Decellularization protocol-dependent damage-associated molecular patterns in rat uterus scaffolds differentially affect the immune response after transplantation

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Abstract
Scaffolds derived from decellularized tissue possess many advantages for bioengineering applications, including for novel infertility treatments. However, the decellularization process results in allogenic-independent damage-associated molecular patterns (DAMPs). This field is poorly studied, in particular for uterus bioengineering applications. An increased knowledge concerning the immune system activation after transplantation of decellularized tissue will enable safer construct development and thereby accelerate translation from research to clinic. We therefore transplanted rat uterus scaffolds produced by three different decellularization protocols based on Triton X-100 (P1 and P2) or sodium deoxycholate (P3) in a syngeneic animal model and assessed the immune response towards DAMPs exposed by the decellularization process. Biopsies were retrieved on day 5, 15, and 30 post transplantation and immunohistochemistry-stained CD45+ (leucocytes), CD4+ (T-cells), CD8a+ (cytotoxic T-cells), CD22+ (B-cells), NCR1+ (NK-cells), CD68+ (pan-macrophages), and CD163+ (M2 macrophages) cells within the grafts were quantified. The gene expression for interferon γ, interleukin (IL)-1β, IL-2, IL-6, and tumor necrosis factor (TNF) eotaxin-2, RANTES, MCP-1, MIP-1α, MIP-3α, IL-8 were also measured. Scaffolds from P1 induced a rapid cell infiltration after transplantation, presumably induced by DNA-based DAMPs. However, this response was only transient. Protocol 3 derived scaffolds induced an early pro-inflammatory cytokine response at the transcript level which remained high throughout the study. This response may be caused by the stronger decellularization detergent that could expose more extracellular matrix-related DAMPs. However, earlier proteomics analysis also identified significantly more abundant heat shock proteins-related DAMPs in this scaffold type. Protocol 2 caused the least immunogenic scaffolds and should thus be the future focus for in vivo uterus bioengineering applications.
INTRODUCTION

Many studies have focused on developing appropriate decellularization protocols for scaffold generation in various bioengineering applications (Brown et al., 2005; Hoganson et al., 2010; Lichtenberg et al., 2006; Totonelli et al., 2012; Uygun et al., 2010; Wang et al., 2013), including uterine tissue (Hiraoka et al., 2016; Kim et al., 2019; Olalekan et al., 2017; Santoso et al., 2014; Young & Goloman, 2013). Whole organ decellularization protocols for the uterus have been established for the rat (Hellström et al., 2014; Miyazaki & Maruyama, 2014; Padma et al., 2017), the rabbit (Campo et al., 2019), the pig (Campo et al., 2017), and the sheep (Daryabari et al., 2019; Tiemann et al., 2020). Collectively, these studies provide an important foundation for translational research on novel uterus bioengineering applications as a mean to restore fertility in women with uterine factor infertility. This is a condition that affects about 1 in 500 women of fertile age (Milliez, 2009) and the only way for these women to become a biological mother is through either gestational surrogacy or to undergo uterus transplantation (UTx). Considering the ethical and legal controversies surrounding gestational surrogacy, UTx was widely recognized when the world’s first live birth was born after this procedure at our center (Brännström et al., 2015). There are now 11 babies born from this fertility treatment in Sweden, and several more births reported from other centers around the world (Akouri et al., 2020; Brännström et al., 2016; Castellon et al., 2017; Ejzenberg et al., 2019; Jones et al., 2019; Testa et al., 2018). As with any organ transplant, UTx is dependent on an allogenic donor source and a carefully balanced immunosuppression to avoid graft rejection. These drugs may cause negative side-effects such as nephrotoxicity, hypertension, and lympho-proliferative disorders (Brännström et al., 2015; Kisu et al., 2020; Samant et al., 2020; Sayegh & Carpenter, 2004). Additionally, most UTx procedures that have resulted in successful deliveries used live donors who were exposed to significant risks associated to the deep pelvic donor surgery.

