The Role of Glycoproteins and HLA markers in Fibrosis of Rheumatic Valvular Disease

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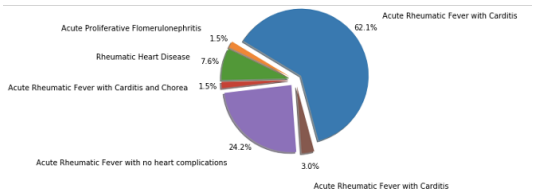
Abstract

Strep throat, a Streptococcus pyogenes bacterial infection is a common cause of rheumatic heart disease. Streptolysin, produced by the Streptococcus pyogenes, causes an autoimmune response in the heart due to molecular mimicry between the lymphatics at the site of the cardiac valve and the antigens expressed on cardiac tissue. The T cell responding to the strep infection infiltrate the valve and secrete IFN-γ, IL-6 and IL-17A, which leads to changes in the extracellular matrix, causing calcification and fibrosis of the mitral valve. Typically, after any injury to a cardiac valve, fibroblasts, connective tissue cells found in the heart, differentiate into myofibroblasts with increased production in TGF-β1. This TGF-β1 production is from the inflammation that occurs after tissue injury. Myofibroblasts migrate to the site of injury, typically the valve, and produce cytokines enhancing the inflammatory response. Previous studies have shown that expression of TGF-β1 and proliferation of myofibroblasts have a potential role in changing the heart tissue into scar tissue leading to rheumatic heart disease. However, scientists have yet to investigate how different subsets of T cells affect differentiation and migrations of myofibroblasts into the valve.

Therefore, the research reported here investigates how the impact of streptolysin treated T cells could influence the "fibrosis" process of differentiated myofibroblasts as evidenced by the production of EDA-fibronectin and proliferation. CD4+, CD8+, and NK-92 T cell subsets were treated with streptolysin to determine the production of IFN-γ, IL-6, and IL-17A. In addition, the secretome produced by the streptolysin treated subsets of these T cells was added to the differentiated myofibroblasts to observe the "fibrosis" process. To examine the impact of contact with the myofibroblasts, CD4+ and CD8+ T cells were cultured together with the myofibroblasts, as well as individually. Results suggested that activated CD4+ T cells caused more significant 'fibrosis' of the myofibroblasts. The secretome of the varying T cell subsets modulated EDA-fibronectin and proliferation independently. When in contact, CD4+ and CD8+ T cells, cultured together, caused more "fibrosis" to the myofibroblasts compared to when the CD4+ T cells and CD8+ T cells were co-cultured independently.

In addition, HLA genes encoding MHC complexes were analyzed using bioinformatics to determine if specific HLA types have a role in rheumatic heart disease. These MHC complexes bind to the T cell receptors, eliciting autoreactivity of the T cell, facilitating fibrosis of the mitral valve. HLA type also appears to have an impact on the fibrosis in rheumatic heart disease. Overall, the results of the research reported here, indicate that various glycoproteins on the T cell surface produce distinct cytokines; specific subsets of T cells have more of an impact on the fibrosis process; and the HLA genes may change the affinity of the binding between the MHC complex and the TCR affecting mitral valve fibrosis.



(a) Ratio of Heart complications after Diagnosis of Rheumatic Fever

(b) MHC-TCR Binding

Figure 1: (a) Diagram depicts the damage that occurs to the heart after an individual is diagnosed with Rheumatic Fever (1). (b) An image was created in Chimera showing the interactions between a peptide-MHC and the TCR A6. The MHC chains are in blue and cyan, the alpha and beta chains of the TCR are in yellow and red, and the peptide is centered in sticks and atoms (PDB 1A07). Figures created by author.

1 Introduction

Rheumatic heart disease (RHD) is the cause of approximately 1.4 million premature deaths annually (2). It is the result of an autoimmune response following a throat infection of Group A Streptococci (GAS). After a few weeks of streptococcal pharyngitis, a short-lived multisystem inflammatory process affects the heart valves, joints, skin, and brain. This multisystem inflammatory process is classified as rheumatic fever, which may immediately lead to the onset of rheumatic heart disease. 76.8% of all cases of rheumatic fever lead to heart damage (1). Most severe of the cardiac complications caused by rheumatic fever include chronic cardiac valvular inflammation, which results in valvular scarring or fibrosis. In RHD, the heart valve scarring is the underlying cause for the disease's morbidity and mortality as it leads to the valve stiffening and blood flow restriction.

Underlying the etiology of RHD is the autoimmune response that is caused by what is referred to as cross-reactive antibodies produced in GAS pharyngitis (3). Following GAS pharyngeal infection, cell wall components of GAS or components of the GAS, such as microbial peptides, overcome the mucosal barrier of the throat and enter venous circulation. This Group A Streptococcal molecule stimulates a response in an antigen- presenting cell (APC). Antigen-presenting cells natively express major histocompatibility complex (MHC) molecules on their surface in both MHC Class I and MHC Class II. MHC Class I molecules are found on all nucleated cells, while MHC Class II molecules are found on macrophages, dendritic cells, and B cells. After the GAS molecule "stimulates" the APCs, their MHC Class II molecules "present" the GAS peptide to the CD4+ T cell receptor (TCR), as shown in **Fig.** 1b. CD4+ is the glycoprotein found on the surface of that subset of T cells. CD4+ T cells assist in the maturation of the B cell to a plasma cell and are often referred to as the instigators of the adaptive immune response. Therefore, Streptolysin, the bacterial exotoxin, facilitates the binding of the MHC complex and TCR, activating the CD4+ T cells. Of heightened significance, these activated T cells are cross-reactive to the GAS bacteria and auto-reactive to the tissue antigens on the mitral valve constituting what is termed "molecular mimicry" (4). This autoreactivity to the mitral valve causes inflammation and the formation of granulomas. These granulomas eventually lead to the scarring of the valve and RHD (**Fig.** 2).

CD4+ T cells are not the only lymphocytes activated after streptococcal infection. Although CD4+ T cells are the most numerous subset of T cells responding to the infection, the presence of GAS can cause activation of other subsets of lymphocytes with variations in glycoproteins on their cell surface, including CD8+ T cells and Natural Killer (NK) cells. Each of these subsets plays a significant role in the immune response. CD8+ T cells are cytotoxic when activated by the APC. They respond by secreting inflammatory cytokines such as TNFα and IFN-γ. They proliferate and can "kill" cells housing invaders such as bacteria and viruses as well as tumor cells by injecting cytotoxic molecules, such as perforins and granzymes, into those cells. They can also kill by binding the Fas receptor on the surface of the target cell as they possess Fas ligand on their cell surface, setting off the extrinsic cell death mechanism. Another subset of T cells is the Natural Killer cells (NK), which are activated through the presence of IL-2. They are also cytotoxic producing molecules similar to the CD8+ T cells (5). The presence of this cytotoxic activity at the site of the mitral valve plays a significant role in the pathology of rheumatic valve disease, most likely causing the scarring of the valve. Of

