Published in final edited form as:

J Cardiovasc Transl Res. 2014 March ; 7(2): 172–181. doi:10.1007/s12265-013-9526-4.

Identification of Streptococcal M-Protein Cardiopathogenic Epitopes in Experimental Autoimmune Valvulitis

Christine A. Kirvan^{1,^}, Jeffrey E. Galvin^{2,^}, Silvia Hilt¹, Stanley Kosanke³, and Madeleine W. Cunningham^{4,*}

¹Department of Biological Sciences, California State University, Sacramento, CA USA

²Zoetis, Lincoln, NE USA

³Department of Pathology, University of Oklahoma, HSC, Oklahoma City, OK USA

⁴Department of Microbiology and Immunology, University of Oklahoma, HSC, Oklahoma City, OK USA

Abstract

The M protein of rheumatogenic group A streptococci induces carditis and valvulitis in Lewis rats and may play a role in pathogenesis of rheumatic heart disease. To identify epitopes of M5 protein that produce valvulitis, synthetic peptides spanning A, B, and C repeat regions contained within the extracellular domain of the streptococcal M5 protein were investigated. A repeat region peptides NT4, NT5/6, and NT7 induced valvulitis similar to the intact pepsin fragment of M5 protein. T cell lines from rats with valvulitis recognized M5 peptides NT5/6 and NT6. Passive transfer of an NT5/6-specific T cell line into naïve rats produced valvulitis characterized by infiltration of CD4+ cells and upregulation of VCAM-1, while an NT6-specific T cell line did not target the valve. Our new data suggests that M protein-specific T cells may be important mediators of valvulitis in the Lewis rat model of rheumatic carditis.

Keywords

autoimmunity; rheumatic heart disease; T cells; M protein

Introduction

Acute rheumatic fever (ARF) is a multisystem inflammatory sequela following group A streptococcal (GAS) pharyngitis in susceptible individuals [1-3]. In ARF, poststreptococcal immune responses target heart, joint, skin, and central nervous system tissues. Carditis is the most serious manifestation of ARF, causing both morbidity and mortality, and is the leading cause of acquired childhood heart disease worldwide [4-11]. Patients with rheumatic heart disease (RHD) develop valvulitis characterized by mitral stenosis and aortic regurgitation [12, 13].

The development of RHD is related to the formation of chronic valvular lesions resulting from cytotoxic antibody deposition and T lymphocyte infiltration that disrupt cardiac function [14-22]. Increased expression of cell adhesion molecules including VCAM-1 on the

*Corresponding author: Dr. Madeleine W. Cunningham, Department of Microbiology and Immunology, Biomedical Research Center Room 217, 975 N.E. 10th Street, Oklahoma City, OK, 73104, USA Phone: 405-271-3128, FAX: 405-271-2217, (madeleine-cunningham@ouhsc.edu).

[^]Contributed equally to work

valve surface has been linked to the expression of proinflammatory cytokines, including IFN γ and TNF α , from invading macrophages and T cells [20, 23-25]. While invading T cells appear to be important mediators of valvular damage, their precise role in the recognition, invasion, and destruction of cardiac valve tissue in rheumatic carditis is incompletely understood.

The pathogenesis of ARF is thought to be mediated through autoimmune mechanisms initiated by cross-reactive immune responses between host and GAS antigens [26-28]. The streptococcal M protein, unique to GAS, is an extracellular virulence factor structurally and immunologically similar to several heart antigens including human cardiac myosin (HCM), tropomyosin, and laminin [16, 29-31]. Variation in M protein sequences is associated with rheumatogenic GAS strains that express a specific subset of M protein serotypes related to the development of ARF [32,33]. Of the known rheumatogenic serotypes, M types 5 (M5) and 6 (M6) have been the most extensively studied for their ability to provoke cross-reactive autoimmune responses against host antigens [3]. The extracellular portion of the M protein can be subdivided into three regions based on amino acid similarities among serotypes: the hypervariable N-terminal A repeat region, which confers serotype specificity on GAS strains, a variable midregion known as the B repeat, and the highly conserved C repeat of the C-terminal region that is shared by most M protein serotypes [34]. Immunization with peptides of the A and B repeat regions elicits rheumatoid lesions characterized by antibody deposition and infiltration of CD4 $^{+}$ cells in the heart [35, 36]. The A, B, and C repeat regions have been implicated in development of cross-reactive T cells in human RHD patients [24, 25, 37, 38], and T cells from rheumatic valves have been shown to proliferate in the presence of peptides from the A and B repeat regions of streptococcal M protein and peptides of light meromyosin and the S2 subfragment of HCM [22, 24, 25].

Animal models have contributed to the elucidation of cross-reactivity between the streptococcal M protein and cardiac antigens. Previously, we have demonstrated that immunization with recombinant M6 protein leads to the development of valvulitis and myocarditis in Lewis rats [39]. Both CD4 $^{+}$ and CD8 $^{+}$ cells were observed in valvular lesions characterized by the presence of verrucae-like nodules and Anitschkow cells. T cells from streptococcal M6 immunized animals proliferated in the presence of HCM and M6 protein. In a companion study, HCM-immunized Lewis rats developed severe myocarditis and valvulitis and generated T cells that recognized peptides of the M5 protein [16]. Recently, animal studies utilizing the Lewis rat model of experimental autoimmune valvulitis revealed that immunization with the streptococcal recombinant M5 protein produced valvular lesions with infiltrates of CD4 $^{+}$ and CD68 $^{+}$ cells that were consistent with findings of inflammatory T cells and macrophages in valves of RHD patients [40, 41]. Together, the work supports the use of the Lewis rat model to uncover the role of the M protein and T cell cross-reactivity in the pathogenesis of RHD.

In the valvulitis model, the cardiopathogenicity of M protein-specific T cells has not been evaluated. Our study used 25 overlapping M5 protein peptides spanning the A, B, and C repeat regions to select potential valve-infiltrating T cell lines. Peptide-specific T cells from the A and B repeat regions were capable of passively transferring valvulitis to naïve animals with characteristic CD4 $^{+}$ cell infiltration and VCAM-1 upregulation. The data suggest that the Lewis rat model of experimental autoimmune valvulitis will provide important insights into streptococcal-induced, cardiopathogenic T cell responses.

