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ORIGINAL RESEARCH ARTICLE

Unmasking features of the auto-epitope essential for β_1-adrenoceptor activation by autoantibodies in chronic heart failure

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Abstract

Aims Chronic heart failure (CHF) can be caused by autoantibodies stimulating the heart via binding to first and/or second extracellular loops of cardiac β_1-adrenoceptors. Allosteric receptor activation depends on conformational features of the autoantibody binding site. Elucidating these features will pave the way for the development of specific diagnostics and therapeutics. Our aim was (i) to fine-map the conformational epitope within the second extracellular loop of the human β_1-adrenoceptor (β_1EC_{II}) that is targeted by stimulating β_1-receptor (auto)antibodies and (ii) to generate competitive cyclopeptide inhibitors of allosteric receptor activation, which faithfully conserve the conformational auto-epitope.

Methods and results Non-conserved amino acids within the β_1EC_{II} loop (compared with the amino acids constituting the EC_{II} loop of the β_2-adrenoceptor) were one by one replaced with alanine; potential intra-loop disulfide bridges were probed by cysteine–serine exchanges. Effects on antibody binding and allosteric receptor activation were assessed (i) by (auto)antibody neutralization using cyclopeptides mimicking β_1EC_{II} ± the above replacements, and (ii) by (auto)antibody stimulation of human β_1-adrenoceptors bearing corresponding point mutations. With the use of stimulating β_1-receptor (auto)antibodies raised in mice, rats, or rabbits and isolated from exemplary dilated cardiomyopathy patients, our series of experiments unmasked two features of the β_1EC_{II} loop essential for (auto)antibody binding and allosteric receptor activation: (i) the $NDPK^{211–214}$ motif and (ii) the intra-loop disulfide bond $C^{209}\leftrightarrow C^{215}$. Of note, aberrant intra-loop disulfide bond $C^{209}\leftrightarrow C^{216}$ almost fully disrupted the functional auto-epitope in cyclopeptides.

Conclusions The conformational auto-epitope targeted by cardio-pathogenic β_1-receptor autoantibodies is faithfully conserved in cyclopeptide homologues of the β_1EC_{II} loop bearing the $NDPK^{211–214}$ motif and the $C^{209}\leftrightarrow C^{215}$ bridge while lacking cysteine C^{216}. Such molecules provide promising tools for novel diagnostic and therapeutic approaches in β_1-autoantibody-positive CHF.

Keywords Antibody/autoantibody; β_1-adrenoceptor/β_1-adrenergic receptor; Chronic heart failure; Conformational auto-epitope; Cyclic peptides/cyclopeptides; Cyclopeptide therapy

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Introduction

Dilated cardiomyopathy (DCM)—defined as progressive cardiac dilatation and dysfunction without coronary heart disease—mostly affects younger adults. It frequently entails severe heart failure and transplantation. Prevalence and annual incidence amount to 0.5% and 0.1%, respectively.[1] According to the 2008 ESC cardiomyopathy classification—within the non-familial forms—autoimmune DCM has been recognized as a proper pathogenetic entity.[2] Autoimmunity arising from acute myocardial inflammation induced by microbial or viral agents is considered one key factor in the pathogenesis of autoimmune DCM. Progress to chronic immune-cardiomyopathy and chronic heart failure (CHF) is common in patients unable to clear the infecting agent,[3] showing persistent inflammation,[4] and/or having a familial pre-disposition to autoimmune reactions.[5] DCM autoimmunity encompasses autoantibodies directed against myocardial antigens[6–8] and cardiovascular G-protein-coupled receptors (for review, see Boivin-Jahns and Jahns[9]), most notably the β_1-AR.[10–13] The latter (termed anti-β_1-aabs) are suspected to play a pivotal pathogenic role because anti-β_1-aab-positive compared with aab-negative DCM patients have a more severely depressed cardiac function[13] and a threefold increased risk for cardiovascular death.[10] The incidence of anti-β_1-aabs can precede DCM symptoms by many years,[14] and selective removal of anti-β_1-aabs can decelerate disease progression and improve outcome in β_1-aab-positive DCM patients.[15,16] Most DCM-relevant anti-β_1-aabs trigger a similar conformational switch of the β_1-AR molecule as the true agonists[17] and elicit detrimental cardiac effects similar to chronic catecholamine challenge. The pathogenic relevance of stimulating anti-β_1-aabs in DCM is corroborated by DCM equivalents induced in rodents by β_1-AR immunization, isogenic antibody transfer, and corresponding rescue experiments.[11,18–20] At bottom line, chronic cAMP stimulation by anti-β_1-aabs appears to play a major role in DCM pathogenesis. Because blockade of sympathetic hormone receptors by beta-receptor antagonists has proven beneficial in CHF, a first logical step was to explore whether these drugs could also (pharmacologically) neutralize the cellular effects of stimulating anti-β_1-aabs.[21] Although β_1-selective (e.g. bisoprolol and metoprolol) and non-selective beta-blockers (e.g. alprenolol and carvedilol) were able to significantly reduce anti-β_1-aab-stimulated cAMP production, in the end, all tested substances—even at saturating concentrations—blocked anti-β_1-aab-mediated effects only partially [resulting in a 50% (alprenolol) up to 70% (carvedilol) reduction of the antibody-induced fluorescence resonance energy transfer (FRET) signals]. Consequently, clinical disease management would greatly profit from diagnostic determination and direct targeted intervention against stimulating anti-β_1-aabs, but the prerequisite knowledge of the structural features of the auto-epitope crucial for cAMP stimulation is yet too limited for that.

