In vivo cellularization of a cross-linked matrix by intraperitoneal implantation: a new tool in heart valve tissue engineering

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This paper was guest edited by Prof. Julie H. Campbell, University of Queensland, Australia

Aim To use in vivo instead of in vitro cell seeding in heart valve tissue engineering.

Methods and results Intraperitoneally preseeded, photo-oxidized bovine pericardial pulmonary valve constructs (group 1) were compared with non-preseeded constructs (group 2) implanted in sheep. All valves functioned normally and were macroscopically intact at explantation (1 week (n = 6) and 1 month (n = 6) in each group), except for one thrombosed leaflet in a group-2 valve at 1 month. Almost 10-fold higher neomatrix deposition and doubling of the leaflet thickness were found in group 1 vs. 2 (P < 0.05). A concomitant significant decrease in leaflet length (15%) was found at 1 month in group 1. The total cross-sectional surface and total amount of collagen of the original matrix remained unchanged in both groups at all times. Immunohistochemistry showed a low immune response, stem/progenitor cell infiltration, appropriated differentiation, and spontaneous endothelialization of the valves. Significantly, increased re-cellularization was found after IP preseeding compared with spontaneous seeding: cell coverage of the leaflet was 71–100 vs. 8–26% (P < 0.05), respectively.

Conclusion Complete re-cellularization can be obtained by IP preseeding of an acellularized cross-linked matrix. Well-functioning valve constructs show cellularization and differentiation into myofibroblast phenotype and concomitant neomatrix deposition.

Introduction

The disadvantages of actual mechanical and bioprosthetic heart valves are related to thromboembolic and bleeding complications as well as to calcification or degenerative valve dysfunction. Recent attempts to overcome these problems by tissue engineering of heart valves focus on the technology of in vitro seeding of host cells on biodegradable synthetic scaffolds or non-cross-linked acellularized matrices. Although some experimental success was claimed, durability of these constructs in terms of the absence of calcification and failure of the prosthetic valve remains to be proved. Furthermore, permanent and complete re-cellularization of the valve structures protecting the valve against degeneration and thrombosis was never demonstrated.

The reasons for these flaws is basically the inherent potential of the scaffold to degenerate and the need for a permanent and complete repopulation of the construct by highly differentiated valve cells capable of producing a new matrix material having natural biophysical properties.

Although it must be theoretically possible to generate a functional heart valve using the concept of in vitro cell seeding on a degradable matrix, we preferred a completely different approach. At first, we used a stable, cross-linked matrix that would not be degraded over time, to guarantee a durable heart valve, having adequate biophysical properties and lasting for a long time without degradation. Second, we applied the concept of endogenous cell seeding using the natural ‘milieu interior’ for attraction, homing, and differentiation of progenitor cells, in contrast to the artificial and limited in vitro seeding conditions. We used the organism as its own bioreactor, based on the work of Campbell et al., who generated a functional vascular graft from an intraperitoneally (IP) deposited capsule around an implanted silastic tube. The capsule comprises...
an internal layer of macrophages, some layers of (myo)fibroblasts and an external layer of mesothelial cells and is generally described as ‘foreign body reaction’. By studying the cell morphology and co-localization of α-smooth muscle actin (ASMA) and CD172a, it has been proven that the entire layer of (myo)fibroblasts is of haematopoietic origin and that the myofibroblasts in those conditions were derived from macrophages. On the other hand, the presence of an undifferentiated cell population in the explants was also reported. ASMA is a generally used marker for myofibroblasts, but CD172a is not an exclusive marker for macrophages and is also expressed in a subpopulation of haematopoietic stem cells. Therefore, the possible presence of stem cells in granulation tissue renders the study of the ‘early’ foreign body reaction as a means to repopulate a biological but stabilized matrix attractive. Based on the presence of approximately 4.5% lineage negative cells on 3 day IP implants in rats (unpublished results), we performed 3 day endogenous IP seeding by the valve recipient itself for this study. As a cross-linked scaffold we have used a non-cytotoxic photo-oxidized bovine pericardium, which is shown to tolerate in vitro cell seeding.

