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Mimicry in Recognition of Cardiac Myosin Peptides by Heart-Intralesional T Cell Clones from Rheumatic Heart Disease

Kellen C. Faé,*‡ Danielle Diefenbach da Silva,* Sandra E. Oshiro,*** Ana C. Tanaka,* Pablo M. A. Pomerantzeff,* Corinne Douay,‡ Dominique Charrant,‡ Antoine Toubert,‡ Madeleine W. Cunningham,§ Jorge Kalil,*† § and Luiza Guilherme²***

Molecular mimicry between Streptococcus pyogenes Ags and human proteins has been considered as a mechanism leading to autoimmune reactions in rheumatic fever and rheumatic heart disease (RHD). Cardiac myosin has been shown as a putative autoantigen recognized by autoantibodies of rheumatic fever patients. We assessed the human heart-intralesional T cell response against human light meromyosin (LMM) and streptococcal M5 peptides and mitral-valve-derived proteins by proliferation assay. Cytokines induced by LMM peptides were also evaluated. The frequency of intralesional T cell clones that recognized LMM peptides was 63.2%. Thirty-four percent of T cell clones presented cross-reactivity with different patterns: 1) myosin and valve-derived proteins; 2) myosin and streptococcal M5 peptides; and 3) myosin, valve-derived proteins and M5 peptides. In addition, several LMM peptides were recognized simultaneously showing a multiple reactivity pattern of heart-infiltrating T cells. Inflammatory cytokines (IFN-γ and TNF-α) were predominantly produced by heart-infiltrating T cells upon stimulation with LMM peptides. The alignment of LMM and streptococcal M5 peptides showed frequent homology among conserved amino acid substitutions. This is the first study showing the cellular response by human heart-infiltrating T cells against cardiac myosin epitopes in RHD patients. The high percentage of reactivity against cardiac myosin strengthens its role as one of the major autoantigens involved in rheumatic heart lesions. T cell reactivity toward myosin epitopes in RHD patients may also trigger the broad recognition of valvular proteins with structural or functional similarities.


Rheumatic fever (RF) occurs after an untreated pharyngeal infection by group A streptococci in susceptible individuals. Arthritis is the first and most common manifestation of RF, affecting ~90% of patients. Carditis is the most serious manifestation and affects 30–45% of RF patients. Although arthritis does not cause permanent injury to the joints, carditis causes heart damage with pericardial, myocardial, and endocardial involvement, followed by progressive and permanent valvular lesions, leading to rheumatic heart disease (RHD) (1, 2).

Molecular mimicry between group A streptococcal Ags and human proteins, mainly heart tissue proteins, has been considered as a plausible mechanism accounting for the association of the streptococcal infection and the autoimmune reactions observed in RF/RHD patients (3–7). It was shown that cross-reactive autoantibodies against group A streptococcal cell wall Ags and heart tissue were present in sera of RF patients, as well as in rabbits immunized with group A streptococci (3–5). The production of mouse and human mAbs against group A streptococcal confirmed these cross-reactions and identified myosin, tropomyosin, and vimentin as heart autoantigens cross-reactive with group A streptococci (6, 8, 9). New autoantigens, such as laminin, were also identified (10). Anti-myosin and anti-streptococcal mAbs not only recognized M protein but also the immunodominant epitope of group A carbohydrate, N-acetylglycosamine (11). Anti-myosin Abs affinity purified from acute rheumatic sera identified a cross-reactive epitope composed of 5-aa residues (Gln-Lys-Ser-Lys-Gln), localized near the pepsin cleavage site of M5 and M6 proteins (9).

The pathogenic role of anti-myosin Abs was suggested in experimental assays, in which anti-myosin Abs from RF patient sera were applied to neonatal rat cardiac myocytes. The anti-myosin Abs caused an increased calcium uptake and retention, leading to myocyte dysfunction and possibly cell death, suggesting that anti-myosin Abs might be extremely deleterious to cardiac function (12).

The link between humoral and cellular immune responses in RHD may be the fact that streptococcal and heart tissue cross-reactive Abs may bind to the valvular endothelium leading to inflammation, cellular infiltration, and valve scarring as described previously (10, 13). Once activated, the valvular endothelium expressed increased amounts of the adhesion molecule VCAM-1, which facilitates the binding/adhesion of T cells and consequently.

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3 Abbreviations used in this paper: RF, rheumatic fever; RHD, rheumatic heart disease; LMM, light meromyosin; pl, isoelectric point; SI, stimulation index.
extravasation into the valves, leading to the cycle of scarring neovascularization, and infiltration of lymphocytes (13).

The presence of CD4+ T cells at the heart lesion sites of RHD patients has been shown (13–15). The functional role of these infiltrating T cells was described by our group. We isolated and identified streptococcal M5- and heart-tissue-protein-cross-reactive CD4+ T cell clones, strengthening the hypothesis of molecular mimicry between β hemolytic streptococci and heart tissue proteins leading to tissue damage in RHD patients (16).

Before heart-infiltrating T cells had been described, peripheral T cell responses from RF patients and mice pointed out several M protein epitopes recognized by CD8+ (17, 18) and CD4+ T lymphocytes (19, 20). Myosin/streptococcal M5 protein cross-reactive T cell epitopes were identified in mice immunized with intact cardiac myosin (21). T lymphocytes from lymph nodes of Lewis rats that developed myocarditis and valvulitis after immunization with streptococcal M protein responded to M protein, intact cardiac myosin, and human cardiac myosin β-chain synthetic peptides (light meromyosin (LMM) fragment) (22). Purified S2 cardiac myosin fragment also induced severe myocarditis as well as valvulitis in the Lewis rats. T cell lines derived from heart-infiltrating T cells of these rats proliferated in response to streptococcal M5 peptides and cardiac myosin (23), supporting the pathogenic role of myosin T cell cross recognition.