Methods

Antigens

Streptococcal peptides were synthesized as 18-mers with 5-aa overlap and purified by high pressure liquid chromatography based on the M protein sequence of M5 Manfredo strain (Table I) [42]. Streptococcal pepM5 was prepared in our laboratory according to previously described procedures [42, 43]. All experiments with animal use were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Oklahoma Health Sciences Center.

Induction of valvulitis in Lewis rats

Female Lewis rats (8-weeks old) were purchased from Harlan-Sprague-Dawley (Indianapolis, IN). Five hundred micrograms (500 µg) of purified pepM5 or M5 synthetic peptides were emulsified in Freund's complete adjuvant 1:1 (v/v) and used to immunize rats by hind footpad injection. One and three days post-immunization, rats were intraperitoneally administered 1×10^{10} killed *Bordetella pertussis* whole organism as an additional adjuvant [16]. Seven days after the primary immunization, rats were boosted subcutaneously with 500 µg of protein or peptide emulsified in Freund's incomplete adjuvant. Negative control rats only received phosphate-buffered saline (PBS) emulsified in Freund's adjuvant and *B. pertussis* injections. Rats were euthanized 21 days after the primary immunization by cardiac exsanguination. Heart, liver, and kidneys were fixed in 10% buffered formalin and embedded in paraffin. All tissues samples were given a 5 digit numerical code prior transfer to the OUHSC Pathology Department to ensure that tissues were assessed for histological changes in an unbiased, blinded fashion. Five-micrometer sections were cut and stained with hematoxylin and eosin for microscopic histological examination. Myocarditis and valvulitis lesions were semi-quantitatively scored as mild (1+) for 10% of tissue affected with focal lesions, moderate (2+) for 25% of tissue affected with focal lesions, severe (3+) for >50% of tissue affected with lesions, or confluent lesions (4+) affecting the majority of the tissue. Liver and kidney from each animal revealed no cellular infiltration.

Generation of rat T cell lines

Popliteal lymph nodes from animals with valvulitis were excised and single cell suspensions were generated by forcing the tissue through a wire mesh. Cells were washed with Iscove's modified Dulbecco's medium (IMDM) (Gibco), separated by Ficoll Hypaque (Sigma Chemical Co.), and lymphocytes recovered after centrifugation. Lymphocytes were cultured in a 96 well plate for 7 days in RPMI 1640-complete supplemented with 2-mercaptoethanol (5×10^{-5} M), L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin, streptomycin, nonessential amino acids, 5% fresh autologous serum and pepM5 at 50 µg/ml. Lymphocytes were restimulated with pepM5 pulsed antigen presenting cells (APCs), which were treated with mitomycin C. Con A stimulated rat spleen cells were added (20% v/v) 48 hours post-stimulation and the cells cultured for an additional 7 days with IL-2. To determine specificity, lymphocytes were stimulated with pepM5, labeled with tritiated thymidine (1 µCi/well), and harvested onto filtermats using a Mach 2 Man cell harvester (Wallac, Gaithersburg, MA). Thymidine uptake was measured by liquid scintillation counting (1025 Rackbeta Counter, Wallac). Stimulation indices were calculated as follows: average CPM of test wells/average CPM of unstimulated control wells. Lines with stimulation indices of 2 or higher were considered positive for pepM5 and specific peptide antigens.

Epitope mapping

Specificity of the generated T cell lines was determined by M5 protein specific-peptide induced proliferation and thymidine incorporation. Cells were cultured with irradiated

splenocytes in the presence of specific peptide then labeled with tritiated thymidine, harvested, and counted as described above. Stimulation indices of 2.0 or higher were considered positive. Cultures were performed in duplicate for each peptide and control wells (medium alone) to examine fine specificities of the T cell lines.

FACS analysis

T cells were analyzed by flow cytometry for cell surface expression of CD3, CD4, and CD8 molecules in addition to $\alpha\beta$ and $\gamma\delta$ T cell receptor proteins. Analysis was performed on a FACScan flow cytometer using CellQuest software (BD Biosciences).

Cytokine Analysis

For analysis of cytokine profile, 1×10^4 cells of each T cell line were cultured with irradiated autologous PBMC (1×10^5 cells/well) in the presence and absence of pepM5 for 24 hours. Resultant supernatants were tested for the presence of IFN- γ and IL-4 using the Cytoscreen ELISA kit (Biosource International, Camarillo, CA) according to manufacturer's instructions. Optical density was detected at 450nm (Dynatech MR 700) and cytokine concentrations detected were compared with known amounts of cytokine standards.

Passive transfer

T cells lines were washed, concentrated by centrifugation, and intravenously injected into naïve rats at 9×10^6 (p1.14 and p1.17) or 1×10^7 (p1.8) cells. Passive transfers with control splenocytes revealed no infiltration of heart tissue. Two weeks post-transfer, hearts were removed and histologically examined for the presence of mononuclear cell infiltrates in valve tissue. All transfers were performed in triplicate. Results were expressed as the number of rats with valvulitis/total number tested.

Immunohistochemistry

Heart, liver, and kidney from both immunized and T cells passive transferred animals were embedded with paraffin to produce slides containing 5- μ m tissue sections. Tissue sections were deparaffinized in a 3:1 mixture of Hemo-D:xylene and rehydrated in graded ethanol. Deparaffinized sections were subjected to antigen retrieval using citrate buffer [20]. Sections were blocked then probed with anti-CD4, anti-CD8, isotype control (Abcam), or VCAM-1 (Santa Cruz Biotechnology) mAbs followed by incubation with a universal secondary biotinylated antibody (JacksonImmunoResearch). Sections were developed with streptavidin alkaline phosphatase and fast red substrate (BioGenex) then counterstained with hematoxylin.

Statistical analyses

Statistical analyses were performed for group peptide and individual peptide immunizations as well as for the passive transfer experiments. P values were calculated by one-way analysis of variance (ANOVA) for comparison of more than two groups.

