Valvular Interstitial Cell Activation in Response to Pro-Inflammatory Cytokine Treatment

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Abstract

Calcific aortic valve disease is the main heart valve disease in the elderly. The disease is characterized by persistence of myofibroblasts, causing extensive remodeling of the extracellular matrix and stenosis of the aortic valve but its regulatory mechanisms are unclear. Recent studies have suggested that myofibroblast differentiation results from an inflammatory process involving pro-inflammatory cytokines, namely transforming growth factor-β1 (TGF-β1), interleukin-6 (IL-6), and interleukin-1β (IL-1β). Here I cultured valvular interstitial cells (VICs) on 8-arm PEG-hydrogels with matrix metalloproteinase (MMP) degradable cross-linkers and a pendant adhesive peptide to allow the cells to grow throughout the hydrogel and adhere to it. I then added each cytokine individually to mimic the inflammatory response of VICs. I found that for two methods of quantification, the addition of TGF-β1 and IL-6 increased the measured level of α-smooth muscle actin (α-SMA), an indicator of myofibroblast activation. This result supported the hypothesis that these cytokines mediate the actively regulated progression of calcific aortic valve disease. IL-1β, though previously implicated in disease pathogenesis, decreased myofibroblast activation, possibly due to its mediation of apoptosis or its regulatory interdependence with MMP, which was present in the hydrogels. In all control conditions, the majority of VICs had a quiescent or deactivated phenotype, indicating the efficacy of PEG-hydrogels as extracellular matrix mimics. This study illuminated possible pathways towards the pathogenesis of calcific aortic valve disease and supported PEG-hydrogels as viable culture substrates.

1 Introduction

Calcific aortic valve disease (CAVD) is associated with stenosis that often leads to valve replacement surgery. It is the most common heart valve disease and the main cause of valve replacement in the elderly.[1] Its main characteristics include calcification, fibrous thickening of valve leaflets, and extensive remodeling of the extracellular matrix.[2] Heart valve contractility is affected due

to valvular interstitial cell (VIC) deposition of collagen and calcified matrix.[3] However, despite the widely held claim that CAVD is purely degenerative, recent studies have suggested that it is instead an actively regulated process in which pro-inflammatory cytokines play a large role.[4] Cytokines are secreted by valvular endothelial cells or inflammatory cells and alter the balance of promoters or inhibitors of extracellular matrix (ECM) degradation.[5] Cytokines may stimulate myofibroblast differentiation, which then contributes to the pathogenesis of CAS through fluctuation of ECM degradation. It is still unclear how these soluble biochemical signals, such as transforming growth factor-β1 (TGF-β1), interleukin-6 (IL-6), and interleukin-1β (IL-1β) regulate VIC function as CAVD progresses, indicating the importance for future studies to examine these cytokines in a suitable microenvironment so as to mimic a possible VIC inflammatory response. VIC myofibroblast differentiation is an important function of a healthy heart valve, but in CAVD, myofibroblast differentiation may persist and contribute to the diseased state.[6]

Myofibroblasts are an activated form of smooth-muscle cells that play a major role in the inflammatory response through avid production of cytokines, chemokines, and growth factors, which control differentiation, proliferation, contraction, healing or tissue remodeling, ECM secretion, and migration to the site of a wound. Consequently, myofibroblasts play an important role in facilitating the pathogenesis of CAVD.[7] These activated forms of smooth muscle cells can be identified immunohistologically by their expression of α-smooth muscle actin (α-SMA), because activated myofibroblasts express α-SMA and damaged or diseased heart valves show a larger level α-SMA.[8] Myofibroblast activation is instrumental in the pathogenesis of CAVD. Therefore, characterizing the mechanisms that lead to myofibroblast activation is important in understanding the progression of CAVD.

