UNIVERSIDAD SAN FRANCISCO DE QUITO USFQ

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Full Expression of Cardiomyopathy is Partly Dependent on B cells: A Pathway that Involves Cytokine Activation, Immunoglobulin Deposition and Activation of Apoptosis.

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RESUMEN

Introducción: Existe información limitada en relación al rol de los mecanismos dependientes de linfocitos B en la progresión de la insuficiencia cardiaca. Sin embargo existe evidencia de que hay deposición de complemento y anticuerpos en el miocardio de pacientes con falla cardiaca. Por lo tanto el propósito de nuestro estudio es determinar la contribución de los linfocitos B en la progresión de insuficiencia cardiaca usando un modelo no quirúrgico en ratones de cardiomiopatía no-isquémica (CMP).

Métodos: El modelo de CMP consiste en dar L-NAME y NACL en el agua e insertar bombas de infusión de Angiotensina II por 35 días. En el día 35 se analizo a los ratones por medio de resonancia magnética, expresión de genes e histología. Se asignaron 4 grupos a los ratones de 12 semanas los cuales fueron: WT CMP, SCID CMP (Deficientes en linfocitos B y T), CD22-CMP (depletados de linfocitos B), y Nude CMP (Deficientes de linfocitos T) con sus controles respectivos. Se realizaron protocolos de depleción y reconstitución de linfocitos B. El efecto protector mediado por la depleción de linfocitos B, se demostró por una reducción significativa de la hipertrofia celular y deposición de colágeno así como una fracción de eyección preservada en el grupo de CD22 CMP versus WT CMP. Al reconstituir los linfocitos B de los ratones SCID CMP se restauro el fenotipo de la cardiomiopatía. Además se demostró que hay deposición de IgG3 y apoptosis en el miocardio de ratones con cardiomiopatía y que linfocitos B activados estimulan producción de colágeno por fibroblastos cardiacos.

Conclusión: :La ausencia de linfocitos B en nuestro modelo de insuficiencia cardiaca resulto en hipertrofia y deposición de colágeno disminuida, conservación de la función cardiaca y en asociación con estos cambios una disminución en la expresión de citoquinas pro-inflamatorias, deposición de IgG y apoptosis en el miocardio. Por lo tanto nuestros resultados demuestran que los linfocitos B juegan un papel importante en el desarrollo y progresión de la cardiomiopatía en un modelo de insuficiencia cardiaca inducido por Angiotensina II.

Palabras clave: sistema inmune, cardiomiopatía, linfocitos, remodelamiento, anticuerpos.

ABSTRACT

Background: Limited information exists on the role of B-cell dependent mechanisms in the progression of heart failure. However, in failing human myocardium there is evidence of deposition of activated complement components as well as anti-cardiac antibodies. We aimed to determine the contribution of B-cells in heart failure progression using a non-surgical mouse model of non-ischemic cardiomyopathy (CMP).

Methods and Results: CMP protocol involved the use of L-NAME and NaCl in the drinking water and Angiotensin-II infusion for 35 days. At day 35 mice were analyzed by cMRI, gene expression and histology. Mice (12 weeks old) were divided into 4 groups, all in C57BL/6 background: WT CMP, SCID CMP (T and B-cell deficient), $CD22^-$ CMP (B-cell depleted) and Nude CMP (T-cell deficient), with their respective controls. We performed B-cell depletion and reconstitution protocols. The protective effect of B-cell depletion was demonstrated by a significant reduction of cell hypertrophy and collagen deposition and a preserved ejection fraction in the $CD22^-$ CMP group compared to WT CMP. Once SCID mice underwent B-cell reconstitution with isolated CMP B-cells, the CMP phenotype was restored. Furthermore, deposition of IgG3 and apoptosis in the myocardium follows the development of CMP; in addition, in vitro studies demonstrated that activated B-cells stimulate collagen production by cardiac fibroblasts.

Conclusions: The absence of B-cells in this model of heart failure resulted in less hypertrophy and collagen deposition, preservation of LV function and in association with these changes a reduction in expression of pro-inflammatory cytokines, IgG deposition and apoptosis in the myocardium. Taken together, these data suggest that B-cells play a contributory role in an Angiotensin-II induced heart failure model.

KEY WORDS: IMMUNE SYSTEM, CARDIOMYOPATHY, LYMPHOCYTES, REMODELING, ANTIBODIES

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(n=15 per group). All p-values compare controls vs all other groups: * p-value <0.05, ** p-value <0.01, *** p-value <0.001.

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Title: Full expression of cardiomyopathy is partly dependent on B cells: A pathway that involves cytokine activation, immunoglobulin deposition and activation of apoptosis.

Short title: B-cells in heart failure

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ABSTRACT

Background: Limited information exists on the role of B-cell dependent mechanisms in the progression of heart failure. However, in failing human myocardium there is evidence of deposition of activated complement components as well as anti-cardiac antibodies. We aimed to determine the contribution of B-cells in heart failure progression using a non-surgical mouse model of non-ischemic cardiomyopathy (CMP).

Methods and Results: CMP protocol involved the use of L-NAME and NaCl in the drinking water and Angiotensin-II infusion for 35 days. At day 35 mice were analyzed by cMRI, gene expression and histology. Mice (12 weeks old) were divided into 4 groups, all in C57BL/6 background: WT CMP, SCID CMP (T and B-cell deficient), $CD22^-$ CMP (B-cell depleted) and Nude CMP (T-cell deficient), with their respective controls. We performed B-cell depletion and reconstitution protocols. The protective effect of B-cell depletion was demonstrated by a significant reduction of cell hypertrophy and collagen deposition and a preserved ejection fraction in the $CD22^-$ CMP group compared to WT CMP. Once SCID mice underwent B-cell reconstitution with isolated CMP B-cells, the CMP phenotype was restored. Furthermore, deposition of IgG3 and apoptosis in the myocardium follows the development of CMP; in addition, in vitro studies demonstrated that activated B-cells stimulate collagen production by cardiac fibroblasts.

Conclusions: The absence of B-cells in this model of heart failure resulted in less hypertrophy and collagen deposition, preservation of LV function and in association with these changes a reduction in expression of pro-inflammatory cytokines, IgG deposition and apoptosis in the

myocardium. Taken together, these data suggest that B-cells play a contributory role in an Angiotensin-II induced heart failure model.

Key Words: Immune system, cardiomyopathy, lymphocytes, remodeling, antibodies

INTRODUCTION-

A number of inflammatory pathways are activated in the course of myocardial injury and in the setting of heart failure[1-4]. Of interest are recent experimental and clinical observations that suggest activation of humoral immune responses following myocardial injury occurs. Indeed, if B-cell produced cytokines or antibodies are directed against the myocardium, as it results from myocardial injury; this pathway of immune activation may contribute to disease progression, and thus represent a new target for therapy.

