Identification of Cardiac Troponin I Sequence Motifs Leading to Heart Failure by Induction of Myocardial Inflammation and Fibrosis

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Background—Despite the widespread use of cardiac troponins for diagnosis of myocyte injury and risk stratification in acute cardiac disorders, little is known about the long-term effects of the released troponins on cardiac function. Recently, we showed that an autoimmune response to cardiac troponin I (cTnI) induces severe inflammation and subsequent fibrosis in the myocardium. This autoimmune disorder predisposes to heart failure and cardiac death in mice.

Methods and Results—To investigate the role of cTnI-specific T cells, T cells were isolated from splenocytes of mice immunized with murine cTnI (mcTnI). Wild-type mice that received mcTnI-specific T cells showed high mcTnI-specific antibody titers, increased production of the proinflammatory cytokines interleukin-1β and tumor necrosis factor-α, severe inflammation and fibrosis in the myocardium, and reduced fractional shortening. To identify the antigenic determinants of troponin I responsible for the observed inflammation, fibrosis, and heart failure, 16 overlapping 16mer to 18mer peptides covering the entire amino acid sequence of mcTnI (211 residues) were synthesized. Only mice immunized with residues 105 to 122 of mcTnI developed significant inflammation and fibrosis in the myocardium, with increased expression of the inflammatory chemokines RANTES, monocyte chemotactic protein-1, macrophage inflammatory protein-1α, macrophage inflammatory protein-1β, macrophage inflammatory protein-2, T-cell activation-3, and eotaxin and the chemokine receptors CCR1, CCR2, and CCR5. Mice immunized with the corresponding human cTnI residues 104 to 121 and the mcTnI residues 131 to 148 developed milder disease.

Conclusions—Transfer of troponin I–specific T cells can induce inflammation and fibrosis in wild-type mice, which leads to deterioration of contractile function. Furthermore, 2 sequence motifs of cTnI that induce inflammation and fibrosis in the myocardium are characterized. (Circulation. 2008;118:2063-2072.)

Key Words: inflammation | heart failure | myocarditis | troponin | immunology

Heart failure has become an increasingly prevalent disorder with considerable morbidity and mortality. Although many causal mechanisms, such as inherited cardiomyopathies, ischemic cardiomyopathy, or muscular overload, are easily identified in clinical practice, the molecular mechanisms that determine the progression of heart failure or ventricular remodeling are largely unknown. The evidence that inflammatory mechanisms may contribute to progressive heart failure is compelling. Thus, myocardial infiltration of lymphocytes and mononuclear cells, increased expression of proinflammatory chemokines and cytokines, and circulating autoantibodies are frequently observed in myocarditis and dilated cardiomyopathy. The antibodies identified in patients with dilated cardiomyopathy are directed against various myocardial constituents.1-3

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Cardiac troponins in blood are the preferred biomarkers of myocardial injury. The fact that they are strictly intracellular proteins that are not found in the circulation of healthy individuals provides a high level of clinical sensitivity and specificity even when cardiac lesions are small. Thus, any significantly detectable troponin in circulation is considered a sign of acute myocardial cell damage.4,5

Nishimura et al6 reported that programmed cell death-1 receptor–deficient mice developed autoantibodies against cardiac troponin I (cTnI) and, as a consequence, severe dilated cardiomyopathy. They further found that administration of monoclonal anti-cTnI antibodies induced myocardial dysfunction, most likely by facilitating Ca2+ influx into cardiomyocytes.7 We and others have shown that autoanti-
bodies to cTnI are present in patients with acute coronary syndrome. These antibodies may interfere with diagnostic assays, leading to unpredictable results, and may have an impact on the improvement in left ventricular ejection fraction after myocardial infarction. These findings indicate that induction of an autoimmune response to cTnI is not a rare event in patients. Recently, we showed that an autoimmune response to murine cardiac troponin I (mcTnI) induces severe event in patients. Recently, we showed that an autoimmune response to murine cardiac troponin I (mcTnI) induces severe inflammation in the myocardium followed by fibrosis and heart failure with increased mortality in mice. Furthermore, we demonstrated that mice immunized with mcTnI before ligation of the left anterior descending coronary artery showed greater infarct size, more fibrosis, higher inflammation scores, and reduced fractional shortening. In the present study, we investigated the role of cTnI-specific T cells in this proinflammatory autoimmune response and identified the antigenic determinants of cTnI responsible.

