Published in final edited form as:

Autoimmunity. 2016 December ; 49(8): 563–570. doi:10.1080/08916934.2016.1217999.

Repeat exposure to group A streptococcal M protein exacerbates cardiac damage in a rat model of rheumatic heart disease

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Abstract

Rheumatic fever and rheumatic heart disease (RF/RHD) develop following repeated infection with group A streptococci (GAS). We used the Rat Autoimmune Valvulitis (RAV) model of RF/RHD to demonstrate that repetitive booster immunization with GAS-derived recombinant M protein (rM5) resulted in an enhanced anti-cardiac myosin antibody response that may contribute to the breaking of immune tolerance leading to RF/RHD and increased infiltration of heart valves by mononuclear cells. With each boost, more inflammatory cells were observed infiltrating heart tissue which could lead to severe cardiac damage. We also found evidence that both complement and anti-M protein antibodies in serum from rM5-immunized rats have the potential to contribute to inflammation in heart valves by activating cardiac endothelium. More importantly we have demonstrated by electrocardiography for the first time in the RAV model that elongation of P-R interval follows repetitive boost with rM5. Our observations provide experimental evidence for cardiac alterations following repeat exposure to GAS M protein with immunological and electrophysiological features resembling that seen in humans following recurrent GAS infection.

Keywords

rheumatic heart disease; rheumatic fever; group A streptococcal M protein; autoimmunity; *Streptococcus pyogenes*

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Declaration of interest

MWC is chief scientific officer at Moleculera Labs, a diagnostic laboratory at the University of Oklahoma Health Sciences Center Research Park, for testing anti-neuronal autoantibodies in neuropsychiatric diseases. The authors alone are responsible for the content and writing of this publication. This study was funded, in part, by grants from the National Heart Foundation (GB06B2497), National Health and Medical Research Council (540419 and 1026753) to NK and CMR and grant HL35280, a Merit Award from the National Heart Lung and Blood Institute to MWC.

Introduction

Multiple infections and high acquisition rates of *Streptococcus pyogenes* (group A streptococcus, GAS) infection contributes to the development of rheumatic fever and rheumatic heart disease (RF/RHD) (1–3). Increased incidence and severity of RF/RHD is attributed to cumulative cardiac damage following recurrent episodes of RF exacerbated by the inflammatory process.

There is overwhelming evidence to support the premise that a variety of GAS antigens including the M protein and group A carbohydrate act as a major immunogen involved in initiating the inflammatory process due to the development of tissue cross-reactive antibodies and T cells generated during a GAS infection (4, 5). In humans, anti-streptococcal heart cross-reactive antibodies directed against the group A carbohydrate and cardiac myosin epitopes are believed to damage the valve endothelium (6, 7) and upregulate VCAM-1 (8), while T cells specific for streptococcal M protein and cardiac myosin epitopes enter through the activated endothelium of the valve (9, 10). T cells and their T cell clones derived from RF have been shown to be directed against specific epitopes of the M protein molecule which share homology with cardiac myosin (9–13). Human cross-reactive T cell clones derived from patients with RHD were primarily reactive with peptides in the B repeat region of the streptococcal M5 protein (9, 10) and this is also supported by prior work which indicated that heart cross-reactivity was found in the B repeat regions of the M protein (14–16).

We have previously established the Rat Autoimmune Valvulitis (RAV) model (17, 18), which is currently the only credible rodent model for investigating the immunopathogenesis of a uniquely human condition. Comparison of the epitopes studied in humans with those found to induce valve lesions in the Lewis rat revealed that the GAS M protein epitopes were similar to those in the human and cross-reacted with cardiac myosin (17–20). M protein specific T cell lines were found to home to the valve and induce VCAM-1 expression in a passive T cell transfer model (20). Lymbury and colleagues demonstrated further that epitopes of the GAS M protein also resided in the C repeat region as C repeat epitopes induced inflammatory lesions in the valve of the Lewis rat (21, 22). It is notable that the GAS M5 protein sequences which are associated with heart lesions appear to be different from sequences which are protective epitopes from the N-terminal or C-repeat regions of GAS M proteins which protect against GAS infection and have been utilized in the GAS vaccines (3, 23–25).

