Focused Issue on K<sub>ATP</sub> channels

DNA immunizations with M<sub>2</sub> muscarinic and β<sub>1</sub> adrenergic receptor coding plasmids impair cardiac function in mice

Luis E.D. Giménez a,b, Ciria C.Q. Hernández b, Elisabete C. Mattos c, Izaira Tincani Brandão d, Bianca Olivieri e, Roberto P. Campelo a, Tânia Araújo-Jorge e, Célio Lopes Silva d, Antônio C. Campos de Carvalho b, Eleonora Kurtenbach a,*

a Departamento de Bioquímica Médica, Instituto de Ciências Biomédicas, Universidade Federal de Rio de Janeiro, Centro de Ciências da Saúde, Cidade Universitária, 21941-590, Rio de Janeiro, RJ, Brazil
b Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, 21449-900, RJ, Brazil
c Ecodata Exames Médicos Ltda., Rio de Janeiro, 22020-120, RJ, Brazil
d Departamento de Bioquímica e Imunologia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Avenida Bandeirantes 3900, Ribeirão Preto, SP, Brazil
e Departamento de Ultra-estrutura e Biologia Celular, Instituto Oswaldo Cruz, Rio de Janeiro, 21045-900, RJ, Brazil

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Abstract

Autoimmune mediated myocardial damage is likely to be a pathogenic mechanism for acquired dilated cardiomyopathies. Evidence confirms that autoantibodies that bind to M<sub>2</sub> muscarinic (M<sub>2</sub>AChR) and β<sub>1</sub> adrenergic receptors (β<sub>1</sub>AR) are present in idiopathic dilated cardiomyopathy and Chagasic patients’ sera. To elucidate the role of these antibodies in cardiac functional impairment, we used a murine model immunized with plasmids encoding the M<sub>2</sub>AChR or β<sub>1</sub>AR via gene-gun bombardment. Anti-M<sub>2</sub>AChR and β<sub>1</sub>AR antibodies were detected over the course of 37 weeks. These antibodies were directed to the second extracellular loop (el2) of both receptors and the third intracellular loop (il3) of the M<sub>2</sub>AChR. Peak antibody titers from weeks 2 to 5 against M<sub>2</sub>AChR-el2 and β<sub>1</sub>AR-el2 as well as elevated titers against M<sub>2</sub>AChR-il3 were detected. Anti-M<sub>2</sub>AChR-il3 and anti-β<sub>1</sub>AR-el2 antibodies were predominant in IgG1 subclass immunoglobulins, suggesting a T-helper-2 biased lymphocyte response. Heart morphology and function was assessed by echocardiography over the course of 42 weeks. Data showed progressive decrease in left ventricular (LV) wall thickness and LV mass that was mostly evident for β<sub>1</sub>AR-immunized mice albeit a small change in LV dimensions. Fractional shortening was altered and values of 41%, 37% and 48% were observed at week 42 for the M<sub>2</sub>AChR, β<sub>1</sub>AR and control groups respectively. In support of autonomic deregulation, a twofold increase in M<sub>2</sub>AChR and a similar decrease in β<sub>1</sub>AR density were observed in radioligand saturation assays for both experimental groups. Histological analysis revealed myofibril disarray and fibrosis, pointing towards remodeling as a consequence of the long-term presence of anti-receptor antibodies.

Keywords: Dilated cardiomyopathy; Autoimmunity; Autoantibody; M<sub>2</sub> muscarinic acetylcholine receptor; β<sub>1</sub> adrenergic receptor; DNA immunization; Small animal echocardiography

1. Introduction

Acquired dilated cardiomyopathies (DCM) constitute a major cause of heart failure and transplantation in developed countries [1]. Following this trend, the burden of DCM in less developed countries has persistently increased due to acute cardiovascular morbidity such as ischemic heart disease [2]. Idiopathic dilated cardiomyopathy (IDC) and chronic Chagas’ disease (CCD) are both acquired forms of DCM for which enterovirus and the parasitic protozoan Trypanosoma cruzi were identified as etiologic agents [3]. However, the pathogenic mechanisms that determine the evolution from the acute myocarditis frequently observed in the initial stages of IDC and CCD to DCM remain unclear [4].

An open question resides in the difficulties of establishing a causative relationship between the persistence of viral genomes or T. cruzi amastigotes in the myocardium and the
process of heart remodeling and dysfunction described in IDC and CCD patients [3,5]. Thus lack of evidence supporting the hypothesis of direct infectious agent action as the sole determinant of chronic myocardial lesions led to the proposal of aberrant cellular and humoral immune responses as coadjuvants in the progression of initial stage myocarditis to chronic DCM [5,6].

In support of this hypothesis, humoral abnormalities were observed in patients with IDC and CCD [3,5]. Antibodies to heart muscle components, such as anti-sarcolemmal and anti-muscarinic adenine nucleotide translocator and against cardiac membrane receptors such as the β1 adrenergic receptor (β1AR) and the M2 muscarinic acetylcholine receptor (M2AChR) were described [7]. Of all the serologic markers of inflammatory heart disease described, only the anti-mitochondrial and the anti-cardiac membrane receptor antibodies showed relevant functional activity [6,8].

