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Localization of CD8 T cell epitope within cardiac myosin heavy chain- $\alpha_{334-352}$ that induces autoimmune myocarditis in A/J mice

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Abstract

Background—Cardiac myosin heavy chain- α (Myhc), an intracellular protein expressed in the cardiomyocytes, has been identified as a major autoantigen in cardiac autoimmunity. In our studies with Myhc₃₃₄₋₃₅₂-induced experimental autoimmune myocarditis in A/J mice (H-2^a), we discovered that Myhc₃₃₄₋₃₅₂, supposedly a CD4 T cell epitope, also induced antigen-specific CD8 T cells that transfer disease to naïve animals.

Methods and Results—In our efforts to identify the CD8 T cell determinants, we localized Myhc₃₃₈₋₃₄₈ within the full length-Myhc₃₃₄₋₃₅₂, leading to four key findings. (1) By acting as a dual epitope, Myhc₃₃₈₋₃₄₈ induces both CD4 and CD8 T cell responses. (2) In a major histocompatibility complex (MHC) class I-stabilization assay, Myhc₃₃₈₋₃₄₈ was found to bind H-2D^d – but not H-2K^k or H-2L^d – alleles. (3) The CD8 T cell response induced by Myhc₃₃₈₋₃₄₈ was antigen-specific, as evaluated by MHC class I/H-2D^d dextramer staining. The antigen-sensitized T cells predominantly produced interferon- γ , the critical cytokine of effector cytotoxic T lymphocytes. (4) Myhc₃₃₈₋₃₄₈ was found to induce myocarditis in immunized animals as determined by histology and magnetic resonance microscopy imaging.

Conclusions—Our data provide new insights as to how different immune cells can recognize the same antigen and inflict damage through different mechanisms.

Keywords

cardiac myosin; CD8 T cells; autoimmunity; myocarditis; MHC class I dextramers

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Conflict of interest

The authors report no relationships that could be construed as a conflict of interest.

1. Introduction

Autoimmunity ensues when immune responses are directed against self-antigens, resulting from the mediation of either autoantibodies or autoreactive T cells. However, when autoreactive T cells are involved, CD4 T helper (Th) cells are commonly implicated because they play a central role in the generation of adaptive immune responses. For example, cytokines produced by CD4 T cells are critical for B cells to produce antibodies to protein antigens, and Th cytokines like interferon (IFN)- γ mediate macrophage activation. On the other hand, interleukin (IL)-2 helps in the differentiation and expansion of CD8 T cells. Because CD4 T cells are needed for B cells to make antibodies, both cell types are expected to recognize the same antigens, but their epitopes could be different. Thus, an argument can be made that therapeutically targeting the pathogenic autoreactive B cells that produce autoantibodies, as well as the CD4 T cells that help the B cells do so, can suppress autoimmune responses in diseases driven by antibodies, such as systemic lupus erythematosus, Hashimoto's thyroiditis, Sjogren's syndrome, and rheumatoid arthritis [1, 2].

CD4 T cells and CD8 T cells are distinct in their recognition of peptides, in that they recognize antigens presented by two different molecules of the major histocompatibility complex (MHC) locus. While MHC class II molecules expressed by professional antigen-presenting cells (APCs) present peptides to CD4 T cells, MHC class I molecules expressed by almost all nucleated cells can present peptides to CD8 T cells. The ends of peptide-binding grooves are open in the class II MHC molecules, enabling them to present long peptides (up to 20 amino acids), as opposed to short peptides of ~8-11 amino acids presented by class I molecules, in which the ends of the MHC-clefts are closed [3, 4]. Therefore, the immunodominant epitopes of protein antigens can be processed and presented in the context of class I or class II molecules through endogenous and exogenous pathways, respectively, leading to the induction of corresponding CD8 and CD4 T cell responses. If such an event happens for self-proteins, then the tissue destruction in the target organs is mediated by CD4 T cells via delayed-type hypersensitivity reaction, whereas CD8 T cells inflict damage through cytotoxicity [2, 5].

To study the immune events of cardiac autoimmunity, rodent models of experimental autoimmune myocarditis (EAM) are commonly employed. These include cardiac myosin heavy chain- α (Myhc)₃₃₄₋₃₅₂-induced EAM in A/J (H-2^a), CBA/J (H-2^k) and B10.A (H-2^k) mice; Myhc₆₁₄₋₆₄₃-induced EAM in Balb/c (H-2^d) mice; and Myhc₁₃₀₄₋₁₃₂₀-induced EAM in Lewis rats (RT1) [6-11]. The disease is induced by immunizing the animals with the immunodominant epitopes of cardiac antigens in complete Freund's adjuvant (CFA). In all the EAM models described above, the disease has been shown to be mediated by CD4 T cells, [8, 10, 11] with a complex disease pathogenesis [12-14]. In our studies with EAM induced by Myhc₃₃₄₋₃₅₂ in A/J mice, however, we noted an unexpected finding that the full-length Myhc₃₃₄₋₃₅₂ (FL-Myhc₃₃₄₋₃₅₂) epitope was also found to contain Myhc₃₃₈₋₃₄₈, which is a determinant for CD8 T cells. By acting as a dual epitope, Myhc₃₃₈₋₃₄₈ induces both CD4 and CD8 T cell responses that are antigen-specific, and induces myocarditis in immunized animals through the predominant production of IFN- γ , the critical cytokine of effector cytotoxic lymphocytes.

2. Materials and Methods

2.1 Mice

Our studies involved the use of 6- to 8-week-old female A/J mice (H-2^a), obtained from the Jackson Laboratory (Bar Harbor, ME) and maintained in accordance with the guidelines of the University of Nebraska-Lincoln. All animals used in the study received humane care, and the Institutional Animal Care and Use Committee approved experimental permit and protocol numbers are A3459-01 and 973, respectively.

2.2 Peptide synthesis and immunization procedures

The indicated peptides, including the controls (bovine ribonuclease [RNase]₄₃₋₅₆, VNTFVHESLADVQA; human immunodeficiency virus glycoprotein 120 [HIV]_{P18-110}, RGPGRFVVTI), were synthesized on 9-fluorenylmethyloxycarbonyl chemistry (Neopeptide, Cambridge, MA). All peptides were HPLC-purified (>90%), identity-confirmed by mass spectroscopy, and dissolved in 1x PBS prior to use. For immunizations, peptides were emulsified in CFA supplemented with *Mycobacterium tuberculosis* H37RA extract to a final concentration of 5 mg/ml, and administered subcutaneously in multiple sites in the flank and sternal regions (100 µg per mouse). For EAM induction, animals were immunized twice as above at 7 day intervals, and pertussis toxin (100 ng/mouse) was administered on day 0 and day 2 after the first immunization [11, 15].

2.3 Measurement of recall responses and derivation of primary T cell cultures

Fourteen days after immunization, animals were euthanized and single cell suspensions were prepared using the draining lymph nodes (maxillary, mandibular, axillary, inguinal and popliteal) and spleens [11, 15]. After lysing the erythrocytes with 1x ammonium chloride potassium buffer (Lonza, Walkersville, MD) and washing, cells were resuspended in 1x IMAG buffer (BD Biosciences, San Diego, CA). CD4 or CD8 T cells were then enriched to a purity of >95 % by negative selection based on magnetic separation using IMAG (BD Biosciences) [15]. Cells were suspended in growth medium containing RPMI medium supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 4 mM L-glutamine, 1x each of non-essential amino acids and vitamin mixture, and 100 U/ml penicillin-streptomycin (Lonza). To measure the recall responses, cells were stimulated for 2 days at a density of 10×10^6 cells/ml in the presence of syngeneic irradiated APCs at a ratio of 1:1, as well as the indicated peptides (0-100 µg/ml). After pulsing with 1 µCi of tritiated ³[H]-thymidine (MP Biomedicals, Santa Ana, CA) for 16 hours, proliferative responses were measured as counts per minute (cpm) using a Wallac liquid scintillation counter (Perkin Elmer, Waltham, MA) [11]. To derive primary T cell cultures, whole cell populations were stimulated with the peptides (50 µg/ml), and supplemented after two days with IL-2 medium (eBioscience, San Diego, CA; Advanced Biotechnologies, Columbia, MD) [11, 15].

2.4 Disease induction by adoptive transfer

A/J mice were immunized with FL-Myhc₃₃₄₋₃₅₂ in CFA, and at termination on day 14, spleens and lymph nodes were harvested to prepare single cell suspensions. CD8 T cells

were then enriched by negative selection based on magnetic separation as described in section 2.3, then stimulated with concanavalin-A (Con-A, 2.5 µg/ml; Sigma Aldrich, St. Louis, MO) for two days. Viable cells were harvested and administered i.p., (14×10^6 cells per animal) into whole body-irradiated (400 rads/mouse) naïve animals [16-19]. Irradiated naïve mice that received $1 \times$ PBS or Con-A activated CD8 T cells obtained from naïve mice, served as controls. Additionally, each mouse received pertussis toxin (100 ng/mouse) i.p., on day 0 and day 2 posttransfer, and the animals were euthanized on day 18 to harvest hearts for histology.