TGF-β1, which is secreted by activated myofibroblasts, has been shown to promote calcification of aortic smooth muscle cells as well as mediate myofibroblast differentiation and activation.[9,10] TGF-β1 is known for its ability to differentiate valvular endothelial cells into mesenchymal cells and regulate multiple aspects of the myofibroblast phenotype through transcriptional activation of α-SMA, collagen, matrix metalloproteinases (MMP), and other cytokines.[11] Due to TGF-β1's implications in calcification and myofibroblast activation, this cytokine is a key point of interest in studying the pathogenesis of CAVD.

IL-1β and IL-6 have both been identified as proinflammatory cytokines that are excreted when the homeostasis of valve tissue is disturbed, as in CAS.[7,12,13] Human valve cusps become progressively thickened, exhibiting enhanced expression of these cytokines.[14,15] IL-1β has been identified in stenotic heart valves as a cytokine important in the activation, transformation, and proliferation of myofibroblasts.[5,7,16,17] IL-6 expression was shown to be stimulated by a bacterial product, lipopolysaccharide, which mimics a diseased response, and it was also implicated in osteogenesis of VICs and upregulation of adhesion molecules between valvular endothelial cells.[22] These recent studies demonstrate that these cytokines are closely interrelated with the development of CAVD and investigating their effects on VICs *in vitro* may be a viable way to investigate the pathogenesis of CAVD.

Many of these studies have been done only by culture on plastic plates, which have been shown to induce myofibroblast activation due to the stiffness of the material. When myofibroblasts constitute the vast majority of cells before a soluble molecular signal is added, the gene expression may be affected and results will not provide an accurate model of in vivo pathogenesis.[18] One study demonstrated that VICs cultured on poly(ethylene glycol) (PEG)-based hydrogels maintain a lower level of activated phenotype when compared to culture on hard plastic plates.[19] In addition, more recent studies have shown that VICs cultured on softer hydrogels with lower Young's moduli have significantly lower levels of myofibroblast activation.[20] Measuring VIC response to TGF-β1, IL-1β, and IL-6 on soft PEG-hydrogels will provide a more accurate picture of VIC response to these cytokines.

My goal for this research was to utilize a hydrogel culture substrate which does not induce myofibroblast activation simply due to the nature of its biophysical properties. Here, I asked the question: How does VIC phenotype change in response to the pro-inflammatory cytokines transforming growth factor-β1 (TGF-β1), interleukin-1β (IL-1β), and interleukin-6 (IL-6)? With many recent studies demonstrating the importance of myofibroblast activation in the pathogenesis of CAS, as well as the role of these molecular signals in the inflammatory regulative process of CAS, I hypothesized that due to the inflammatory nature of all four of these cytokines, they all contribute to a diseased phenotype in the heart valve. Therefore, I predicted all three of these molecular signals would increase the percentage of activated myofibroblasts in cell culture. Looking at the variable of cytokine or growth factor concentration, I expected to find a positive correlation with myofibroblast activation.

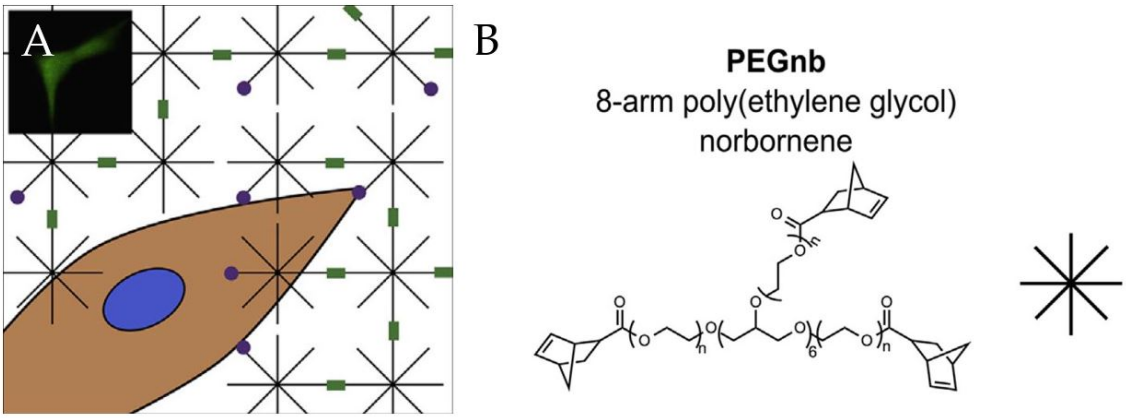

Figure 1:

