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Comparative immunoproteome analysis of the response of susceptible A.BY/SnJ and resistant C57BL/6 mice to Coxsackievirus B3-infection

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Abstract

Both, innate and cell-mediated immunity contribute to prevention of chronic myocarditis and consecutively, cardiomyopathy. Thus, in resistant C57BL/6 mice myocarditis induced by Coxsackievirus B3 (CVB3)-infection is abrogated by immune-mediated mechanisms. However, susceptible A.BY/SnJ mice develop dilated cardiomyopathy (DCM) due to chronic myocarditis. Cardiac auto-antibodies have been shown to play a pivotal role in the initiation and/or progression of inflammatory DCM. In order to investigate differences in the autoimmune response of susceptible and resistant mice to infection with CVB3, the patterns of autoantibodies reacting with heart proteins in A.BY/SnJ and C57BL/6 mice were profiled by 2-D Western blot analysis during the acute and chronic phases of myocarditis up to three months, when the pathophysiological phenotype in the susceptible mice has progressed to DCM. In the early phase of infection both mouse strains displayed similar autoantibody patterns. In contrast, at later time points compared to the resistant C57BL/6 strain susceptible A.BY/SnJ mice displayed a much stronger autoimmune response against proteins associated with cell structure, protein transport as well as primary metabolic processes such as energy production. During chronic myocarditis strong antibody responses against myosin heavy chain 6, mitochondrial and heat shock proteins were observed in A.BY/SnJ mice. Antibodies directed against alpha-enolase, serotransferrin, radixin and two processed myosin protein species accumulated late and only in A.BY/SnJ mice suffering from inflammatory DCM. Functional assignment of the target proteins of cardiac autoantibodies indicates that these might be directly involved in cardiac dysfunction.

Keywords: Coxsackievirus B3-induced dilated cardiomyopathy; Murine model; Cardiac auto-antibodies; 2-D Western Blot .

1. Introduction

Dilated cardiomyopathy is a heart disease with an extremely poor clinical outcome. Besides genetic factors, mechanical stress and intoxication, virus infection-induced abnormalities in cellular and humoral immunity also contribute to the overall pathogenesis, such as cardiac enlargement and impaired systolic dysfunction.[1] Recent reports have provided evidence that autoimmune reactions against certain myocyte antigens may play a pivotal role in the initiation and/or progression of DCM. The pathogenic potential of cardiac auto-antibodies has been proven in animal models either by active immunization with proteins like troponin I or by transfer of antibodies against the corresponding epitopes, both leading to dysfunction of the heart.[2-4] Furthermore, preliminary clinical data suggest that a few of these auto-antibodies are indeed "pathogenic", actually causing cardiac dysfunction and heart failure.[5-6] Depending on the individual genetic pre-disposition such harmful autoimmune reactions are supposed to emerge as a consequence of heart muscle damage induced by viral triggers, ischemia or exposure to cardiotoxins leading to myocyte necrosis/

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apoptosis and subsequent liberation of a "critical amount" of self-antigens previously hidden to the immune system.[7-8]

Since extensive studies of human cardiac autoimmune reactions are limited by the restricted availability of appropriate tissue specimen such as endomyocardial biopsies, appropriate animal models are necessary. So far, only a few animal models that facilitate the analysis of pathogenesis of DCM following viral infection have been established.[9-11] Murine models of Coxsackievirus B3 (CVB3)-induced myocarditis excellently mimic the human disease patterns and are thus very well suited for the exploration of mechanisms leading to chronic myocarditis and finally to the dilatation of the heart. Development of severe myocarditis and synthesis of viral proteins during the acute phase of viral infection have been demonstrated in susceptible mouse strains such as A.BY/SnJ (H-2^b), DBA/2 (H-2^d) and SWR/J (H-2^q). In contrast, resistant C57BL/6 (H-2^b) and DBA/1J (H-2^q) mice eliminate the virus after an acute phase, do not show severe cardiac lesions and lack the chronic phase of the disease.[10, 12-15]

In order to investigate differences in the autoimmune response between susceptible and resistant mice infected with CVB3, the autoantibody patterns directed against heart proteins were followed by 2-D Western blot analysis over a period of 84 d post infection (p.i.) with CVB3 in A.BY/Sn and C57BL/6 mice. Over this time course of 3 months mice of the susceptible strain A.BY/SnJ displayed a much stronger autoimmune response than C57BL/6 mice. This response was primarily directed against proteins associated with cell structure, protein transport as well as primary metabolism. In chronic myocarditis strong antibody signatures against myosin heavy chain 6, mitochondrial and heat shock proteins were observed and their functional relevance is discussed.