2.5 Staining for markers of effector CD8 T cells

Groups of A/J mice were immunized with Myhc₃₃₄₋₃₅₂, and after 10 days, lymph nodes were obtained and lymph node cells (LNCs) were prepared. Cells were stimulated with the peptide (50 µg/ml) for 2 days, and the cultures were maintained in IL-2 medium. On days 4, 6, 8 and 10 post-stimulation, cells were stained with CD8 and CD107a/b or Rat-IgG2a (isotype control) antibodies (eBioscience), and 7-amino-actinomycin-D (7-AAD; Invitrogen, Carlsbad, CA). After washing, cells were acquired by flow cytometry (FACS Calibur, BD Biosciences, San Diego, CA), and the frequencies of CD107a/b⁺ cells were enumerated in the live (7-AAD⁻) CD8 T cell subset using Flow Jo software (Tree Star, Ashland, OR) [20, 21]. Similarly, on the indicated days, after staining with CD8 antibodies and 7-AAD, cells were fixed and permeabilized, then stained with granzyme B or perforin antibodies and Rat-IgG2a (isotype control) [22-24]. After washing, cells were acquired by flow cytometry and the frequencies of granzyme B⁺ and perforin⁺ cells were enumerated in the live (7-AAD⁻) population as above.

2.6 MHC class I stabilization assay

To identify the epitopes that bind MHC molecules, we used a MHC class I stabilization assay [25], which involved the use of a transporter associated with antigen-processing 2-deficient RMA-S cell lines that were stably transfected with these MHC class I genes: H-2D^d (kind gift from David H. Raulet, University of California, Berkeley), H-2K^k (kind gift from Peter Cresswell, Yale School of Medicine, New Haven), and H-2L^d (kind gift from Ted Hansen, Washington University, St. Louis). RMA-S-D^d cells were cultured in Kappler-Marrack complete tumor medium [26]. RMA-S-K^k and RMA-S-L^d cells were cultured in RPMI medium supplemented with 10% FBS, 10 mM HEPES, 2 mM L-glutamine and 100 U/ml penicillin-streptomycin. Briefly, cells were plated in 96-well plates at a density of 2×10^6 /ml and were incubated with or without Trunk A-4 and Trunk A-5 peptides (0 to 400 µM) at 26°C/5% CO₂ for 18 hours. The plates were then transferred to a 37°C incubator for 1.5 hours, and after washing, cells were stained with fluorophore-labelled antibodies for H-2D^d (BD Pharmingen, San Diego, CA), H-2K^k (BD Pharmingen), and H-2L^d (Cedarlane, Burlington, NC). After washing, cells were acquired by flow cytometry, and the mean fluorescence intensities (MFI) were calculated using CellQuest software (BD Biosciences). Finally, MFI ratios were derived using the formula: MFI ratio = (MFI observed in the presence of peptide at 37°C/MFI observed in the absence of peptide at 26°C) – (MFI observed in the absence of peptide at 37°C/MFI observed in the absence of peptide at 26°C) × 100 [25].

2.7 Derivation of MHC dextramers and determination of antigen-specificity of Myhc-reactive T cells by dextramer staining

The antigen specificity of Myhc-reactive CD4 T cells was determined by MHC class II/IA^k dextramer staining. MHC class II/IA^k dextramers (Myhc₃₃₄₋₃₅₂ and RNase₄₃₋₅₆), were prepared as we have described previously [27]. CD4 T cells obtained from mice immunized with Myhc₃₃₄₋₃₅₂ were stimulated with the peptide (50 µg/ml) for 2 days, and the cells were maintained in IL-2 medium. On day 8, cells were stained with dextramers for two hours at room temperature followed by staining with anti-CD4 and 7-AAD [27]. After washing, cells were acquired by flow cytometry, and the percentages of dextramer⁺ cells were determined in the live (7-AAD⁻) cell populations using Flow Jo software.

The antigen specificity of Myhc-reactive CD8 T cells was determined by MHC class I/H-2D^d dextramer staining. The process followed three stages.

2.7.1 Expression of MHC class I heavy chains and β2-microglobulin (β2m)—

Derivation of MHC class I dextramers required the expression of the class I heavy chain for H-2D^d and the light chain β2m. The expression vector for the mouse MHC class I heavy chain of H-2D^d was constructed as previously described [28]. Briefly, the full-length extracellular domains of the H-2D^d allele were amplified from the cDNA synthesized from the splenocytes of naïve A/J mice using the sequence-specific forward and reverse primers 5'GGGAATTCATATGGGCTCACACTCGCTGAGG3' and 5'AGAGGATCCGGATGAAGGAGGCTCCTCCTTGCC3'. The forward and reverse primers were designed to include the in-frame *Nde*1 site/start codon and in-frame *Bam*H1 sites, respectively. The amplified fragments were digested with the respective enzymes and cloned into a pET22b⁺ vector (Novagen, Madison, WI). Finally, the sequence for the biotinylation site (5'GATCCCTGCATCATATTC TGGATGCACAGAAAATGGTGTGGAATCATCGTTAAA3') was cloned immediately after the codon for the 280th amino acid of the MHC class I heavy chain [28].

The construct for human β2m (pHN1-β2m) was received as a kind gift from Dr. David Garboczi [29]. MHC class I monomers and β2m were expressed in BL21(DE3)pLysS competent cells (Promega, Madison, WI) induced by isopropyl β-D-1-thiogalactopyranoside (ThermoScientific, Waltham, MA) at a final concentration of 1 mM and 0.4 mM, respectively [28]. The proteins expressed as insoluble inclusion bodies were purified, solubilized in urea and stored snap-frozen in aliquots at -80°C until use [28].

2.7.2 Preparation of H-2D^d dextramers—

The heavy chain of H-2D^d was refolded with β2m and Trunk A-4, Trunk A-5 or Trunk A-6 peptides in individual reactions as described previously [28]. To prepare control dextramers, we used HIV_{P18-I10}, which binds the H-2D^d allele, as reported by others [30]. Briefly, peptides (1.5 mg), β2m (2000 nM), and MHC heavy chain (1000 nM) dissolved in injection buffer (3 mM guanidine HCl, 10 mM sodium acetate, 10 mM EDTA, pH 4.2) were injected sequentially into a beaker containing folding buffer (100 mM Tris base, 0.4 M L-arginine, 2 mM EDTA, pH 8.0, supplemented with 5 mM reduced glutathione, 0.5 mM oxidized glutathione and 100 mM phenylmethylsulfonyl fluoride) at 4°C with continuous stirring. At 12 hours and 18 hours post incubation, the

reaction mixtures were supplemented with additional amounts of heavy chain protein (1000 nM), and the incubation was continued for a total of 36 hours. The precipitate containing unfolded protein complexes was removed by centrifuging the samples at 13,000 rpm for 20 minutes, and the MHC heavy chain/ β 2m/peptide complexes in the supernatant were concentrated using ultracentrifugal filters (Millipore, Billerica, MA). These complexes were then desalted using gel-filtration columns (GE Healthcare Bio-Sciences, Pittsburgh, PA) and biotinylated using the biotin ligase, BirA ([25 μ g or 10 nM; Avidity, Denver, CO]/ 2.5 mg/ml of the MHC/peptide complexes) at 30°C overnight. After desalting, biotinylated MHC/peptide monomers were assembled with dextran-streptavidin-phycoerythrin molecules (kindly provided by Immudex Aps, Copenhagen, Denmark) at a molar ratio of 20:1 by incubating the reaction mixtures at room temperature (RT) for 30 minutes in the dark, followed by reconstitution in the dextramer buffer (50 mM Tris HCl, pH 7.2). The reagents were stored at 4°C until use.

2.7.3 Dextramer staining—To ascertain the antigen specificity of the CD8 T cells, we used H-2D^d dextramers, which included specific (H-2D^d/Trunk A-4, H-2D^d/Trunk A-5 and H-2D^d/Trunk A-6) and control (H-2D^d/ HIV_{P18-110}) dextramers [27]. Briefly, LNCs obtained from the animals immunized with FL-Myhc₃₃₄₋₃₅₂ were stimulated with the immunizing peptide (50 μ g/ml), and on day 5 post stimulation, viable cells were harvested and maintained in IL-2 medium. Cells harvested on day 8 were washed and chilled on ice for 20 minutes, followed by staining with the MHC class I dextramers using FBS (2.5%)-supplemented IL-2 medium, pH 7.63, at RT for 1 hour. Cells were washed and stained with anti-CD8, anti-CD4 and 7-AAD. After washing, cells were acquired by flow cytometry, and the percentages of dextramer⁺ CD8⁺ cells were determined in the live (7-AAD⁻) population using Flow Jo software.