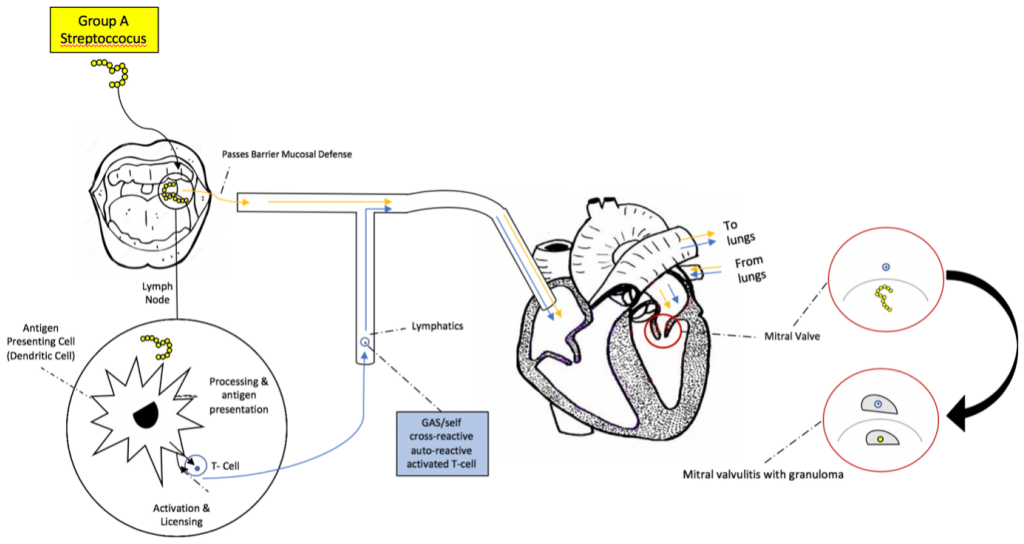


Figure 2: Diagram illustrates the pathway of the streptolysin bacteria and the T cell in Rheumatic Heart Disease. After a Group A Streptococcus (GAS) bacterial infection, the bacteria are found at the site of the antigen-presenting cell, which activates the T cell. The bacteria and lymphatic cells extravasate through the endothelium of the mitral valve and cause valvulitis leading to granuloma, which leads to granuloma and further scarring of the valve (3). Figure created by author.

particular interest, while traditionally requiring the presence of B cells for T cell subset activation, Streptolysin has been shown to independently activate p38 MAPK in mast cells, a type of granulocyte which is also part of the immune system, causing their activation (6). Transcription of p38MAPK has also been reported to cause CD8+ T cell activation (7). These activated T cells are the underlying cause of the disease.

In response to any cardiac injury, the heart, itself, has repair mechanisms. However, in RHD, the repair mechanisms may underlie the disease itself. Four major cell types comprise the healthy heart: myocytes, endothelial cells, cardiac smooth muscle cells, and fibroblasts. The fibroblasts represent the most substantial portion of the cellular mass. Fibroblasts of the heart are arranged as sheets and strands along the cardiac myocytes. Their role is to respond to both mechanical and chemical stimuli through paracrine factors. The active role of these fibroblasts is to produce and maintain the extracellular matrix by signaling myocytes through mechanical stress. Another form of fibroblasts, myofibroblasts, are large cells with ruffled membranes and highly active endoplasmic reticulum. They are not found in healthy cardiac tissue and are found only after cardiac injury. Myofibroblasts are migratory and are responsive to cytokines released at the site of cardiac injury (8). Myofibroblasts produce and secrete several cytokines themselves as well as maintain the inflammatory response to injury. Investigations support the role of increased expression of TGF-β1 in the differentiation of fibroblasts into myofibroblasts (9). In particular, the increased expression of TGF-β1 in the valve microenvironment and the aberrant proliferation of myofibroblasts could have a potentially significant role in tissue scarring in RHD. This presence of increased levels of TGF-β1 can lead to valvular fibrosis typical of rheumatic heart disease (10).

The in vitro research reported here investigated how both activated CD4+, CD8+, and NK T cells (Helper T cells, Cytotoxic T cells, and Natural Killer cells) stimulated with Streptolysin, the protein produced by GAS, affected the T cells themselves and the myofibroblasts. The model entailed the differentiation of fibroblasts into myofibroblasts through the administration of exogenous TGF-β1 and the resulting upregulation of EDA-fibronectin (11). Cytokine production was analyzed in the context of the various T cell subsets, and the secretome of the T cell subsets was administered to the myofibroblasts to examine the potential to cause the "fibrosis" of the "valve," the underlying cause of RHD. Fibrosis was measured as a function of extracellular matrix protein and proliferation of the

myofibroblasts. Research using this model allows for a better understanding of the injury which takes place to the mitral valve during RHD, including the contributions of the various T cell subsets. This model was created with the hopes to design more effective therapeutic options for this disease, which seems to target the mitral valve uniquely. The results would indicate how various activated T cells when treated with streptolysin may cause fibrosis to myofibroblasts and may reveal a potential target for therapy.

In addition to understanding RHD through an in vitro model, it has long been suspected that there is a genetic predisposition to RHD. However, explaining this susceptibility has been difficult. Because antigen-presenting cells have a substantial role in the etiology of RHD, HLA markers on antigen-presenting cells in RHD populations were analyzed to determine more susceptible HLA types. HLA types are significant in encoding for the MHC complexes that are responsible for the presentation of the antigen on the surface of the antigen-presenting cells. Case studies of HLA marker data from various countries(12)(13)(14), of individuals with RHD, were collected and compared to analyze the role HLA-markers have on the etiology of RHD. HLA markers of individuals diagnosed with RHD from Brazil, Turkey, and India determined the tendency for specific genetic HLA markers to cause rheumatic heart disease. The results across the different HLA markers would suggest the common HLA types of diagnosed RHD patients, indicating specific tendencies of RHD.

2 Materials and Methods

2.1 HFF-1 Cells

2.1.1 Cell Culture

HFF-1 foreskin fibroblast cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). All cells were cultured at 37°C and 5% CO2. HFF-1 were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Grand Island, NY). The cell culture medium was supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin (Invitrogen). Upon reaching confluency, cells were trypsinized and expanded using 0.05% trypsin-EDTA (Invitrogen). Cell proliferation was determined using an MTS assay (CellTiter® 96 AQueous One Solution Reagent, Promega, Madison, WI) as per the manufacturer's protocol. Determination of cell viability was done similarly using an MTS assay or a Vi-CELL XR Cell Viability Analyzer (Beckman Coulter, Indianapolis, IN).

2.1.2 MTS assay for Measuring Proliferation and Viability of HFF-1 after treatment of TGF-β1

An MTS-based CellTiter® 96 AQueous assay was performed in order to measure the proliferation of HFF-1 over time (48, 72, 96 hours) after treatment of TGF-β1 in concentrations of 0, 0.1, 1, 10 ng/mL. Cell Titer® 96 AQueous One Solution Reagent (Promega, Madison, WI) was added at 15 μL/well. The plate was incubated for 1h at 37°C and 5% CO2 and absorbance was measured using a BioTek ELX808 microplate reader at 490 nm.

2.1.3 Preparation of Lysates of HFF-1

HFF-1 was placed in T25 flasks at 0.1×10^6 cells/mL in 5mL of media. After treatment for 72 hours with TGF-β1 (0, 0.1, 1, 10 ng/mL), cells were trypsinized and centrifuged (7min at 1200rpm). The cell pellets were resuspended in 2mL PBS (Invitrogen) and centrifuged (7min at 1200rpm). Cells were resuspended in 1X lysis buffer #9803 (Cell Signaling Technology, Danvers, MA) supplemented with protease cocktail inhibitor #P8340 (Sigma, St. Louis, MO) at 1×10^6 cells per 1mL solution. Samples were placed on ice for 10min and then centrifuged (15min at 13,000rpm at 4°C). Lysates were collected and stored at -80°C.