Results

Induction of valvulitis in Lewis rat by streptococcal M protein peptides

Examination of heart sections from pepM5-immunized rats revealed regions of valvulitis. Sections of rat hearts were stained with hematoxylin and eosin and microscopically evaluated for cellular infiltrates in the valves. Cardiac valvulitis found in two-thirds of immunized rats was characterized by infiltrating mononuclear cells comparable to previous results using recombinant M6 protein [39]. Cellular infiltrates were observed in approximately 25% (score 2+) of the valvular endothelium and in deeper valve tissue

containing interstitial accumulations of mononuclear cells along with pronounced edema (Fig. 1A and inset). No lesions or cellular infiltrates were observed in the valves of adjuvant controls (Fig. 1B). The presence of both CD4+ (Fig. 1C) and CD8+ (Fig. 1D) T cells was confirmed in valves from pepM5 immunized rats, but not in adjuvant control animals (Fig. 1E). The data was similar to valve tissue removed from human RHD patients in which CD4+ and CD8+ cells were found in necrotic Aschoff body lesions [20].

To identify cardiopathogenic epitopes, overlapping synthetic peptides representing the A (N-terminal), B, and C repeat regions of the group A streptococcal M5 protein were used to immunize Lewis rats (Table 1). The specificity of M protein-induced carditis was initially determined by immunization with group combinations of five peptides (Table 2). Hearts from immunized rats were examined for lesions and compared to valves from animals immunized with intact group A streptococcal pepM5 or adjuvant alone (Fig. 2 and Table 3). M protein peptides from Groups 1 and 2 of N-terminal peptides (A repeat region) induced 2+ valvulitis similar to pepM5 with cross-sections of valves showing mononuclear cell invasion and macrophage-like cells in approximately 25% of the valve tissues (Fig. 2 A-D and Table 3). Valves from Group 2 (NT5-8)-immunized rats revealed pronounced edema in addition to mononuclear cells (Fig. 2E). While cellular infiltrates penetrated the valve endothelium, myocarditis was not observed in Group 1 or 2 immunized animals. In contrast, Group 3 containing B repeat peptides (B1A-B2B3A) was found to induce mild myocarditis as seen by multifocal mononuclear cell infiltrates in the right ventricular free wall in one of three animals tested (Fig. 2F). The observed lesions were similar in placement to the severe myocarditis found in human cardiac myosin-immunized animals [16]. Valvular and myocardial cellular infiltrates appeared to be heart specific; no lesions were present in tissue sections from livers and kidneys. Heart tissues from rats immunized with peptides groups 4 (B3A-C1B) and 5 (C1C2-C3) as well as the adjuvant control were histologically normal with no detectable presence of cellular infiltrates (data not shown). The data demonstrate that peptides from the A repeat region of streptococcal M5 protein produce valvulitis in Lewis rats.

To further localize specific cardiopathogenic N-terminal M5 epitopes, Lewis rats were immunized with individual M protein peptides from the A repeat region (Fig. 3 and Table 4). Histological examination of heart tissue revealed that N-terminal peptide NT4 (residues 40-58) was capable of inducing valvulitis in approximately 25% of valve tissues characterized by mononuclear cell infiltrates and edema (Fig. 3A). The NT4 peptide also produced multifocal myocarditis with infiltration of both neutrophils and mononuclear cells (Fig. 3B) in approximately 10% of tissues and was comparable to the results observed for intact pepM5. Immunization with peptide NT5/6 (residues 67-84) (Fig. 3C) and NT7 (residues 85-102) (Fig. 3D) revealed cellular infiltration in over 25% of the mitral valve through the endothelium at the valve surface. Lymphocytes and monocytes likely infiltrated the valve through the endothelial surface and not from the myocardium as no lesions were observed in the myocardium in contrast with peptide NT4 and pepM5 immunizations. While NT4, NT5/6, and NT7 all induced valvulitis characterized by moderate to severe mononuclear cell infiltration, only valves from NT 7-immunized animals were found to have Anitschkow-like cells similar to previously reported data using M6 protein [39]. Anitschkow cells are characteristically seen in hematoxylin-and eosin-stained sections of rheumatic hearts, and are termed “owl-eye” cells because of their appearance due to a condensed nucleus. The data demonstrate that individual M protein epitopes from the N-terminal region of M5 protein are capable of producing valvulitis in rats similar to the intact pepM5 or the recombinant M5 proteins and share characteristics of valves from RHD patients.

Passive transfer of M5-specific T cell lines to naïve animals

To further characterize streptococcal M protein induced valvulitis, T cell lines were established from pepM5 immunized animals. A total of 13 lines were generated (Supplemental Table 1). The cell lines were characterized as CD3+, CD4+, $\alpha\beta$ TCR+ and all lines recognized intact pepM5. Two T cell lines recognized the N-terminal peptides of the A repeat region of the group A streptococcal M5 protein. Line p1.14 reacted strongly with both NT5/6 and NT6 while line p1.8 recognized NT6 alone (Supplemental Table 2). Line p1.14 was of particular interest due to its ability to recognize peptide NT5/6 as immunization with this specific peptide caused moderate valvulitis in the Lewis rat model (Fig. 3C). Three other lines, p1.15, p1.17, and p1.22 were specific for streptococcal M5 protein B repeat region peptides B2 and B3A, which share a high degree of sequence homology (data not shown—See Table 2).

PepM5-induced cytokine production was evaluated in the five T cell lines. All five lines secreted IFN- γ in response to the pepM5 fragment (range 400-2200 pg/ml), while none of the T cell lines produced IL-4. T cells/clones derived from RHD also produced IFN- γ and no IL-4 [44]. T cell lines p1.8 (NT6-specific) and p1.14 (NT5/6-specific) both secreted 750 pg/ml IFN- γ with pepM5 stimulation. The pattern of cytokine secretion by T cell lines indicates a strong T_H1 response to pepM5 stimulation and is identical to reported pro-inflammatory cytokine secretion by human T cell clones from RHD patients [24, 25].

