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CD133 antibody conjugation to decellularized human heart valves intended for circulating cell capture

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Abstract

The long term efficacy of tissue based heart valve grafts may be limited by progressive degeneration characterized by immune mediated inflammation and calcification. To avoid this degeneration, decellularized heart valves with functionalized surfaces capable of rapid *in vivo* endothelialization have been developed. The aim of this study is to examine the capacity of CD133 antibody-conjugated valve tissue to capture circulating endothelial progenitor cells (EPCs). Decellularized human pulmonary valve tissue was conjugated with CD133 antibody at varying concentrations and exposed to CD133 expressing NTERA-2 cl.D1 (NT2) cells in a microflow chamber. The amount of CD133 antibody conjugated on the valve tissue surface and the number of NT2 cells captured in the presence of shear stress was measured. Both the amount of CD133 antibody conjugated to the valve leaflet surface and the number of adherent NT2 cells increased as the concentration of CD133 antibody present in the surface immobilization procedure increased. The data presented in this study support the hypothesis that the rate of CD133⁺ cell adhesion in the presence of shear stress to decellularized heart valve tissue functionalized by CD133 antibody conjugation increases as the quantity of CD133 antibody conjugated to the tissue surface increases.

Keywords

CD133 antibody; heart valve; decellularization; endothelial progenitor cells; regenerative medicine

1. Introduction

Heart valve disease affects over 5.2 million people (2.5% prevalence) in the United States [1–3]. The treatment of choice for clinically significant heart valve disease is surgical repair or replacement [4]. Approximately 100 000 heart valve replacements are performed in the US annually, and more than 250 000 worldwide [2, 5–8]. The long-term efficacy of these

operations depends on the material used for valve replacement. Mechanical heart valve substitutes are limited by the need for lifelong anticoagulation and the morbidity thereof [9, 10]. Currently available biologic heart valve prostheses may be limited by progressive degeneration by immune mediated inflammation and calcification, which ultimately lead to thrombosis, dysfunction, and failure [7–20]. Heart valve bioprostheses engineered to be immunologically inert and mechanically durable with the ability to grow, repair, and regenerate could solve these problems [13, 17].

Decellularization of heart valves reduces surface antigenicity, preserves mechanical properties, maintains natural extracellular matrix (ECM) characteristics, and produces a scaffold that is theoretically capable of being repopulated with native vascular cells [16–18, 20–27]. However, decellularization does not completely eliminate the immune mediated degeneration of some tissue valve grafts [12, 14, 17, 25, 28]. Growth and remodeling of implanted decellularized bioprostheses require repopulation of the acellular graft [25, 29]. It has been demonstrated that the re-endothelialization can enhance biological stability by reducing thrombogenicity and calcification of cardiovascular bioprostheses [11, 14, 15, 30–35]. Unfortunately, there are currently no heart valve substitutes with the capacity for growth or regeneration [9, 36, 37]. Current biologic valve replacements, including decellularized valves, demonstrate poor cellular attachment, proliferation, and biocompatibility, all of which are necessary for self-repair and regeneration [9, 10, 17, 18, 23–25, 28, 33, 37–40].

The production of *in vitro* cell-seeded composite bioprostheses is technically difficult, labor intensive, and time consuming, which limits their clinical practicality and precludes them from use in emergencies [31, 36, 41]. Thus, the concept of bioprosthetic heart valves with functionalized surfaces capable of *in vivo* re-cellularization through the recruitment of blood circulating endothelial progenitor cells (EPCs) has been proposed as a feasible approach to solving these problems [14, 24, 31, 36, 37, 42]. Cardiovascular tissues functionalized with various bioactive molecules to interact the circulating EPCs and mature endothelial cells (ECs) have shown promising results both *in vitro* and *in vivo* [37, 41–44].

Mature ECs are terminally differentiated and quiescent, which limits their capacity to repair damaged endothelium [45]. On the other hand, circulating EPCs, expressing CD133⁺, CD34⁺, VEGFR2⁺, CD14⁺, VE-cadherin⁺, eNOS⁺, are capable of adhering to non-endothelialized intravascular surfaces, differentiating into ECs, and forming a functional endothelium [34, 45–48]. Therefore, CD133 appears to be a useful target for the selective capture of EPCs. As a proof of this concept, previously, decellularized heart valves functionalized with CD133 antibody showed superior capacity to generate an endothelium *in vivo* compared to non-functionalized valves after 3 months in a sheep model [37].