Antibodies with adrenergic (positive chronotropic effect in cultured spontaneously beating rat cardiomyocytes) or muscarinic agonistic (negative chronotropic effect and impairment of L type Ca2+ currents in isolated cardiomyocytes) functional activities were detected in the sera of IDC and CCD patients [9–12]. The antibody binding properties to these receptors were also characterized [9,12]. The epitopes of these anti-receptor antibodies were mapped using short overlapping peptides in the context of neutralization experiments in spontaneously beating cardiomyocyte cultures. In these experiments, dominant epitopes were identified in the first and second extracellular loops (el1, el2) of the β1AR [13]. Roughly 80% of IDC patient sera showed binding to β1AR-el1 or el2 [13]. The observed incidence of anti-M2AChR antibodies in IDC patients was 36–50% and in this case, the dominant epitope was mapped to the el2 [11]. On the other hand, the reported occurrence of anti-M2AChR antibodies in CCD patients was significantly higher than the values reported for IDC [14] and recognition of alternative epitopes on the third intracellular loop (i13) by antibodies from sera of different stage CCD patients that correlate with disease progression was also reported [15].

Previous reports [16–19] dealt with the establishment of animal models of autoimmune cardiomyopathy. Elevated antibody titers were obtained from the sera of these animals by immunizing against synthetic peptides corresponding to the el2 of the human β1AR and M2AChR [16,18,19]. In these studies, the rabbit raised antibodies recognized in situ myocardial adrenergic receptors and inhibited muscarinic antagonist binding [16,19]. Moreover, the anti-β1AR-el2 antibodies had a positive chronotropic effect and the anti-M2AChR-el2 antibodies had a negative chronotropic effect on rat cardiomyocytes in culture as seen for affinity-purified antibodies from IDC patients [16,18].

The limitations associated to these studies reside in the short-lived humoral responses and the elevated cost of continued synthetic peptide immunizations [20]. Thus, we explored a different approach to immunize our animals, employing the helium-driven dermal bombardment of gold particles coated with plasmid DNA coding for the M2AChR and β1AR proteins. Immune autoreactive responses should be enhanced by this immunization procedure, representing an attractive alternative for the establishment of an autoimmune model of DCM [20].

In this study, BALB/c mice were immunized with plasmid DNA coding for the human M2AChR or β1AR. To determine the effect of the immunizations, heart function was directly assessed by real-time echocardiographic imaging and receptor antibody titer profiling. Histopathological analysis to assess tissue architecture and radioligand binding assays to determine heart muscarinic and adrenergic receptor expression were also performed.

2. Materials and methods

2.1. DNA Plasmids

The pcDNA3-hM2 construct coding the human M2AChR sequence was prepared by subcloning a 1950 bp AvrII restriction fragment from the pcD-hM2 plasmid that originally included the M2 muscarinic receptor sequence (from Dr. Edward Hulme, NIMR, London, UK) into the XbaI unique site in the pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA, USA). The pBC121BI empty plasmid and the pBC121BI-hβ1AR plasmid coding for the human β1AR were from Dr. Bryan R. Cullen and Dr. Robert J. Lefkowitz respectively (Duke University Medical Center, Durham, NC, USA). The plasmids were grown in DH5α E. coli strains and purified with Qiagen Giga Kits (Qiagen, Chatsworth, CA, USA) following the manufacturer’s instructions.

The el2 from the mouse and the human M2AChR and β1AR share a 100% sequence identity. The overall identity between the human and mouse M2AChR is 92% and 70% among the β1AR from both species. These high identities allowed for the use of human receptor-coding plasmids in this study.

2.2. Animals and immunizations

Male BALB/c mice, 7 weeks old and weighing 20.4 ± 4.6 g were randomly assigned to a control group immunized either with the pcDNA3.1 or the pBC121BI plasmid (n = 19), to a group immunized with the pcDNA3-hM2 plasmid construct (n = 18, M2AChR group) and to a group immunized with the pBC121BI-hβ1AR plasmid (n = 19, β1AR group). The animals from each group were individually identified and kept at the faculty animal facility. All animal procedures were carried out in accordance with the guidelines established by the Fiocruz Committee for Ethics for the Care and Use of Laboratory Animals (Resolution 24 2/95).

Immunizations were performed using a helium-driven gene gun (Bio-Rad, Hercules, CA, USA). DNA-coated particles were prepared by combining 25 mg of 1.6 µm gold microcarriers and 100 µl of 0.05 mol/l spermidine with plasmid DNA...
with 0.1 µg per well of a fusion peptide of the human overnight at 4 °C. Alternatively microtitre plates were coated bicarbonate buffer (50 mmol/l, pH 9.6) and incubated

D-E-A-R-R-C-Y-N-D-P-K-C-C-D-F-V-T-N221) in carbonate–

b control. Additionally a purified rabbit antibody against the lar Biology, Strasbourg, France[21]) were used as a positive

in this study.

the ratio (%) of each animal's paired immune/pre-immune

(50 µg) and 1 mol/l CaCl2 (100 µl). After precipitation and washing, the mixture was loaded into tubing and dried. As a result, each cartridge contained 1 µg of plasmid DNA. The DNA was delivered to each animal’s shaved ventral region with a discharge pressure of 400 psi. Mice from both groups were primed and boosted three times with a 14-day interval between boosts. The immunizations were repeated 20 weeks after the first three boosts.

2.3. Sera collection and enzyme-linked immunosorbent assays (ELISA)

Mice blood samples were collected from the orbital sinus at a mean interval of 6 weeks over the course of 37 weeks (immune samples) including samples prior to the first immunizations (pre-immune samples). The samples were incubated for 1 h at 37 °C and overnight at 4 °C, centrifuged twice at 15,000 × g for 15 min for serum separation and stored in small aliquots at −20 °C.

