

# Inflammatory Response Assessment of a Hybrid Tissue-Engineered Heart Valve Leaflet

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**Abstract**—Despite substantial research in the past few decades, only slight progress has been made toward developing biocompatible, tissue-engineered scaffolds for heart valve leaflets that can withstand the dynamic pressure inside the heart. Recent progress on the development of hybrid scaffolds, which are composed of a thin metal mesh enclosed by multi-layered tissue, appear to be promising for heart valve engineering. This approach retains all the advantages of biological scaffolds while developing a strong extracellular matrix backbone to withstand dynamic loading. This study aims to test the inflammatory response of hybrid tissue-engineered leaflets based on characterizing the activation of macrophage cells cultured on the surfaces of the tissue construct. The results indicate that integration of biological layers around a metal mesh core—regardless of its type—may reduce the evoked inflammatory responses by THP-1 monocyte-like cells. This observation implies that masking a metal implant within a tissue construct prior to implantation can hide it from the immune system and may improve the implant's biocompatibility.

**Keywords**—Hybrid heart valve, THP-1 cell line, Inflammatory response, Metal mesh scaffold.

## INTRODUCTION

Since the early 1980s, scientists have been looking for ideal valve substitutes and engineered tissues to replace natural leaflets.<sup>7,23–25,27,34,38</sup> However, despite substantial research in the past three decades, only slight progress has been made toward developing tissue-engineered scaffolds for heart valve leaflets. Most of the currently engineered tissue materials lack bioactive sites and are unable to coordinate the interaction between cells

and scaffolds. As a result, superficial cells, particularly endothelial cells, cannot adhere firmly enough to the structure and are more easily lost to the blood flow. Therefore, the inflammatory cells and platelets aggregate and activate, which results in thrombosis and degradation.<sup>6,12</sup> Additionally, engineered tissues that were previously developed and implanted in animals are either thicker, stiffer, and less pliable than native valves<sup>30,31</sup> or exhibit size remodeling<sup>17</sup> and partial degeneration with no interstitial tissue reconstitution.<sup>32</sup> Other challenges involve an insufficiency or excess in composition and tenacity that makes the valve unable to withstand the pressure within the systematic circulation.<sup>37,14</sup>

We have recently developed a novel heart valve leaflet composed of a hybrid scaffold<sup>1</sup> with a thin core of super elastic Nitinol mesh tightly enclosed within multi-layer biological tissue. This approach retains all the advantages of biological scaffolds while providing a strong extracellular matrix (ECM) backbone, which allows the valve to withstand the dynamic pressure loads inside the heart. Additionally, the high-resolution mesh pattern ensures the structural integrity of the surrounding tissue, which allows cells and ECM components on both sides of the mesh to actively interact with each other.

In the present study, we tested the inflammatory response of this leaflet *in vitro* by characterizing the activation of macrophage cells cultured on its surfaces, which was found to be well-correlated with post-implantation inflammation.<sup>20,22</sup>

## MATERIALS AND METHODS

### *Hybrid Leaflet Concept*

We have developed tissue layers similar to a natural heart valve leaflet to create hybrid tissue-engineered

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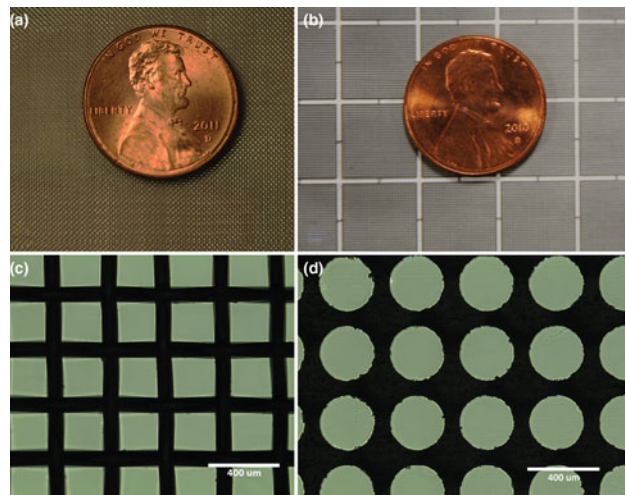
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leaflets, as previously described.<sup>1</sup> The leaflet is composed of a thin metal mesh enclosed by layers of smooth muscle cells and fibroblast cells that are covered by an endothelial layer. We have currently used two types of metals, Nitinol and T316 Stainless Steel meshes, to test and compare the biocompatibility of the leaflet material.

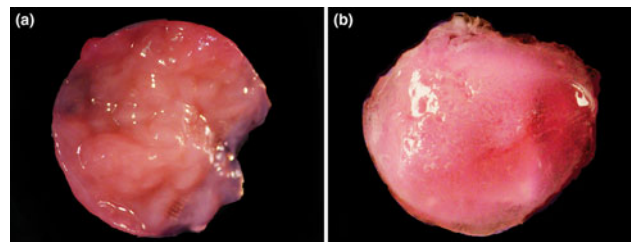
The flat mesh of T316 Stainless Steel (Figs. 1a, 1c) is woven from 76  $\mu\text{m}$  round wires, with a target size of 80 EPI  $\times$  80 PPI<sup>1</sup> (TWP Inc., Berkeley, CA). Nitinol meshes (Figs. 1b, 1d) were manufactured through acid etching of flat, superelastic, Nitinol sheets. The Nitinol meshes have a thickness of 76  $\mu\text{m}$  etched with a network of holes of 240  $\mu\text{m}$  diameter with a central distance of 320  $\mu\text{m}$ . The base material was polished with a subsequent ultrasonic wash in ethanol and glow discharge for 40 s before autoclaving. Both meshes were cut into pieces with an area of 1  $\text{cm}^2$  to be used for cell culture.

