

# Unmasking features of the auto-epitope essential for $\beta_1$ -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  $\beta_1$ -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  $\beta_1$ -adrenoceptor ( $\beta_1$ EC<sub>II</sub>) that is targeted by stimulating  $\beta_1$ -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  $\beta_1$ EC<sub>II</sub> loop (compared with the amino acids constituting the EC<sub>II</sub> loop of the  $\beta_2$ -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  $\beta_1$ EC<sub>II</sub>  $\pm$  the above replacements, and (ii) by (auto)antibody stimulation of human  $\beta_1$ -adrenoceptors bearing corresponding point mutations. With the use of stimulating  $\beta_1$ -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  $\beta_1$ EC<sub>II</sub> loop essential for (auto)antibody binding and allosteric receptor activation: (i) the NDPK<sup>211–214</sup> motif and (ii) the intra-loop disulfide bond C<sup>209</sup>  $\leftrightarrow$  C<sup>215</sup>. Of note, aberrant intra-loop disulfide bond C<sup>209</sup>  $\leftrightarrow$  C<sup>216</sup> almost fully disrupted the functional auto-epitope in cyclopeptides.

**Conclusions** The conformational auto-epitope targeted by cardio-pathogenic  $\beta_1$ -receptor autoantibodies is faithfully conserved in cyclopeptide homologues of the  $\beta_1$ EC<sub>II</sub> loop bearing the NDPK<sup>211–214</sup> motif and the C<sup>209</sup>  $\leftrightarrow$  C<sup>215</sup> bridge while lacking cysteine C<sup>216</sup>. Such molecules provide promising tools for novel diagnostic and therapeutic approaches in  $\beta_1$ -autoantibody-positive CHF.

**Keywords** Antibody/autoantibody;  $\beta_1$ -adrenoceptor/ $\beta_1$ -adrenergic receptor; Chronic heart failure; Conformational auto-epitope; Cyclopeptides/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.<sup>1</sup> According to the 2008 ESC cardiomyopathy classification—within the non-familial forms—autoimmune DCM has been recognized as a proper pathogenetic entity.<sup>2</sup> 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,<sup>3</sup> showing persistent inflammation,<sup>4</sup> and/or having a familial pre-disposition to autoimmune reactions.<sup>5</sup> DCM autoimmunity encompasses autoantibodies directed against myocardial antigens<sup>6–8</sup> and cardiovascular G-protein-coupled receptors (for review, see Boivin-Jahns and Jahns<sup>9</sup>), most notably the  $\beta_1$ -AR.<sup>10–13</sup> The latter (termed anti- $\beta_1$ -aabs) are suspected to play a pivotal pathogenic role because anti- $\beta_1$ -aab-positive compared with aab-negative DCM patients have a more severely depressed cardiac function<sup>13</sup> and a threefold increased risk for cardiovascular death.<sup>10</sup> The incidence of anti- $\beta_1$ -aabs can precede DCM symptoms by many years,<sup>14</sup> and selective removal of anti- $\beta_1$ -aabs can decelerate disease progression and improve outcome in  $\beta_1$ -aab-positive DCM patients.<sup>15,16</sup> Most DCM-relevant anti- $\beta_1$ -aabs trigger a similar conformational switch of the  $\beta_1$ -AR molecule as the true agonists<sup>17</sup> and elicit detrimental cardiac effects similar to chronic catecholamine challenge. The pathogenic relevance of stimulating anti- $\beta_1$ -aabs in DCM is corroborated by DCM equivalents induced in rodents by  $\beta_1$ -AR immunization, isogenic antibody transfer, and corresponding rescue experiments.<sup>11,18–20</sup> At bottom line, chronic cAMP stimulation by anti- $\beta_1$ -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- $\beta_1$ -aabs.<sup>21</sup> Although  $\beta_1$ -selective (e.g. bisoprolol and metoprolol) and non-selective beta-blockers (e.g. alprenolol and carvedilol) were able to significantly reduce anti- $\beta_1$ -aab-stimulated cAMP production, in the end, all tested substances—even at saturating concentrations—blocked anti- $\beta_1$ -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- $\beta_1$ -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- $\beta_1$ -aabs target the first ( $\beta_1$ -EC<sub>I</sub>) and/or second extracellular loops ( $\beta_1$ -EC<sub>II</sub>) of the  $\beta_1$ -AR.<sup>22,23</sup> IgG binding to immobilized linear peptides representing these domains has been used to determine the prevalence of anti- $\beta_1$ -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- $\beta_1$ -aabs target specific conformations of  $\beta_1$ -EC<sub>I</sub> and/or  $\beta_1$ -EC<sub>II</sub> that are not properly represented by immobile linear peptides. To date, DCM-relevant anti- $\beta_1$ -aabs can only be detected by native assays or functional readouts.<sup>19,24,25</sup> However, the many patients potentially to be screened for anti- $\beta_1$ -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  $\beta_1$ -AR activation.<sup>17,24</sup> Interestingly, circular peptide representations of  $\beta_1$ -EC<sub>II</sub> (alone or as an add-on to cardioselective  $\beta_1$ -blockers) could stop progression and even revert CHF in rats subjected to  $\beta_1$ -EC<sub>II</sub> immunization(s), whereas cardioselective  $\beta_1$ -blockers alone were only able to stabilize the dilated cardiomyopathic phenotype.<sup>19,20</sup> 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  $\beta_1$ -EC<sub>II</sub> that are essential and specific for allosteric receptor activation by anti- $\beta_1$ -aabs and required in cyclopeptides for a neutralization of that effect.

