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FTY720 (Gilenya) treatment prevents spontaneous autoimmune myocarditis and dilated cardiomyopathy in transgenic HLA-DQ8-BALB/c mice

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Abstract

Although dilated cardiomyopathy (DCM) is often caused by viral infections, it frequently involves autoimmune mechanisms associated with particular HLA-DR and DQ alleles. Our homozygous HLA-DQ8.Ab⁰ transgenic mice in the BALB/c background (HLA-DQ8^{BALB/c}-Tg) developed early and progressive fatal heart failure from 4–5 weeks of age. Clinical signs of the disease included cyanotic eyes, tachycardia with dyspnea, from pale to cyanotic limbs and terminal whole body edema. Sick mice had extremely dilated hearts, enlarged liver and spleen, and pleural/peritoneal effusion. Histology of the heart showed extensive heart muscle destruction with signs of fibrosis. The autoimmune nature of the disease was shown by high titers of anti-myosin antibodies in the sera and IgG deposits in sick heart muscles, as well as focal neutrophil, T-cell and macrophage infiltration of the heart muscle. The sera of the sick mice showed a granular staining pattern on sections of healthy heart muscle. Quantitative analyses of DCM-specific gene expression studies revealed that sets of genes are involved in inflammation, hypoxia and fibrosis. Treatment with FTY720 (Fingolimod/Gilenya) protected animals from the development of cardiomyopathy. HLA-DQ8^{BALB/c}-Tg mice represent a spontaneous autoimmune myocarditis model that may provide a useful tool for studying the autoimmune mechanism of DCM and testing immunosuppressive drugs.

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Conflict of interest

The authors declare they have no conflict of interest.

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1. Introduction

Dilated cardiomyopathy (DCM), the most frequent cause of cardiac transplantation, is considered the most severe complication of myocarditis [1]. Myocarditis is the inflammation of the heart muscle, which is caused by both viral- and non-viral infections, drug-induced hypersensitive reactions or autoimmune reactions [1]. Despite the extensive research, no definitive cure exists for myocarditis; thus, seeking new model systems and treatment modalities are of special importance.

Several mouse models mimic particular aspects of myocarditis leading to DCM in humans. Immunization of mice with cardiac myocyte-specific antigens such as cardiac troponin I [2] or myosin heavy chain [3,4] leads to an autoimmune reaction and myocarditis in susceptible mice. Coxsackie virus B3-induced myocarditis is another commonly used model system [5]. Additionally, HLA-DQ8 Tg mice in non-obese diabetic (NOD) background develop autoimmune myocarditis spontaneously [6,7]. The involvement of autoantibodies, T cells and proinflammatory cytokines has been shown in these autoimmune myocarditis mouse models [6,7].

Scientists at the Mayo Clinic (Rochester, MN) generated a mouse strain carrying the HLA-DQ8 transgene, and later, this human transgene was transferred into NOD mice [8]. In contrast with the original NOD strain, the newly created transgenic line (NOD.DQ8Ab⁰) does not develop spontaneous diabetes because the original mouse MHC (H-2A^{g7}) involved in spontaneous diabetes in NOD mice was replaced by the HLA DQ8 transgene [8]. However, approximately 90% of female NOD.DQ8 mice died due to a primary myocarditis followed by DCM at the age of 15–21 weeks [9]. Historically, we received mice from the original (master) HLA-DQ8Ab⁰ colony from Dr. C. David (Mayo Clinic, Rochester) and backcrossed it onto the BALB/c background to map arthritis-specific proteoglycan (PG) epitopes, presented by the HLA-DQ8 allele in our PG-induced arthritis (PGIA) model [10]. Years after the HLA-DQ8Ab⁰ homozygous inbred colony with the BALB/c background (HLA-DQ8^{BALB/c}-Tg) was established and tested for arthritis [10] (>20–25 additional generations), multiple sub-colonies showed high mortality when brother-sister breeding was consistently used.

Here, we report that the HLA-DQ8^{BALB/c}-Tg mice develop spontaneous autoimmune myocarditis and DCM in the early age of life. Among the anti-inflammatory or immunosuppressive treatments that we tested, FTY720, a sphingosine-1-phosphate receptor antagonist (Fingolimod/Gilenya), was the only treatment that could successfully prevent the development of the disease and subsequently protected animals from DCM up to adulthood.

2. Materials and methods

2.1. Chemicals

All chemicals, unless indicated otherwise, were purchased from Sigma/Aldrich Chemical Co. (St. Louis, MO) or Fischer Scientific (Chicago, IL). Phosphate-buffered saline (PBS; pH 7.4) was used to perfuse hearts and wash frozen sections and NUNC microplates when

enzyme-linked immunosorbent assay (ELISA) was used. Antibodies, monoclonal antibodies (mAbs) and FcBlock were purchased from BD Biosciences (San Diego, CA).

2.2. Mice

Homozygous humanized HLA-DQ8^{BALB/c}Tg mice lacking their I-A^d molecule (native mouse MHC) described earlier [10] were used. All animal procedures were conducted under the protocol approved by the Institutional Animal Care and Use Committee of Rush University Medical Center (IACUC 05-002, 08-002 and 11-004).