The goal of this study was to determine whether commercially used decellularized human heart valve tissue could be functionalized by CD133 antibody conjugation to attract the circulating EPCs *in vivo*. Additionally, we sought to confirm that the mechanism of EPC attraction is indeed mediated by the presumed CD133 antigen-CD133 antibody interaction. We hypothesized that the rate of CD133⁺ cell adhesion in the presence of shear stress to decellularized human heart valve tissue functionalized by CD133 antibody conjugation

would increase as the quantity of CD133 antibody conjugated to the valve tissue surface increases.

2. Materials and methods

2.1. NT2 cell culture and characterization

NTERA-2 cl.D1 (NT2) cells expressing CD133 were obtained from ATCC (Manassas, VA, USA) and cultured in Dulbecco's modified eagle's medium (DMEM) with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a humidified 5% CO₂ atmosphere at 37 °C. To confirm the CD133 expression, NT2 cells were plated on culture slides and maintained as above for 1 week and then fixed with 10% neutral buffered formalin (NBF). Nonspecific binding was blocked with serum-free protein block (Dako, Glostrup, Denmark) for 15 min at room temperature. The cells were then incubated with mouse monoclonal anti-CD133 IgG antibody (EMD Millipore, Billerica, MA, USA) diluted in antibody diluent (Dako) for 1 h at room temperature. The cells were then washed with phosphate buffered saline (PBS) (Thermo Fisher Scientific, Inc.) and incubated goat anti-mouse IgG with AlexaFluor 488 (Thermo Fisher Scientific, Inc.) for 30 min in the dark at room temperature. The cells were then covered with glass coverslips using Vectashield mounting medium for fluorescence with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA). Fluorescence images were obtained using a Leica Biosystems DM 4000B microscope (Buffalo Grove, NY, USA) equipped with a QImaging Retiga-2000RV camera (Surrey, BC, Canada).

For the flow cytometric analysis, NT2 cells were mechanically released from their adhesion to culture plates with a sterile scraping device and suspended in PBS at 10×10^6 cells mL⁻¹. Two samples were created from this cell suspension (Marked and Unmarked). The Marked sample was then incubated with mouse monoclonal CD133 IgG antibody with PE (Miltenyi Biotec, Inc., Bergisch Gladbach, Germany) added in a 1 to 50 volumetric ratio for 30 min in the dark at 0 °C. The unmarked sample was incubated with for 30 min in the dark at 0 °C. Both samples were then washed with PBS and re-suspended in PBS at 5×10^6 cells mL⁻¹. Flow cytometry data was acquired using a FACSCalibur flow cytometer with CellQuest Pro software (BD Biosciences, San Jose, CA, USA). The fluorescence intensity of the PE fluorophore was measured using a 488 nm excitation laser and 575/25 emission filter. The number of events analyzed for each sample was 10 000. Flow cytometry data was analyzed using FlowJo VX 10.0 software (FlowJo, LLC, Ashland, OR, USA).

2.2. CD133 antibody conjugation

All reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) excluding 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), which was obtained from Thermo Fisher Scientific, Inc. The carboxyl groups of the cryopreserved decellularized human pulmonary valve tissue (CryoLife, Inc., Kennesaw, GA, USA) was activated in 20 mL of 10 mM 2-morpholinoethane sulfonic acid (MES) aqueous solution containing 10 mM EDC and 10 mM N-hydroxysuccinimide (NHS) in for 30 min at 37 °C. Mouse monoclonal CD133 IgG antibody (EMD Millipore) was then added to the reaction in a quantity sufficient to achieve the desired antibody concentration and incubated for 2 h at 37 °C. The

tissue samples were then submerged in 100 mL of termination buffer (500 mM ethanolamine and 500 mM sodium chloride) for 1 h at room temperature. The antibody-conjugated tissue samples were then washed by submerging in 100 mL of washing buffer containing 100 mM trizma hydrochloride for 1 min followed by submerging in 100 mL of washing buffer containing 100 mM sodium acetate trihydrate and 500 mM sodium chloride for 1 min. This washing was repeated three times. The antibody-conjugated valve tissues were then washed with PBS and stored in PBS at 4 °C.