The aim of this study was primarily to prove complete re-cellularization of the scaffolds and to demonstrate appropriate cell differentiation and phenotype. On the other hand, we also wanted to show that the valves remain haemodynamically stable up to 1 month after implantation in the pulmonary position, free of thrombosis in the absence of anti-coagulation therapy and free of calcification.

At present, it was not our intention to compare this valve to any type of existing bioprosthesis in terms of durability because the actual design of the valve is experimental and meant only as a tool to prove re-cellularization potential.

Methods

Animals and study design

Lovenal sheep (n = 24, ewes 60 kg (45–75)] were selected and cared for in accordance with the ‘Guide for the Care and Use of Laboratory Animals’ (NIH publication 85-23, revised 1985). The study was approved by the local Ethics Committee.

Photo-oxidized (Cardiofix™, Sulzer Carbomedics, TX, USA) pericardium was used.

The study comprised four groups to which the sheep were randomly assigned and the groups contained six animals each: group 1, spontaneous seeding and 1 week in pulmonary position; group 2, spontaneous seeding and 1 month in pulmonary position; group 3, IP preseeding and 1 week in pulmonary position; and group 4, IP preseeding and 1 month in pulmonary position. The sheep of groups 3 and 4 were implanted in the peritoneal cavity with a pericardial patch suspended in a stainless steel cage. Three days later, the implant was aseptically retrieved and a valve (Figure 1) was made, similar to the one described by White et al. In short, a 6.8 × 3.8 cm pericardium patch was folded along the long axis leaving an extra 2 mm at on end. The fold was fixed with one 5-0 prolene suture at each side, the whole flap was folded along the short axis, and the short axes were sewn together with a running suture. The double-walled tube was suspended on a large forceps with the suture on one of the tips. A left thoracotomy was performed, cardio-pulmonary bypass installed, the native pulmonary valve excised, and the

Figure 1 Finalised 21 mm diameter valve construct ready for implantation.

Histology and immunohistochemistry

After overnight fixation in 4% paraformaldehyde, the samples were incubated overnight in 20% sucrose in PBS. The samples were snap-frozen in Neg-50 medium (Prosan, Belgium) and stored at −80 °C. Seven micrometre cryosections of the samples were placed on poly-L-lysine-coated slides and stored at −20 °C until staining.

Cross-linked matrix stability assessment was performed by means of microsoriasis red staining allowing measurement of the total collagen as well as the organized collagen (Figure 2). We measured the total area of the pericardium (A0, between green lines) and the area of the red stained material within that area (A1). An additional picture was taken using polarized light to measure the organization of the collagen. To obtain this picture, the section was placed in between two perpendicularly orientated polarization filters, the collagen bundles within the section themselves also act as polarization filters thus causing these bundles to be visible again. On this image, the area of the red illuminated bundles (A2) is measured. The collagen density and organized collagen are calculated by the following formulas.

(1) Collagen density: \[(A1/A0) \times 100\]
(2) Organized collagen: \[(A2/A0) \times 100\]

Both are expressed as a percentage: collagen density as a percentage of the total area and organized collagen as a percentage of the collagen area.
This method allows studying the matrix integrity both on a microscopic and molecular organization level. On the microscopic level, it allows assessment of gross structural damages such as lacerations and increase or decrease in spongiosity. On the molecular organization level, it allows the study of bundle breakdown, an important feature that might occur during cellular infiltration.