Several groups have therefore evaluated uterus bioengineering as an alternative treatment regime to uterine factor infertility, and showed that small bioengineered grafts derived from decellularized tissue could restore fertility in a partial uterus injury model in the mouse and the rat (Hellström et al., 2016; Hiraoka et al., 2016; Miyazaki & Maruyama, 2014; Santoso et al., 2014). The animal model of using bioengineered uterine segments/parts is clinically relevant in cases with large uterine defects such as after partial uterine resection secondary to treatment for invasive placentation or large/ repeated myomectomy.

There are surprisingly few bioengineering studies that investigated the immunological response following engraftment of decellularized tissue (Morris et al., 2017). It is generally assumed that the close homology between the amino-acid chains that make up the extracellular matrix (ECM) structure in mammals remain immunologically inert between species following engraftment (Dziki et al., 2017b). Yet, the decellularization process itself involves chemicals and detergents that denature proteins that inevitably also produce damage-associated molecular patterns (DAMPs). Fragmented ECM molecules, including fibronectin, heparin sulfate and hyaluronic acid have been shown to act as DAMPs that can affect the host immune response after implantation (Kono & Rock, 2008; Tang et al., 2012). Additionally, damaged DNA remnants from the decellularization process can also act as DAMPs (Gong et al., 2020). These complex mechanisms are often overlooked in bioengineering studies and may affect graft quality and functional outcome. In the present study, we therefore evaluated the immune response using a syngeneic rat model to elucidate if there were any allogenic-independent immunological consequences potentially caused by the decellularization processes.

MATERIALS AND METHODS

2.1 Animal experiments

A total of 45 female rats (8–10 weeks old, 140–180 g; Janvier labs) were used for the study. Twelve Lewis rats and three Sprague Dawley (SD) rats were used as donors while the remaining 30 rats were divided into five groups as viewed in Table 1. One of these groups consisted of six Lewis rats receiving SD uterine tissue and served as an allogenic control group. The remaining animals were syngeneic to the decellularized donor tissue grafts (inbreed Lewis rats). This experimental setting allowed us to study any allogenic-independent immunological consequences potentially caused by the decellularization process. The animal work was approved by the local animal welfare committee at Gothenburg University, Sweden (114-2014).

2.2 Uterus isolation and decellularization

Each of the 12 uteri that were decellularized and used as grafts for the study was isolated with an intact vasculature from the aorta to the vena cava, which facilitated decellularization by whole organ perfusion (flow rate = 2 ml/min) as detailed earlier (Hellström et al., 2014; Padma et al., 2017). Each uterus was decellularized according to one of the following decellularization protocols (P; four organs per protocol): P1 and P2 were decellularized by using dimethyl sulfoxide (4%) and Triton-X100 (1%) for 4 h, respectively, followed by 16 h of washing with phosphate-buffered saline (PBS; P1) or with distilled water (DW; P2); P3 was decellularized with...
Each decellularized rat uterus was thawed from long-term storage and was cut up into full-thickness uterus tissue patches 10 × 5 mm in size. To avoid potential infection that would interfere with the interpretation of our results, the patches were sterilized by gamma radiation at 25 kGy/hour for 3 min 25 s.

Under isoflurane anesthesia (Baxter), three patches from the respective protocols were grafted subcutaneously on the nape of the rat according to the animal groups listed in Table 1. A 6.0 prolene suture (Ethicon) was placed in each corner of every patch to secure it to the underlying tissue and to facilitate triangulation of the grafts at the later time points (Figure 1). The skin was then closed with three stitches of 4.0 silk suture (S&T). Each rat was administered subcutaneously with buprenorphine, 0.05 mg/kg, RB Pharmaceuticals.