Although our earlier studies established that immunization of Lewis rats with GAS M5 or M6 protein (17) or M5 peptides from GAS M5 protein triggered the development of primarily valvulitis which is histologically and immunologically similar to RF/RHD (21), the sequence of events by which this process is initiated and exacerbates remains unclear. One hypothesis has been that repeated streptococcal infections would incite greater immune responses against the heart and induce RF/RHD. Here we provide evidence that in rats immunized and boosted one, two or three times with recombinant GAS M5 (rM5) protein to mimic repetitive infection with GAS there was incremental increase in antibody responses that could contribute to cardiac damage. Furthermore using rat cardiac endothelial cells in an

in vitro culture system, we established that serum from rM5-immunized rats may have the propensity to initiate the inflammatory response including VCAM-1 upregulation that could pave the way for T cells to infiltrate cardiac tissue. These new studies in the Lewis rat further support an inflammatory process at the valve endothelium as well as the role of streptococcal M protein as an antigen propagating T cells which infiltrate the valve after streptococcal infection as well as a basis for the hypothesis that recurrent GAS infections are important in breaking tolerance.

Materials and methods

Animals

Female Lewis rats (LEW/ SsN; Albino: a,h,c: RT¹) at 6–8 weeks were used. Experimental procedures were conducted with approval (#A1326) from James Cook University Animal Ethics Committee.

Antigens

rM5 protein, derived from GAS serotype M5, was cloned and purified as described previously (21). Briefly, extracellular domain of GAS M5 protein (amino acid residues 1 to 450) was amplified by PCR from genomic DNA (strain M5T5/B/PS) using standard techniques. The M5 DNA was ligated into pQE-30 expression vector (Qiagen, Chadstone Centre, Australia) to form the pQE30.m5 construct and subsequently transformed to *Escherichia coli* BL21 (pREP4). Plasmid DNA, extracted from positive clones was sequenced. The histidine-tagged rM5 was overexpressed in *E. coli* and purified by Ni-nitrilotriacetic acid affinity chromatography according to the manufacturer's instructions (QiaExpressionist; Qiagen). Protein concentration and purity were assessed by bicinchoninic acid and sodium dodecyl sulphate-polyacrylamide gel electrophoresis, respectively. No endotoxin was found in the purified rM5 (tested by the Limulus amoebocyte lysate method using an E-Toxate kit (Sigma, Castle Hill, Australia). The peptides were reconstituted in sterile phosphate-buffered saline (PBS) prior to use.

Immunization of rats

Following anaesthetization with isoflurane (5% induction, 1.5% maintenance), Lewis rats were immunized subcutaneously (s.c.) with 0.5 mg rM5 protein, or PBS for controls, in complete Freund's adjuvant and boosted seven days later s.c. in the flank with the antigen in incomplete adjuvant as previously described (26). In subsequent experiments a total of 24 rats were used (4 rats per group) and boosted 1, 2 or 3 times at seven day intervals with either rM5 or PBS. All rats were euthanized 14 days after final booster and hearts, spleens and serum were taken for analysis. Rats undergoing ECG (n = 13 in each group with PBS and rM5) were primed and given 3 boosts and ECG recordings taken at weekly intervals over a 30 day period.

Enzyme-linked immunosorbent assays (ELISA)

Immunoglobulin G (IgG) reactivity in sera was determined by ELISA. Briefly, Nunc Maxisorp plates (Roskilde, Denmark) were coated overnight with 10 µg/ml of GAS M protein antigen (rM5) or heat denatured rat cardiac myosin. After blocking, rat sera were

applied in triplicate at 1:100 dilution for rM5 and 1:4 dilution for cardiac myosin for 1 h at 37 °C. Secondary antibody goat anti-rat IgG (H+L) (Jackson ImmunoResearch, West Grove, PA) conjugated with horseradish-peroxidase was added for 1 h at 37 °C. After washing, 2,2'-azino-di(3-ethylbenzthiazoline)-6-sulphonate (ABTS) was added for 30 min before OD was measured at 405 nm. Controls included a positive serum sample, normal rat serum as negative control and antibody conjugate alone.