2.1.4 Indirect Enzyme-Linked Immunosorbent Assay (ELISA) to Measure αSMA (alpha-smooth muscle actin) and EDA-Fibronectin (Extra Domain-A Fibronectin)

100μL lysate samples in a 96-well ELISA plate were incubated overnight at 4°C. The plate was emptied. 300μL 1X BSA Diluent/Blocking Solution (KPL, Gaithersburg, MD) was used to block wells. A rabbit anti-human primary antibody (1:300) against αSMA and EDA-Fibronectin (Abcam,

Cambridge, MA) was added to wells (1h at RT). After a wash with a 1X wash solution (KPL), an anti-rabbit secondary antibody (Abcam) (1:300) was added to wells (1h at RT). After a wash, 100μL substrate solution (KPL) was added to wells. Absorbance at 405nm was measured (BioTek ELx808).

2.2 H9/TK-1/NK T Cells

2.2.1 Cell Culture

H9 (derivative of HuT 78) cutaneous T lymphocyte, TK-1 T lymphocyte, and NK-92 peripheral blood natural killer cells were obtained from the American Type Culture Collection (ATCC). All cells were cultured at 37°C and 5% CO2. H9 were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin (Invitrogen). TK-1 were maintained in RPMI-1640 (Invitrogen) supplemented with 2mM L-glutamine (Invitrogen), 1.5 g/L sodium bicarbonate (Invitrogen), 4.5 g/L glucose (Invitrogen), 10mM HEPES (Invitrogen), 1.0 mM sodium pyruvate (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), 0.05 mM 2-mercaptoethanol (Invitrogen), 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin (Invitrogen). NK-92 were maintained in RPMI-1640 (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin (Invitrogen). Cell proliferation was determined using an MTS assay (CellTiter® 96 AQueous One Solution Reagent, Promega) as per manufacturer's protocol. Determination of cell viability was done similarly using an MTS assay or a Vi-CELL XR Cell Viability Analyzer (Beckman Coulter).

2.2.2 MTS assay for Measuring Proliferation and Viability of H9, TK-1, NK Cells with Streptolysin

An MTS-based CellTiter® 96 AQueous assay was performed in order to measure the proliferation of H9, TK-1, NK cells over 24 h after treatment of Streptolysin in concentrations of 0, 0.1, 1, 10 U/ml (Kraakman, 1995). Cell Titer® 96 AQueous One Solution Reagent (Promega) was added at 15 μL/well. The plate was incubated for 1h at 37°C and 5% CO2 and absorbance was measured using a BioTek ELX808 microplate reader at 490 nm.

2.2.3 Indirect Enzyme-Linked Immunosorbent Assay (ELISA) to Measure IL-6, IFN-γ, IL-17

100μL of the secretome of the treated T cells with Streptolysin in a 96-well ELISA plate were incubated overnight at 4°C. The plate was emptied. 300μL 1X BSA Diluent/Blocking Solution (KPL) was used to block wells. A rabbit anti-human primary antibody (1:300) against IL-6, IFN-γ, IL-17 (Abcam) was added to wells (1h at RT). After a wash with 1X wash solution (KPL), an anti-rabbit secondary antibody (Abcam) (1:300) was added to wells (1h at RT). After a wash, 100μL substrate solution (KPL) was added to wells. Absorbance at 405nm was measured (BioTek ELx808).

2.3 HFF-1 Treated with H9, TK-1, NK T cells

2.3.1 MTS assay for Measuring Proliferation and Viability of H9, TK-1, NK Cells with Streptolysin

An MTS-based CellTiter® 96 AQueous assay was performed in order to measure the proliferation of HFF-1 cells over 24 h after treatment of secretome of H9, TK-1, NK in concentrations of the streptolysin in 0, 0.1, 1, 10 U/ml. Cell Titer® 96 AQueous One Solution Reagent (Promega) was added at 15 μL/well. The plate was incubated for 1h at 37°C and 5% CO2 and absorbance was measured using a BioTek ELX808 microplate reader at 490 nm.

2.3.2 Preparation of Lysates of HFF-1

HFF-1 was placed in T25 flasks at 0.1×10^6 cells/mL in 5mL of media. After treatment for 72 hours with the secretome of the T cells (0, 0.1, 1, 10 U/mL), cells were trypsinized and centrifuged (7min at 1200rpm). The cell pellets were resuspended in 2mL PBS (Invitrogen) and centrifuged (7min at 1200rpm). Cells were resuspended in 1X lysis buffer #9803 (Cell Signaling Technology) supplemented with protease cocktail inhibitor #P8340 (Sigma) at 1×10^6 cells per 1mL solution. Samples were placed on ice for 10min and then centrifuged (15min at 13,000rpm at 4°C). Lysates were collected and stored at -80°C.

2.3.3 Indirect Enzyme-Linked Immunosorbent Assay (ELISA) for EDA-Fibronectin

100μL lysate samples in a 96-well ELISA plate was incubated overnight at 4°C. The plate was emptied. 300μL 1X BSA Diluent/Blocking Solution (KPL) was used to block wells. A rabbit anti-human primary antibody (1:300) against EDA-Fibronectin (Abcam) was added to wells (1h at RT). After a wash with 1X wash solution (KPL), an anti-rabbit secondary antibody (Abcam) (1:300) was added to wells (1h at RT). After a wash, 100μL substrate solution (KPL) was added to wells. Absorbance at 405nm was measured (BioTek ELx808).

2.3.4 MTS assay for Measuring Proliferation and Viability of HFF-1

An MTS-based CellTiter® 96 AQueous assay was performed in order to measure the proliferation of HFF-1 cells over 24 h after treatment of the H9, TK-1, H9 and TK-1 and NK with 0.1 U/mL and 0 U/ml. Cell Titer® 96 AQueous One Solution Reagent (Promega) was added at 15 μL/well. The plate was incubated for 1h at 37°C and 5% CO2 and absorbance was measured using a BioTek ELX808 microplate reader at 490 nm.

2.4 HLA Types

2.4.1 HLA Types in the Different Countries

Data was extracted from the different papers describing the HLA types in the different patients diagnosed with rheumatic fever. Python Spyder was used to graph the number of patients with the HLA types that have and do not have rheumatic fever as described by the papers in Turkey, Brazil, and India.

2.4.2 Comparing HLA Types

From the data extracted for the people in Turkey, Brazil, and India, the HLA types described were analyzed to different MHC Class II molecules. The graph was made through Python Spyder to determine the frequency of different HLA types in the cases of rheumatic fever.

2.5 Statistical Analysis

Assays were performed three or more times with n=5. A Student's t-test (unpaired, two-tailed) was conducted to determine statistical significance with an α-value of 0.05. Bars means are ± standard deviation. All figures and diagrams presented were created by the author. Figures 1a, 6b, 10, and 11 were made using Python Spyder, an open-source environment using python language to conduct scientific programming. **Fig.** 1b was made using Chimera, an interactive visualization platform of molecular structures. **Fig.** 2 was made using lines and circles in Microsoft PowerPoint. Figs. 3- 9c were made using Microsoft Excel. Pictures 9b-d were taken at 100x magnification.

3 Results and Discussion

3.1 The Effect of TGF-β1 on HFF-1

HFF-1 treated with TGF-β1 was used to demonstrate fibroblast differentiation into myofibroblasts. Varying concentration of TGF-β1 was used to treat the HFF-1. The variations in concentrations over three periods of TGF-β1 were assessed in order to determine which concentration allowed for the highest rate of proliferation, as shown in **Fig.** 3. HFF-1 proliferated in all concentrations of TGF-β1 (0, 1, 10, 100ng/ml), demonstrating a faster proliferation rate at an increased dosage of TGF-β1 and over a larger period. However, HFF-1 cells were shown to have the most significant proliferation at 72 hours rather than 48 hours or 96 hours. Also, HFF-1 cells were shown to proliferate the greatest with a treatment of 10 ng/ml of TGF-β1 ($p<0.05$). Therefore, 10 ng/ml of TGF-β1 at 72 hours was used to model the differentiation of HFF-1 into myofibroblasts.