Considering all these data that confirmed myosin as one of the major autoantigens involved in rheumatic heart lesions, in this study we analyzed the reactivity of human heart-infiltrating T cell clones from severe RHD patients against human cardiac myosin β-chain peptides and searched for valve-derived proteins and M5 protein cross-reactivity. We also analyzed the cytokine production of intralesional T cell clones responsive to human cardiac myosin and verified their clonality by TCR usage and the CDR3 sequencing. The definition of the autoantigens recognized by the heart-infiltrating T lymphocytes will contribute to understanding more fully the mechanisms underlying the autoimmune lesions leading to RHD.

Materials and Methods

Patients

We studied six severe RHD patients who were followed for a period of 2–5 years by a cardiologist from the Heart Institute at the University of São Paulo (São Paulo, Brazil). The medium age of the patients was 10 years (SD ± 4.6). The samples collected were surgical fragments obtained during valve correction surgery. The surgical fragment collection procedures were approved by the Heart Institute Ethics Committee (Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo), and informed consent was obtained from parents of the patients participating in this study. We analyzed one heart fragment for each severe RHD patient, except for patient no. 1 from whom we obtained two heart fragments; one derived from the mitral valve and the other from papillary muscle.

Establishment of heart-infiltrating T cell lines and T cell clones

Heart-infiltrating T cell lines were established from seven surgical fragments from six RHD patients. Heart tissue was finely minced with injection needles and small scissors, placed in flat-bottom 96-well plates (BD Biosciences) with DMEM (Invitrogen Life Technologies) supplemented with 2 mM L-glutamine (Invitrogen Life Technologies), 10% pooled normal human serum, antibiotics (Gentamicin and Penicillin at the concentration of 40 and 20 μg/ml, respectively), and 40 U/ml human IL-2 (PrepoTech) on a HLA-DR-matched feeder layer of PBMC (105 cells/well), irradiated at 5000 rad as described previously (16). All T cell lines were further expanded with irradiated HLA-DR-matched feeder cells and PHA-P (2.5 μg/ml).

T cell clones were obtained by the limiting dilution method in the presence of 105 HLA-DR-matched PBMC-irradiated, PHA-P (2.5 μg/ml) and IL-2 (40 U/ml) as mentioned above. For T cell clones expansion, we also used IL-7 and IL-15 (2.5 ng/ml; PrepoTech). Of 160 T cell clones generated, 38 were tested against LMM and streptococcal M5 peptides and also against heart tissue-derived proteins isolated by m.w. and isoelectrical point (pl).

Immunohistochemistry

Sections of 4 μm were cut from cardiac tissue prepared from frozen fragments and specimens embedded in OCT 4583 (Miles). Anti-CD4 (MT 310) and anti-CD8 (DK 25) (Dakopats) mAbs were used to define T cell subpopulations. Peroxidase-coupled avidin (Dakopats) was added later, and the reaction was developed with diamino-benzidine (Sigma-Aldrich).

Flow cytometry analysis

T cell lines and T cell clones were analyzed using the following mAbs: anti-αβ TCR (FITC), anti-CD3 (PE), anti-CD4 (PE), and anti-CD8 (FITC) (all obtained from BD Pharmingen). A total of 10 × 10⁶ events gated on lymphocytes region was determined by flow cytometry using a FACSscan cytometer with CellQuest software (BD Biosciences).

Peptide synthesis

Fifty peptides of the LMM fragment (Table I) were designed based on the cardiac myosin β-chain protein sequence (24) and were synthesized as 18-mer peptides with 5-mer overlaps as described previously (23). M5/streptococcal peptides were synthesized by the tea bag method using t-BOC chemistry (25) and were checked by mass spectrometry and purified by high pressure liquid chromatography. Eight N-terminal overlapping M5 peptides were synthesized as 15- to 20-mers, based on the streptococcal M5 protein sequence (26, 27) (Table II).

Preparation of mitral valve proteins

Mitral valve-derived proteins were obtained from lysates of postmortem normal human mitral valve tissue and separated by two-dimensional electrophoresis (28). Briefly, the technique combines urea isoelectrofocusing electrophoresis using a broad range carrier ampholytes mixture (pH 3.6–9.2) in the first dimension with SDS-PAGE (5–15% polyacrylamide gel) electrophoresis in the second dimension to resolve heart tissue proteins by size and m.w.

Mitral valve microfragments were homogenized in lysis buffer (50 nM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1 mM PMFS, 100 μg/ml N-tosyl-L-phenylalanine chloromethyl ketone, 1 μg/ml leupeptin) for 30 min, on ice. The supernatant was collected after centrifugation at 2000 × g for 10 min, and 200 μg/ml mitral valve proteins were isolated by isoelectrofocusing electrophoresis followed by SDS-PAGE electrophoresis. Thirty proteins were blotted onto nitrocellulose membranes. The blots were cut and solubilized with DMSO (Merck) precipitated in sodium carbonate/bicarbonate buffer 0.05 mol/l (pH 9.6) and washed with RPMI 1640 medium (Sigma-Aldrich) (29), yielding a fine suspension of protein-loaded nitrocellulose particles that were used as Ags for the proliferation assay.