Lymphocyte proliferation assays

Specificity and reactivity of lymphocytes from immunized rats were determined by measuring proliferative responses to antigen stimulation in a [³H] thymidine incorporation assay (21). Pooled splenic mononuclear cells from each group of rats, were cultured in triplicate at 10⁵ cells/well in RPMI-1640 medium with 2.5% heat-inactivated rat serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 10 mM Hepes buffer. Test cells were stimulated with 50 µg/ml rM5. Baseline readings were obtained from unstimulated cells in culture medium only. After 120 h of culture, cells were pulsed for 4 h with 0.25 µCi ³H-thymidine (GEHealthcare, Rydalmere, Australia). Proliferative response is reported as stimulation index (SI), calculated as a ratio between mean counts per minute (cpm) in stimulated cells and cpm in unstimulated cells (cpm of test wells/cpm of control wells).

Histology

Formalin-fixed paraffin-embedded (FFPE) sections of rat hearts were stained with haematoxylin and eosin (H&E) and examined microscopically (Olympus BH2) for infiltration of inflammatory cells, fibrosis or necrosis as evidence of myocarditis or valvulitis. FFPE sections were immunostained using antibodies against pan T cell marker CD3 (clone G4.18, eBiosciences, Kensington, Australia), T cytotoxic cell CD8 (OX8, Cedarlane Laboratories, Burlington, ON, Canada) and macrophage lysosomal membrane molecule CD68 (ED1, Chemicon, Boronia, Vic, Australia). After antigen retrieval in citrate buffer (pH 6.0), sections were blocked with 0.3% hydrogen peroxide, followed by 1.5% horse serum. Sections were incubated with primary antibody at 4 °C overnight. Biotinylated horse anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA, USA) at 1:50 or 1:100 dilutions was applied for 30 min followed by incubation with alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch). Positively stained cells were visualized with Fast Red (BioGenex, San Ramon, CA, USA). Histological examination was performed sequentially in groups by an experienced researcher blinded to the sample identity. Staining was scored according to the number of infiltrating inflammatory cells observed per 10 high-power fields with valves graded as 0, no staining; 1, <5 cells in a valve; 2, >5 cells but no foci; 3, 1 or 2 focal lesions; 4, >2 lesions and myocardium graded as 0, isolated cells throughout tissue; 1, 1 or 2 small foci; 2, >2 small foci; 3, 1 large focal lesions; 4, Aschoff-type lesion.

Flow cytometric analysis of endothelial cell activation

We chose vascular cell adhesion molecule (VCAM) –1 as a measure of endothelial cell activation as basal expression of this adhesion molecule is very low in the absence of inflammation but increases to maximum levels within 4–6 h upon stimulation. Rat aortic

endothelial cells (RAOEC) (GenLantis, San Diego, USA) at passage 6 were grown in Rat Endothelial Growth Medium (GenLantis) until sub-confluent before changing to Rat Endothelial Basal Medium (GenLantis) for 24 h to induce cell quiescence prior to performing the assays. In the first experiment, RAOEC were stimulated for 4 h with 2% or 5% sera taken from rM5-immunized or non-immune control rats. In the second experiment, 2% sera or 2% heat-inactivated (56 °C for 30 min) sera was added to RAOEC to determine whether complement plays a role in initiating valve inflammation. In a third experiment, to determine whether rM5 specific antibodies contribute RAOEC were incubated with 2% heat-inactivated non-immune or immune sera, which had been pre-absorbed overnight at 4 °C with 100 µg/ml rM5 protein or rM5 protein alone used as a control to determine if antigen alone increases VCAM-1 expression. Each experiment included controls with either unstimulated RAOEC (negative control) or stimulated with 10 ng/ml TNF- α (positive control). All samples were tested in triplicate wells and experiments were repeated at least twice on different days. After 4 h of stimulation, RAOEC were washed in staining buffer (PBS/2% FBS/0.05% NaN₃, pH 7.4), harvested and stained for 30 min on ice with mouse anti-rat CD106-PE (Biolegend, cat. no. 200403) to detect adhesion molecule VCAM-1 or mouse IgG-PE isotype control (Biolegend, cat. no. 400113). After washing in staining buffer, cells were resuspended in cold PBS and immediately subjected to flow cytometry. Data were acquired using a BD FACSCalibur flow cytometer using CellQuest software. Cell debris, characterized by low forward and side scatter, were excluded from analysis and cells stained with isotype control antibodies were used to set VCAM-1 positive gates. For each sample, 10 000 events were recorded.