DCM-relevant anti-β_1-aabs target the first (β_1-EC_I) and/or second extracellular loops (β_1-EC_{II}) of the β_1-AR.[22,23] IgG binding to immobilized linear peptides representing these domains has been used to determine the prevalence of anti-β_1-aabs in various diseases. However, these assays exhibit low sensitivity/specificity and, in general, fail to discriminate patients from healthy subjects in a non-random fashion, because the disease-relevant anti-β_1-aabs target specific conformations of β_1-EC_I and/or β_1-EC_{II} that are not properly represented by immobile linear peptides. To date, DCM-relevant anti-β_1-aabs can only be detected by native assays or functional readouts.[19,24,25] However, the many patients potentially to be screened for anti-β_1-aabs (i) require clinical diagnostics based on conventional procedures [e.g. enzyme-linked immunosorbent assay (ELISA)] for measuring IgG binding to synthetic representations of the very auto-epitope conformation specifically targeted in the course of allosteric β_1-AR activation.[17,24] Interestingly, circular peptide representations of β_1-EC_{II} (alone or as an add-on to cardioselective β_1-blockers) could stop progression and even revert CHF in rats subjected to β_1EC_{II} immunization(s), whereas cardioselective β_1-blockers alone were only able to stabilize the dilated cardiomyopathic phenotype.[19,20] Therefore, cyclopeptides might provide an adequate synthetic mimic of the DCM-relevant conformational auto-epitope. Following up on this idea, we unmask here the structural features within β_1-EC_{II} that are essential and specific for allosteric receptor activation by anti-β_1-aabs and required in cyclopeptides for a neutralization of that effect.

Methods

Anti-β_1EC_{II} antibodies

Anti-β_1EC_{II} antibodies were raised in male Lewis rats[11] or rabbits (Dianova, Hamburg, Germany) or mice (mouse Mab 23-6-7, BioGenes, Berlin, Germany[26]) by immunization with β_1-EC_{II}/glutathione *S*-transferase (GST) fusion proteins. In addition, a monoclonal rat antibody was generated by fusing spleen cells from anti-β_1EC_{II}-positive immunized rats with a multiple myeloma Sp2/0-Ag14 cell-line (rat Mab 13F6[26]). Hybridoma cells expressing mouse Mab 23-6-7 and rat Mab 13F6 were submitted to DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig). Depositing was carried out according to the rules of the Budapest Treaty (accession numbers DSM ACC3121 and ACC3174).

Human anti-β_1-aabs were gained from representative CHF patients.[21] Because of the relatively large amounts of serum necessary to isolate human IgG for the serial functional assays, as a *proof of concept* in the present work,

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IgG was isolated from three DCM patients only {two men [56a, New York Heart Association (NYHA) IV, left ventricular ejection fraction (LVEF) 29%, and 53a, NYHA III, LVEF 31%] and one woman (29a, NYHA IV, LVEF 26%)}. In all three patients, LVEF was determined by ventriculography, and coronary heart disease was excluded by coronary angiography. At the time of invasive diagnostics, all three patients were stable under standard CHF medication including angiotensin-converting enzyme inhibitor/AT1 receptor blockers, beta-blockers, and aldosterone antagonists. In none of the patients, exposure to cardio-toxic substances, myocarditis, or other systemic heart diseases was evident from clinical history. IgG was freshly isolated from the respective (human or animal) sera and dialyzed against the appropriate assay buffers. Immuno-reactivity of rodent IgG against a linear 25 amino acid (AA) β_1EC_{II} peptide (residues 199–123) was determined by ELISA.[12,13]

Cyclopeptides

Cyclopeptides corresponding to AA residues 200–220 of the human β_1-AR and constituting the entire β_1-EC_{II}[27] were cyclized between the C-terminal glutamate and the free N-terminal amino group (Peptide Speciality Laboratories, Heidelberg, Germany). Non-conserved residues differing between the β_1-AR and β_2-AR were sequentially substituted by alanine. Cysteine^{216} (C^{216}) was replaced with α-aminobutyric acid (B) to prevent aberrant disulfide bridging to C^{209}. Corresponding 22-mer cyclopeptides of the second extracellular loop of the human β_2-adrenergic receptor (β_2-EC_{II}) served as negative controls. In 18-mer cyclopeptides representing a minimal EC_{II} structure encompassing residues 204–219 of the human β_1-AR, C^{215} or C^{216} was replaced with serine to selectively disrupt potential intra-loop disulfide bridges (for further details, see *Figure* 2 and *Table* S1). Cyclopeptides were freeze-dried, reconstituted in water, and stored at −20°C. For the functional assays, anti-β_1-abs/β_1-aabs were pre-incubated with 40 mol of cyclopeptides/mol IgG, assuming a 1:1 stoichiometry between the cyclic 22AA-EC_{II} peptide variants (~3.0 kDa) and one IgG molecule (~150 kDa). In fact, the molar excess was only 20-fold, assuming two variable (Fab) chains with two antigen-binding regions per IgG molecule. Antigen-binding regions are further subdivided into hypervariable (HV) and framework (FR) regions; HV regions comprise about five to eight AAs directly contacting a portion of the antigen's surface.[28] IgG was freshly isolated from the respective (human or animal) sera by caprylic acid precipitation, dialyzed against the appropriate assay buffers, and allowed to interact with the Cyclopeptides (CPs) for 16 h at 4°C (cold room) in an Eppendorf cup on a gently rotating incubator. After short spin, the supernatant was used for the assays.

cAMP measurements by a fluorescence resonance energy transfer sensor

cAMP measurements by a FRET sensor using HEK-β_1E_1 cells stably co-expressing the human β_1-AR and a FRET sensor for intracellular cAMP followed published procedures.[21] Cells were grown on poly-D-lysin-coated 96-well plates (Ibidi, Martinsried, Germany). Endogenous β_2-AR was blocked with ICI-118551 (0.1 μM). Cells were imaged with a fluorescence microscope optimized for the cyan fluorescent protein (CFP)/yellow fluorescent protein (YFP) donor/acceptor pair of the cAMP sensor (iMIC 2000, TILLPhotonics, Martinsried, Germany); CFP/YFP emissions were continuously recorded for 32 min. FRET efficiency reflecting intracellular cAMP levels was calculated every 6 s using TillvisION v4.5.59 (TILLPhotonics) and OriginPro 8G (OriginLab, Northampton, USA). Antibody effects normalized to agonist maximum were derived from successive determinations of baseline (10 min) and effect of the antibody tested (20 min) and of (−)-isoproterenol (1 μM, 2 min) performed on sets of ≈500 cells.

