19 immunohistochemical analysis (Fig. 7a and b).

### 3.4. Latent EBV levels are low but unchanged after EVLP

To assess latent EBV antigens, RNAScope, a variant of *in-situ* hybridization (ISH) was used to quantify EBER1, an EBV RNA molecule found in latently infected cells [26]. Overall, the quantity of EBER1 was low (median EBER1+ frequency of 0.0154% and 0.0152% in lung tissues and lymph nodes, respectively) (Fig. 8a and b).



**Fig. 3.** RTX delivery through EVLP does not significantly increase cell death or inflammatory cytokines. A) Concentrations of cytokines (in pg/mL) were measured before and after perfusion in perfusions with RTX ( $n = 7$ ) and in control perfusions ( $n = 7$ ) with a magnetic bead ELISA and are expressed as a ratio of post- to pre-EVLP concentrations of cytokines. B) The change in cell death was expressed as a ratio of post- to pre-EVLP TUNEL positive cells according to HALO image quantification. Displayed are the individual ratios for each perfusion of lymph nodes ( $n = 10$  with RTX or  $n = 5$  on EVLP alone) and lung tissue ( $n = 8$  with RTX or  $n = 10$  on EVLP alone) with their median and associated ranges between the maximum and minimum values shown by the error bars. A Mann–Whitney U statistical analysis was conducted with significance defined at  $p < 0.05$ . (Abbreviations: Ex-vivo lung perfusion – EVLP, Rituximab – RTX).



**Fig. 4.** Perfusion with RTX does not adversely affect lung function within EVLP. Physiological parameters were collected and expressed as fold changes, post- to pre-EVLP. There was no statistical difference between the RTX ( $n = 3$ ) and control groups ( $n = 3$ ) in regards to oxygenation (P/F ratio, mmHg), static and dynamic compliance (mL/cm of H<sub>2</sub>O), PawP (cm of H<sub>2</sub>O), plateau airway pressure (cm of H<sub>2</sub>O) and PVR (mmHg). Ratios are presented with the colored bars illustrating the median values and interquartile ranges, and error bars denoting the range from the minimum to maximum values. Parameters were collected at every hour for 6 h (red = RTX group, blue = EVLP alone). Mann–Whitney U statistical analysis was conducted with significance defined at  $p < 0.05$ . (Abbreviations: Ex-vivo lung perfusion – EVLP, Rituximab – RTX). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

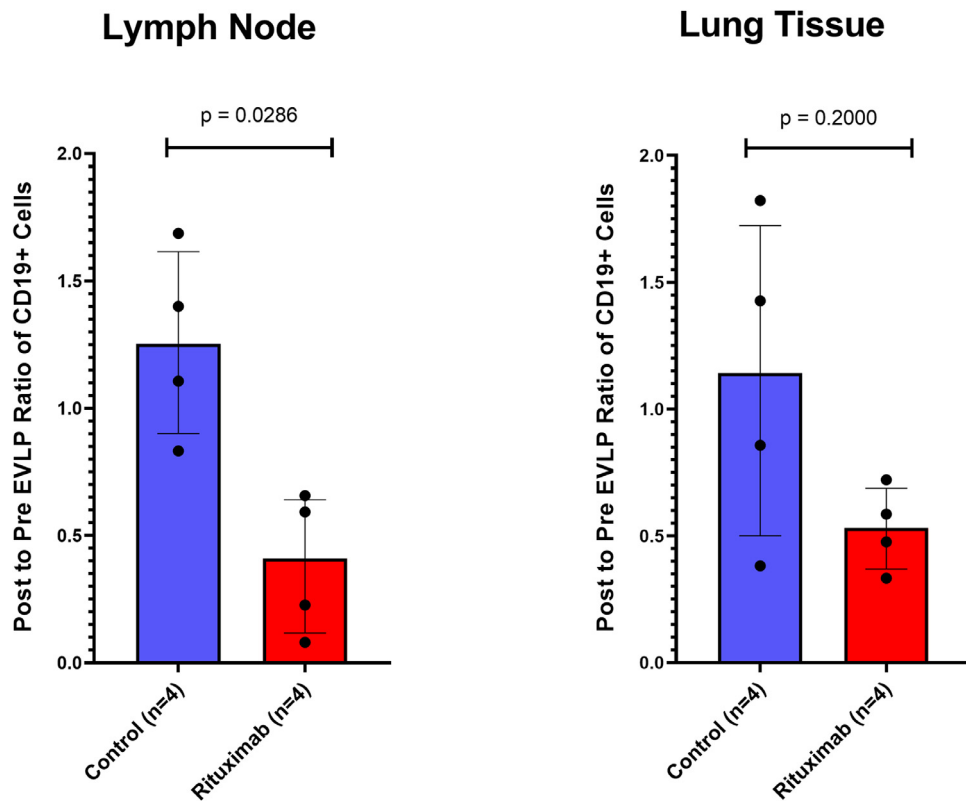
Furthermore, the ratios of EBER1 cells post- to pre-EVLP were not significantly different between samples perfused with RTX or EVLP alone (Fig. 8c). This is consistent with experiments above where B-cell depletion is only likely to occur in the presence of complement exposure as would occur in the post-transplant scenario. Given the low starting amounts of EBER1 staining, a 24 h post-tissue culture sample was not evaluated by ISH. Instead an EBV PCR of the supernatant was performed but was negative in both control and treated samples (data not shown).