Electrocardiography following repeat boosts with recombinant M protein

To determine whether rM5 immunized rats had conduction abnormalities, we immunized Lewis rats on day 0, 7, 14 and 21 as described above with either rM5 (n = 13) or PBS (n = 13). At weekly intervals, electrocardiography was performed under general anaesthesia (AD Instrument, Power Laboratories). Rats were held in supine position following anaesthesia and maintained with 2% isoflurane (LASER Animal Health) with an induction dose of 5% and 1–1.5 L/min of oxygen. Standard limb leads were constructed from electrodes of 26-gauge needles placed subcutaneously for 1 cm at the right and left forepaws and the medial left thigh region. The negative electrode was placed carefully in the left front paw, ground electrode in the right paw and the positive electrode in the left hind paw. The electrical potentials were recorded for 30 seconds with *LabChart 6* software. Time at peak points of P and R waves were recorded and the P-R interval was calculated (27) in three randomly chosen segments from the ECG trace and the mean values recorded.

Statistical analysis

The statistical differences between treatment groups were evaluated using GraphPad Prism 5 statistical software. Differences in antibody responses between booster groups were tested using one-way analysis of variance (ANOVA) corrected with Bonferroni's post hoc test and results are reported as mean $\pm$ standard error (SEM). Flow cytometric data to assess VCAM-1 expression in RAOEC were tested by one-way ANOVA with Bonferroni or Tukey's post-hoc tests as appropriate. Differences in P-R intervals in ECG between rM5

immunized and controls over time were assessed by two-way ANOVA with Tukey's multiple comparisons test. A probability value of 0.05 was considered significant.

Results

IgG antibody levels against M5 are enhanced by repeated boosters and react with cardiac myosin

ELISA was used to determine IgG antibody response to rM5 in sera of rats boosted 1, 2 or 3 times at seven day intervals. Regardless of the number of boosts, all rM5-immunized rats showed significant ($p < 0.01$) IgG reactivity to rM5 protein compared to PBS-control rats. Those rats that were boosted twice and thrice had significantly higher responses ($p < 0.05$ and $p < 0.01$) compared to rM5 animals immunized with a single boost (Figure 1A). Studies have shown that shared epitopes between cardiac myosin and streptococcal M protein contribute to antigenic cross-reactivity and subsequent heart disease (13, 14, 17, 28). We found that sera from all rM5-immunized rats but not PBS-immunized control rats reacted with cardiac myosin ($p < 0.01$) (Figure 1B). rM5-immunized rat groups boosted three times reacted more strongly than those boosted either once or twice ($p < 0.05$) with differences between booster groups approaching statistical significance.

Repeat exposure to streptococcal M protein results in increased lymphocyte response

RF/RHD is considered a Th1-mediated response to GAS infection, with numerous studies describing antibody and CD4 T cell involvement (29–32). Regardless of the number of boosters, rats which had been immunized with rM5 protein responded specifically to rM5 protein whilst control rats did not respond (Figure 1C). Significantly higher proliferation ($p < 0.05$) was observed in rats boosted three times compared to those boosted one or two times.