The evidence in support of B-cell activation following myocardial injury is as follows: First, in patients with acute heart failure, there is activation of B-cells. Markers of B-cell activation increase in hospitalized patients with HFrEF and return to normal following treatment [5]. Second, a variety of anti-cardiac antibodies have been found in patients following myocardial infarction and in patients with various forms of heart failure [6-9]; in these settings, the initial myocardial injury was not immunologically mediated. Suggesting that following myocardial ischemia or necrosis new antigens are exposed that trigger an immune response and antibody production. Third, a large proportion of patients with end-stage cardiomyopathy, regardless of the etiology, have anti-cardiac antibodies deposited in the myocardium. In a study of 100 samples of failing myocardium it was found that 70% were positive for IgG3 with equal proportion among ischemic and non-ischemic patients. Interestingly, at least one of the antibodies was directed against a mitochondrial protein [10]. Fourth, increased levels of activated complement components are found in the circulation of patients with advanced disease and, more importantly, present in failing myocardium [11, 12]; a finding that gives credence to the potential pathological role of antibody deposition in failing myocardium. And finally, limited

clinical observations suggest that strategies to remove antibodies may have an impact on the course of heart failure [5, 13]. For example, studies using immune-adsorption in patients with non-ischemic cardiomyopathy that have circulating anti-beta receptor antibodies appear to improve clinically as well as recover cardiac function[14]. Also as a proof of principle, therapeutic plasma exchange in patients with heart failure has been performed and appears to be safe [5].

These observations taken together are consistent with the hypothesis that following myocardial injury, B-cell activation occurs that triggers downstream effects that result in anti-cardiac antibody formation, complement deposition and further myocardial injury. Accordingly, the purpose of these studies was to test, in an animal model of non-ischemic cardiomyopathy (CMP), the role of B-cells in the progression of heart failure.

METHODS

Mouse Model of CMP Induction

Male wild type (WT; strain C57BL/6J; Harlan Laboratories), Nude (strain J:NU, Foxn1^{nu}/Foxn1^{nu}; Jackson Laboratories) and severe combined immunodeficient (SCID; strain B6.CB17-Prkdc^{scid}/SzJ C57BL/6 background; Jackson Laboratories) mice were used in a CMP model modified from Oestreicher et al [15]. Cardiomyopathy groups were given drinking water containing 0.1 mg/ml L-NAME (Sigma) and 1% NaCl (Sigma). After 1 week of acclimatization to the water, mice were anesthetised with inhaled isoflurane and implanted with subcutaneous osmotic mini-pumps (Alzet model 1004) that delivered Angiotensin-II (Ang-II; Sigma) at a rate of 0.7 mg/kg/day for an additional 4 weeks. All mice were sacrificed at the end of the fifth week.. All experiments

used 3 month old mice, which demonstrated decreased function but a higher survival rate compared to older mice (5-8 month old). All experiments were performed under approval of the Houston Methodist Hospital Research Institute Institutional Animal Care and Use Committee (IACUC).

B-cell depletion

Male WT mice were treated with two doses of a mouse CD22 antibody (BioXcell) 3 days before initiation of the CMP protocol and again 21 days into the protocol. The antibody was injected intraperitoneally on both occassions with a final concentration of 0.2mg/33 ul and diluted in PBS for a total volume of 200 ul. A non-specific IgG1 antibody (BioXcell) was used as a control. Initial B-cell depletion of 90% was confirmed by flow cytometric analysis of mouse spleens, while the other cellular compartments (i.e. T-cells) remained unchanged.

B-cell reconstitution

Mice underwent CMP protocol as described and at day 35 they were sacrificed and the spleens removed. A single-cell suspension from the spleens was prepared using 100µl ACK lysis buffer followed by 300 µl phosphate buffered saline (PBS) and then filtered through a 70 micron cell strainer. B-cell isolation was performed from this cell suspension using a B-cell isolation kit (Miltenyi Biotec), following the manufacturers instructions. Briefly, the cell suspension was centrifuged at 300x g for 10 minutes, supernatant removed and pellet resuspended in buffer. Biotin-antibody cocktail was added at 10 µl per 10^7 total cells, and the solution incubated for 15 minutes at 2-8°C. After incubation, 30 µl buffer and 20 µl anti-biotin Microbeads per 10^7 total

cells were added. The incubation process was repeated, and cells were washed and magnetically separated to obtain the unstimulated, purified, B-cells. These purified B-cells were then diluted in PBS and injected intraperitoneally (IP) in SCID mice. Three days after IP injection the heart failure protocol was initiatied in WT mice, SCID mice, and SCID mice with reconstituted B-cells (SCID+B-cells). B-cell reconstitution was confirmed by flow cytometric analysis of mouse spleens.

Histological analysis

Mouse hearts were removed and sectioned mid-heart, with apex portions used for PCR studies and base portions fixed in 2% paraformaldehyde, processed, paraffin embedded and cut into 5 micron sections. To measure fibrosis, sections were stained using a Trichrome kit (Sigma-Aldrich), according to manufacturer's instructions. The slides were then cover slipped and analyzed at 20x magnification using an Olympus AX70 microscope. Pictures were taken of all regions of the left ventricle and analyzed for fibrosis using Image Pro Plus v4.0 analysis software (Media Cybernetics, Silver Spring, MD). Color cube-based selection criteria were used to denote positive staining (within the color spectrum of blue dye) and stained/unstained areas were measured. The results expressed are the average percent tissue area (pixels) stained by the dye. Analysis was performed by an observer blinded to the sample identities. Myocyte size was measured as previously described by measuring myocyte diameter at the level of the nucleus in H&E stained sections [16].

Immunohistochemistry and immunofluorescence

Briefly, we performed antigen retrieval in rehydrated sections with 1% sodium citrate, after which sections were blocked for 30 minutes using 1% horse serum in PBS, followed by washing in PBS alone for 15 minutes. Samples were then incubated at a 1:100 dilution for 30 minutes against antibody subclasses: IgG3-FITC (Abcam), IgG1-FITC (eBioscience), IgG2a-FITC (eBioscience), IgG2b-FITC (eBioscience) and IgM-FITC (eBioscience). Then samples were washed three times with PBS for 10 minutes. Finally, each section was incubated for 5 minutes in 3% Sudan Black to eliminate endogenous fluorescence and cover slipped in aqueous mounting media. For dual fluorescence, staining was performed using IgG3-FITC (Abcam) and BAX-TRITC (Santa Cruz Biotechnology). Photomicrographs were taken using a Diagnostic Instruments SPOT II digital camera mounted on an Olympus AX70 fluorescent microscope by an observer blinded to the source of each specimen. Pre-set exposure settings were unchanged for all photomicrographs. Two blinded observers analyzed the photomicrographs, which were later decoded for analysis. The samples were considered positive or negative based on the presence of fluorescence in the sarcolemma. Apoptosis was assessed by immunohistochemistry staining using an anti-ssDNA/Apostain monoclonal antibody assay (eBiosciences), according to the manufacturer's instructions.