2.3. Evaluation of the clinical symptoms of heart failure

A clinical scoring system was established to follow the physical signs and progression of heart failure. Score 1 was given when the first sign of sickness, dark cyanotic eyes appeared; score 2 when dark eyes with fuzzy fur appeared; score 3 when excess fluid and body edema were seen; and score 4 when the animal was terminally sick and could not move.

2.4. Measurement of physical heart parameters

Weights of hearts were measured on an analytical scale after excessive perfusion of the hearts with ice-cold PBS. The sizes of the hearts (length (x), width (y) and depth (z)) were measured with a digital caliper and expressed as a sum (x+y+z).

2.5. Serum anti-myosin antibody measurement and Western blotting

Anti-myosin antibodies were measured as described earlier [11] with slight modification. Briefly, ELISA plates were coated with 5 µg/ml porcine cardiac myosin in PBS overnight at room temperature and then blocked with 2% bovine serum albumin in washing buffer (PBS containing 0.1% Tween 20). Two-fold dilutions of sera harvested from sick or healthy control mice were used in the range of 1:1,000–64,000. Sera were incubated for two hours, followed by one hour incubation with peroxidase-labeled goat anti-mouse IgG1 (1:4,000). Color reactions were developed with TMB (3,3',5,5'-tetramethylbenzidine) substrate and the optical density was read at 450 nm.

Western blot analysis was performed to detect the presence of (auto)antigenic components in heart extracts of healthy (wild-type BALB/c), asymptomatic and sick HLA-DQ8^{BALB/c}-Tg mice, as well as (auto)antibodies in the sera of asymptomatic and sick HLA-DQ8^{BALB/c}-Tg mice with DCM. Hearts were extensively perfused with cold PBS and then homogenized in PBS containing protease inhibitor cocktail cOmpleteTM (Roche Diagnostics, Indianapolis, IN). Supernatants of heart extracts were separated on 8% sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose membrane, and incubated with the sera of normal (wild-type) BALB/c, or asymptomatic or sick mice with DCM. Peroxidase-labeled goat anti-mouse IgG1 was used to detect tissue-reactive (circulating) antibodies, and the peroxidase reaction was developed by Pierce's enhanced chemiluminescence western blotting substrate.

2.6. Histology, immunohistochemistry and confocal microscopy

Hearts from animals, which did not show signs of heart failure, were fixed with formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin (HE) and analyzed

using a Nikon Microphot-FXA photomicroscope equipped with a *CoolSNAP* digital camera (Photometrix, Tucson, AZ) controlled by the MetaMorph software package (v7.7., Universal Imaging Corp., Molecular Devices, Downingtown, PA). For immunohistochemistry, hearts were cut into two pieces, and frozen and cryostat sections of hearts of healthy (wild-type) BALB/c mice and HLA-DQ8^{BALB/c}-Tg mice were treated with FcBlock (1:100) and 10% goat serum in PBS for one hour at room temperature. These frozen sections were stained with (i) cell-specific mAbs, such as AlexaFluor 488 or PE-labeled anti-Gr1 for neutrophils, anti-CD3-AlexaFluor 488 for T cells, or anti-F4/80-AlexaFluor 488 antibodies for macrophages/dendritic cells (all at 1:100 dilutions). (ii) Frozen sections of healthy and sick HLA-DQ8^{BALB/c}-Tg, as well as control (wild-type) BALB/c mice were incubated with sera (at 1:500 dilution) of naïve BALB/c and sick (HLA-DQ8^{BALB/c}-Tg) mice for one hour at room temperature and then stained with rat mAb to mouse-IgG1-FITC diluted 1:100. (iii) To detect *in vivo* antibody depositions, frozen sections of sick HLA-DQ8^{BALB/c}-Tg hearts were blocked with 10% goat serum and FcBlock (1:100) and stained with rat mAb against mouse-IgG1-FITC without primary antibodies or sera. All immunofluorescence-stained sections were mounted with DAPI-containing Vectashield (Vector Laboratories, Burlingame, CA) and examined with a Nikon Microphot-FXA photomicroscope in epifluorescence mode or Nikon Eclipse confocal microscope. Images were acquired at various magnifications (4x-20x) and analyzed by the MetaMorph software package.

2.7. RNA isolation and PCR array

Total RNA was isolated from PBS-perfused DCM hearts of homozygous HLA-DQ8^{BALB/c}-Tg mice and wild-type (control) animals using TRI reagent. A qualitative evaluation of RNA samples was conducted on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), and samples with RIN8.5 (or higher) were used for subsequent quantitative analyses. Mouse “PrimePCR Pathways” (Cardiomyopathy–Dilated M96, Bio-Rad, Hercules, CA) PCR array platforms were used to study DCM-associated gene expression (<http://www.bio-rad.com/en-us/prime-pcr-assays/predesigned-plate/sybr-green-cardiac-hypertrophy-h96>). For the cDNA synthesis and qRT-PCR experiments, we used an iScript cDNA Synthesis kit and SsoAdvanced Universal SYBR[®] green qRT-PCR mixtures (Bio-Rad). Quantitative RT-PCR analyses were performed on a CFX real time PCR machine (Bio-Rad), and PCR array data were analyzed with “CFX manager” software (Bio-Rad). Enrichment in various biological pathways was determined using the Database for Annotation, Visualization and Integrated Discovery (DAVID) Web-based annotation tool v6.7 [12].