**Methods**

**Mice**

Female A/J mice (5 to 6 weeks of age) obtained from Harlan Winkelman GmbH (Barchen, Germany) and female severe combined immunodeficiency (SCID) mice (5 to 6 weeks of age) obtained from Charles River (Sulzfeld, Germany) were maintained in the animal facility at the University of Heidelberg and used in all experiments. The animal work was approved by the Animal Care and Use Committee of the University of Heidelberg.

**Preparation of Recombinant mcTnI**

The murine cardiac troponin subunit mcTnI was expressed in *Echerichia coli* and purified as described previously. In addition to purification via ion-exchange chromatography, mcTnI was applied to a cardiac troponin C affinity column as a second purification step. Isolated mcTnI fractions were dialyzed extensively against 1 mmol/L HCl, then lyophilized and stored at -80°C.

**Cell Sorting**

CD90+, CD8+, and CD4+ T cells were enriched to 90% purity from the spleen by magnetically activated cell sorting with anti-CD90-anti-CD8-, and anti-CD4-conjugated microbeads (Miltenyi Biotec, Auburn, Calif).

**Transfer of T Cells**

For the transfer experiments, 4 groups of mice that were treated differently were used. Two groups of mice were first immunized with mcTnI on days 0 and 7. On day 21, purified T cells from 1 group of mice were reconstituted in vitro in the presence of dendritic cells and monocytes with 100 µg/mL mcTnI for 48 hours, whereas T cells from the second group were not restimulated with mcTnI. Additionally, 2 other groups of mice were immunized, first with adjuvant alone on days 0 and 7. On day 21, purified T cells from 1 group of mice were restimulated in vitro in the presence of dendritic cells and monocytes with 10 µg/mL mcTnI for 48 hours, whereas T cells from the second group were not restimulated with mcTnI. Next, 10⁶ to 10⁷ stimulated T cells were injected intraperitoneally into wild-type (WT) recipient mice irradiated with 600 rad or into nonirradiated SCID mice. To survey the effect of the number of T cells transferred, 3 additional groups of WT recipient mice irradiated with 600 rad or into nonirradiated SCID mice. To survey the effect of the number of T cells transferred, 3 additional groups of WT recipient mice irradiated with 600 rad or into nonirradiated SCID mice. To survey the effect of the number of T cells transferred, 3 additional groups of WT recipient mice irradiated with 600 rad or into nonirradiated SCID mice. To survey the effect of the number of T cells transferred, 3 additional groups of WT recipient mice irradiated with 600 rad or into nonirradiated SCID mice. To survey the effect of the number of T cells transferred, 3 additional groups of WT recipient mice irradiated with 600 rad or into nonirradiated SCID mice. To survey the effect of the number of T cells transferred, 3 additional groups of WT recipient mice irradiated with 600 rad or into nonirradiated SCID mice. To survey the effect of the number of T cells transferred, 3 additional groups of WT recipient mice irradiated with 600 rad or into nonirradiated SCID mice.

**Determinant of Autoantibody Titers**

Antibody titers were determined essentially as described previously. In brief, to measure serum anti-peptide or troponin I (TnI) titers, plates were coated either with 100 µL of each peptide per well or cTnI (5 µg/mL) in bicarbonate buffer (pH 9.6) and incubated overnight. Antibody secondary antibody diluted to 1:5000 for IgG (Sigma, St. Louis, Mo) was used for detection. Serum samples from test mice were diluted to 1:100, 1:500, 1:2500, and 1:12 500. Normal mouse serum was used as control. Optical densities were determined at 450 nm. Antibody end-point titers for each individual mouse were calculated as the greatest positive dilution of antibody that yielded a positive signal.

**cTnI-Dependent Cytokine Production by Splenocytes**

For in vitro cytokine production, splenocytes were cultured at 5×10⁶ per well in RPMI 1640 complete medium in the presence of either cTnI (10 µg/mL) or medium alone for 48 hours. Supernatant was collected, separated into aliquots, and frozen at -20°C. Cytokines (IL-1β, IL-2, IL-4, IL-6, IL-10, IL-13, IL-17, interferon-γ, and tumor necrosis factor [TNF]-α) were measured by DuoSet ELISA Development Systems (R&D Systems) according to the manufacturer's instructions.