β_1-AR mutants

cDNA constructs of the human β_1-AR bearing point mutations E202A, R207A, D212N, P213A, or V219A were generated by site-directed mutagenesis (*Table* S2), confirmed by sequencing, transiently expressed in HEK293 cells, and characterized by Western blotting and radioligand displacement.[21] To ensure reproducibility (and sufficient supply) of the test agent 'anti-β_1EC_{II} antibody' for our experiments with human β_1-AR mutants, we used monoclonal anti-β_1EC_{II}-abs raised in mice (Mab 23-6-7[26]) or rats (Mab 13F6[26]), thereby reducing the variability due to potentially different HV regions of polyclonal rabbit, rat, or human IgGs used in the experiments before.[28] HEK293 cells expressing human β_1-AR with the indicated point mutations were exposed (60 min, 37°C) to anti-β_1EC_{II}-Mabs (0.2 μg/μl) or (−)-isoproterenol (1 μM) in the presence of 3-isobutyl-1-methylxanthine (100 μM) and ICI-118551 (37.5 pM). Cellular cAMP was measured by RIA (Coulter-Immunotech, Krefeld, Germany).

Statistics

After testing for normal distribution (comparable variances on the means of each group), data were analysed by one-way analysis of variance (ANOVA) with subsequent Dunnett's post-hoc test for multiple comparisons (comparing all means against the control/reference mean) using GraphPad PRISM 8 (version 8.3.1, GraphPad Software Inc., USA).

Ethics

Animal experiments were approved by the animal ethics committee of the Government of Lower Franconia (Vote No. 55.2-2531.01-52/08, Experimental Animal Use and Care Committee, Government of Lower Franconia, Bavaria, Germany). The study of patient's biomaterials complies with the *Declaration of Helsinki* and was approved by the Medical Ethics Committee of the Medical Faculty of the University of Würzburg (Vote No. 186/07). Informed consent of the donors was obtained.

Results

Role of intra-loop disulfide bridges in auto-epitope conformation

The β_1-EC_{II} contains three cysteines significantly determining loop conformation: C^{209} and C^{215} form an intra-loop disulfide bridge, while C^{216} forms a bridge to β_1-EC_I.[29] In cyclopeptides, a possible aberrant bridge $C^{209}\leftrightarrow C^{216}$ can alter the physiological conformation. To address whether this plays a role for binding and allosteric receptor activation by anti-β_1EC_{II}-abs, we synthesized two minimal (18-mer) cyclopeptides representing AA residues 204–219 of the β_1-EC_{II}, which had either C^{215} (termed 18 C/C/S) or C^{216} (termed 18 C/S/C) replaced by serine, thereby allowing either the physiological bridge $C^{209}\leftrightarrow C^{215}$ or the aberrant bridge $C^{209}\leftrightarrow C^{216}$ (*Table* S1). The reactivity of these cyclopeptides with stimulating anti-β_1EC_{II}-abs was assessed using sera of 99 rats having developed high titres of anti-β_1EC_{II}-abs and a cardiac DCM phenotype after repeated immunizations with β_1EC_{II}/GST fusion proteins.[19,20] First, we investigated pre-adsorption of these rat anti-β_1EC_{II}-abs to either 18-mer cyclopeptide (at 40-fold molar excess) prior to ELISA with linear 25-mer β_1EC_{II} peptides. Binding of the large majority of sera (83/99 = 84%) was neutralized by 18 C/C/S (bearing the physiological bond $C^{209}\leftrightarrow C^{215}$), but not by 18 C/S/C (bearing the aberrant bond $C^{209}\leftrightarrow C^{216}$). Only four sera were neutralized by 18 C/S/C, and 12 rat sera could not be fully blocked with either cyclopeptide (*Table* 1).

Because β_1-AR-mediated cAMP stimulation is the readout delineating the clinically relevant cardio-pathogenic potential of anti-β_1EC_{II}-abs,[10] we next analysed neutralization of the cAMP stimulatory effect of rabbit anti-β_1EC_{II}-abs using HEK-β_1E_1 cells co-expressing human β_1-AR with a cAMP FRET reporter.[21] In these cells, intracellular cAMP levels can be continuously monitored via the CFP/YFP emission ratio of the FRET sensor, and cAMP stimulation by anti-β_1EC_{II}-abs can be normalized to the maximal stimulation obtained with 1 μM (−)-isoproterenol, measured in the same batch of cells. Rabbit anti-β_1EC_{II}-abs increased cAMP levels by about 18.8 ± 1.2% of the maximum achieved with the full agonist (−)-isoproterenol. Pre-absorption of rabbit anti-β_1EC_{II}-abs to 18 C/C/S (the 18-mer cyclopeptide with the physiological bond $C^{209}\leftrightarrow C^{215}$; 40-fold molar excess) reduced the stimulatory effect of the antibodies by more than 50% (9.4 ± 2.2%; $P < 0.01$). By contrast, pre-absorption of rabbit anti-β_1EC_{II}-abs to 18 C/S/C (the cyclopeptide with the aberrant bond $C^{209}\leftrightarrow C^{216}$; 40-fold molar excess) failed to block the antibody-induced increases in intracellular cAMP (18.1 ± 2.0%, n.s.; *Figure* 1). In conclusion, inhibition of anti-β_1EC_{II}-induced β_1-AR activation by β_1EC_{II}-mimicking cyclopeptides requires the natural intra-loop disulfide bridge $C^{209}\leftrightarrow C^{215}$ and is disrupted by the non-physiological $C^{209}\leftrightarrow C^{216}$ bond.