Before immunohistochemistry (IHC) was performed, the presence of cells was verified using standard haematoxylin and eosin (H&E) staining and microscopic screening. Additional MOVAT pentachrome staining was performed to evaluate the composition of connective tissue elements in the newly deposited matrix.13

The samples were immunohistochemically stained for the following markers: CD45 (clone: 1.11.32, Serotec, Oxford, UK), CD44 (clone: BAT31A, A&E Scientific SPRL, Marcq, Belgium); major histocompatibility complex (MHC)-I (clone: HS8A, A&E Scientific SPRL, Marcq, Belgium); MHC-II (clone: TH14B, A&E Scientific SPRL, Marcq, Belgium); phosphohistone H3 [rabbit polyclonal (06-570 Upstate), Biognost, Heule Belgium]; vimentin (clone: V9, DAKO, Heverlee, Belgium); ASMA (clone: 1A4, DAKO, Heverlee, Belgium); smooth muscle heavy chain (SMMS-1, clone: SMMS-1, DAKO, Heverlee, Belgium); CD172a (clone: DH59B, A&E Scientific SPRL, Marcq, Belgium); CD34 (clone: QBEnd10, DAKO, Heverlee, Belgium); CD117 [rabbit polyclonal (ab5633), Abcam, Cambridge, UK]; and endothelial nitric oxide synthase (eNOS, clone: 3, BD Pharmingen, Erembodegem, Belgium). The cells from one complete leaflet section were counted for each valve, the length of the leaflet was measured as well as the thickness and surface of the deposited new material. Length, thickness, and surface area of the neomatrix were measured on calibrated micrographs by image analysis, using an Axioplan 2 imaging microscope (Zeiss, Belgium) and the Axiovision 4.3 software package (Zeiss, Belgium). For neomatrix surface calculation, the surface of the original matrix was subtracted from the total leaflet surface, both measured on the same picrosirius red stained section. For cellular phenotyping, a total of 500 cells were assessed for each leaflet and the results were expressed as a percentage. To avoid bias, several pictures from each leaflet were taken, divided into quarters, and counted according to a randomization list.

### Figure 2
Microscopy images of picrosirius red stained photo-oxidized bovine pericardium with clear field illumination (upper left) and polarized illumination (upper right). The lower panels are representative micrographs of each group. Spontaneous, non-IP-seeded valve group. Each individual micrograph is showing the picrosirius red stained collagen and in blue pseudo-color the organized collagen bundles photographed with polarized light.

The IP-preseeded leaflets showed a significant 15% shortening after being 1 month in the pulmonary position. Leaflet thickness of IP-preseeded valves is twice that of the spontaneously seeded valves. De novo deposited or

### Results

#### Functionality of the ovine valve implants

As shown in Table 1, all animals were within the same range of age and weight, a requirement for implantation of a 21 mm diameter valve construct. As shown in the same table, all except one valve construct were adequately implanted. In one valve, one leaflet was caught by the distal running suture of the end-to-end anastomosis with the native pulmonary artery, resulting in one small and two large leaflets. Assessment of the haemodynamic function was performed but showed no differences between any of the groups. Equal gradients and pulmonary insufficiency scores were observed. Regarding thrombus deposition, none of the six non-preseeded valves, explanted at 1 week, showed valve thrombosis. At 1 month, however, one out of six valves presented an obstructive thrombus. All preseeded valves were free of thrombosis at 1 week and at 1 month.

#### Re-cellularization and remodelling of the ovine valve implants

A four- to seven-fold higher cell count was found in the IP-seeded group as compared with the spontaneously seeded group, at 1 week and 1 month, respectively (Table 2 and Figure 3; H&E stain). In and on spontaneously seeded valve leaflets, cellularization remained constant. Although a further numerical increase in cell count was apparent in the IP-preseeded valves at 1 month, this increase did not reach a statistical significance.

Cell density and the amount of leaflet covered by the new material were estimated, as shown in Figure 4. A significantly higher cell density was found in the IP-preseeded groups as compared with the spontaneously seeded groups (upper panel). A similar result was found regarding the coverage of the implanted material with newly deposited material (lower panel). Figure 3 shows H&E or MOVAT pentachrome stained representative sections of both control and IP-preseeded valves after 1 week or 1 month in the pulmonary position.