ELISA assays were performed by coating microtitre plates (High binding, Corning Inc., Corning, NY, USA) with 1 µg per well of synthetic peptides (Sigma-Genosys, The Woodlands, TX, USA) corresponding to the el2 of the human M1AChR (168 V-R-T-V-E-D-G-E-C-Y-I-Q-F-F-S-N-A-A-V-T-F-G-T-A-I192) or the human β2AR (195H-W-W-R-A-E-S-D-E-A-R-R-C-Y-N-D-P-K-C-C-D-F-V-T-N197) or the human M2AChR-el2 (residues V-R-T-V-E) (a gift from Dr. Johan Hobeke, Institute for Molecular and Cellular Biology, Strasbourg, France[21]) were used as a positive control. Additionally a purified rabbit antibody against the

1AR-el2 or a rabbit raised antibody that recognized the

M2AChR-il3 were also used as positive controls. Primary antibodies were incubated with 0.1 µg per well of a fusion peptide of the human M2AChR-el2 (residues 267–381) and glutathione S-transferase (GST) of Schistosoma japonicum as previously described [15]. Afterwards wells were saturated with PBS + 5% (v/v) fetal bovine serum + 0.1% (v/v) Tween 20 (PBS–FBS–T) solution for 2 h at room temperature. Sample sera were diluted to 1:400 in PBS–FBS–T solution. Serial dilutions of a monoclonal antibody raised against the N-terminus of the M2AChR-el2 (residues V-R-T-V-E) (a gift from Dr. Johan Hobeke, Institute for Molecular and Cellular Biology, Strasbourg, France[21]) were used as a positive control. Additionally a purified rabbit antibody against the β2AR-el2 or a rabbit raised antibody that recognized the M2AChR-il3 were also used as positive controls. Primary antibodies were incubated for 2 h at 37 °C. Afterwards goat biotinylated anti-mouse and anti-rabbit IgG (H + L) antibodies (1:5000 in PBS–FBS–T) or rabbit biotinylated anti-mouse IgGl and IgG2a isotype antibodies (1:5000 in PBS–FBS–T) were allowed to react for 1 h at 37 °C. The bound antibodies were detected with streptavidin-horseradish peroxidase conjugate (1 µg/ml) solution in PBS–T. TMB (3,3',5,5'-tetramethyl benzidine) was used as substrate for the peroxidase. Color development was measured as absorbance at 450 nm. All reagents used in the ELISA assays were acquired from Zymed (San Francisco, CA, USA). All ELISA data were plotted as the ratio (%) of each animal’s paired immune/pre-immune sera O.D. allowing for a time-course profile delineation of the antibody response subsequent to gene gun immunization in this study.

2.4. Serial ultrasonic echocardiography

Serial transthoracic echocardiography was performed in four sessions starting before the immunizations and in weeks 15, 30 and 42 after DNA priming. Light anesthesia was induced by an i.p. injection of ketamine and xylazine (60 and 5 mg/kg, respectively) 10 min prior to the procedure. The images were acquired with the animals placed in a heating pad in a shallow lateral decubitus position by a single blinded observer. A 10 MHz annular phased-array electronic transducer connected to a Megas GPX (Esaote SpA, Genova, Italy) echocardiographic system was used. Penetration depth was set to 4 cm and sector angle to 30°. Standard lead II electrocardiograms were recorded for heart rate (HR) measurements and parasternal long- and short-axis views were obtained after adjusting gain settings for optimal epicardial and endocardial wall visualization. Images were digitally stored for offline analysis.

The following parameters were measured on the M mode tracings and averaged from three cardiac cycles: left ventricular (LV) end systolic and end-diastolic diameters (LVESd, LVEDd); anterior and posterior wall thickness of systole and diastole (Aws th, Awd th, Pws th and Pwd th). The measurements were made using the leading edge method [22]. LV mass was calculated by: LV mass = [(LVEDd + Awd th + Pwd th)3 – LVEDd3] × 1.055. The derived LV mass was normalized by the body weight (BW) and expressed in mg/g. LV fractional shortening (FS) was calculated by FS% = [(LVEDd – LVESd)/LVEDd] × 100. Aortic (Ao) and left atrium (LA) diameters were obtained from M mode traces at the short-axis base level and the LA/Ao ratios were plotted.

2.5. Histopathological analysis

Mice (n = 4 from each group) were sacrificed by CO2 inhalation at week 45 and hearts were collected for histopathological analysis. The specimens were processed in paraffin-embedded sections and stained with hematoxylin–eosin (H and E) or Masson Trichrome for fibrosis detection. Images were collected on a Zeiss Axiosvert 200 2TV microscope coupled to a digital acquisition system (Thornwood, NY, USA). The analysis of inflammatory foci was performed using an ×40 objective by the observation of an average of 30 fields from the base, the papillary and the apex sections of the heart. The quantification of inflammatory infiltrates was done by counting the number of foci containing at least five mononuclear cells in each field. The quantification of fibrosis was done by the digital determination of the area of blue stained nuclei in each field. The quantification of fibrosis was done by the digital determination of the area of blue stained fibrosis (Image Pro Plus 5.01, Media Cybernetics, Inc. Silver Spring, MD, USA) from 30 fields (×20 objective) in sections corresponding to those used for the inflammatory foci quantification. The results were expressed as the relative area of fibrosis in the fields analyzed.

2.6. Membrane preparation and binding assays

Mice (n = 5) from each group were sacrificed by cervical dislocation at week 45. The hearts from each group were
pooled and transferred to ice-cold Tyrode solution. The steps that follow were performed at 4 °C. Heart samples were minced and suspended in buffer A (25 mmol/l HEPES, 1 mmol/l EDTA, 0.1 mmol/l PMSF, and 5 mmol/l MgCl₂, pH 7.5) and then homogenized with three 15 s strokes with a Tissumizer (Tekmar Company, Cincinnati, OH, USA) and 60 s resting intervals. The suspensions were filtered through cheesecloth and centrifuged at 4 °C for 15 min at 48,000 × g. The resulting pellets were resuspended in buffer B (similar to buffer A, except for 2 mmol/l MgCl₂) with the aid of a Teflon-glass homogenizer. Next, the suspensions were centrifuged again for 30 min at 48,000 × g. Finally, the pellets were resuspended in buffer B and stored at −70 °C.