On day 5, 15 and 30 post Tx, one of the grafted tissue pieces was retrieved by reopening the incision on the nape under isoflurane anesthesia. Each specimen was carefully isolated, then cut into two pieces: one was placed in 4% formaldehyde (Histolab) for 24 h that was used for histological analysis, and the other piece was trimmed further where as much host tissue as possible was removed from the graft before being placed in RNALater (Qiagen) for further gene expression analysis of pro-inflammatory markers.

### 2.3 Preparation of grafting material, transplantation procedure, and biopsy retrieval

Biopsies were dehydrated and embedded in paraffin and then cut in 5 μm sections. Histological analysis with hematoxylin and eosin and 4',6-diamidino-2-phenylindole was performed using standard methods. Immunohistochemistry analysis was performed using the Mach 3 and vulcan fast red kits (Biocare Medical) using a specific antibody for T-cells, cytotoxic T-cells, B-cells, macrophages, M2 macrophages, or NK-cells as summarized in Table 2. Following antigen retrieval with citric acid buffer (pH = 6.0), slides were processed with each antibody for 1 h at room temperature. All slides were then cover-slipped with Pertex (Histolab) and scanned in a microscope slide-scanner (Leica SCN400; Leica Microsystems CMS GmbH). In the scanning software, six areas per stained slide were randomly selected at 200× magnification (0.141 mm²/field). The total number of all cells (hematoxylin+ cells), and the total number of positively stained cells within the transplanted tissue was counted manually for respective antibody, excluding stained cells located in the lumen of blood vessels. The cell counts were then averaged for each animal, time point and experimental animal group.

### 2.4 Histology and image analyses

On day 5, 15 and 30 post Tx, one of the grafted tissue pieces was retrieved by reopening the incision on the nape under isoflurane anesthesia. Each specimen was carefully isolated, then cut into two pieces: one was placed in 4% formaldehyde (Histolab) for 24 h that

| Group | Description                                      | Notes                                      |
|-------|--------------------------------------------------|--------------------------------------------|
| 1     | Autologous uterus to Lewis recipient, n = 6      | (gold standard control)                    |
| 2     | SD donor uterus to Lewis recipient, n = 6        | (allogenic control)                        |
| 3     | Lewis decellularized uterus transplant (P1)      | to Lewis recipient, n = 6                  |
| 4     | Lewis decellularized uterus transplant (P2)      | to Lewis recipient, n = 6                  |
| 5     | Lewis decellularized uterus transplant (P3)      | to Lewis recipient, n = 6                  |

Notes: Three different Lewis rat uterus scaffolds produced by different decellularization protocols (P1, P2, and P3; Group 3, 4 and 5, respectively) were grafted into a Lewis rat recipient (inbred rat strain) to evaluate any potential negative immunogenic effects caused by the decellularization process itself.
**TABLE 2** Markers and the associated antibodies (Abcam, Amsterdam, Netherlands) used for immunohistochemistry to detect immune cells in grafted tissue

| Marker | Target               | Catalogue #   | Dilution |
|--------|----------------------|---------------|----------|
| CD4    | T-cells              | ab33775       | 1:50     |
| CD8a   | Cytotoxic T-cells    | ab33786       | 1:100    |
| UC22   | B-cells              | ab197650      | 1:500    |
| UC68   | Macrophages          | ab125212      | 1:1000   |
| UC163  | M2 macrophages       | ab182422      | 1:200    |
| UC1    | NK-cells             | ab214468      | 1:500    |
| CD45   | Protein tyrosine phosphatase | ab10558 | 1:800 |