2. Material and Methods

2.1 Virus infection and animal model of ongoing myocarditis

In this study we used a CVB3 stock which was derived from the infectious cDNA copy of the cardiotropic Nancy strain as previously described.[12] A.BY/SnJ mice (H-2^b) susceptible for the development of ongoing myocarditis and dilation of the heart following CVB3 infection and the resistant strain C57BL/6 were kept under specific pathogen-free conditions at the animal facilities of the Department of Molecular Pathology, University Hospital Tübingen. Experiments were conducted according to the German animal protection law. Mice (4-5 week-old, n=7 per time point) were infected intraperitoneally with 5 x 10^4 plaque-forming units (PFU) of purified CVB3 as described.[16] At different time points p.i., mice were sacrificed and hearts were collected for analysis. Histology and *in situ* hybridization was performed on transverse tissue sections covering the right and left ventricle taken below the level of the valves which were fixed in 4 % phosphate-buffered paraformaldehyde (pH 7.2) and embedded in paraffin. The rest of the left and right ventricles were snap frozen in liquid nitrogen and used for proteomic analysis.

2.2 *Histology and in situ hybridization*

Paraffin-embedded hearts were cut into 5 cm thick tissue sections and stained with hematoxylin/eosin (H&E) to evaluate the extent of myocardial injury and inflammation at day 0, 8, 28, and 84 p.i. For the detection of CVB3 positive-strand genomic RNA and interleukin-6 mRNA in hearts we used single-stranded ^{35}S-labeled RNA probes. Virus-specific probes were synthesized from the dual-promoter plasmid pCVB3-R1 as previously described.[12] Slide preparations were subjected to autoradiography, exposed for 3 weeks at 4 °C, and counterstained with H&E.

2.3 *Preparation of protein extracts from heart tissues*

Heart tissues were collected at four different time points representing different stages of disease: control – day 0, day 8 p.i., day 12 p.i., and day 84 p.i. Snap frozen heart tissue probes (n=2 per group) were disrupted to fine powder using a Mikro dismembrator (Braun, Melsungen, Germany) at 2.600 rpm for 2 min. The powder was dissolved in extraction buffer containing 8 M urea, 2 M thiourea, 2% CHAPS (Sigma, Taufkirchen, Germany) and sonicated on ice 3 times for 3 sec each with 9 cycles at 80 % energy using a Sonoplus (Bandelin, Berlin, Germany). Subsequently, the lysate was centrifuged at 16.000 g for 1 h at 4 °C and protein concentrations of the supernatants were determined using a Bradford assay kit (Bio-Rad, Munich, Germany). Sample aliquots were stored at -80 °C.

2.4 *Preparation of immunoglobulins G (IgG) from murine sera*

In order to estimate the dynamics of immune response to CVB3 infection in mice, pooled sera of seven mice were collected from the different time points (day 0, day 4 p.i., day 8 p.i., day 12 p.i., day 28 p.i., and day 84 p.i.) and used to isolate IgGs. Serum (250 µL) was mixed 1:1 (vol/vol) with Protein G SepharoseTM 4 Fast Flow (GE Healthcare, Munich, Germany) equilibrated with PBS buffer (138 mM NaCl, 2 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4, at 4 °C for 2 h) and incubated for 2 h with gentle shaking. After 5 washes with PBS buffer, the bound IgGs were eluted by adding 100 mM glycine, pH 2.5 for 15 min. The supernatant was collected and used to determine the amount of IgG using a Bradford assay kit (Bio-Rad, Munich, Germany).

2.5 *2-D Western blot analysis*

For 2-D Western blot analysis, a pool of heart proteins of the A.BY/SnJ strain from 4 different time points (0, 8, 12 and

84 days p.i.) was separated by 2-D gel electrophoresis using IPG strips of the pH-ranges 4-7 and 6-11, 11 cm length (GE Healthcare) and 12.5 % SDS-PAGE, and transferred onto PVDF membranes for Western blotting using a conventional semi-dry blotting device (Milliblot Graphic Electroblotter II, Millipore, Billerica, MA, USA). For visualization of equal loading of protein, membranes were washed for 30 min in water and then stained with ink solution for 15 min before scanning of images with an Epson Expression 1680 Pro Scanner (Epson, Meerbusch, Germany). After destaining by soaking in TBS-T buffer (Tris-buffered saline-Tween, 20 mM Tris-HCl, 137 mM NaCl, and 0.1 % Tween 20), membranes were blocked with a solution of 5 % non-fat dry milk (Carl Roth, Karlsruhe, Germany) in TBS-T buffer for 90 min at room temperature. After 6 washes with TBS-T buffer, each membrane was incubated with a serum pool of seven individual mice from each time point as primary antibody mixture overnight at 4 ˚C. Membranes were washed 6 times in TBS-T buffer before the incubation with secondary antibody Immunopure peroxidase goat anti mouse IgG (H+L, 1:50.000) prepared in 5 % non-fat dry milk in TBS-T buffer at room temperature for 1 h. Following 6 washes in TBS-T buffer, membranes were incubated with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Thermo Scientific, Bonn, Germany) for 5 min, before signals were detected with a Lumi-Imager (Roche, Mannheim, Germany).