#### 4. Discussion

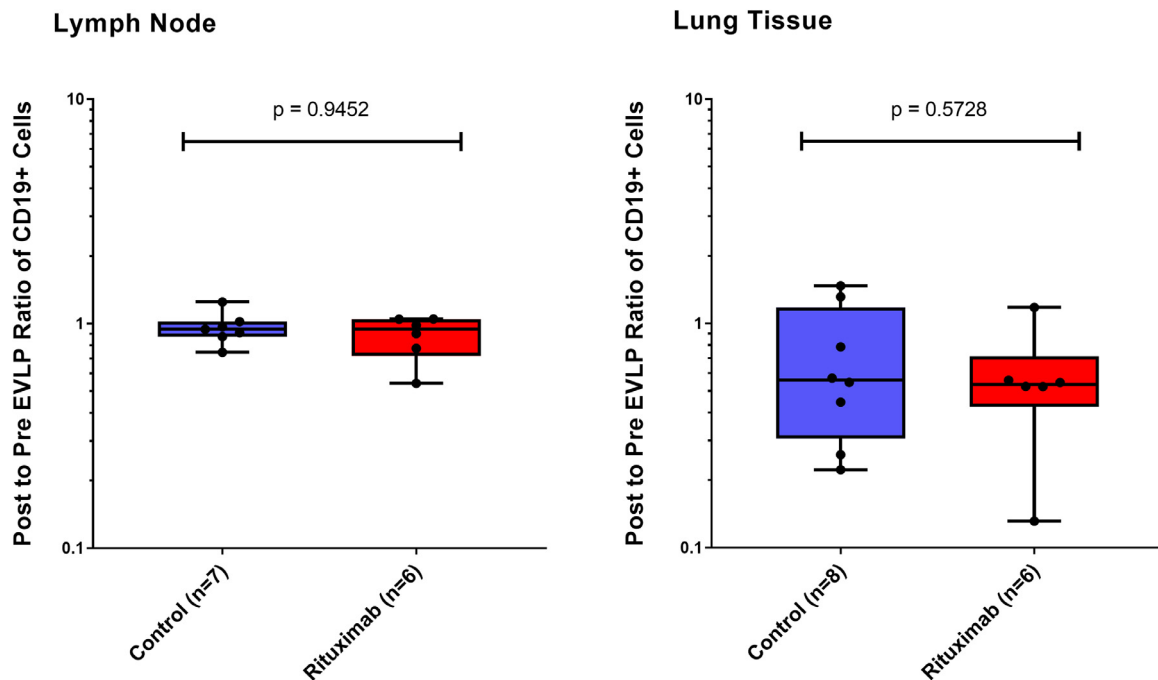
Ex-vivo organ perfusion is an innovative modality that can recondition sub-optimal grafts. Here we illustrate, that monoclonal antibody therapies can be successfully delivered, and bound to their targets within the donor graft via EVLP. We also demonstrated that bound RTX was capable of inducing B-cell depletion *in vitro* by 24 h post-perfusion once exposed to complement. This would be analogous to the clinical setting where bound RTX should begin depleting