## Methods

### Anti- $\beta_1$ EC<sub>II</sub> antibodies

Anti- $\beta_1$ EC<sub>II</sub> antibodies were raised in male Lewis rats<sup>11</sup> or rabbits (Dianova, Hamburg, Germany) or mice (mouse Mab 23-6-7, BioGenes, Berlin, Germany<sup>26</sup>) by immunization with  $\beta_1$ -EC<sub>II</sub>/glutathione *S*-transferase (GST) fusion proteins. In addition, a monoclonal rat antibody was generated by fusing spleen cells from anti- $\beta_1$ EC<sub>II</sub>-positive immunized rats with a multiple myeloma Sp2/0-Ag14 cell-line (rat Mab 13F6<sup>26</sup>). 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- $\beta_1$ -aabs were gained from representative CHF patients.<sup>21</sup> 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,

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)  $\beta_1$ EC<sub>II</sub> peptide (residues 199–123) was determined by ELISA.<sup>12,13</sup>

## Cyclopeptides

Cyclopeptides corresponding to AA residues 200–220 of the human  $\beta_1$ -AR and constituting the entire  $\beta_1$ -EC<sub>II</sub><sup>27</sup> 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  $\beta_1$ -AR and  $\beta_2$ -AR were sequentially substituted by alanine. Cysteine<sup>216</sup> (C<sup>216</sup>) was replaced with  $\alpha$ -aminobutyric acid (B) to prevent aberrant disulfide bridging to C<sup>209</sup>. Corresponding 22-mer cyclopeptides of the second extracellular loop of the human  $\beta_2$ -adrenergic receptor ( $\beta_2$ -EC<sub>II</sub>) served as negative controls. In 18-mer cyclopeptides representing a minimal EC<sub>II</sub> structure encompassing residues 204–219 of the human  $\beta_1$ -AR, C<sup>215</sup> or C<sup>216</sup> 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  $-20^\circ\text{C}$ . For the functional assays, anti- $\beta_1$ -abs/ $\beta_1$ -aabs were pre-incubated with 40 mol of cyclopeptides/mol IgG, assuming a 1:1 stoichiometry between the cyclic 22AA-EC<sub>II</sub> 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.<sup>28</sup> 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  $4^\circ\text{C}$  (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- $\beta_1$ E<sub>1</sub> cells stably co-expressing the human  $\beta_1$ -AR and a FRET sensor for intracellular cAMP followed published procedures.<sup>21</sup> Cells were grown on poly-D-lysine-coated 96-well plates (Ibidi, Martinsried, Germany). Endogenous  $\beta_2$ -AR was blocked with ICI-118551 (0.1  $\mu\text{M}$ ). 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  $\mu\text{M}$ , 2 min) performed on sets of  $\approx 500$  cells.