**Histopathological Evaluation**

For histopathological evaluation of myocardium, mice were euthanized on day 21 after transfer of T cells and on day 28 after immunization with peptides, respectively. Sections of 5-µm thickness were cut at various depths in the myocardial tissue section and stained with hematoxylin and eosin (to determine the level of inflammation) and Masson's trichrome (to detect collagen deposition). Evidence of myocarditis and fibrosis was evaluated in a blinded manner by 2 independent investigators who used light microscopy, according to the following scoring system: grade 0, no inflammation; grade 1, cardiac infiltration in up to 5% of the cardiac sections; grade 2, infiltration in 6% to 10%; grade 3, infiltration in 11% to 30%; grade 4, infiltration in 31% to 50%; and grade 5, infiltration in >50% of cardiac sections.

**RNase Protection Assay**

The mCR-5 cytokine receptor multiprobe template set (BD Biosciences Pharmingen, Heidelberg, Germany) was used to measure mouse mRNAs encoding the chemokine receptors CCR1, CCR2, CCR1b, CCR3, CCR4, and CCR5. The mCK-5c multiprobe template set (BD Biosciences Pharmingen) was used to measure mouse mRNAs encoding lymphotoxin (Lyn), RANTES (regulated on activation, normal T-cell expressed, and secreted chemokine), macrophage inflammatory protein (MIP)-1b, MIP-1a, MIP-2, interferon-γ-inducible protein (IP)-10, monocyte chemotactic protein (MCP)-1, T-cell activation-3 (TCA-3), and eotaxin. The measurement was done according to the manufacturer's guidelines.

**Echocardiography**

Transsthoracic echocardiography was performed as described previously. The investigator who conducted the echocardiography was unaware of the treatment status.

**Synthesis of Oligopeptides**

Sixteen overlapping 16mer to 18mer peptides covering the entire amino acid sequence of mcTnI (211 residues), the corresponding human cTnI residues 104 to 121 (VDKVDGERDYEIAEKVTKN), residues 73 to 90 (VEVVVDDERDYEIAEKCLHN) and 99 to 116 (LKVLDLRGKFKRPLLRKV) of fast skeletal TnI were synthesized and purified by high-performance liquid chromatography (Peptide Specialty Laboratories, Heidelberg, Germany). The purity of the peptides was >90%.

**Antigen Preparation and Administration**

Each mouse was injected subcutaneously with an emulsion of 120 µg of 1 of the peptides or TnI as positive control, or control buffer and adjuvant as negative control. The peptide/protein was supplemented...
Figure 1. Transfer of T cells into WT mice. Mice were immunized with mcTnI or adjuvant alone. On day 21, T cells were isolated from splenocytes and restimulated in vitro in the presence of dendritic cells and monocytes with 10 μg/mL mcTnI (mcTnI mcTnI or mcTnI/no) for 48 hours or were not restimulated with mcTnI (mcTnI/no or CFA/no). Then, 10^6 to 10^7 T cells per mouse were transferred to WT mice irradiated with 600 rad. A through G, Effects on production of mcTnI-specific autoantibodies (total IgG [A], mean±SEM) and cytokines (B, mean±SEM), inflammation and fibrosis (C), left ventricular end-diastolic (D, mean±SEM) and end-systolic (E, mean±SEM) diameter, fractional shortening (F, mean±SEM), and expression of chemokines and chemokine receptors in the myocardium (G). Antibody end-point titers for each individual mouse were calculated as the greatest positive dilution of antibody above normal mouse serum levels for day 21. *P<0.05, Kruskal-Wallis test and U test. CFA indicates complete Freund’s adjuvant; Nr, number; and L32, ribosomal protein L32.
with complete Freund’s adjuvant containing 5 mg/mL *Mycobacterium tuberculosis* H37Ra (Sigma) on days 0, 7, and 14. The mice were euthanized on day 28 for histopathological evaluation, RNA protection assay analysis, and measurement of cytokines and antibody titers.