intra-graft B-cells shortly after transplantation. In the EBV D+/R- lung transplant, patients are at very high risk of early PTLD due to donor transmission of EBV from B-cells within the allograft. As such, early depletion of those B-cells should mitigate the risk of PTLD. Administration of RTX did not induce any adverse changes in lung parameters, proinflammatory cytokines levels, or cell death. In fact, although not all statistically significant, RTX administration led to a global trend in decreasing inflammatory cytokine production, notably in IL-8, IL-1 $\beta$ , TNF $\alpha$  and IL-6.

When delivered intravenously to a patient, RTX-mediated depletion of B-cells is not appreciable until two or more weeks post-administration, with several courses of treatment being required [27]. However, we observed an effect much sooner (24 h) suggesting that a high locally delivered dose is perhaps more efficient in binding target B-cells. The mechanisms of RTX mediated B-cell depletion likely include complement dependent cellular cytotoxicity and antibody dependent cellular cytotoxicity [28]. The former mechanism is consistent with the effect we observed after the addition of serum (complement) and 24-h tissue culture. This is also support by lack of

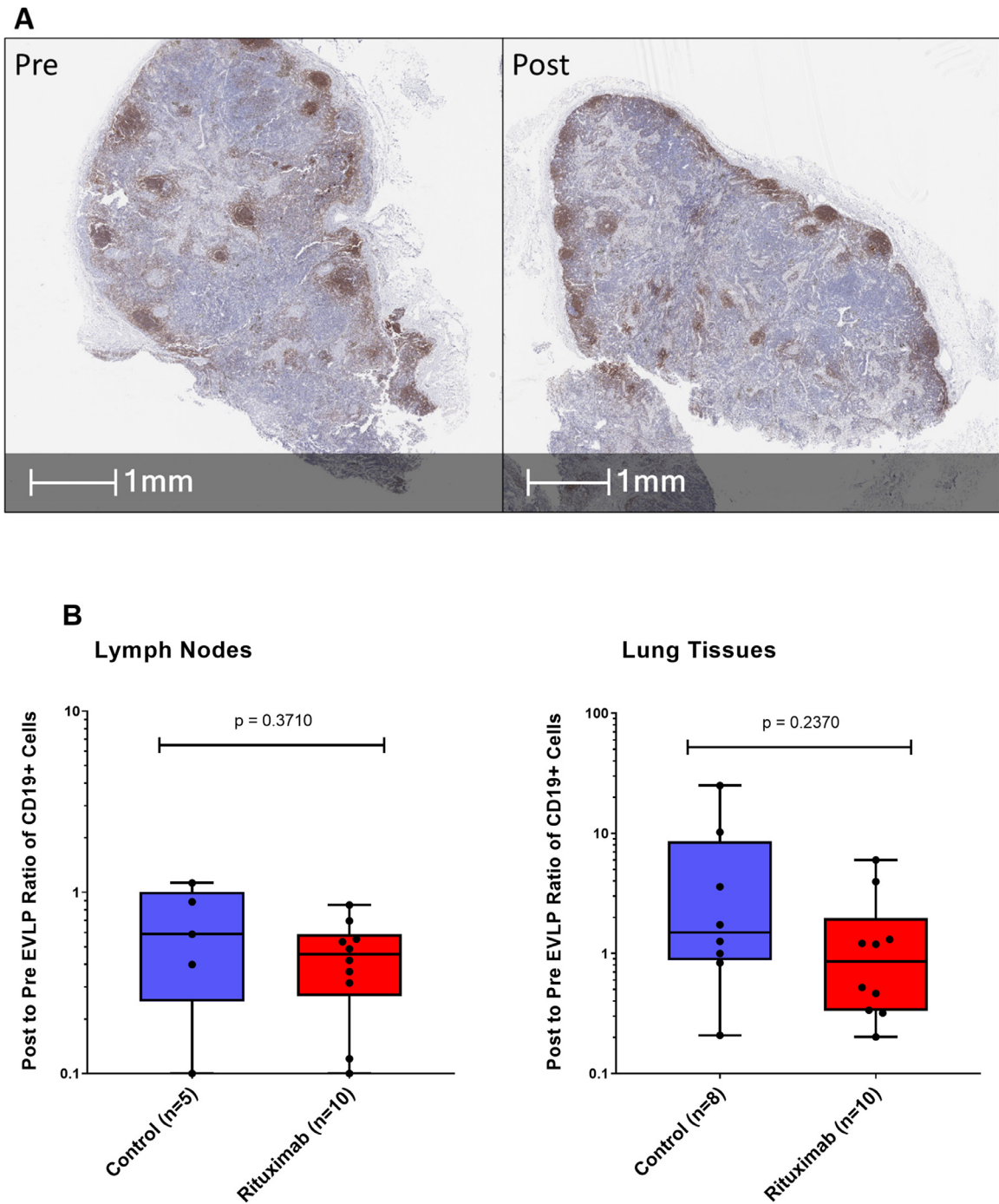