Repeat rM5 boosters exacerbate inflammation in heart tissue

Examination of H&E stained sections of hearts revealed that some rats immunized with rM5 had evidence of carditis, with mononuclear cells (CD3+ and CD68+) infiltrating into valvular tissue. Heart sections were then immunostained (Figure 2A–C) and examined to ascertain whether valvulitis and/or myocarditis were present and whether repeated boosts with rM5 resulted in more severe lesions. Myocardium and valves of control rats that were immunized with PBS in adjuvant were all normal in appearance. We found that the number of infiltrating T cells (CD3+) and macrophages (CD68+) increased with each rM5 booster, indicating exacerbation of pathology. Of the rats immunized with rM5 protein and boosted once, all developed mild myocarditis with small foci of inflammatory cells and two developed valvulitis. Three rats that were boosted twice with rM5 protein developed myocarditis and two developed valvulitis. Three of 4 rats boosted three times developed both myocarditis and valvulitis. Although valvulitis was apparent in the same proportion (2/4) of rM5-immunized rats boosted either once or twice, the mean inflammatory score was lower in rats boosted once compared to twice boosted rats (2.72 vs 3.16 respectively).

VCAM-1 expression is up-regulated in endothelial cells by serum from immunized rats

VCAM-1 expression increased significantly in cells stimulated with 2% serum from rM5 immunized rats compared to cells stimulated with non-immune (Figure 3A&B). Moreover, heat inactivation of the rM5 immunized serum significantly reduced VCAM-1 expression (Figure 3C). Additionally pre-absorption of anti-rM5 antibodies from rM5 immunized sera resulted in partial reduction of VCAM-1 expression on RAOEC (Figure 3D). rM5 protein alone did not induce VCAM-1 expression. TNF- α was shown to strongly upregulate VCAM-1 as a positive control.

Prolongation of P-R interval following repeat exposure to rM5

There were no significant ($p > 0.05$) differences in P-R intervals in non-immunized rats over a 28 day period of observation (Figure 4). However significant prolongation in P-R interval was observed following the 2nd boost. P-R interval was significantly prolonged in ECGs in rM5 immunized rats compared to controls ($p = 0.024$) at 28 days.

Discussion

It is known that RF and RHD are limited to 0.3–3% (33, 34), of those 3.7% may have streptococcal pharyngitis (35). The Lewis rat was chosen originally as a model because it is highly susceptible to developing autoimmune diseases such as myocarditis (36) and experimental autoimmune encephalitis (37). The Lewis rat may express autoimmune genes which may provide the increased susceptibility to RF/RHD. The rat model has also been used successfully to study Sydenham chorea, the neurologic manifestation of RF (38). In our previous studies of the Lewis rat model (17, 20, 21), immunization with intact M protein molecules or the more potent peptides of M protein led to consistent valvulitis in approximately two-thirds of Lewis rats (17, 20, 21).

Our studies conducted in a RAV model of RF/RHD revealed that repeat exposure to GAS M5 leads to increased cellular infiltration of cardiac tissue and increased antibody and T cell responses towards GAS M protein. In rheumatic carditis patients, cellular infiltration and damage to the valves and valvular endothelium is more marked leading to long-term effects compared to the cellular infiltration observed in the myocardium (7). The group of rats boosted three times with rM5 had both a higher proportion of animals with valvulitis and a higher mean inflammatory score than rats that received one or two boosts. Most importantly, we found that rats immunized and boosted repeatedly (three times) with GAS rM5 protein developed higher antibody cross-reactivity to cardiac myosin compared to those that received fewer boosts, further establishing parallels between this model and RF/RHD.

Several early experimental models of RF/RHD trying to emulate rheumatic carditis by repetitive GAS immunization failed to produce lesions which are unequivocally rheumatic in nature (39). Although, more recently, experiments in a rat model using killed or sonicated GAS have shown increased anti-myocardial antibodies and increased cellular infiltration into cardiac tissue (40), T cell reactivity and the type of inflammatory lesions observed in these animals were not investigated.