β-Adrenergic receptor density (B_max) was determined by [3H] dihydroalprenolol hydrochloride ([3H]-DHA, 120 Ci mmol, New England Nuclear, Boston, MA, USA) saturation binding assays. The homogenates (150 µg) were incubated in buffer C (25 mmol/l Tris–HCl, 1 mmol/l EDTA, 0.1 mmol/l PMSF, and 5 mmol/l MgCl₂, pH 7.5) with increasing amounts of [3H]-DHA (5–80 nmol/l) at 25 °C for 1 h in a final volume of 400 µl. The reaction was stopped by rapid vacuum filtration using a cell harvester (Brandel, Gaithersburg, MD, USA) through GF/B glass fiber filters pre-soaked in 0.3% polyethylenimine followed by three fast washes with ice-cold 10 mmol/l phosphate buffer, pH 7.4. The trapped radioactivity was determined by liquid scintillation counting (efficiency of 45%, Packard Instruments Co., Meriden, CT, USA). Nonspecific binding was defined as the bound radioactivity in the presence of unlabeled propranolol (10 µmol/l). Specific [3H]-DHA binding activity was estimated by subtracting the nonspecific binding from the total binding.

Muscarinic receptor density was determined by L-[N-methyl-3H] scopolamine methyl chloride ([3H]-NMS, 84 Ci mmol, Amershams Biosciences, Piscataway, NJ, USA) saturation binding assays. Accordingly homogenates (150 µg) were incubated in buffer C with increasing concentrations of [3H]-NMS (10–1200 pmol/l) at 37 °C for 2 h in a final volume of 2 ml. Nonspecific binding was defined by unlabeled atropine (2 µmol/l). Subsequent procedures were performed as previously described for the β-adrenergic receptor. Estimates of maximal bound (B_max) and equilibrium dissociation constant (K_d) were obtained from least square curve fitting analysis according to the rectangular hyperbolic model using the GraphPad Prism 4.02 software (San Diego, CA, USA).

2.7. Statistics

Statistical significance (P < 0.05) was evaluated using one way analysis of variance (ANOVA) with Bonferroni’s multiple comparisons test for the histological analysis data and one-site saturation binding parameters. Paired two-way ANOVA with Bonferroni’s multiple comparisons test was used for the analysis of ELISA and echocardiographic parameters. The difference between the radioligand saturation binding experiments were also compared using a global fitting algorithm based on Akaikes’s information criterion. Normal distribution was confirmed for all data sets. These analyses were performed with the aid of the GraphPad Prism 4.02 personal computer software.

3. Results

3.1. Anti-β1AR and M2AChR specific antibody response in the immunized mice

Synthetic peptides corresponding to the M1AChR-el2 and the M2AChR-il3-GST fusion protein were used to detect the antibody response against these peptides in the animals immunized with the M1AChR encoding plasmid. These assays are depicted in Fig. 1A, B respectively. Peak antibody production (63 ± 8% above pre-immune antibody titers) against the M2AChR-el2 was observed at week 5 (Fig. 1A). Following this first peak, a second and discrete rise was observed after the administration of the second set of boosters (at week 20). In another set of experiments, anti-M2AChR-il3 antibodies were readily detectable (134 ± 19%) 2 weeks after the initial immunizations (Fig. 1B). Antibody titers for M2AChR-il3 then remained elevated and after the boosters administered at week 20, the anti-il3 titers rose again through the last time point tested (126 ± 31%). The overall titers of antibodies against the M2AChR-il3 were greater than the titers for the antibodies against the M2AChR-el2 for the dilutions of sera tested. Moreover anti-il3 antibodies were readily detectable over the course of the study (37 weeks) whereas the anti-el2 antibodies were mostly present in the first weeks analyzed.

In the animals immunized with the β1AR plasmid DNA, anti-β1AR-el2 specific antibody (Fig. 1C) titers peaked at weeks 2–5 (55 ± 5% to 63 ± 11%) and showed a moderate increase after the administration of the second set of boosters that then decreased over time. Cross specific IgG production was not detected for β1AR-el2 on sera collected from the M2AChR group of animals or for M2AChR-el2 on the sera collected from the β1AR group (data not shown). Anti-GST from the M2AChR-il3-GST fusion peptide) antibodies were also not detected (data not shown).

An increase in IgG1 and IgG2a subclass antibodies against the M2AChR-il3 was detected in sera samples from the animals immunized with the M1AChR coding plasmid collected at week 20 (Fig. 1D). An increment of 130 ± 25% over the pre-immune titers was observed for the IgG1 subclass and of 70 ± 8% for the IgG2a subclass. These results strongly suggest that a Th2 biased response might be operating in the M1AChR plasmid DNA immunized animals. The immunoglobulin subclass distribution was also skewed, favoring the IgG1 subclass production in the β1AR group. This is demonstrated by a 134 ± 48% increase in anti-β1AR-el2 IgG1 production (Fig. 1D). These results confirmed the Th2 biased response induced by gene gun immunization also observed in the M2AChR group.
3.2. Assessment of heart morphology and function by echocardiography

The effects of M2AChR and β1AR plasmid DNA immunizations on cardiac morphology and function were evaluated by serial echocardiography. The results are summarized in Table 1 and Figs. 2–5. LVESd and LVEDd cavity dimensions are highlighted in Table 1. A significant increase in mean cavity size was observed for the LVESd but not for the LVEDd when comparing the three groups at a given time point. This was evident for both experimental groups (Table 1) that showed significantly larger LVESd than the control group at week 42.