3.2 Differentiation of HFF-1 into myofibroblasts

After the treatment of TGF-β1, it was necessary to show the differentiation of fibroblasts into myofibroblasts. It has been shown that myofibroblasts have an increase in the production of αSMA

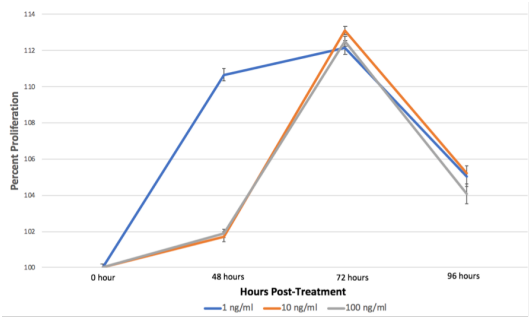
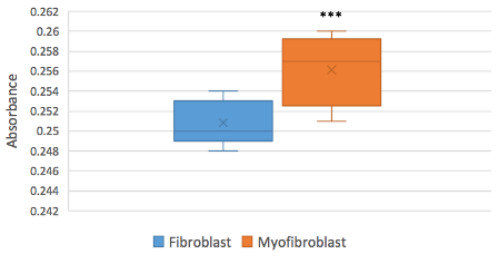
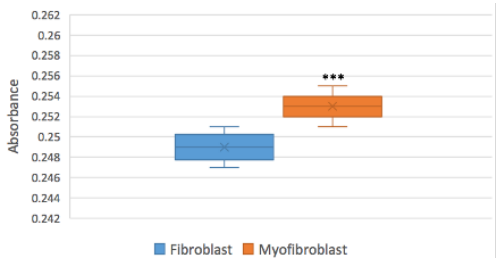


Figure 3: HFF-1 cells were plated at 0.1 million cells/mL in varying concentrations of TGF-B1. An MTS assay was conducted at 0, 48, 72, and 96 hours post-treatment to measure proliferation at all concentrations. Lines are means ± STDEV (n=5). Figure created by author.



(a) αSMA Expression



(b) EDA-Fibronectin Expression

Figure 4: HFF-1 Cells were lysed, and (a) αSMA expression and (b) EDA-Fibronectin was measured via ELISA. The data shows that differentiated myofibroblasts have an increased expression level of αSMA. and EDA-Fibronectin. Box and whisker plot made from the absorbance values (n=5). ***p<0.001. Figures created by author.

(alpha smooth muscle actin) and EDA-Fibronectin (Extra Domain-A Fibronectin) (Cucoranu, 2005). Therefore, as suggested by **Fig.** 3, HFF-1 were treated with 10 ng/ml of TGF-β1 at 72 hours, the concentration and time point at which the greatest viability occurred. The treated fibroblasts over 72 hours were compared to untreated fibroblasts as undifferentiated control. Treatment of TGF-β1 showed an elevated expression in αSMA and EDA-Fibronectin suggesting differentiation toward myofibroblast (Hsia, 2016), as demonstrated in Figures 5 and 6 (p<0.001).

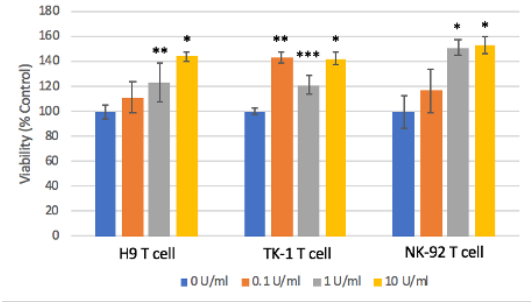


Figure 5: H9, TK-1, and NK-92 cells were plated at 1 million cells/mL in varying concentrations of streptolysin. An MTS assay was conducted at 24 hours post treatment to measure proliferation. Cells proliferated across concentrations. Bars are means ± STDEV (n=5). *p<0.05 **p<0.01 ***p<0.001. Figure created by author.

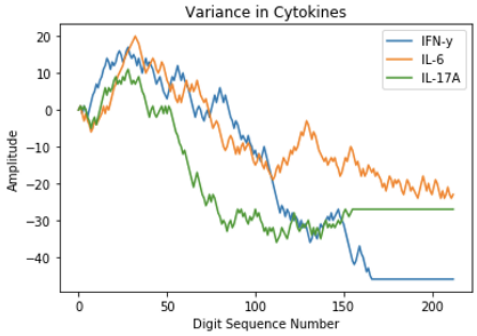


Figure 6: Sequences of the different cytokines measured were taken by UniProt. Based on the digit sequence, the amplitude was given either a value of +2 if they were strong hydrophobic, +1 if they were weak hydrophobic, -1 if they were weak hydrophilic and -2 if they were strong hydrophilic. Note that since IL-6 is a larger compound than IFN-γ and IL-17A, the amplitude flatlines after their sequence terminates. Figure created by author.

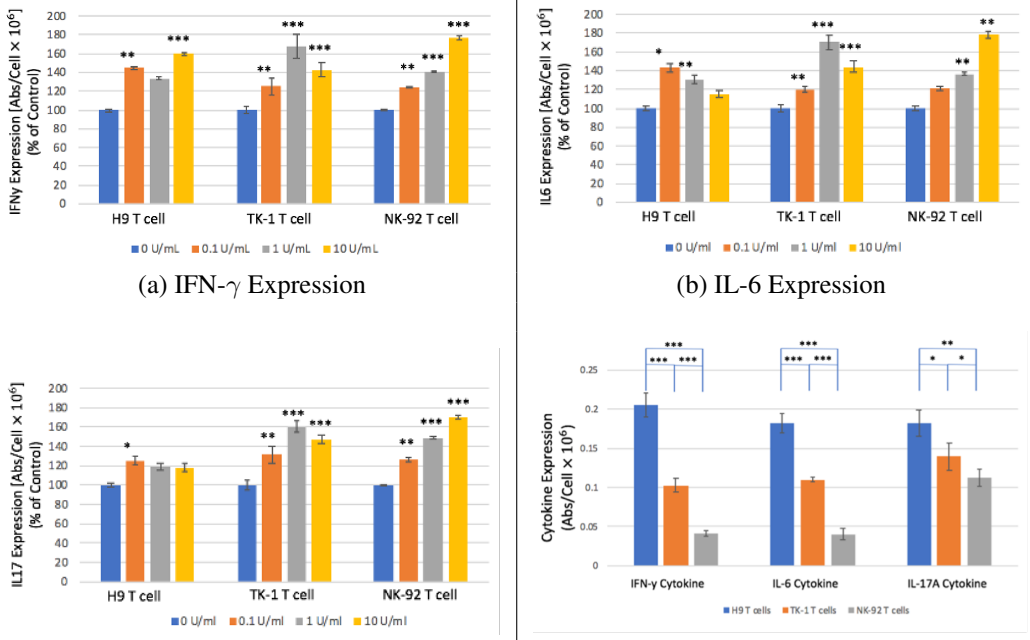
3.3 Proliferation of T cells after treatment of Streptolysin

The subsets of T cells were stimulated with streptolysin to either activate or facilitate activation. The H9 (CD4+ T cells) were activated as the CD4+ T cells used were HIV permissive, suggesting activation (Pan, 2013). TK-1 (CD8+ T cells) were activated by the exogenous administration of streptolysin activating p38MAPK suggesting activation. NK-92 (NK T cells) were activated by 0.1 µL/ml of IL-2 for 48 hours. After activation, the proliferation of the different subsets of T cells at different concentrations of streptolysin (0, 0.1, 1, 10 U/ml) was measured, as proliferation is a marker of activation (Wieland, 2016). H9, TK-1, and NK-92 cells proliferated across all concentrations of streptolysin (0, 0.1, 1, 10 U/ml) in a dose-response fashion, as shown in **Fig.** 5. TK-1 (CD8+ T cells) were unique in that they respond with increased proliferation even at low concentration (0.1 U/ml) of streptolysin ($p<0.01$) while other cells did not.