Proliferation assay

Proliferation assays were performed in 96-well plates incubating 3 × 10⁵ T cell clones, 105-irradiated (5000 rad) HLA-DR-matched mononuclear cells with 6 μg/ml LMM peptides, 5 μg/ml streptococcal M5 synthetic peptides, and 50 μl/well of mitral valve proteins identified by m.w. and pl and blotted into nitrocellulose as described above, for 96 h at 37°C in a humidified 5% CO2 incubator. Negative controls were a suspension of lymphoblasts and irradiated PBMC in DMEM for the peptides and 50 μl of a protein-free nitrocellulose suspension for valve protein experiments. PHA-P (2.5 μg/ml) was used as positive control for proliferative responses. All Ags were tested in triplicate and pulsed-labeled with 0.5 μCi/well of tritiated thymidine (Amersham Biosciences) for the final 18 h of culture. Cells were then harvested and analyzed in an automated beta counter (Beta plate 1205-LKB). Proliferative response was considered positive when the stimulation index (SI) was ≥2.5. The SI value 2.5 was determined based on the pattern of reactivity of patients and controls (healthy individuals) previously analyzed (data not shown). T cell clones positive with the 2.5 SI cutoff were consistently significant (Student’s t test) when compared with the negative control (absence of Ag). Background responses of T cell proliferation assays were considered based on cpm obtained from irradiated APCs and T cell clones in the absence of Ag (negative control). The mean of all negative controls was 174.8 cpm. For positive controls (PHA-stimulated cells) the mean was 13318.7 cpm.

Ag-specific cytokine production

T cell clones (5 × 10⁵) were stimulated with 6 μg/ml LMM peptides, in the presence of 5 × 10⁵-irradiated HLA-DR-matched mononuclear cells for
Peptide | Amino Acid Sequences | Residues Location | Peptide | Amino Acid Sequences | Residues Location
---|---|---|---|---|---
LMM1 | KEALISSLTRGKLTYYQQ | 1295–1312 | LMM26 | BGDLMEMITGSHANPM | 1620–1637
LMM2 | TYYQGLEDRLQLLEEVRK | 1308–1325 | LMM27 | ANRMAAQQKVDVLQLSL | 1633–1650
LMM3 | EEEVKARNALALOSAR | 1321–1338 | LMM28 | SSLSLLKQTGQMDHAYR | 1646–1663
LMM4 | LOSARDCDKLLRQYEEK | 1334–1351 | LMM28B | SFYAVROQSEHNAEKAI | 1659–1677
LMM5 | EYBTEETAKALRQVLSK | 1346–1364 | LMM30 | RANDDLKENIAVYBHNN | 1663–1680
LMM6 | BVLKSKANSAVQMTKKE | 1360–1377 | LMM31 | EELRRAVQDTEQSGSLK | 1685–1702
LMM7 | ETYYDEAIDIQRKAECSLE | 1373–1390 | LMM32 | RSRLKQEJLITSVQR | 1698–1715
LMM8 | ELEAKKALKLQIARASE | 1386–1403 | LMM33 | SERVQLHNSQNSLNIQNC | 1711–1728
LMM9 | QAEABEAVIRHCASE | 1399–1416 | LMM34 | LNIQKRMNADLQSLQTE | 1724–1741
LMM10 | CSSELETKHLRQNEEOL | 1412–1429 | LMM35 | EESIQUEEILAPNEEKQ | 1740–1755
LMM11 | KIDLMGVORERNGAAA | 1425–1442 | LMM36 | LTEQKADENATLPDIWG | 1750–1767
LMM12 | AAAALDKQKDRFDKTLA | 1438–1455 | LMM37 | AAMAEELKKEQTDSSAH | 1763–1780
LMM13 | DKLAAEMQKARYEESQESL | 1451–1468 | LMM38 | TSAHELRRKMNQETKIQD | 1776–1794
LMM14 | QSEOESQKARSLST | 1464–1481 | LMM39 | TYYDIQLRDIAEOQAIK | 1790–1807
LMM15 | SLSTELFKKNAYESLES | 1477–1494 | LMM40 | RJQIAEKQLQOEALREV | 1802–1820
LMM16 | KSELEHLETPRKENQLQ | 1490–1507 | LMM41 | KARLEVRELEHESAQVR | 1821–1840
LMM17 | NRNMLEEISDLYGLODG | 1503–1520 | LMM42 | KERANAEKVRQMQEKER | 1829–1846
LMM18 | KEGQGGCTPHTVEGERQ | 1515–1533 | LMM43 | RSEKKEFKTLYTDER | 1842–1859
LMM19 | KVKEKLEAKMEQSLAE | 1529–1546 | LMM44 | TEEDKNIOLRQLDVLKDL | 1855–1872
LMM20 | LOSALEERAEASRHEGKT | 1541–1559 | LMM45 | LVDKQLOKVAYKQEEA | 1868–1885
LMM21 | REKGLQACLEPQNIKAE | 1555–1572 | LMM46 | QABERSAQQANTNSLKR | 1881–1898
LMM22 | NQKAEERKQLEDEEEM | 1567–1585 | LMM47 | LSRKFPQVHELQEEA | 1894–1911
LMM23 | DEEMQEEARNKLRLVSDL | 1581–1598 | LMM48 | AEBRADLAEQSVNLRAK | 1907–1924
LMM24 | VDNLQTVSDAATKSNB | 1594–1611 | LMM49 | KLEBAKGIDRTKLLNL | 1920–1936
LMM25 | BESEHKEAKGKEMSHBN | 1607–1624

*Peptides based on the cardiac myosin β-chain protein sequence published by Diederich et al. (24). Shared amino acid sequences were underlined.