2.8. Treatment protocols

In preventive approaches, all treatment modalities (determined in preliminary experiments) started at the age of 4 weeks when the mice had no sign of heart failure and finished 5 weeks later. All treatment regimens were applied three times a week. The doses of compounds/drugs were used as before [13–15] and pretested in preliminary experiments using half or double doses of compounds listed below. Mice were examined daily during the treatments for the clinical signs of heart failure and subsequent mortality. Treatment groups were as follows (Table 1): (1) Remicade (anti-TNF mAb) was given intraperitoneally (ip.) at a dose of 150 µg/mouse; (2) IM7 (anti-CD44 mAb) was given ip. at a dose of 100 µg/mouse; (3) Cyclosporine A (calcineurin inhibitor) was given subcutaneously at a dose of 15 mg/kg; and

(4) FTY720 (S1P receptor antagonist) was given orally in water at a dose of 1 mg/kg. (5–6) Mice of control groups rat IgG (three times a week) ip or were left untreated (Table 1).

In a second set of experiments, FTY720 treatment of HLA-DQ^{BALB/c}-Tg mice started (dosage as above) when the first sign of heart failure (Score 1) was diagnosed and lasted until their death.

2.9. Statistical analysis

Descriptive statistics were used to determine group means and the standard error of the mean (mean $\pm$ SEM) unless otherwise stated. The difference between two groups was tested for statistical significance using Student's *t*-test. A $p < 0.05$ value was considered statistically significant.

3. Results

3.1. HLA-DQ8^{BALB/c}- Tg mice develop fatal heart failure at early age

HLA-DQ8Ab⁰ homozygous inbred transgenic mice with a C57Bl/6 background were originally backcrossed into the BALB/c genetic background in our laboratory to study autoimmune arthritis (PGIA) [10]. During the maintenance of the colony using a brother-sister mating process, we observed that HLA-DQ8^{BALB/c}-Tg homozygous mice had early mortality (at 2–3 months of age) due to the heart failure that started at the age of 4–5 weeks (Fig. 1A). Initial signs of the disease were loss of weight, decreased physical activity, and pale or cyanotic limbs and eyes, and these symptoms were gradually replaced by edema. Terminally, most animals showed extensive whole body edema (Fig. 1B). Autopsy showed voluminous pleural and peritoneal effusions, extremely dilated heart, and swollen dark liver (Fig. 1C) mimicking DCM.

For quantitative measurements, we removed the hearts of sick animals and measured the physical parameters after extensive washing. Hearts from HLA-DQ8^{BALB/c}-Tg mice were enlarged (Fig. 1F) and weighed significantly more than age- and gender-matched wild-type animals (Fig. 1G). Inflammatory cell infiltration, fibrotic areas and hemorrhages were seen in histology sections (data not shown).

To determine the cause of the cardiomyopathy, we performed histological analysis of the heart muscle. First, we examined young, symptom-free HLA-DQ8^{BALB/c}-Tg mice expecting that the histopathological abnormalities might precede the appearance of the clinical symptoms. Indeed, HE staining revealed that mono- and polymorphonuclear cells have already infiltrated the heart muscle mostly from the pericardial side in seemingly healthy 3–5 week old HLA-DQ8^{BALB/c}-Tg mice (Figures 2A–F). Thus, we concluded that the DCM was caused by myocarditis. Subsequent immunofluorescence analysis identified the infiltrating cell types in the myocardium. Anti-Gr1 staining showed focal neutrophil infiltration of the heart muscle both superficially (pericardial side) and in deeper regions (Figures 2G–I). Additionally, CD3 positive T cells (Fig. 2I) and F4/80 positive macrophages (Fig. 2J) were also detected in the heart muscle, but no B cells could be seen (data not shown).

3.2. Dilated cardiomyopathy was due to autoimmune myocarditis

The most common cause of myocarditis is a viral infection. We sent 4 blood samples from both sick and asymptomatic HLA-DQ8^{BALB/c}-Tg animals for detailed infectious disease analysis to Charles River Laboratories (Frederick, MA). No virus or any other infection or antibodies was verified using the common rodent microbiological panel [16](data not shown). Because the myocarditis may also be the consequence of pathologic immune reactions, such as in NOD.DQ8 Tg mice [6,7,9,17], we tested the sera of sick HLA-DQ8^{BALB/c}-Tg mice for circulating (auto)antibodies against heart muscle antigens. The sera of sick animals showed a strong positive reaction in ELISA; the sera reacted with porcine cardiac myosin antigen up to a dilution of 1:64,000 (Fig. 3A). To examine whether the sera of sick HLA-DQ8^{BALB/c}-Tg mice reacted with other potential autoantigens of heart, we performed a Western blot analysis (Fig. 3B). In addition to the two bands of cardiac myosin at ~180–220 kDa, we found two additional bands at approximately 30 and 40 kDa when heart extracts were “stained” with the sera of either asymptomatic or sick HLA-DQ8^{BALB/c}-Tg mice (Fig. 3B). Although the exact identification of these two protein bands has not been performed, based on the literature [18,19], the ~30 kDa band might correspond to cardiac troponin (33 kDa), identified by another DCM study in PD-1 deficient mice [18,19].