Here we found that repeated streptococcal exposure not only leads to cardiac tissue damage but is also correlated with a significant elongation of the P-R interval, a hallmark of RF in humans, and significantly elevated antibody cross-reactivity to cardiac myosin and streptococcal M protein. While non-pathogenic auto-antibodies, including anti-cardiac myosin antibodies, are routinely found in low levels in the periphery of non-rheumatic individuals (41), antibody cross-reactivity between GAS and cardiac myosin in patients with RF/RHD has been well documented (6). In addition, M5 protein-heart cross-reactive T cells, demonstrated in T cell cloning experiments, have been isolated from heart valves of patients with RHD (9, 10). Several groups have proposed that sequence or structural mimicry between M protein and cardiac myosin or other heart proteins may directly or indirectly lead to epitope spreading and disease progression (12). Anti-cardiac myosin antibodies have also been shown to recognize laminin in valve tissue, potentially leading to T cell entry into the immune privileged valve (6). The pathogenesis of RF/RHD is multifaceted and complex.

Evidence suggests that GAS M protein and group A carbohydrate are involved in the breaking of tolerance and induction of autoimmunity in RF/RHD (24). It has also been suggested that anti-collagen antibodies generated from exposure of collagen to the immune system when the valve is initially damaged. Another hypothesis suggests that binding of M protein to collagen type IV may result in inflammation of the valvular endothelial tissue that could potentially initiate an immune response (25). Since both anti-cardiac myosin and anti-collagen autoantibodies are found in rheumatic fever, development and progression of RF/RHD like several other autoimmune diseases, likely involves multiple antigens and mechanisms leading to autoimmune pathology.

Our findings demonstrate that in a susceptible host, repeated GAS exposure increases the inflammatory response leading to more severe valvular changes. Repeated streptococcal exposure was investigated by Wang *et al.* (42) where streptococcal infections of mouse nasal associated lymphoid tissue (NALT) led to TGF- β and IL-6 production and increased Th17 responses only after multiple infections in the mice. Th17 responses have also been linked to autoimmune disease (43). It is possible that repeated exposure to GAS M protein may drive the expansion of CD4⁺ T helper cells and antibodies that are cross-reactive with cardiac myosin surpassing a critical threshold sufficient to break peripheral tolerance. Support for our hypothesis of cross-reactivity comes from previous observations in human T cell clones which cross-react with cardiac myosin and streptococcal M protein (9, 10) and from evidence demonstrating that rat T cell clones from RAV model transfer disease to naive rats (20). Future studies should also aim to generate T cell clones from M protein immunized rats that could provide evidence of cardiac myosin cross-reactivity in this model.

Studies have demonstrated the expression of VCAM-1 on valve endothelium in ARF patients with infiltration of T cells into the valve (8). To determine if the effect of soluble factors in rM5 hyperimmune serum on valvular endothelium, we performed *in vitro* assays with cultured rat endothelial cells. Serum from rM5-immunized rats significantly increased ($p < 0.005$) the expression of VCAM-1. This observation supports the hypothesis that an early event in the inflammatory process involving the endothelium and the subendothelial tissue may be partially due to the ability of cross-reactive anti-GAS antibodies to activate VCAM-1 on endothelial cells. Heat-inactivation of immune sera significantly reduced

VCAM-1 expression suggesting a possible role for heat-labile molecules such as complement which may be activated by immune complexes in immune sera involved in endothelial cell activation. These data may suggest that antibodies and heat labile serum factors such as complement and immune complexes in the hyperimmune may contribute to the activation of VCAM-1 which could lead to the infiltration of T cell into the valvular tissue.

The P-R interval in an ECG denotes the time from the beginning of atrial depolarization to the onset of ventricular depolarization. Prolongation of P-R interval in an ECG shows features of conduction abnormality and is a hallmark of cardiac damage (44). In RF/RHD, prolongation of the P-R interval is also a Minor Criteria for diagnosis (45, 46). In this investigation we provide the first electrophysiological evidence of cardiac damage in the RAV model of RF/RHD. Successive rM5 boost increased P-R interval suggestive of increased cardiac pathology over time following immunization and heart damage which was confirmed by immunohistological studies in our model.