Flow cytometry analysis

Blood was obtained by cardiac puncture at the end of each experiment and centrifuged on a ficoll gradient, and the buffy coat layer was washed twice in PBS. Spleens were removed and prepared as described above (B-cell reconstitution protocol) for cell labeling. Cells were then

stained and incubated for 30 minutes with antibodies to CD22-PE, CD45-FITC, CD3-PE (Santa Cruz Biotechnology), CD69-FITC (Biolegend). CD19-FITC, CD86-PE, CD80-FITC (eBioscience) Samples were analyzed by a BD LSRII flow cytometer using FACSDiva software (BD Biosciences).

Mean Arterial Pressure Measurement

Blood pressure measurements were taken on conscious animals 3 times each week throughout the protocol using a tail cuff CODA non-invasive blood monitoring system (Kent Scientific). This system used 10 cuff inflations and internal software to calculate and record average mean arterial pressures. Initial experiments used telemetry monitoring of mean arterial pressure to validate tail cuff measurements, but telemetry was not used routinely due to the invasiveness and inflammatory effects of the implantation.

Magnetic Resonance Imaging

MRI was performed using a 9.4T (89 mm bore) superconducting magnet with a Bruker AVANCE console that has live animal imaging capabilities. For mouse imaging, the system is equipped for isoflurane anesthesia administration and cardiac gating, which is performed as a service by the imaging core at our facility. The identities of all animals were blinded before imaging and uncoded following analysis. MRI analysis was performed at the completion of the 35-day CMP protocol, just prior to sacrifice.

Analysis of Gene Expression

Total mRNA was isolated from cardiac tissues using RNA-binding columns (RNaqueous-4-PCR, Ambion), according to manufacturer's protocols. RNA was immediately reverse transcribed into

cDNA using a kit employing random primers (iScript, Bio-Rad), according to the manufacturer's directions. Real-time reaction mixtures contained 11 µl H_2O, 12 µl IQ SYBR Green Supermix (Bio-Rad), 1 µl primers, and 1 µl cDNA template (25 µL total). Sense and antisense primers were chosen from the RTPrimerDB public primer database for mouse. Reactions were aliquoted into 96-well plates, plates were sealed, centrifuged at 500× g for 60 seconds, and then samples were amplified for 40 cycles of 10 seconds at 95ºC, 30 seconds at 55ºC, and 10 seconds at 72ºC. Real-time PCR was performed in a MyIQ5 iCycler (Bio-Rad). All samples were measured in triplicates and normalized to GAPDH controls run on the same plate for each mouse cDNA sample that was tested. Gene expression levels were calculated by using $(2^{Ct\ (gene)}/2^{Ct\ (GAPDH)})*1000$ to standardize to GAPDH and express as relative transcript numbers. Positive gene expression was additionally visualized by conventional PCR and ethidium bromide stained agarose gel electrophoresis using appropriate primer sets.

Plasma immunoassays

At day 35, mice were sacrificed and blood collected in tubes containing EDTA. Blood was then centrifuged at 13000 rpm for 10 minutes, and plasma collected and frozen at -80°C. Cytokine profile was then measured using a ProcartaPlex multiplex immunoassay (eBioscience), according to manufacturer's instructions, and analyzed with a Luminex 200 instrument.

***In Vitro* experiments**

Control and heart failure mice were produced using the protocol outlined above. At day 35 mice from both groups were sacrificed and B-cells obtained from spleens as described in the B-

cell reconstitution protocol section. Control B-cells were stimulated with LPS and CpG ODN (ODN M362) for 12 hours while heart failure derived B-cells sat in media without stimulation. After 12 hours all tubes were centrifuged and the supernatants collected. These supernatants were co-cultured with previously isolated and cultured cardiac fibroblasts, which had reached confluence and were maintained in Media (RPMI 1641 with penicillin/streptomycin) supplemented with 10% fetal calf serum. All three supernatants were added to separate fibroblast cultures along with their respective controls. The supernatant stimulated fibroblast reaction was stopped at 6 hours using lysis buffer provided with the RNAqueous RNA isolation kit (Ambion) according to manufacturers instructions. cDNA was obtained and RT-PCR was run for genes associated with proliferation, collagen production and nuclear activation.

Statistical Analysis

All experiments had 5 animals per group and each experiment was repeated three times making a total of 15 animals per group once all experiments were completed. Data reported as mean ± standard deviation. Analysis was done using GraphPad Prism Software. ANOVA with Tukey comparison as a post-test with a 95% confidence interval were used when analyzing more than 3 groups. A p-value <0.05 was considered significant.

RESULTS

Cytokine and B cell activation in a mouse model of non-ischemic cardiomyopathy

As shown in **figure 1A** the expression of a variety of inflammatory cytokines in failing myocardium and controls; there is increased myocardial expression of pro inflammatory

cytokines such as IL-1β, INF-γ, TNF-α and IL-6, as well as a reduction in the anti-inflammatory cytokine IL-10. **Figure 1B** depicts the cytokine profile in peripheral blood samples from heart failure and normal mice. As shown, the only cytokine significantly up regulated is B-cell activating factor (BAFF). **Figure 1C**, shows the expression of a variety of B-cell activation markers in splenic B-cell isolates obtained from heart failure mice and controls. There is a marked increase in the expression CD69, and CD22, while the total B-cell compartment is unchanged in CMP mice. **Figure 1D** illustrates myocardial tissue from heart failure mice and controls that was stained for the presence of immunoglobulins. As shown, antibody deposition in myocardial tissue is primarily of the IgG3 subclass while the other subclasses IgG1, IgG2a, IgG2b are not present.

B-cells are not required for hypertensive response but are necessary to produce cardiomyopathy phenotype

Figure 2 shows the hemodynamic response of control untreated mice and cardiomyopathy (CMP) treated mice in the following groups: wild type (WT CMP), mice that lack B- and T-cells (SCID CMP), mice treated with an antibody to eliminate CD22+ cells ($CD22^-$ CMP) or mice that lack T-cells (Nude CMP). As shown, in **Figure 2A**, the hypertensive response was equal among all the treated groups (controls mean 75 mmHg vs. 160 mmHg for all CMP treated groups at day 21, $p<0.001$). **Figure 2B** shows the percent change in heart weight per tibia length among the CMP treated groups. As shown, greater increases in heart weight were observed in the WT CMP and Nude CMP groups (32.2% ± 2.7 and 40.9% ± 15, respectively) compared to the SCID CMP and $CD22^-$ CMP groups (14.6% ± 8 and 15.5% ± 6.6, respectively). **Figure 2C** shows left ventricular ejection fraction (LVEF), as measured by cMRI, at the end of the cardiomyopathy

protocol. As shown, there is a significant reduction in LVEF in WT CMP and Nude CMP mice compared to controls (49.4% ± 7 and 53% ± 10, respectively, vs. 67% ± 3 in untreated control). The SCID CMP and $CD22^-$ CMP groups did not have reduction in LVEF compared to controls (63% ± 4 and 59% ±7, respectively, vs. 67% ± 3 in untreated controls). **Figure 2D** shows the changes in LV mass, which follow the same pattern.