A cell pattern similar to a natural heart valve was selected to develop the hybrid leaflets. Three different cell types were isolated and used for the preliminary assay. Human aortic smooth muscle cells and human aortic adventitial fibroblast/myofibroblast cells were used to fulfill the role of valvular interstitial cells (VICs),<sup>10,11</sup> and human umbilical vascular endothelial cells (all from Lonza Group, Allendale, NJ) were used to act as the valvular lining cells. The basal media for culturing cells contained DMEM (Dulbecco's Modified Eagle Medium; Gibco, Carlsbad, CA), 10% fetal bovine serum (FBS, HyClone, Rockford, IL), 1% penicillin/streptomycin (Gibco, Carlsbad, CA) and 1% L-glutamine (Gibco, Carlsbad, CA), with appropriate growth factors added to the media for enhancement of growth and proliferation. The cultured cells were fed every 2–3 days, and split 1 to 3 at confluence. Cells were used at passages 3 to 5 for the experiment.

The tissue engineered constructs were cultured, as described in our previous publication.<sup>1</sup> Briefly, a collagen gel mixture composed of bovine type I collagen was neutralized using proper amounts of NaOH. After combining the mixture with smooth muscle cells to form the first layer, collagen constructs were formulated by casting collagen solution in circular molds. The cells in the next layers were then plated around these collagen scaffolds at 1 week intervals to achieve a phenotype similar to a natural heart valve *in vivo*.<sup>1</sup> Figures 2a and 2b show the engineered hybrid tissue after 3 months of cell culture for both the Stainless Steel and Nitinol mesh scaffolds, respectively.



**FIGURE 1.** (a) and (c) The woven stainless steel mesh sheet with 76  $\mu\text{m}$  round wires; (b) and (d) The Nitinol mesh with a thickness of 76  $\mu\text{m}$  developed through acid-etching a medical grade super-elastic Nitinol sheet. Both meshes were divided into multiple 1  $\text{cm} \times 1 \text{cm}$  blocks for tissue culture.



**FIGURE 2.** The engineered hybrid leaflet with (a) Stainless Steel mesh core, and (b) Nitinol mesh core enclosed within three layers of cells after 3 months of cell culture.

### Immunogenicity Assay

The immune responses of THP-1 cells (ATCC, Manassas, VA) to the leaflet constructs were tested *in vitro*. It has been shown that these cells, which are of human origin and well-characterized, mimic human monocyte behavior and are frequently used in biocompatibility assays.<sup>5,13,16,19,40,41</sup> We cultured the THP-1 monocyte-like cells at  $0.5\text{--}7 \times 10^5$  cells/mL in RPMI 1640 with 10% FBS, 50  $\mu\text{mol L}^{-1}$  of  $\beta$ -mercaptoethanol (Gibco, Carlsbad, CA), 100 units  $\text{mL}^{-1}$  penicillin, 100  $\mu\text{g L}^{-1}$  streptomycin and 2  $\text{mmol L}^{-1}$  glutamine. These monocytes were seeded at a density of 150,000 cells/ $\text{cm}^2$  over the tissue samples and incubated at 37  $^\circ\text{C}$  overnight. THP-1 cells were added to the surface of the fully formed hybrid tissues, which were comprised of all three cell layers (i.e., smooth muscle, fibroblasts and endothelial cells). Additionally, THP-1 cells were added to the bare metal meshes (i.e., Stainless Steel and Nitinol) to test and compare the inflammatory responses evoked

<sup>1</sup>EPI: end per inch; PPI: pick per inch.

onto the isolated metal mesh. We also compared the biocompatibility of the fully formed hybrid leaflet with glutaraldehyde-treated bovine pericardial tissue, which is widely used in bioprosthetic heart valve leaflets. To do so, THP1 cells were added to the surface of the bovine pericardial leaflets (Neovasc, Inc, Richmond, BC, Canada).