48 h. Supernatant from T cell cultures were assayed for IFN-γ, TNF-α, IL-10, and IL-4 by cytometric bead array kit (BD Biosciences). Cytokine detection by cytometric bead array was performed according to manufacturer’s instructions. Acquisition and analysis were performed in a FACS-Calibur flow cytometer (BD Biosciences). Cytokine analysis was performed based on a standard cytokine concentration curve, with the detection limit of 10–5000 pg/ml for all cytokines tested. Spontaneous cytokine productions from T cell clones and irradiated mononuclear cells in the absence of Ags were considered to calculate the cytokine production of each LMM peptide tested.

**TCR-BV BJ gene usage**
RNA was extracted from cell pellets of T cell clones. The cDNA was prepared from 1 μg of total RNA with AMV reverse transcriptase (cDNA cycle kit; Invitrogen Life Technologies) as described by the manufacturer. cDNA samples were then amplified for 40 cycles with specific BV and BJ (13 families) primers and an internal fluorescent BC primer. Results were analyzed by Immunoscope software (30).

**DNA sequencing for CDR3 determination**
BVBC PCR products were cloned into pCR2.1-TOPO vector (Invitrogen Life Technologies) and transformed into *Escherichia coli* supercompetent cells (Invitrogen Life Technologies). After blue/white screening of recombinant plasmids on X-galactoside indicator plates, plasmids were purified by alkaline lysis followed by phenol/chloroform/iso-amyl alcohol. Inserts were checked by agarose gel electrophoresis after BVBC PCR amplification, and both strands were sequenced with BigDye Terminator v3.0 Ready Reaction Cycle sequencing kit (Applied Biosystems). Products were loaded on 4.25% acrylamide sequencing gels (377A DNA sequencer; Applied Biosystems). Reaction Cycle sequencing products were analyzed and with Chromas version 1.45 software from School of Healthy Science (Griffith University, Southport, Queensland, Australia) and nucleotide translation to protein with ExPASy (Expert Protein Analysis System) protein sequence tools of the Swiss Institute of Bioinformatics.

**Results**

**T cell subsets in heart tissue and intralesional T cell lines**
CD4⁺ and CD8⁺ T cell subsets were determined by immunohistochemical analysis on heart tissue fragments. Seven intralesional T cell lines were generated, in which four were from mitral and aortic valves, one from papillary muscle, and two from left atrium fragments. CD4⁺ T cells were predominantly found in situ (Table III). Heart tissue-infiltrating T cell lines were CD3⁺ αβTCR⁺, and most of them, 6 of 7, were predominantly CD4⁺. Only one T cell line (Lu7.1, patient no.2) presented similar amounts of CD4⁺ and CD8⁺ T cells (49.4 and 38.5%, respectively) (Table III). Intralesional T cell clones derived from these T cell lines were CD4⁺ αβTCR⁺, except for two clones (3.1.21, patient no. 1 and 10.2.13, patient no. 3) characterized as CD8⁺ αβTCR⁺.

**Cross-reactive Ag recognition by heart-infiltrating T cell clones**
Seven heart-infiltrating T cell lines were established from heart tissue fragments of six RHD patients in the presence of IL-2 without addition of exogenous Ags. Thirty-eight heart-infiltrating T cell clones were tested against 50 peptides derived from the LMM fragment of the human cardiac myosin β-chain, 30 mitral valve-derived proteins identified by m.w. and pI, and eight N-terminal overlapping M5 peptides to verify the presence of cross-reactivity between streptococcal Ags and heart proteins.

Among the intralesional T cell clones studied, 24 recognized at least one of the LMM peptides tested showing a very high reactivity (63.2%), despite not having been previously stimulated in vitro with self Ags (Table IV). Interestingly, among the 63.2% of
positive intraleisonal T cell clones, we observed that the reactivity against LMM peptides was 36.8% for T cell clones derived from aortic/mitral valve lesions and 26.3% for myocardium-derived T cell clones (Table IV).

T cell reactivity against LMM peptides was spread along the sequence of the LMM fragment, and among the 50 LMM peptides only 10 were not recognized. Although we have not identified an immunodominant region, some LMM peptides encompassing amino acid residues 1477–1494 (LMM15), 1672–1689 (LMM30), 1724–1758 (LMM34 and LMM35), 1790–1807 (LMM39), and 1920–1936 (LMM49), were preferentially recognized (Table IV).

Interestingly, we observed that 16 LMM peptides (16 of 40, 40%) were exclusively recognized by aortic and mitral valve-derived T cell clones, whereas only eight LMM peptides (8 of 40, 20%) were exclusively recognized by myocardium-derived T cell clones. However, 16 (16 of 40, 40%) were recognized by both mitral valve and myocardium-derived T cell clones (data not shown).

The recognition of the LMM peptides by intraleisonal T cell clones involved two major points: 1) cross-reactivity with valvular proteins and streptococcal M protein; and 2) multiple peptide reactivity. In Table V, we showed the reactivity of five selected intraleisonal T cell clones focusing on these major points. Three patterns of cross-reactivity were observed: 1) myosin and valve-derived proteins (first pattern); 2) myosin and streptococcal M5 peptides (second pattern); and 3) myosin, valve-derived proteins and streptococcal M5 peptides (third pattern). The first pattern is illustrated by a cross-reactive mitral valve-derived T cell clone (10.2.13) that presented very high SI (SI >20.0) against the LMM 36 and 38 peptides encompassing 1750 through 1794 aa residues and a response against two different regions (LMM 14 and 40) with SI >6.0. This clone also recognized the 37 kDa/PL 5.12 and 58 kDa/PL 5.12 valve-derived proteins, and emphasized the molecular mimicry between self-Ag (myosin and valvular proteins). These proteins (37 kDa/PL 5.12 and 58 kDa/PL 5.12) were analyzed by peptide mass fingerprinting (data not shown) and presented a high percentage of sequence coverage with a myofibril associated glycoprotein-4 (48%) and vimentin with 86% of sequence coverage, respectively. Another example of this cross-reactivity is a myocardium-derived T cell clone 3.2.12.9 that recognized a 56-53 kDa/PL 6.76 valve-derived protein SI (5.9) and the LMM 41(1816–1833) peptide. The second pattern, cross-reactivity between myosin and streptococcal M5 peptides, is represented by the high reactivity of 7.1.8 T cell clone against the M5(1–20) peptide (SI = 14.6) and the LMM 4(1334–1351) (SI = 4.5). This T cell clone displayed the molecular mimicry between self-Ag and Streptococcus pyogenes Ags. T cell clone 3.1.17 illustrated the third pattern that includes cross-reactivity between self-Ags (myosin and valvular proteins) and S. pyogenes Ags, in which the highest reactivity was against 59-56 kDa/PL 7.76 valvular protein (SI = 5.4) (Table V).