2.8 Intracellular cytokine staining

Single cell suspensions were prepared from mice immunized with FL-Myhc₃₃₄₋₃₅₂, Trunk A-4 peptide or Trunk A-5 peptide on day 14 post immunization. Cells (5x10⁶/ml) were stimulated with the immunizing peptides (50 μ g/ml) for two days, and IL-2 medium was then added. On day 6, viable cells were stimulated briefly with phorbol-12-myristate 13-acetate (PMA; 20 ng/ml) and ionomycin (300 ng/ml; Sigma Aldrich) in the presence of monensin (2 mM; Golgi stop; BD Pharmingen) for 5 hours. Cells were washed and stained with antibodies for CD4 and CD8 (eBioscience) and 7-AAD. After washing and fixing, cells were permeabilized and stained with cytokine antibodies or their respective isotype controls (rat IgG1, rat IgG2a and rat IgG2b). Cells were acquired by flow cytometry, and frequencies of cytokine-producing CD4 or CD8 T cells were analyzed in the live (7-AAD⁻) population using Flow Jo software by utilizing the gates drawn for isotype controls corresponding to each cytokine [11]. The following clones of cytokine antibodies were used: IL-2 (JES6-5H4); IFN- γ (XMG1.2); IL-4 (11B11); IL-10 (JES5-16E3); IL-17A (eBio 17B7); IL-17 F (eBio 18F10); and tumor necrosis factor (TNF)- α (MP6-XT22) (all from eBioscience) and IL-22 (140301; R&D Systems, Minneapolis, MN).

2.9. Histopathology

Hearts were collected at termination on day 21 post immunization with the peptides, and the tissues were fixed by immersion in 10% phosphate buffered formalin [11, 31]. Three longitudinal sections 5 μ m thick were made from each heart and stained with hematoxylin and eosin (H and E). The sections were examined by a board-certified pathologist blinded to treatment. Inflammation and severity scores were obtained based on the total number of inflammatory foci added across the three sections, deriving the percent inflammation as reported previously [9].

2.10 Magnetic resonance microscopy (MRM imaging)

To determine the structural and functional abnormalities of hearts in mice affected with myocarditis, we used MRM imaging as a non-invasive tool [32]. Briefly, mice were anaesthetized using 2% isoflurane; a rectal thermometer was placed in the rectum to monitor body temperature, and a pulse oximeter was connected to monitor the heart rate. An animal monitoring system involving respirometry and pulse oximetry was used to minimize image blurring due to respiratory and cardiac movements. Animals were subjected to MRM imaging using a wide-bore (89 mm) 9.4 T vertical-bore magnet (Varian, Inc. Walnut Creek, CA) equipped with triple axis gradients of 100 G/cm and a 4 cm radio-frequency imaging coil as described previously [32]. Using an echo-based cine pulse sequence, short-axis slices of hearts were captured in eight time frames and stored. The images were analyzed using Segment software (Segment v1.8 R1430, Medviso, Sweden) to determine the changes, if any, in the structural (left ventricular [LV] wall thickness) and functional parameters (end diastolic volume and ejection fraction).

2.11 Statistics

Student's t-test was used to determine differences in T cell proliferative responses, MHC class I stabilization assay, cytokine-producing cells, and degree of inflammation between groups. $p < 0.05$ values were considered significant.

3. Results

3.1 A/J mice immunized with FL-Myhc₃₃₄₋₃₅₂ show generation of antigen-specific CD4 and CD8 T cell responses

EAM induced with FL-Myhc₃₃₄₋₃₅₂ has been used to study the cellular mechanisms of cardiac autoimmunity that involve the mediation of Myhc-reactive CD4 T cells [6, 11]. However, by characterizing the infiltrates of the hearts, we noted that CD8 T cells contributed a proportion of T cells (CD3⁺ CD8⁺: 3.50 \pm 1.40%; CD3⁺ CD4⁺: 11.84 \pm 5.09%), leading us to conduct tests to determine their antigen specificity. Groups of A/J mice were immunized with Myhc₃₃₄₋₃₅₂, and after 14 days, CD4 and CD8 T cells were enriched by negative selection with a purity of more than 95% (Fig. 1, top panel). Cells were stimulated with irradiated APCs pulsed with Myhc₃₃₄₋₃₅₂ to measure the proliferative responses based on ³[H]-thymidine-incorporation assay. Predictably, CD4 T cells specifically and significantly responded to Myhc₃₃₄₋₃₅₂ ($p < 0.0053$), since the responses to a control peptide, RNase₄₃₋₅₆, were absent (Fig. 1, bottom left panel). Their antigen specificity

was later confirmed by staining the cells with MHC class II/IA^k dextramers. In brief, LNCs from mice immunized with Myhc₃₃₄₋₃₅₂ were stimulated with the peptide and the cells were rested in IL-2 medium. Viable cells harvested on day 8 were stained with IA^k/Myhc₃₃₄₋₃₅₂ and RNase₄₃₋₅₆ (control) dextramers, leading to the detection of Myhc-specific CD4 T cells (2.59±0.32%) since the staining for control dextramers was negligible (0.24±0.073%) (Supplemental fig. 1; p=0.00016). However, unexpectedly, CD8 T cells obtained from immunized animals also responded to Myhc₃₃₄₋₃₅₂, dose- and antigen-dependently, and the responses were significant when compared with control (p<0.00058; Fig. 1, bottom right panel). Importantly, the magnitude of CD8 T cell responses was comparable to that of CD4 T cells, as the responses between the two did not differ significantly.

We then asked if the CD8 T cells were to respond to Myhc, whether the antigen-sensitized cells would produce IFN- γ , the signature cytokine of CD8 T cells. To test this possibility, LNCs from immunized mice were stimulated with Myhc₃₃₄₋₃₅₂, and their cytokine-producing abilities were tested in relation to CD4 and CD8 T cell subsets. Supplemental figure 2 shows that the cells capable of producing Th1 (IFN- γ) and Th17 (IL-17A, IL-17F and IL-22) cytokines were predominant, followed by IL-10-producing Th2 cells. In contrast, under the same conditions, the IFN- γ -producing ability of CD8 T cells was enhanced four-fold (p = 0.0032), but Th17 cytokines were limited to IL-17F and IL-22, and IL-10-producing cells were negligible. The enhanced IFN- γ -producing ability of CD8 T cells further supported our proposal that they might be antigen-specific and play a role in disease mediation.

To determine the pathogenic role of Myhc-reactive CD8 T cells, lymphocytes were obtained from animals immunized with Myhc₃₃₄₋₃₅₂, and the CD8 T cells were enriched. After stimulating with Con-A for two days, cells were administered into naïve animals. Upon evaluating hearts at termination on day 18, we detected inflammatory foci accompanied by hemorrhagic necrosis and mineralization in the recipients of antigen-primed CD8 T cells (Fig. 2ai and 2aaii), but not in the saline group (Fig. 2aaiii) or the recipients of naïve CD8 T cells (Fig. 2aaiiv). This finding suggested that Myhc-reactive CD8 T cells are pathogenic, and the effector mechanisms might involve their cytolytic functions. Consistent with this notion, we verified that Myhc₃₃₄₋₃₅₂-reactive CD8 T cells express granzyme B (Fig. 2b left panel; p<0.027) and perforin (Fig. 2b middle panel; p<0.026) intracellularly, and express CD107a/CD107b (Fig. 2b right panel; p<0.020), the surface markers of degranulation process of effector cells [20-22, 24, 33, 34].

3.2 Localization of CD8 T cell epitopes within the FL-Myhc₃₃₄₋₃₅₂

CD8 T cells generally recognize epitopes with a length of 8 to 11 amino acids, although they can recognize longer peptides [3, 35-37]. However, in our case, the finding that the 19-mer FL-Myhc₃₃₄₋₃₅₂ induced CD8 T cell responses suggested that the CD8 T cell epitopes must be localized within this peptide sequence, and identification of these determinants required three levels of screening.

In the primary screening, we sought to use the truncated peptides Trunk A (Myhc₃₃₄₋₃₄₉), Trunk B (Myhc₃₃₈₋₃₅₂), Trunk C (Myhc₃₃₄₋₃₄₇) and Trunk D (Myhc₃₄₀₋₃₅₂), generated from FL-Myhc₃₃₄₋₃₅₂ in a proliferation assay, in which the truncations resulted from the deletions

of 3 to 6 amino acids from the N- or C-terminal ends in each (Table 1). Briefly, CD4 and CD8 T cells were enriched from animals immunized with Myhc₃₃₄₋₃₅₂, as above, and the cells were tested for their responses to Myhc₃₃₄₋₃₅₂ (positive control), truncated peptides and RNase₄₃₋₅₆ (negative control), leading us to make two observations.