The RAV model is the only credible model currently in use to dissect the multiple mechanisms that may play a role in the immunopathogenesis of RF/RHD (47). Currently using this model we aimed to determine whether repeated exposure to different GAS M serotypes, both 'rheumatogenic' and 'non-rheumatogenic' or non-group A streptococci, are sufficient to exacerbate rheumatic carditis with symptomatology. In summary, our experimental work presented herein demonstrates that increases in antibodies and T cells stimulated in response to repeated exposure of GAS M protein amplify and exacerbate cardiac tissue damage and increase P-R interval. Moreover, this process may be partly initiated by damage activation of the endothelium followed by infiltration of inflammatory cells leading to valvular and myocardial pathology.

Acknowledgments

The authors wish to thank Sabine Tacke (Radboud University Nijmegen, Netherlands) and Jacinta Power (James Cook University, Australia) for their assistance with the serology and RAV work.

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Figure 1. IgG and cellular responses after repetitive exposure to GAS rM5 protein

Lewis rats were immunized with rM5 and boosted one to three times at seven day intervals. A) Anti-rM5 IgG responses were significantly higher after the second and third boosts ($p < 0.05$ and $p < 0.01$). B) Reactivity towards cardiac myosin also increased after a third boost, with the difference approaching significance. Data points indicate mean absorbance $\pm$ SEM from $n = 7$ animals from two independent experiments. * denotes significantly higher ($p < 0.01$) response compared to PBS-immunized control rats. C) The proliferative response in PBMC in the presence of rM5 is shown as median stimulation index with interquartile range. * $p < 0.05$ vs PBS-immunized control rats.

Figure 2. Inflammation in heart tissue increases with each additional boost

Rat hearts were stained using antibodies against CD3 to detect infiltrating T cells (arrows) and CD68 to detect macrophages (arrowheads). Rats that had been immunized with rM5 protein in adjuvant and boosted A) once only had fewer infiltrating cells than those that had been boosted B) three times, suggesting that additional boosters increased inflammation. C) In contrast, control rats immunized with PBS in adjuvant and boosted three times had no evidence of valvulitis or myocarditis, with minimal staining for T cells or macrophages. Bars = 50 μm.

Figure 3. VCAM-1 expression on the surface of rat aortic endothelial cells (RAOEC)
 Quiescent cells were stimulated in triplicate wells for 4 h with pooled non-immune (NI) serum or pooled immune sera from rM5-immunized rats (IMM). Unstimulated cells and cells stimulated with 10 ng/ml TNF- α were used as controls. Activation of RAOEC was determined by flow cytometric analysis of VCAM-1 expression following staining with anti-rat CD106-PE. Plots were gated on forward and side light scatter characteristics to exclude dead cells and cell debris. A) Representative density plots showing gating on VCAM-1 positive cells. B) VCAM-1 expression increased significantly in cells stimulated with 2% IMM serum compared to cells stimulated with 2% NI serum. C) Heat inactivation of serum complement significantly reduced VCAM-1 expression in IMM-stimulated cells. D) Overnight pre-absorption of anti-rM5 antibodies in IMM sera resulted in reduced VCAM-1 expression on RAOEC. rM5 protein alone did not induce VCAM-1 expression. Data are representative of two separate experiments with similar results. * $p < 0.05$, *** $p < 0.005$, ANOVA with Tukey's multiple comparison post hoc test.

Figure 4. Prolongation of the P-R interval in rats immunized with rM5

The changes in P-R interval of female Lewis rats immunized (day 0, 7, 14 & 21) with rM5 ($n = 13$) or PBS ($n = 13$) was determined by weekly electrocardiogram assessment. After 28 days, rM5-immunized rats demonstrated a significantly prolonged P-R interval ($p = 0.024$) compared to PBS-immunized rats. Data points for each individual rat are depicted with horizontal bars depicting mean $\pm$ SEM. NS – no significance. P values were determined by two way ANOVA with Tukey's post-hoc multiple corrections.