The mitral valve-derived proteins 56-53 kDa/PL 6.76, 36 kDa/PL 7.64 seem to be either cytoskeleton proteins or enzymes that match with plasminogen precursor and enzymes such as lactate dehydrogenase. The protein 59-56 kDa/PL 7.76 could not be identified.

In addition, the second major point as mentioned above was the multiple peptide reaction that was observed for some of the positive T cells 3.1.7, 4.2.3.39, and 4.2.3.45 (Table IV). The 4.2.3.45 T cell (Table V) conceived as prototype of cross-reactivity recognized six LMM peptides, with different levels of reactivity, in which the highest reactivity was against the LMM 41(1816–1833) peptide (SI = 6.8). This T cell also recognized a mitral valve-derived protein (36 kDa/PL 7.64) as described for the first pattern of cross-reactivity. Because we were unable to analyze the clonality of T cell 4.2.3.45, the possibility could not be excluded that it contained more than one clone.

Among the cross-reactive T cell clones (13 of 38, 34.2%), seven T cell clones presented the first pattern of cross-reactivity (myosin and valvular proteins), three clones the second pattern (myosin and M protein), and three clones the third pattern (myosin, valvular proteins and M protein). The multiple LMM reactivity (more than three LMM peptides) was detected for nine cross-reactive T cell clones (Table IV). As we mentioned above, we could not affirm that all of them were clonal populations. However, among those seven T cell clones in which we could define the TCR usage, we verified that at least three of the clones (3.1.3, 3.1.8, and 10.2.13) with multiple LMM reactivity were monoclonal populations (see Table VIII). Thus, the three patterns of cross-reactivity observed are in fact the prototypes of all the cross-reactive intraleisonal T cell clones studied. Considering all LMM-positive T cell clones (24 of 38, 63%) (Table IV), 18 T cell clones presented multiple LMM reactivity, in which nine of them were cross-reactive T cell clones as shown.

### LMM peptides induced inflammatory cytokine production

The Ag-specific cytokine production was evaluated in four LMM-reactive intraleisonal T cell clones (Table VI). The cytokines assessed were IFN-γ, TNF-α, IL-10, and IL-4. Among the eight LMM peptides tested, all induced TNF-α and seven induced IFN-γ. IL-4 was not produced. We also observed that three T cell clones also produced IL-10 when stimulated by some LMM peptides (Table VI).

---

**Table III. T cells subsets of surgical samples and intraleisonal T cell lines from RHD patients**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Tissue</th>
<th>Positive Cells/Field</th>
<th>T Cell Line</th>
<th>Positive Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mi.v.</td>
<td>3.2 2.4 1.3</td>
<td>Lu 3.1</td>
<td>94.4 99.8 87.2 7.4</td>
</tr>
<tr>
<td>2</td>
<td>F.M.</td>
<td>9.4 3.3 2.8</td>
<td>Lu 3.2</td>
<td>95.0 94.5 76.2 19.1</td>
</tr>
<tr>
<td>3</td>
<td>Mi.v.</td>
<td>1.2 1.2 1.0</td>
<td>Lu 7.1</td>
<td>94.2 91.1 49.4 38.5</td>
</tr>
<tr>
<td>4</td>
<td>L.A.</td>
<td>2.4 1.7 1.4</td>
<td>Lu 10.2</td>
<td>NT 87.7 86.4 0.6</td>
</tr>
<tr>
<td>5</td>
<td>L.A.</td>
<td>6.1 1.2 5.0</td>
<td>Lu 4.2</td>
<td>93.0 99.9 76.6 8.5</td>
</tr>
<tr>
<td>6</td>
<td>Ao.v.</td>
<td>0.7 0.3 2.3</td>
<td>LG 34.1</td>
<td>NT 99.0 80.0 9.6</td>
</tr>
</tbody>
</table>

* Positive patterns: CD4 **4**, CD8 **3**, CD4/CD8 **1.5**.

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*a* Tissue samples from these patients were analyzed by immunohistochemistry and T cell lines by flow cytometry. Intraleisonal T cell clones derived from these patients were mainly CD41* T* CD81*, except the clones 3.1.21 from patient no. 1 and 10.2.13 from patient no. 3 characterized as CD41* T* CD81*. Mi.v.: Mitral valve; Ao.v.: Aortic valve; F.M.: Papillary muscle; L.A., left atrium; NT, not tested.