**Statistical Analysis**

Results are expressed as mean±SEM. Data were analyzed with the Kruskal-Wallis test followed by the Mann–Whitney *U* test to explore the significance between treatment groups. *P* values <0.05 were considered significant. SPSS statistical software (version 15.0; SPSS, Inc, Chicago, Ill) was used for all calculations.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**Transfer of T Cells**

Mice that received T cells from mcTnI-immunized mice that had been restimulated in vitro with mcTnI showed high mcTnI-specific total IgG antibody titers (Figure 1A), severe inflammation (histoscore 1.8±0.4) and fibrosis (histoscore 1.9±0.4) in myocardium (Figure 1C), and increased mcTnI-induced production of the cytokines IL-1β, IL-4, IL-13, IL-17, and TNF-α (Figure 1B) compared with mice that had received T cells from mcTnI-immunized mice that had not been restimulated in vitro with mcTnI or mice that received T cells from mice given adjuvant alone, irrespective of their in vitro restimulation, did not show any signs of inflammation, fibrosis, or alteration of heart function. One of 6 mice that received T cells from mcTnI-immunized mice without in vitro restimulation showed slight inflammation and fibrosis but no significant alteration of heart function. To examine the expression of different chemokines and chemokine receptors, we measured mRNA expression levels of the chemokines Ltn, RANTES, MIP-1α, MIP-1β, MIP-2, IP-10, MCP-1, TCA-3, and eotaxin, as well as levels of the chemokine receptors CCR1, CCR2, CCR1b, CCR3, CCR4, and CCR5, in the myocardium of the recipient mice. We were able to detect mRNA levels for RANTES, MIP-1β, MIP-1α, MIP-2, MCP-1, TCA-3, and eotaxin only in mice that received mcTnI-specific T cells, unlike the otherwise-treated mice (Figure 1G). In addition, we found that mRNAs for CCR1, CCR2, and CCR5 were expressed only in the myocardium of mice that received mcTnI-specific T cells and not in the other groups of mice (Figure 1G).

Later, to study the effect of the number of T cells transferred, 3 additional groups of WT recipient mice irradiated with 600 rad were injected intraperitoneally with either 10⁶ to 10⁷, 10⁵ to 10⁶, or 10⁴ to 10⁵ T cells. When we reduced the number of T cells transferred, the severity of disease decreased. Transfer of 10⁴ to 10⁵ or a lower number of T cells revealed no significant inflammation in the recipient mice (Figure 2A). On the other hand, transfer of 10⁶ to 10⁷ T cells in nonirradiated SCID mice induced inflammation in 10 of 11 recipients. Overall, the histoscores in SCID mice were lower than those in irradiated WT recipient mice (histoscore 0.8±0.1, data not shown).

To study the effect of T-cell subsets, CD4⁺ and CD8⁺ T cells were isolated from the spleens of immunized mice and restimulated in vitro with mcTnI; in the case of CD8⁺, T cells were restimulated with both mcTnI and supplemental IL-2. Transfer of whole T cells (CD90⁺; histoscore 2.1±0.5)
and transfer of the CD4+ subset of T cells (histoscore 2.4) induced moderate to severe disease, whereas transfer of the CD8+ subset of T cells alone did not induce any signs of inflammation in the recipient mice (Figure 2B).

Identity of Peptides
To identify the antigenic sequences of mcTnI that triggered inflammation and fibrosis, 16 overlapping 16mer to 18mer peptides covering the entire amino acid sequence of murine TnI (211 residues) were synthesized, followed by high-performance liquid chromatography purification (Figure 3).

Immunization and Humoral Immune Response
The mice developed significant autoantibody titers against all injected peptides by day 28. However, total IgG autoantibody titers were significantly higher in mice immunized with peptides 7, 8, 10, and 14 (data not shown).

Immunization With Residues 105 to 122 (Peptide 9) and 131 to 148 (Peptide 11) of mcTnI Induces Myocardial Inflammation
Four of 5 mice immunized with residues 105 to 122 of mcTnI (peptide 9: VDKVDEERYDVEAKVTKN) showed inflammation, with a histoscore ≥1 (Figures 3 and 4A through 4D). One of 5 mice immunized with residues 131 to 148 of mcTnI (peptide 11: QKIYDLRGKFKRPTLRRV) showed signs of inflammation (histoscore = 1; Figures 3 and 4E). No inflammation was observed in the myocardium of mice immunized with the other synthesized mcTnI residues or control buffer and adjuvant alone (Figures 3 and 4F).