The IP-preseeded leaflets showed a significant 15% shortening after being 1 month in the pulmonary position. Leaflet thickness of IP-preseeded valves is twice that of the spontaneously seeded valves.
neomatrix surface is approximately 10-fold larger when comparing IP-preseeded with spontaneously seeded valves (Table 2 and Figure 3; MOVAT stain). Due to one outlier in the spontaneously seeded valves at 1 week after implantation, statistical significance between both 1 week groups could not be attained. With respect to the new matrix deposition, we found that collagens were present (picrosirius red, data not shown), but that organization into bundles was not yet achieved. The blue-green staining of the new matrix material also revealed the presence of ground substance and proteoglycans.

Matrix stability

Table 2 also summarizes the matrix stability data. No differences in collagen density could be observed. All these data were within the range of collagen density observed in the test samples, which were only implanted in the peritoneal cavity for 3 days [84.1 (73.2, 90.1)%]. Regarding the percentage of organized collagen, we found a limited but significant decrease in collagen organization in the spontaneously seeded implants in the 1 month group as compared with the 1 week group. This decrease was already apparent in the 1 week IP-preseeded group but did not change with time. Additionally, we also found that this decreased collagen organization was already present after 3 days of IP implantation [90.3 (77.6, 94.8)%].

Cell phenotyping in valve constructs

Table 3 summarizes the immunohistochemical cellular phenotyping of spontaneously seeded and IP-preseeded valves, and IP test sample implanted in the sheep's peritoneal cavity for 3 days. Since these are percentages, only the fraction of positive cells is shown, keeping in mind the large

### Table 1 Age, weight, and valve function

<table>
<thead>
<tr>
<th></th>
<th>Spontaneous</th>
<th>IP-preseeded</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 week</td>
<td>1 month</td>
<td>1 week</td>
</tr>
<tr>
<td>Age (days)</td>
<td>737 [384, 753]</td>
<td>705 [368, 713]</td>
<td>575 [458, 756]</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>67.5 [66.2, 68.2]</td>
<td>66.0 [46.8, 70.5]</td>
<td>65.0 [59.8, 69.5]</td>
</tr>
<tr>
<td>Normal function</td>
<td>6/6</td>
<td>6/6</td>
<td>6/6</td>
</tr>
<tr>
<td>Peak gradient (mmHg)</td>
<td>9.0 [8.3, 22.0]</td>
<td>11.4 [8.0, 18.0]</td>
<td>11.4 [10.3, 23.0]</td>
</tr>
<tr>
<td>Thrombus</td>
<td>0/6</td>
<td>1/6</td>
<td>0/6</td>
</tr>
</tbody>
</table>

Data are presented as median [interquartile range]. Spontaneous, non-IP-seeded valve group. KW indicates Kruskal–Wallis test.

### Table 2 Image analysis and matrix stability data

<table>
<thead>
<tr>
<th></th>
<th>Spontaneous</th>
<th>IP-preseeded</th>
<th>KW</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 week</td>
<td>1 month</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell count</td>
<td>3753 [1450, 7531]</td>
<td>3345 [1928, 3737]</td>
<td>12126 [7146, 25373]</td>
<td>20404 [4894, 27898]</td>
</tr>
<tr>
<td>Leaflet length (mm)</td>
<td>16.5 [14.6, 18.2]</td>
<td>16.2 [15.6, 18.2]</td>
<td>17.2 [15.8, 18.7]</td>
<td>14.3 [13.6, 15.4]</td>
</tr>
<tr>
<td>Leaflet thickness (mm)</td>
<td>0.60 [0.43, 1.18]</td>
<td>0.60 [0.50, 0.69]</td>
<td>1.23 [0.63, 1.31]</td>
<td>1.32 [0.91, 1.68]</td>
</tr>
<tr>
<td>Neomatrix surface (mm²)</td>
<td>0.94 [0.44, 7.74]</td>
<td>1.03 [0.37, 2.04]</td>
<td>10.99 [2.47, 12.58]</td>
<td>9.38 [4.11, 15.57]</td>
</tr>
<tr>
<td>Collagen density (%)</td>
<td>87.7 [82.1, 94.9]</td>
<td>86.6 [80.9, 88.3]</td>
<td>83.3 [78.3, 88.5]</td>
<td>82.3 [74.0, 88.3]</td>
</tr>
<tr>
<td>Organized collagen (%)</td>
<td>96.7 [94.9, 97.6]</td>
<td>92.7 [89.8, 94.8]</td>
<td>93.3 [88.1, 95.4]</td>
<td>92.3 [85.1, 95.4]</td>
</tr>
</tbody>
</table>

Data are presented as median [interquartile range]. Spontaneous, non-IP-seeded valve group. KW indicates Kruskal–Wallis test.