To determine the tissue localization of serum reactivity (i.e., autoantigen localization), we immune-stained heart frozen sections of healthy (wild-type) BALB/c mice with the sera of sick HLA-DQ8^{BALB/c}-Tg mice (Figures 4A–F). A strong positive reaction was observed (Figures 4A and 4C) when compared to the normal mouse serum controls (Figures 4B and 4D). To visualize the sub-cellular localization of autoantigenic components, sections were also analyzed with confocal microscopy. Autoantibodies of sick HLA-DQ8^{BALB/c}-Tg mice reacted with antigenic components at the membrane proximal regions of cardio-myocytes (Fig. 4E) together with a strong granular staining in the cytoplasm (Fig. 4F).

After confirming that the sera of sick HLA-DQ8^{BALB/c}-Tg mice showed a strong reaction with myocardial antigens, we examined whether there was *in vivo* antibody deposition in the heart muscle of these sick HLA-DQ8^{BALB/c}-Tg mice (Figures 4G–L). Cell-bound antibodies can activate various effector mechanisms including complement activation- or Fc receptor-dependent cytotoxicity. Moreover, this can also explain the neutrophil infiltration observed in the heart muscle (Figures 2G–I): the local complement activation could attract neutrophils to the acute inflammation by the production of chemotactic molecules (C3a, C5a) respectively. In turn, neutrophil activation could contribute to the severe myocardium damage with necrosis and reactive fibrosis, which we observed in the late stages of the disease. Thus, these auto-antibodies may contribute substantially to the development of the pathology of DCM.

To detect whether the autoantibodies of sick mice with DCM can bind to myocardial components *in vivo*, frozen sections of hearts of sick HLA-DQ8^{BALB/c}-Tg mice were stained with rat anti-mouse IgG-FITC. We observed a strong positive reaction (Figures 4G, 4I, 4K and 4L) compared to the heart sections of healthy control BALB/c mice (Figures 4H and 4J), which indicated that there was *in vivo* antibody deposition in the heart of sick mice. Confocal microscopic analysis showed mostly cell surface positivity (Fig. 4K) together with

granular staining (Fig. 4L) similar to what was seen in normal heart sections incubated with the sera of sick animals (Figures 4E and 4F).

3.3. Disease-associated gene expression changes in the hearts of HLA-DQ8^{BALB/c}-Tg mice with DCM

To further characterize the molecular background of the spontaneous DCM in HLA-DQ8^{BALB/c}-Tg mice, we employed DCM-specific PCR array platforms. This type of platform is designed for monitoring gene expression changes of 48 DCM-related genes. We found that 26 genes were differentially expressed in at least 3 of the 4 investigated DCM samples; 14 genes were significantly up- and 12 were down-regulated in mice with myocarditis compared to the healthy control group (Fig. 5 and Table 2). PCR array data were analyzed for pathway representation using the DAVID gene-annotation enrichment analysis tool, which noted that extracellular matrix (ECM)-receptor interaction (Suppl. Fig. 1) and insulin/IGF signaling pathways (Suppl. Fig. 2) were the most frequently targeted pathways in diseased myocardium of HLA-DQ8^{BALB/c}-Tg mice. Altered expression showing genes play pivotal roles in pathways, which are implicated in fibrosis, myocardial hypoxia and inflammation (Table 2).

3.4. FTY720 protected HLA- DQ8^{BALB/c}-Tg mice from developing autoimmune myocarditis

Based on the observations described above, we were in favor of the possibility that the myocarditis and subsequent DCM in HLA-DQ8^{BALB/c}-Tg mice was due to an autoimmune reaction. To further support this conclusion and also to test potential treatment modalities in autoimmune DCM, we treated these transgenic mice with various immune modulatory molecules. Fundamentally, we applied two approaches: we gave various immune modulatory treatments in a preventive fashion (Table 1 and Fig. 6A) and also for treatment of an established disease. The preventive treatments started in 4-week old, symptom-free mice and finished after 5 weeks, when the survival rate was calculated (Table 1). We tested anti-CD44 and anti-TNF α monoclonal antibodies as well as Cyclosporine A and FTY720 treatments (Table 1 and Fig. 6A). TNF α blockade with Remicade, inhibition of calcineurin with Cyclosporine A, or anti-CD44 (IM7) treatments had no significant effect on the survival rate (60%, 50% and 61.5% survival, respectively) compared to the 54.5% and 50% survival rate in the control IgG-treated and untreated mice (Table 1 and Fig. 6A), respectively. However, FTY720, a S1P-receptor blocker increased the survival rate to 100% (Table 1 and Fig. 6A). The animals of the treatment groups were followed after completing the 5-week FTY720 preventive treatment. The clinical symptoms of DCM gradually developed in these animals during the treatment-free period, and only 2 of 8 and 4 of 9 animals survived at the end of 4–8 additional weeks.