A) Diagram of a VIC on a PEG-hydrogel. Small purple circles indicate adhesive peptide (CRGDS) and green rectangles indicate MMP-degradable crosslinking peptide. 8-arm star-like shapes indicate PEG backbones. B) Structure of PEG-norbornene, which makes up the 8-arm PEG backbone. Both graphics were obtained from K.M. Mabry et al. / *Biomaterials 49* (2015) 47-56.

2 Methods

2.1 Procedures performed by qualified scientist before experimentation

VIC Isolation Porcine hearts were obtained from a Hormel Food slaughterhouse, where the death of the pigs is not for the production of heart valves and the hearts are a by-product of the pork industry that would otherwise go to waste. Other researchers isolated the valves from the hearts and removed valvular endothelial cells using collagenase digestion in a 37°C incubator. After digestion, cells were seeded on tissue culture plates for 3-5 days, and then frozen down at passage one until ready to use.

Functionalizing Poly(ethylene glycol) with Norbornene The reagents norbornene carboxylic acid and HATU were used in a solvent of dimethylformamide (DMF) to functionalize PEG with norbornene. After that reaction was complete, 8-arm PEG-thiol (PEG-SH) was added. Then, in an ice bath, DIEA (N,N-Diisopropylethylamine) was added. After at least two hours of reaction time, the PEG-NB was precipitated three times from ice-cold ether. Next, the PEG-NB was dissolved in water to 20% by weight, dialyzed, frozen in liquid nitrogen or -70°C, then lyophilized.

2.2 Procedures I personally performed

Gel Preparation I obtained matrix metalloproteinase cross-linkers (MMP), CRGDS adhesive peptide, and lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) (photoinitiator), from the American Peptide Company. I then mixed these reagents to 2 mM, 19 mM, and 0.05% by weight, respectively, in a deionized water solvent (units were decided according to which unit each solution was received in). The 8-arm PEG-NB, synthesized in the previous step, was dissolved in phosphate buffered saline to 10% by weight. The MMP, RGDS, LAP, and 8-arm PEG-NB were mixed together according to the volumes and concentrations specified in Appendix A. Gels were polymerized on thiolized glass coverslips under a UV light for 3 minutes.

I calculated concentrations and volumes based on gel volume (12μL) and thiol to ene ratio, which was 0.7:1. I chose this thiol to ene ratio because the ratio affects the stiffness of the hydrogel and therefore VIC response, and in previous studies, a 0.7:1 cross-linking ratio yielded low levels of myofibroblast activation. Additionally, this ratio is not soft enough to allow the cells to degrade the matrix so much as to create difficulties when imaging.[20]

A rheometer was used with a 0.5 N axial force to find Young's Modulus E and quantify the stiffness of the gels.

Cell Culture VICs from passage 2 or 3 were seeded on PEG-hydrogels in a density of 200 cells/mL. They were allowed to proliferate in 15% fetal bovine serum (growth media) for 24 hours.

All cytokines (TGF-β1, IL-1β, and IL-6) were added to cell culture wells in 0, 0.1, 1.0, 10.0, and 100 ng/mL concentrations, along with 1% fetal bovine serum. There were three gels for each concentration.

Fixing Cells and Immunostaining After 48 hours of culture on gels, VICs were fixed with 4% paraformaldehyde, permeabilized with 0.1% Tritonx100, and non-specific antibody staining was blocked with 5% bovine serum albumin. Gels were then incubated with the antibody ms mAv to α-SMA ab7187 (for cytoplasm or f-actin). Following antibody coupling, samples were washed and incubated with Alexa 488 goat mouse IgG (for α-SMA staining), tetramethylrhodamine (TRITC) for cytoplasm staining, and 4',6-diamidino-2-phenylindole (DAPI) for nuclei staining.