dRnoCPE5177931) and expression levels were measured using primer probe mix for the following pro-inflammatory cytokine genes: interferon γ (IFN-γ, dRnoCPE5173726), interleukin 1β (IL-1β, dRnoCPE5171240), interleukin 2 (IL-2, dRnoCPE5150062), interleukin 6 (IL-6, dRnoCPE5187348), tumor necrosis factor (TNF, dRnoCPE5152044), eotaxin-2 (dRnoCPE5152090), RANTES (regulated on activation, normal T cell expressed and secreted; dRnoCPE5175661), monocyte chemoattractant protein 1 (MCP-1, dRnoCPE512142), macrophage inflammatory protein 1-alpha (MIP-1α, dRnoCPE5146925), macrophage Inflammatory Protein-3 (MIP-3α, dRnoCPE5148903), and IL-8 (dRnoCPE5182084). Each reaction was partitioned into 16,000–21,000 droplets and the PCR reactions were then performed in a C1000 thermal cycler. The fluorescence intensity of each droplet was measured in a droplet reader and the data analysis was done with Quantasoft. Negative and positive droplet populations were detected and number of copies/µl were normalized with the reference gene and data were transformed to fold change.

### 2.6 Statistical analysis

GraphPad Prism 8 (GraphPad) was used for statistical analyses. All data sets were tested for normality using the Shapiro–Wilk test. When data were normally distributed, Welch’s t-test was used (two group comparison) or one-way ANOVA with Tukey’s corrections for multiple group comparison. For non-parametric data, Mann–Whitney U-test was used (for two group comparison), or the Kruskal–Wallis test with Dunn’s post-hoc test to correct for multiple group comparison was used to assess significant differences.

### 3 RESULTS

#### 3.1 Rat uterus scaffold generation

We were able to reproduce uterus scaffolds by decellularization using earlier established protocols (Hellström et al., 2014, 2016; Padma et al., 2017). As evident by the histological observations following uterus decellularization, we detected more nuclear remnants as hematoxylin smears in P1-derived scaffolds, while scaffolds produced by P2 or P3 did not show any histological signs of remaining DNA. The ECM structure was generally well preserved by each protocol (Figure 1a–d).
3.2 | Macroscopic observations during biopsy retrieval

On Day 5, autologous grafts looked well integrated with the host tissue with no apparent swelling or discoloration. On the other hand, animals with allografts were swollen at the surgery site caused by subcutaneous accumulation of blood. The grafts in recipients of P1, P2, or P3 scaffolds were less integrated in the host tissue compared with autografts, but each implant looked clean with no particular fibrous tissue covering the implants at this stage (Figure 1f). On Day 15, we noted that the allografts were significantly more encapsulated within fibrous tissue, compared with the syngeneic groups, where the decellularized grafts seemed more integrated with signs of granulation (Figure 1g). The P1–P3 scaffolds were thinner and seemed to have reduced in volume compared to their appearance on the engraftment day. On Day 30 post Tx, this scaffold degradation was more evident and the marking sutures, aided us to identify the grafting sites and to distinguish host tissue from graft tissue in animals that received decellularized tissue grafts (P1–P3; Figure 1h).

However, the grafts were easily identified in the two control groups receiving autologous or allogenic grafts, particularly concerning the allografts, which had been encapsulated and would detach easily from the surrounding tissue.

3.3 | Cell counts and cytokine gene expression

3.3.1 | Day 5 post transplantation

A panel of representative images taken on stained sections from biopsies isolated five days after Tx is presented in Figure 2. Quantification of hematoxylin+ cells on Day 5 after transplantation showed that significantly more cells had infiltrated into the allografts compared with autografts (p = 0.037; Figure 3a). For the transplanted decellularized tissue, P1-derived grafts contained higher numbers of hematoxylin+ cells compared with the other two scaffold types. This cell density was similar to that in the allograft group and was significantly higher than the cell densities found in P2 and P3 grafts (P1 vs. P2, p = 0.0001; P1 vs. P3, p = 0.0007). The number of infiltrating CD45+ leucocytes were significantly higher in the allografts, P1- and P3-derived scaffolds compared with P2-derived scaffolds and autografts (Figure 3b). The number of CD4+ T-cells was also higher in the allografts compared to the autografts (p = 0.011; Figure 3c). We also found that grafted P2 and P3-derived scaffolds contained more T-cells compared with autografts, but due to the large intra group variance, there was no significant difference. For example, there were only a few biopsies in the P1-derived scaffolds that contained any CD4+ T-cells at all, while half the number of biopsies taken from P2-derived grafts, and four out of six biopsies taken from P3-derived grafts had many infiltrating T-cells on day five (Figure 3c). The number of CD8a+ cytotoxic T-cells (Figure 3d), CD22+ B-cells (Figure 3e), NCR1+ NK-cells (Figure 3f) and CD68+ macrophages (Figure 3g) were not significantly different between any graft types on Day 5 after Tx. When the class M2 macrophage marker CD163 was quantified in each graft, we found a higher presence of this cell type in allografts compared with autografts (p = 0.038; Figure 3h). As a high proportion of class M2 macrophages (CD163+ cells) in relation to the total number of macrophages (CD68+ cells) in the tissue can indicate a favorable tissue regeneration milieu, we also assessed the CD163+ and CD68+ ratio. However, no major skewing of macrophages to a regenerative M2-stage was noticed in any of the groups at this time point. However, there was a trend towards a higher ratio in allografts and P2 grafted animals (Figure 3i).