Because the preventive treatment with FTY720 proved to be fully protective, we tested the therapeutic effect of FTY720 treatment (Fig. 6B). We collected mice at the time of the earliest clinical sign of myocarditis (score 1: cyanotic and dark eyes) and treated them with FTY720 in similar dosages as determined earlier and was effective for the prevention of the disease. The control group of mice did not receive FTY720 treatment, only the same volume of water orally. The average age of the mice in both groups was 50 $\pm$ 3 days. Although FTY720-treated mice showed slightly slower disease progression at the beginning of

treatment and slightly prolonged survival compared to untreated HLA-DQ8^{BALB/c}-Tg mice, this effect was only marginal and not significant (Fig. 6B).

4. Discussion

We report here that spontaneous myocarditis developed at an early age of life, and it leads to fatal DCM in homozygous HLA-DQ8^{BALB/c}-Tg mice. The autoimmune nature of the pathological process was supported by the following data: inflammatory cell infiltration of the myocardium, including T cells, neutrophils and macrophages; the sera of sick animals reacted with cardiac myosin and two other myocardial proteins and resulted in antibody deposition in the heart of sick mice. Additionally, FTY720 treatment, which abrogates the egress of lymphocytes from the peripheral lymphatic tissues, prevented the disease development and protected animals (tested so far) up to four months of life.

Previously, two independent research groups described the role of the HLA-DQ8 transgene in the development of autoimmune myocarditis [6,7]. Elliott and his colleagues reported spontaneously appearing autoimmune myocarditis and heart block in the HLA-DQ8 Tg I-A β knockout NOD mice [6]. Progressive heart abnormalities were reported together with anti-cardiac myosin antibodies and T cell response [6], but they could not detect *in vivo* deposited antibodies in the heart muscle [6]. Preventive treatment with Cyclosporine A delayed the onset of the disease, but after the discontinuation, mice gradually developed autoimmune myocarditis, showing that the treatment had only a temporal effect [6]. Taylor and colleagues from the Joslin Diabetes Center (Boston, MA) reported similar results in their independently generated HLA DQ8-Tg NOD mice; progressive heart failure developed from 4 months of age with inflammatory cell infiltration in the heart muscle (B and T lymphocytes, macrophages) and cardiac myosin specific antibodies [7]. Despite the strong evidence that the spontaneous myocarditis in both of these models is due to an autoimmune reaction against the heart muscle, no data are available currently on which specific cardiac antigen could trigger the pathology. Additionally, there is only speculation on how the HLA-DQ8 transgene could contribute to this process. The hypothesis is that this particular HLA type can present cardiac protein peptides, or alternatively, during the thymic selection in these transgenic mice, particular CD4⁺ T cells are generated, which are prone to recognize cardiac peptides [7].

Although there are similarities between our findings in the HLA-DQ8^{BALB/c}-Tg mice and the two above-mentioned HLA-DQ8-Tg NOD models [6,7], the most important difference is the different genetic background. So far, the NOD background seemed to be critical for the disease development in all previously described models of DCM [17], and it was thought to be as important as the presence of the HLA-DQ8 allele [17]. However, here, we report that the presence of the HLA-DQ8 transgene in the BALB/c background was also favorable for the development of autoimmune heart disease; thus, the exclusivity of the NOD background could not be confirmed.

Consistent with this finding, Nishimura and colleagues described another spontaneous dilated cardiomyopathy model in PD-1 deficient BALB/c mice [18] where circulating autoantibodies were detected, together with IgG and C3 depositions in the heart [18]. The

sera of sick PD-1 deficient mice recognized exclusively a 33 kDa protein band, which later proved to be cardiac troponin [19]. Although this model was slightly different from our HLA-DQ8^{BALB/c}-Tg mice [6,7], importantly, the BALB/c background supported the development of the abnormal autoimmune reaction against the heart muscle. Susceptibility to autoimmune diseases, in general, is determined by genetic variations of MHC and non-MHC genes. Both the NOD and the BALB/c strain is known to be “autoimmune-prone” which is due to the H-2^{g7} or the H-2^d MHC haplotypes [20,21], respectively, together with the combination of several other genes. Overall, these data suggest that there should be either shared or strain-specific non-MHC key genetic elements in these mouse strains, which support the development of autoimmune myocarditis. Identification of such genes together with human gene analogues could contribute to the better understanding of the immunogenetics of autoimmune myocarditis.

Despite the relatively detailed characterization of the available models, the exact role of the HLA-DQ8 transgene and the genetic background is still not clear in the pathogenesis of autoimmune myocarditis and DCM. Therefore, we incorporated potential new elements (either causative or consequential) of the pathology by focusing on gene expression changes in the myocardium of sick HLA-DQ8^{BALB/c}-Tg mice, using a PCR-based gene expression array containing DCM-related genes selected in previous studies (<http://www.biocompare.com/en-us/prime-pcr-assays/pre-designed-plate/sybr-green-cardiac-hypertrophy-h96>). The differentially expressed genes could be divided into three characteristic subgroups: insulin/IGF (insulin-like growth factor) pathway-related, inflammation-linked and fibrosis-associated genes (Table 2, Supplemental Figures 1 and 2) according to the DAVID gene annotation analysis.