## $\beta_1$ -AR mutants

cDNA constructs of the human  $\beta_1$ -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.<sup>21</sup> To ensure reproducibility (and sufficient supply) of the test agent 'anti- $\beta_1$ EC<sub>II</sub> antibody' for our experiments with human  $\beta_1$ -AR mutants, we used monoclonal anti- $\beta_1$ EC<sub>II</sub>-abs raised in mice (Mab 23-6-7<sup>26</sup>) or rats (Mab 13F6<sup>26</sup>), thereby reducing the variability due to potentially different HV regions of polyclonal rabbit, rat, or human IgGs used in the experiments before.<sup>28</sup> HEK293 cells expressing human  $\beta_1$ -AR with the indicated point mutations were exposed (60 min,  $37^\circ\text{C}$ ) to anti- $\beta_1$ -EC<sub>II</sub>-Mabs (0.2  $\mu\text{g}/\mu\text{l}$ ) or (–)-isoproterenol (1  $\mu\text{M}$ ) in the presence of 3-isobutyl-1-methylxanthine (100  $\mu\text{M}$ ) 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  $\beta_1$ -EC<sub>II</sub> contains three cysteines significantly determining loop conformation: C<sup>209</sup> and C<sup>215</sup> form an intra-loop disulfide bridge, while C<sup>216</sup> forms a bridge to  $\beta_1$ -EC<sub>I</sub>.<sup>29</sup> In cyclopeptides, a possible aberrant bridge C<sup>209</sup>↔C<sup>216</sup> can alter the physiological conformation. To address whether this plays a role for binding and allosteric receptor activation by anti- $\beta_1$ EC<sub>II</sub>-abs, we synthesized two minimal (18-mer) cyclopeptides representing AA residues 204–219 of the  $\beta_1$ -EC<sub>II</sub>, which had either C<sup>215</sup> (termed 18 C/C/S) or C<sup>216</sup> (termed 18 C/S/C) replaced by serine, thereby allowing either the physiological bridge C<sup>209</sup>↔C<sup>215</sup> or the aberrant bridge C<sup>209</sup>↔C<sup>216</sup> (Table S1). The reactivity of these cyclopeptides with stimulating anti- $\beta_1$ EC<sub>II</sub>-abs was assessed using sera of 99 rats having developed high titres of anti- $\beta_1$ EC<sub>II</sub>-abs and a cardiac DCM phenotype after repeated immunizations with  $\beta_1$ EC<sub>II</sub>/GST fusion proteins.<sup>19,20</sup> First, we investigated pre-adsorption of these rat anti- $\beta_1$ EC<sub>II</sub>-abs to either 18-mer cyclopeptide (at 40-fold molar excess) prior to ELISA with linear 25-mer  $\beta_1$ EC<sub>II</sub> peptides. Binding of the large majority of sera (83/99 = 84%) was neutralized by 18 C/C/S (bearing the physiological bond C<sup>209</sup>↔C<sup>215</sup>), but not by 18 C/S/C (bearing the aberrant bond C<sup>209</sup>↔C<sup>216</sup>). 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  $\beta_1$ -AR-mediated cAMP stimulation is the readout delineating the clinically relevant cardio-pathogenic potential of anti- $\beta_1$ EC<sub>II</sub>-abs,<sup>10</sup> we next analysed neutralization of the

cAMP stimulatory effect of rabbit anti- $\beta_1$ EC<sub>II</sub>-abs using HEK- $\beta_1$ E<sub>1</sub> cells co-expressing human  $\beta_1$ -AR with a cAMP FRET reporter.<sup>21</sup> 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- $\beta_1$ EC<sub>II</sub>-abs can be normalized to the maximal stimulation obtained with 1  $\mu$ M (–)-isoproterenol, measured in the same batch of cells. Rabbit anti- $\beta_1$ EC<sub>II</sub>-abs increased cAMP levels by about 18.8 ± 1.2% of the maximum achieved with the full agonist (–)-isoproterenol. Pre-absorption of rabbit anti- $\beta_1$ EC<sub>II</sub>-abs to 18 C/C/S (the 18-mer cyclopeptide with the physiological bond C<sup>209</sup>↔C<sup>215</sup>; 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- $\beta_1$ EC<sub>II</sub>-abs to 18 C/S/C (the cyclopeptide with the aberrant bond C<sup>209</sup>↔C<sup>216</sup>; 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- $\beta_1$ EC<sub>II</sub>-induced  $\beta_1$ -AR activation by  $\beta_1$ EC<sub>II</sub>-mimicking cyclopeptides requires the natural intra-loop disulfide bridge C<sup>209</sup>↔C<sup>215</sup> and is disrupted by the non-physiological C<sup>209</sup>↔C<sup>216</sup> bond.