(a) D4 T cells responded vigorously to Myhc₃₃₄₋₃₅₂ ($p=0.03$) as well as Trunk A ($p=0.02$) and Trunk B ($p=0.02$) peptides, but their responses to Trunk C and Trunk D peptides were progressively reduced (Fig. 3, top panels). The responses to Myhc peptides were specific, since the antigen-sensitized T cells did not respond to the control. These patterns were expected because the putative MHC class II (IA^k)-binding residue Asp at position 338 (Asp₃₃₈), as well as the two CD4 T cell receptor (TCR)-contact residues (Ala₃₄₈, major; and Ala₃₄₄, minor), for CD4 T cells are preserved in both Trunk A and Trunk B (Table 1, [6]). As such, the relative reduction in response to Trunk C was expected because the major TCR-contact residue Ala₃₄₈ is lacking (Table 1). Although the Trunk D peptide contains both TCR-contact residues, the response was low, perhaps because the peptide lacked the putative MHC-binding residue, Asp₃₃₈.

(b) Like CD4 T cells, CD8 T cells also responded to various truncated peptides of Myhc, but not the control, with similar trends in a decreasing order (Trunk A > Trunk B > Trunk C > Trunk D; Fig. 3, bottom panels). The magnitude of responses to Trunk A and Trunk B peptides, was significantly higher than responses to the control (RNase 43-56, $p<0.05$). Since, the Trunk A peptide (with deletions of three amino acids [VYK] at the C-terminus) showed a comparable response to that obtained with the Trunk B peptide (with deletions of four amino acids [DSAF] at the N-terminus) (Table 1), we focused on the central core region (12-mer: DVLSFTAEEKAG), which was common to both in order to identify shorter peptides that could potentially induce CD8 T cell responses. In support of this proposal, we noted that responses to the Trunk C peptide, with deletion of two additional residues (AG) at the C-terminus, and to the Trunk D peptide, with deletion of six residues (DSAFDV) at the N-terminus, remained low (Fig. 3, bottom panels).

In the secondary screening, within the Trunk A peptide we designed six 10-mer to 12-mer peptides labelled Trunk A-1 to Trunk A-6 (Table 2). We enriched CD4 and CD8 T cells from mice immunized with FL-Myhc₃₃₄₋₃₅₂ and evaluated their responses to these six peptides.

As shown in figure 4 (top panels), Trunk A-4 and Trunk A-5 peptides induced the responses in CD8 T cells ($p = 0.009$), and if any, the responses for Trunk A-4 were better than FL-Myhc₃₃₄₋₃₅₂ ($p=0.02$), whereas responses for Trunk A-6 were absent. Likewise, responses to three other peptides (Trunk A-1, A-2 and A-3) also were marginal (data not shown).

Furthermore, our attempts to reduce the length of Trunk A-4, and A-5 peptides to fewer than 10 amino acids did not yield better results. To this end, we designed 9-mer peptides, based on the epitope prediction database [38]. Proliferative responses, as indicated by stimulation indices, were marginal compared with responses obtained with the control peptide (RNase₄₃₋₅₆) (Supplemental table 1).

In contrast to CD8 T cells however, while all three peptides (Trunk A-4, A-5 and A-6) induced responses in the CD4 T cells comparable to responses induced by Myhc₃₃₄₋₃₅₂, responses to Trunk A-4 and Trunk A-5 ($p = 0.005$), but not Trunk A-6 ($p = 0.061$), were significantly higher than responses to the control (Fig. 4, bottom panels). We had expected the responses to Trunk A-5 (with a deletion of major TCR-contact residue Ala₃₄₈) and Trunk A-6 (with a deletion of putative MHC-contact residue Asp₃₃₈) to be lower than responses to Trunk A-4, which contained both the MHC-contact residue Asp₃₃₈ and two TCR-contact residues (Ala₃₄₄; and Ala₃₄₈; Table 1). But that was not the case.

We thus identified Trunk A-4 and Trunk A-5 as the putative Myhc peptides that induce both CD4 and CD8 T cell responses. The immunogenicity of these peptides was later confirmed by immunizing groups of mice. As expected, CD4 T cells responded comparably to both Trunk A-4 and A-5 peptides, whereas the response to Trunk A-6 was weak (Supplemental fig. 3, top panels). In contrast, CD8 T cells responded to a greater degree to Trunk A-4 followed by Trunk A-5, and the least response was obtained for Trunk A-6 peptides (Supplemental fig. 3, bottom panels).

3.3 Identification of MHC class I allele that binds CD8 T cell epitopes

It has been previously shown that the FL-Myhc₃₃₄₋₃₅₂ binds the IA^k (MHC class II) allele in A/J mice. Since this mouse strain expresses three MHC class I alleles – H-2D^d, H-2K^k and H-2L^d – it was necessary to determine the specific allele that presents the Myhc peptides to CD8 T cells. This was examined via an MHC class I stabilization assay that involved the use of RMA-S cell lines expressing H-2D^d, H-2K^k and H-2L^d alleles, which stabilize MHC molecules on their surface when the MHC-expressing cells bind with peptides [39]. We used fluorophore-conjugated allele-specific antibodies to ascertain MHC stability, determining the difference in the MFI ratios between cells loaded with or without peptides by flow cytometry. By using the peptides that induced the CD8 T cell responses (Trunk A-4 and Trunk A-5), we found that the MFI ratios obtained with RMA-S-D^d cells were significantly higher for Trunk A-5 peptides ($p = 0.03$) compared to cells incubated with no peptide (Fig. 5). Although MFI ratios obtained for Trunk A-4 were lower than those obtained with Trunk A-5 peptides, the ratios were significantly higher than those obtained with the control peptide, especially at a concentration of 200 μ M or more ($p = 0.05$). Under similar conditions, however, neither of the two peptides bound to RMA-S-K^k- and RMA-S-L^d-expressing cells (data not shown). Thus, we identified Trunk A-4 and Trunk A-5 peptides as the binders of the H-2D^d molecule. These results were further corroborated using T2-D^d cells (kind gift from Ted Hansen, Washington University, St. Louis; and Joyce Solheim, University of Nebraska Medical Center, Omaha), which also showed stronger affinity for Trunk A-5 than Trunk A-4 (data not shown).

3.4 Antigen-specificity of CD8 T cells sensitized with FL-Myhc₃₃₄₋₃₅₂

After identifying Trunk A-4 and Trunk A-5 peptides as the putative CD8 T cell epitopes based on peptide screening and MHC-stabilization assay, we sought to create MHC class I dextramers and we recently showed that the MHC dextramers were found to be superior to tetramers in various experimental settings [27, 40, 41]. We generated H-2D^d dextramers by conjugating the peptide-loaded, biotinylated H-2D^d protein assembled with the β 2m as

described previously [27]. Essentially, we prepared dextramers for Trunk A-4, Trunk A-5 and Trunk A-6 peptides (control) and used them to determine the specificity of CD8 T cells generated in response to Myhc₃₃₄₋₃₅₂. The expectation was that H-2D^d dextramers of Trunk A-4 and A-5, but not A-6, peptides would bind CD8 T cells, but none would bind CD4 T cells. Briefly, LNC cultures were prepared from A/J mice immunized with Myhc₃₃₄₋₃₅₂, and the cells were stained with the dextramers and CD4 and CD8 antibodies. As shown in figure 6, H-2D^d/Trunk A-4 ($2.74 \pm 0.34\%$; $p = 0.0014$) and H-2D^d/Trunk A-5 ($1.79 \pm 0.29\%$; $p = 0.0042$) dextramers stained the Myhc₃₃₄₋₃₅₂-sensitized CD8 T cells, whereas the staining for control (Trunk A-6 dextramers) was negligible ($0.05 \pm 0.008\%$). Similarly, dextramers prepared using the irrelevant control antigen HIV_{P18-110} also did not stain the cells ($0.04 \pm 0.0058\%$), indicating that the staining obtained with H-2D^d /Trunk A-4 and A-5 dextramers was specific. Additionally, none of the H-2D^d dextramers stained the CD4 T cells generated in mice immunized with Myhc₃₃₄₋₃₅₂, further ensuring the specificity of the MHC class I dextramers (Fig. 6). Taken together, the patterns of staining obtained with H-2D^d dextramers and the MHC-stabilization assay were identical, indicating that the two CD8 T cell epitopes (Trunk A-4 and Trunk A-5) are localized within Myhc₃₃₄₋₃₅₂.