Figure 3A shows the change in myocyte size in untreated controls and in WT CMP, SCID CMP, $CD22^-$ CMP, and Nude CMP groups. Myocyte size for controls was 26.0 ± 3.7 μm. The greatest increase in myocyte size was observed in the WT CMP group (32.9 ± 1.1 μm) and the Nude CMP group (32.3 ± 2.1 μm). For mice in the $CD22^-$ CMP and in SCID CMP groups, the increase in myocyte size was significantly lower than that observed in the WT CMP group (28.0 ± 1.4 μm and 27.8 ± 0.5 μm for $CD22^-$ CMP and SCID CMP, respectively) and not significantly different than that of controls. **Figure 3B** shows the change in collagen content in untreated controls and in WT CMP, SCID CMP, $CD22^-$ CMP and Nude CMP groups. As shown, a large increase in collagen content was observed in WT CMP and Nude CMP (21.8% ± 2.5 and 26.0% ± 7.1 area stained, respectively) whereas only a modest increase in collagen content was observed in the SCID CMP and $CD22^-$ CMP groups (12.5 % ± 2.8 and 14.7% ± 2.6 area stained, respectively) compared to untreated controls.

The cardiomyopathy phenotype is restored after B-cell reconstitution

Figure 4 shows the effect of B-cell reconstitution on SCID mice treated to develop cardiomyopathy. The hypertensive response was the same in the reconstitution group (SCID+B-cells CMP) as the other CMP groups (**figure 4A**). The figure shows untreated controls or mice

treated to produce cardiomyopathy in the following backgrounds: WT, SCID and SCID with B-cell reconstitution. We observed that once we reconstituted B-cells in SCID mice and induced CMP, myocyte size in the SCID+B-cells CMP group (28.2 ± 2.6 μm) was increased compared to SCID CMP (23.8 ± 1.8 μm) and similar to WT CMP (28.8 ± 2.1 μm), while the controls showed minimal change (23.7 ± 0.9 μm) **(Figure 4C)**. This effect was also observed with the percent change in the heart weight to tibia length ratio (SCID+B-cells CMP: 47% ± 6.1, SCID CMP: 8% ± 3.2, WT CMP: 50% ± 4, and controls: 5% ± 0.1; **Figure 4B**) and collagen content (SCID+B-cells CMP: 29.4% ± 2.7, SCID CMP: 11.2% ± 1.4, WT CMP: 36.2% ± 7.8, and controls: 3.4% ± 1.3 area stained; **Figure 4D)**. Finally, BNP expression was high in WT CMP group, low in B-cell deficient mice ($CD22^-$ or SCID CMP), and elevated in SCID with B-cell reconstitution compared to controls (**Figure 4E**).

Intact B-cell function in cardiomyopathy is associated with increased levels of inflammatory cytokines, IgG deposition and Bax expression in myocardium

Figure 5 shows gene expression profiling of inflammatory markers in cardiac tissue of control and the CMP treated mice measured at the end of the treatment protocol. As shown, the WT CMP group was characterized by increases in IL-1β (**Figure 5A**), TNF-α (**Figure 5B**), IL-6 (**Figure 5C**) and IL-10 **(Figure 5D)** expression, relative to the control group. In the groups with B-cell deficient mice, SCID CMP, $CD22^-$ CMP, there was reduced expression of these genes. The SCID+B-cell CMP group showed increased expression of TNF-α, IL-6 and IL-1β. IL-10 expression was significantly lower in the WT CMP group and elevated to levels comparable to the WT control in the SCID CMP and $CD22^-$ CMP groups (**Figure 5D**).

Figure 6A shows analysis of IgG3 and Bax in myocardium of untreated control mice and CMP groups. IgG3 protein expression was present in most mice in the WT CMP group (7/8). Fewer mice in the B-cell deficient groups were positive for IgG3: 0/8 of SCID CMP and 3/8 $CD22^-$ CMP mice. In contrast, higher numbers of mice were positive for IgG3 in the T-cell deficient Nude CMP (6/8) and SCID+B-cell CMP (5/8) groups. Bax expression was present in most mice in the WT CMP group (7/8). Fewer mice in the B-cell deficient groups were positive for Bax: 0/8 of SCID CMP and 1/8 of $CD22^-$ CMP mice. In contrast, higher numbers of mice in the T-cell deficient Nude CMP (6/8) and SCID+B-cell (5/8) groups were positive for Bax. In **Figure 6B,** the association of anti-ssDNA and IgG3 deposition is depicted. These findings support the hypothesis that cardiomyocyte cell death is occurring via apoptotic mechanisms and not necrosis. In **Figure 6C** 2x2 tables demonstrate the association of IgG3 staining with Bax and anti-ssDNA in the myocardial tissue samples of all groups studied, which emphasizes that almost all had concomitant staining but apoptosis was never present without IgG3, suggesting the important role that IgG3 deposition plays in this CMP model. **Figure 7** shows representative images where BAX-positive areas stain red and IgG3-positive areas stain green, with dual staining in the bottom panel in yellow.

B-cell mediated mechanisms are associated with increased fibroblast proliferation

Figure 8 shows the change in gene expression of a variety of markers in cardiac fibroblasts following stimulation with supernatants of either untreated B-cells or activated B-cells with LPS or CpG ODN or untreated B-cells obtained from heart failure mice (HF B-cells). As shown, B-cell supernatants of activated B-cells and HF B-cells were capable to stimulate cardiac fibroblast to

increase expression of NF-κB (2-fold), PCNA (4-fold), Ki-67 (6-fold), IL-6 (12-fold) and collagen (2-fold). Of interest, collagen expression was highest following fibroblast stimulation with HF B-cell supernatant (4- fold increase) compared to control.

DISCUSSION

This study demonstrates that in a mouse model based on Angiotensin-II and L-NAME there is a consistent CMP phenotype characterized by hypertension and left ventricular dysfunction with an associated activation of the inflammatory cascade, as shown by myocardial expression of inflammatory cytokines, IgG3 antibody deposition, up-regulation of apoptotic signals in myocardial tissue and peripheral B-cell activation. By utilizing mice with different genetic modifications as well as selectively depleting B-cells from the periphery, we demonstrated that for full expression of the CMP phenotype B-cells are required. The role of B-cells in cardiomyopathy development was independent of the hypertensive response and associated with attenuation of the inflammatory response. These observations demonstrate that, in this model of non-ischemic cardiomyopathy, B-cells play a central role in the establishment of cardiac injury, independent of the hypertensive response.