2.6 *Quantitative analysis of 2-D Western blots*

Detection and quantification of spot signals on the Western blot images were performed using the Delta2D software version 3.4 (Decodon, Greifswald, Germany) with some adjustments. In order to suppress effects of the Western blot background, two parameters in the Delta2D software including "image filter closing" and "image filter opening" were set to 1 and 10, respectively. The mean absolute volume of 15 background spots evenly distributed on the blots of each time point was used for background subtraction. For further calculations only spots identified in both technical replicates were considered. The volumes of 6 different time points (day 0 and 4, 8, 12, 28, and 84 days p.i.) were used to evaluate the changes during the time course. The changes were determined by calculating the ratios of volumes of the corresponding time points divided by volumes at day 0.

2.7 *Matrix assisted laser desorption ionization time-of-flight tandem mass spectrometry (MALDI-TOF-MS/MS)*

In order to reveal which cardiac proteins were preferentially recognized by auto-antibodies following viral infection, spots of a 2-D gel loaded with 300 µg of protein and stained with Coomassie brilliant blue (Sigma, Taufkirchen, Germany) were matched with the corresponding Western blot signals via the corresponding protein map on the membranes recorded after ink staining before immunoblotting. Spots of interest were manually excised and subsequently subjected to mass spectrometry for identification. Preparation of peptide extracts and MALDI-TOF-MS/MS analysis on a Proteome Analyzer 4800 (Applied Biosystems, Darmstadt, Germany) were carried out as described previously.[17] Identification of proteins was based on peptide mass fingerprint data confirmed by at least one protein specific peptide fragmentation pattern. Identifications with the International Protein Index (IPI) for mouse v. 3.26 using the Mascot algorithm via the GPS Explorer software package version 3.6 (Applied Biosystems) were considered to be statistically significant when the protein and peptide ion scores exceeded 60 and 32, respectively, which corresponds to $p<0.05$. Methionine oxidation was set as variable and carbamidomethylation as fixed modification.

3. Results and discussion

3.1 *Histopathological characterization of susceptible and resistant mice following CVB3 infection*

Histopathological examination of A.BY/SnJ and C57BL/6 mouse hearts at different time points -0, 4, 8, 28, and 84 days p.i.- was performed to evaluate myocardial injury as well as virus infection and the disease state throughout the course of infection. Monitoring of the virus infection by radioactive *in situ* hybridization with ^{35}S-labeled CVB3-specific probes provided proof for strong focal viral replication in the acute phase of infection in both mice strains as already reported.[12, 18] Virus load was highest at day 8 p.i. in C57BL/6 mice, but declined very fast in the following days leading to complete virus clearance. Thus, no viral RNA was detectable in C57BL/6 mice at day 28 and 84 p.i.. In A.BY/SnJ mice virus load reached its maximum at day 8 p.i. as well. Subsequent to the acute phase of infection the intensity of virus replication dropped down, however, low amounts of viral RNA were still detectable at later stages of infection, documenting the well known CVB3 persistence in chronic myocarditis in A.BY/SnJ mice.[19-20] The magnitude of viral replication was paralleled in histological and immunohistological findings. At day 8 p.i., staining with H&E revealed that the extent of myocardial lesions and inflammation was higher in A.BY/SnJ than in C57BL/6 mice. Only in A.BY/SnJ mice ongoing inflammation and a significant fibrosis was noted which was followed by a dilated phenotype with a significant thinning of the ventricular walls at day 84 p.i. [21], whereas, no significant remodeling was observed in the resistant mouse strain. In C57BL/6 mice an early and adequate release of cytokines (interleukin (IL)-10, IL-6, tumor necrosis factor-alpha) by dendritic cells (DCs) was reported, which might contribute to prevention of chronic CVB3-induced myocarditis.[18] Additionally, the chemokines interferon-inducible protein 10 (IP-10) and chemokine (C-C motif) ligand 5 (RANTES) were found to be secreted by DCs from resistant C57BL/6 mice earlier in infection and at significantly higher levels, indicating a protective role of IP-10 and RANTES in

CVB3-induced myocarditis.[18]

3.2 *Humoral immune response of susceptible and resistant mouse strain after CVB3-infection*

In order to minimize the contribution of interindividual variations among individual mice, sera from seven different mice of each time point were pooled for the isolation of IgG and the determination of its total concentration. Figure 1 illustrates that the total IgG did not change dramatically immediately after infection (day 4 p.i.) compared to the concentration observed before infection. However, IgG level significantly increased afterwards, with A.BY/SnJ displaying a faster increase (day 8 p.i.) then C57BL/6 mice (day 12 p.i.). In both strains sera contained the highest amount of IgG antibodies at day 12 p.i. Thereafter, the amount of IgG decreased at day 28 p.i. in both mouse strains. However, the total amount of IgG was higher throughout the whole course of myocarditis in the A.BY/SnJ strain compared to C57BL/6 mice which is in contrast to the findings for CVB3-specific antibodies, where equal levels were observed in both mouse strains.[22] Moreover, in A.BY/SnJ mice the early response to virus infection was much stronger, displaying a 4.5-fold increase in IgG amount from day 4 to 8 p.i.. In C57BL/6 mice the IgG level increased only 2.9-fold in that time window, but at day 12 p.i. a more than 7-fold higher level compared to that before infection was reached. In summary, a lower IgG antibody level was observed in C57BL/6 mice prior to infection, which raised delayed compared to that of the A.BY/SnJ mouse strain and was not maintained at the same high level. Since the C57BL/6 mice started with a lower basal level, fold changes were larger following infection with CVB3 in this strain compared to A.BY/SnJ mice. Maintenance of higher levels of IgG in the A.BY/SnJ mice might represent the continuing interaction with CVB3 and the damaged host tissue.