**Figure 3** Representative H&E and MOVAT stained sections of valves from each group. Spontaneous, non-intraperitoneally seeded valve group.
differences in total cell observed, large differences in the absolute cell type count are apparent. CD44⁺ cells, absent in IP test samples, showed a significant increase from 1 week to 1 month in spontaneously seeded valves only. Expression of MHC-I and -II was low. At 1 week, MHC-I expression significantly differed between spontaneously seeded and IP-preseeded valves. Phosphohistone H3 showed a low but consistent presence of dividing cells. In the spontaneously seeded constructs, ASMA⁺ or vimentin⁺ cells increased significantly from 1 week to 1 month to levels already attained by IP-preseeded valves at 1 week. At both time points, there was an insignificant presence of SMMS-1⁺ cells. CD34⁺ cells were present in low percentages in all groups but not in all individual valves. CD117⁺ cells, in all groups but not in all individual valves.

Table 3: Immunohistochemical data

<table>
<thead>
<tr>
<th>In situ period</th>
<th>Spontaneous</th>
<th>IP test samples</th>
<th>IP-preseeded</th>
<th>KW</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 week</td>
<td>1 month</td>
<td>3 days</td>
<td>1 week</td>
</tr>
<tr>
<td>CD44 (%)</td>
<td>4.81 [1.75, 9.16]</td>
<td>17.03 [8.81, 32.40]</td>
<td>0.00 [0.00, 0.00]</td>
<td>4.65 [2.02, 8.05]</td>
</tr>
<tr>
<td>MHC-I (%)</td>
<td>0.69 [0.00, 2.36]</td>
<td>1.47 [0.11, 2.27]</td>
<td>0.17 [0.09, 3.97]</td>
<td>2.55 [2.03, 4.15]</td>
</tr>
<tr>
<td>MHC-II (%)</td>
<td>0.00 [0.00, 0.00]</td>
<td>0.00 [0.00, 0.83]</td>
<td>0.00 [0.00, 1.10]</td>
<td>0.10 [0.00, 1.11]</td>
</tr>
<tr>
<td>Vimentin (%)</td>
<td>1.29 [0.41, 1.57]</td>
<td>0.74 [0.42, 2.28]</td>
<td>1.30 [0.27, 3.64]</td>
<td>1.13 [0.51, 1.47]</td>
</tr>
<tr>
<td>Phosphohistone H3 (%)</td>
<td>18.73 [12.48, 27.96]</td>
<td>86.78 [60.61, 91.93]</td>
<td>13.25 [8.32, 53.74]</td>
<td>59.49 [29.64, 85.09]</td>
</tr>
<tr>
<td>ASMA (%)</td>
<td>4.83 [1.74, 5.78]</td>
<td>33.07 [21.89, 38.92]</td>
<td>0.78 [0.00, 5.22]</td>
<td>34.12 [13.56, 51.91]</td>
</tr>
<tr>
<td>SMMS-1 (%)</td>
<td>0.00 [0.00, 0.45]</td>
<td>0.06 [0.00, 0.68]</td>
<td>0.00 [0.00, 0.00]</td>
<td>0.00 [0.00, 0.00]</td>
</tr>
<tr>
<td>CD34 (%)</td>
<td>0.00 [0.00, 0.44]</td>
<td>1.69 [0.00, 4.71]</td>
<td>1.49 [0.28, 2.87]</td>
<td>1.09 [0.00, 2.79]</td>
</tr>
<tr>
<td>CD117 (%)</td>
<td>0.00 [0.00, 0.00]</td>
<td>0.93 [0.30, 3.01]</td>
<td>0.37 [0.21, 1.69]</td>
<td>0.86 [0.52, 1.61]</td>
</tr>
<tr>
<td>eNOS</td>
<td>1/6</td>
<td>3/6</td>
<td>0/5</td>
<td>5/6ᵇ,ᵃ</td>
</tr>
</tbody>
</table>