LV wall thickness measurements in systole and diastole are shown in Fig. 2A–D. A significant decrease in anterior wall thickness in diastole (Awd th) for the muscarinic and the adrenergic groups was evident starting at week 30 (Fig. 2A). This difference increased through week 42 when mean muscarinic and adrenergic group wall thickness in diastole was 10% and 21% thinner, respectively, when compared to the control group. Posterior wall measurements (Pwd th, Fig. 2B) followed the same pattern as anterior wall measurements in diastole; while wall thickness at 42 weeks was smaller in both groups compared to controls. When the same measurements were taken in systole (Aws th and Pws th), the symmetry was lost as clearly seen by comparing Fig. 2C, D. Although the effect of M2AChR plasmid immunization was modest (9% and 15% for anterior and posterior walls, respectively, at week 42), β1AR plasmid immunization induced a 27–31% decrease in wall thickness in diastole (Awd th) for the muscarinic and the adrenergic groups was evident starting at week 30 (Fig. 2A).

Fig. 1. Time course of the production of anti-M2AChR and β1AR antibodies in the sera of plasmid DNA immunized mice. Panel A: screening of anti-M2AChR-el2 antibodies in control and pcDNA3-hM2 immunized (M2AChR group) mice. Panel B: screening of anti-M2AChR-il3 antibodies on the same groups of animals shown in panel A. Panel C: screening of anti-β1AR-el2 antibodies in control and pBC12BI-hβ1AR-immunized (β1AR group) mice. Panel D: IgG subclass (IgG1 and IgG2a) relative titers against the M2AChR-el2 in the control and M2AChR groups (left panel) or against the β1AR-el2 in the control and β1AR groups (right panel) in samples collected at week 20. In all cases, the ordinate shows the absorbance ratio at 450 nm of sera collected over the course of 37 weeks (or at week 20 for the graphs of panel D) and pre-immune sera. Data points (or bars) represent the mean ± S.E.M. from three independent experiments. A significant difference (P < 0.05) is shown by an asterisk (*) when comparing each experimental group with the control group.

### Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control group</th>
<th>M2AChR group</th>
<th>β1AR group</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>Pre-immune 22.5 ± 1.0</td>
<td>42 weeks 23.5 ± 2.1</td>
<td>Pre-immune 20.0 ± 1.1</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>Pre-immune 176 ± 51</td>
<td>42 weeks 232 ± 22</td>
<td>Pre-immune 173 ± 50</td>
</tr>
<tr>
<td>LVESd (mm)</td>
<td>Pre-immune 1.2 ± 0.2</td>
<td>42 weeks 2.3 ± 0.1</td>
<td>Pre-immune 1.1 ± 0.1</td>
</tr>
<tr>
<td>LVEDd (mm)</td>
<td>Pre-immune 3.1 ± 0.3</td>
<td>42 weeks 4.4 ± 0.2</td>
<td>Pre-immune 3.1 ± 0.2</td>
</tr>
</tbody>
</table>

Values for each parameter are indicated as mean ± S.E.M. A significant difference (P < 0.05) is shown by an asterisk (*). The P values were calculated by comparing each measured mean from each experimental group with the control group for a given time. Two-way ANOVA and Bonferroni’s multiple comparison tests were used to establish the statistical significance between the measured values. BW indicates body weight; bpm, beats per minute; LVESd, and LVEDd, left ventricular end systolic and end-diastolic internal diameters.
in posterior and anterior wall thickness during systole at week 42. When comparing each time point after the immunizations onset, anterior wall thickness in systole appeared to be more affected than the posterior free wall. Interestingly, this tendency was readily observed for the β1AR plasmid immunized mice as early as week 15 after the first set of boosters.

LV mass estimates normalized by BW are shown in Fig. 2E. The group of animals immunized with the plasmid coding the M2AChR showed no significant change in LV mass when compared with the control group. In contrast, the LV relative mass of the β1AR plasmid immunized group decreased after week 30 when the difference reached 45% of the mean relative LV mass of the control group.

Atrial dimensions directly reflect ventricular diastolic failure. Thus, an index of atrial diameter modifications was determined taking the aorta as a measurement standard. This procedure is suitable because the aorta is not susceptible to diameter modifications due to myocardial intrinsic hypertrophic mechanisms. Shifts in atrial cavity size are shown as the ratios of LA cavity dimension and the diameter of the aorta (Ao) in Fig. 3. An early and persistent increase in relative LA cavity size was observed in the β1AR group for all subsequent time points analyzed after the initiation of the immunization regime. LA dilatation was smaller in the M2AChR group, suggesting that volume overload was not present and diastolic function was better preserved in this group of animals when compared with the β1AR group.

Several systolic function parameters are summarized in Fig. 4. Both anterior and posterior systolic wall thickenings were impaired in the β1AR plasmid immunized group. Control group anterior wall thickening was 100 ± 9% as compared to 46 ± 9% for the β1AR group at week 42 (Fig. 4A). The LV free posterior wall was better preserved from this effect and β1AR animal wall thickening only fell to 64 ± 7% at the same time point (Fig. 4B). At week 15, the M2AChR plasmid immunized group showed a higher anterior and posterior systolic thickening when compared to control animals. These values showed a significant decrease when acquired at weeks 30 and 42. In fact, the variations observed in this period for both groups are significantly higher than those observed for the control group.
Fig. 4. Impairment of LV contractility by $M_2$AChR and $\beta_1$AR DNA immunization. Serial echocardiographic estimates of LV wall thickening and LV FS. Panel A: serial estimates of LV anterior wall (Aw) systolic thickening. Panel B: serial estimates of LV posterior wall (Pw) systolic thickening. Panel C: LV FS calculated from LV end-diastolic and end-systolic internal dimensions.