### $\beta_1$ EC<sub>II</sub> loop residues essential for proper conformation of the auto-epitope

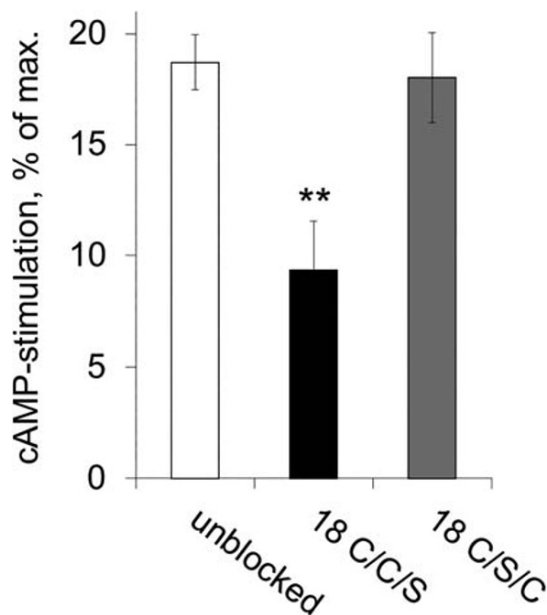
Even the 18-mer cyclopeptide with the appropriate disulfide bridge yielded only incomplete inhibition, indicating that an optimal representation of the auto-epitope conformation might require longer constructs with a less bulky C<sup>216</sup> substitution. Accordingly, a 22-mer cyclopeptide was generated, representing the entire predicted  $\beta_1$ EC<sub>II</sub> loop and having cysteine C<sup>216</sup> replaced by  $\alpha$ -aminobutyric acid (B), an artificial cysteine homologue not engaging in disulfide bridges. This optimized construct (22 C/C/B- $\beta_1$ , depicted in Figure 2) inhibited anti- $\beta_1$ EC<sub>II</sub> antibody-induced stimulation by ≥80% (Figure 3, first black column on the left) and served as a template to map further residues within  $\beta_1$ EC<sub>II</sub> essential for the proper conformation of the auto-epitope. Nine variants of 22 C/C/B- $\beta_1$  were synthesized, each having a different non-conserved AA (compared with the AA constituting the EC<sub>II</sub> loop of the  $\beta_2$ -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- $\beta_1$ -EC<sub>II</sub>-abs<sup>a</sup>

Neutralizing cyclopeptides (40 mol/mol IgG)	Effective pre-adsorption of anti- $\beta_1$ EC <sub>II</sub> rat sera ( <i>n</i> ) (≥90% reduction in ELISA reactivity with $\beta_1$ -EC <sub>II</sub> peptides)
18-mer C/C/S (C209↔C215)	83 (84%)
18-mer C/S/C (C209↔C216)	4 (4%)
Not fully blocked	12 (12%)

<sup>a</sup>Determined by enzyme-linked immunosorbent assay (ELISA) of IgG binding to a linear 25 AA  $\beta_1$ -EC<sub>II</sub> peptide.<sup>12,13</sup>

**Figure 1** Neutralization of the stimulatory effects of rabbit anti- $\beta_1$ EC<sub>II</sub>-abs by pre-incubation with cyclopeptides containing intra-loop bonds C<sup>209</sup>↔C<sup>215</sup> (18 C/C/S) or C<sup>209</sup>↔C<sup>216</sup> (18 C/S/C): Stimulatory effects of polyclonal rabbit anti- $\beta_1$ EC<sub>II</sub>-abs on the human  $\beta_1$ -AR coupled to a CFP/YFP-FRET sensor for intracellular cAMP, normalized to maximal stimulatory effects obtained by 1  $\mu$ M (–)-isoproterenol. Rabbit anti- $\beta_1$ EC<sub>II</sub>-abs were pre-absorbed with the indicated cyclopeptides (40 mol CP/mol IgG). Unblocked: effect of stimulating rabbit anti- $\beta_1$ EC<sub>II</sub>-abs alone. Data are given as mean  $\pm$  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- $\beta_1$  variants with clinically relevant stimulating anti- $\beta_1$ EC<sub>II</sub>-abs was determined by (i) pre-absorption of autoantibody binding to the linear 25 AA  $\beta_1$ EC<sub>II</sub> peptide (determined by ELISA) and by (ii) pre-absorption of the receptor-stimulating capacities of anti- $\beta_1$ EC<sub>II</sub>-abs (determined with cAMP FRET reporter cells). Sera of  $\beta_1$ EC<sub>II</sub>/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- $\beta_1$  vs. P213A: 87.9  $\pm$  3 vs. 7.2  $\pm$  8%,  $P < 0.0001$ ). The same was observed with a stimulating monoclonal mouse anti- $\beta_1$ EC<sub>II</sub> antibody (Figure S1).