To examine the effect of antibody concentration on the relative quantity of antibody ultimately conjugated to the tissue surface and subsequent capacity to capture CD133⁺ cells from a flowing suspension, four groups with one valve each were created: control, 1, 10, and 100 $\mu\text{g mL}^{-1}$. In addition, an antibody conjugation concentration of 100 $\mu\text{g mL}^{-1}$ was used to evaluate the cryopreservation on the above mentioned outcomes. To determine the antibody conjugation, three samples from each region [valve leaflet (valve), pulmonary artery (PA), and sinus] of each valve were frozen-sectioned to 6 μm thick sections. Sections were then dried at room temperature overnight and washed with PBS. Nonspecific binding was blocked with serum-free protein block for 15 min at room temperature. Sections were then incubated with goat anti-mouse IgG with AlexaFluor 594 for 45 min at room temperature. Nine fluorescence microscopy images were collected from each sample using the Leica Biosystems DM 4000B microscope equipped with a QImaging Retiga-2000RV camera. Images were processed by ImagePro Express 6.3 (Media Cybernetics, Inc., Rockville, MD, USA) and analyzed with ImageJ software (National Institutes of Health, Bethesda, MD, USA). For each image, the fluorescence intensity of the signal image and the autofluorescence image was calculated as the average pixel density of the tissue area. The fluorescence intensity of the autofluorescence images was then used to normalize the fluorescence intensity of the signal image.

2.3. Microflow chamber study

Three tissue samples from each region (valve, PA, and sinus) of each valve were placed in a parallel plate microflow chamber with a 0.010 in thick gasket (GlycoTech, Gaithersburg, MD, USA) and exposed to a suspension of NT2 cells (10^5 cells mL^{-1}) in DMEM with 10% FBS with a shear stress of 0.4 dynes cm^{-2} for 1 h at room temperature. The samples were then gently rinsed with PBS, fixed with 10% NBF, mounted on glass slides and covered with glass coverslips using Vectashield mounting medium for fluorescence with DAPI. To examine the capacity of EC capture, fluorescence images were obtained using the Leica Biosystems DM 4000B microscope. The number of cells per unit area was calculated by counting the number of cells present in the image and dividing by the area of tissue captured in the image.

2.4. Valve tissue cryopreservation

In order to investigate the long-term functionality, the CD133-conjugated decellularized human heart valve tissues were cryopreserved by CryoLife, Inc. using their proprietary cryopreservation technology and stored at -196 °C for 3 months. The relative quantity of antibody that remained conjugated to the valve tissue surface and the capacity of the tissue

to capture CD133 expressing cells from a flowing suspension were then measured in the same manner as described above.

2.5. Statistics

All data is presented as mean $\pm$ standard deviation. A two-tailed Welch–Aspin *t*-test was used to test the statistical significance of differences between groups. A *p*-value of <0.05 was considered to be statistically significant.

3. Results

3.1. NT2 characterization

The data presented in figure 1 verifies the CD133 expression profile of NT2 cells. The intensity of the green fluorescence signal in figure 1(A) demonstrates the strong expression of CD133 by NT2 cells, and the location of the green fluorescence signal at the cellular periphery supports the idea that CD133 is expressed on the cell surface. The 1D gate used in figure 1(C) was set to exclude 99.0% of the unmarked NT2 cells. With this gate, 58.2% of labeled NT2 cells were included. While the strict interpretation of this result is that no more than 60% of NT2 cells express CD133, closer inspection of the CD133-PE fluorescence intensity profile of the marked cells in figure 1(C) indicates that the percentage of NT2 cells that express CD133 is likely much higher.

3.2. CD133 antibody conjugation

The effect of varying the CD133 antibody presented in the antibody surface immobilization procedure is displayed in figure 2. Figure 2(A) shows a qualitative increase in red immunofluorescence as the concentration of CD133 antibody used in the antibody conjugation procedure is increased. Figure 2(B) quantitatively expresses the increase in red immunofluorescence as the concentration of CD133 antibody used in the antibody conjugation procedure is increased. This increase is statistically significant ($p < 0.05$) between all groups for each tissue type except between the sinus control and sinus $1 \mu\text{g mL}^{-1}$ groups ($p = 0.334$). These data support the conclusion that the quantity of CD133 antibody-conjugated to decellularized human pulmonary valve tissue increases as the concentration of antibody used in the conjugation procedure is increased. Additionally, qualitative assessment of the immunofluorescence images of the $100 \mu\text{g mL}^{-1}$ group in figure 2(A) reveals that the majority of antibody conjugation occurred at the surface of the tissues rather than within the tissue matrix.