3.3 *Time-resolved analysis of the antibody patterns in A.BY/SnJ and C57BL/6 mice after infection with Coxsackievirus B3.*

To explore possible differences in the autoimmune response of susceptible (A.BY/SnJ) and resistant (C57BL/6) mice at the different time points after infection with CVB3, the autoantibody repertoire was profiled using 2-D immunoblotting. The Western blot results document that applying regular standard limits of detection only very few weak signals were observed in pools of control sera of both mouse strains (Fig.2 A, B). Thus, laboratory animals kept under defined conditions did not seem to display any unspecific infections. In contrast, Western Blot signals strongly increased in intensity after infection with CVB3 from day 4 p.i. onwards (Fig.2 C, D, Supporting information Fig. S1).

For the permissive A.BY/SnJ mouse strain, a strong autoimmune response against cardiac autoantigens was observed up to day 28 p.i. (Fig. 2). Only at day 84 p.i. (Fig.2 K) a decrease in signal strength was recorded. For DCM patients fading of the cardiac auto-antibodies was noted with disease progression.[23]

In the resistant mouse strain C57BL/6, the strongest response was seen at day 4 p.i., followed by a continuous decrease of autoimmune response signals over time reflecting the reported virus elimination in these mice within 28 days p.i. [18] Importantly, significantly weaker signals were detected at later time points (day 28 and 84 p.i.), which was associated with complete recovery (Fig. 2 J, L). These different cardiac autoimmune response patterns and the differences in the total IgG patterns highlight different immune responses after viral infection in the two mouse strains. The observation of higher levels of IgG in A.BY/SnJ mice before infection fits with the hypothesis that higher frequencies of circulating auto-antibodies predispose subjects to develop more severe cardiac diseases like DCM. [24] This susceptibility to viral infection or immunization with heart-specific

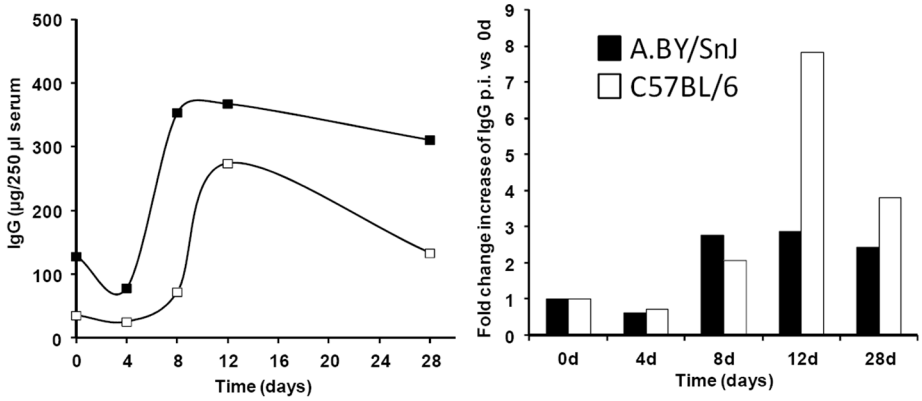

Fig. 1. Total IgG content in serum of A.BY/SnJ and C57BL/6 mice before and after infection with CVB3. A) Kinetics of changes of IgG concentrations during the time course of infection of a susceptible mouse strain A.BY/SnJ (white symbols) and a resistant mouse strain C57BL/6 (black). B) Relative changes in IgG-content compared to non-infected control samples.

auto-antigens of A.BY/SnJ mice is likely genetically determined [25] but the precise genetic alterations responsible have not been determined yet.