When we investigated the gene expression levels for the proinflammatory cytokines IFN-γ, IL-1β, IL-2, IL-6, and TNF on Day 5, we detected significantly higher level of IFN-γ in the allografts compared with autografts (p = 0.007) and P2-derived grafts (p = 0.003; Figure 4a). Interleukin 1-β expression was significantly elevated in transplanted scaffolds produced by P1 and P3 compared with autografts (p = 0.006 and p = 0.043, respectively; Figure 4b). Gene expression analysis also indicated a higher presence of IL-2 mRNA in P3-derived grafts compared with autografts (p = 0.018; Figure 4c).

No significant difference in IL-6, IL-8, or TNF expression was detected between the groups at this time point (Figure 4d–f, respectively). The gene expression levels were also not significantly different between the groups for the chemoattractants eotaxin-2, RANTES, MIP-1α, and MIP-3α at this time point. However, the MCP-1 expression was higher in scaffolds produced by P1 and P2 compared with P3-derived scaffolds (Figure 4g–k).

The same data from this time point is also summarized in Figure S1.

3.3.2 | Day 15 post transplantation

A panel of representative images taken on stained sections from biopsies isolated 15 days after Tx is presented in Figure S2. The cell density quantifications from this time point revealed that there was no difference in the total number of cells (hematoxylin+) between any of the grafts (Figure 3a). The number of CD45+ leucocytes were still high in the allografts, P1- and P3-derived scaffolds while the number of immune cells remained low in P2-derived scaffolds and in autografts (Figure 3b). The number of CD4+ T-cells found in the tissue was very different at Day 15 compared with Day 5 (Figure 3c). Two P3-derived grafts isolated from the animals at this time point had many infiltrated T-cells, which is mirrored by the large error bar for this group. When we assessed the presence of CD8+ cytotoxic T-cells, we noticed that there were three P3 grafts that had a substantial infiltration of this cell type (Figure 3d). But again, due to the large intra group variation, these data were not significantly different compared with the other groups. However, allografts contained significantly more cytotoxic T-cells compared with autografts (p = 0.020). The CD22+ B-cell population was lower in P1 and P2 scaffolds compared with the numbers found on Day 5 post Tx in the same graft types (Figure 3e), and B-cell were significantly fewer in these two groups on Day 15 post Tx compared with the other groups. Natural killer cells identified as NCR1+ cells
3.3.3 | Day 30 post transplantation

Quantified labelled cells from grafts isolated 30 days after Tx (Figure S4) revealed that the total number of cells in autografts were higher than in any of the other groups (p = 0.015 vs. allograft; p < 0.01 vs. P1–P3), and it was evident that the original high cell number from autografts (normal uterus tissue) had declined significantly from Day 5 (Figure 3a; p = 0.0004). The number of infiltrating CD45+ leukocytes were still significantly higher in the P3-derived scaffolds compared with the autografts, P1- and P2-derived scaffolds, and by Day 30, the number of leucocytes had significantly declined in P1-derived scaffolds compared to the first time point (Figure 3b; p = 0.004). Very few CD4+ T-cells were found in any of the grafts at this late time point (Figure 3c), and the general immune cell presence seemed to have normalized since we were unable to detect any significant difference between the numbers of CD8a+ cytotoxic T-cells, CD22+ B-cells, NCR1+ NK-cells, or the macrophages (Figure 3d–i).