3.4 Production of Cytokines after T cells are treated with Streptolysin

After the different subsets of T cells (H9, TK-1, and NK-92) were treated with varying concentrations of streptolysin as shown in **Fig.** 5, the secretome secreted by the individual T cell subsets was evaluated for cytokine production. The cytokines measured included IFN-γ, IL-6, and IL-17A. Production of these cytokines demonstrates the T cell subsets response to the streptolysin. As demonstrated in **Fig.** 6, different cytokines are composed of different sequences that determine the polarity of the molecule. The polarity of the cytokine determines the cytokines' ability to invade the membrane of the target cell (15). Cytokines produced by the T cells would be an immune response that can target the cells, including myofibroblasts. The greater hydrophilic properties of a cytokine allow for transport across the cell membrane affecting functional responses to immune deposition. **Fig.** 6 compares the polarity of the cytokines measured. IFN-γ can inhibit viral replication. IFN-γ is predominately produced by the natural killer cell as a part of innate immune response.

In addition, IFN-γ is released by CD4+ Th1 and CD8 + cytotoxic T lymphocytes during antigen-specific immunity (16). IFN-γ production increased when the different T cells (H9, TK-1 and NK-92) were exposed to streptolysin, as shown in **Fig.** 7a. IFN-γ expression increased significantly even at 0.1 U/ml concentration of streptolysin compared to the control ($p<0.01$). IL-6 has been shown to be produced by T cells to stimulate inflammatory and auto-immune processes. IL-6 was particularly increased when CD4+ T cells differentiate into Th2 cells. IL-6 production was also increased when the different T cells when exposed to streptolysin, as shown in **Fig.** 7b. The pro-inflammatory response was noticed significantly in TK-1 cells for 0.1 U/ml ($p<0.01$), 1 U/ml ($p<0.001$) and 10 U/ml ($p<0.001$). IL-17A is a cytokine produced by the T helper cell. IL-17A's role is the involvement in inducing and mediating the proinflammatory response. IL-17A, like the other cytokines, increased in production when the different T cell subsets were exposed to streptolysin, as shown by **Fig.** 7c, especially at 0.1 U/ml in H9 ($p<0.05$), TK-1 ($p<0.01$), and NK-92 ($p<0.01$). The increase of these cytokines suggest that the T cell immune response has the potential to affect the myofibroblasts. This



(a) IFN-γ Expression

(b) IL-6 Expression

(c) IL-17 Expression

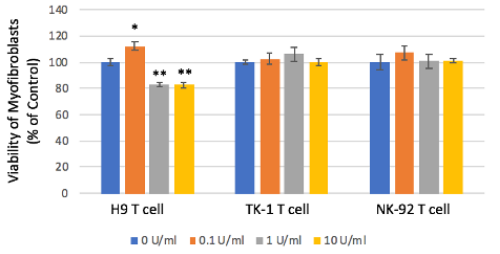
(d) Cytokine Expression across the T cells

Figure 7: Cell culture supernatants were used to measure (a) IFN-γ, (b) IL-6 and (c) IL-17 secretion via ELISA . The data shows that subsets of T cells have increased expression levels of IFN-γ, IL-6 and IL-17. This expression is representative that IFN-γ illicits an immune response. IL6 is a pro-inflammatory cytokine that facilitates the immune response. IL-17 is a pro-inflammatory cytokine that facilitates the immune response.(d) The cytokine expression for IFN-y, IL-6, and IL-17A collected in a, b, and c were compared at 0.1 U/ml of streptolysin. Bars are means $\pm$ STDEV (n=5). *p<0.05 **p<0.01 ***p<0.001. Figures created by author.

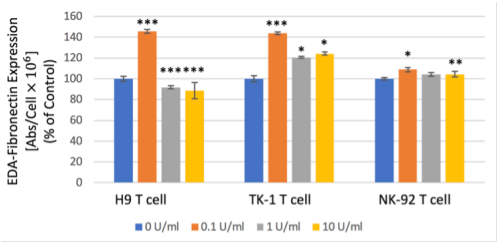
is shown in Figure 13 with the viability of the myofibroblasts when treated with the secretome of the T cells as shown in Figure 13. Comparing the expression of the different cytokines at 0.1 U/ml of streptolysin would suggest how cytokine expression across the different T cells varied when treated with that concentration of streptolysin. As shown in **Fig.** 7d, after stimulated with streptolysin at the same concentration, H9 T cells produced the greatest amount of all of the cytokines: IFN-γ ($p<0.001$), IL-6 ($p<0.001$), IL-17A ($p<0.01$). NK-92, when stimulated by the streptolysin, followed TK-1 in production of the cytokines. This indicates that different T cell populations can respond to streptolysin and release inflammatory cytokines; H9 T cells are more responsive compared to TK-1 and NK-92.

3.5 Proliferation of myofibroblasts after treatment of secretome produced by T cells

HFF-1 were treated with 10 ng/ml for 72 hours as determined necessary to differentiate toward a myofibroblast. The T cell subsets were treated with varying concentrations of streptolysin (0, 0.1, 1, 10 U/ml). The secretomes secreted by the T cells for the varying concentrations of streptolysin were used. The myofibroblasts were treated with the secretome of the different T cell subsets. Results, depicted in **Fig.** 8a, show that the secretome of H9 cells decreased the viability of myofibroblasts ($p<0.01$) except at 0.1U/ml ($p<0.05$) where it caused significant proliferation. This is an unexplained finding and warrants further investigation. The secretome of TK-1 and NK-92 T cells showed a slight increase in the viability of the myofibroblasts but not statistically significant. This can be explained by the relative abundance of cytokine production per cell type; H9 T cells expressed much higher levels of IFN-γ, IL-6 and IL-17A per 1 million cells compared to TK-1 and NK-92 T cells. However, the viability of the myofibroblasts after treatment of the secretome doesn't necessarily indicate fibrosis of the connective tissue. Fibrosis of tissue occurs when there is an increase in the production of EDA-Fibronectin which is shown in **Fig.** 8b.



(a) The effect of the secretome of streptolysin treated T cell subsets on the proliferation of myofibroblasts



(b) EDA-Fibronectin expression by myofibroblasts

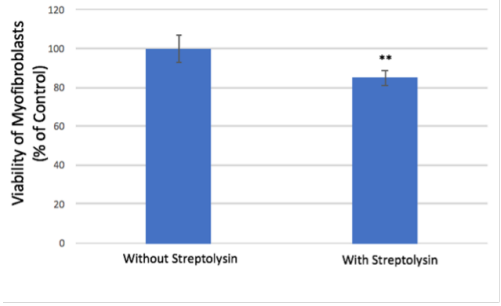
Figure 8: HFF-1 cells were plated at 1 million cells/ml and treated with TGF-B1. The secretomes of the T cells treated with different concentrations of streptolysin was administered. (a) An MTS assay was conducted at 24 hours post treatment to measure myofibroblast proliferation. (b) Cell were lysed and EDA-Fibronectin was measured via ELISA. This expression is representative of the proteins that cause fibrosis. Bars are means $\pm$ STDEV (n=5). *p<0.05 **p<0.01 ***p<0.001. Figure created by author.