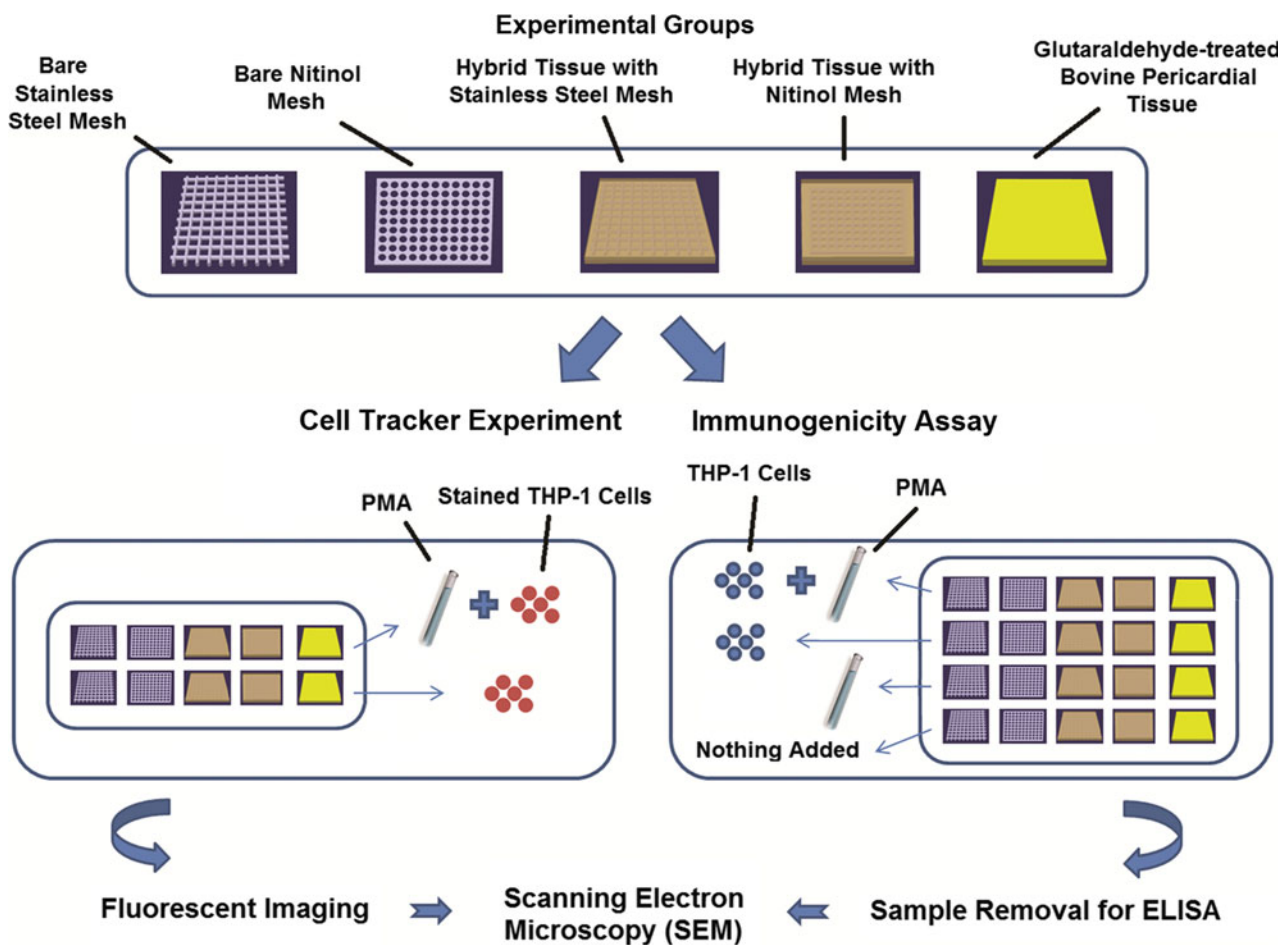
The THP-1 cell line can be differentiated to macrophages by factors such as phorbol 12-myristate 13-acetate (PMA).<sup>36</sup> PMA is not particularly relevant in an *in vivo* environment, therefore, it was only used to differentiate THP-1 monocytes into macrophage cells *in vitro*. Different conditions based on the presence or absence of PMA—as a positive control—were considered. The activation of THP-1 cells was determined using ELISA (Enzyme-Linked Immunosorbent Assay) for quantifying the secretion level of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) by immune cells, in response to the biomaterials. The experimental procedure is schematically shown in Fig. 3. Two constructs made of cells from different sources were considered for each

condition, while the sample removal in ELISA was performed three times for each construct.

THP-1 cells were seeded for 24 h, and the cell culture supernatant was removed and analyzed by ELISA (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. In some instances, as indicated, 50 ng/mL PMA (Sigma, St. Louis, MO) was added 1 h after the addition of THP-1 cells to enhance the differentiation of the cells into macrophage cells (Fig. 3).

#### Cell-Tracker Experiment

Activation and adhesion of the monocytes were visually verified using a cell-tracker dye experiment (Invitrogen, Carlsbad, CA) that stained the THP-1 cells before adding them to different experimental groups. These cell tracker reagents can be loaded into cells by adding the reagent to the culture media and then washing the cells briefly with fresh medium prior



**FIGURE 3.** Schematic representation of the experimental procedure used for the biocompatibility assay. The experimental groups and the conditions considered for both immunogenicity assay and cell tracker probe are shown. There were two constructs for each condition, and the sample removal in ELISA was performed three times per construct.

to analysis. THP-1 cells were harvested by centrifuging the supernatant with subsequent aspiration. The dye was added to the THP-1 cells with fresh, pre-warmed media that was incubated for 30 min at 37 °C. Finally, the dye-contained medium was replaced, and the cells were washed with PBS.

#### Scanning Electron Microscopy (SEM)

The ultra-structural features of cell adhesion and spreading were visualized at various magnifications ranging from 10 to 1000 $\times$ , using a low-voltage, high-resolution SEM (Quanta 3D FEG Electron Microscope, FEI, Hillsboro, OR) at 10 kV. Hybrid tissue groups as well as bare metal groups were used for SEM analysis. To prime the constructs, the tissue samples were dehydrated in ascending concentrations of ethanol (30–100%), followed by critical-point drying in CO<sub>2</sub>. Then, all of the samples were mounted on carbon stubs, and sputtered using a 4 nm coating of gold (VCR Group, San Francisco, CA).