The IFN-γ expression was still high in half of the allografts on Day 30 post Tx, and for one animal grafted with P1 decellularized tissue. However, the means were not significantly different between the groups (Figure 4a). Grafts that were based on P3 had elevated IL1β expression compared with both autografts (p = 0.002) and allografts (p = 0.032; Figure 4b). Furthermore, animals grafted with decellularized tissue from P3 had a tendency towards an elevated
expression of the inflammatory cytokines IL-2, IL-6, and TNF, but this was not significantly different from the other groups at this time point (Figure 4c–f). The gene expression levels for the chemokine attractants eotaxin-2, RANTES, MIP-3A, MCP-1, and IL-8 were not significantly different between the groups at this time point. But there was a slight increase in the MIP-1α expression in scaffolds produced from P3 (Figure 4e–k). Compared with expression levels from Day 5, there had been an upregulation of eotaxin-2 in all groups, and of IL-8 in autografts, allografts, and P2.

The same data from this time point is also summarized as bar graphs in Figure 55.

4 | DISCUSSION

Bioengineering principles have been developed for many organs, including the female genital organs. Most research on uterus bioengineering has so far achieved successful transplantation of engineered patches from scaffolds derived from natural decellularized uterine tissue. However, scaffolds developed by decellularization contain DAMPs as a consequence of the generation process. This includes residues of nucleotides and damaged ECM macromolecules such as fragmented hyaluronic acid, collagen, elastin or fibronectin (Houghton et al., 2006; Kono & Rock, 2008; Postlethwaite & Kang, 1976; Tang et al., 2012). Hence, the immunological consequences following engraftment of such scaffold can be allo-independent and be affected by the variety of reagents used for the decellularization process. Even if biomaterials based on ECM are considered to cause only a modest immunological response following engraftment, it has been shown that DAMPs can act as immunogens through the activation of toll-like receptors and receptors for advanced glycation end-products presented on infiltrating host cells and cause inflammation by increased expression of IL-1, IL-6, TNF, and IFN-γ (Malik et al., 2011; Tian et al., 2007). These immunological events can be detrimental and may compromise several steps in the regeneration process, including scaffold
integration, homing, and survival of endogenous cells, adverse phenotypic changes of immune cells and undesirable scarring formation. We therefore evaluated the presence of infiltrated immune cells in transplanted decellularized uterus tissue using a syngeneic animal model—an experimental setting that allowed us to distinguish if the decellularization processes may affect the immunogenicity of our produced uterus scaffolds. It is necessary to optimize decellularization protocols and investigate such scaffold attributes since a minimal reactive DAMP content can facilitate the translation of uterus bioengineering applications to larger animal models, and also in the future, to the human.

As we did not see any distinct differences in densities of resident macrophages, T, B, or NK cells in the grafts 5 days after Tx, the prominent cell infiltration in P1-derived scaffolds was likely caused by neutrophils, dendritic cells, fibroblasts and mast cells that normally respond quickly to wound healing and/or implanted biomaterials (Morris et al., 2017). Together with scaffolds produced by P3, P1-derived scaffolds had a significant amount of CD45+ leucocytes during the first 15 days after engraftment. It is likely that this robust response was initiated by multiple DAMPs, including DNA-based DAMPs (i.e., oligodeoxynucleotides) in P1 produced scaffolds since we know from earlier studies that these scaffolds contain up to 30% of