Immunization With Residues 105 to 122 (Peptide 9) of mcTnI Causes Myocardial Fibrosis
Comparable to the observed inflammation, 4 of 5 mice immunized with residues 105 to 122 of mcTnI (peptide 9) showed significant deposition of collagen in the myocardium, with a fibrosis score ≥1 (Figures 4G through 4I), whereas no fibrosis was detected in mice immunized with other peptides or control buffer and adjuvant alone. Myocardial fibrosis was indicated by bright blue staining for collagen deposition and was associated with myocardial inflammation.

Immunization With Residues 105 to 122 (Peptide 9) of mcTnI Induces Expression of Chemokines and Chemokine Receptors in Myocardium
To investigate the expression of different chemokines and chemokine receptors, we measured mRNA expression levels of the chemokines Ltn, RANTES, MIP-1β, MIP-1α, MIP-2, IP-10, MCP-1, TCA-3, and eotaxin and of the chemokine receptors CCR1, CCR2, CCR1b, CCR3, CCR4, and CCR5 in the myocardium of the immunized mice. We were able to detect mRNA levels for RANTES, MIP-1β, MIP-1α, MIP-2, MCP-1, TCA-3, and eotaxin only in mice immunized with residues 105 to 122 of mcTnI (peptide 9) and not in mice immunized with the other peptides or control buffer and adjuvant alone (Figure 5A). In addition, we found that the mRNAs for the chemokine receptors CCR1, CCR2, and CCR5 were expressed solely in the myocardium of mice immunized with residues 105 to 122 of mcTnI (peptide 9; Figure 5B).

Immunization With Residues 105 to 122 (Peptide 9) of mcTnI Induces Production of Antibodies That React With the Complete Protein mcTnI
We tested whether immunization with the complete protein mcTnI also induced antibodies to residues 105 to 122 of mcTnI (peptide 9). On day 28, mice immunized with mcTnI developed antibody not only against the whole mcTnI protein but also against residues 105 to 122 of mcTnI (peptide 9;
Figure 5C). Conversely, immunization with residues 105 to 122 of mcTnI (peptide 9) induced production of antibodies that reacted with the complete protein mcTnI (Figure 5D).

**Human cTnI and Other Proteins With Similar Amino Acid Sequences as the Antigenic Murine Amino Acid Sequences 105 to 122 and 131 to 148**

When comparing the antigenic residues 105 to 122 and 131 to 148 of mcTnI with the corresponding amino acid sequences of human cTnI (hcTnI) or other proteins with similar amino acid sequences, we found that residues 104 to 121 and residues 130 to 147 of hcTnI differed in only 1 amino acid from the major (residues 105 to 122) and minor (residues 131 to 148) epitopes of mcTnI, respectively (Figure 6). The next protein with amino acid sequences similar to both mcTnI epitopes (105 to 122 and 131 to 148) was skeletal TnI (residues 73 to 90 and 99 to 116 in slow skeletal troponin and residues 98 to 115 in fast skeletal troponin). Residues 73 to 90 of slow skeletal troponin differed in 6 amino acids from the main epitope of mcTnI (105 to 122; peptide 9; Figure 6). Residues 98 to 115 of fast skeletal TnI differed in 3 amino acids, and residues 99 to 116 of slow skeletal TnI differed in 4 amino acids from the minor epitope of mcTnI (131 to 148; peptide 11).