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| Table IV. Reactivity of intralesional T cell clones against LMM peptides*
<table>
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<tbody>
<tr>
<td>Patient</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>LMM</td>
</tr>
<tr>
<td>1.2.9</td>
</tr>
</tbody>
</table>

*Total positive T cell clones/total tested: 63.2% (24/38); Mi. and Ao.v.-positive clones/total tested: 36.8% (14/38); myocardium-positive clones/total tested: 26.3% (10/38); T cells clones were tested against 50 synthetic peptides derived from the LMM fragment from human cardiac myosin β-chain (Table I) at the concentration of 6 μg/mL. Only peptides recognized by at least one T cell clone were shown. Proliferative responses of T cell clones are shown as SI, and values ≥2.5 were considered positive and are underlined and bold typed. Mean of cpm for negative controls was 174.8 and for positive controls 15245.8.

Clones from the same patient; Mi.v., mitral valve; Ao.v., aortic valve; L.A., left atrium; P.M., papillary muscle.
Amino acid residue homology between M5 and LMM epitopes recognized by heart-infiltrating T cell clones

Homology between cardiac myosin (LMM fragment) and M5 streptococcal peptides was searched using the Clustal W program for protein multiple sequence alignment server of the European Bioinformatics Institute (European Molecular Biology Laboratory) ([http://www.ebi.ac.uk/clustalw/]). We analyzed the degree of homology between M5 and LMM cross-reactive peptides recognized by three intralesional T cell clones (3.1.17, 7.1.8, and 7.1.10) (Table VII) by taking into consideration amino acid identities and conserved or semiconserved substitutions. The highest percentage of homology between peptide sequences were obtained with the peptides LMM10 (1413–1430) aligned with M5 (83–103) (87% of homology), followed by LMM12 (1439–1456) that aligned with M5 (83–103) and LMM25 (1607–1624) aligned with M5 (163–177) (both with 67% of homology) and LMM4 (1334–1351) that aligned with M5 (1–20) (53% of homology) (Table VII). The analysis of the alignment between the cross-reactive LMM and M5 peptides showed that conserved substitutions were the most frequent characteristic followed by identity (Table VII).

BVBJ usage and CDR3 sequences of some intralesional T cell clones that were reactive with human LMM peptides, mitral valve-derived proteins, and streptococcal M5 peptides

Molecular analysis first confirmed T cell monoclonality based on detection of a single BVBJ usage and a single β-chain CDR3 amino acid sequence (Table VIII). Several BVBJ families (Table VIII) capable of recognizing different cross-reactive Ags were found (Tables IV and V). Interestingly, we found three T cell clones (3.2.12.9, 3.1.3, 3.1.8) expressing the same BVBJ family (BV13 BJ257) together with the same CDR3 β-chain size and sequence (Table VIII), suggesting homologous structural recognition. T cell clone 3.2.12.9 is a prototype of the first pattern of cross-reactivity (recognition of myosin and valvular proteins); 3.1.8 and 3.1.3 T cell clones presented the same pattern, although they recognized different LMM Ags (Table IV) and the 35 kDa/pI 8.4 mitral valve-derived protein (data not shown).

Table VI. Ag-specific cytokine production by myosin primed heart-infiltrating T cell clones

<table>
<thead>
<tr>
<th>T Cell Clones</th>
<th>Ag</th>
<th>IFN-γ (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
<th>IL-10 (pg/ml)</th>
<th>IL-4 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.2.13</td>
<td>LMM14 (1464–1481)</td>
<td>24</td>
<td>476.9</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>LMM36 (1750–1767)</td>
<td>67.6</td>
<td>1036.3</td>
<td>51</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>LMM38 (1776–1794)</td>
<td>27.8</td>
<td>360.8</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>LMM40 (1802–1820)</td>
<td>10</td>
<td>245</td>
<td>20.9</td>
<td>–</td>
</tr>
<tr>
<td>4.2.3.45</td>
<td>LMM11 (1425–1442)</td>
<td>56</td>
<td>13</td>
<td>63</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>LMM49 (1920–1936)</td>
<td>97</td>
<td>27</td>
<td>123</td>
<td>–</td>
</tr>
<tr>
<td>7.1.8</td>
<td>LMM4 (1334–1351)</td>
<td>–</td>
<td>44.2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3.1.17</td>
<td>LMM25 (1607–1624)</td>
<td>73.6</td>
<td>252.1</td>
<td>73.2</td>
<td>–</td>
</tr>
</tbody>
</table>

* Cytokines production was calculated subtracting from baseline production (T cell and feeder). Level of detection for all cytokines was 10–5000 pg/ml. –, Cytokine production not detected.