Fig. 5. Impaired contractility was observed in M mode tracings of the left ventricle of experimental animals. Panel A shows representative bidimensionally oriented M mode echocardiograms obtained at mid papillary level at baseline (pre-immune), weeks 15 and 42 after the initial plasmid DNA priming. The top row shows the echocardiograms of a control animal (animal number 9) at the mentioned time points. The middle row corresponds to an $M_2$AChR plasmid DNA immunized animal (animal 32) and the bottom row to a $\beta_1$AR group animal (animal 47). The horizontal bar represents 0.4 s and the vertical bar represents 5 mm. Panel B. Posterior wall systolic thickening of the individual animals and the time points depicted in panel A.
LV FS was also affected by DNA immunizations. By week 42 values of 37 ± 3% and 41 ± 3% were observed for the β1AR and the M2AChR group, respectively, versus a control group value of 48 ± 2% (Fig. 4C) indicating, once more, a cardiac contractile impairment.

The time course of the impairment reflected by the fall in LV FS can be assessed in the series of M mode echocardiograms in Fig. 5. This effect appeared as a diminished systolic thickening and decreased contractile velocity of the anterior and the posterior walls of the β1AR plasmid DNA immunized animal (animal number 47). In this case, a decrease of 115–67% in the Pw systolic thickening from the pre-immune measurement (baseline) to 42 weeks was observed. The same profile was observed for a representative animal from the M2AChR plasmid DNA immunized group depicted in the middle row panels (animal number 32). For this animal, a decrease of 100–83% was observed in the same period. It is worth pointing out that the M mode echocardiograms of the control animal chosen (animal number 9) shown in the top row series did not show any change in this parameter over the course of the experiment (114%, 140% and 142% at baseline, weeks 15 and 42, respectively).

3.3. Histopathological analysis

Histopathological samples from the experimental groups were analyzed by both H and E and Masson’s trichrome staining, revealing important alterations (Fig. 6). In the M2AChR animals (Fig. 6E, F) organization was maintained but Masson’s trichrome staining revealed diffuse collagen deposits (blue stained areas in Fig. 6E, F) indicative of fibrosis of the myocardium. These alterations were largely observed in the β1AR group (Fig. 6G–I) where the H and E stained preparation clearly showed myocyte disarray with moderate leukocyte infiltrate (Fig. 6G). The Masson’s trichrome analysis revealed continuous areas of fibrosis interrupting myocyte continuity as shown in the photomicrographs depicted in Fig. 6H, I. Quantification of mean fibrotic areas in the specimens examined (30 fields) is represented in Fig. 6J. Approximately 20% of the area of the observed fields was taken by fibrosis in both experimental groups. No significant differences concerning the presence of inflammatory foci were found among the three groups (quantification of data not shown).

It is worth pointing out that although no patent clinical signs of heart failure were observed (hepatomegaly, dyspnea, edema and decreased locomotor activity among others) for experimental groups, the BW follow-up revealed a relative increase for this variable mainly in the β1AR group of animals (Table 1). However, postmortem examinations of immunized mice did not show appreciable fluid accumulation in any body cavity.

Fig. 6. Evidence of myofibril disarray and fibrosis in experimental mice. Representative photomicrographs of mid papillary section of the heart from a control (panels A–C, top row), an M2AChR (panels D–F, middle row) and a β1AR (panels G–I, bottom row) group animal. The sections depicted in panels A, D, and G were stained with H and E and the sections shown in panels B, C, E, F, H and I were stained with Masson’s trichrome. Panels B, E and H were photographed at ×100 magnification (horizontal bar corresponds to 50 µm) and panels A, C, D, F, G and I were photographed at ×400 magnification (bar corresponds to 10 µm). Panel J represents the mean relative area of fibrosis per field (±S.E.M.) quantified as blue pixels in digitally acquired images from 30 fields representative of the entire hearts of the animals analyzed (see “Section 2” for details).
Here we provide evidence for a direct causative effect of the density \((\alpha)\) antagonist \([3H]\)-DHA using the same heart homogenates from mice sacrificed 45 weeks after initial DNA priming. Saturation binding parameters from control and experimental mice heart homogenates

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Control group</th>
<th>M2AChR group</th>
<th>(\beta_1)AR group</th>
</tr>
</thead>
<tbody>
<tr>
<td>([3H])-NMS</td>
<td>92.8 ± 10.5</td>
<td>77.8 ± 6.7</td>
<td>103.6 ± 13.0</td>
</tr>
<tr>
<td>([3H])-DHA</td>
<td>17.0 ± 3.0</td>
<td>15.8 ± 2.9</td>
<td>15.2 ± 1.7</td>
</tr>
</tbody>
</table>

The values were obtained from non-linear regression analysis of radioligand saturation experiments (refer to “Section 2” for more details). Values for each parameter are indicated as the mean ± S.E.M. from three independent experiments. Equilibrium dissociation constant \((K_D)\) values are given in pM units for \([3H]\)-NMS binding and in nM units for the binding of \([3H]\)-DHA. A significant difference \((P < 0.05)\) is shown by an asterisk (*) when comparing each binding parameter for a given radioligand with the experimental groups with the control group. Statistical significance was estimated using one-way ANOVA and Bonferroni’s multiple comparison tests. \([3H]\)-NMS indicates L-\([N\text{-methyl-}\text{H}]\) scopoline methyl chloride; \([3H]\)-DHA indicates \([H]\) dihydrolphenol hydrochloride and \(B_{\text{max}}\) maximal bound ligand.