Data are presented as medians [interquartile range]. Spontaneous, non-IP-seeded valve group; IP test samples, 3 day IP-implanted control patches. KW indicates Kruskal–Wallis test and P indicates Pearson’s χ² test.
ᵃSignificant difference between either both control groups or between both IP-seeded groups.
ᵇSignificant difference between 1 week groups.
ᶜNo statistical analysis could be performed because n < 6.
ᵈSignificantly different from IP test samples.
absent in 1 week spontaneously seeded valves, increased significantly at 1 month, to the level already attained by IP-preseeded valves at 1 week of pulmonary implantation.

We realize that the valve tissue treatment as we performed is not optimal for maintaining the endothelium: ideally the valves should have been fixed in situ with paraformaldehyde. However, the initial H&E stains revealed that we could have an endothelial cell layer, which we stained for endothelial NO synthase (ecNOS). Figure 5 shows different aspects of the re-cellularization. Panels A–C show the stainings for vimentin, ASMA, and SMMS-1, respectively. These micrographs illustrate the presence of blast-like and spindle-shaped vimentin$^+$ cells (A), ASMA$^+$ spindle-shaped cells, and only limited presence of SMMS-1$^+$ cells. Panels D and E illustrate the presence of CD117$^+$ or CD34$^+$ cells, respectively. The DAPI-stained section in panel F shows completely re-cellularized photo-oxidized bovine pericardium. Panel G shows an incompletely organized endothelial layer on an IP-seeded valve construct that was implanted in the pulmonary artery for 1 week. However, at 1 month we found organized endothelial layers on the wall and fibrosa side of the leaflet (H) and on both sides of the leaflet (I). Presence of endothelium (ecNOS) was scored as present (1) or absent (0) and summarized in Table 3. At 1 week, IP-preseeded valves had a significantly higher proportion of endothelialized valves than the spontaneously seeded valves. There was also a significant increase in endothelialization when compared with the IP test samples, which were completely devoid of any endothelial cell.

**Discussion**

To provide evidence that the combination of photo-oxidized bovine pericardium and cells seeded by the short IP implantation is suitable as heart valve prosthesis, we implanted this material in the pulmonary valve position and compared them to valves made from the same material but without IP cell seeding. Despite the fact that the valves were handcrafted and therefore prone to variations, no abnormal valve function could be detected. This was independent of the treatment of the valves, that is, IP-preseeded or not. Only the absence of valve thrombosis suggests the superiority of the IP-preseeded valves.

Re-cellularization was clearly dependent upon prior IP seeding of the matrix for three days. IP-preseeding covered the valves almost completely with new tissue containing more cells and new extracellular matrix than could be achieved by spontaneous seeding of similar constructs. On the other hand, an average 15% shrinkage of the leaflets was observed in IP-seeded valves after being 1 month in the circulation. The actual valves were designed to assure adequate leaflet coaptation in spite of this 15% shrinkage. Since cells have infiltrated the original matrix, it was important to look at the structural integrity of the matrix. No decrease in
the amount of collagen was observed, only a small decrease in organization. However, this was coinciding with the repopulation of the material and can be caused by the cells growing and migrating in between the collagen fibres, inducing a limited dissociation of the collagen structure.

Cells having a role in inflammation and the immune response are present in low percentages. It is interesting that in spontaneously seeded valves cells positive for CD44, a receptor for hyaluronic acid involved in homing, of among others, primitive cells, increase three times from 1 week to 1 month after implantation in the pulmonary artery and are three times higher than the CD45 cells, a panleukocytic marker. The IP test samples were completely devoid of CD44 cells, whereas the IP-preseeded valves had a significantly larger proportion of these cells. This suggests either a differentiation of these cells or an attraction of cells from the blood. In this respect it is also noteworthy to mention the existence of CD45/CD44 cells, of bone marrow stromal origin, also expressing markers like Stro-1.