**Fig. 5.** RTX retains its activity 24 h after perfusion. Lymph node and tissue biopsies ( $n = 4$  with RTX or  $n = 4$  on EVLP alone for both sample types) collected were cultured in media with human serum for 24 h. Following the 24-h incubation, the cells were collected and analyzed with flow cytometry. Illustrated are the ratios of live B-cells post- to pre-EVLP CD19+ cells after the 24-h culture. The individual ratios are displayed with the colored bars showing the median values and the error bars denoting the range between the maximum and minimum. Mann–Whitney U statistical analysis was conducted with significance defined at  $p < 0.05$ . (Abbreviations: Ex-vivo lung perfusion – EVLP, Rituximab – RTX).



**Fig. 6.** Flow cytometry analysis of CD19 illustrates no change in B-cell quantities after RTX perfusion. Proportions of CD19+ cells were taken as a percentage of live CD45+ CD3- cells. Ratios of post- to pre-EVLP CD19+ cells were generated and compared. The ratio of live CD19+ cells were observed in lymph nodes ( $n = 6$  with RTX and  $n = 7$  on EVLP alone) and lung tissue ( $n = 6$  with RTX and  $n = 8$  on EVLP alone). Displayed are the individual ratios for each perfusion with their median and associated ranges from the maximum to minimum values demonstrated by the error bars. Mann–Whitney U statistical analysis was conducted with significance defined at  $p < 0.05$ . (Abbreviations: Ex-vivo lung perfusion – EVLP, Rituximab – RTX).