Quantification of Myofibroblast Activation Cells were imaged using a Zeiss fluorescent microscope and exposure times were controlled among each experiment. In the first method of quantification, and from the suggestion of my mentors, I manually counted cells for the presence of α-SMA and stress fibers to determine myofibroblast activation.

I used histograms as a second method of quantification. I used Adobe Photoshop to select all pixels above a brightness level of 50 on the histogram and isolate the area of the image that consisted of cytoplasm. Then, I removed the rest of the image (as it does not contain cells) and overlayed the α-SMA channel onto the area that was selected previously. Now the image consists solely of the α-SMA channel on the same area which contained the cells. Then, using all the data points of the histogram, levels of α-SMA expression were measured by calculating the percent of pixels that had a brightness level over 50.

Data Analysis Statistical significance was determined using analyses of variance and post-hoc 2-sample t test. Effect size was calculated using Cohen's *d*. I also calculated 95% confidence intervals. I considered results significant compared with the untreated control if $p < 0.05$.

2.3 Hazardous Chemicals and Devices

- **Paraformaldehyde (4%)** flammable, toxic if inhaled or ingested, causes skin irritation, carcinogenic
- **Ultraviolet light** can cause eye or skin damage, carcinogenic with high levels of exposure

2.4 Safety Procedures and Protective Methods

I have been trained in the use of hazardous chemicals and disposal of hazardous waste, and while handling paraformaldehyde I was supervised by my qualified scientist and used a chemical fume hood with personal protective equipment. When using the UV light, eye and skin proection were used, as well as covers for the UV light itself. Splashing was minimized with mechanical pipettes. There was no eating or drinking in the work area. Emergency eye and face wash, shower stations, and fire extinguishers were placed around the lab. Paraformaldehyde waste was placed into specially designated waste containers.

3 Results

Analyses of variance (ANOVAs) for each cytokine and for both methods of quantification indicated a statistically significant affect of cytokine concentration on measured myofibroblast activation ($p < 0.01$, see Table 1 for individual statistics). Post-hoc t-tests were then conducted to compare each concentration of cytokine with the control condition for that experiment.

In the TGF-β1 experiment (see Figure 2), it was found that there was an statistically significant increase in measured myofibroblast activation for all concentrations when compared to the control condition (0 ng/mL), with the exception of the 1 ng/mL condition in the histogram analysis. There were large effect sizes (Cohen's d) of 1.6 and 2.2 for histogram and counting analysis between the control and 100 ng/mL conditions, respectively. A similar result was found in the IL-6 experiment (see Figure 3), with a statistically significant increase in activation for all conditions except the 0.1 and 1 ng/mL conditions. There were large effect sizes of 1.1 and 3 between the control and 100 ng/mL conditions, respectively.

In the IL-1β experiment (see Figure 4), there was a statistically significant decrease in measure myofibroblast activation for each concentration of cytokine when compared to the control condition, except for the 1 ng/mL condition in the histogram analysis and the 0.1 ng/mL condition in the manual counting analysis. There were large effect sizes of 2 and 0.6 for histogram and counting analysis, respectively.

Table 1: ANOVA results for each cytokine between all 5 conditions (0, 0.1, 1, 10, and 100 ng/mL concentrations). All p-values indicate statistical significance; at least one condition for each cytokine experiment is statistically different from the other conditions.

Cytokine	Method of analysis	df	F	p
TGF-β1	Histogram	4, 84	14.28	<0.001
	Counting	4, 83	23.93	<0.001
IL-1β	Histogram	4, 85	4.84	0.001
	Counting	4, 84	5.45	<0.001
IL-6	Histogram	4, 80	14.96	<0.001
	Counting	4, 81	5.66	<0.001