To confirm that pepM5 immunization produced cross-reactive, pathogenic T cells that could recognize and invade cardiac tissues, T cell lines p1.14 and p1.8 were passively transferred by intraperitoneal route into naïve Lewis rats. NT5/6-specific line p1.14 was capable of migrating to and infiltrating heart tissues causing valvulitis in the Lewis rat model with cellular infiltrates present in over 25% of valve tissues (Fig. 4A and B and Supplemental Table 3). In contrast, T cell line 1.8, which recognized only the NT6 epitope of pepM5, did not invade heart tissues (Fig. 4C) even when a million additional cells (1×10^6) were transferred into naïve animals in comparison to T cell line p1.14 (Fig. 4A and B). Normal splenocytes from PBS injected animals did not infiltrate the heart (Supplemental Table 3). To ensure that passive transfer results of p1.8 and p1.14 were not solely due to differences in pepM5 stimulation indices, T cell line p1.17 specific for the B2 and B3A epitopes was used as a control as its pepM5 stimulation index was similar to that of T cell line p1.8 (Supplemental Table 1). Although peptide Group 3 that contained B repeat peptides did not cause valvulitis, T cell line p1.17 did home to the valve (Fig. 4D) supporting previous work that demonstrates T cell responsiveness to B repeat region peptides [25].

The presence of T cell line p1.14 in hearts from passively transferred animals was confirmed by immunohistochemistry. CD4+ cells were observed in cardiac tissues of p1.14 infused animals (Fig. 5A), but not from animals passively transferred with line 1.8 (Fig. 5B). The cell adhesion molecule VCAM-1 is induced by pro-inflammatory cytokines including IFN γ and TNF α secreted by activated T_H cells and is required to promote T cell infiltration into tissues during immune responses. Heart tissues from the T cell line p1.14 passive transfer revealed detectable VCAM-1 staining by immunohistochemistry (Fig. 5C) in comparison to T cell line p1.8 transferred tissues (Fig. 5D). The ability of the NT5/6-specific p1.14 T cell line to passively transfer valvulitis is significant, not only in that it demonstrated pepM5-specific T cells target the heart valve *in vivo*, but that the p1.14 T cell line is also capable of potentially inciting endothelium activation to aid in the infiltration and subsequent damage of the valve. The relevance of the Lewis rat passive transfer model is in its comparison to derived intralesional T cell clones generated from human rheumatic valve. A human T cell clone was found to be specific for the M protein epitope DKLKQQRDTLSTQKET that overlaps with the NT5/6 epitope recognized by p1.14 (Fig. 6) [24] and is the same epitope that has been associated with greater severity of disease in RHD [45]. The data provide

direct evidence that streptococcal M protein specific T cells migrate to the heart *in vivo* and likely contribute to valvulitis in the Lewis rat model.

Discussion

To our knowledge, this is the first report to demonstrate the passive transfer of streptococcal M protein-specific T cell migration and infiltration to cardiac tissues *in vivo*. The experimental design enabled identification of dominant cross-reactive epitopes located in the A and B repeat regions of M5 protein. Three groups of M protein peptides representing all N-terminal peptides (A repeat region) and the more centrally located B repeat region peptides were capable of inducing mononuclear cell invasion of heart valves or myocardium in the Lewis rat model of autoimmune valvulitis (Fig. 2 A-F and Table 3). None of the peptides induced changes in other organs. Although other reports have found valvulitis from immunization with C repeat region peptides of M5 protein, we did not identify M5 C repeat region peptides capable of producing valvular lesions with Lewis rat immunizations of peptide Group 4 (B3A-C1B) and Group 5 (C1-C3) (Table 2) [38, 40, 41]. The disparity of results between the immunization studies may be due to differences in our peptide length and amino acid sequence overlap.

Individual epitopes of the rheumatogenic serotype M type 5 protein were capable of initiating valvulitis in the Lewis rat model producing changes that are consistent with lesions from rheumatic valves. M5 protein peptides NT4 (40-58), NT5/6 (67-84), and NT7 (85-102) induced valvulitis in Lewis rats (Fig. 3 A-D and Table 4). Tissue sections from animals immunized with individual M protein peptides revealed moderate to severe mononuclear cell infiltration into the valve as well as the presence of Anitschkow cells identical to that found with intact pepM5 immunization. The results mirror those from previous studies using recombinant M5 and M6 proteins [39, 40]. The ability of the NT4 peptide to induce both valvulitis and myocarditis is of special interest as epitope mapping studies from rheumatic carditis patients show high antibody reactivity to this peptide [46].

Previously we have shown mice developed mononuclear cellular infiltrates in myocardium after immunization with specific M5 peptides located within the A and B repeat regions of the M5 protein molecule. Immunization of mice with M5 peptides NT4, NT5, and NT6 led to the formation of focal myocarditis characterized by cellular infiltrates in hearts and all three peptides share sequence homology with cardiac myosin [35, 47]. In addition, antibody from animals immunized with NT7 strongly reacted with human cardiac myosin. [35, 47]. In our study, Lewis rat immunizations with peptide NT5/6 produced cellular infiltration of the valve, but not NT5 or NT6 suggesting that the pathogenic epitope is found only in the overlapping peptide sequence. This is supported by reports from RHD where the NT5/6 peptide sequence is recognized by intralesional T cell clones [24]. Our previous data suggests that regions of M proteins that share homology exclusively with cardiac myosin, tropomyosin, or laminin may break tolerance and induce heart disease [25, 35, 48].

To further characterize cell-mediated pathology, CD4⁺ T cell lines were established from Lewis rats immunized with pepM5 (Supplemental Table 1). Of the five peptide-specific T cell lines produced, p1.14 and p1.17 were shown to transfer valvulitis to naïve animals. Most importantly, T cell line p1.14, which recognized the N terminal peptides NT5/6 and NT6, was capable of invading valve tissue upon passive transfer whereas T cell line p1.8, which only recognized NT6, did not produce cellular infiltration of the valve, even in the presence of tenfold more cells (Fig. 4 A-C, Tables 6 and 7). Immunohistochemistry for CD4⁺ T cells revealed valvular infiltration by T cell line p1.14 (NT5/6), but not by line 1.8 (NT6) (Fig. 6 A-B). The transfer results are supported by individual peptide immunization

studies and strongly suggest that the NT5/6 M5 protein peptide was capable of provoking a cardiopathogenic T cell response.