To confirm the clinical relevance of these results, comparable neutralization experiments were performed with

stimulating anti- $\beta_1$ -aabs isolated from selected DCM patients. Figure 4A shows a representative recording of cAMP reporter signals obtained with patient-derived anti- $\beta_1$ -aabs, which induced an increase in  $\beta_1$ -AR-mediated cAMP production of  $\approx$ 30% of the maximal signal obtained with saturating concentrations (1  $\mu$ M) of the full agonist (–)-isoproterenol. That stimulatory effect was almost fully abolished by pre-incubation with the (non-mutated) cyclopeptide 22 C/C/B- $\beta_1$ , whereas the mutant P213A (earlier found ineffective after pre-absorption of rodent anti- $\beta_1$ EC<sub>II</sub>-abs; Figure 3 and Figure S1) also failed to block the stimulatory effect of human anti- $\beta_1$ -aabs (Figure 4A). The blocking profile of the entire set of mutated cyclopeptides on cAMP stimulation by human anti- $\beta_1$ -aabs isolated from selected antibody-positive DCM patients (male/female) is shown in Figure 4B.

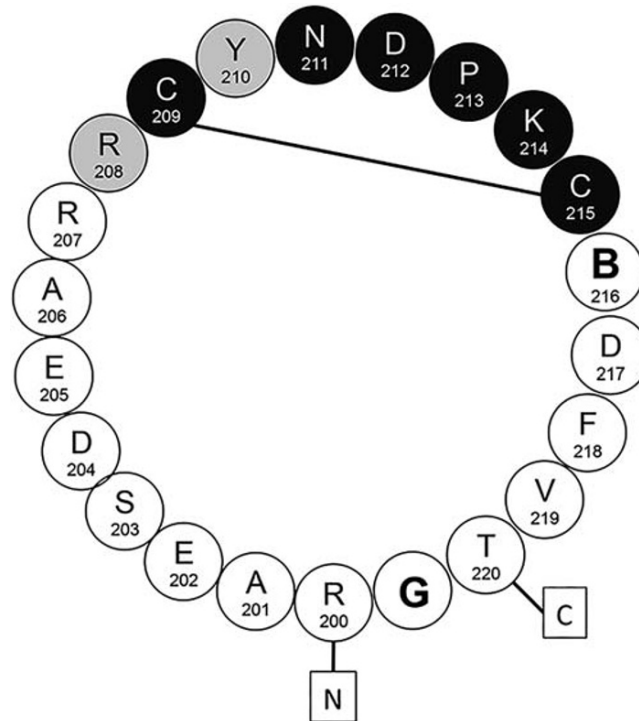
In summary, our data suggest that the NDPK<sup>211–214</sup> motif within  $\beta_1$ -EC<sub>II</sub> is of paramount relevance for  $\beta_1$ -AR-induced cAMP stimulation by potentially cardio-pathogenic anti- $\beta_1$ -aabs. The stimulatory effect of human DCM-associated autoantibodies appeared even more sensitive to disruptions of the NDPK<sup>211–214</sup> motif than the effect of anti- $\beta_1$ EC<sub>II</sub>-abs raised in rodents. Moreover, cAMP stimulation by human anti- $\beta_1$ -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- $\beta_1$ -aabs is slightly larger, encompassing the motif R (CY)NDPK<sup>208–214</sup>. 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,<sup>30</sup> which human DCM patients most probably have not undergone (explaining the generally lower levels and lower avidity of human anti- $\beta_1$ -aabs<sup>25</sup>).

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- $\beta_1$ -aabs. Minimal requirements for such agents encompass a core domain of eight essential AA ranging from positions 208 to 215 of the human  $\beta_1$ -AR sequence, which harbours the essential intra-loop bridge C<sup>209</sup>↔C<sup>215</sup>. Aberrant disulfide bridging to C<sup>216</sup> must be precluded. The core domain should be embedded in a protein circle encompassing the entire  $\beta_1$ EC<sub>II</sub> loop to be fully efficient.