### 3.4. Adrenergic and muscarinic antagonist binding assays

The non selective muscarinic antagonist \([3H]\)-NMS was used in saturation binding assays with heart homogenates from mice sacrificed 45 weeks after initial DNA priming. Saturation binding parameters for this ligand are shown in Table 2. Both the M2AChR and the \(\beta_1\)AR-immunized groups showed an approximately twofold increase in muscarinic receptor density \((B_{\text{max}})\) (Table 2) with no apparent change in the equilibrium dissociation constants \((K_D)\). On the other hand, an opposite effect was observed for the saturation of the \(\beta_1\)AR-specific antagonist \([3H]\)-DHA using the same heart homogenates from the M2AChR and \(\beta_1\)AR groups that were used for the \([3H]\)-NMS binding assays. The \([3H]\)-DHA saturation assays showed a twofold decrease (Table 2) in adrenergic receptor density \((B_{\text{max}})\) for both experimental groups and again no significant change in \(K_D\) values. Binding experiments were best fitted by a one-site binding hyperbola. Taken together, saturation-binding data indicate that adrenergic and muscarinic receptor expressions were regulated in opposite ways in both the M2AChR and the \(\beta_1\)AR animal groups.

### 4. Discussion

In the present study, murine models of autoimmune cardiomyopathy were created, for the first time, by gene gun DNA dermal bombardment using plasmids encoding the entire sequences coding for the entire receptor genes were able to maintain elevated antibody levels that would otherwise result in a single and discrete response peak.

Following a different trend, persistently elevated titers of M2AChR-il3 specific antibodies were detected in the M2AChR group (Fig. 1B). Interestingly, these anti-il3 antibody titers were readily elevated at the first measurements performed (2 weeks), then fell to lower levels (lowest level at week 20) and finally rose again persisting throughout the last measurement performed (Fig. 1B). The biphasic behavior observed in the assays depicted in Fig. 1B could be initially due to the priming of the animals with DNA and in line with Retondo et al. [15], the second progressive elevation in il3 titers could be related to progressive heart injury as also shown by the other functional and pathological parameters analyzed in this study.

The results from the assays depicted in Fig. 1 (panel D) suggest that receptor DNA immunizations evoked a mixed Th1 and Th2 phenotype response with a probable predominance of the Th2 phenotype as an increase of 130 ± 25% for the IgG1 subclass and 70 ± 8% for the IgG2a subclass at week 20 was observed for the animals from the muscarinic receptor immunized group and a 134 ± 48% increase in anti-\(\beta_1\)AR-el2 IgG1 production (Fig. 1D) for the \(\beta_1\)AR group. DNA immunization can effectively induce MHC class I-restricted cellular immunity in the form of CD8+ cytotoxic T lymphocytes and elicit humoral immune reactions dependent on MHC class II-restricted activation of Th helper cells [20]. Actually, the efficiency and type of immune response elicited after DNA immunization is mostly dependent on the administration route and method used as discussed by various authors [24–26]. After intramuscular or intradermal DNA immunization, the humoral response is characterized by the predominant production of specific IgG2a antibodies and cytokines such as IL-2 and IFN-γ, whereas dermal gene gun immunization yields a preponderance of IgG1 specific antibodies and IL-4, suggesting Th1 and Th2 biased responses [24]. Equally mouse inbred strain choice has also been shown to influence Th helper cell response [27] upon pathogen challenge. Incidentally, BALB/c mice commonly respond secreting Th2-associated cytokines after the first set of boosters were administered (Fig. 1A, C). This response was of a similar extent to the responses described in previous reports based on M2AChR or \(\beta_1\)AR-el2 peptide immunization in rabbit [16,17,19] or rat [23] models, although these earlier reports relied on continuous peptide immunization to maintain elevated antibody levels that would otherwise result in a single and discrete response peak.
pathogen challenge in opposition to other strains (e.g., C57BL/6) that display Th1 biased responses after pathogen challenge or DNA immunization [27]. Thus, thorough analysis of the immune responses in different mouse strains should be performed in order to establish the preferred T-helper cell bias in autoimmune dilated cardiomyopathy animal models derived from different DNA immunization routes.

DNA immunizations were used in attempts to create autoimmune animal models of cardiomyopathy. Levitus et al. [28] using i.m. direct needle injections as a route for the administration of P2β T. cruzi ribosomal protein encoding plasmid DNA observed a predominance of IgG2a subclass production. Although a Th1 biased response was obtained, as observed in previous works using the same immunization route [24–26] and for other autoimmune diseases [29], the observed in previous works using the same immunization route did not show any cardiac alterations and the antibodies raised did not present functional activity. In contrast, recent data [30] demonstrated that in a well-established murine model of autoimmune myocarditis based on cardiac myosin immunizations, IL-4 secretion and a Th2 biased immune response are important for heart lesion establishment in a period of at least 30 weeks. Accordingly we were able to document heart functional impairment in animals that predominantly produced IgG1 subclass antibodies (thus probably a Th2 biased response), in a similar interval, as discussed below.

The echocardiographic measurements performed on the left ventricle point to a significant decrease in wall thickness in diastole and systole that is present in both experimental groups (Fig. 2A–D). The extent of this effect was greater in the β1AR animal group in which a small increase in LV cavity size was also observed (Table 1). Immunization of rabbits with synthetic peptides corresponding to the sequence of β1AR and M2AChR-el2 was previously used in attempts to recreate the alterations observed in IDC and CCD patients [17–19]. Two of these studies were based on gross anatomy analysis to determine ventricular wall and cavity measurements [18,19]. Fu et al. [18] immunized rabbits for a period of 6 months with the M2AChR-el2 peptide and failed to report any alterations in ventricular size. A later report [19] reinstated the same model adding a second group of animals now immunized with the β1AR-el2. In this work, moderately increased ventricular cavity sizes were described for the β1AR-el2 immunized group accompanied by septal and free ventricular wall thinning. Curiously, only a slight right ventricular cavity enlargement was documented for the M2AChR-el2 immunized group in the referred work [19]. This data found support in work by Jahns et al. [23] that after continuously immunizing inbred rats with the β1AR-el2 for a period of 15 months observed significant LV cavity dilatation. Opposite effects on LV morphology and dimensions were observed in a later report that characterized rabbits immunized for a 6-month period with β1AR-el2 [17]. In this report, echocardiographic measurements of LV cavity size, LV wall thickness and whole heart gross anatomy implied that immunization with the β1AR-el2 peptide exerted a hypertrophic effect on rabbit hearts.