3.3. Microflow chamber study

The effect of the concentration of CD133 antibody used in the antibody surface immobilization procedure on the rate of NT2 cell capture following microflow exposure is demonstrated in figure 3. Figure 3(A) qualitatively demonstrates that the number of NT2 cells captured by the tissue surface increases as the concentration of CD133 antibody used in the immobilization procedure is increased. Figure 3(B) expresses this finding in a quantitative fashion. This finding is statistically significant between all groups for each tissue type. This data supports the conclusion that the rate of CD133⁺ cell adhesion to a tissue surface increases as the concentration of CD133 antibody used in the antibody

immobilization procedure increases. Interestingly, the improvement in cell adhesion between the $1 \mu\text{g mL}^{-1}$ and the $10 \mu\text{g mL}^{-1}$ groups is much greater than the improvement between the $10 \mu\text{g mL}^{-1}$ and the $100 \mu\text{g mL}^{-1}$ groups despite the much larger increase in the quantity of antibody conjugated to the tissue between the $10 \mu\text{g mL}^{-1}$ and the $100 \mu\text{g mL}^{-1}$ groups as shown in figure 2.

3.4. Valve tissue cryopreservation

The effect of cryopreservation on the quantity of CD133 antibody presents on the valve tissue surface and the rate of NT2 cell capture following microflow exposure is demonstrated in figure 4. Qualitatively (figure 4(A)) and quantitatively (figure 4(B)) the quantity of CD133 antibody present on the valve tissue surface and the rate of NT2 cell capture following microflow exposure is the same for the pre-cryopreservation and post-cryopreservation tissue samples. These data support the conclusion that cryopreservation does not decrease the quantity of CD133 antibody presented on the valve tissue surface, nor does it decrease the rate of NT2 cell capture following microflow exposure.

4. Discussion

The two basic options for valve replacements, mechanical and biological, both have their unique shortcomings. Mechanical valves require indefinite anticoagulation, accompanied by numerous office visits and blood checks, to offset their risk of thromboembolism [49]. Biological valves have less risk for thrombosis, but unfortunately have greater material deterioration over time [50]. Common to both of these current options is their lack of viability, potential to grow and the ability to significantly remodel themselves as the patient changes, most notably in rapidly growing children [51]. The promise of tissue-engineered heart valves lies in the ability to recapitulate normal cellularity, structure and function of native valves. Significant efforts have been made in the field of tissue engineering as reviewed by many [52–54]. However, most tissue-engineering valve attempts require cell harvesting from patients, passive seeding and *in vitro* maturation; all time-consuming and expensive methods.

Structurally, the heart valve leaflets consist of luminal ECs surrounding interstitial cells. At the structural and functional level, valve ECs resemble other vascular ECs. They express markers for CD31, and von Willebrand factor (vWF) and endothelial nitric oxide synthase (eNOS) and can be invoked to express intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin [55, 56]. As such, valve ECs lie at the nexus of valve function and growth. We previously reported that conjugation of decellularized porcine heart valves with CD133 antibody was faster, easier and superior to pre-seeding decellularized scaffolds in promoting cellularization and biomechanical development [37]. In this study, we evaluated the capacity of commercially available decellularized human heart valve tissue functionalized by CD133 antibody-conjugation to attract CD133⁺ cells. Additionally, we aimed to confirm that the mechanism of EPC attraction is indeed mediated by the presumed CD133 antigen-CD133 antibody reaction. Taken together, we demonstrated that the CD133 antibody-conjugated valve tissue had a superior capacity to selectively adhere to CD133⁺ cells than non-functionalized valve tissue

under flow condition, and that this capacity is mediated by the antigen-antibody reaction (figures 2 and 3). This combined with the findings reported previously [37] supports the idea that tissue surface functionalization, particularly by CD133 antibody, aimed at capturing circulating EPCs represents an effective means of creating heart valve bioprosthesis with the capacity for *in vivo* re-endothelialization.