The Igf pathway (Supplemental Fig. 2) has been implicated in regulating myocardial function, metabolism, cell survival and apoptosis in the heart [22]. Particularly, insulin-like growth factor-I (*Igf1*), which was found to be cardioprotective [23], was up-regulated in the heart of sick HLA-DQ8^{BALB/c}-Tg mice, whereas three downstream signaling molecules, insulin receptor substrate 1 (*Irs1*) [24] and *Akt1/2* [25] were down-regulated. Interestingly, another isoform of the *Irs* family (*Irs2*) was significantly up-regulated in myocardial tissues, which might be because *Irs2* plays a role in the hypoxic stress-buffering mechanism [26,27,27] caused by the destruction of cardiomyocytes and vasculature in the inflamed myocardium. The main regulators of *Igf* activity and availability are the insulin-like growth factor binding proteins (IGFBPs). *Igfbp6* [28] was up-regulated in sick HLA-DQ8^{BALB/c}-Tg mice, which can also contribute to the lower *Irs1* and *Akt1* expression resulting in decreased anti-apoptotic signaling. Together, changes in the IGF pathway in the myocardium of sick HLA-DQ8^{BALB/c}-Tg mice suggest a complex myocardial repair response to inflammation; however, multiple questions remain regarding the implicated genes and their roles in DCM.

The second group of genes influences the severity of inflammation and myocarditis. Toll-like receptor-4 (*Tlr4*) could be activated by the binding of heat shock proteins and extracellular breakdown products released from the inflamed myocardium [29,30]. Cathepsin-K (*Ctsk*), a lysosomal/endosomal enzyme participating in the degradation of these breakdown products [31], was up-regulated in sick HLA-DQ8^{BALB/c}-Tg group together with proinflammatory cytokine IL-6 (*Il6*). Moreover, the up-regulation of serpin peptidase

inhibitor (*SerpinA3*) could also augment the inflammation by complement activation and promoting cell death [32]. Previous studies revealed the cardio-protective (i.e., anti-inflammatory) effects of adiponectin (*Adipoq*) [29], and the elevated expression levels of this protective protein could indicate the activation of anti-inflammatory pathways. Osteopontin (*Spp1*), described as a key stress mediator and pro-inflammatory protein in injured cardiac tissues [33,34], was up-regulated in HLA-DQ8^{BALB/c}-Tg mice with DCM.

DCM is characterized by extensive fibrotic remodeling of the myocardium [35] [36], which is supported by our data inasmuch as PCR array revealed elevated expression of known fibrosis-associated extracellular matrix genes. More specifically, *Col1a1/Col3a1* and *Fnl* genes encode collagen type Ia1 and type IIIa1 and fibronectin, the main components of fibrotic myocardium produced by fibroblast-to-myofibroblast differentiation [37]. The development of these cells is promoted by galectin-3 (*Lgals3*) [38], produced by macrophages [39], which was up-regulated in sick mice, as well as IL-6 (*Il6*), which can also support myofibroblast formation [39]. Two additional genes, *Nppa* and *Nppb* (natriuretic peptide A and brain natriuretic peptide), were shown to be secreted in response to excessive stretching of cardiomyocytes in cardiovascular disease and myocarditis [40,41] and were also up-regulated in sick HLA-DQ8^{BALB/c}-Tg mice. Clearly, pro-inflammatory genes were up-regulated simultaneously with weakened anti-inflammatory mechanisms in mice with DCM, in addition to the increased expression of fibrosis- and hypoxia-associated genes (Table 2).

The most important goal of this study, however, was to find therapeutic approaches to prevent the disease or treat sick animals. Unfortunately, our therapeutic approaches failed, whereas the preventive application of FTY720 was very promising, which was in line with two previous studies [42,43]. The blockade of the TNF α effect with remicade, or CD44 with IM7, or calcineurin with Cyclosporine A had no effect on the development of the disease, showing that these pathways had no significant contribution to the pathology. In contrast, FTY720 treatment completely prevented the development of DCM when the treatment started at early age in symptom-free animals. However, this beneficial effect lasted only until the treatment ended because a few weeks after the discontinuation of the therapy, the clinical symptoms appeared, and the animals died of heart failure similarly to untreated littermates. FTY720 had only marginal effect in animals in which the first symptoms were already present at the time when the treatment was started. The preventive effect of FTY720 has already been demonstrated earlier in cardiac myosin-induced rat myocarditis [42] and encephalomyocarditis virus-induced myocarditis in mice [43].