3.5 Determination of the pathogenic potential of Myhc-sensitized CD8 T cells

To determine the pathogenic potential of antigen-sensitized CD8 T cells, we analyzed their cytokine-producing abilities and compared the responses with CD4 T cells [11]. In brief, LNCs from mice immunized with Myhc₃₃₄₋₃₅₂ were restimulated with the Trunk peptides (A-4, and A-5) and Myhc₃₃₄₋₃₅₂, and the frequencies of cytokine-producing cells were evaluated for Th1, Th2 and Th17 cytokines and TNF- α . As shown in figure 7 (left panels), cells capable of producing Th1 (IFN- γ) cytokines and TNF- α were predominantly present in both CD4 and CD8 T cell cultures, followed by Th17 and Th2 cytokines. Importantly, when the cytokine profiles of CD4 and CD8 T cell subsets were compared, it was evident that the frequencies of IFN- γ -secreting cells were consistently greater in the CD8 T cell cultures, regardless of peptides used (Myhc₃₃₄₋₃₅₂: 11.19 ± 1.08 vs 1.98 ± 0.43 , $p = 0.02E-02$; Trunk A-4: 12.87 ± 0.46 vs 2.85 ± 0.54 , $p = 8.08E-06$; Trunk A-5: 14.40 ± 1.62 vs 2.96 ± 0.48 , $p = 4.91E-05$). Conversely, percentages of cells producing Th2 cytokines tended to be greater in CD4 T cells stimulated with Trunk A-4 and A-5 peptides (Fig. 7, right panels). No other major differences were noted for other cytokines except that CD8 T cell cultures produced fewer IL-17F-producing cells than did CD4 T cells ($p = 0.009$). The findings that the cytokine patterns obtained in CD8 T cell cultures with the Trunk peptides A-4 and A-5 were comparable to those produced with Myhc₃₃₄₋₃₅₂, and that their responses were largely similar to those produced in CD4 T cells suggest that the A-4 and A-5 peptides might be able to induce disease.

3.6 Evaluation of disease induced with Trunk A-4 and Trunk A-5 peptides

We evaluated the disease-inducing abilities of peptides by immunizing groups of mice with Trunk A-4 and Trunk A-5 and compared the disease severity, as assessed by histology, with that of Myhc₃₃₄₋₃₅₂, which was used as a positive control (Fig. 8). While all three peptides induced myocarditis showing infiltrations of mononuclear cells, the disease severity induced with Trunk A-4 and A-5 peptides was lower than that induced by Myhc₃₃₄₋₃₅₂ (Fig. 8; Myhc₃₃₄₋₃₅₂: incidence, 5/5; percent area inflamed, 12.8 ± 5.54). However, among the two

truncated peptides, the disease incidence (Trunk A-4, 6/13; Trunk A-5, 3/9) and the homogeneity of histological changes (percent area inflamed: Trunk A-4, 3 ± 0.75 ; Trunk A-5, 10.7 ± 7.22) were more consistent with Trunk A-4 than Trunk A-5. We also made efforts to determine whether the disease induced with the truncated peptides could also be captured non-invasively. To provide a proof-of-principle for this analysis, we subjected the animals immunized with Trunk A-4 peptides to MRM imaging to evaluate structural and functional abnormalities. The data revealed that the LV wall thickness in the Trunk A-4-immunized mice was increased by ~ 1.35 -fold ($p = 2.25E-05$) in relation to age-matched healthy mice (Supplemental fig. 4a). Likewise, measurement of cardiac output parameters indicated that both end diastolic volumes ($p = 3.45E-05$) and ejection fractions ($p = 5.67E-07$) were low in the myocarditic mice compared to healthy controls (Supplemental fig. 4b). Taken together, the data suggest that the Trunk A-4 and Trunk A-5 peptides can induce myocarditis, and the histological features are similar to those of myocarditis induced by Myhc₃₃₄₋₃₅₂.

4. Discussion

We report here the localization of the CD8 T cell determinant, Myhc₃₃₈₋₃₄₈ that induces EAM in A/J mice. Previously, it has been shown that Myhc₃₃₄₋₃₅₂, an immunodominant epitope that binds the MHC class II allele IA^k, induces myocarditis in IA^k-expressing A/J mice through the generation of Myhc-reactive CD4 T cells [6, 10, 11, 15]. We recently created MHC class II/IA^k tetramers and dextramers and demonstrated that Myhc₃₃₄₋₃₅₂-specific CD4 T cells are generated in the peripheral repertoire and infiltrate the hearts of immunized animals [15, 27]. However, by examining the heart infiltrates, we noted that CD8 T cells also formed a component of the T cell fraction ($\sim 35\%$ of T cells), leading us to determine their potential role in the mediation of cardiac autoimmunity.

We first verified that the responses of CD8 T cells from the animals immunized with Myhc₃₃₄₋₃₅₂ were comparable with responses of CD4 T cells (Fig. 1). Importantly, we confirmed that Myhc-sensitized CD8 T cells produce IFN- γ (Supplemental fig. 2) and express markers CD107a/CD107b, granzyme and perforin, the markers of their effector functions, and transfer disease to naïve animals (Fig. 2). Based on these observations, we decided to localize the CD8 T cell epitopes within FL-Myhc₃₃₄₋₃₅₂ (DSAFDVLSFTAEEKAGVYK). In this peptide, three amino acid residues were previously identified to be critical (Asp₃₃₈, MHC class II/IA^k-anchor; and Ala₃₄₄-minor; and Ala₃₄₈-major TCR-contacts; Table 1), where deletion of major TCR contact residue Ala₃₄₈ was found to reduce CD4 T cell responses [6]. Hence, during the primary screening, we took an approach to ablate the CD4 T cell responses by designing peptides to retain or to delete the MHC (Asp₃₃₈)- or the major TCR (Ala₃₄₈)-contact residues. These include trunk A and trunk B (both contain MHC- and TCR-contact residues); trunk C (lacks the TCR-contact residue); and trunk D (lacks the MHC-contact residue) (Table 1). Predictably, we noted the responses for Trunk A and Trunk B in CD4 T cells, whereas the responses for Trunk C and Trunk D were low (Fig. 3). In contrast, the two truncated peptides (Trunk A and Trunk B), containing all three residues essential for the induction of CD4 T cell responses, also induced comparable responses in CD8 T cells (Fig. 3). These data pointed to a possibility that the central core region (DVLSFTAEEKAG) may encompass the CD8 T cell epitope/s,

with the further possibility that CD4 T cell responses could still be preserved, since the MHC class II anchor and the TCR-contact residues for the generation of CD4 T cells were still conserved (Table 1).

Reports indicate that the peptides binding to MHC class I molecules can be of variable lengths (8 to 11 amino acids), but long peptides up to 15 amino acids can also bind class I molecules under the conditions of conformational change of their peptide-binding sites. Therefore, we designed a total of six peptides with a length of 10 to 12 amino acids, and found that two peptides (Trunk A-4: DVLSFTAEEKA, and Trunk A-5: DVLSFTAEEK) induced responses in CD8 T cells comparable to those obtained with FL-Myhc₃₃₄₋₃₅₂, whereas response for Trunk A-6 was absent (Fig. 4). But surprisingly, all three peptides, including Trunk A-5, which was missing the major TCR-contact residue Ala₃₄₈ for CD4 T cells, and Trunk A-6, which was missing the class II MHC-anchor residue Asp₃₃₈ at the N-terminus, stimulated the CD4 T cells, and their responses were comparable to those stimulated by Myhc₃₃₄₋₃₅₂. These data support the notion that shorter peptides of 10-mers can also be presented by MHC class II molecules to CD4 T cells as reported by others [42-44]. While our data also suggest the possibility of the existence of an alternative MHC class II-anchor residue, the display of major or minor TCR-contact residues in the short peptide sequences may still be similar to FL-Myhc₃₃₄₋₃₅₂. Alternatively, the minor TCR-contact residue Ala₃₄₄ may get displayed differently with an altered binding register in the absence of the major TCR-contact residue as in Trunk A-5 (Table 2), and such a compensatory change may be adequate to facilitate peptide-recognition by TCR. Overall, since Trunk A-4 and Trunk A-5 peptides showed comparable responses in CD8 T cells, we decided to use these for further experimentation. Finally, after confirming that the MHC class I allele H-2D^d presents both Trunk A-4 and Trunk A-5 peptides, we created their dextramers and confirmed that the CD8 T cells reacting to Myhc were antigen-specific, suggesting they play a role in the development of myocarditis.

To evaluate the pathogenic potential of CD8 T cell epitopes, we first analyzed the cytokine-producing ability of CD8 T cells based on intracellular staining using panels of Th1, Th2 and Th17 cytokines and TNF- α . Essentially, the Myhc-reactive CD8 T cell cultures contained cells capable of producing all cytokines tested, and their profiles were comparable to those of CD4 T cells. Importantly, the frequencies of IFN- γ -producing cells were significantly greater in CD8 than in CD4 cultures, except that IL-17F-producing cells were low in the cultures responsive to Trunk A-4 and A-5 peptides. However, regardless of cell type, Th2 cytokines were consistently low. However, it is to be noted that conflicting reports were available as to the role of cytokines in the mediation of autoimmune myocarditis, in part, due to the variation in the genetic backgrounds of the mouse strains used [12, 13]. For example, IL-13-deficient and IL-12p40-deficient Balb/c mice showed opposing disease phenotypes, in that the disease severity was enhanced in IL-13-deficient mice, whereas in IL-12p40-deficient mice, the severity was relatively low [13, 45-47]. Conversely, while, Balb/c mice deficient for IFN- γ showed more severe disease, the IL-4-deficient A/J mice showed an attenuated myocarditis [31, 47]. It is currently held that the cytokines represented by both Th1 (IFN- γ) and Th17 (IL-17 family cytokines and IL-22) are critical to the mediation of autoimmune myocarditis, since cytokines other than IL-12 produced by APCs, such as IL-1, IL-6, IL-23 and transforming growth factor- β , favor the development of

proinflammatory Th17 cytokines [13, 46]. However, it is to be noted that IL-17A appears not critical for the development of myocarditis as IL-17A-deficient mice can develop severe disease [48]. Adding to this complexity is the possibility of plasticity for the interconversion of Th1 to Th17 and vice versa [49, 50]. Finally, by verifying the disease-inducing potential, we noted that the histological changes observed with myocarditis resulting from Trunk A-4 and Trunk A-5 peptides were comparable to those resulting from FL-Myhc₃₃₄₋₃₅₂, but the disease severity induced by the Trunk peptides remained mild to moderate (Fig. 8). The relative lower ability of CD8 T cells to produce IL-17F (Fig. 7), which otherwise contributes to the aggravation of inflammation by promoting neutrophil/monocyte migration [51, 52], might explain the lower disease severity induced with the determinants for CD8 T cells.