FIGURE 4 When the cytokine gene expression of the pro-inflammatory cytokines interferon γ (IFN-γ), interleukin (IL)-1β, IL-2, IL-6, and tumor necrosis factor was quantified, the allografted tissue (labelled black) showed a high initial INF-γ response. However, P3 (labelled green) and to some degree also P1 (labelled red) had an elevated expression of the other cytokines evaluated. The expression levels for the chemokines IL-8, monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1-alpha (MIP-1α), RANTES, eotaxin-2 and MIP-3α were largely stable during the experiment with few group differences. #, significant difference from autograft; ¥, significantly different to allograft (G); *, significantly different to P1; ¥, significantly different to P2; ‡, significantly different to P3; *, significantly different to all [Colour figure can be viewed at wileyonlinelibrary.com]
the original donor DNA content as a consequence to the milder and less complete decellularization protocol (Hellström et al., 2014). Furthermore, cytokine gene expression analysis at Day 5 after engraftment revealed that both P1- and P3-derived scaffolds had an increase of the pro-inflammatory cytokines IL-1β and IL-2. Yet, the measured gene expression levels of the neutrophil chemoattractants showed no differences between the groups that could explain the higher initial cell populations in P1 and P3 grafts. However, mRNA expression can rapidly be regulated and differences in the chemoattractant levels may have occurred at an earlier time point than measured herein.

The immunological response in P3-derived scaffolds could be a consequence of more ECM-based DAMPs from the slightly more aggressive detergent (SDC) that is used to produce this scaffold compared with the other two protocols. However, these scaffolds have also been shown to contain about 6% of fragmented donor DNA (Hellström et al., 2014). Thus, our results may suggest an immune response towards nuclear remnant acting as DAMPs in both P1 and P3 scaffolds (Ishii et al., 2001; Park et al., 2004). Our earlier proteomics studies conducted on the P1-, P2- and P3-derived uterus scaffolds used herein also identified significantly more fibronectin and versican core proteins in P3-derived scaffolds compared with scaffolds produced by P1 and P2 (Hellström et al., 2014). In the same study, we also found that this scaffold type contained significantly more amounts of heat shock protein 70, 71, and 90 (4.68, 1.7, and 2.3 times more than what was found in P2-derived scaffolds, respectively). These molecules are well known DAMPs and can for example activate toll-like receptors 2 and 4 which are both linked to potentiate the immune response. Consequently, it is plausible that the immune activation seen in transplanted scaffolds developed by P3 was driven by an increased DAMPs content caused by the SDC detergent used during the decellularization process. The high cytokine expression in P1- and P3-derived scaffolds may also have skewed the macrophage polarization towards a pro-inflammatory class M1 macrophage phenotype for these groups, as indicated by the lower (but not significantly different) ratio of CD163+/CD68+ cells for these groups compared with P2 grafted animals. However, the general mild response of infiltrating cells and the stable IFN-γ, TNF, and IL-6 gene expression suggest that there was only a modest innate immune response after the engraftment of our decellularized tissue, with P2 derived scaffolds being the least immunogenic. Similarly, the expression levels for the chemoattractants eotaxin-2, RANTES, MCP-1, MIP-1α, MIP-3α, IL-8 were largely stable during the entire experiment with very few group differences. However, additional studies that also investigate the pro-inflammatory mediators at the protein level are needed to confirm these gene products in the decellularized tissue after engraftment, especially in recipients of P3-derived scaffolds.

Based on several parameters, it was evident that the early immune response seen in P1-derived scaffolds had ceased after 15 days post Tx. It was also clear that the number of CD8α+ cytotoxic T-cells in the allogenic group was significantly higher compared with autologous grafts at this intermediate time point, while implanted scaffolds from decellularized uterus tissue had similar cytotoxic T-cell numbers to that of the gold standard (autografts). However, some animals in group P3 showed indications towards a potentially harmful immunological response. This may indicate a scaffold production variability and a need for a quality control measurement in future bioengineering applications. The immunological response in P3-derived scaffolds not only included a high infiltrate of CD45+ leukocytes and an increased CD8α+ cytotoxic T-cell density, but also involved a significantly higher presence of CD22+ B-cells. Such response may indicate immune progression towards graft rejection (Graca, 2020; Julien et al., 2017; Wrenshall et al., 1999) and/or a plausible course towards unfavorable tissue regeneration (Choi et al., 2001; Theill et al., 2002).