#### Statistical Analysis

Quantitative data from the immunogenicity assay were reported as mean  $\pm$  SD. The R software package for Windows (Lucent Technologies Inc., Costa Mesa, CA) was used for statistical analysis. The unpaired

Student's *t* test was performed. A *p* value of less than 0.05 was considered statistically significant.

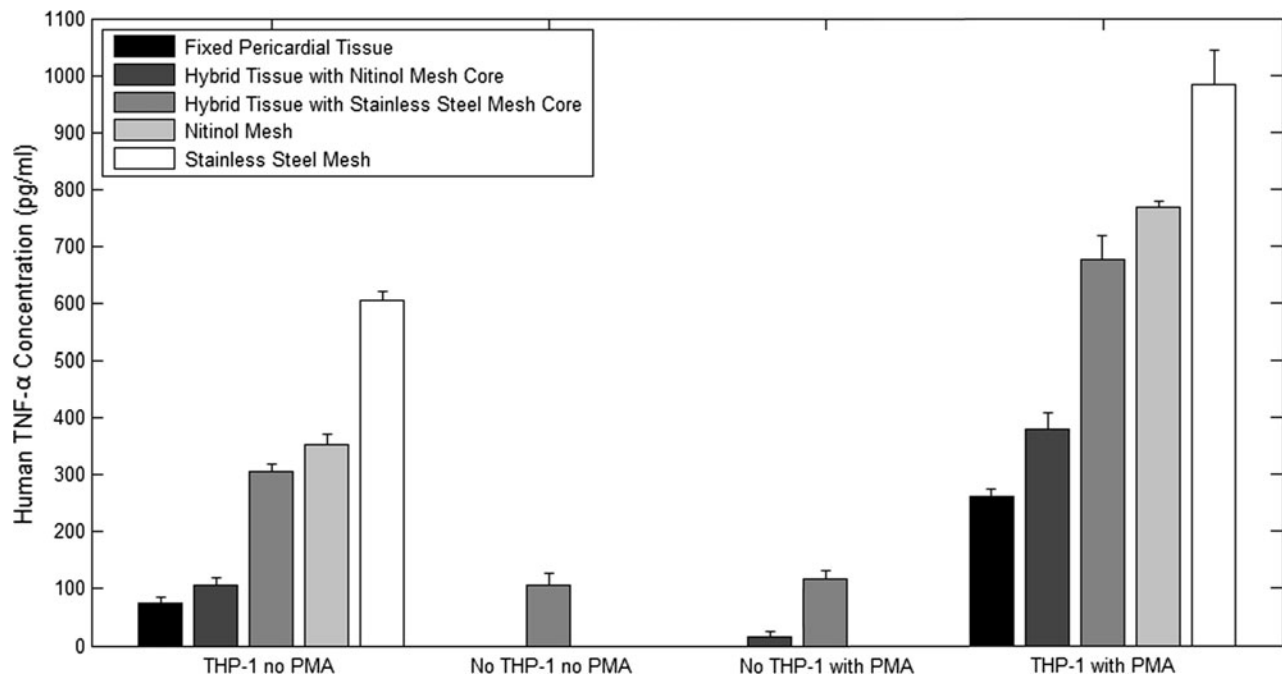
## RESULTS

After culturing the third layer (i.e., the endothelium) around the hybrid leaflets, the mesh was completely concealed by formation of a smooth, confluent surface that lined the construct. The collagen scaffold shrinkage stopped after the tissue was fully formed at the end of 8 weeks of producing sufficient ECM components. The endothelial layer seemed to be participating in the remodeling activities of the tissue and was strongly attached to the inner two layers.<sup>1</sup>

Four different conditions were considered: (1) the addition of THP-1 cells with PMA, (2) the addition of THP-1 cells without PMA, (3) groups without THP-1 cells or PMA to see whether the cells in the construct secrete any cytokines by themselves, and finally (4) groups without THP-1 cells but with PMA addition. The results of the ELISA for the levels of monocyte activation in all the experimental groups are shown in Fig. 4.

#### Groups Containing Nitinol Mesh

We found that the level of TNF- $\alpha$  secretion was significantly lower in the hybrid tissue constructs with



**FIGURE 4.** Secretion of human TNF- $\alpha$  was measured in different experimental groups using ELISA. The groups contained: hybrid tissue with Nitinol core, hybrid tissue with Stainless Steel core, Nitinol mesh alone, Stainless Steel mesh alone, fixed pericardial sheets and cell culture media as the control group. The tests were performed in four different conditions: (1) THP-1 cells were added to the groups without addition of PMA, (2) THP-1 cells were added to the groups along with addition of PMA at proper time step, (3) groups with no THP-1 cells addition but with PMA, and finally (4) a condition with no added THP-1 cells and no PMA.