Cardiac injury as induced in this model is characterized by myocyte hypertrophy, extensive collagen deposition, LV dilatation with reduced ejection fraction, and increases in markers of disease (i.e. BNP). In the initial phase there is a compensatory hypertrophy, followed by LV dilatation and a marked decrease in ejection fraction at week 5 of the cardiomyopathy protocol. This model with the use of L-NAME and Angiotensin II demonstrates a severe

phenotype of cardiomyopathy with a non-surgical approach and a human-like non-ischemic cardiomyopathy. Other characteristics of this model, include edema, decreased In addition, the profile of inflammatory cytokines was consistent with observations in humans, where there is elevation of TNF-α, INF-γ, IL-1β and IL-6 and a decrease in IL-10, an anti-inflammatory cytokine, in heart failure. In this animal model, the initial injury was not infectious, inflammatory, or ischemic, and, thus, the observations suggest that following hypertensive injury a series of mechanisms are activated that ultimately lead to cardiomyopathy. But of great interest was the observation that mice depleted of B-cells that developed an intact hypertensive response did not have the same degree of adverse remodeling observed in immune-competent mice. The implications of this observation are important because it will direct research to further characterize and intervene in these inflammatory responses but also suggest that hypertension, as a sole hemodynamic insult, may not be sufficient to fully promote cardiac injury. Alternatively, injury mediated by a hypertension-signaling pathway is magnified by the addition of a second pathway that induces increased expression of BAFF, which in turn leads to B-cell activation and a cascade of B-cell responses.

With regards to the role of B-cells in the development of non-ischemic cardiomyopathy, the findings of the present study are important because we demonstrated that, even when the initial injury is not immunologically mediated, activation of B-cells was necessary to fully express the extent of myocardial injury. This suggests that B-cell-mediated responses, whether they result from antibody production or B-cell-produced cytokines and B-cell interactions, cause cardiac injury or amplify an inflammatory response that is detrimental to cardiac function. In fact, the recognition that, in animals with intact B-cell function and the phenotypic

expression of heart failure, there was deposition of IgG3 in the myocardium and expression of Bax, a pro-apoptotic molecule, suggests that a pathway of cell injury, in addition to the fibrotic response, accompanies the remodeling process.

Limited information exists on the role of B-cells in the development of cardiomyopathy with the exception of murine and human models where the mechanism of cardiac injury is immunologically mediated, such as animal models of virally-mediated injury or cases of acute myocarditis and some forms of dilated cardiomyopathy in humans [17, 18]. But new observations provide credence to the role of B cells in pathological process in the cardiovascular system. Zouggari et al. demonstrated in a murine model of myocardial infarction, that B-cells were activated and responsible for monocyte migration and amplification of myocardial injury[19]. Additionally, in models of atherosclerosis, B-cells contribute to plaque formation via antibody production, cytokine release and cell-mediated interactions[20]. The observations described above as well as the one described in these studies, are consistent with the contribution of B-cells in conditions wherein the initial cardiac injury is not immunologically mediated and represent a major contribution of this investigational area. In addition, we now provide evidence that soluble factors produced from B-cells obtained from heart failure mice increase collagen expression in cardiac fibroblasts.

In this study, we utilized SCID mice, which lack T- and B-cells, as well as Nude mice, which lack T-cells, and treated them to induce cardiomyopathy. Furthermore, we treated WT mice with an antibody to eliminate CD22+ positive cells, a marker expressed on mature B-cells, as a way to eliminate B-cells from the periphery. The consistent findings presented in this study demonstrate that mice without B-cells, whether genetically determined as in the SCID mice or

by antibody depletion of CD22+ cells, did not develop LV dysfunction, myocyte hypertrophy and fibrosis; nor did they have evidence of IgG3 deposition or Bax expression in the myocardium. Additionally, experiments wherein we reconstituted SCID mice with CMP B-cells and subsequently treated to produce cardiomyopathy demonstrated that the failing phenotype was present.

There are several potential contributions of B-cells to cardiac injury in this model. First, dysregulation of B-cell subpopulations (B1 vs B2 and Bregs) may occur, therefore activating pro-inflammatory pathways that promote cell hypertrophy and fibrosis with increased expression of TNF-α, INF-γ, IL-1β, and IL-6 and blunting of anti-inflammatory pathways with reductions in IL-10 [21, 22]. Consistent with this hypothesis are the observations from our studies with B-cell supernatants obtained from activated B-cells and from HF B-cells wherein following incubation with cardiac fibroblasts, there was increased expression of collagen gene. Second, the presence of IgG3 in failing myocardium is consistent with the possibility of cell injury being mediated by non-specific activation of Fc gamma receptor (FcgR), a receptor for the Fc portion of IgG, and triggering of apoptotic pathways[23], as suggested by an increase in Bax expression. Finally, IgG3 may recognize specific receptors of antigenic determinants that decrease cardiac cell function or activate functional receptors that promote cell death[24]. Germane to this discussion are the observations of a study in human subjects with advanced heart failure in which we found that 70% of the myocardial samples studied regardless of the etiology, were positive for anti-cardiac antibodies and at least 50 % also were positive for activated complement components, an association suggesting that antibody formation in advanced heart failure, regardless of the initial mechanisms of cardiac injury, may participate in the progression of the disease state [10].

The consistency of the observations in failing human myocardium and in this murine model of non-ischemic cardiomyopathy strongly suggests that B-cells play a central role in disease progression.

In this murine model of non-surgically-induced non-ischemic CMP, intact B-cells were required for full expression of injury. This implies that B-cell-mediated responses, whether due to antibody formation or B-cell produced cytokines are activated following myocardial injury, which in turn contributes to myocyte hypertrophy, fibrosis and activation of apoptotic pathways that amplify the initial cardiac injury. The precise mechanism by which B-cell activation occurs is unknown and remains the focus of continued investigations. These findings support the idea that modulation of B-cell mediated responses, as for example by the administration of an anti-B-cell antibody as performed in this experiments, may prevent the progression of heart failure and give credence to continued clinical investigations aimed at prevent B-cell activation in heart failure.

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None

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FIGURE LEGENDS-

Figure 1. Immune system changes in a mouse model of non-ischemic cardiomyopathy. (A) Pro-inflammatory cytokines are up regulated in the myocardial tissue milieu, while (B) cytokines in the circulation only show a significant increase in BAFF expression. (C) Surface marker profiles of splenic B-cells of CMP mice compared to controls show an overall increase in activated B-cells (CD19/CD69) but not total B-cell numbers and/or total leucocytes (CD19/CD45) side scatter horizontal was used for B-cell markers (CD19) and side scatter vertical was used for other markers (CD86, CD69, CD45). (D) Immunofluorescence assays determined the deposition of immunoglobulins in myocardial tissue: only IgG3 was present and distributed along the sarcolemma. All p-values compare controls vs all other groups: * p-value <0.05, ** p-value <0.01, *** p-value <0.001.