3.6 Fibrosis of the myofibroblasts

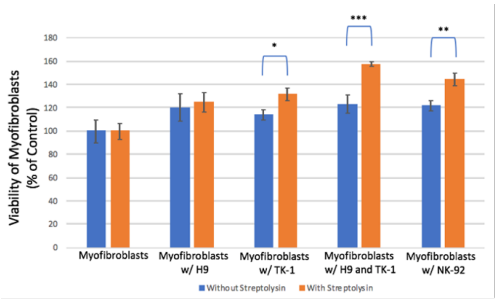
Myofibroblasts were treated with the secretome of the streptolysin treated T cell subsets. Upon treatment, there was an increase in protein production of EDA-Fibronectin when the myofibroblasts increased in viability. Fibrosis, as mentioned previously, is the formation of excessive connective tissue. In the heart, this causes scarring and granulomas which can cause damage to the mitral valve. EDA-Fibronectin plays a role in cell adhesion especially in the extracellular matrix. After tissue injury, fibroblasts begin repair in producing EDA-Fibronectin to remodel the area. In **Fig.** 8b, the production of EDA-Fibronectin is demonstrated. An increase in EDA-Fibronectin was seen, in particular at 0.1 U/ml of streptolysin for the T cells: H9 ($p<0.001$), TK-1 ($p<0.001$), NK-92 ($p<0.05$). The reason for the less significant increase in EDA-Fibronectin for NK-92 cells can be explained by NK-92 cell mediated cell cytotoxicity resulted in decreased viability of the myofibroblasts.

3.7 Treatment of myofibroblasts with T cell subset co-culture and their secretome

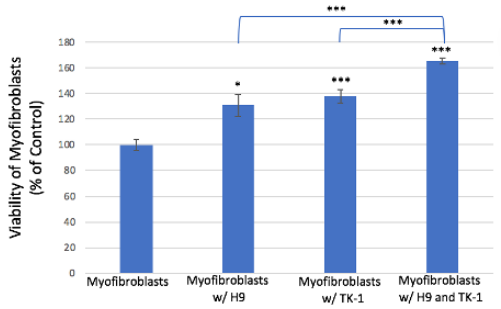
Myofibroblasts thus far have been treated with the secretome of the T cells, which contain the cytokines produced. However, many T cells cause damage to tissue directly via cell-cell contact. Therefore, the myofibroblasts were cocultured with the T cells. **Fig.** 9a shows the treatment of myofibroblasts with and without streptolysin. Myofibroblasts that were treated with streptolysin demonstrated a decrease in viability ($p<0.01$). H9, TK-1 and NK-92 T cells were treated with streptolysin at 0.1 U/ml and cultured with the myofibroblasts. In **Fig.** 9b, the viability of the myofibroblasts after co-culture were represented. The viability of the myofibroblasts increased when treated with the supernatant of the cells exposed to streptolysin as shown by **Fig.** 8a, however **Fig.** 9b shows that the viability of the myofibroblasts increased when treated with the T cells and streptolysin as opposed to the myofibroblasts cultured with the T cells alone. This maintained true even when the coculture of the H9 and TK-1 when exposed to streptolysin were cultured with the myofibroblasts ($p<0.001$). **Fig.** 9c showed a statistical significance when exposed to the T cells: H9 ($p<0.05$) and TK-1 ($p<0.001$) treated with streptolysin as opposed to streptolysin alone. There is also a statistically significant increase in viability in the co-culture of H9 and TK-1 compared to the culture alone ($p<0.001$). This combined treatment suggests an increase in EDA-Fibronectin. This is further supported by the myofibroblasts treated with H9 and TK-1 (**Fig.** 9f) display more convoluted myofibroblasts in the background than the myofibroblasts treated with H9 (**Fig.** 9d) and TK-1 (**Fig.** 9e) exclusively. The greater quantity of myofibroblasts indicate an increase in the viability of the connective tissue matrix as supported by production of EDA-Fibronectin.



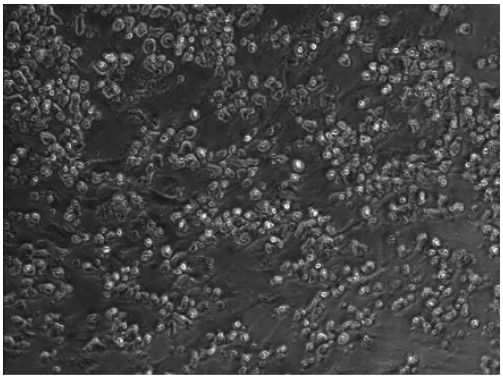
(a) Viability of myofibroblasts treated with streptolysin.



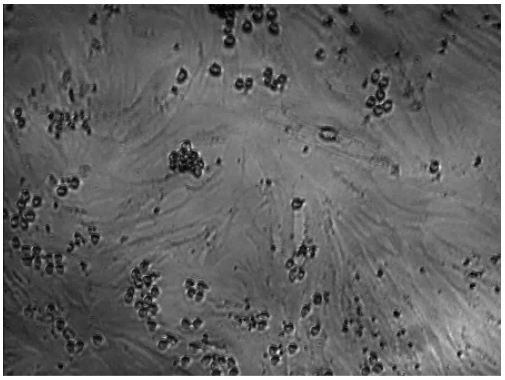
(b) The effect of streptolysin on the proliferation of the myofibroblasts



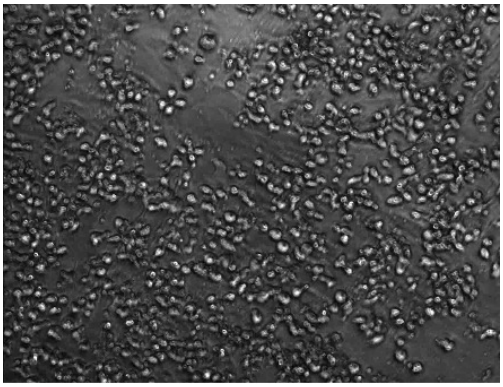
(c) The Effect of T cells on the proliferation of myofibroblasts.



(d)Photomicrograph of myofibroblasts and H9 T cells.

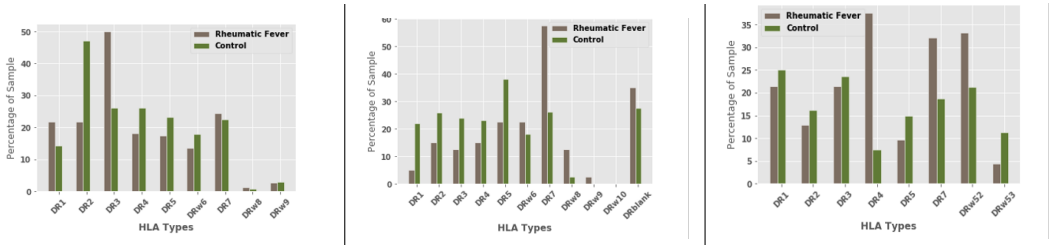


(e)Photomicrograph of myofibroblasts and TK-1 T cells.



(f) Photomicrograph of myofibroblasts and H9 and TK-1 T cells.

Figure 9: (a) Myofibroblasts treated with 0.1 U/ml of streptolysin and without streptolysin. An MTS assay was conducted at 24 hours post treatment to measure proliferation. (b,c) Myofibroblasts plated at 1 million cells/ml. The T cells cocultured with 0.1 U/ml of streptolysin. An MTS assay was conducted at 24 hours post treatment of the cells to measure proliferation. Bars are means ± STDEV (n=5). *p<0.05 **p<0.01 ***p<0.001. Figures created by author. (d) Myofibroblasts treated with H9 T cells and secretome. (e) Myofibroblasts treated with TK-1 T cells and secretome. Note the background long stranded are the myofibroblasts while the rounder cells are the T cells. Images taken at 100x magnitude.