a Nitinol core when compared to bare Nitinol mesh ( $p < 0.05$ ; Table 1). Furthermore, it was observed that the addition of PMA significantly increases the inflammatory responses in both the bare Nitinol mesh ( $p < 0.05$ ; Table 1) and the hybrid tissue construct with Nitinol core groups ( $p < 0.05$ ; Table 1). No TNF- $\alpha$  secretion was found in the absence of THP-1 cells without PMA, while the response with PMA was negligible. This indicated that the source of TNF- $\alpha$  secretion was merely due to differentiated THP-1 cells and not due to the cells in the tissue portion. The results of the Cell Tracker probe showed adhesion of THP-1 cells on the metal/tissue surfaces. After the addition of PMA, the fluorescent images showed that the THP-1 cells attached to the Nitinol mesh with a higher rate of attachment (Figs. 5a, 5b). The amount of the cells that attached to the same area at the top of the mesh was almost 9 and 23 cells, without and with PMA, respectively, which demonstrated a nearly 2.5 fold increase in the rate of attachment ( $p < 0.05$ ; data were extracted from 6 images per condition). The images of the same probe for the hybrid tissues with the Nitinol mesh core were taken under normal light microscopy (Figs. 6a, 6b). The average amount of attached cells was almost 13 and 7 cells, with and without PMA, respectively ( $p < 0.05$ ). The thick tissue (~0.6 mm) around the mesh did not allow the fluorescent light to effectively penetrate through. Therefore, it was not possible to take clear fluorescent images for those groups. To address this issue and clearly image the surface of the tissue, SEM was performed for all the hybrid tissue constructs to test whether the THP-1 monocytes were firmly attached to the surface (Fig. 7). It can be observed in Figs. 7a and 7b that the THP-1 cells were attached and embedded onto the surfaces of the hybrid tissue leaflets.

#### *Groups Containing Stainless Steel Mesh*

Activation of the monocytes, denoted by secretion of TNF- $\alpha$ , was significantly lower in tissue constructs with a Stainless Steel mesh core, compared to the bare Stainless Steel mesh ( $p < 0.05$ ; Table 1). A significantly higher response was observed in the presence of PMA in both groups ( $p < 0.05$ ; Table 1). It was also found that the level of monocyte activation in the bare Stainless Steel mesh groups and hybrid tissue constructs with Stainless Steel groups was almost 2-fold and 1.3-fold higher, respectively, compared to the similar Nitinol groups. Without the addition of THP-1 cells the same ( $p \sim 0.24$ ) response was observed with or without the addition of PMA. The fluorescent images of the Cell Tracker probe confirmed that the groups, in the presence of PMA, have more THP-1 cells attached to the Stainless Steel mesh (28 cells, Fig. 5d) compared to

those without PMA (19 cells, Fig. 5c). These quantitative data confirmed that the rate of activation is higher for these groups compared to those with a Nitinol core because the microscope field-of-view and the surface area of the meshes are the same in both situations. The same results were achieved from the Cell Tracker probe experiment, as observed in Figs. 6c and 6d. Here, the average amount of attached cells was almost 21 and 9 cells, with and without PMA, respectively ( $p < 0.05$ ).

#### *Groups Containing Glutaraldehyde-treated Bovine Pericardial Tissue*

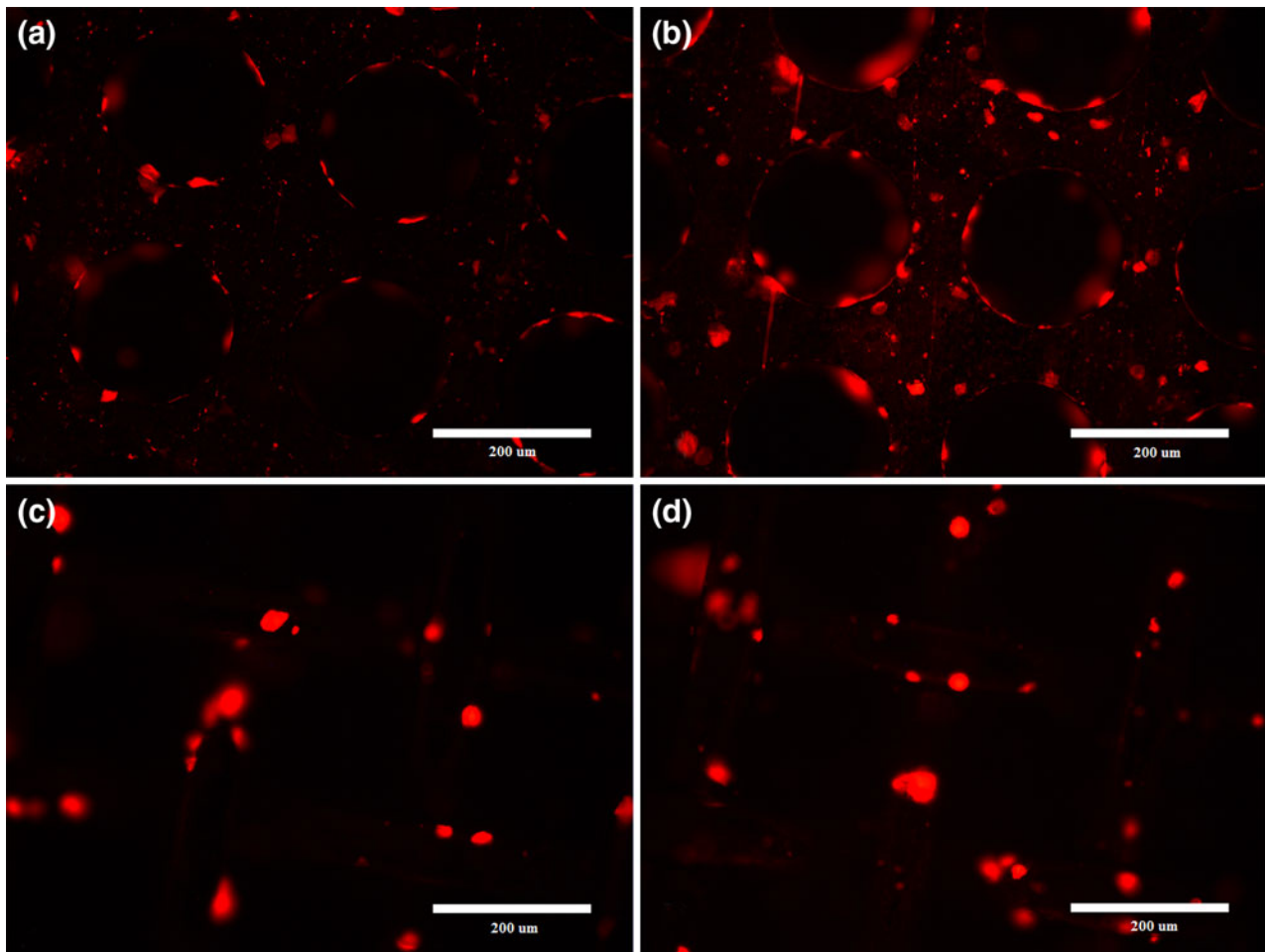
The result of the ELISA for this group showed a comparable ( $p \sim 0.32$ ) level of TNF- $\alpha$  secretion compared to the hybrid tissue constructs with a Nitinol core. However, this immunogenic response was found to be significantly lower compared to the other experimental groups (Table 1).