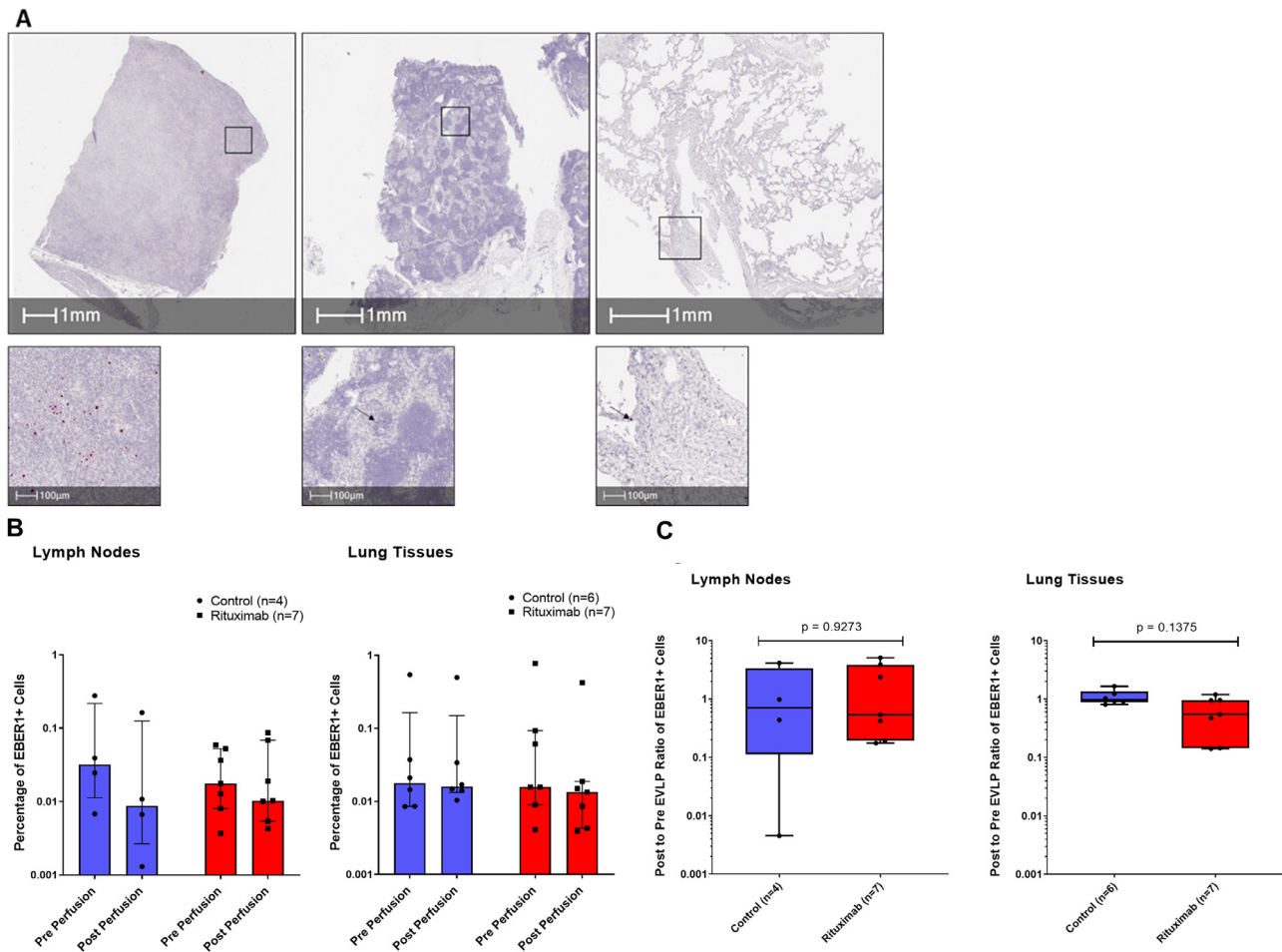




**Fig. 7.** Immunohistochemical analysis illustrates no decrease in CD19+ cells after perfusion with RTX. A) Representative section of a lymph node from before and after a perfusion with RTX stained with an antibody against CD19 (brown). B) The proportional change of CD19 in lymph node ( $n = 10$  on RTX perfusions and  $n = 5$  on EVLP alone) and lung tissue samples ( $n = 10$  on RTX perfusions and  $n = 8$  on EVLP alone) was expressed as a ratio of post- to pre-EVLP CD19+ percentages. Percentages were acquired by taking the medium and strong CD19 positively stained cells as determined by HALO image quantification. Displayed are the individual ratios for each perfusion with their median and associated ranges between the maximum and minimum values shown by the error bars. Mann–Whitney U statistical analysis was conducted with significance defined at  $p < 0.05$ . (Abbreviations: Ex-vivo lung perfusion – EVLP, Rituximab – RTX).