At 30 days post Tx, the total number of cells in all grafted scaffold types remained unchanged compared with the total cell numbers found on Day 15. This may suggest ongoing wound healing and tissue regeneration rather than any rejection events (L. Chen et al., 2019). The cell density in autologous grafts was significantly higher compared with the other groups which may be explained by the high number of containing uterus cells at the day of engraftment and that the grafts at this time point have been revascularized enough to enable cell support. This is further supported by the low amount of CD45+ cells found in this group. However, the high CD45+ resident cells in P3-derived scaffolds suggest a continued presence of DAMPs in these scaffolds. However, we noticed that the elevated IL-1β gene expression in P3-derived scaffolds was still present at 30 days post Tx which may indicate that there is a slow detrimental immunological progression towards the destruction of particular components of this scaffold type (Asea et al., 2000; W. Chen et al., 1999; Dunn et al., 2006).

In general, we detected a low ratio of the anti-inflammatory class M2 macrophage phenotype in all scaffolds at all time points. Hence, the macrophage population seemed skewed towards a more pro-inflammatory M1 phenotype in all scaffold types. This is somewhat surprising since grafted decellularized small intestinal submucosa and urinary bladder ECM led to a more profound M2 polarization (Dziki et al., 2017a). M1 polarization is initiated by high levels of INF-γ, TNF-α, and IL-6 (Krzyszczuk et al., 2018; Novak & Koh, 2013) which we did not detect at any time point. However, M1 phenotype polarization has also been associated with slow degrading implants where they seem to play an important role in stimulating angiogenesis through an M1-dependent VEGF signaling (Morris et al., 2017). Hence, the M1 polarization found herein may, at least partly, be due to the nature of ischemia and a delayed scaffold degradation. However, more studies would be needed to elucidate this further, and it would be advantageous to also include specific antibodies towards the M0 and M1 phenotypes in such studies. Furthermore, a more detailed analysis of the involved T-cell sub groups would enable a better characterization of the immune response. For example, by mapping the T-regulatory cells and the T-helper 2 cells, and their expression of anti-inflammatory cytokines (e.g., IL-4, IL-10, IL-13, and transforming growth factor–β) that are known to skew macrophages into a regenerative and anti-fibrotic M2 phenotype (Wynn & Vannella, 2016).

This study aimed to investigate if immune stimulating DAMPs in uterus scaffolds produced by three different decellularization
protocols impact the early and late local immunologic response. Collectively, our results showed that the scaffolds developed by the milder decellularization protocol (P2) contained fewer DAMPs than the scaffolds produced by the slightly more aggressive detergent (P3), while we also detected a mild initial response in P1-derived scaffolds, presumably generated by remaining nucleotide-associated DAMPs. Although additional work needs to be done to determine the complete immunogenic profile of the decellularized tissues used herein, our results clearly showed that P2-derived scaffolds caused the lowest immunological response. Hence, this scaffold type and decellularization protocol should continue to be the focus for future uterus bioengineering applications to develop novel fertility treatments.

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CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHORS CONTRIBUTIONS

Arvind Manikantan Padma, Ahmed Baker Alshaikh, Mihai Oltean, Mats Brännström, and Mats Hellström designed the study outline. Arvind Manikantan Padma, Ahmed Baker Alshaikh, Mihai Oltean, Min Jong Song, and Randa Akouri were responsible for the collection of data. All authors contributed to the data analysis and drafting the manuscript to its final version.

DATA AVAILABILITY STATEMENT

Original data generated for this article are available from the corresponding author on reasonable request.

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