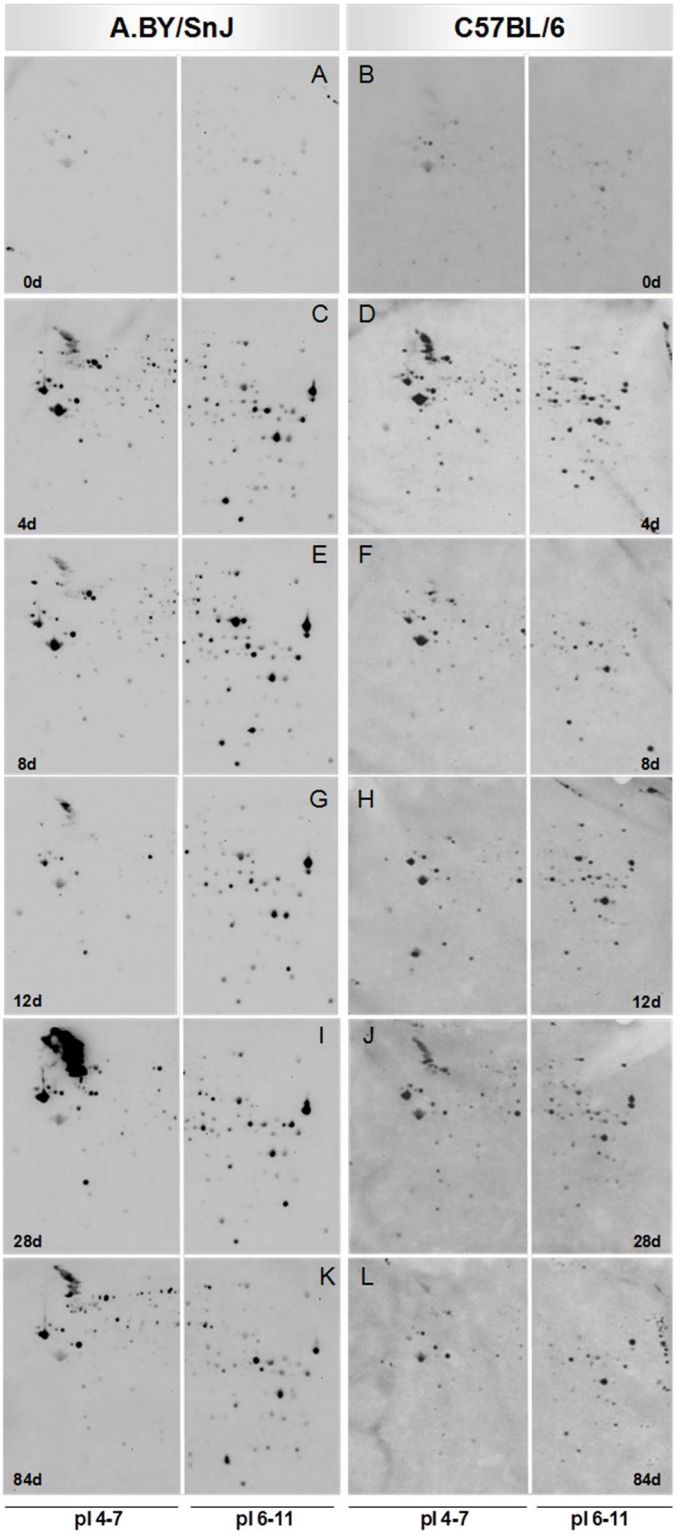

Fig. 2. Kinetics of autoimmune response of A.BY/SnJ and C57BL/6 mice against Coxsackievirus infection. 2D Western Blot images using IPG strips pH 4-7 and pH 6-11 were combined and display the autoimmune responses in both mice strains compared to non-infected controls (day 0).

A quantitative analysis of Western blot signals with the aid of the Delta2D software revealed a total of 227 spot signals for A.BY/SnJ mice, while only 169 spot signals were detected for the C57BL/6 strain (Supporting information Table S1). 131 spot signals were observed in the autoantibody patterns of both strains, whereas, 96 and 38 spot signals were only present in A.BY/SnJ and C57BL/6 mice, respectively. Due to the very low intensities of antibody signals observed in the non-infected mice, for further quantitative analysis only spot signals exceeding a fold increase >5 in the intensity ratio between the particular time point and the non-infected control group were considered. The number of significantly increased signals was higher in A.BY/SnJ mice compared to C57BL/6 mice during the whole course of infection (Supporting information Table S1). The autoimmune response due to viral infection was highest at day 4 p.i. in both mouse strains with 82 % (A.BY/SnJ) and 69 % (C57BL/6) of all antibody signals exceeding 5-fold increase. Remarkably, this peak in the cardiac autoimmune response clearly preceded the increase in total IgG level in both strains (see Fig. 1). However, while the level of most antibodies declined rapidly in the resistant strain, in A.BY/SnJ mice about 50 % of all antibodies displayed such high levels throughout the chronic phase up to day 84 p.i. (Fig. 3). High titers of autoantibodies can influence cardiac function by negative chronotropic and/ or negative inotropic effects mediated by binding to surface receptors, forming immune complexes or influencing downstream processes. [26]

3.4 *Identification of protein targets for auto-antibodies*

Identification of antigens by mass spectrometric analysis revealed protein identities for 209 of 227 spots visible on the Coomassie-stained gels (92 %) representing 122 distinct protein species.[27] For A.BY/SnJ mice 8 spots contained 2 proteins, and the remaining 201 spots contained only one major protein (Supporting information Fig. S2, Table S2). Similarly for strain C57BL/6, proteins have been identified in 125 of 169 spots (74 %) representing 124 proteins (87 distinct species). Here, only one spot contained 2 proteins, and the remaining 124 spots each contained only one predominant protein (Supporting information Table S3). Thus, the identifications of antibody targets by MALDI-mass spectrometry indeed permitted an evaluation of the protein groups targeted. The majority of proteins representing antigens in both mouse strains were of mitochondrial origin (44 %), followed by cytoplasmic (19 %), cytoskeletal (7 %) and plasma membrane proteins (7 %). The low coverage of surface antigens was likely caused by the fact that proteins with multiple transmembrane domains cannot be resolved efficiently on 2-D gels.