## Directed mutational analysis of the presumed auto-epitope in intact human $\beta_1$ -ARs

Our above results regarding the auto-epitope of stimulatory anti- $\beta_1$ EC<sub>II</sub>-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  $\beta_1$ -ARs. To

**Figure 2** Schematic representation of the 22-mer C/C/B- $\beta_1$  cyclopeptide mimicking the human  $\beta_1$ EC<sub>II</sub> 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  $\beta_1$ -AR. 'B' stands for the artificial amino acid  $\alpha$ -aminobutyrate substituting C<sup>216</sup> in order to prevent a non-physiological S-S-bond C<sup>209</sup>↔C<sup>216</sup>. Boxed letters N and C indicate the termini of the predicted  $\beta_1$ EC<sub>II</sub> loop. The full line between C<sup>209</sup> and C<sup>215</sup> indicates the essential intra-loop disulfide bridge; black circles depict amino acid residues essential for the neutralization of stimulating rodent anti- $\beta_1$ EC<sub>II</sub>-abs. Grey circles represent additional amino acid residues supposed to be relevant for the neutralization of stimulating human anti- $\beta_1$ EC<sub>II</sub>-abs. Amino acid residues R<sup>207</sup>, E<sup>205</sup>, S<sup>203</sup>, and T<sup>220</sup> were not found essential by directed mutational analysis.



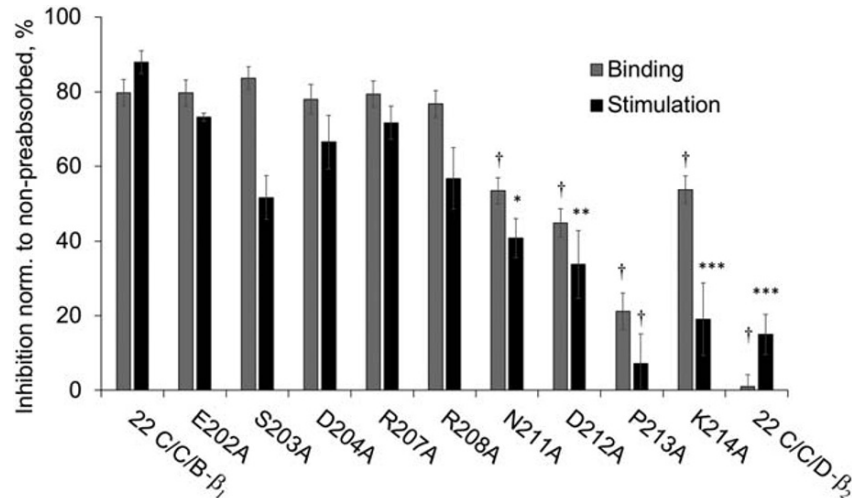
confirm this assumption, we then investigated whether the very same AA residues essential for antibody neutralization by cyclopeptides would also be relevant for the interaction of stimulating anti- $\beta_1$ EC<sub>II</sub>-abs with full-length human  $\beta_1$ -ARs. For this purpose, we generated  $\beta_1$ -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  $\beta_1$ -ARs were expressed in HEK293 cells. Expression and functionality of the  $\beta_1$ -AR mutants were ascertained (i) by ligand binding and (ii) by cAMP responses compared with the wild-type  $\beta_1$ -AR expressed under same conditions (Table S3).

To ensure reproducibility of the test agent anti- $\beta_1$ EC<sub>II</sub> antibody, we then measured the cAMP responses induced via the mutant  $\beta_1$ -ARs by two different monoclonal anti- $\beta_1$ EC<sub>II</sub>-abs (Mab 23-6-7 or Mab 13F6, raised in mice or rats, respectively<sup>26</sup>). Whereas the cardio-noxious potential of anti- $\beta_1$ EC<sub>II</sub>-Mabs generated in mice (as Mab 23-6-7) has been clearly shown independently by many research groups,<sup>30–32</sup>