Discussion

To our knowledge, this is the first report showing the cellular immune response and mimicry of human heart-infiltrating T cells against human cardiac myosin β-chain epitopes in RHD patients. We showed a very high frequency (63.2%) of heart-infiltrating T cell clones recognizing myosin peptides, despite not priming in vitro with self Ags. Although these T cell clones exhibited a high frequency of recognition of LMM peptides, it was not possible to identify an immunodominant epitope along the LMM sequence. However, six regions were preferentially recognized as defined by the LMM peptides encompassing amino acid residues 1477–1494 (LMM15), 1672–1689 (LMM30), 1724–1758 (LMM34 and 35), 1790–1807 (LMM39), and 1920–1936 (LMM49) (see Table IV). Interestingly, some of these regions (LMM39 and 49), were described as potential inducers of myocarditis and valvulitis in Lewis rats (23). The fact that cardiac myosin β-chain is the dominant isoform in human ventricle tissue and that immune responses to cardiac myosin are sufficient to induce myocarditis in animal models in the absence of infection (31) strengthens the role of cardiac myosin as an immunodominant cardiac autoantigen.
The results presented in this study from RHD patients showed the recognition of human LMM regions by both myocardium and valve-derived intralesional T cell clones that may contribute to the development of rheumatic valvular disease in humans. Despite the fact that myosin is an intracellular protein and is found in small amounts in valvular tissue (32), curiously we observed that 16 LMM peptides were exclusively recognized by T cell clones from mitral valve, whereas only eight LMM peptides were exclusively recognized by T cell clones from the myocardium, but 16 LMM peptides were recognized by T cells from both sites of the lesions. The reactivity against LMM peptides of these infiltrating T cells from both myocardium and valvular tissue may be attributable to the stimulation of these cells initially by the α-helical coiled-coil streptococcal M protein. Probably, cardiac myosin-autoreactive infiltrating T cells enter the valves through the activated valve surface endothelium during RHD (13). Then, with the progression of the lesions, scarring, and neovascularization in the valve, and exposure of new antigenic determinants, these streptococcal M protein/cardiac myosin cross-reactive T cells may recognize several other LMM peptides as well as valvular proteins, probably due to homology or structural similarities. This hypothesis is supported by the fact that myosin is not a predominant autoantigen in the myocardium, whereas only eight LMM peptides were exclusively recognized by T cell clones from the myocardium, but 16 LMM peptides were recognized by T cells from both sites of the lesions. The reactivity against LMM peptides of these infiltrating T cells from both myocardium and valvular tissue may be attributable to the stimulation of these cells initially by the α-helical coiled-coil streptococcal M protein. Probably, cardiac myosin-autoreactive infiltrating T cells enter the valves through the activated valve surface endothelium during RHD (13). Then, with the progression of the lesions, scarring, and neovascularization in the valve, and exposure of new antigenic determinants, these streptococcal M protein/cardiac myosin cross-reactive T cells may recognize several other LMM peptides as well as valvular proteins, probably due to homology or structural similarities. This hypothesis is supported by the fact that myosin is not a predominant autoantigen in the myocardium, whereas only eight LMM peptides were exclusively recognized by T cell clones from the myocardium, but 16 LMM peptides were recognized by T cells from both sites of the lesions.

Table VII. Alignment of cross-reactive M5 and LMM epitopes recognized by three heart-infiltrating T cell clones

<table>
<thead>
<tr>
<th>T Cell Clones</th>
<th>Epitopes Recognized</th>
<th>SI</th>
<th>Amino Acid Sequence Alignment</th>
<th>Homology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1.17</td>
<td>LMM25(1607–1624)</td>
<td>3.3</td>
<td>R S R N E A L R – V K K K M E G D L N</td>
<td>67</td>
</tr>
<tr>
<td>M5 (163–177)</td>
<td>3.0 – – – – E T I G T L K I L D E T V K</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.1.8</td>
<td>LMM4 (1334–1351)</td>
<td>4.5</td>
<td>L Q S A R H D C D L R E Q Y E E E</td>
<td>53</td>
</tr>
<tr>
<td>M5 (1–20)</td>
<td>14.6 T V T R G T I S D P Q R A K E A L D K Y</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.1.10</td>
<td>LMM10 (1413–1430)</td>
<td>3.0</td>
<td>C S S L E K T K H R L Q N E I E D L</td>
<td>87</td>
</tr>
<tr>
<td>M5 (83–103)</td>
<td>4.1 L K Q Q R D T L S T Q K E T L E R E V Q N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.1.10</td>
<td>LMM12 (1439–1456)</td>
<td>3.3</td>
<td>A A A A A L D K K Q R N F K I L A –</td>
<td>67</td>
</tr>
<tr>
<td>M5 (83–103)</td>
<td>4.1 L K Q Q R D T L S T Q K E T L E R E V Q N</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

aAmino acid residues identified with * are identical, : means conserved substitutions, and . means semiconserved substitutions. T cell clones 3.1.17 and 7.1.8 were prototypes of cross-reactivity patterns (Table V), and T cell clone 7.1.10 recognized the M5 (81–103) peptide, identified as a streptococcal M protein immunodominant region, as previously described (16, 35).

Table VIII. TCR usage and CDR3 sequences of heart-infiltrating T cell clones reactive against LMM or streptococcal M5 peptides and mitral valve-derived proteins

<table>
<thead>
<tr>
<th>T Cell Clones</th>
<th>BV Family</th>
<th>CDR3 Region (amino acid sequence and length)</th>
<th>BJ Family</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1.17*</td>
<td>BV5</td>
<td>SPGSHEQY (8 aa)</td>
<td>BJ2S7</td>
</tr>
<tr>
<td>10.2.13*</td>
<td>BV1</td>
<td>SISGGAWTDQY (12 aa)</td>
<td>BJ2S3</td>
</tr>
<tr>
<td>3.2.12.9*</td>
<td>BV13</td>
<td>SGRQGRQYEQY (10 aa)</td>
<td>BJ2S7</td>
</tr>
<tr>
<td>3.1.16*</td>
<td>BV13</td>
<td>SGRQGRQYEQY (10 aa)</td>
<td>BJ2S7</td>
</tr>
<tr>
<td>3.1.8*</td>
<td>BV13</td>
<td>SGRQGRQYEQY (10 aa)</td>
<td>BJ2S7</td>
</tr>
<tr>
<td>3.1.21*</td>
<td>BV14</td>
<td>STDSTSYEQY (10 aa)</td>
<td>BJ2S7</td>
</tr>
<tr>
<td>3.1.79*</td>
<td>BV3</td>
<td>SFTGRLDNEQF (11 aa)</td>
<td>BJ2S1</td>
</tr>
</tbody>
</table>

aT cell clones identified as prototype of cross-reactivity (Table V).
b,c,d T cell clones that also presented the first, second, and third pattern of cross-reactivity, respectively (data not shown).
Beside the high frequencies of recognition by human T cells of LMM peptides observed in this study and those of M5 peptides described previously (36), our data did not allow us to know whether these peptides are processed and presented in vivo. Data recently published by Cunningham and colleagues (33) showed that peripheral T cell clones obtained from a RF patient recognized cardiac myosin peptides and the whole myosin protein as well as the intact recombinant streptococcal M protein and M peptides. These results suggest that peptides similar to our synthetic peptides may be generated during Ag processing and presented in vivo. However, the exact sequences of peptides processed and presented by APC/MHC in vivo are unknown.