Residues 104 to 121 of hcTnI but Not Residues 73 to 90, 98 to 115, or 99 to 116 of Skeletal Troponin I Induce Myocardial Inflammation and Fibrosis

To study whether either hcTnI or skeletal TnI, different peptides with the most similarity in amino acid sequence to both pathogenic epitopes of mcTnI (105 to 122 and 131 to 148), has a pathogenic effect on the myocardium, mice were immunized with either hcTnI (104 to 121), slow skeletal TnI (residues 73 to 90 or 99 to 116), or fast skeletal TnI (residues 98 to 115). Residues 105 to 122 (peptide 9) of mcTnI were used as a positive control (Figures 7A through 7G). All mice immunized with residues 105 to 122 of mcTnI developed mild to severe inflammation and fibrosis (Figures 7A through 7E). Four of 8 mice immunized with residues 104 to 121 of hcTnI showed mild inflammation and fibrosis (Figures 7A, 7F, and 7G), some located perivascularly (Figure 7G). In contrast, none of the mice immunized with skeletal TnI residues 73 to 90 (Figure 7A), residues 98 to 115, or residues 99 to 116 (data not shown) showed signs of inflammation.

**Three-Dimensional Structure of Troponin I**

Localization of the peptide 9 sequences is marked with an arrow in a rendering of the actual 3-dimensional structure of TnI in association with troponin T (Figure 8).

**Discussion**

Here, we showed for the first time that transfer of TnI-specific T cells led to severe inflammation and fibrosis in healthy recipient WT mice, which resulted in enlarged hearts, increased end-systolic and end-diastolic diameters, reduced fractional shortening, inflammation, fibrosis, and heart failure. Furthermore, we identified 2 amino acid sequences of murine TnI that led to heart failure by inducing inflammation and fibrosis. In this regard, residues 105 to 122 (peptide 9) of mcTnI were the strongest inducer of inflammation and fibrosis in the myocardium, which was accompanied by increased expression of the inflammatory chemokines RANTES, IP-10, MCP-1, MIP-1α, MIP-1β, MIP-2, TCA-3, and eotaxin and of the chemokine receptors CCR1, CCR2, and CCR5. McTnI residues 131 to 148 (peptide 11) were a minor epitope that induced milder inflammation. Although the corresponding hcTnI residues 104 to 121, which differed in 1 amino acid from the mcTnI residues 105 to 122, also induced milder inflammation in mice, none of the mice immunized...
with skeletal TnI showed significant signs of inflammation. Furthermore, we demonstrated that mice immunized with mcTnI also developed antibodies directed against residues 105 to 122 of mcTnI (peptide 9). Conversely, immunization with residues 105 to 122 of mcTnI (peptide 9) induced production of antibodies that reacted with the complete protein mcTnI. Thus, residues 105 to 122 of mcTnI must be immunogenic in the complex structure of the whole mcTnI protein as well. This epitope becomes more interesting because it forms a parallel helix (residues 90 to 135 of TnI) with the helix of troponin T (residues 226 to 271), and it has been suggested that this coil between TnI and troponin T has important physiological roles that are characteristic of troponin.13

It has been reported that autoantibodies against cTnI induced heart failure by chronic stimulation of Ca\textsuperscript{2+} influx in cardiomyocytes.6,7 Recently, we reported that a humoral and cellular autoimmune response against mcTnI induced severe inflammation and fibrosis in the myocardium of mice, with persistent, prominent inflammation and fibrosis over 270 days and reduced long-term survival.8 We also demonstrated that mice preimmunized with TnI before ligation of the left anterior descending coronary artery showed greater infarct size, more fibrosis, higher inflammation scores, and reduced fractional shortening than mice without preimmunization.9 These results indicate that an autoimmune response against TnI aggravates the outcome of acute cardiac damage and may have a significant influence on postinfarct remodeling. Now, we demonstrate that troponin-induced heart failure is T-cell dependent. Furthermore, CD4+ T cells are necessary for successful transfer of disease, as opposed to CD8+ T cells, which appear not to play a significant role. We could not transfer disease with CD8+ T cells alone, nor was the severity of disease higher when both CD4+ and CD8+ were transferred than with transfer of CD4+ T cells alone. In addition, we found significantly elevated levels of mcTnI-specific TNF-α, IL-1, IL-4, IL13, and IL-17 production. This cytokine profile suggests that mcTnI-induced myocarditis in A/J mice exhibits a Th2-like (IL-4, IL13) and Th17-like (IL-17) phenotype. In the past, we demonstrated that experimental autoimmune myocarditis in A/J mice induced by cardiac myosin has a Th2 phenotype.14 Others described an important role for Th1 subsets in experimental autoimmune myocarditis, so that the relative contributions of the CD4+ Th1 and Th2 subsets are still unclear.14–16 Recently, Rangachari et al17 demonstrated a significant role for another subset of CD4+ T cells characterized by IL-17 production in Balb/c mice with experimental autoimmune myocarditis induced by cardiac myosin peptide. We believe that the strain of mice used in the experiments is a crucial factor in deciding which subsets of CD4+ cells are involved in disease induction and progression.