Figure 2. B-cells are not required for hypertension but for cardiomyopathy. Shown is the hemodynamic response of untreated mice and cardiomyopathy treated mice in the following groups: wild type (WT CMP), mice that lack B- and T-cells (SCID CMP), mice treated with an antibody to eliminate CD22+ cells ($CD22^-$ CMP) or mice that lack T-cells (Nude CMP). (A) Hypertensive response was equal among all the CMP-treated groups, independent of B and T-cell compartments, compared to controls. (B) As shown by the percent change in heart weight per tibia length, greater increases were observed in the WT CMP and Nude CMP groups compared to the SCID CMP and $CD22^-$ CMP groups. (C) LVEF, as measured by cMRI at the end of the cardiomyopathy protocol, is significantly reduced in WT CMP and Nude CMP mice compared to controls. The SCID CMP and $CD22^-$ CMP groups have a significantly higher LVEF than the WT CMP mice. (D) Changes in LV mass followed an inverse pattern to the changes in

LVEF, with LV mass elevated in WT CMP compared to controls (n=15 per group). All p-values compare controls vs all other groups: * p-value <0.05, ** p-value <0.01, *** p-value <0.001.

Figure 3. Lack of B-cells attenuates adverse remodeling in cardiomyopathy. (A) The greatest increase in myocyte size compared to untreated controls was observed in the WT CMP and Nude CMP groups, while in mice of the $CD22^-$ CMP and SCID CMP groups the increase in myocyte size was insignificant, compared to controls. (B) A significant increase in collagen content was observed in WT CMP and Nude CMP mice compared to controls, whereas only a modest increase in collagen content was observed in the SCID CMP and $CD22^-$ CMP groups (n=15 per group). (C) Representative pictures of myocardial tissue sections stained for trichrome in all 5 groups. All p-values compare controls vs all other groups: * p-value <0.05, ** p-value <0.01, *** p-value <0.001.

Figure 4. Reconstitution of B-cells in SCID mice restores the heart failure phenotype. The figure shows untreated controls and mice treated to produce cardiomyopathy in the following backgrounds: WT, SCID and SCID with B-cell reconstitution (SCID+B-cells). Once we reconstituted B-cells in SCID mice and induced CMP, (A) hypertensive response was the similar, (C) myocyte size was increased, and this effect was also observed with the (B) percent change in heart weight to tibia length and (D) collagen content. (E) BNP expression was high in WT CMP group, lower in B-cell deficient mice ($CD22^-$ CMP (not shown) or SCID CMP) and elevated in SCID mice with B-cell reconstitution (n= 15 per group). All p-values compare controls vs all other groups: * p-value <0.05, ** p-value <0.01, *** p-value <0.001.

Figure 5. Inflammatory cytokine profile in B-cell-deficient mice and B-cell-reconstituted mice compared to controls with cardiomyopathy. Quantitative gene expression of inflammatory markers in cardiac tissues at the end of the treatment protocol the WT CMP group was characterized by increase in (A) IL-1β, (b) TNF-α, and (c) IL-6 expression, compared to untreated controls. In the groups with B-cell deficient mice (SCID CMP, $CD22^-$ CMP and Nude CMP), there was reduced expression of these genes. Following B-cell reconstitution in the SCID CMP group, expression of TNF-α, IL-6 and IL-1β increased. (D) IL-10 expression was significantly reduced in the WT CMP group and increased to levels comparable to the untreated controls in the SCID CMP and $CD22^-$ CMP groups. All p-values compare controls vs all other groups: * p-value <0.05, ** p-value <0.01, *** p-value <0.001.

Figure 6. Markers of antibody deposition and activation of apoptosis in myocardial tissue of control and CMP mice. (A) IgG3 expression was present in most mice in the WT CMP group. Fewer mice in the B-cell deficient groups, SCID CMP and $CD22^-$ CMP were positive for IgG3. In contrast, higher numbers of mice in the T-cell depleted but with intact B-cell function groups, Nude CMP or SCID with B-cell reconstitution CMP, were positive for IgG3. Bax expression in myocardium of control mice and various CMP treated groups is also shown, with a pattern of expression similar to IgG3. (C) The 2x2 tables show the distribution of IgG3 and Bax or IgG3 and anti-ssDNA staining in all the pooled analyzed myocardial tissue samples (n=8 per group).

Figure 7. Co-localization of IgG3 with markers of apoptosis- Figure shows representative images where BAX-positive areas stained red (A) and IgG3-positive areas stained green (B), with dual staining in the bottom panel in yellow (C). Scale bar 100 μm.

Figure 8. Markers of proliferation and collagen expression in isolated cardiac fibroblasts after stimulation with activated B-cell products. Figure shows gene expression profile in cultured cardiac fibroblasts where there is increased expression of NF-κB, PCNA, ki-67, IL-6 and collagen I and III with either HF B-cell supernatant or stimulated B-cell (LPS, CpG-ODN) supernatant.

APPENDIX A -FIGURES

Figure 1.