β_1EC_{II} loop residues essential for proper conformation of the auto-epitope

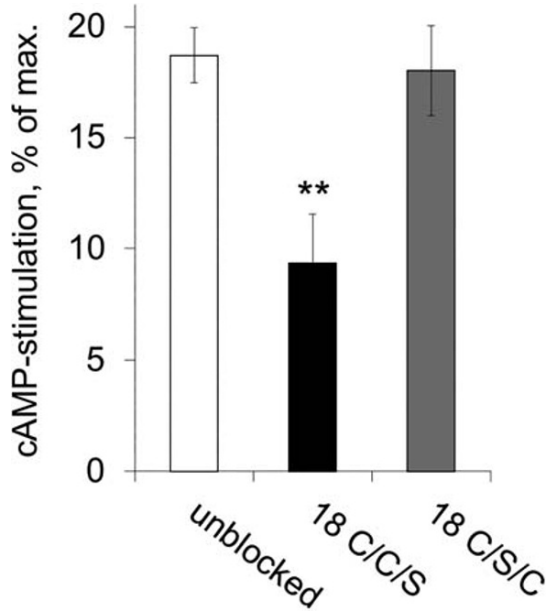
Even the 18-mer cyclopeptide with the appropriate disulfide bridge yielded only incomplete inhibition, indicating that an optimal representation of the auto-epitope conformation might require longer constructs with a less bulky C^{216} substitution. Accordingly, a 22-mer cyclopeptide was generated, representing the entire predicted β_1EC_{II} loop and having cysteine C^{216} replaced by α-aminobutyric acid (B), an artificial cysteine homologue not engaging in disulfide bridges. This optimized construct (22 C/C/B-β_1, depicted in *Figure* 2) inhibited anti-β_1EC_{II} antibody-induced stimulation by ≥80% (*Figure* 3, first black column on the left) and served as a template to map further residues within β_1EC_{II} essential for the proper conformation of the auto-epitope. Nine variants of 22 C/C/B-β_1 were synthesized, each having a different non-conserved AA (compared with the AA constituting the EC_{II} loop of the β_2-AR) sequentially replaced by alanine (E202A, S203A, D204A, R207A, R208A, N211A, D212A, P213A, and K214A). Conserved AA residues essential for

Table 1 Effect of intra-loop disulfide bonds on the binding of anti-β_1-EC_{II}-abs[a]

Neutralizing cyclopeptides (40 mol/mol IgG)	Effective pre-adsorption of anti-β_1EC_{II} rat sera (*n*) (≥90% reduction in ELISA reactivity with β_1-EC_{II} peptides)
18-mer C/C/S (C209↔C215)	83 (84%)
18-mer C/S/C (C209↔C216)	4 (4%)
Not fully blocked	12 (12%)

[a]Determined by enzyme-linked immunosorbent assay (ELISA) of IgG binding to a linear 25 AA β_1-EC_{II} peptide.[12,13]

Figure 1 Neutralization of the stimulatory effects of rabbit anti-β_1EC_{II}-abs by pre-incubation with cyclopeptides containing intra-loop bonds $C^{209}\leftrightarrow C^{215}$ (18 C/C/S) or $C^{209}\leftrightarrow C^{216}$ (18 C/S/C): Stimulatory effects of polyclonal rabbit anti-β_1EC_{II}-abs on the human β_1-AR coupled to a CFP/YFP-FRET sensor for intracellular cAMP, normalized to maximal stimulatory effects obtained by 1 μM (−)-isoproterenol. Rabbit anti-β_1EC_{II}-abs were pre-absorbed with the indicated cyclopeptides (40 mol CP/mol IgG). Unblocked: effect of stimulating rabbit anti-β_1EC_{II}-abs alone. Data are given as mean ± SEM ($n \geq 5$ per experiment; differences between the conditions were analysed by one-way ANOVA with subsequent Dunnett's post-hoc test for multiple comparisons; $^{**}P < 0.01$).



adrenoceptor function *per se* were omitted from the scan (for details, see *Table* S1).

The reactivity of the 22-mer C/C/B-β_1 variants with clinically relevant stimulating anti-β_1EC_{II}-abs was determined by (i) pre-absorption of autoantibody binding to the linear 25 AA β_1EC_{II} peptide (determined by ELISA) and by (ii) pre-absorption of the receptor-stimulating capacities of anti-β_1EC_{II}-abs (determined with cAMP FRET reporter cells). Sera of β_1EC_{II}/GST-immunized cardiomyopathic rats served as a source for DCM-relevant autoantibodies. Mean results obtained with sera from 20 individual rats are summarized in *Figure* 3, demonstrating that receptor binding (grey columns) and receptor stimulation (black columns) were efficiently pre-absorbed (>80%) by all cyclopeptide mutants tested, except for those having disrupted an NDPK motif encompassing AA residues 211–214. The most disruptive mutation was P213A, which almost completely abolished the scavenger effect of the respective cyclopeptide (blocking capacity 22 C/C/B-β_1 *vs*. P213A: 87.9 ± 3 *vs*. 7.2 ± 8%, $P < 0.0001$). The same was observed with a stimulating monoclonal mouse anti-β_1EC_{II} antibody (*Figure* S1).

To confirm the clinical relevance of these results, comparable neutralization experiments were performed with stimulating anti-β_1-aabs isolated from selected DCM patients. *Figure* 4A shows a representative recording of cAMP reporter signals obtained with patient-derived anti-β_1-aabs, which induced an increase in β_1-AR-mediated cAMP production of ≈30% of the maximal signal obtained with saturating concentrations (1 μM) of the full agonist (−)-isoproterenol. That stimulatory effect was almost fully abolished by pre-incubation with the (non-mutated) cyclopeptide 22 C/C/B-β_1, whereas the mutant P213A (earlier found ineffective after pre-absorption of rodent anti-β_1EC_{II}-abs; *Figure* 3 and *Figure* S1) also failed to block the stimulatory effect of human anti-β_1-aabs (*Figure* 4A). The blocking profile of the entire set of mutated cyclopeptides on cAMP stimulation by human anti-β_1-aabs isolated from selected antibody-positive DCM patients (male/female) is shown in *Figure* 4B.