FTY720 (Fingolimod) is a S1P antagonist, which blocks the egress of lymphoid cells from the peripheral lymph nodes, thus inhibiting their normal peripheral trafficking. FTY720 as an immune modulatory molecule has a beneficial effect in multiple autoimmune diseases or models [13,44–48] and, most recently, was approved (Gilenya) for the treatment of multiple sclerosis by the FDA. Here, the preventive application of the FTY720 completely blocked the development of the DCM, inhibiting the development of autoimmunity against the heart muscle. This complex mechanism might include multiple target points such as the inhibition of lymphocyte recirculation, thus decreasing the chance of antigen recognition, the activation of potentially autoreactive cells, and their migration to the peripheral tissues

where they could trigger cellular effector mechanisms. Another yet unknown component of the effectiveness of FTY720 preventive treatment might be that FTY720 may ameliorate heart fibrosis by the down regulation of several genes [49], genes which were upregulated in our study (*Col1a1*, *Col3a1*, *Fn1*, *Il6*, *Tgfb1*, *Igf1*). Thus, the promising preventive effect of FTY720 might be due to the combination of blocked lymphocyte recirculation and the direct anti-fibrotic effect on the heart muscle. Moreover, FTY720 treatment increased the PD-1 expression of follicular helper T cells in MS patients [50]. PD-1 is a co-receptor molecule found on T lymphocytes, which negatively regulates the T cell receptor-derived activation signals, and, in the absence of PD-1, uncontrolled activation signals lead to an autoimmune reaction against the heart muscle [18]. Although we do not have data about PD-1 in our case, it is tempting to speculate that the FTY720 treatment not only inhibited the lymphocyte trafficking and fibrosis but also interfered with the co-stimulatory pathways.

In conclusion, here we report a HLA-DQ8^{BALB/c}-Tg spontaneous autoimmune myocarditis model which adds a few new aspects to the previous NOD.DQ8 models. All of these models offer outstanding possibilities to study the development of autoimmune heart disease and also to test new treatment modalities. To our knowledge, this study is the first to demonstrate the effectiveness of FTY720 treatment in a spontaneous model of autoimmune myocarditis. Although these results are preliminary, they might precipitate further studies with FTY720/ Fingolimod in autoimmune myocarditis for which no definitive treatment currently exists.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DCM	dilated cardiomyopathy
NOD	non-obese diabetic
DAVID	Database for Annotation, Visualization and Integrated Discovery
ECM	extracellular matrix

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Figure 1.

HLA-DQ8^{BALB/c}-Tg mice develop severe heart failure at early age. **A:** Survival curve of HLA-DQ8 myocarditis. Mice died in terminal heart failure or were euthanized before reaching moribund condition (stage 4—according to our scoring system). The survival curve was calculated from the data of 62 animals. Approximately 50% of the animals died before 10 weeks of age. **B:** Sick mice developed extreme whole body edema terminally. **C:** Macroscopic pathology showed dilated heart and enlarged dark liver. **D, E:** Corresponding images of healthy control mice. **F, G:** Physical parameters (size and weight) of sick HLA-DQ8^{BALB/c}-Tg hearts (black columns), compared to healthy age and gender matched wild-type controls (white columns). Size (**F**) of the hearts was calculated by summing the length, width and depth parameters measured with a digital caliper. Heart weight (**G**) was measured on an analytical scale after extensive perfusion of hearts with PBS. Diagrams show the

average $\pm$ SEM values calculated from the data of sick (n=10) or healthy (n=6) mice.
Significant differences are indicated (*P<0.001, **P<0.00001).

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Figure 2. Inflammatory cells infiltrate the heart muscle of healthy (no sign of heart failure yet) HLA-DQ8^{BALB/c}-Tg mouse. **A–F:** Heart sections were stained with HE. Panel series A–C and D–E depict representative pathologic lesions from two independent heart sections, respectively. Original images at 4x (**A, D**), 10x (**B, E**) and 20x (**C, F**) magnifications of the indicated regions (black rectangular regions) are shown. Cellular infiltrates were analyzed with immunofluorescent labeling using AlexaFluor 488-labeled anti-Gr1 mAb (**G**–20x magnification, **H**–40x magnification), anti-CD3-Alexa-488 + Gr-1-PE (**I**), or F4/80-Alexa488 (**J**) antibodies, respectively, counterstained with DAPI, and the images were overlaid using MetaView software.

Figure 3. Myocardium-specific autoantibody production in HLA-DQ8^{BALB/c}-Tg mice. **A:** Anti-myosin antibody titer was tested on porcine cardiac myosin coated ELISA plates using serial 2-fold serum dilution. Sera from sick animals (n=32) and healthy controls (n=7) were tested individually at 7 different dilutions. Data points represent mean OD ± SEM values of sick (black symbols) or healthy control (open symbols). **B:** Western blot analysis detected the presence of antigenic components, which were stained with antibodies present only in the sera of mice with DCM. Protein extracts from hearts were separated on 8% SDS-PAGE, transferred onto nitrocellulose membrane, incubated with the sera of normal (wild type) BALB/c (left side) or DCM sick mice. “W”- wild-type BALB/c sample, “H” healthy (symptom-free) and “S”- sick HLA-DQ8^{BALB/c}-Tg heart extracts. “CM” refers to cardiac myosin-specific bands detected only by the sera of sick animals. At least two additional bands (arrows with “?”) were also stained with the serum of DCM mouse. Asterisks (*) indicate the immunoglobulin heavy- and light-chains.

Figure 4.