In rheumatic carditis, expression of cell adhesion molecules including VCAM-1 is necessary for the recruitment of pathogenic T cells to heart tissues [20]. IFN- γ and other pro-inflammatory cytokines such as TNF α secreted by antigen-activated T cells may play an important role in the development or maintenance of valvulitis by inducing increased expression of VCAM-1 on valvular endothelium, which in turn, increases the rate of mononuclear cell recruitment to the valve surface.

In ARF and RHD, a majority of T cells isolated from heart and peripheral blood were found to secrete IFN- γ ; in contrast, the cells were found to secrete only small amounts of the T_H2 type cytokine IL-4 [44, 49]. In this study, we evaluated M5-specific T cell lines for IFN- γ and IL-4 secretion patterns. Both the p1.14 and p1.8 T cell lines secreted identical amounts of IFN- γ and no IL-4 with pepM5 stimulation, however only the NT5/6-specific p1.14 T cell line induced an upregulation of VCAM-1 on the valvular surface (Fig. 5 C and D), indicating most importantly that epitope specificity as well as IFN- γ secretion may be required to promote attachment and invasion of valve endothelium. Additional studies will be needed to clarify the cellular and molecular factors necessary to mediate M protein-specific T cell infiltration into the heart.

Mimicry between streptococcal M protein and cardiac myosin has been reported in our laboratory as well as many others (for review, [4, 27]). In ARF, T cell mimicry is defined as recognition of related epitopes that arise from similarities of the α -helical coiled-coil structure in streptococcal M proteins and human cardiac myosin, tropomyosin, and laminin of the heart. T cell cross-reactivity to distinct α -helical coiled-coil proteins may involve the repetitive seven amino acid residue periodicity that maintains α -helical structure and thus creates a high number of degenerate possibilities for recognition by T cells [29]. Recent crystal structure analysis of the streptococcal M protein supports how the alpha helical structure is related to both virulence and cross-reactivity [50]. Studies of ARF/RHD T cell clones have demonstrated that cross-reactive streptococcal M protein and heart antigens are recognized by an individual T cell [24, 25]. While this study did not evaluate cross-reactivity to heart antigens, an intralesional human T cell clone from ARF/RHD with epitope specificity for streptococcal M5 NT5/6 was found to also recognize an epitope of human cardiac myosin light meromyosin that shared an 87% amino acid homology with the NT5/6 sequence [24]. The M5 epitope DKLKQQRDTLSTQKET corresponding to NT5/6 may also distinguish severe and mild forms of ARF/RHD [45]. The M5 peptide NT5/6 sequence was preferentially recognized by 46% of T cells from patients with severe RHD, in comparison to 27 % of mild RHD and 8.6% healthy subjects [45]. While the data suggest that streptococcal M5 NT5/6 peptide is an important mediator of valvulitis and RHD, further analyses will be required to explain its role in autoimmune carditis.

The new data demonstrates that the Lewis rat is a robust model to simulate pathogenic valvulitis using specific epitopes of the streptococcal M protein. The elucidation of the individual steps of rheumatic valvular heart disease will be important for understanding the role of streptococcal M protein in driving autoimmune cell-mediated immune attack against heart valve and myocardium. The clinical relevance of the new data is the characterization of the M5 peptide NT5/6-specific T cell line p1.14, which will serve as a useful tool in dissection of the experimental autoimmune valvulitis model and rheumatic carditis pathogenesis. A greater understanding of the cross-reactive and immunopathological events in valvulitis and RHD may provide new insights into the diagnostic markers and treatment options. While the study only evaluated a small number of animals, we were able to consistently induce and passively transfer valvulitis using specific M5 peptides NT 4, NT

5/6, NT 7, and B2-B3A. The collective evidence suggests that amino acid sequences in M5 protein which share homology with cardiac myosin may break tolerance and promote T-cell-mediated inflammatory heart disease in our Lewis rat model of autoimmune valvulitis as well as RHD in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Janet Heuser, Trekka Guyer, Kylie Perrich, and Kathy Alvarez for technical assistance, Dr. Nadia Ellis for helpful discussions and instruction, and Dr. Christin Bendorf for review of the manuscript.

Financial support: M.W.C holds grants from the National Heart, Lung, and Blood Institute (HL35280 and HL56267) and the Oklahoma Center for the Advancement of Science and Technology.