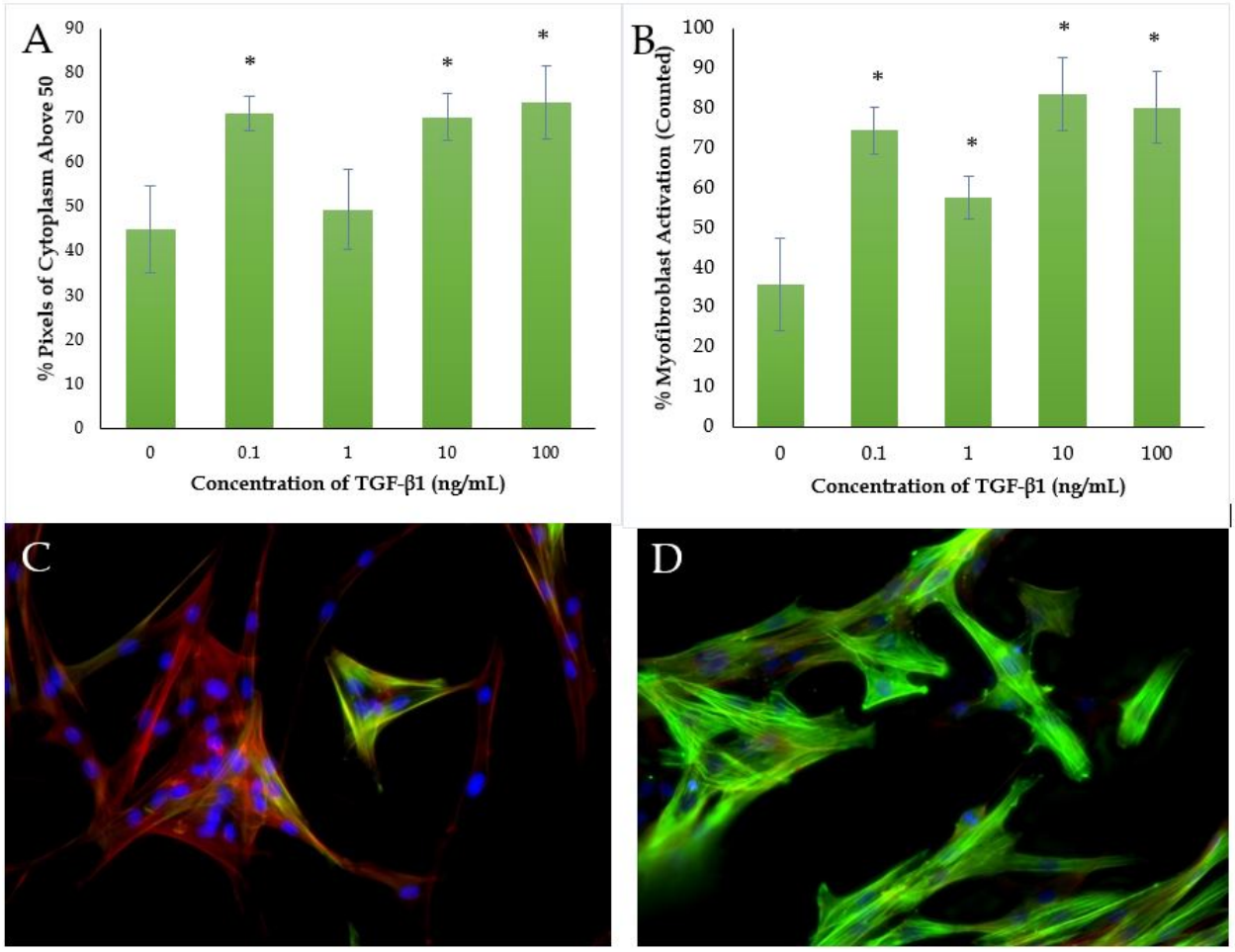

Figure 2: TGF-β1 experiment

Example images from TGF-1 experiment where red = F-actin (cytoplasm) , blue = DAPI (nuclei), and green = α-SMA. Images were obtained from z-stack extended focus projections at 20x magnification and are from the conditions of A) 0 ng/mL and B) 100 ng/mL. C) Image analysis by histogram and D) by manually counting. Error bars indicate 95% confidence intervals.

* Indicates significance ($p < 0.05$) when compared with t-test to 0 ng/mL condition.