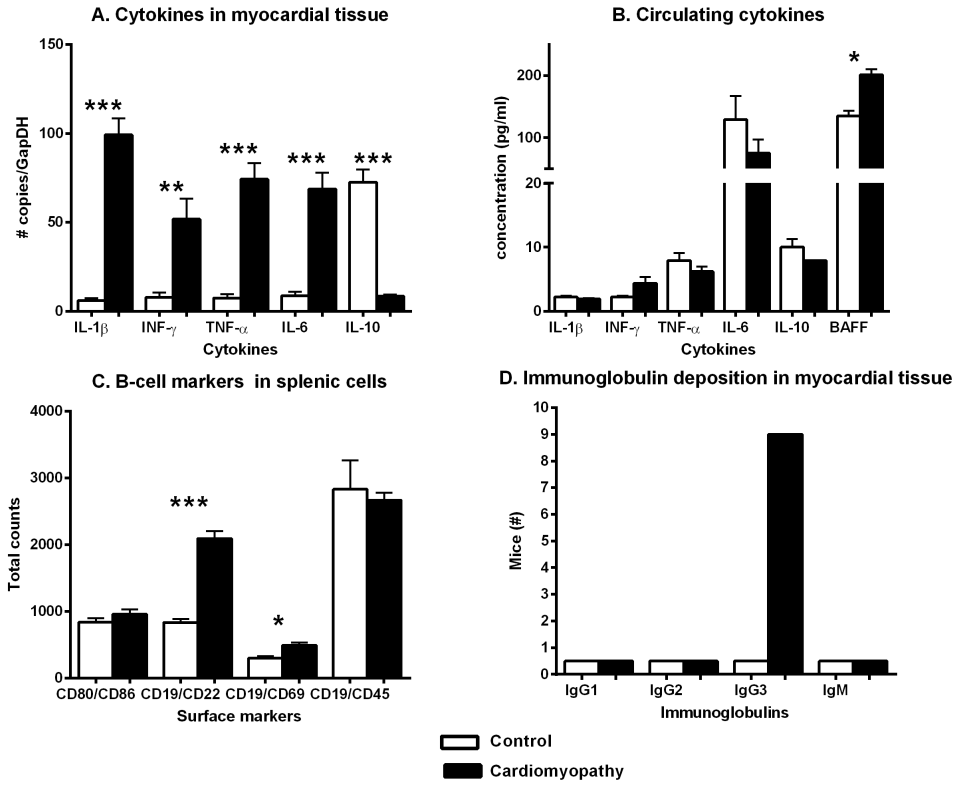
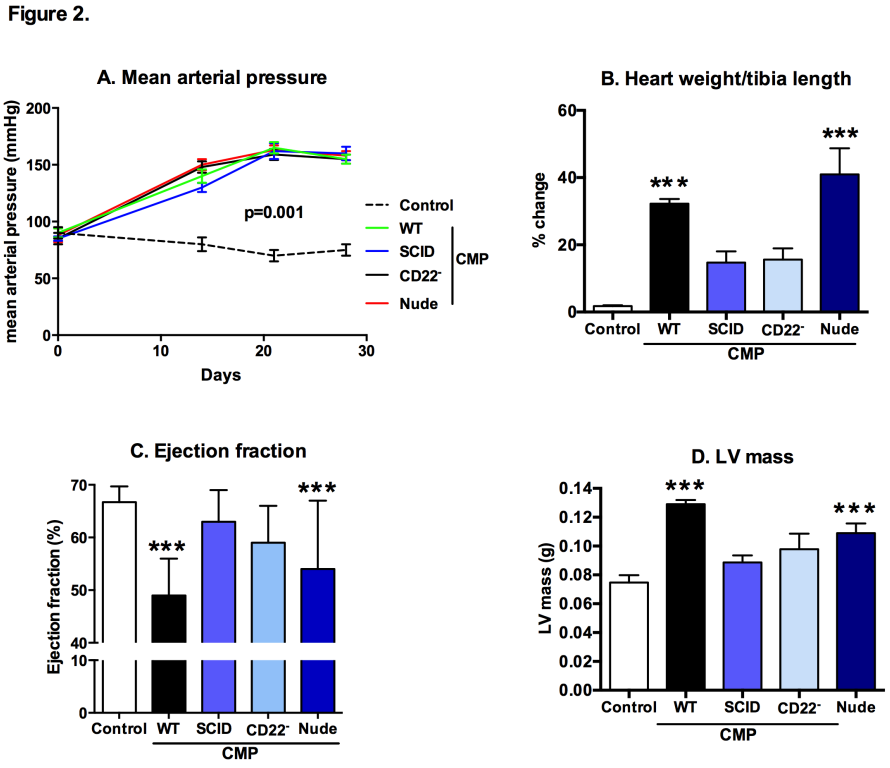


Figure 2



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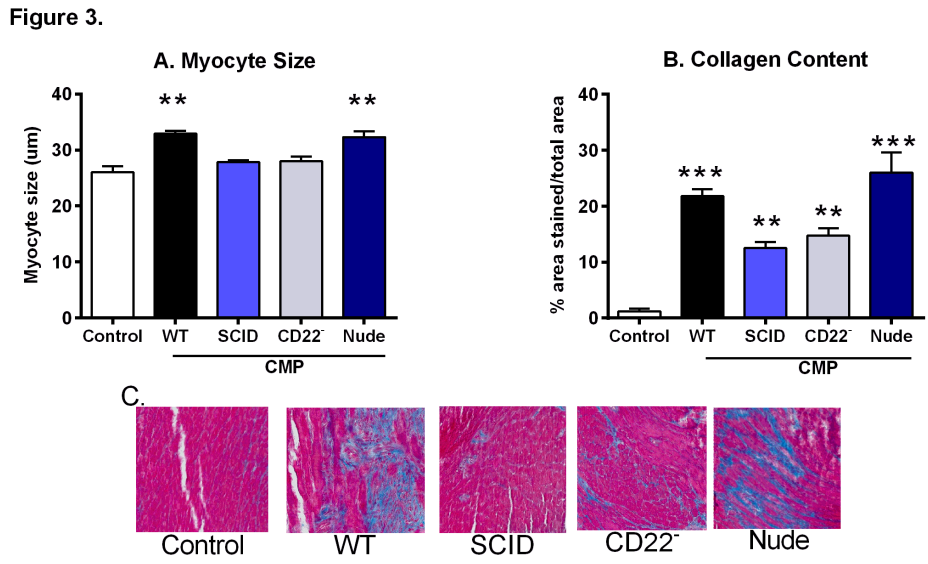

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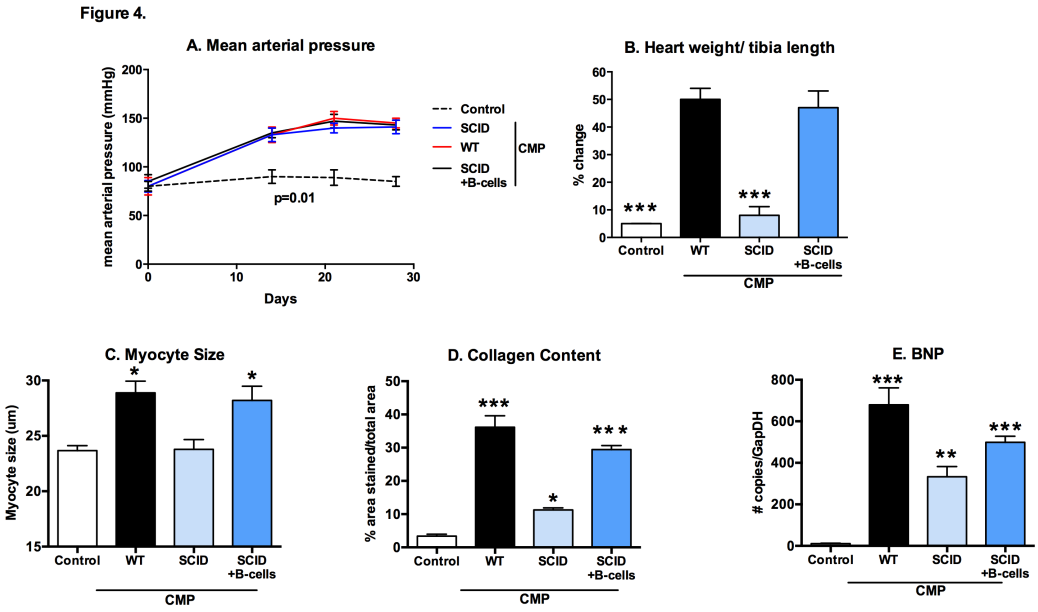