In the present work, estimates of the LV relative mass (Fig. 2E) revealed a decrease in this parameter for the β1AR group owing to a loss in LV wall thickness. The changes observed in LV mass suggest that LV remodeling in the β1AR group might correspond to a direct dilatation-remodeling effect that was particularly evident for this group. The M2AChR group also revealed a direct dilatation trend as evidenced by the relative mass estimates, but to a lesser extent than the decreased mass observed in the β1AR-immunized group.

Early increments in LA dimensions were readily observed (Fig. 3) in both experimental groups, although again LA enlargement was more severe in the β1AR group. This variable was used to aid in the diagnosis of congestive heart failure in rats [31]. In regard to this, LA diameter was increased in large extent myocardial infarcted rats owing to congestive heart failure [32]. The pathogenic significance and the prognostic impact of LA enlargement was analyzed in the studies of left ventricular dysfunction (SOLVD) population [33]. Patients with a reduced LV ejection fraction had an increased risk proportional to the increase in the size of the LA, which was independent of age or symptomatic status. However, it is unclear whether the prognostic power of the enlarged LA might be the result of LV diastolic dysfunction or the presence of mitral regurgitation or atrial fibrillation. This is noteworthy because all of these pathophysiologic variables contribute to the enlargement of the atrial chambers [34]. Although the prognostic significance of this variable was established in humans, the determination of this variable was rarely exploited in previous works dealing with animal models of DCM and heart function other than experimental large extent myocardial infarcts [31,32].

Our echocardiographic analysis also revealed a hampered LV contractile function. This effect was evident in the FS values shown in Fig. 4C. In this figure, a decrease in this parameter is shown in both the M2AChR and the β1AR-immunized animals and, at least for the adrenergic group, our results are in line with those presented by Jahns et al. [23]. Furthermore, when the systolic thickening of the anterior and posterior LV walls was analyzed, an important impairment in contractility was highlighted as a consequence of the immunizations used in this work (Fig. 4A, B and Fig. 5). Taken together the echocardiographic data presented in this work showed that both the M2AChR and the β1AR DNA immunizations significantly altered LV systolic function and probably diastolic function as well, in spite of a moderate effect on LV dimensions (Table 1).

Histological analysis revealed diffuse fibrosis deposits for the M2AChR immunized group and patch-like deposits for the β1AR-immunized group (Fig. 6). These results are in agreement with the results from the three reports cited above where rabbits were immunized with either M2AChR or β1AR-el2 [17–19]. In support of these findings, Redfern et al. [35] described a transgenic mouse that conditionally expressed receptors that constitutively activated the Gi protein signaling pathway. These authors described a fibrotic response that
is characteristic of dilated cardiomyopathy and was documented by DNA-array data, showing that fibronectin, laminin, and collagen were all up-regulated, in these animals [35]. Activation of the fibrotic pathway is probably common not only to Gi protein over-stimulation but also to β1-AR agonist continuous stimulation and increased internalization, since in the β1-AR-el2 peptide immunized models [17,19] and transgenic animals that superexpress the β1-AR [36] or the GRK2 [37], remodeling of the myocardium with fibrosis is shown to occur.

The possibility that the persistent presence of anti-M2AChR and β1-AR antibodies could lead to changes in the number of membrane receptors was also evaluated in our work. We showed that muscarinic receptor density in hearts of M2AChR and β1-AR-immunized mice was significantly up-regulated whereas the equilibrium dissociation constants (Kd) for the radioligand remained unchanged (Table 2). These results confirmed previous findings obtained with M2AChR-el2 immunized rabbits [18,19] that also showed muscarinic receptor up-regulation. An increase in muscarinic receptor number was also observed in mice chronically infected with T. cruzi attaining 72 fmol/mg of total protein as compared to uninfected mice with only 47 fmol/mg of total protein (L.E. Giménez, unpublished data). These results also agree with those obtained for a canine model of heart failure [38] and an in vivo PET study of IDC patients with congestive heart failure by Le Guludec et al. [39].

On the other hand, β-adrenergic receptor density was similarly decreased in both experimental groups by a twofold factor (Table 2). These results were in line with a large body of evidence found in patients with severe heart failure. In these patients, a decrease in membrane-bound cardiac β1-AR density accompanied by β-adrenergic receptor uncoupling as a consequence of increased β-adrenergic receptor kinase activity was demonstrated [40–42]. This was also observed in rats immunized with β1-AR-el2 as noted before [23].

A relevant response concerning this group of patients was an increase in Gi protein and transcript levels [43,44]. This fact may explain the desensitization of adenyl cyclase activity [45], a pathway used by M2AChR to inhibit atrium force via activation of KAC and the accentuated antagonism of β-adrenergic positive inotropy in the ventricles. Here we showed for the first time a prevalence of M2AChR expression in hearts of β1-AR immunized animals that were immunized with DNA plasmids coding for the M2AChR and β1-AR. These results suggest that a long-term cardiac imbalance favoring Gi overstimulation and a reduced ratio of cAMP synthesis in the context of agonist-like anti-GPCR antibodies is of significant importance in the pathogenesis of DCM and heart failure.

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