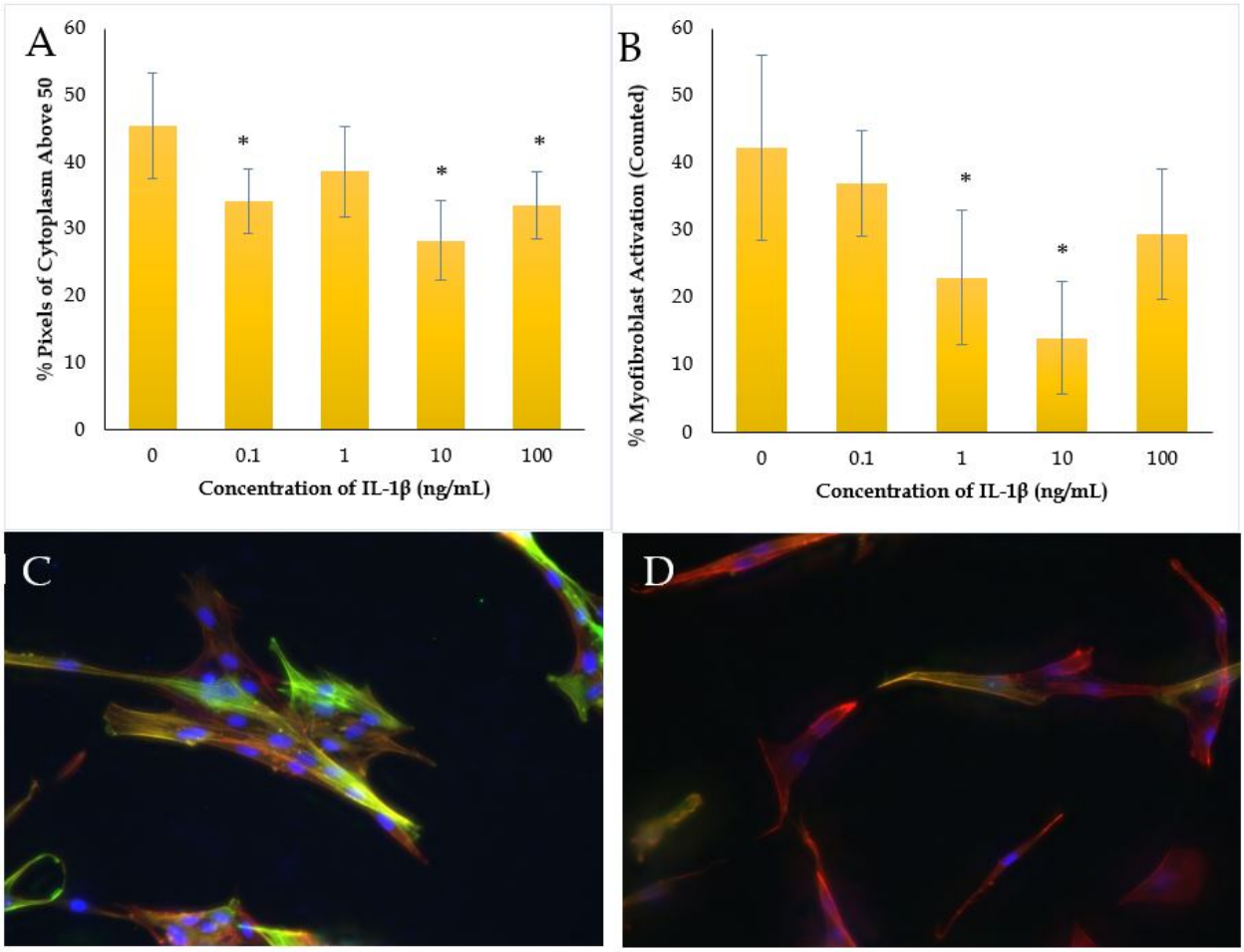

Figure 3: IL-1β experiment

Example images from the IL-1β experiment. See Figure 2 for image description. Images are examples from the conditions A) 0 ng/mL and B) 100 ng/mL. C) Image analysis by histogram and D) by manually counting. Error bars indicate 95% confidence intervals.