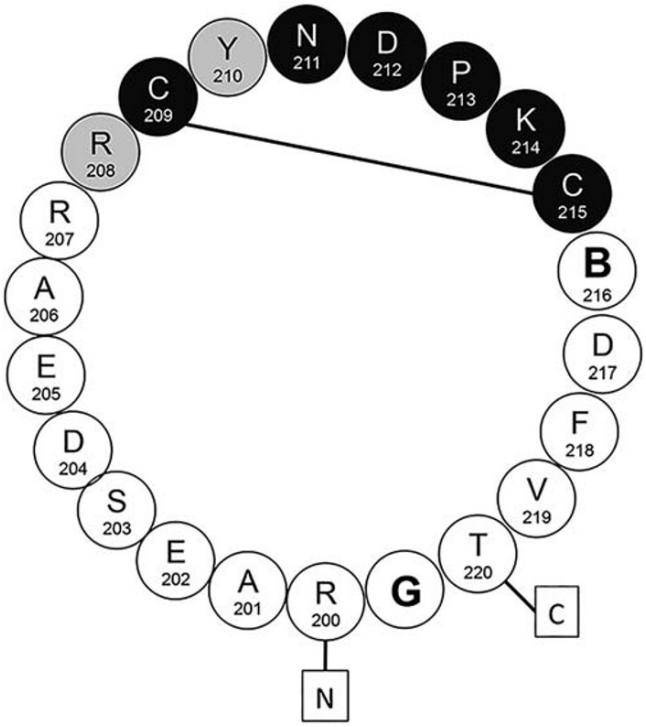
In summary, our data suggest that the $NDPK^{211–214}$ motif within β_1-EC_{II} is of paramount relevance for β_1-AR-induced cAMP stimulation by potentially cardio-pathogenic anti-β_1-aabs. The stimulatory effect of human DCM-associated autoantibodies appeared even more sensitive to disruptions of the $NDPK^{211–214}$ motif than the effect of anti-β_1EC_{II}-abs raised in rodents. Moreover, cAMP stimulation by human anti-β_1-aabs was also affected by the adjacent mutation R208A (*Figure* 4B vs. *Figure* 3, black columns), suggesting that the auto-epitope targeted by human anti-β_1-aabs is slightly larger, encompassing the motif R (CY)$NDPK^{208–214}$. This finding is not unexpected because our rodents through repeated immunizations have undergone a maturation process in their adapted immune system, entailing increases in antibody avidity and epitope narrowing,[30] which human DCM patients most probably have not undergone (explaining the generally lower levels and lower avidity of human anti-β_1-aabs[25]).

The black labels in *Figure* 2 summarize the results obtained in our experiments aiming at a further refinement of cyclopeptides intended as synthetic targets or therapeutic scavengers of stimulating anti-β_1-aabs. Minimal requirements for such agents encompass a core domain of eight essential AA ranging from positions 208 to 215 of the human β_1-AR sequence, which harbours the essential intra-loop bridge $C^{209}\leftrightarrow C^{215}$. Aberrant disulfide bridging to C^{216} must be precluded. The core domain should be embedded in a protein circle encompassing the entire β_1EC_{II} loop to be fully efficient.

Directed mutational analysis of the presumed auto-epitope in intact human β_1-ARs

Our above results regarding the auto-epitope of stimulatory anti-β_1EC_{II}-abs led to the assumption that the generated neutralizing (scavenger) cyclopeptides represent competitive inhibitors faithfully mimicking the three-dimensional structure of the auto-epitope as presented on native β_1-ARs. To

Figure 2 Schematic representation of the 22-mer C/C/B-β_1 cyclopeptide mimicking the human β_1EC_{II} bearing the essential motif of the clinically relevant auto-epitope: circles with capitals specify amino acids (single letter code). Numbers specify their position in the sequence of the human β_1-AR. 'B' stands for the artificial amino acid α-aminobutyrate substituting C^{216} in order to prevent a non-physiological S-S-bond $C^{209} \leftrightarrow C^{216}$. Boxed letters N and C indicate the termini of the predicted β_1EC_{II} loop. The full line between C^{209} and C^{215} indicates the essential intra-loop disulfide bridge; black circles depict amino acid residues essential for the neutralization of stimulating rodent anti-β_1EC_{II}-abs. Grey circles represent additional amino acid residues supposed to be relevant for the neutralization of stimulating human anti-β_1EC_{II}-aabs. Amino acid residues R^{207}, E^{205}, S^{203}, and T^{220} were not found essential by directed mutational analysis.



confirm this assumption, we then investigated whether the very same AA residues essential for antibody neutralization by cyclopeptides would also be relevant for the interaction of stimulating anti-β_1EC_{II}-abs with full-length human β_1-ARs. For this purpose, we generated β_1-AR constructs bearing point mutations of AA residues either presumed to be essential components of the core auto-epitope (D212N and P213A) or flanking the core auto-epitope while not essentially contributing to its formation (R207A and V219A). The mutant β_1-ARs were expressed in HEK293 cells. Expression and functionality of the β_1-AR mutants were ascertained (i) by ligand binding and (ii) by cAMP responses compared with the wild-type β_1-AR expressed under same conditions (*Table* S3).

To ensure reproducibility of the test agent anti-β_1EC_{II} antibody, we then measured the cAMP responses induced via the mutant β_1-ARs by two different monoclonal anti-β_1EC_{II}-abs (Mab 23-6-7 or Mab 13F6, raised in mice or rats, respectively[26]). Whereas the cardio-noxious potential of anti-β_1EC_{II}-Mabs generated in mice (as Mab 23-6-7) has been clearly shown independently by many research groups,[30–32] the clinical relevance of rat anti-β_1EC_{II}-Mabs remained to be demonstrated. Thus, according to our previously described strategy[11]—after purification from the cell supernatant—we (intravenously) transferred Mab 13F6 into healthy rats every 4 weeks. Compared with animals receiving a rat IgG isotype control, rats receiving Mab 13F6 within (6 to) 9 months of Mab transfer developed a dilated cardiomyopathic phenotype (*Figure* S2), demonstrating its disease-inducing potential.

To rule out effects of the mutations on β_1-AR function *per se*, increases in cAMP were again normalized to the maximal stimulation achieved for each β_1-AR mutant by the full agonist (−)-isoproterenol (1 μM). As summarized in *Figure* 5, β_1-ARs bearing mutations within the presumed auto-epitope (D212N and P213A) were significantly ($P < 0.0001$) less sensitive to stimulation by rat or mouse anti-β_1EC_{II}-Mabs than the wild-type β_1-AR. In contrast, cAMP stimulation by rat or mouse anti-β_1EC_{II}-Mabs was not significantly ($P \geq 0.01$) affected by mutations in the flanking regions (R207A and V219A), previously found to be not involved in the scavenger