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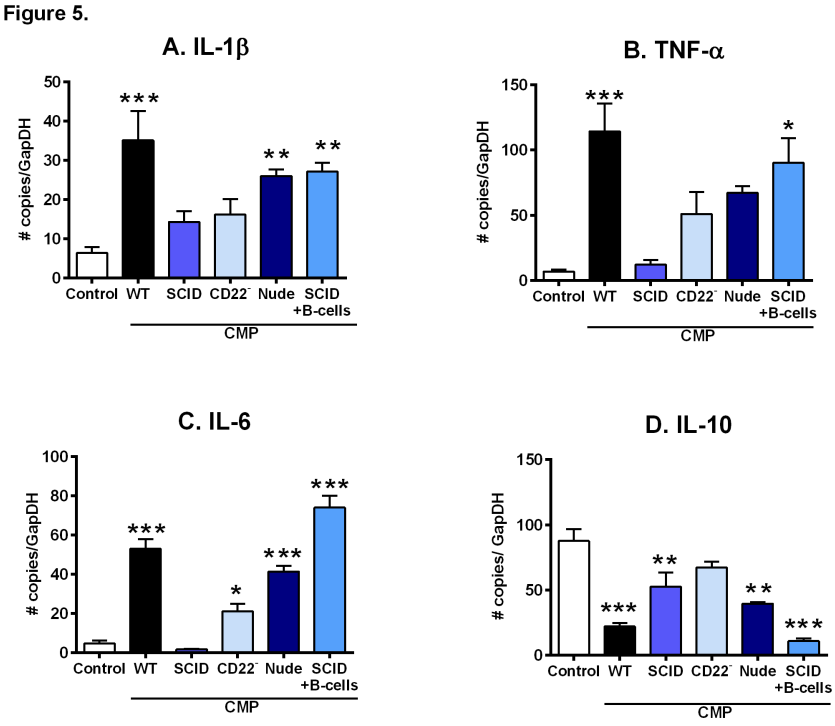
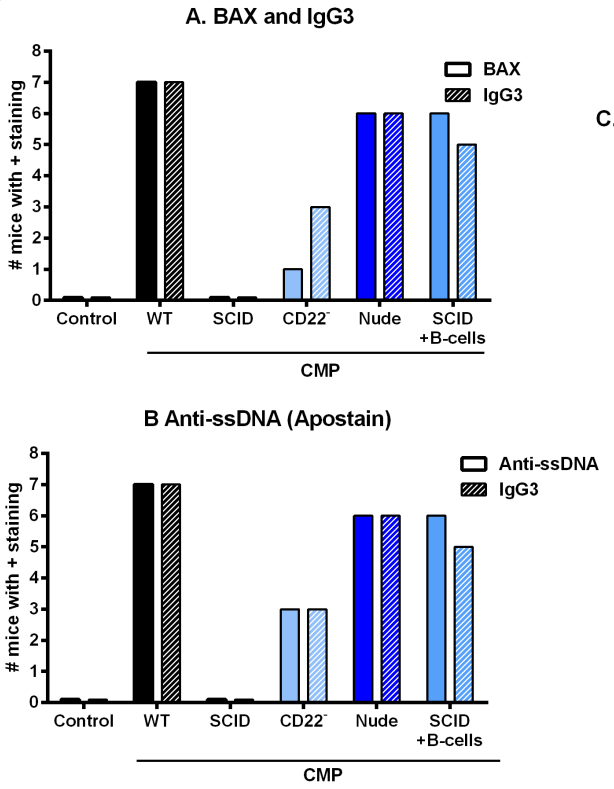


Figure 6-

Figure 6.



C.

	IgG3+	IgG3-
BAX+	19	0
BAX-	2	27

	IgG3+	IgG3-
Apo+	21	0
Apo-	0	27

Figure 7-

Figure 7.

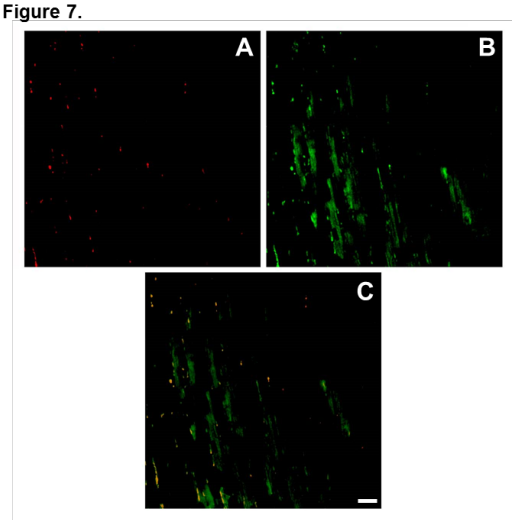
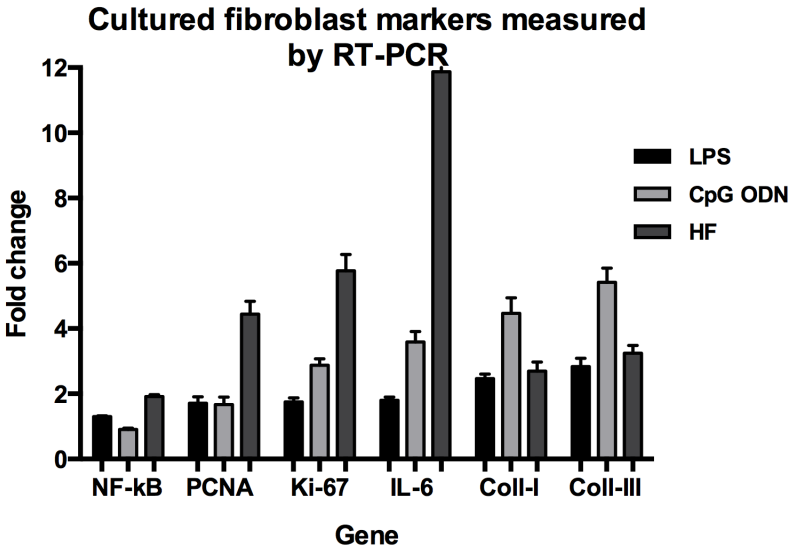


Figure 8-


Cultured fibroblast markers measured by RT-PCR
Fold change
12
10
8
6
4
2
0
NF-kB
PCNA
Ki-67
IL-6
Coll-I
Coll-III
Gene
LPS
CpG ODN
HF