The choice of antibody surface immobilization technique deserves careful consideration. There are three general approaches to antibody surface immobilization: non-covalent adsorption, random covalent immobilization, and oriented covalent immobilization. The simplest method of antibody immobilization is randomly oriented non-covalent adsorption, which relies on ionic bonds, electrostatic and hydrophobic interactions and van der Waals forces [57–59]. Non-covalent antibody surface adsorption results in randomly oriented antibodies, which decreases both the number of available antigen binding sites and the antigen binding capacity of the surface [59–62]. Non-covalent interactions are typically very weak and are sensitive to changes in pH, temperature, fluid dynamic conditions, and salt concentrations, all of which may result in poor functional performance, antibody leaching, and poor storage stability [58, 59]. Random covalent antibody immobilization adequately binds antibodies to the surface, but also results in a large number of surface binding orientations, which may decrease the number of active antigen binding sites by up to 14-fold [60, 61]. In fact, the chemistry used for random covalent antibody immobilization may even be biased toward sub-optimal antibody orientation [61]. Despite being randomly oriented, it has been shown that covalent antibody immobilization not only prevents degradation of surface functionalization but also results in a more favorable antibody orientation profile than non-covalent adsorption [58, 60]. Covalent immobilization of antibodies in a controlled and oriented fashion maximizes the number of exposed, functional antigen-binding sites [58, 60, 61]. The effect of uniformly oriented covalent antibody immobilization versus randomly oriented covalent antibody immobilization on surface capacity to capture antigen ranges from negligible to greater than a 200-fold increase [61]. All oriented antibody immobilization techniques show improvements over randomly oriented immobilization techniques, however, the choice of technique should be tailored to the goals of each application [57, 58]. Despite being theoretically advantageous, oriented antibody immobilization suffers from a number of limitations including being difficult to achieve, difficult to evaluate on irregular surfaces, and being highly dependent on a large number of factors such as the nature of the antibody-antigen interaction, which can be widely variable [57, 58, 61]. For these reasons, the present study employed a random covalent immobilization technique as to avoid the functional limitations of a non-covalent adsorption technique, and the difficulties of an oriented covalent immobilization technique.

As an additional step toward demonstrating the clinical utility of CD133 antibody-conjugated valve tissue, we investigated the functional stability of this system to cryopreservation (figure 4). The ability to cryopreserve these antibody-conjugated heart valve bioprostheses would allow individual institutions to maintain a sizeable supply without concern for spoilage. This can provide institutions with the ability to use biologic heart valve prosthesis capable of *in vivo* re-cellularization in emergent cases, which is a significant advantage over *in vitro* cell-seeded composite bioprostheses or bioprostheses that do not support regeneration of a native endothelium.

A large hurdle that must be cleared prior to the clinical translation of anti-CD133 antibody tissue conjugation is the fact that only a very small number of circulating cells are CD133⁺ EPCs [63]. In this study, the tissue was exposed to a fluid containing a very high concentration of CD133⁺ cells, and every cell captured was considered to be a successful cell capture. However, *in vivo*, the vast majority of circulating cells are not CD133⁺ EPCs [63]. In fact, circulating EPCs are known to lose their CD133 expression shortly after leaving the bone marrow [64]. The low concentration of EPCs in circulation combined with the propensity for other circulating cells to attach to non-endothelialized surfaces leaves anti-CD133 functionalized graft surfaces vulnerable to coverage by non-ECs long before enough EPCs can be immobilized to cover and protect the surface. This technology will require extensive optimization to overcome this shortfall. The solution will likely require a multi-pronged approach that includes inducing greater EPC mobilization from the bone marrow during the peri-implantation period, optimizing the quantity and stability of CD133 antibody affixed to the surface, adding additional surface modifications for greater cell capture specificity, and decreasing the propensity for platelet and other non-EC attachment.

This study possesses several limitations. The specificity of anti-CD133 surface functionalization was not tested. It is possible that anti-CD133 surface functionalization imparts an increase in cell capture capacity for all cell types. However, results reported by Jordan *et al* [37] would seem to counter this notion. The shear stress of 0.4 dynes cm⁻² used in the microflow experiment is far below the 10–15 dynes cm⁻² considered to be normal arterial shear stress. This limitation may change the expected rate of *in vivo* cell capture, but not the relative improvement of cell capturing capacity versus control. Plouffe, *et al* [65] demonstrated that an increase in shear stress does indeed decrease the quantity of cells captured, but not the relative improvement in cells captured versus control. Finally, the underlying reason for the development of this technology remains to be addressed. Biologic heart valve grafts fail due to immune mediated degeneration and calcification, which ultimately lead to valve dysfunction, a process that takes years, if not decades, to occur. This study assessed the potential for anti-CD133 functionalized grafts to promote rapid *in vivo* endothelialization, which we hypothesize will accelerate native tissue remodeling and prevent or minimize immune mediated degeneration and calcification. Further studies will be required to test this hypothesis.