(a) Percentage of sample with specific HLA Types in India

(b) Percentage of sample with specific HLA Types in Brazil.

(c) Percentage of sample with specific HLA Types in Turkey.

Figure 10: Data extracted were plotted for the percentage of samples in Control and Rheumatic Fever patients from (a) India (13), (b) Brazil (12), (c) Turkey (14). Figure created by author.

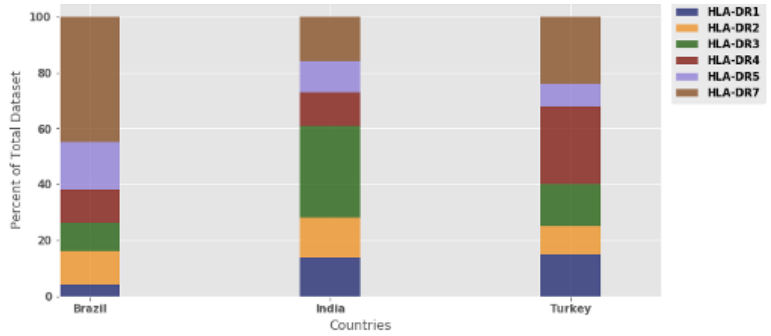


Figure 11: Data extracted were plotted for the percentage of rheumatic fever samples from the different countries combined from **Fig.** 10 for specific HLA types. Figure created by author.

3.8 HLA Molecules of Different Countries

Previously, the focus in RHD has been on the role of the T cell surface protein and its role in causing fibrosis to the valve. However, the genetics of the HLA molecules of the individual's cells have been proposed to play as much of a role in the cardiac injury after a GAS infection. Previously there has been shown that for class I antigens, HLA-A and HLA-C group antigens between patients with RHD and controls have no significant difference affect. Instead it has been shown that HLA-B13 antigen has been shown to be higher compared to controls without reaching statistical significance. Instead the more prominent significant change compared to the control were the findings for class II antigens. **Fig.** 10 shows the class II HLA molecules of the individuals who have been diagnosed with rheumatic fever compared to control. Data from patients from three countries were compared for their different HLA molecules. HLA molecule presentations in India (**Fig.** 10a), Brazil (**Fig.** 10b) and Turkey (**Fig.** 10c) that were induced by rheumatic fever were compared to the control population. **Fig.** 10 shows that in Turkey, there is a maximum of about 36 percent of the samples to be diagnosed with rheumatic fever to have a specific HLA type. Furthermore, **Fig.** 10 also shows that in Brazil about 55 percent of a sample has a specific HLA type.

3.9 Comparing HLA molecules from different countries

The HLA molecules that have been modeled in **Fig.** 10 from the different scientific databases were combined into one graph (Figure 19). Figure 19 again presents the percentages of patients in different countries that are diagnosed with rheumatic fever. The data suggest that in Brazil, the most common HLA molecule in RHD patients is HLA-DR7. In India, the data suggest that the most common HLA molecule type is HLA-DR3. The data for the patients in Turkey suggest that HLA-DR4 is the most common HLA molecule type. However, in each country, all of the 6 HLA types measured were present. The variability in the HLA type indicates that

Table 1: This is a summary of the findings for the assays performed describing the results when myofibroblasts were treated with streptolysin or the secretome of the T cells. The "+" indicates the level of magnitude of the results. More "+" means a greater increase.

Configuration of Cells and Their Treatment

Myofibroblasts	+	+	+	+
Streptolysin	+			
Secretome of T cells exposed to streptolysin	-	CD4+	CD8+	NK
Experiment	**Results**			
Cell Proliferation				
Viability of Myofibroblasts	↓	↑	↑	↑
Viability of T cells		↑ (+)	↑ (++)	↑ (+++)
Cytokines by T cells				
IFN-γ		↑ (+++)	↑ (++)	↑ (+)
IL-6		↑ (+++)	↑ (++)	↑ (+)
IL-17A		↑ (+++)	↑ (++)	↑ (+)
Fibrosis Marker				
EDA-Fibronectin	↓	↑ (++)	↑ (++)	↑ (+)

Table 2: This is a summary of the findings for the assays performed when the cells were co-cultured. The "+" indicates the level of magnitude of the results. More "+" means a greater increase.

Configuration of Cells and Their Treatment

Myofibroblasts	+	+	+	+
Co-Culture of T cells incubated with streptolysin	CD4+	CD8+	CD4+ and CD8+	NK
Experiment	**Results**			
Cell Proliferation				
Myofibroblasts viability	↑ (+)	↑ (+)	↑ (+++)	↑ (++)
Fibrosis Marker				
EDA-Fibronectin	↑	↑	↑	↑

4 Conclusion and Future Work

This study suggests how variations in the glycoprotein on the T cell and the HLA molecules of individuals may exacerbate the preferential damage to the mitral valve after rheumatic fever. Through a series of investigations of myofibroblasts exposed to T cells treated with streptolysin, the research conducted in this study demonstrates the role of T cells and inflammatory cytokines in the injury process of rheumatic heart disease. These results are summarized in **Table** 1 and **Table** 2.

In the bloodstream, there are various different types of T cells present. CD4+, CD8+ and NK T cells are commonly stimulated after infection. In the research reported here, each of these T cell subtypes proliferated when exposed to different concentrations of streptolysin indicating activation and mitogenic response of the T cell. Once activated, T cells produce different cytokines after exposure to the exotoxin. Each of the T cells demonstrated increase expression of IFN-γ, IL-6 and IL-17A in response to streptolysin exposure. In CD4+ T cells, the increase in production of IFN-γ can differentiate CD4+ T cells into Th1 T cells, IL-6 production can differentiate CD4+ T cells into Th2 T cells, and IL-17A production can differentiate CD4+ T cells into Th17 T cells (17). In addition, this investigation demonstrated IFN-γ is more hydrophilic than the other cytokines. The hydrophilic properties of IFN-γ may help facilitate the transport of the IFN-γ through the cell membrane. IFN-γ is also cytokine involved in the chronic inflammatory response while IL-6 and IL-17A are both involved in the acute and chronic inflammatory response. The greater production of inflammatory cytokines in CD4+ compared to CD8+ and NK T cells suggest that CD4+ T cells may play an important role in rheumatic heart disease compared to other cells. Myofibroblasts produced an increase in EDA-Fibronectin when exposed to the secretome of CD4+ T cells suggesting fibrosis. The CD8+ and NK cells had little to no effect on the viability of the myofibroblasts. This little

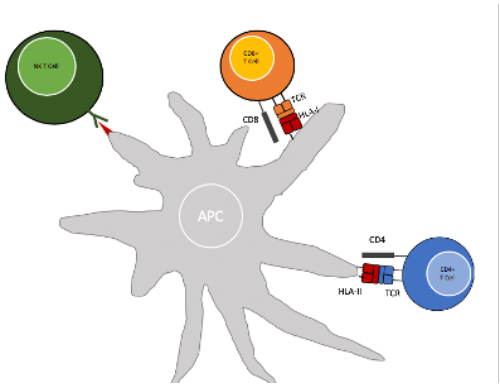


Figure 12: A picture was created showing the binding of the CD4+, CD8+, NK T cells to the antigen presenting cell through the HLA complexes (18). The blue T cell is the CD4+ T cell, the orange T cell is the CD8+ T cell and the green T cell is the NK T cell. The red proteins on the gray antigen presenting cell are bound to those blue, orange and green T cells. The study here demonstrates the binding of interest that causes damage in RHD. The binding described is of the TCR of the CD4+ T cell and HLA Class II molecules. Figure created by author.

increase in viability translated to a little change in the production of EDA-Fibronectin which marks fibrosis. In summary, CD4+ T cells caused more fibrosis than CD8+ and NK T cells.