References

1. Stollerman GH. Rheumatic fever in the 21st century. *Clin Infect Dis*. 2001; 33(6):806–14. [PubMed: 11512086]
2. Stollerman GH. Rheumatic Fever. *Lancet*. 1997; 349:935–942. [PubMed: 9093263]
3. Cunningham, MW. Pathogenesis of Acute Rheumatic Fever. In: Stevens, DS.; Kaplan, EH., editors. *Streptococcal Diseases*. first. New York, NY: Oxford Press; 2000. p. 102-132.
4. Cunningham MW. Streptococcus and Rheumatic Fever. *Current Opinion in Rheumatology*. 2012; 24:408–416. [PubMed: 22617826]
5. Dajani AS. Guidelines for the diagnosis of rheumatic fever (Jones criteria, 1992 update). *Journal of the American Medical Association*. 1992; 268:2069–2073. [PubMed: 1404745]
6. Kaur S, et al. Ethnic differences in expression of susceptibility marker(s) in rheumatic fever/ rheumatic heart disease patients. *Int J Cardiol*. 1998; 64:9–14. [PubMed: 9579811]
7. Veasy LG, Tani LY, Hill HR. Persistence of acute rheumatic fever in the intermountain area of the United States. *J Pediatrics*. 1994; 124:9–16.
8. Veasy LG, Wiedmeier SE, Orsmond GS. Resurgence of acute rheumatic fever in the intermountain area of the United States. *New England Journal of Medicine*. 1987; 316(8):421–427. [PubMed: 3807984]
9. Veasy LG, et al. Temporal association of the appearance of mucoid strains of *Streptococcus pyogenes* with a continuing high incidence of rheumatic fever in Utah. *Pediatrics*. 2004; 113(3 Pt 1):e168–72. [PubMed: 14993572]
10. Kaplan MH, Johnson DR, Cleary PP. Group A streptococcal serotypes isolated from patients and sibling contacts during the resurgence of rheumatic fever in the United states in the mid-1980's. *Journal of Infectious Diseases*. 1989; 159:101–103. [PubMed: 2642516]
11. Bisno AL. Acute pharyngitis. *N Engl J Med*. 2001; 344(3):205–11. [PubMed: 11172144]
12. Unny, SK.; Middlebrooks, BL. Streptococcal rheumatic carditis. In: Rickenberg, HV., editor. *Microbiological Reviews*. 1983.
13. Cunningham, MW. Streptococci and rheumatic fever. In: Rose, NR.; Friedman, H., editors. *Microorganisms and autoimmune disease*. Plenum Publishing Corp; New York: 1996. p. 13-66.
14. Zabriskie JB, Hsu KC, Seegal BC. Heart-reactive antibody associated with rheumatic fever: characterization and diagnostic significance. *Clinical and Experimental Immunology*. 1970; 7:147–159. [PubMed: 4920603]
15. Krisher K, Cunningham MW. Myosin: a link between streptococci and heart. *Science*. 1985; 227(4685):413–5. [PubMed: 2578225]
16. Galvin JE, et al. Induction of myocarditis and valvulitis in Lewis rats by different epitopes of cardiac myosin and its implications in rheumatic carditis. *Amer J Pathol*. 2001; 160:297–306. [PubMed: 11786423]

17. Martins TB, et al. Comprehensive analysis of antibody responses to streptococcal and tissue antigens in patients with acute rheumatic fever. *Int Immunol.* 2008; 20(3):445–52. [PubMed: 18245783]
18. Kemeny E, et al. Identification of mononuclear cells and T cell subsets in rheumatic valvulitis. *Clinical Immunology and Immunopathology.* 1989; 52:225–237. [PubMed: 2786783]
19. Fraser W, et al. Rheumatic Aschoff nodules revisited. II: Cytokine expression corroborates recently proposed sequential stages. *Histopathol.* 1997; 31:460–464.
20. Roberts S, et al. Immune mechanisms in rheumatic carditis: Focus on valvular endothelium. *J Infect Dis.* 2001; 183:507–511. [PubMed: 11133385]
21. Raizada V, et al. Tissue distribution of lymphocytes in rheumatic heart valves as defined by monoclonal anti-T cell antibodies. *American Journal of Medicine.* 1983; 74:90–96. [PubMed: 6336893]
22. Guilherme L, et al. Human heart-filtrating T cell clones from rheumatic heart disease patients recognize both streptococcal and cardiac proteins. *Circulation.* 1995; 92:415–420. [PubMed: 7634457]
23. Grundtman C, H I, Førre OT, Saatvedt K, Mikkelsen K, Lundberg IE. Cardiovascular disease in patients with inflammatory rheumatic disease is associated with up-regulation of markers of inflammation in cardiac microvessels and cardiomyocytes. *Arthritis Rheum.* 2010; 62:667–73. [PubMed: 20131226]
24. Fae KC, et al. Mimicry in recognition of cardiac myosin peptides by heart-intralesional T cell clones from rheumatic heart disease. *J Immunol.* 2006; 176(9):5662–70. [PubMed: 16622036]
25. Ellis NMJ, et al. T cell mimicry and epitope specificity of crossreactive T cell clones from rheumatic heart disease. *J Immunol.* 2005; 175:5448–5456. [PubMed: 16210652]
26. Cunningham MW. Autoimmunity and molecular mimicry in the pathogenesis of post-streptococcal heart disease. *Front Biosci.* 2003; 8:s533–43. [PubMed: 12700052]
27. Cunningham, MW. Group A streptococcal sequelae and molecular mimicry. In: Cunningham, MW.; Fujinami, RS., editors. *Effects of Microbes on the Immune System.* first. Philadelphia, PA: Lippincott, Williams and Wilkins; 2000. p. 123-149.
28. Guilherme, L.; Kalil, J. Rheumatic Fever: How streptococcal throat infection triggers an autoimmune disease. In: Rose, YSaNR., editor. *Infection and Autoimmunity.* Elsevier; 2004. p. 321-331.
29. Manjula BN, Trus BL, Fischetti VA. Presence of two distinct regions in the coiled-coil structure of the streptococcal Pep M5 protein: relationship to mammalian coiled-coil proteins and implications to its biological properties. *Proceedings of the National Academy of Sciences of the United States of America.* 1985; 82(4):1064–8. [PubMed: 3856248]
30. Manjula BN, Fischetti VA. Tropomyosin-like seven residue periodicity in three immunologically distinct streptococcal M proteins and its implications for the antiphagocytic property of the molecule. *Journal of Experimental Medicine.* 1980; 151(3):695–708. [PubMed: 6987328]
31. Bronze MS, et al. Protective and heart-cross-reactive epitopes of type 19 streptococcal M protein. *Transactions of the Association of American Physicians.* 1987; 100:80–4. [PubMed: 2457979]
32. Bisno, AL. The concept of rheumatogenic and non-rheumatogenic group A streptococci. In: Read, SE.; Zabriskie, JB., editors. *Streptococcal diseases and the immune response.* Academic Press Inc; New York: 1980. p. 789-803.
33. Bisno, AL. *Streptococcus Pyogenes.* In: Mandell, GL.; Bennett, RG.; Dolin, R., editors. *Principles and Practice of Infectious Diseases.* Churchill Livingstone; New York: 1995. p. 1786-1799.
34. Fischetti VA. Streptococcal M protein: molecular design and biological behavior. *Clinical Microbiology Reviews.* 1989; 2(3):285–314. [PubMed: 2670192]
35. Huber SA, Cunningham MW. Streptococcal M protein peptide with similarity to myosin induces CD4+ T cell-dependent myocarditis in MRL/++ mice and induces partial tolerance against coxsackieviral myocarditis. *Journal of Immunology.* 1996; 156(9):3528–34.
36. Cunningham MW. T cell Mimicry in inflammatory heart disease. *Mol Immunol.* 2004; 40:1121–1127. [PubMed: 15036918]