Proinflammatory cytokines are important in the development of autoimmune myocarditis. We previously showed that administration of either IL-1 or TNF-α promoted virus- and myosin-induced myocarditis in genetically resistant B10.A mice.18 Recently, we demonstrated that the presence of myocarditis is associated with increased levels of TNF-α from cardiac myosin–stimulated splenocytes in culture.19 Furthermore, when A/J mice were infected with coxsackie virus...
B3 and treated with an IL-1 receptor antagonist, myocardial injury was diminished. Thus, IL-1 and TNF-α are clearly critical in the pathogenesis of autoimmune myocarditis.

We have identified 2 pathogenic epitopes of the TnI molecule responsible for the induction of inflammation, fibrosis, and heart failure. All of the peptide sequences used to immunize mice led to an increase in total IgG autoantibody titers. Even though mice immunized with peptide sequences 7, 8, 10, and 14 showed higher total IgG antibody titers than mice immunized with the other peptides used in the experiments, only mice immunized with peptides 9 and 11 showed inflammation and fibrosis in the myocardium. We conclude that in our model, the initiation of the inflammatory process in the myocardium, followed by fibrosis and alteration of heart function, is primarily T-cell dependent. This is supported by our findings that we can transfer disease to healthy WT mice with isolated T cells alone and by the findings by Smith and Allen, who demonstrated that myosin-induced myocarditis is a T-cell–mediated disease. Additional support is provided by the fact that Okazaki et al and Nishimura et al described no inflammation in the myocardium in mice treated with antibodies against troponin. They demonstrated that antibodies to cTnI (humoral immune response only) induced heart dysfunction due to chronic stimulation of Ca2+ influx in cardiomyocytes by these antibodies. We also recognize the possibility that once the inflammatory process has been induced, antibodies may have additional effects in the broadening of this ongoing inflammatory process that is followed by fibrosis and alteration of heart function.

When mice were immunized with the whole troponin protein (mcTnI), we expected to induce an immune response (cellular and humoral) directed against different peptide sequences (epitopes) of this protein. By testing the sera of mice immunized with the whole troponin protein for the presence of antibodies directed against the pathogenic peptide 9, we demonstrated that an immune response against peptide 9 could be induced not only by immunization with the synthesized peptide 9 alone but also

**Figure 7.** A, Groups of mice were immunized with either mcTnI (residues 105 to 122), hcTnI (residues 104 to 121), or skeletal troponin (residues 73 to 90). Effects on severity of inflammation and fibrosis are shown on day 28 (Kruskal-Wallis test and U test). B through G, Histological examination of the hearts (stained with hematoxylin and eosin). Representative heart section of mice immunized with mcTnI (residues 105 to 121; peptide 9) with inflammation scores of 3 and 5 (B and D) and 2 and 5 (C and E). Representative heart section of mice immunized with hcTnI (residues 104 to 121) and inflammation score of 1 (F) and with perivascular inflammation (G) on day 28. B and C, Magnification ×10; D through G, magnification ×20. Nr indicates number.
sequences of TnI did not induce inflammation or fibrosis in the myocardium. The use of the whole troponin protein will identify all antibodies directed against different sequences of troponin, including those that might have no pathogenic effect.

Chemokines, such as RANTES, MCP-1, MIP1-α, and their major receptors, CCR2 and CCR5, play important roles in the pathogenesis of many inflammatory diseases.22–24 MCP-1 mRNA expression has been shown in endomyocardial biopsy specimens from patients with dilated cardiomyopathy, which suggests a significant role of this chemokine in the regulation of inflammatory cell infiltration into the myocardium.25 Recently, we described an important role for MCP-1 and MIP1-α and their major receptors, CCR2 and CCR5, in the initiation of autoimmune myocarditis.23 In our experiments, only mice immunized with residues 105 to 122 of mcTnI (peptide 9) showed increased expression of these chemokines, which correlated with increased myocardial inflammation.