4.1 Limitations

In this study, we noted the following limitations: (i) for intracellular cytokine staining, we could not compare the cytokine profiles obtained with Myhc peptide-stimulations, with the cells cultured in medium-alone, or the cells stimulated with the irrelevant peptide, since the cells in the latter two conditions were not expected to be viable in the cultures for six days. Although, we evaluated the cytokine-secretion based on cytokine bead array analysis in the whole cell cultures specific to Myhc peptides (data not shown), we could not analyze the cytokines in the individual CD4 and CD8 subsets enriched by magnetic separation; (ii) we performed the MHC class I stabilization assay based on published protocols, but these protocols do not involve the use of a positive control peptide; its use otherwise would have been helpful to compare the affinity of Myhc peptides; (iii) the animals immunized with Trunk A-5 peptide were not subjected for MRM imaging since the incidence of disease induced with this peptide was low; and (iv) we made no efforts to identify the amino acid residues within Myhc₃₃₈₋₃₄₈ that are critical for binding MHC class I (H-2D^d) and the TCR of CD8 T cells.

5. Conclusion

We have demonstrated that Myhc₃₃₈₋₃₄₈ acts as a dual epitope for both CD4 and CD8 T cells, and the CD8 T cell epitopes induce myocarditis through the generation of Myhc-specific IFN- γ -producing cells. Interestingly, the shorter peptides, Trunk A-5 and A-6, lacking the major TCR-contact residue and MHC class II-anchor, respectively, also induced CD4 T cell responses comparable to those produced by FL-Myhc₃₃₄₋₃₅₂. These observations may lead to the conclusion that a proportion of antigen-specific CD4 T cells that arise in response to Myhc₃₃₄₋₃₅₂ may be the result of recognition of shorter peptides possibly generated by the processing of FL-Myhc₃₃₄₋₃₅₂ by APCs. In this scenario, the presence of the single TCR-contact residue Ala₃₄₄ in FL-Myhc₃₃₄₋₃₅₂, supposedly a minor TCR-contact residue in Trunk A-5, may be sufficient to induce activation of CD4 T cells. During this process, the cardiac-reactive CD8 T cells can also be produced and participate in the disease pathogenesis. Thus, the hierarchical generation of cardiac-reactive CD4 and CD8 T cells may be possible, but they may collectively contribute to the tissue destruction. Although the disease induced by CD4 cells is typically mediated by Th1 and Th17 cytokines, in addition to their cytolytic functions, cytokines produced by CD8 T cells similar to Th1 and Th17 phenotypes [53, 54] also have been implicated. Furthermore, Myhc, being an intracellular

protein, is expressed by cardiac myocytes, and the resident APCs in A/J mice have also been shown to display fragments of Myhc [6]. Yet, the animals do not develop myocarditis spontaneously, possibly because of self-tolerance due to the lack of costimulatory signals. In inflammatory conditions, however, upregulation of costimulatory molecules may favor the presentation of peptides, leading to a break in self-tolerance, and cardiac autoimmunity may ensue, possibly involving the generation of Myhc-reactive CD4 and CD8 T cells. Of note, the generation of pathogenic, dual T cell-specific responses through the recognition of a single epitope similar to Myhc₃₃₄₋₃₅₂ has been described for at least three other self-antigens, myelin oligodendrocyte glycoprotein 35-55, cancer testes antigen and interphotoreceptor retinoid-binding protein 201-216 [42, 55-57]. However, reports also indicate that induction of antigen-specific CD8 T cells can be potentially beneficial to controlling autoimmune responses, because CD8 T cells that lack the expression of CD28 can behave as regulatory T (Treg) cells, as shown in systemic lupus erythematosus and rheumatoid arthritis [58-60]. This was not the case, however, with Myhc-specific CD8 T cell responses, because the Myhc-reactive cells were positive for CD28, and did not express other markers of Treg cells like forkhead box P3 (data not shown). Nonetheless, the finding that a solitary peptide fragment bearing the epitopes for two different T cell types induced myocarditis provides new insights as to how different immune cells can recognize the same antigen and mediate cardiac damage by different mechanisms. In autoimmune settings, identification of the disease-inducing epitopes and their cellular mechanisms may possibly create opportunities to target antigen-specific T cells for therapy. As to human relevance, IgG autoantibodies for various cardiac antigens like cardiac myosin, β 1-adrenergic receptor, cardiac troponin I, Na-K-ATPase and heart-specific mitochondrial inner membrane proteins M7 type A and cardiac adenine-nucleotide transporter have been demonstrated in patients with myocarditis/dilated cardiomyopathy, and experimentally, in animal models of adjuvant and viral myocarditis, but their pathological significance continues to be uncertain [61-65]. However, the requirement that T cell help is critical for B cells to produce IgG antibodies suggests that cardiac-reactive T cells might play a pathogenic role. Our data may point to this possibility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

Myhc	cardiac myosin heavy chain- α
MHC	major histocompatibility complex
Th	T helper
IFN	interferon

IL	interleukin
APCs	antigen-presenting cells
EAM	experimental autoimmune myocarditis
CFA	complete Freund's adjuvant
FL-Myhc	full-length Myhc
RNase	bovine ribonuclease
HIV	human immunodeficiency virus envelope glycoprotein
³[H]-thymidine	tritiated-thymidine
cpm	counts per minute
Con-A	concanavalin-A
LNCs	lymph node cells
7-AAD	7-amino-actinomycin-D
MFI	mean fluorescence intensities
β2m	β2microglobulin
PMA	phorbol-12-myristate 13-acetate
Bir A	biotin ligase
RT	room temperature
TNF	tumor necrosis factor
H and E	hematoxylin and eosin
MRM	magnetic resonance microscopy
LV	left ventricular
TCR	T cell receptor
Treg	regulatory T cells

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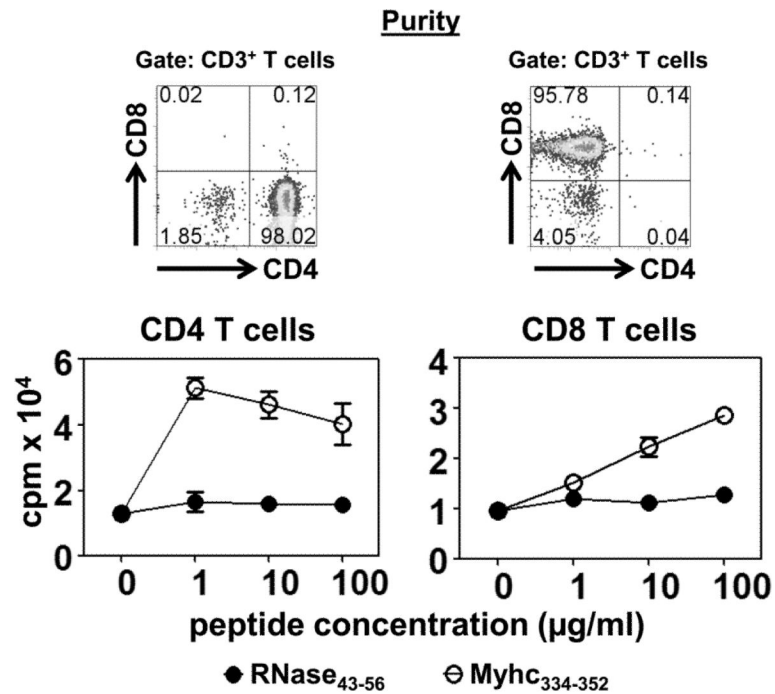


Figure 1. FL-Myhc₃₃₄₋₃₅₂ induces both CD4 and CD8 T cell responses

A/J mice were immunized with FL-Myhc₃₃₄₋₃₅₂ in CFA; after 14 days animals were euthanized to prepare single cell suspensions from spleens and lymph nodes, from which CD4 and CD8 T cells were enriched by magnetic separation. The purity of CD4 and CD8 T cell preparations were ascertained by staining with antibodies for CD3, CD4 and CD8, and 7-AAD, where the percentages of respective cell populations were determined within the live (7-AAD⁻) cells (top panel). Cells were stimulated with the irradiated APCs pulsed with the indicated peptides for two days. After pulsing with ³[H]-thymidine (1µCi/well) for 16 hours, proliferative responses were measured as cpm. Mean ± SEM values derived from five experiments, each involving 5 to 8 mice are shown (bottom panels). RNase₄₃₋₅₆ (control).