However, in vivo there are multiple different T cells that are present. Each of these different T cells work together to cause injury and promote fibrosis of the valve. When the CD4+ and CD8+ T cells were cultured together there was an increase in viability of the myofibroblasts, suggesting an increase in fibrosis compared to the control and compared to each cell individually. This happens because the activated CD4+ T cells activate the CD8+ T cell. The activated T cells cause greater damage than each T cells alone. This co-culture, in vivo and in vitro, of all of the different T is what causes injury in rheumatic heart disease.

The antigen presenting cells, express MHC complex, encoded by the HLA gene and these vary from individual to individual. HLA Class I molecules that bind to the CD8+ T cells have not been shown to be associated with propensity for rheumatic heart disease. However, HLA Class II molecules that bind to CD4+ T cells, seem to play more of a role in the propensity for rheumatic heart disease. However, one specific HLA molecule has not been linked with rheumatic heart disease across different populations. For example, in India, the most common HLA type in patients with rheumatic fever is HLA-DR3. In Turkey and Brazil, the most common HLA type is HLA-DR4 and HLA-DR7 respectively. This data suggests that perhaps different social and environmental factors stimulate different HLA types in rheumatic fever patients. These tendencies that are shown in rheumatic heart disease patients demonstrate the role of HLA in the damage of the mitral valve after rheumatic fever.

More research is necessary to understand fibrosis of the mitral valve in rheumatic heart disease. Future studies on the interaction of different cytokines expressed by the T cells would help demonstrate the role other cytokines beyond IFN-γ, IL-6 and IL-17A have on fibrosis of the mitral valve in rheumatic heart disease. T lymphocytes produce at least 16 different cytokines. Some of these cytokines are involved only in chronic inflammatory responses and fibrosis. Cytokines that are involved in acute inflammatory responses might be increased less extensively compared to the chronic inflammatory response similar to the response of IL-6 and IL-17A (19). Vimentin has been shown to play a role in renal fibrosis (20). In addition, other molecules such as PAI-1 have also been shown to play a role in fibrosis, specifically PAI-1 suppresses the degradation of extracellular matrix which in other words prevents apoptosis of myofibroblasts (21). PAI-1 may play an important role in mitral valve fibrosis by inhibiting ECM clearance similar in renal fibrosis. The hypothesis is that in rheumatic heart disease, PAI-1 would be increased and PAI-1 levels would be associated with the fibrosis of the mitral valve. The ratio of T cell subsets at the site of the mitral valve also require consideration in future in vitro research. Overall, these in vitro investigations reported here suggest the important role of T cells, specifically CD4+ T cell, in the mechanism of injury in rheumatic heart disease and could be used to develop additional therapeutic options in the future.

References

[1] Fauchier, T. *et al.* Acute rheumatic fever: A population-based study in wallis, a south pacific island. *International Journal of Cardiology* **181**, 30–31 (2015).

[2] Talwar, K. K. & Gupta, A. Predictors of mortality in chronic rheumatic heart disease. *Indian J Med Res.* **144**, 311–313 (2016).

[3] Bright, P. D. *et al.* An immunological perspective on rheumatic heart disease pathogenesis: More questions than answers. *Heart* **102**, 1527–1532 (2016).

[4] Cunningham, M. W. Molecular mimicry between streptococcal m protein and cardiac myosin and the immunopathogenesis of rheumatic fever. In *Molecular Mimicry, Microbes, and Autoimmunity*, 39–56 (2014).

[5] Lehmann, C., Zeis, M. & Uharek, L. Activation of natural killer cells with interleukin 2 (il-2) and il-12 increases perforin binding and subsequent lysis of tumor cells. *British Journal of Haematology* **114**, 660–665 (2001).

[6] Stassen, M. *et al.* The streptococcal exotoxin streptolysin o activates mast cells to produce tumor necrosis factor alpha by p38 mitogen-activated protein kinase- and protein kinase c-dependent pathways. *Infection and Immunity* **71**, 6171–6177 (2003).

[7] Wu, C.-C., Hsu, S.-C., Shih, H.-M. & Lai, M.-Z. Nuclear factor of activated t cells c is a target of p38 mitogen-activated protein kinase in t cells. *Molecular and Cellular Biology* **23**, 6442–6454 (2003).

[8] Baum, J. & Duffy, H. S. Fibroblasts and myofibroblasts: What are we talking about? *Journal of Cardiovascular Pharmacology* **57**, 376–379 (2011).

[9] Midgley, A. C. *et al.* Transforming growth factor-β1 (tgf-β1)-stimulated fibroblast to myofibroblast differentiation is mediated by hyaluronan (ha)-facilitated epidermal growth factor receptor (egfr) and cd44 co-localization in lipid rafts. *Journal of Biological Chemistry* **288**, 14824–14838 (2013).

[10] Kim, L. *et al.* Overexpression of transforming growth factor-β1 in the valvular fibrosis of chronic rheumatic heart disease. *Journal of Korean Medical Science* **23**, 41 (2008).

[11] Cucoranu, I. *et al.* Nad(p)h oxidase 4 mediates transforming growth factor-β1–induced differentiation of cardiac fibroblasts into myofibroblasts. *Circulation Research* **97**, 900–907 (2005).

[12] Guilherme, L., Weidebach, W., Kiss, M. H., Snitcowsky, R. & Kalil, J. Association of human leukocyte class ii antigens with rheumatic fever or rheumatic heart disease in a brazilian population. *Circulation* **83**, 1995–1998 (1991).

[13] Jhinghan, B. *et al.* Hla, blood groups and secretor status in patients with established rheumatic fever and rheumatic heart disease. *Tissue Antgens* **27**, 172–178 (1986).

[14] Khosroshahi, H., Kahramanyol, O. & Doganci, L. Hla and rheumatic fever in turkish children. *Pediatr Cardiol* **13**, 204–207 (1992).

[15] Yang, N. J. & Hinner, M. J. Getting across the cell membrane: An overview for small molecules, peptides, and proteins. In *Site-Specific Protein Labeling Methods in Molecular Biology*, 29–53 (2014).

[16] Bhat, P., Leggatt, G., Waterhouse, N. & Frazer, I. H. Interferon-γ derived from cytotoxic lymphocytes directly enhances their motility and cytotoxicity. *Cell Death Disease* **8** (2017).

[17] Bhatnagar, Grover & Ganguly. Superantigen-induced t cell responses in acute rheumatic fever and chronic rheumatic heart disease patients. *Clinical and Experimental Immunology* **116**, 100–106 (1999).

[18] Domagala-Kulawik, J. The role of the immune system in non-small cell lung carcinoma and potential for therapeutic intervention. *Translational Lung Cancer Research* **4** (2015).

[19] Feghali, C. A. & Wright, T. M. Cytokines in acute and chronic inflammation. *Frontiers in Bioscience* **2**, d12–26 (1997).

[20] Nakatsuji, S., Yamate, J. & Sakuma, S. Relationship between vimentin expressing renal tubules and interstitial fibrosis in chronic progressive nephropathy in aged rats. *Virchows Archiv* **433**, 359–367 (1998).

[21] Huang, W.-T., Vayalil, P. K., Miyata, T., Hagood, J. & Liu, R.-M. Therapeutic value of small molecule inhibitor to plasminogen activator inhibitor–1 for lung fibrosis. *American Journal of Respiratory Cell and Molecular Biology* **46**, 87–95 (2012).