Although antibody formation against intracellular proteins is not expected, high abundance of heart specific mitochondria targeting antibodies (M7) was already reported in DCM patients in 1984.[28] The functional relevance of antibody production was also shown in a CVB3 infection model

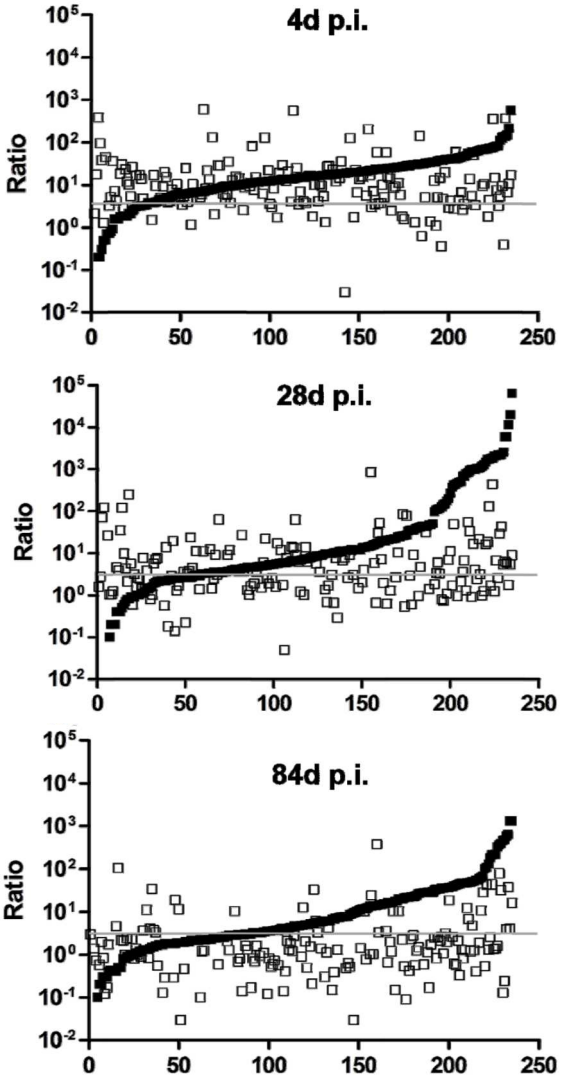

Fig. 3. Kinetics of Western Blot signal intensities at 4, 28 and 84 days p.i. with CVB3 in comparison to non-infected mice (day 0). Ratios of each time point vs. day 0 of A.BY/SnJ mice (black) were sorted by size and the corresponding ratio of each signal in C57BL/6 mice (white) was plotted. The horizontal gray line marks a 5 fold increase of the antibody signal in comparison to non-infected mice.

where a strong increase of antibodies directed against the mitochondrial adenine-nucleotide transporter led to alterations in cellular energy consumption and calcium homeostasis. [29, 30] The localization of the antigens for which antibody signals were identified in A.BY/SnJ mice only (48 protein species) did not differ significantly from that of C57BL/6 mice except for a slightly increased percentage of cytoskeletal antigens (10 %).

Functional categorization of all identified proteins (115 different protein species mapped to Uniprot accession numbers) disclosed carbohydrate metabolism (30 proteins), respiratory electron transport chain (19), cellular component organization (12) and tricarboxylic acid cycle (9) as the major biological processes targeted by auto-reactive antibodies (Supporting information Table S4). In the acute phase of infection (day 4 and 8 p.i.) the antigens targeted by the cardiac autoantibodies were assigned to similar biological processes in both mouse strains (according to PANTHER-www.pantherdb.or). However, a comparison of the proteins recognized by autoantibodies in A.BY/SnJ and C57BL/6 mice at particular time points revealed a higher number and stronger signal intensities in the permissive strain for targets involved in primary metabolic processes like respiration, carbohydrate metabolism and tricarboxylic acid cycle but