B-cell depletion during the 5–12 h timespan of EVLP which is performed with an acellular perfusate and lack of added blood or serum. One could expect that following transplantation, exposure to recipient serum complement factors would then result in significant intra-graft donor B-cell depletion. A longer duration of EVLP could potentially allow B-cell depletion to occur during the procedure itself, but may also result in allograft injury. As ex-vivo organ perfusion techniques improve over time, long duration clinical EVLP will likely be very feasible, and more amenable to this approach.

In several cases, RTX treatment has even shown efficacy in reducing the effects of rejection through B-cell ablation [29,30]. The presence of donor-derived B-cells has been associated with benefits and detriments. Regulatory donor cells can assist in reducing inflammation and contribute to tolerance [31]. Yet in contrast, these populations are similarly linked to negative outcomes such as graft vs. host disease [32]. Donor B-cells serve as an additional target for the alloimmune response arising from the recipient [33,34]. Watanabe et al. have further illustrated within a murine model that depletion of



**Fig. 8.** Administration of RTX does not decrease the latent viral load of EBV during the timespan of EVLP. A) Representative image of a positive control (left), lymph node (middle) and lung tissue section (right) stained with the EBER1 RNAScope probe. The positive control is a section of splenic tissue from an individual with Hodgkin's lymphoma. The section was scanned at 20X magnification and was further magnified to visualize the punctate positive signal exhibited by the EBER1 probe (denoted by the arrows). B) Shown are the proportions of EBER1 medium and strong positive cells as quantified by HALO in lymph nodes ( $n = 7$  from RTX perfusions and  $n = 4$  from EVLP alone perfusions) and lung tissue ( $n = 7$  from RTX perfusions and  $n = 4$  from EVLP alone perfusions). The median is expressed by the bar and the interquartile range is presented through the error bars. C) The percentages are expressed as a ratio post- to pre-EVLP (EVLP alone = blue box, RTX = red box). Displayed are the individual ratios for each perfusion with their median and error bars displaying the associated ranges between the maximum and minimum values. A Mann–Whitney U statistical analysis was conducted with significance defined at  $p < 0.05$ . (Abbreviations: *Ex vivo* lung perfusion – EVLP, Rituximab – RTX). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

CD20+ cells may reduce graft ischemia-reperfusion injury [35]. Though our study could not show direct benefit to allograft tolerance, it did illustrate that RTX can induce a significant reduction in IL-13 and MCP-1, both of which are cytokines associated with B-cells [36,37]. Both IL-13 and MCP-1 have been suggested to play a role in rejection with their involvement in cases of lung inflammation as well as bronchiolitis obliterans syndrome [38–41]. RTX may therefore serve a role in reducing the inflammatory profile of the donor graft. The success of this study illustrates the potential to use alternative immunotherapies within EVLP to target inflammation and improve graft transplant outcomes. Potential applications could include using anti-thymocyte globulin or alemtuzumab to reduce graft-versus-host disease and rejection as well as the use of other monoclonals to target microbial infections [42–44].

In recent studies, our team and others have demonstrated success in using EVLP to administer high-dose broad spectrum antibiotics through EVLP to reduce bacterial and microbial infections [45,46]. Similarly, EVLP has been recently utilized to target viruses through the application of ultraviolet light therapy to inactivate circulating hepatitis C virus within the donor organs both experimentally and in a clinical trial [5,47]. In this study, we target another such virus in EBV. EBV-related diseases, notably PTLD, remain a major problem

after organ transplantation in the high-risk EBV D+/R- setting, and lung transplant recipients in particular, seem to be uniquely predisposed. Pediatric patients who are more commonly seronegative pre-transplantation also have a much higher rate of PTLD [48]. Despite the success of current treatment methods, the number of available preventative strategies for EBV associated PTLD in D+/R- scenarios is lacking. The potential to decrease latent EBV through EVLP may serve to significantly reduce the risk of EBV-associated PTLD within D+/R-recipients and address the need for an adequate preventative strategy. However, it should be acknowledged that we are uncertain to what degree of B-cell depletion within the graft would be sufficient to prevent transmission of EBV. Carefully conducted clinical trials may be the only way to satisfactorily answer this question.