Figure 3 Neutralization of the functional effects of polyclonal rat anti-β_1EC_{II}-IgG by 22-mer cyclopeptides mimicking the human β_1-EC_{II} with non-conserved amino acids (compared with the amino acids constituting the EC_{II} loop of the β_2-AR) sequentially replaced by alanine: sera of 20 cardiomyopathic Lewis rats (immunized with β_1EC_{II}/GST fusion proteins) were pre-absorbed with the indicated cyclopeptide mutants (40 mol CP/mol IgG, 4°C, 16 h). Grey bars: IgG binding to the linear 25 AA (199–223) β_1EC_{II} peptide as determined by ELISA (triplicates). Black bars: β_1-AR-mediated cAMP stimulation in HEK293 cells expressing native β_1-AR functionally coupled to a cAMP FRET sensor. Decreases in ELISA reactivity (grey bars) or receptor activation/cAMP stimulation (black bars) following pre-absorption with the different cyclopeptide mutants are shown, normalized to the values obtained without blocking cyclopeptides. Columns represent mean values ± SEM of n = 20 rat sera. Differences between the non-mutated 22 C/C/B-β_1 cyclopeptide and CP mutations were analysed by one-way ANOVA with subsequent Dunnett's post-hoc test for multiple comparisons; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{\dagger}P < 0.0001$. Internal negative control: non-mutated 22C/C/D-β_2 (sequence and alignment, see *Table* S1).

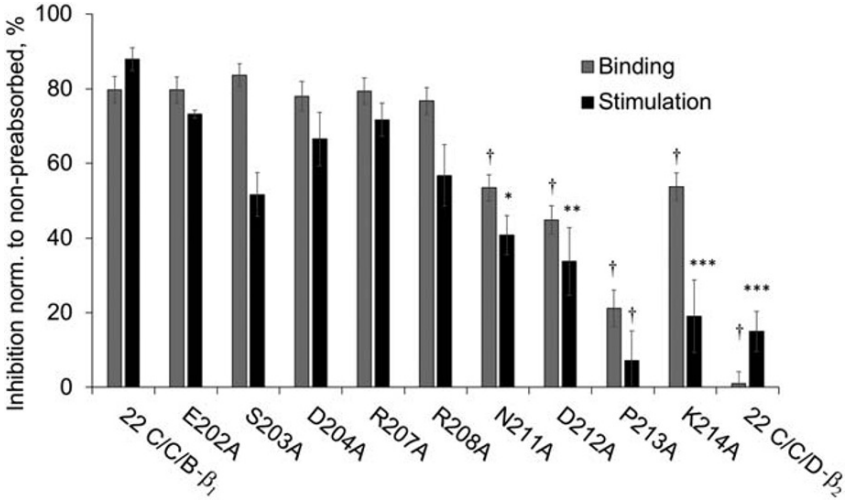
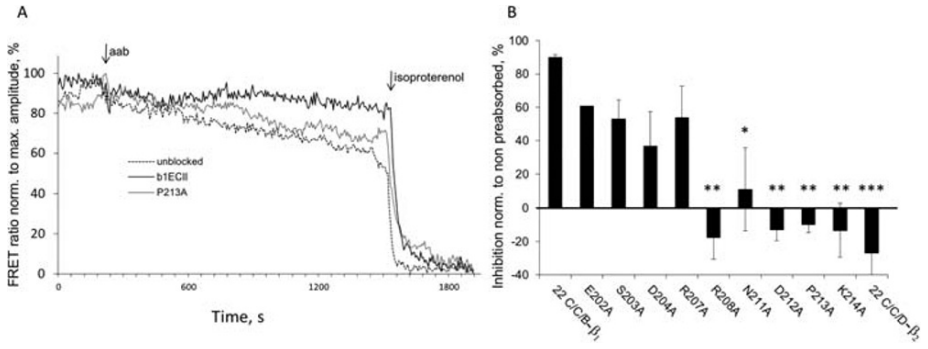
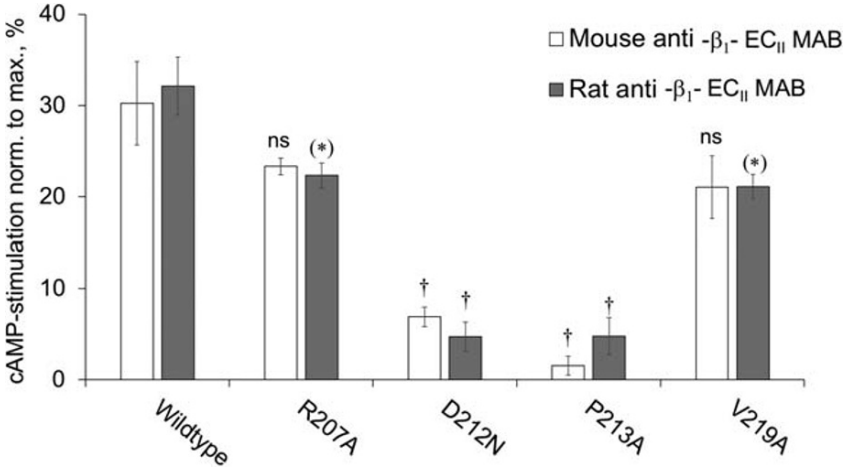


Figure 4 Neutralization of human anti-β_1-aabs from DCM patients by 22-mer cyclopeptides mimicking β_1EC_{II} with non-conserved amino acids (compared with the amino acids constituting the EC_{II} loop of the β_2-AR) sequentially replaced by alanine: human anti-β_1-aabs (IgG fractions) were pre-absorbed with the indicated cyclopeptide mutants (40 mol/mol IgG, 4°C, 16 h). Anti-β_1-aab-induced cAMP production was measured in HEK293 cells expressing the native β_1-AR functionally coupled to a cAMP FRET sensor. (A) Representative recordings of FRET ratios obtained upon addition of anti-β_1-aabs prepared from a male DCM patient followed by the maximal signal achieved with 1.0 μM of (−)-isoproterenol (Iso). (B) Prevention of cAMP stimulation after pre-absorption of patient anti-β_1-aabs. Results are normalized to the values without pre-absorption. Columns represent mean ± SEM from at least three to four independent experiments with IgG prepared from different exemplary DCM patients (two men and one woman). Differences between the non-mutated 22 C/C/B-β_1 cyclopeptide and CP mutations were analysed by one-way ANOVA with subsequent Dunnett's post-hoc test for multiple comparisons; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Internal negative control: non-mutated 22C/C/D-β_2 (sequence and alignment, see *Table* S1).



effect of cyclopeptides (*Figures* 2 and 3B). In conclusion, the same AA residues essential for antibody neutralization by cyclopeptides (*Figures* 3 and 4) appear also essential for the anti-β_1EC_{II}-induced activation of native human β_1-AR (*Figure* 5). Consequently, cyclopeptides that faithfully mimic the auto-epitope conformation relevant for allosteric β_1-AR activation should also efficiently block the effects of stimulating anti-β_1EC_{II}-abs.