37. Fae K, et al. Heart infiltrating T cell clones from a rheumatic heart disease patient display a common TCR usage and a degenerate antigen recognition pattern. *Mol Immunol*. 2004; 40(14-15): 1129–35. [PubMed: 15036919]
38. Lymbury RS, et al. Induction of autoimmune valvulitis in Lewis rats following immunization with peptides from the conserved region of group A streptococcal M protein. *J Autoimmun*. 2003; 20(3):211–7. [PubMed: 12753806]
39. Quinn A, et al. Induction of autoimmune valvular heart disease by recombinant streptococcal M protein. *Infect Immun*. 2001; 69(6):4072–4078. [PubMed: 11349078]
40. Gorton D, Govan BL, Ketheesan N. B and T cell responses in group A streptococcal M protein/peptide induced experimental carditis. *Infection and Immunity*. 2009; 77:2177–2183. [PubMed: 19273562]
41. Gorton DE, et al. An alternative technique for the induction of autoimmune valvulitis in a rat model of rheumatic heart disease. *J Immunol Methods*. 2010; 15(355):80–85. [PubMed: 20206182]
42. Cunningham, MW. Group A streptococci: molecular mimicry, autoimmunity and infection. In: Hook, M.; Switalski, L.S., editors. *Microbial adhesion and invasion*. Springer Verlag; New York: 1992. p. 147-167.
43. Quinn A, et al. Autoantibody germ-line gene segment encodes VH and VL regions of a human anti-streptococcal monoclonal antibody recognizing streptococcal M protein and human cardiac myosin epitopes. *Journal of Immunology*. 1995; 154(8):4203–12.
44. Guilherme L, et al. Rheumatic heart disease: pro-inflammatory cytokines play a role in the progression and maintenance of valvular lesions. *Am J Pathol*. 2004; 165:1583–1591. [PubMed: 15509528]
45. Guilherme L, et al. T-cell reactivity against streptococcal antigens in the periphery mirrors reactivity of heart-infiltrating T lymphocytes in rheumatic heart disease patients. *Infect Immun*. 2001; 69(9):5345–51. [PubMed: 11500404]
46. Ellis NMJ, et al. Priming the immune system for heart disease: a perspective on group A streptococci. *J Infect Dis*. 2010; 202:1059–1067. [PubMed: 20795820]
47. Cunningham MW, et al. Molecular analysis of human cardiac myosin-cross-reactive B- and T-cell epitopes of the group A streptococcal M5 protein. *Infection and Immunity*. 1997; 65:3913–3923. [PubMed: 9284171]
48. Adderson EE, et al. Molecular analysis of polyreactive monoclonal antibodies from rheumatic carditis: human anti-N-acetyl- glucosamine/anti-myosin antibody V region genes. *Journal of Immunology*. 1998; 161:2020–2031.
49. Guilherme L, Kalil J, Cunningham M. Molecular mimicry in the autoimmune pathogenesis of rheumatic heart disease. *Autoimmunity*. 2006; 39(1):31–9. [PubMed: 16455580]
50. McNamara C, et al. Coiled-coil irregularities and instabilities in group A streptococcus M1 are required for virulence. *Science*. 2008; 319:1405–1408. [PubMed: 18323455]

Abbreviations

GAS	Group A streptococcus
RHD	Rheumatic heart disease
VCAM-1	Vascular cell adhesion molecule-1
IFNγ	gamma interferon

Figure 1 (A-E).

Histopathologic features of valvulitis in Lewis rat heart sections stained with hematoxylin and eosin. A. Valvulitis is shown in rats immunized with group A streptococcal pepM5 (magnification, ×20) with the presence of mononuclear cell infiltrates directly under the valvular endothelium and in deeper valve tissue (small arrow) and inset (magnification, ×200). Areas of edema are seen throughout the valve tissue (large arrows). Unaffected myocardium is shown (dashed arrow). B. Animals that received adjuvant alone had no detectable histopathologic lesions or edema (magnification, ×20). Valves from rats immunized with pepM5 demonstrated the presence of both CD4+ (C) and CD8+ (D) T cells in comparison to the adjuvant control (E) (magnification, ×40).

Figure 2 (A-F).

Induction of valvulitis and cellular infiltration in hematoxylin- and eosin-stained heart valves from Lewis rats immunized with group A streptococcal M5 peptide groups (Table 2).

A. Lewis rats immunized with M5 peptide group 1 (NT1-4/5) revealed the presence of mononuclear cells (arrows) within mitral valve (magnification, $\times 40$) in relation to surface endothelial cells (dashed arrow). B. Cellular infiltrates (arrows) in valvular tissue from group A streptococcal M5 peptide group 1-immunized animals (magnification, $\times 400$). No cellular infiltrates were observed in the myocardium. C and D. Group 2 M5 peptides (NT 5-8) induced mononuclear cell invasion (arrows, magnification, $\times 40$ and $\times 400$) and provoked a strong inflammatory response observed by the presence of edema in the valve (E, large

arrows). F. In contrast, group 3 peptides (B1A-B2B3A) induced small focal lesions within the myocardium with mononuclear cell infiltrates.

Figure 3 (A-D).

Identification of specific streptococcal M5 peptides that induced valvulitis and myocarditis from the A repeat region of pep M5. Peptide NT4 induced both valvulitis (A) and myocarditis (B) similar to group A streptococcal pepM5 protein. Valvulitis produced by peptide NT5/6 (C) is characterized by invasion of both small mononuclear cells and macrophages. Prominent mononuclear cell infiltrates are present in streptococcal M5 peptide NT7-induced valvulitis (D). Small arrows indicate invading mononuclear cells and large arrows denote the presence of edema within the valvular tissues. Valves from NT7 immunized rats revealed the presence of Anitschkow-like cells (dashed arrow). Magnification at $\times 40$ for all tissues.

Figure 4 (A-D).