One of the limitations involved with our study is the variability of allograft quality. The lungs used within the study consisted of organs that may have suffered from edema, injury, infection or factors that could influence outcome parameters. In this study, we administered a dose of 500 mg of RTX based on one of the standard regimens used in clinical practice [49,50]. As our primary goal was penetration of RTX into the graft, this dosage was chosen as we felt that this full body dose would adequately target all B-cells in the comparably smaller graft. Despite variability in lung size, the dosage was not

adjusted for internal surface area. Additional studies will aim to elucidate the optimal dosage to be used for maximum efficacy while minimizing potential adverse effects.

Secondly, we did not demonstrate a reduction of EBV viral load. This was likely because the measurable baseline of EBV viral burden by EBER *in-situ* hybridization analysis was so low. This is despite the fact that lung transplants seem to have a higher preponderance for EBV-related PTLD compared with other organs. Due to the low amount of biopsied tissue that was used for culture, histological analysis could not be performed. qPCR analysis of the *ex situ* culture supernatant was done and illustrated that EBV was not produced at a detectable level (data not shown), thus quantitation was not possible. This result may have occurred due to a lack of reactivation or the absence of an appropriately sensitive assay for the small amounts of EBV present. Nevertheless, in an immunosuppressed posttransplant patient, even a low viral burden can give rise to uncontrolled EBV DNAemia.

Lastly, though we have shown that high-dose RTX does not adversely affect lung physiology or increase cell death and inflammation, it is unclear whether the drug will negatively affect the recipient once transplanted. Porcine EVLPs are often utilized to illustrate the preclinical efficacy of a proposed application of the platform [51]. Although a porcine EVLP model is available, a porcine equivalent of RTX is lacking, and the chimeric human-mouse antibody RTX is unlikely to bind to porcine lymphocytes, making this large-animal model less ideal. Using pigs genetically modified to express human CD20 may be a potential avenue for assessment of this approach in an animal model. However, we believe that sufficient clinical experience exists with RTX use in the organ transplant setting (including its use in desensitization protocols) that further pre-clinical data are not required to proceed to a pilot clinical trial. We have recently initiated a small safety trial at our institution in EBV D+/R- lung transplant recipients.

In summary, we provide a proof of concept study showing that administration of a monoclonal antibody to the donor lung *ex vivo* could potentially be used to treat a prevalent donor viral infection. This approach could be further expanded to other targets aiming at not only treating infections, but also to precondition donor organs leading to improved outcomes after transplantation.

### Author contributions

T.K. conducted the flow cytometry, RTX ELISA, and culture assays, interpretation of the data and writing the report; R.v.p.R facilitated the long EVLP experiments and contributed to the study design; V.h. F. assisted with flow cytometry, contributed to the study design, interpretation of the data, and writing the report; M.G. assisted in the EVLP experiments and study design; S.K. and D.K. assisted in the interpretation of the data and writing the report; M.C. and A.H. participated in the study design, coordination of logistics, interpretation of the data and writing the report. All authors have read and approved the final version of the manuscript.

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### Declaration of Interests

D.K. consulted with Atara Bio and received a grant from Roche. S. K. and M.C. are both founders of Perfusix Canada, XOR Laboratories Toronto and consulted with Lung Bioengineering. A.H. received grant support from Roche, Astellas and consulted with Astellas, Merck and Sanofi.

### Supplementary materials

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