Eriksson et al8 showed that troponin autoantibodies are present in patients and can produce false-negative results by blocking the binding of troponin antibodies used in analytical assays to the target protein. The report of the presence of autoantibodies against cTnI in patients with acute coronary syndrome8 points to an early induction of an autoimmune response to cTnI in these patients. In addition to analytical interference in the assays, TnI autoantibodies may have clinical consequences. Okazaki et al7 showed that troponin autoantibodies induced dilation and cardiac dysfunction by the chronic stimulation of calcium influx in cardiomyocytes. Recently, we showed that provoking an autoimmune response to cTnI induces severe inflammation in the myocardium followed by fibrosis and heart failure with increased mortality in mice.9 These results suggest that some of the patients who develop an autoimmune response to released cTnI may have a higher risk of heart failure due to inflammation in the myocardium. These autoimmune responses may also explain the discrepancy observed in some patients with involvement of 1 or 2 coronary arteries even though the entire heart is diffusely hypokinetic. Furthermore, the role of autoantibodies in heart failure has been supported by many clinical studies demonstrating that the removal of immunoglobulins by immunoadsorption can improve ejection fraction and reduce morbidity in patients with dilated cardiomyopathy.10 Now, we have identified 1 major and 1 minor epitope of TnI (peptides 9 and 11, respectively) that appear to be responsible for disease induction. Using these newly identified epitope sequences of TnI instead of the whole troponin molecule, a more specific screening test of patients with high risk for progressive autoimmune inflammatory heart failure may be possible. Furthermore, we have demonstrated the important role of troponin-specific T cells in inducing autoimmune inflammation. These findings may aid in the development of new approaches to the early treatment of heart failure in some patients and in the initiation of further (clinical) studies to investigate the role of TnI release, induction of an autoimmune response to released TnI during acute cardiac damage, and its role in postinfarct remodeling and heart failure.

Figure 8. Localization of the peptide 9 amino acid sequences is marked with an arrow in a rendering of the actual 3-dimensional structure of TnI in association with troponin T. TnC and troponin T are shown in red and yellow, respectively. TnI is shown in cyan, except for the 2 stretches of amphiphilic helices (TnC-binging sites), which are dark blue. Reprinted by permission from Macmillan Publishers Ltd: Takeda et al,15 copyright © 2003.
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Disclosures
Dr Katus developed the troponin T assay and holds a patent on this assay jointly with Roche Diagnostics. Drs Katus and Kaya have applied for a patent entitled “measuring troponin antibodies to assess cardiovascular risk” jointly with Roche Diagnostics. The remaining authors report no conflicts.

References

CLINICAL PERSPECTIVE
Despite the widespread use of cardiac troponin I (cTnI) for diagnosis of myocyte injury and risk stratification in acute cardiac disorders, little is known about the precise role of an autoimmune response to the troponins on cardiac function. Recently, investigators made the surprising discovery that mice treated with monoclonal anti-cTnI antibodies developed myocardial dysfunction. Shortly afterward, it was reported that autoantibodies to cTnI are also present in patients with acute coronary syndrome. These findings indicate that induction of an autoimmune response to cTnI is not a rare event in patients. Recently, we demonstrated that the prevalence of cTnI antibodies in patients with acute coronary syndrome has an impact on improvement in left ventricular ejection fraction. Furthermore, the role of autoantibodies in heart failure has been supported by clinical studies demonstrating that the removal of immunoglobulins by immunoadsorption can improve ejection fraction in patients with dilated cardiomyopathy. Recently, we showed that inducing an autoimmune response to cTnI leads to severe inflammation in the myocardium followed by fibrosis and heart failure with increased mortality in mice. Now, we demonstrate that this disease is primarily a CD4+ T-cell–dependent disease with a Th2/Th17 phenotype, and we identify the antigenic determinants of cTnI that are responsible. We believe that these findings can fundamentally change the understanding of the pathophysiology of inflammatory cardiovascular diseases and postinfarct remodeling.
Identification of Cardiac Troponin I Sequence Motifs Leading to Heart Failure by Induction of Myocardial Inflammation and Fibrosis

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