## DISCUSSION

The ideal heart valve substitute should be a tissue-engineered valve durable enough to remain functional in the heart during the lifetime of the patient. Evoking an immune response on the surface of the constructs may lead to immature degradation of the tissue engineered construct and ultimately immune rejection. Therefore, the leaflets should be biocompatible and non-thrombogenic, with a regenerative capacity to eventually become part of the body.<sup>23</sup> This study aimed to assess the immunogenicity of an engineered heart valve leaflet we recently developed.<sup>1</sup> To reduce the unfavorable effect of scaffold components on biocompatibility, we cultured hybrid tissues using biological matrix components (bovine type I collagen in this study). Due to the ability of collagen to accommodate cellular ingrowth without cytotoxic degradation products, they are considered biocompatible for *in vivo* applications.<sup>21</sup> As a result, we expect that the combined use of bovine collagen and human cells do not have any adverse immunological effect on cell–cell or cell–ECM interaction/signaling.

Several factors such as surface roughness and porosity, chemical reactions at the surface, corrosion properties of the material, and the toxicity of the metal may affect biocompatibility of the implants.<sup>18</sup> With regards to the immune reactions to bare metal meshes without surface modifications, we found that the THP-1 monocytes secrete less TNF- $\alpha$  in response to Nitinol vs. Stainless Steel (Figs. 4, 5). Our results also confirmed the superior biocompatibility of Nitinol compared to Stainless Steel, which is in good agreement with previous studies.<sup>26,35</sup> Enclosing the Nitinol