5. Conclusions

The data presented in this study support our hypothesis that the rate of CD133⁺ cell adhesion in the presence of shear stress to decellularized human heart valve tissue functionalized by CD133 antibody conjugation increases as the quantity of CD133 antibody conjugated to the tissue surface increases. We also demonstrated the functional stability of CD133 antibody-conjugated decellularized heart valve tissues system to cryopreservation. These findings suggest that commercially available decellularized human heart valve prostheses can be functionalized with anti-CD133 antibodies to improve *in vivo* endothelialization. Despite its current limitations, anti-CD133 surface functionalization remains a promising technology for future cardiovascular bioprosthesis engineering and clinical translation.

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Figure 1.

NT2 CD133 expression. (A) Fluorescence microscopy images of NT2 cells at $200\times$ magnification. Green: CD133, Blue: DAPI. Scale bar = $100\ \mu\text{m}$. (B) Dot plot for unmarked sample of NT2 cells. The region encircled by the black polygon represents the gate used to select NT2 cells from both samples (marked and unmarked). (C) Histogram of CD133-PE (488 nm excitation laser, 575/25 nm emission filter) fluorescence intensity of marked and unmarked NT2 cells. 1D gate excludes 99.0% of unmarked cells and includes 58.2% of marked cells.

Figure 2.

CD133 antibody conjugation. (A) Fluorescence microscopy images of decellularized human pulmonary valve tissue (valve leaflet (valve), pulmonary artery (PA), and sinus) following anti-CD133 antibody conjugation at various antibody concentrations. Magnification = 200 $\times$. Scale bars = 100 μm . Green = autofluorescence. Red = anti-CD133. (B) Relative quantity of anti-CD133 antibody conjugated to decellularized human pulmonary valve tissue (valve, PA, and sinus, respectively) following anti-CD133 antibody conjugation at various antibody concentrations expressed as fluorescence intensity of fluorophore conjugated secondary antibody per tissue area normalized to autofluorescence intensity. Error bars = SD. *: $p < 0.05$ all of the valve tissues were compared to one another, **: $p < 0.05$ all of the PA tissues

were compared to one another, ***: $p < 0.05$ all of the sinus tissues were compared to one another. ‡: $p = 0.334$.

Figure 3.

NT2 cell capture. (A) Representative fluorescence microscopy images of decellularized human pulmonary valve leaflet tissue conjugated with CD133 antibody at various antibody concentrations following microflow exposure to NT2 cells. Magnification = $400\times$. Scale bars = $100\text{ }\mu\text{m}$. Blue = DAPI. (B) Number of NT2 cells captured per mm^2 following microflow exposure to NT2 cells by CD133 antibody conjugated to decellularized human pulmonary valve tissue (valve, PA, and sinus, respectively) at various antibody concentrations. Error bars = SD. *, *: $p < 0.05$ all of the valve tissues were compared to one another, **: $p < 0.05$ all of the PA tissues were compared to one another, ***: $p < 0.05$ all of the sinus tissues were compared to one another.

Figure 4.

The effect of cryopreservation. (A) Pre- and post-cryopreservation fluorescence microscopy images of decellularized human pulmonary valve leaflet tissue conjugated with CD133 antibody at $100 \mu\text{g mL}^{-1}$. Top row: Magnification = $400\times$. Scale bars = $100 \mu\text{m}$. Green = autofluorescence. Red = CD133 antibody. Bottom row: post microflow exposure to NT2 cells. Magnification = $200\times$. Scale bars = $100 \mu\text{m}$. Blue = DAPI. (B) Number of NT2 cells captured per mm^2 following microflow exposure to NT2 cells by CD133 antibody conjugated to decellularized human pulmonary valve tissue at $100 \mu\text{g mL}^{-1}$ pre- and post-cryopreservation. Error bars = SD.