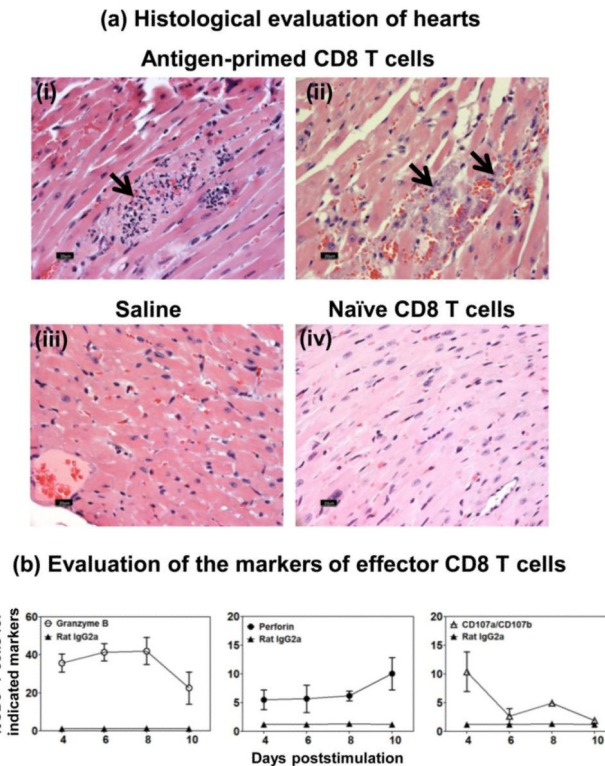


Figure 2. The effector FL-Myhc₃₃₄₋₃₅₂-sensitized CD8 T cells transfer disease to naïve recipients

(a) Histological evaluation of hearts. A/J mice were immunized with FL-Myhc₃₃₄₋₃₅₂; after 14 days, CD8 T cells were enriched from spleens and lymph nodes. Cells were stimulated with Con-A for two days, and viable lymphoblasts were administered i.p. into naïve irradiated mice; each mouse also received pertussis toxin i.p. Whereas, naïve irradiated mice that received $1 \times$ PBS or Con-A stimulated CD8 T cells from unimmunized A/j mice served as controls. The animals were euthanized on day 18 post transfer, and hearts were examined by H and E staining. Panel-a (i and ii; Antigen-primed CD8 T cell-recipients) shows lymphocyte infiltrations, hemorrhagic necrosis and mineralization; multifocal necrotic areas and degenerative leukocytes, respectively; panel-a (iii and iv) shows normal sections from recipients of saline or naïve CD8 T cells respectively.

Representative sections are shown from experiments involving four to six mice per group. **(b) Evaluation of the markers of effector CD8 T cells.** LNC were isolated from mice immunized with Myhc₃₃₄₋₃₅₂, and after stimulating with peptide for two days, cultures were maintained in IL-2 medium. CD8 T cells were tested for granzyme B and perforin intracellularly, and also for surface expression of CD107a/CD107b by flow cytometry using anti-mouse antibodies, and their frequencies were enumerated in the CD8 subset. Mean \pm SEM values derived from seven experiments each involving three to six mice are shown.

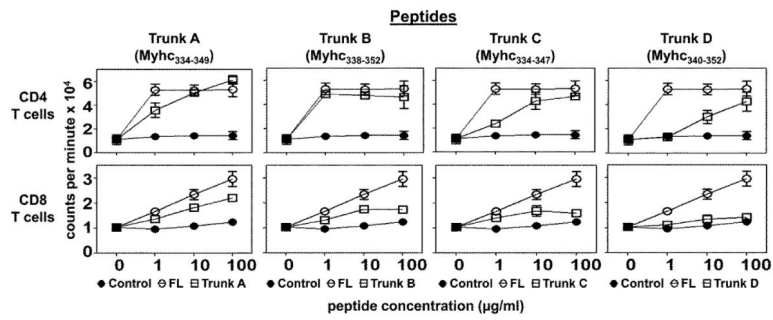


Figure 3. Evaluation of CD4 and CD8 T cell responses to the truncated peptides of FL-Myhc₃₃₄₋₃₅₂ (13 to 16-mers) in the primary screening

A/J mice were immunized with Myhc₃₃₄₋₃₅₂ in CFA; after 14 days, single cell suspensions were prepared from spleens and lymph nodes, from which CD4 and CD8 T cells were enriched by magnetic separation. Cells were stimulated with Myhc₃₃₄₋₃₅₂ and its truncated peptides (13 to 16-mers: Trunk A to D) in the presence of irradiated APCs for two days, followed by pulsing with ³[H]-thymidine. After 16 hours, proliferative responses were measured as cpm. RNase₄₃₋₅₆ (control). Mean ± SEM values obtained from two individual experiments, each involving 10 to 11 mice are shown.

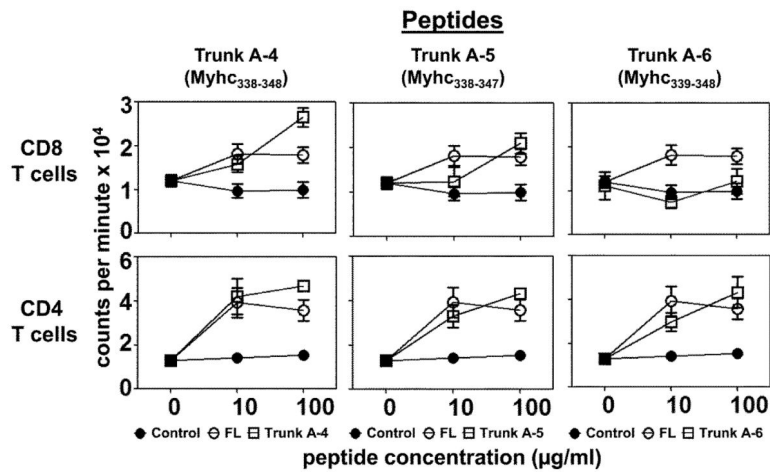


Figure 4. Evaluation of CD4 and CD8 T cell responses to the short peptides of FL-Myhc₃₃₄₋₃₅₂ (10 to 11-mers) in the secondary screening

A/J mice were immunized with Myhc₃₃₄₋₃₅₂ in CFA; after 14 days, CD4 and CD8 T cells were enriched by magnetic separation using the cell suspensions obtained from spleens and lymph nodes. Cells were stimulated with Myhc₃₃₄₋₃₅₂ and short peptides (10 to 11-mers: Trunk A-4 to A-6) in the presence of irradiated APCs for two days, followed 16 hours later by pulsing with ³[H]-thymidine, the incorporation of which was measured as cpm. RNase₄₃₋₅₆ (control). Mean ± SEM values obtained from two to four individual experiments, each involving 9 to 11 mice are shown.

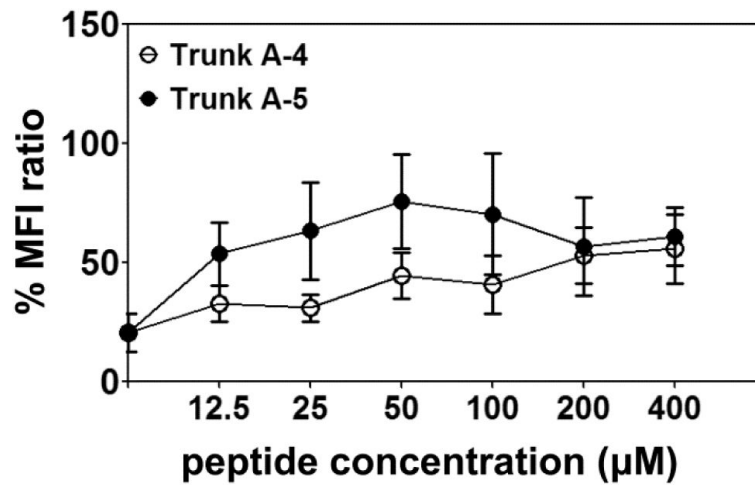


Figure 5. Determination of the binding of CD8 T cell epitopes to H-2D^d allele by MHC class I-stabilization assay

RMA-S-D^d cells were incubated in the presence or absence of the indicated peptides (0 to 400 µM) for 18 hours at 26°C; the plates were then transferred to a 37°C incubator and incubation continued for an additional 1.5 hours. After washing, cells were stained with fluorophore-conjugated H-2D^d antibody and acquired by flow cytometry. MFI values were obtained using CellQuest software. The MFI ratios were then derived using the formula as described in the section 2.6. Mean ± SEM values derived from five to six experiments are shown.