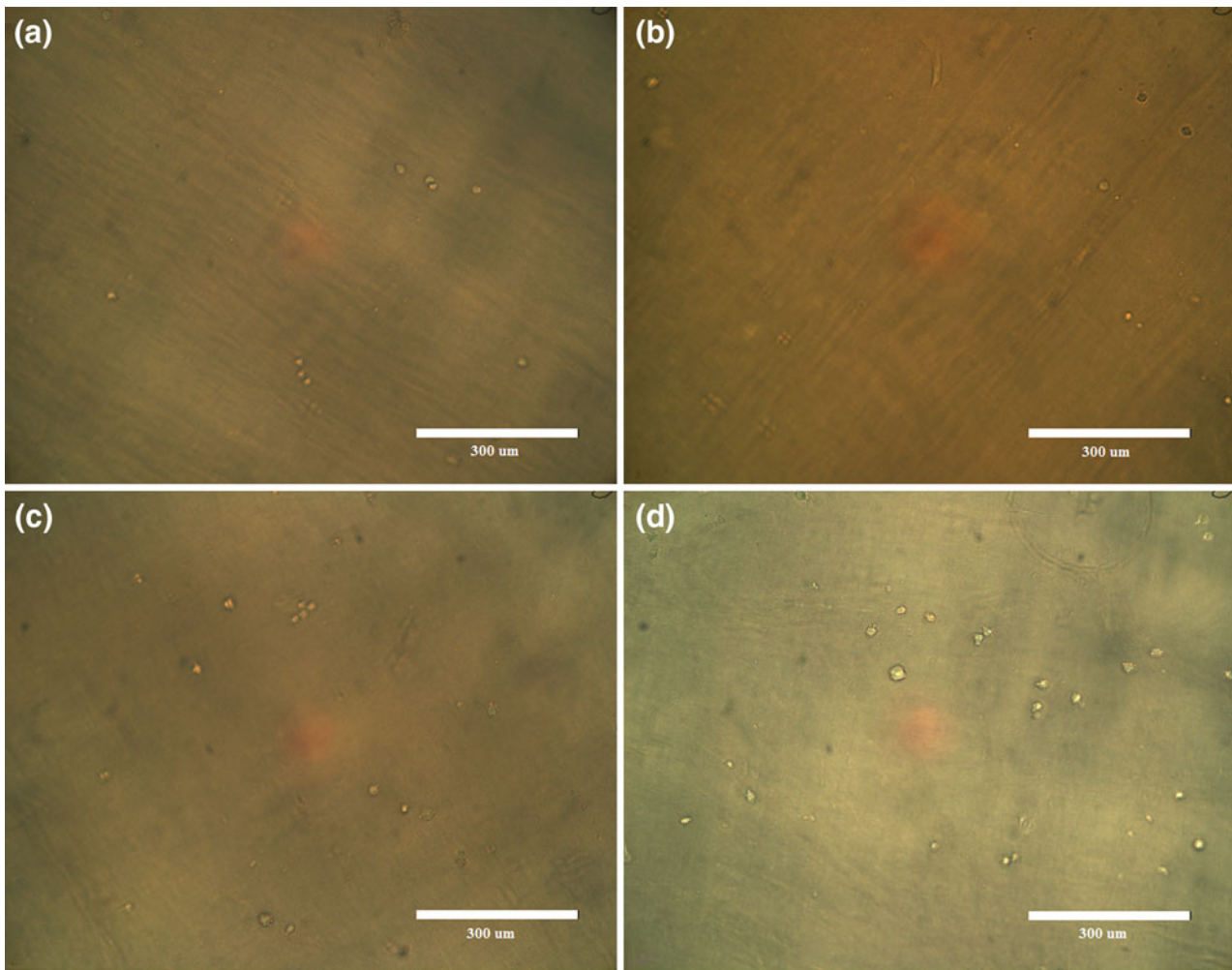


**FIGURE 5.** The result of the Cell Tracker experiment shows the attachment of THP-1 cells to the mesh. The cells were stained as red with cell tracker dye solution. Nitinol mesh (a) without and (b) with PMA; Stainless Steel mesh (c) without and (d) with PMA.

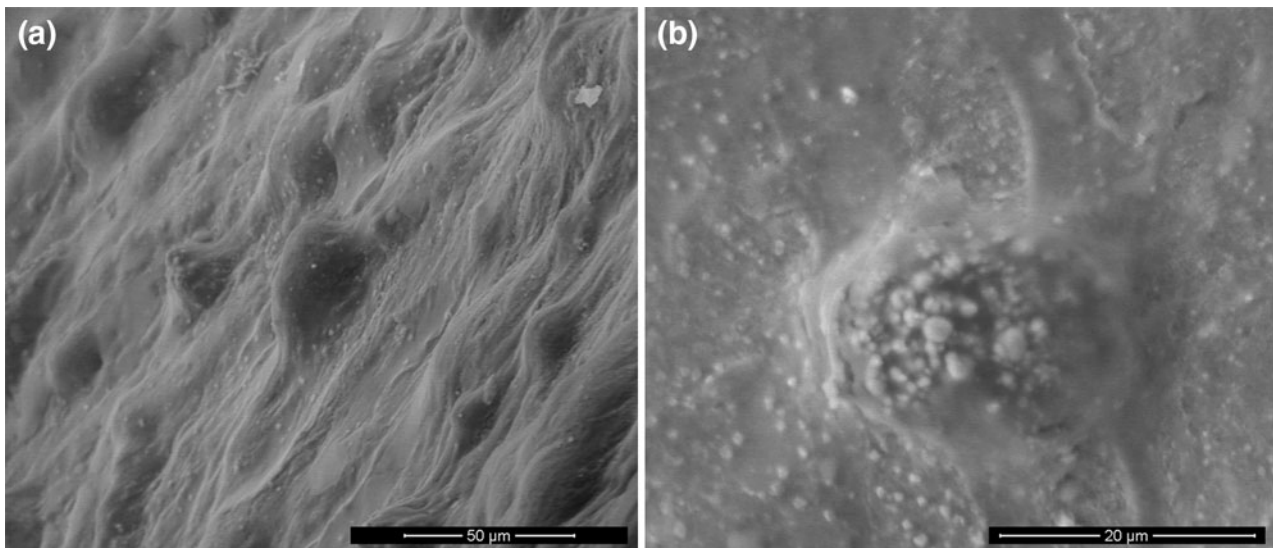
and Stainless Steel meshes within the tissue profoundly reduced the activation of macrophage cells that seeded over the surface of the tissue. This can be considered a major improvement in the biocompatibility of metal scaffolds, as the adhesive proteins of the collagen scaffold in the hybrid construct mask the mesh to the monocytes. The results not only indicate that the hybrid leaflets with a Nitinol core provoke less of an immune response, but also show that these constructs evoke a similar ( $p \sim 0.32$ ) immunologic response to processed bovine pericardial tissue membranes, which are frequently used in bioprosthetic heart valves and are well-known for their superior biocompatibility<sup>15</sup> (Fig. 4). The addition of PMA to all the experimental groups increased the level of TNF- $\alpha$  secretion. In the absence of THP-1 cells, we found a trace level of TNF- $\alpha$  only in the hybrid constructs with a Stainless Steel mesh core while the rest of the constructs did not secrete a significant amount of TNF- $\alpha$ . This confirms that the main source of TNF- $\alpha$  is due to the THP-1 cells rather than the cultured cells.