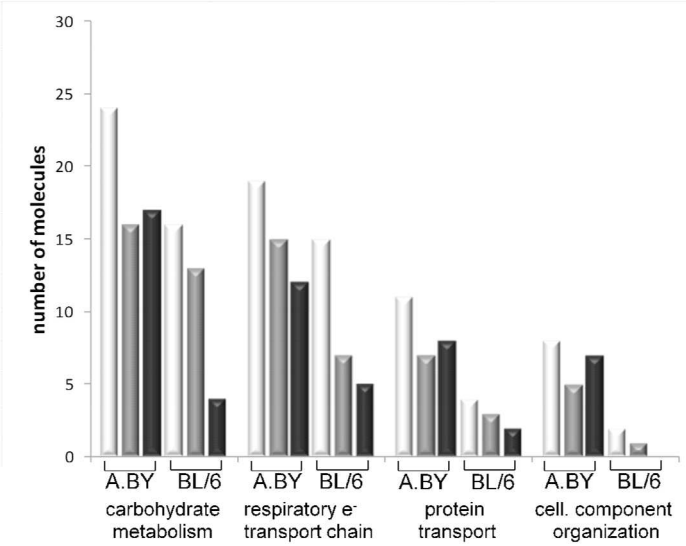

Fig. 4. Biological processes displaying differences in the number of assigned autoantibodies (assigned by the identification of the corresponding antigen) between the susceptible strain A.BY/SnJ and the resistant strain C57BL/6 at day 84 p.i. (black bars). Acute myocarditis (day 4 and day 8 p.i.) white bars; chronic myocarditis (day 28 p.i. grey bars).

also protein transport and cellular component organization (Supporting information Table S5). This higher number of protein targets in the susceptible strain A.BY/SnJ (Fig. 4, Supporting information Table S1 and S5) might reflect more pronounced cardiomyocyte lysis even in the early phase of infection.[31]

In agreement with observations for various animal models [5, 32] myosin heavy chain 6 was the most pronounced cardiac antigen in the permissive strain for which signal intensities increased up to 1800-fold at day 28 p.i. compared to the non-infected controls. A similar extremely strong signal was also reported for CVB3 infected SWR/Ola (H-2q) mice at day 25 p.i. suffering from subsequent myocarditis but without mortality.[33] However, rather weak anti-myosin antibody signals were observed in C57BL/6 mice at day 28 p.i., Molecular mimicry between Coxsackievirus and myosin was hypothesized to play a role in pathogenesis.[34-35] However, anti-myosin antibodies were also detected after troponin I-induced myocardial damage [36] or immunization with myosin itself.[37] These reports as well as the marked production of anti-myosin antibody in the permissive strain support a causative role of released cardiac antigens and their

corresponding auto-antibodies in pathogenesis of cardiomyopathies.[1] Auto-antibodies against troponin I or T, which were reported in other studies (reviewed by [38]), could not be detected in this study although the respective antigens were abundantly present in the 2-DE (data not shown). These differences might be caused be different genetic background because analyses of human sera from patients suffering from DCM and ICM against known cardiac auto-antibodies –among them anti-troponin I- revealed only frequencies of 15-20 % presence of specific antibodies [39] supporting the notion of a highly variable autoimmune response following myocardial damage. In our study we cannot address the prevalence of auto-antibodies directed against cardiac G-protein coupled receptors [40-41] since these membrane proteins were not resolved on the 2-D-gels [42] used for pre-fractionation of proteins prior to immunoblotting.

In A.BY/SnJ mice an autoantibody profile very similar to the one just described for myosin heavy chain 6 auto-antibodies, i.e. highest intensities at day 28 p.i. followed by a substantial drop in titer during active DCM (day 84 p.i.), was also observed for a number of mitochondrial proteins like components of complex I, II, III, and V of the respiratory electron transfer chain as well as for acyl-CoA-dehydrogenases. A variety of these proteins are flavoproteins (medium-chain specific acyl-CoA dehydrogenase, long-chain specific acyl-CoA dehydro-genase, isovaleryl-CoA dehydrogenase, electron transfer flavoprotein subunit beta, succinate dehydrogenase flavoprotein subunit Sdha and [ubiquinone] iron-sulfur protein Sdhb) which have already been described as cardiac antigens of auto-antibodies after CVB3-infection in mice.[43] Since mitochondria are highly abundant in cardiomyocytes, protein release in case of myocardial damage might lead to substantial antibody formation with functional relevance. The high number of gap-junctions facilitating the penetration of plasma membrane allows antibodies to gain access even to intracellular targets [30] and might contribute to energy limitations supposed already from altered levels of transcripts and proteins involved in lipid metabolism [21] and respiratory electron transport chain.[44-45]

Also for stress proteins like mitochondrial stress-70 protein and heat shock protein 8 strong increases in Western Blot signal intensities were detected predominantly at day 28 p.i. in comparison to non-infected mice. Cardiac autoantibodies against heat shock proteins have also been found in serum of patients suffering from DCM [46], supporting the similarities in the autoimmunity-related processes observed in animal models and in patients.

With the manifestation of the cardiomyopathy phenotype a drop in cardiac autoantibody levels was detected, although the titers in the permissive mice remained at much higher level than those in the recovered strain C57BL/6 (Figure 2I, J, Fig. 5). Most of the intense signals decreased significantly up to day 84 p.i. as already mentioned.

However, for a small group of cardiac proteins like radixin, alpha enolase, sero transferrin and 2 myosin peptides high antibody titers were detected only in the susceptible mouse strain and only at 84 days p.i. (Supporting information Table S1). In contrast, no antibodies against these proteins were present in C57BL/6 mice at any time point of myocarditis. Thus, a specific role for these auto-antibodies in virus-induced cardiomyopathy can be speculated, but has to be proven by use of age matched controls and the analysis of sera from different animal models as well patient samples.