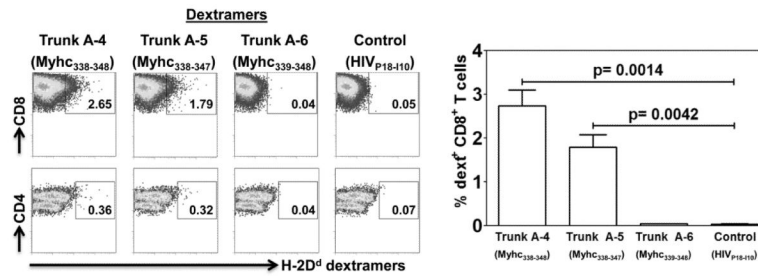


Figure 6. Evaluation of antigen-specificity of CD8 T cells using MHC class I dextramers
LNC were obtained from animals immunized with Myhc₃₃₄₋₃₅₂ and after cells were stimulated with the peptide for two days, IL-2 medium was supplemented. On day 8, cells were stained with the indicated H-2D^d dextramers, followed by anti-CD8, anti-CD4 and 7-AAD. After washing, cells were acquired by flow cytometry, and the percentages of dextramer⁺ cells were analyzed in relation to CD4 and CD8 T cells by Flow Jo software. H-2D^d/Trunk A-6 and H-2D^d/HIV_{P18-110} dextramers, control. Representative flow cytometric plots from one of the six experiments are shown (left panel). Mean \pm SEM values derived from six experiments are shown (right panel).

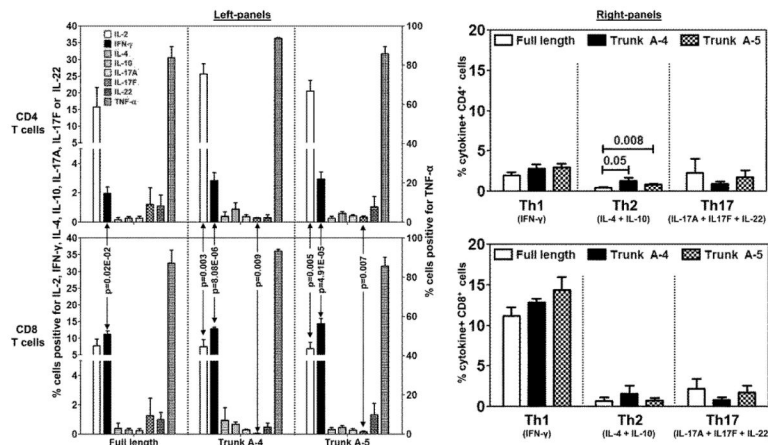


Figure 7. Cytokine responses to dual epitopes of FL-Myhc334-352 in CD4 and CD8 T cells LNCs obtained from A/J mice immunized with Myhc334-352, Trunk A-4, Trunk A-5 or Trunk A-6 were stimulated with the immunizing peptides for two days, and the cultures were maintained in IL-2 medium. Cells were briefly stimulated on day 6 with PMA and ionomycin for 5 hours in the presence of monensin. After staining with anti-CD4, anti-CD8 and 7-AAD, cells were fixed and permeabilized, and then stained with cytokine antibodies or their respective isotype controls. Cells were acquired by flow cytometry and the percentages of cytokine-producing cells in the live (7-AAD⁻) CD4 or CD8 T cell subsets were determined using Flow Jo software in relation to the gates drawn for isotype controls corresponding to each cytokine. Mean \pm SEM values from four to six independent experiments are shown. In the left panel, cells positive for individual cytokines are shown (left, y-axis: IL-2, IFN- γ , IL-4, IL-10, IL-17A, IL-17F and IL-22; right, y-axis: TNF- α). The right panel shows the cytokine⁺ cells with respect to Th1 (IFN- γ), Th2 (IL-4 + IL-10) and Th17 (IL-17A + IL-17F + IL-22) subsets.

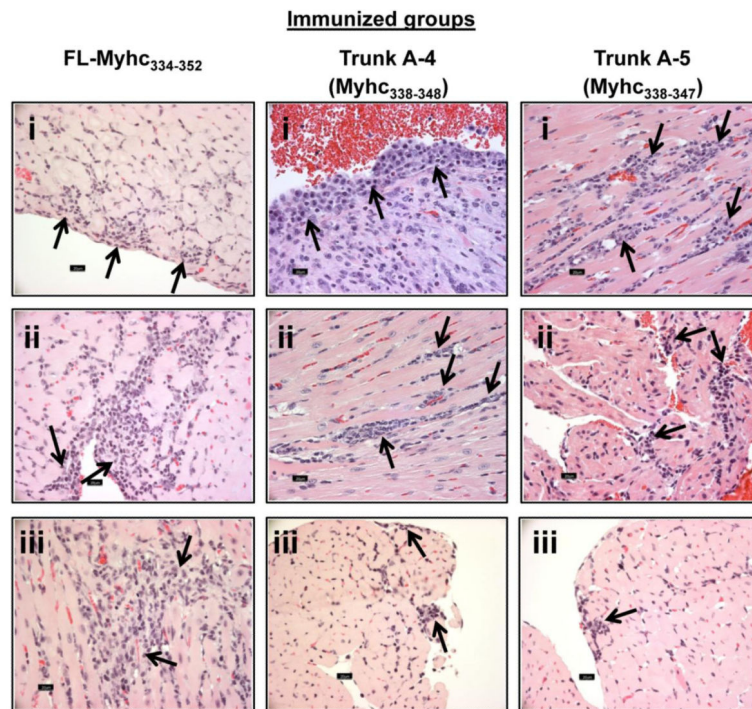


Figure 8. Histological evaluation of hearts obtained from mice immunized with FL-Myhc₃₃₄₋₃₅₂, and its truncated-derivatives, Trunk A-4 and Trunk A-5

Groups of A/J mice were immunized with Myhc₃₃₄₋₃₅₂, Trunk A-4 or Trunk A-5 in CFA on day 0 and day 7, and the animals received pertussis toxin on day 0 and day 2 after the first immunization. Animals were euthanized 21 days later, and hearts were examined for inflammation by H and E staining. Arrows indicate lymphocytic infiltrations. Left panels (Myhc₃₃₄₋₃₅₂-immunized group): epicardial infiltrates of lymphocytes and macrophages (i); subendocardial infiltrates of lymphocytes and macrophages extend into myocardium (ii); and interstitial myocardial infiltrates of macrophages and lymphocytes (iii). Middle panels (Trunk A-4-immunized group): subendocardial plasma cells, lymphocytes and macrophages extending into the myocardium (i); perivascular and interstitial lymphocytic and histiocytic infiltrates in the myocardium (ii); and subendocardial lymphocytes and macrophages (iii). Right panels (Trunk A-5-immunized group): interstitial lymphocytes and macrophages in myocardium (i); subendocardial lymphocytes and a few macrophages and rare plasma cells infiltrate the atria (ii); and small foci of lymphocytes in subendocardium (iii).

Table 1

The list of peptides used to evaluate CD4 and CD8 T cell responses in the primary screening

Peptide	Sequence	minor	major
	TCR contacts	344	348
		↑	↑
FL-Myhc ₃₃₄₋₃₅₂	DSAFDVLSFTAEKAGVYK [†]		
	↓		
	338		MHC-anchor
Trunk A (Myhc ₃₃₄₋₃₄₉)	DSAFDVLSFTAEKAG		
Trunk B (Myhc ₃₃₈₋₃₅₂)	DVLSFTAEKAGVYK		
Trunk C (Myhc ₃₃₄₋₃₄₇)	DSAFDVLSFTAEK		
Trunk D (Myhc ₃₄₀₋₃₅₂)	LSFTAEKAGVYK		

[†] putative MHC class II (I^A^k)-binding residue is shown with dotted arrow and two TCR-contact residues are shown with bold arrows

Table 2

The list of peptides used to evaluate CD4 and CD8 T cell responses in the secondary screening

Peptide	Sequence
Trunk A (Myhc ₃₃₄₋₃₄₉)	DSAFDVLSFTAEEKAG
Trunk A-1 (Myhc ₃₄₀₋₃₄₉)	LSFTAEEKAG
Trunk A-2 (Myhc ₃₃₉₋₃₄₉)	VLSFTAEEKAG
Trunk A-3 (Myhc ₃₃₈₋₃₄₉)	DVLSFTAEEKAG
Trunk A-4 (Myhc ₃₃₈₋₃₄₈)	DVLSFTAEEKA
Trunk A-5 (Myhc ₃₃₈₋₃₄₇)	DVLSFTAEEK
Trunk A-6 (Myhc ₃₃₉₋₃₄₈)	VLSFTAEEKA

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