Attachment of the THP-1 cells to the meshes and surface of the hybrid tissues are shown in Figs. 5 and 6, respectively. This attachment is an indication of differentiation of these cells to macrophages since adhesion is a phenotypic characteristic of macrophage cells and not monocytes. These monocytes attached to the mesh and the surface of the tissue even without addition of PMA (Figs. 5a/5c and 6a/6c). SEM was used to assess whether the monocytes were embedded into the endothelial surface of the tissue constructs (Fig. 7). This phenomenon is equivalent to the migration and chemotaxis of the differentiated monocytes *in vivo*.

Our results indicate that integration of biological layers around a Nitinol mesh core minimizes the evoked inflammatory response. In other words, establishing this environment *in vitro* somehow masked the immunogenic reactions of the metal scaffolds while creating the hybrid tissue leaflet. Therefore, enclosing the thin metal mesh within a soft tissue shell not only results in a biocompatible surface but also does not interfere with the overall elasticity and durability of the mesh.<sup>1</sup>



**FIGURE 6.** The result of the Cell Tracker probe with normal light microscopy shows attachment of THP-1 cells to the hybrid tissues; hybrid tissue with Nitinol mesh core (a) without and (b) with PMA; hybrid tissue with Stainless Steel mesh core (c) without and (d) with PMA.



**FIGURE 7.** (a) SEM image of the hybrid tissue with Nitinol mesh core. THP-1 cells were embedded into surface of the construct; (b) SEM image shows one THP-1 cell that was spread and attached to the endothelial surface of the tissue construct.



### Cell Masking Improves Biocompatibility of Metal Implants

Immediately upon embedding a metal implant inside the body, an inflammatory process is initiated that eventually encapsulates the implant with granulation tissue. The inflammatory reaction results in the migration of polymorphonuclear granulocytes and, soon after, monocytes to the site of implant. Monocytes differentiate into macrophages, which create a confining fibrous capsule around the implant.<sup>2</sup> It is thought that the prolonged activation of macrophage cells near the implant site is critical to the long term response of implanted devices.<sup>3</sup> Our results suggest that prior enclosing of a metal implant within tissue constructs effectively masks it from the immune system and may reduce the inflammatory response by converting the implants to biologically inactive or inert materials.

Based on the properties of the implanted material, the surrounding cells may form scar material or regenerate new natural tissue. Because the implanted metals would be covered by migratory local cells, the response of the neighboring tissues is the most important aspect of biocompatibility.<sup>39</sup> When the implant is completely inert or biocompatible, the body provokes a normal healing process. By growing tissue around a metal mesh *in vitro*, we mimicked the ideal *in vivo* implantation situation by pre-formation of granulation tissue. This observation implies that masking metal implants within a tissue construct prior to implantation can effectively hide it from the immune system and improve the implant's biocompatibility. We expect that prior growing of an autologous tissue construct around an implant guarantees masking the implant and minimizes the formation of additional adverse granulation tissue *in vivo*.

#### Whether or Not to Cross-link?

For *in vivo* implantation, the bovine collagen-based tissues may need to be cross-linked in some manner. The reason for cross-linking treatments is to tailor the mechanical and degradation properties of collagen implants since these biological scaffolds are mechanically fragile and degrade quickly. However, the cross-linking procedure is a potential cause of calcification and degeneration.<sup>28</sup> Cross-linking does not stabilize valvular glycosaminoglycans (GAGs), which play important roles in regulating the mechanical behavior of the native cuspal tissue during dynamic motion of the leaflet.<sup>29</sup> As a result, the current bioprosthetic heart valves are associated with immune reaction and progressive deterioration that limit their durability.<sup>4,33</sup> Here, the hybrid scaffold concept retains the advantages of using biological scaffolds while maintaining a

stronger ECM backbone. Moreover, we have shown that after culturing the third layer (i.e., endothelial layer), the tissue completely formed after 8 weeks such that no further degradation or shrinkage was observed; the cells and ECM components on both sides of the mesh interacted with each other.<sup>1</sup> As a result, we expect that the hybrid scaffold can be used as an alternative to cross-linked tissues. These hybrid valve leaflets possess a smooth, confluent surface that comprises of endothelial cells. The biological portion of the scaffold does not demonstrate any shrinkage after the tissue matures and produces enough ECM components. This behavior decreases the risk of potential thrombosis after *in vivo* implantation.

### LIMITATIONS

The source of the cell lines used in this study cannot exactly emulate the role of VICs.<sup>8,9</sup> However, it would have been extremely difficult to extract and culture the patients' valvular cells for *in vivo* applications. Therefore, we used vascular cells that are easily obtainable from the peripheral vasculature and can be used for future therapeutic applications.

We have not yet tested for potential thrombosis, which is an important factor to consider in developing any type of heart valve. It is anticipated that the hybrid tissue will also reduce the secretion of other pro-inflammatory cytokines. However, further studies are required to determine whether or not the hybrid tissue material will in fact induce macrophage cells to undergo polarization toward a pro-healing phenotype. In addition to the assessment of the inflammatory responses discussed here, the blood compatibility of the hybrid composites in this study should be tested in the future.

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