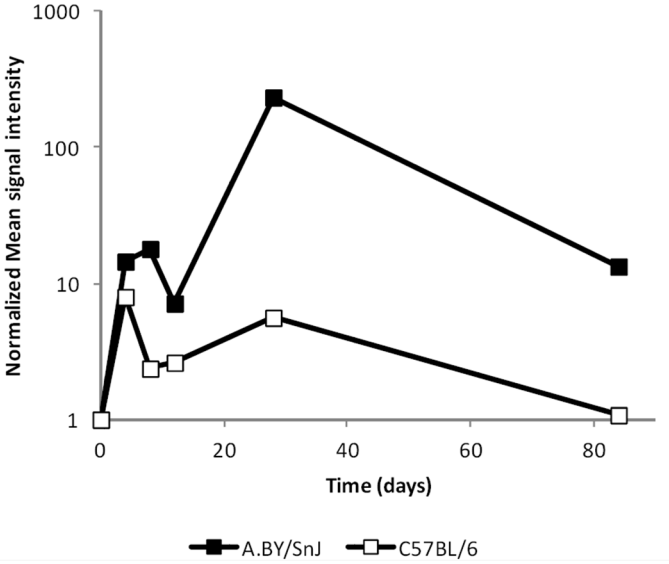

Fig. 5. Kinetics of mean Western Blot signal intensities in comparison to non-infected mice (day 0) of A.BY/SnJ mice (black symbols) and C57BL/6 mice (white symbols).

4. Concluding Remarks

In this study we investigated two mouse strains differing in their immune response after infection with CVB3, thus displaying a different cardiac autoantibody profile and repertoire. In the resistant C57BL/6 strain which is able to eliminate the virus within a few days due to effective cytokine production and activation of immune cells only short term accumulation of cardiac auto-antibodies was observed, the titer of which then declined fast and significantly. In contrast, in the permissive strain A.BY/SnJ virus persistence and ongoing myocardial injury were associated with persistent high titers of cardiac auto-antibodies. Here, a certain genetic predisposition probably causes alterations in the adaptive immune system. Furthermore, linkage of the major histocompatibility complex to susceptibility to chronic viral infections has been hypothesized. More extensive comparative genetic studies of mice strains presenting extensive autoimmune reactions with those lacking strong auto-antibody formation after viral infection are necessary, and the two strains of the present study seem to be potential candidates for such studies. The time resolved profile of the autoimmune response of A.BY/SnJ mice was characterized by the presence of massive antibody titers against cardiac myosin and mitochondrial proteins during chronic inflammation

(day 28 p.i.). The supposed lack of energy due to inference of antibodies with primary metabolism as well as loss of sarcomer integrity due to targeting of cell structure proteins might contribute to further myocyte loss leading later on to the manifestation of cardiomyopathy as a late consequence of enterovirus infection.

5. Supplementary material

Supplementary data and information is available at: http://www.jiomics.com/index.php/jio/rt/suppFiles/96/0.

Supplementary material includes:

- Supplementary Fig. S1. Number of cardiac autoantibodies detected in serum of mice infected with CVB3.
- Supplementary Fig. S2 Fused images of 2-D Western Blot images of all time points of pooled serum.
  - A. Labeled (spot signal numbers) fused image of 2-D Western Blot images of all time points of pooled serum in the pH range 4-7.
  - B. Labeled (Gene name or UniProt accession number) fused image of 2-D Western Blot images of all time points of pooled serum in the pH range 4-7.
  - C. Labeled (spot signal numbers) fused image of 2-D Western Blot images of all time points of pooled serum in the pH range 6-11.
  - D. Labeled (Gene name or UniProt accession number)fused image of 2-D Western Blot images of all time points of pooled serum in the pH range 6-11.
- Supplementary Table S1. List of all autoantigens identified by MALDI-TOF-MS/MS corresponding to autoantibodies generated in two mouse strains.
- Supplementary Table S2. Protein identification details of cardiac protein targets of autoantibodies detected by 2-D Western Blot in serum of A.BY/SnJ mice.
- Supplementary Table S3. Protein identification details of cardiac protein targets of autoantibodies detected by 2-D Western Blot in serum of A.BY/SnJ mice.
- Supplementary Table S4. List of biological processes displaying significant enrichment ($p<0.05$) among the list of proteins identified as antigens for autoantibodies in the hearts of A.BY/SnJ or C57BL/6 mice infected with CVB3.Enrichment of biological processes among the regulated proteins was calculated in the PANTHER software by comparison with the reference set of NCBI mouse database.
- Supplementary Table S5. List of biological processes displaying significant enrichment ($p<0.05$) among the list of proteins identified as antigens for autoantibodies in the hearts of A.BY/SnJ or C57BL/6 mice infected with CVB3resolved per time point. Enrichment of biological processes among the regulated proteins was calculated in the PANTHER software by comparison with the reference set of NCBI mouse database.Color defines significance of enrichment: dark red: highly significant; bright red >0.01< 0.05; yellow no significant enrichment.

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