Figure 5 Stimulation of mutant β_1-ARs bearing point mutations within or flanking the presumed auto-epitope of functional anti-β_1EC_{II} antibodies: to ensure reproducibility of the test agent anti-β_1EC_{II} antibody, HEK293 cells expressing human β_1-AR with the indicated point mutations were exposed to monoclonal anti-β_1EC_{II}-abs raised in mice (mouse Mab 23-6-7,[26] white columns) or rats (rat Mab 13F6,[26] grey columns). cAMP levels were determined in the cell lysates by radio-immunoassay. For each experiment, increases in cAMP following antibody exposure were normalized to the maximal responses achieved with 1.0 μM of the full agonist (−)-isoproterenol. Data are given as mean ± SEM of at least six independent experiments per column. Differences between the wild-type β_1-AR and the indicated mutants were analysed by one-way ANOVA with subsequent Dunnett's post-hoc test for multiple comparisons; $^{**}P < 0.01$; $^{\dagger}P < 0.0001$.



Discussion

We here provide a set of direct and indirect evidence delineating the minimal structure and conformation of the β_1-AR epitope targeted by anti-β_1-aabs supposed to play an important role in various cardiovascular disorders.[9] Allosteric β_1-AR activation by these autoantibodies is considered a severe risk factor if not a cause of CHF.[10,11] It is long known that such stimulating aabs mostly target the β_1-EC_{II}.[23] However, attempts at further narrowing the target epitope have yielded ambiguous results: autoantibodies involved in Chagas cardiomyopathy are thought to target residues 201–205[33]; in post-partum cardiomyopathy, the autoantibody target has been placed at residues 200–210; and for DCM, a whole series of overlapping sequences have been published (residues 183–208, 197–202, 206–212, and 213–218[22,23,33]), encompassing almost the entire β_1-EC_{II} and even portions of the adjacent transmembrane domain VI. Almost all these data have been obtained with linear peptides as probes. However, the surface of a protein is essentially a continuum of potential epitopes, but the fact that functional epitopes are often found to be discontinuous shows that mapping by linear peptide scanning may not be generally useful for comprehensive mapping or analysis of functional (energetic) epitopes.[34] By now, it seems quite clear that disease-relevant anti-β_1-aabs are only those that stimulate the β_1-AR[21] and that such aabs bind to a conformational epitope, thereby stabilizing an active conformation and prolonging the active state of the β_1-AR.[17] Consequently, stimulating anti-β_1-aabs poorly cross-react with linear peptides,[12,13,24] which probably explains why epitope mapping with linear peptides has not yielded a clearer picture of the relevant β_1-AR auto-epitope(s). However, such a clearer picture is desperately needed for the development of specific diagnostics and therapeutics addressing this disease mechanism.

One crucial and potentially clinically relevant finding of our study is the identification of a minimal peptide structure that efficiently hinders receptor stimulation by DCM-associated human anti-β_1-aabs in an experimental approach using cells expressing functionally coupled human β_1-ARs. We have previously demonstrated that cyclopeptides mimicking that structure can stop progression of CHF and even partially revert the CHF phenotype in a human-analogous rat model of autoimmune DCM.[19,20] This therapeutic effect is conveyed not only by scavenging (and thus neutralizing) cardio-pathogenic anti-β_1EC_{II}-abs but possibly also by induction of B-cell tolerance.[35] In support of this hypothesis, we observed in the above therapy model not only a reduction of autoantibody titres due to *in vivo* scavenging of circulating anti-β_1EC_{II}-abs but also a reduction of anti-β_1EC_{II}-secreting B cells.[19] While long-lived plasma cells express very little or no immunoglobulins on the cell surface,[36] B cells do and could thus also serve as targets of β_1EC_{II}-CPs. To detect the few antigen-specific memory B cells within splenocytes of CP-treated vs. untreated immunized rats, in our previous study, we differentiated memory B cells into short-lived plasma blasts by boosting

the rats with β_1EC_{II}/GST-FPs 3 days prior to the analysis of the spleens. Whereas long-lived plasma cells were not targeted by β_1EC_{II}-CPs, preventive as well as therapeutic application of the same CPs resulted in a ~ 80% reduction of the frequencies of splenocytes secreting anti-β_1EC_{II}-abs.[19] This finding indicates that in immunized anti-β_1EC_{II}-positive rats, repeated injections of β_1EC_{II}-CPs may lead to impaired B-cell receptor (BCR)-mediated β_1EC_{II}-specific memory B-cell expansion (and differentiation into anti-β_1EC_{II}-producing plasma cells). Thus, cyclopeptides based on the criteria outlined in the present study may complement therapeutic strategies that exclusively aim at (non-specific) autoantibody scavenging, such as extracorporeal IgG absorption[37] or the systemic application of aptamers.[38]

Furthermore, the very same peptide core conveying to cyclopeptides the potency to prevent allosteric β_1-AR activation by anti-β_1EC_{II}-aabs is obviously also a crucial prerequisite of the allosteric stimulation mechanism itself, which implies that the cyclopeptide actually mimics the 3D structure of the auto-epitope that provides the allosteric trigger. The proposed antibody-binding region as outlined in *Figure* 2 comprises the essential motif $NDPK^{211–214}$ framed by the equally essential disulfide bridge between cysteines $C^{209}\leftrightarrow C^{215}$. In the intact receptor, this structure is constrained by another adjacent disulfide bridge linking C^{216} to the transmembrane region next to EC_I. Both pairs of disulfide bridges are relevant for receptor function.[39,40] According to the 3D structure of the turkey β_1-AR,[41] the assumed auto-epitope is located at the C-terminal end of the backward-oriented α-helix constituting β_1-EC_{II}. Several features pre-dispose the $NDPK^{211–214}$ motif as an autoantigen and allosteric trigger: N^{211}, D^{212}, and K^{214} are prone to bond with the binding surface of the antibody, while P^{213} is needed to interfere with the H-bond formation to neighbouring helix residues, thereby bending the local α-helix[42] and creating a unique structure for antibody recognition. The allosteric activation trigger is most probably provided by D^{212}, the exchange of which to asparagine is known to cause paradoxical receptor activation by antagonistic ligands.[43]