Most of the T cell clones analyzed in our study displayed multiple Ag reactivity, despite the fact that some T cell populations had been identified as monoclonal expansions. Three intralobular T cell clones (3.2.12.9, 3.1.3, 3.1.8) presented exactly the same BV13BJ2S7-CDR3 (SGRQGRYEQY), compatible with monoclonal expansions; however, they recognized several LMM peptides, except for the 3.2.12.9 T cell clone that recognized two LMM peptides. Other T cells displayed a similar pattern as shown in Table IV, but because some of them were not assayed for TCR usage and CDR3 sequences, we could not affirm their monoclonality. In addition, several cross-reactive intralobular T cell clones from one patient (no. 1) displayed different amino acid sequences in the CDR3 (Table VIII), compatible with the diversity of Ags recognized (Tables IV and V). These results reinforce the degenerate pattern of Ag recognition by the TCR as previously described (37–39). Another important finding was the fact that the intralobular T cell clones bearing BV3, BV5, BV13, and BV14 families capable of recognizing several cross-reactive Ags were found among the mononuclear cells that infiltrated the heart tissue as oligoclonal expansions and also in the periphery (40). These data indicate that such clonal populations migrate from the periphery to the heart lesions through valve endothelium. The fact that these clonal populations cross-recognized human LMM peptides, streptococcal M5 peptides, and mitral valve-derived proteins demonstrates the role of these cross-reactive Ags in the development of the rheumatic heart lesions.

Considering that cytokines are likely to be important second signals following an infection triggering effective immune responses in most of individuals and probably a deleterious response in autoimmune disease, we analyzed the cytokine production by cross-reactive intralobular T cell clones upon specific Ag stimulation with LMM peptides. These peptides predominantly induced inflammatory cytokine production (IFN-γ and TNF-α). Although these T cell clones are monoclonal populations, when in vitro stimulated by some LMM peptides they also produced low levels of IL-10. Similar results in animal models showed that hemagglutinin-specific T cell clones bearing identical (Vdj) β and (Vj) α rearranged sequences could release both Th1/Th2 cytokines (41, 42).

Recently, it was demonstrated that peripheral T cell clones from RF patients produced IFN-γ when stimulated with cardiac myosin peptides (33), giving support to the idea that these cells migrate from the periphery to the heart (39, 40). In addition, we showed that mononuclear cells from rheumatic heart lesions predominantly secret IFN-γ, TNF-α, and IL-10 in both myocardium and valvular tissue, with a scarcity of cells producing IL-4 in the valves (43). The fact that Ag-specific cross-reactive infiltrating T cell clones displayed the same cytokine pattern, such as observed in situ, reinforces the role of proinflammatory cytokines in the progression of RHD.

The cross-reactive LMM and M5 peptides recognized by intralobular T cell clones showed high homology, and conserved amino acid substitutions were frequently observed. These results are in agreement with previous reports, showing that self Ags and viral and bacterial proteins that present structural similarities without sequence identities are able to activate T cell clones, and that identity of a few amino acid residues are enough for triggering T cell cross-recognition (44, 45). In contrast, some results in animal models showed that a single modification in the TCR peptide contact region or in the MHC anchor residues abolished peptide recognition or changed the T cell function, from pathogenic to regulatory profile (46, 47). In type 1 diabetes animal model, diabetogenic T cells primed for one glutamic acid decarboxylase epitope could generate functionally distinct T cell populations, depending on the extension of amino acid residues. One T cell population induced a regulatory response preventing diabetes in NOD mice, and another population was islet-invasive (46, 47).

Recent studies showed that lymph node-derived T cells from BALB/c mice immunized with human cardiac myosin recognized M protein peptides (21). The M5 peptides, B2 (150–167) and NT4 (40–58) demonstrated 47% of homology with cardiac myosin peptides from the human LMM(1313–1329) region and identity with 4-aa residues of the human S2(1279–1286) region, respectively (48). S2 epitopes, located in the rod region of cardiac myosin, induced myocarditis and valvulitis in Lewis rats (23). It is important to note that these cross-reactive streptococcal M5 peptides aligned with the sequences of the M5 peptides recognized by T cell clones from RHD patients, showing that some regions of streptococcal M5 protein are involved with the development of inflammatory heart disease, leading to myocarditis/valvulitis in both animals and humans.

Altogether, we showed for the first time, mimicry and recognition of human cardiac myosin peptides by heart-infiltrating T cell clones from RHD patients. We also showed that myosin, a myocardium/streptococcal cross-reactive protein, potentially has the ability to generate cellular cross-reactivity with valvular tissue proteins initially by mimicry and then by an epitope spreading mechanism. The data suggest that myosin may trigger the broad recognition of valvular proteins with structural or functional similarities. The further molecular characterization of the autoantigens recognized by heart-infiltrating T cells certainly will contribute to a better understanding of the autoimmune reactions in RHD and in the future could be used as a modulator of the aggressive response against the valve.

Disclosures
The authors have no financial conflict of interest.

References
with streptococcal M protein and myosin recognize the sequence GLN-LYS-