* Indicates significance ($p < 0.05$) when compared with t-test to 0 ng/mL condition.

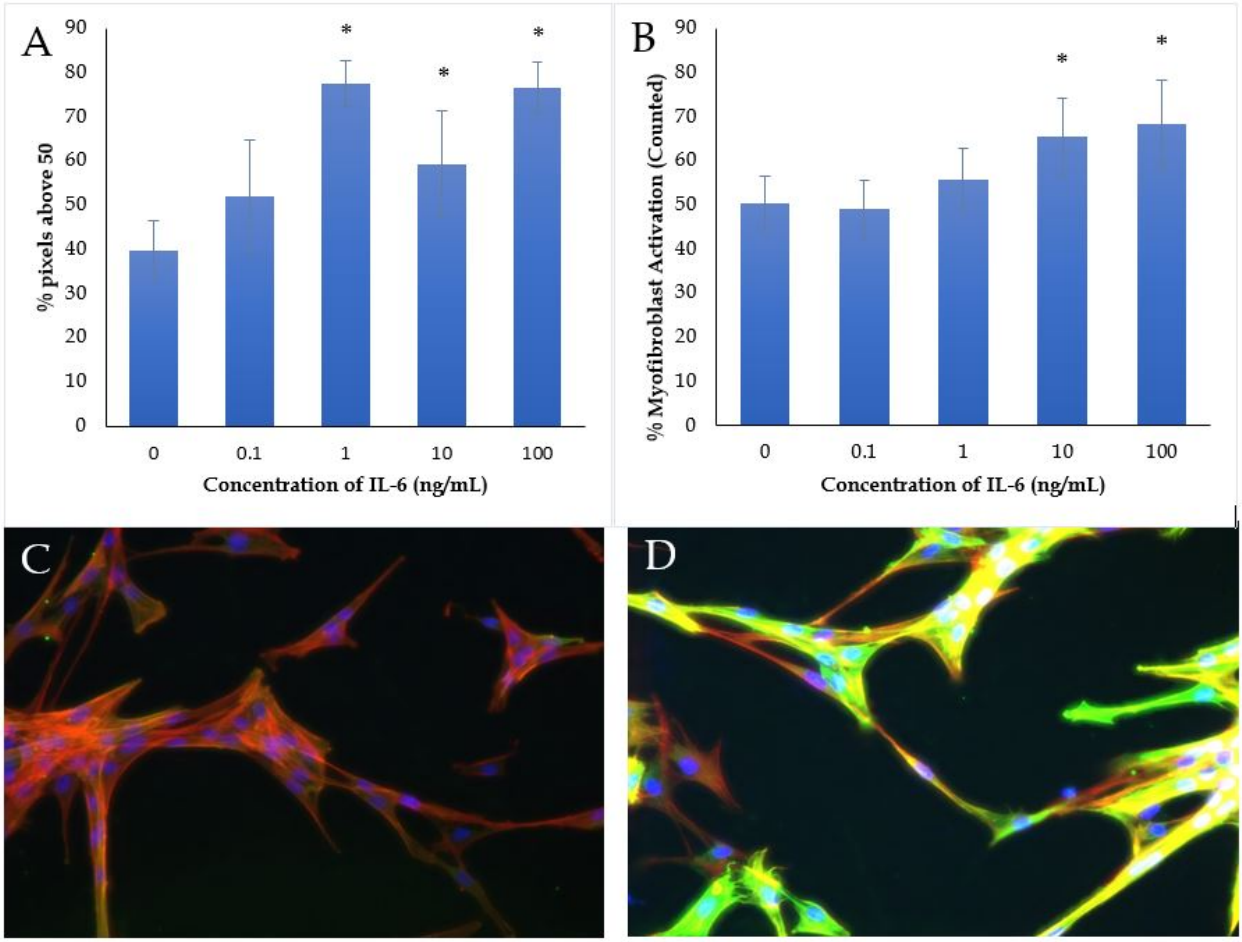

Figure 4: IL-6 experiment

Example images from the IL-6 experiment. See Figure 2 for image description. Images are examples from the conditions A) 0 ng/mL and B) 100 ng/mL. C) Image analysis by histogram and D) by manually counting. Error bars indicate 95% confidence intervals.