Limitations

The fact that human anti-β_1-aabs are polyclonal and that the concentration of specific anti-β_1-aabs in the circulation of a patient is probably much lower than in specifically immunized anti-β_1EC_{II}-positive rodents represents a major challenge for a therapeutic use of the here described cyclopeptides in the future. Immunoglobulin G after maturation by its (antigen-binding) HV regions assumingly recognize stretches of five (to eight) AAs as a target epitope.[28,34] The EC_{II} loop of the β_1-AR is thought to be composed of 30 AAs (AA 195–225 of the receptor protein), and most functionally active human anti-β_1-aabs were shown to be directed against this segment of the β_1-AR membrane protein, representing a readily accessible target on the cell surface. Thus, it seems conceivable that *polyclonal* human anti-β_1-aabs may target different epitopes (stretches of five to eight AAs) even within the 30 AAs that constitute β_1EC_{II}, which might result in diverging functional effects and/or downstream effects.[12] Moreover, not only the *specific target epitope* (at the surface of a same target protein) but also the *target protein* itself (which might be sarcolemmal proteins, such as myosin or troponin, or myocyte membrane proteins such as β_1-AR, β_2-AR, β_3-AR, M2-muscarinergic, and/or angiotensin II AT_1 receptors), or *cross-reactions* (*or molecular mimicry*[44]) between the target protein and other functionally relevant proteins may contribute to the diversity of functional effects and/or downstream effects of human aabs. In case of cross-activation of β_1-AR by human anti-myosin aabs,[45] β_1-derived cyclopeptides would probably fail to hinder anti-myosin-aab-induced stimulation of cardiac β_1-AR. Assessment of the effect of human aabs on downstream signalling might be even further complicated by the *co-existence* of aabs against different target proteins, which could then enhance (additive effect) or inhibit (opposing effect) downstream signalling and would require a panel of different epitope-mimicking CPs to be therapeutically used on a case-to-case basis (e.g. human anti-β_1-aabs were shown to increase cAMP production, whereas human anti-M2-aabs inhibit cAMP production,[46] or myocardial damage induced by anti-β_1-aabs in heart failure was found to be alleviated by human anti-β_2-aabs[47]).

Next steps and clinical perspective

As a consequence (and because of the pilot character of our study, testing only a few exemplary DCM patients), it will be necessary to fine-map the target epitopes of anti-β_1EC_{II}-aabs of a larger number of CHF patients (with different aetiologies, e.g. DCM vs. ICM vs. hypertensive heart disease) to confirm our findings. Because human anti-β_1-aabs are polyclonal, their target epitope(s) might encompass not only the here unmasked structural motif in β_1-EC_{II}, but also structural motifs in other extracellular domains of the human β_1-AR, for example, in β_1-EC_I,[10,22] which were not addressed in the present study. Moreover, in future (prospective) CHF studies, the patients should be systematically screened also for the presence of autoantibodies against other cardiac membrane proteins. The functional effects and the specific target epitopes of each of these aabs should then be analysed more in detail (e.g. for functional tests by adapting the here described FRET approach and for epitope fine-mapping by adapting the here described Ala-scan approach). In the end, this could lead to the identification of functionally relevant (targetable) key epitopes also in other G-protein-coupled membrane receptors. Fine-mapping of functionally relevant key epitopes would

allow to tailor epitope-specific cyclopeptides that (i) act as autoantibody scavengers in the circulation and—in the light of the data obtained by β_1EC_{II}-CP treatment of anti-β_1EC_{II} antibody-positive rats[19]—might equally (ii) impair BCR-mediated autoantibody-specific memory B-cell expansion. Because the general risk of non-specific immune (e.g. T-)cell activation increases with the size of a given (cyclo-)peptide from >25 AAs on,[48] the exact knowledge of a key epitope required to neutralize (or scavenge) functional human aabs would allow to design much shorter (*per se* less immunogenic) cyclopeptides, as we started in the present study by testing 22-mer instead of 25-mer β_1EC_{II}-CPs (used in our previous therapy study in anti-β_1EC_{II} antibody-positive rats[19]). Even shorter CPs could be imagined, comprising 18, 16, or even less AAs.

With the availability of a wide panel of autoantibody-specific (preferably short) scavenger CPs, a future therapeutic strategy could—in the end—comprise a kind of personalized approach in autoantibody-associated CHF, that is, the injection of an individualized mixture of tailored cyclopeptides, depending on the autoantibody profile and the downstream 'net effect' of cardio-noxious vs. cardio-protective autoantibodies in an individual CHF patient.

Conflict of interest

The University of Würzburg has filed for patent protection of the HEKβ_1E_1 cells (DE 102009 019578.5.2009) and for the methods and substances described in the present manuscript (EP 05 00 7056.4, WO 2006/103101 A2, EP 07 01 6637.6, WO 2009/027063 A2, WO 2010/61/2006.091, EP 11 01 2535.6). R.J. and M.J.L. are stockholders of the biotech company AdvanceCor GmbH (formerly CorImmun GmbH), Martinsried, Germany. The remaining authors have no conflicts of interest to declare.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 - Amino-acid sequences and denomination of cyclopeptides.
Table S2 - Primer for site directed mutagenesis by nested PCR
Table S3 - Characteristics of β_1AR-mutants expressed in HEK293-cells
Figure S1 - Neutralization of a monoclonal mouse anti-β_1EC_{II}-antibody by 22mer-cyclopeptides corresponding to the human β_1-EC_{II}, each having a different non-conserved amino-acid (compared to the amino-acids constituting the EC_{II}-loop of the β_2-AR) sequentially replaced with alanine.
Figure S2 - Time course of left ventricular diameters from rats immunized against β_1EC_{II} or receiving a monoclonal rat anti-β_1EC_{II} and corresponding control animals, determined by echocardiography.

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