Autoantibody deposition in the heart of HLA-DQ8^{BALB/c}-Tg mice. **A-F:**

Immunohistochemistry shows that the sera of sick HLA-DQ8^{BALB/c}-Tg mice reacted with normal heart tissue. Frozen sections of the ventricular heart from healthy BALB/c mice were incubated with 1:500 diluted sera from HLA-DQ8^{BALB/c}-Tg mice with DCM (**A, C, E, F**) or the sera of healthy control mice (**B, D**). Autoantibodies bound to the heart tissue were detected with rat anti-mouse IgG-FITC. Sections were analyzed using either conventional (**A-D**) or confocal fluorescence microscopy (**E, F**). **G-L:** Immunohistochemistry showed IgG depositions in the heart tissue of HLA-DQ8^{BALB/c}-Tg mice with DCM. Frozen heart sections from sick HLA-DQ8^{BALB/c}-Tg (**G, I, K, L**) or healthy BALB/c (**H, J**) mice were stained with rat anti-mouse IgG-FITC. Sections were analyzed using either conventional- (**G-J**) or confocal fluorescent microscopy (**K, L**).

Figure 5. Gene expression analysis in myocardium of sick HLA-DQ8^{BALB/c}-Tg mice DCM-associated gene expression changes presented using heat-map diagram. Red squares indicate up-regulated genes; green squares indicate down-regulated genes. RNA samples from four sick mice were compared to those from four control (wild-type) BALB/c mice.

Figure 6.

Immunomodulatory (preventive and therapeutic) treatments of HLA-DQ8^{BALB/c}-Tg mice.

A: Preventive treatment with Remicade (anti-TNF α mAb), Cyclosporine A, IM-7 (anti-CD44 mAb) and FTY720. The summary of treatment is detailed in Table 1. Bars show the percentage of survival at the end of the treatments calculated from 3–4 independent experiments. **B:** FTY720 treatment of sick HLA-DQ8^{BALB/c}-Tg mice delayed disease progression but did not cure. The treatment of mice started when they developed dark, cyanotic eyes, the first sign of the disease (score 1). Bars show the mean age of mice $\pm$ SEM (FTY720-treated group n=7, control group n=9). Severity score of the disease is shown on the horizontal axis (score 2: dark eyes, fuzzy fur; score 3: starting edema; score 4: terminally sick, no movement, breathing problems).

Table 1

Summary of preventive treatments of HLA-DQ8^{BALB/c}-Tg mice.

<u>Therapy</u>	<u>Mechanism</u>	<u>Treatment (3x/week)</u>	<u>* Live/All animals</u>
Remicade	anti-TNF mAb	150 µg/mouse; ip.; 3x/week	6/10
IM7	anti-CD44 mAb	100 µg/mouse; ip.; 3x/week	8/13
Cyclosporine A	calcineurin inhibitor	15 mg/kg; sc.; 3x/week	3/6
FTY720	S1P receptor antagonist	1 mg/kg; po; 3x/week	23/23
Rat IgG	normal/naive rat IgG-	100 µg/mouse; ip.; 3x/week	6/11
<u>No treatment</u>	=	=	<u>9/18</u>

All treatment modalities were started at the age of 4 weeks, when the mice had no sign of heart failure yet.

* The number of surviving mice at the end of the 5-week treatment period. Injections: ip, intraperitoneally; sc, subcutaneously; po, oral treatment in water.

Table 2

Differentially expressed genes in the myocardium of mice with DCM

Gene	Change	Inflammation	Hypoxia	Fibrosis	Reference(s)
<i>Akt3</i>	↑	+	–	–	
<i>Ctsk</i>	↑	+	–	–	[41]
<i>Fhl</i>	↑	+	–	–	[51]
<i>Lgals3</i>	↑	+	–	–	[52]
<i>Serpina3n</i>	↑	+	–	–	[44]
<i>Spp1</i>	↑	+	–	–	[46,47]
<i>Ttr4</i>	↑	+	–	–	[39,40]
<i>Irs2</i>	↑	+	+	–	
<i>Igf1</i>	↑	+	+	+	[53–55]
<i>Nppa/b</i>	↑	–	+	+	[36,38]
<i>Col1a1</i>	↑	–	–	+	[29,30]
<i>Col3a1</i>	↑	–	–	+	[29,30]
<i>Igfbp6</i>	↑	–	–	+	[52]
<i>Myh9</i>	↑	–	–	+	[56]
<i>Adcy5</i>	↓				
<i>Adipoq</i>	↓	+	–	–	[39,40]
<i>Agr1a</i>	↓				
<i>Atf1</i>	↓				
<i>Akt1</i>	↓	+	–	–	[19]
<i>Akt2</i>	↓	+	–	–	
<i>Dlg1</i>	↓				
<i>Hk2</i>	↓				
<i>Irs1</i>	↓		+		[18]
<i>Myh10</i>	↓	–	+	+	[30]
<i>Myh6</i>	↓	–	+	+	[30,31,34]
<i>Pdk4</i>	↓	–	±	–	

Genes differentially expressed in the myocardium of DCM were categorized into 3 major functional groups: inflammation, hypoxia and fibrosis.

Genes belonging to the insulin-like growth factor (IGF) pathway are boldfaced.
↑: up-regulated; ↓: down-regulated; +/-: has role; —: no role.

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