The low presence or even absence of antigen presenting molecules MHC-I and -II also shows that the material is only minimally immunogenic. To define whether we have a proliferative tissue, we assessed the proliferation rate of the cells by staining for phosphohistone H3. In general, we found a low mitotic index (around 1%) in all valves, which was not different from the mitotic index of the IP test samples. It is known that in foreign body reaction the cells are rather attracted to the material than proliferating. Therefore, we also studied two markers expressed by stem/progenitor cells. Regarding CD34 cells, representing 0.5% of the cells in peripheral blood, we found already an enrichment in the IP test samples. Despite the fact that some numerical variation was observed, these cells were not differently present in any of the other samples. More interesting were the findings with CD117 cells. During spontaneous seeding, these cells could only be detected after the valves remained in the pulmonary position for 1 month. On the contrary, the IP seeding primed the matrix with a small fraction of these cells, which remained present in the IP-preseeded valves at both time points.

For the identification of endothelial cells, we chose an antibody directed against the ecNOS. ecNOS provides additional information regarding the endothelial function, but more importantly the commonly used markers, von Willebrand Factor (vWF) or CD31, might yield false positives in this particular setting. Being part of the blood clotting cascade, vWF can insulate the implant surface resulting in aspecific staining of the external cell layer. CD31 can also be expressed by T-cells and macrophages, one of the key cell types in the early foreign body reaction. Scoring the presence of endothelial cells revealed that IP-preseeded valves had a better potential towards re-endothelialization than the nude matrix material. Because of the morphology and patchy development of the endothelial layer in the preseeded valves, we think that the cells are originating from circulating progenitors that home to the newly deposited matrix or are attracted by the deposited cells. In controls, on the other hand, the observed layer of endothelial cells is the external layer of pannus, originating from native vessel cell overgrowth. This is an important finding since it is known that in normal aortic and pulmonary valves the endothelium regulates the anti-thrombogenicity and causes an NO-dependent regulation of the intrinsic contractility of the leaflets. Furthermore, myofibroblasts enable this intrinsic contractility by acting on NO, endothelin-1, and prostaclin released by the endothelium.

These findings clearly illustrate the potential of these cells to reseed the stabilized biological matrix with myofibroblasts. Furthermore, the 1 month implants already showed signs of spontaneous re-endothelialization. The process of re-cellularization seems to be self-limiting, since the amount of newly deposited material is not continuously increasing and is getting covered by endothelium. This should prevent new cells from adhering and contributing to the re-cellularization process.

Although we have used a stabilized biological matrix, the cells obtained by peritoneal seeding are able to modify this matrix as well as to deposit their own. An important issue that remains to be studied in this regard is whether or not repopulation is improving the compliance of the material. This biohybrid valve constructed out of xenogeneic matrix material and autologous cells combines the reliability of the matrix with the viability of the cells. At present, the origin of these cells and the mediators, such as interleukins,
integrins, etc. are largely unknown. We know that the cells are of mesenchymal origin and can differentiate into the appropriate phenotype, i.e. myofibroblast, for cell repopulation. Considering the mediators, we also know that the cells and possibly the differentiation are not sustained by a standard cell-culturing medium. At present, the group of Campbell3,6 has shown that the cells are possibly derived from macrophages attracted to the material. The present study provides evidence for our alternative hypothesis that the repopulation, although mediated or initiated by macrophages, is (haematopoietic) stem cell derived.

Acknowledgements
The project was partially funded by Fonds voor Wetenschappelijk Onderzoek (G.0549.06). The authors wish to thank lab assistants Ruth Plusquin, Veerle Leunens, Monique Vercalsteren, and Kristof Reyniers.

Conflict of interest: none declared.

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