* Indicates significance ($p < 0.05$) when compared with t-test to 0 ng/mL condition.

4 Discussion

The first goal in this study was to mimic the ECM environment that exists *in vivo* through use of degradable and adhesive PEG-hydrogels. The low level of myofibroblast activation in the control conditions indicates that the deactivated quiescent phenotype was maintained, because the gel conditions effectively mimicked the native elasticity of the ECM. The cells responded to the culture substrate by adhering to the CRDGS adhesive peptide and degrading the MMP cross-linkers as they grew. The stiffness of each gel was 5-7 kPa (Young's Modulus E). In all control conditions for each separate experiment and for both methods of quantification, the measured level of myofibroblast activation was around 30-40%, indicating that the majority of cells were not contributing to the diseased state. The culture substrate stiffness and structure provided effective ECM mimics.

The second goal of this study was to mimic the inflammatory response of VICs in the valve. With an effective ECM mimic developed, the inflammatory cytokines TGF-β1, IL-1β, and IL-6 were added individually and myofibroblast activation was measured using two different methods. TGF-β1 has been implicated in VIC myofibroblast differentiation using immunostaining imaging and characterization of activation by α-SMA fiber formation.[10,19] It is considered a positive control for many cell culture studies of myofibroblast activation. The results from the TGF-β1 experiment support these studies, with large effect sizes and statistics indicating a significant increase in myofibroblast activation for each concentration of TGF-β1.

IL-1β has also been implicated in valve disease pathogenesis, and is considered a strong pro-inflammatory cytokine.[19] However, the data analysis indicates that an increase in IL-1β concentration significantly decreased the level of myofibroblast activation. There are several possible explanations for this result. It has been previously found that IL-1β and MMP may have a regulatory interdependence.[21] Due to the presence of MMP cross-linkers in the gels, the MMP proteases in the VICs may be interfering with the inflammatory signal provided by the IL-1β. Additionally, IL-1β mediates apoptosis, which also could have decreased cell number and affected the measured myofibroblast activation of the VICs.[21]

Finally, IL-6 was previously implicated in the disease pathways of valvular endothelial cells,[22] as well as VICs, but it had never been added to a culture system similar to the one used in this study. The results indicate a significant increase in myofibroblasts, as well as a large effect of IL-1β addition on myofibroblast activation. IL-6 is a possible inflammatory signal that contributes to aortic valve disease.

The primary limitation of these results is that this research was conducted on a 2D hydrogel environment in which the VICs were attached to the top of the gels, creating a polarized environment: one side of the VICs was adhered to the hydrogel, while the other side was in contact with the liquid media. Therefore, encapsulating the VICs within the hydrogel, creating a 3D matrix environment, may be a step toward a more complex system and a better ECM mimic.

The results supported the hypothesis that two pro-inflammatory cytokines (TGF-β1 and IL-6) mediate the actively regulated progression of cardiac aortic valve disease, an overwhelmingly common disease in the elderly. However, the results did not support the hypothesis that IL-1β plays an inflammatory role in disease progression. The data do further support previous findings of regulatory interdependence between IL-1β and MMP. Ultimately, with this study I also demonstrated the efficacy of PEG-hydrogels as physiologically relevant matrix environments for studying cardiac aortic valve disease, and possibly other inflammatory diseases.

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Appendix A

Table 2: Volumes of materials needed for gel preparation (See Section 2.2). Units were decided according to the unit of concentration that each material was received in.

	Stock Concentration	Desired Concentration	Volume (μL)
LAP	2 wt%	0.05 wt %	5.00
RGDS	73.61 mM	2 mM	5.43
8-arm PEG-NB	20 wt%	10 wt%	100.00
MMP	95.28 mM	6 mM	12.59
PBS			76.97
TOTAL	—	—	198