Passive transfer of streptococcal M protein specific T cell lines produced valvulitis in naïve Lewis rats. NT5/6-specific T cell line p1.14 migrated to the mitral valve of naïve Lewis rats (A and B) in contrast to NT6-specific T cell line p1.8, which did not infiltrate heart valves (C). T cell line 1.17, which was M5-specific and recognized M5 peptides B2 and B3A, migrated to cardiac valves (D), similar to p1.14 (small arrows indicate endothelium). Large arrows indicate mononuclear cells (A-D). Magnification at $\times 40$ for all tissues.

A. CD4⁺ expression in recipient of p1.14 T cell line

B. CD4⁺ expression in recipient of p1.8 T cell line

C. VCAM-1 expression in recipient of p1.14 T cell line

D. VCAM-1 expression in recipient of p1.8 T cell line

Figure 5 (A-D).

A. Cardiac tissues illustrate infiltration of CD4⁺ cells and detectable upregulation of VCAM-1 (C) after passive transfer of NT5/6-specific T cell line p1.14. Passive transfer of NT6-specific T cell line 1.8 did not reveal the presence of CD4⁺ cells (B) or upregulation of VCAM-1 (D) in the valve. Magnification, $\times 40$.

Rat T cell line p1.14 (NT5/6): **DKLKQORDTLSTQKETLE**-----

Human T cell clone 7.1.10 - - **LKQORDTLSTQKETLEREVQN**

Rat T cell line p1.8 (NT6) **QORDTLSTQKETLEREVQN**

Figure 6.

Amino acid sequence of alignment of streptococcal M5 protein amino acid sequence recognized by Lewis rat M5 specific T cell lines associated with passive transfer of valvulitis compared with the streptococcal M5 protein amino acid sequence recognized by an intralesional T cell clone isolated from valve in human rheumatic carditis [24].

Table 1
Amino acid sequence of group A streptococcal M5 protein peptides*

Peptide	Sequence	Residue positions
NT-1 ⁺	AVTRGTINDPQRAKEALD	1-18
NT-2	KEALDKYELENHDLKTKN	14-31
NT-3	LKTKNEGLKTENEGLKTE	27-44
NT-4	GLKTENEGLKTENEGLKTE	40-58
NT-4/5	GLKTEKKEHEAENDKLK	54-70
NT-5	KKEHEAENDKLKQQRDTL	59-76
NT-5/6	DKLKQQRDTLSTQKETLE	67-84
NT-6	QRDTLSTQKETLEREVQN	72-89
NT-7	REVQNTQYNNETLKIKN	85-102
NT-8	KIKNGDLTKELNKTRQEL	98-115
B1-A	TRQELANKQQESKENEKAL	111-129
B1-B	ENEKALNELLEKTVKDKI	124-141
B1-B2	VKDIAKEQENKETIGTL	137-154
B2	TIGTLKKILDETVKDKIA	150-167
B2-B3A	KDKIAKEQBNKETIGTLK	163-180
B3A	ITGLKKILDETVKDKLAK	176-193
B2-B3B	DKLAKEQKSKQNIGALKQ	189-206
B3B	GALKQELAKKDEANKISD	202-219
C1A	NKISDASRKGLRRDLAS	215-232
C1B	DLDASREAKKQLEAEHKQ	228-245
C1-C2	AEHQKLEEQNKISEASRK	241-258
C2A	EASRKGLRRDLASREAK	254-271
C2B	SREAKKQLEAEQQKLEEQ	267-284
C2-C3	KLEEQNKISEASRKGLRR	280-297
C3	KGLRRDLASREAKKQ	293-308

* Predicted amino acid sequence of serotype 5 M protein from *S. pyogenes*, strain Manfredo.

⁺ Peptides represent the sequence found in the A repeat amino terminal region (NT), B (B), and C (C) repeat regions of the M5 protein

Table 2
Group A streptococcal M5 protein peptide groups

Peptide Group	Peptides
Group 1 (A repeat)	NT1, NT2, NT3, NT4, NT4/5
Group 2 (A repeat)	NT5, NT5/6, NT6, NT7, NT8
Group 3	B1-A, B1-B, B1-B2, B2, B2-B3A
Group 4	B3A, B2-B3B, B3B, C1A, C1B
Group 5	C1-C2, C2A, C2B, C2-C3, C3

Table 3
Streptococcal M5 Protein Peptide Groups 1 and 2 Induce Valvulitis in Lewis Rats

Treatment	Myocarditis (positive/tested)	Score (infiltrates [#])	Valvulitis (positive/tested)	Score (infiltrates [#])
M5 Group 1	0/3	0	2/3	2 ⁺
M5 Group 2	0/3	0	2/3	2 ⁺
M5 Group 3	1/3	1	0/3	0
M5 Group 4	0/3	0	0/3	0
M5 Group 5	0/3	0	0/3	0
pepM5	1/3	1 ⁺	2/3	2 ⁺
Adjuvant control [*]	0/9	0	0/9	0

Statistical analysis by one way ANOVA, p= 0.045.

[#] Presence of inflammatory cells

^{*} PBS

⁺ Freund's complete adjuvant

Table 4
Streptococcal M5 peptides induce valvulitis in Lewis rat model

Peptide antigen	Myocarditis (positive/tested)	Score (infiltrates [#])	Valvulitis (positive/tested)	Score (infiltrates [#])
NT1	0/3	0	0/3	0
NT2	0/3	0	0/3	0
NT3	0/3	0	0/3	0
NT4	1/3	1.5	2/3	2+
NT4/5	0/3	0	0/3	0
NT5	0/3	0	0/3	0
NT5/6	0/3	0	1/3	2+
NT 6	0/3	0	0/3	0
NT7	0/3	0	1/3	0
NT8	0/3	0	0/3	0
pepM5	1/3	1	2/3	2+
PBS	0/3	0	0/3	0

Amino terminal peptides of M5 protein induce valvulitis in Lewis rat model. M5 peptides NT4, NT 5/6, and NT7 induced valvulitis and NT 4 also induced myocarditis.

Statistical analysis by one way ANOVA $p = 0.10$.