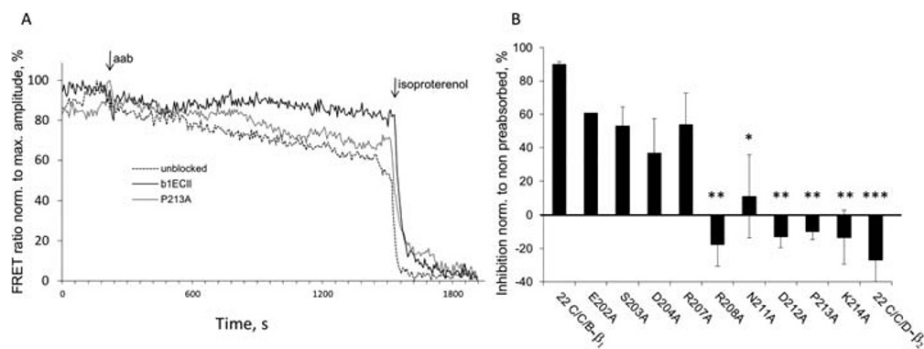
the clinical relevance of rat anti- $\beta_1$ EC<sub>II</sub>-Mabs remained to be demonstrated. Thus, according to our previously described strategy<sup>11</sup>—after purification from the cell supernatant—we (intravenously) transferred Mab 13F6 into healthy rats every 4 weeks. Compared with animals 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  $\beta_1$ -AR function *per se*, increases in cAMP were again normalized to the maximal stimulation achieved for each  $\beta_1$ -AR mutant by the full agonist (–)-isoproterenol (1  $\mu$ M). As summarized in Figure 5,  $\beta_1$ -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- $\beta_1$ EC<sub>II</sub>-Mabs than the wild-type  $\beta_1$ -AR. In contrast, cAMP stimulation by rat or mouse anti- $\beta_1$ EC<sub>II</sub>-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- $\beta_1$ EC<sub>II</sub>-IgG by 22-mer cyclopeptides mimicking the human  $\beta_1$ EC<sub>II</sub> with non-conserved amino acids (compared with the amino acids constituting the EC<sub>II</sub> loop of the  $\beta_2$ -AR) sequentially replaced by alanine: sera of 20 cardiomyopathic Lewis rats (immunized with  $\beta_1$ EC<sub>II</sub>/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)  $\beta_1$ EC<sub>II</sub> peptide as determined by ELISA (triplicates). Black bars:  $\beta_1$ -AR-mediated cAMP stimulation in HEK293 cells expressing native  $\beta_1$ -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  $\pm$  SEM of  $n = 20$  rat sera. Differences between the non-mutated 22 C/C/B- $\beta_1$  cyclopeptide and CP mutations were analysed by one-way ANOVA with subsequent Dunnett's post-hoc test for multiple comparisons; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; † $P < 0.0001$ . Internal negative control: non-mutated 22C/C/D- $\beta_2$  (sequence and alignment, see Table S1).



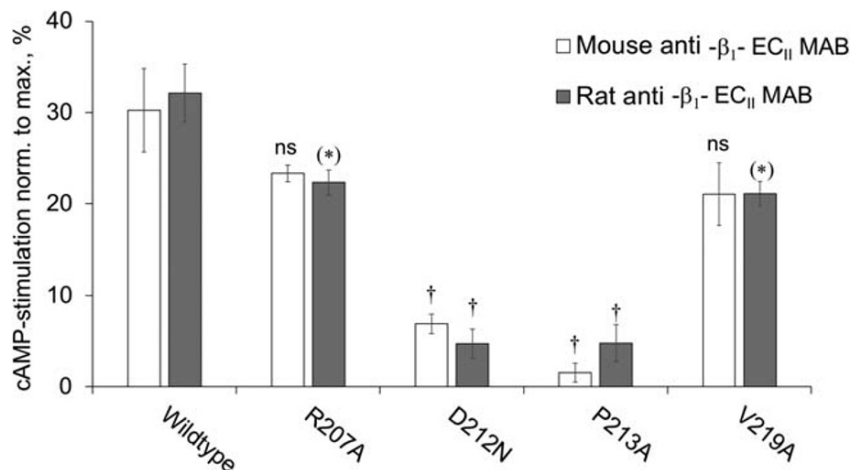
**Figure 4** Neutralization of human anti- $\beta_1$ -aabs from DCM patients by 22-mer cyclopeptides mimicking  $\beta_1$ EC<sub>II</sub> with non-conserved amino acids (compared with the amino acids constituting the EC<sub>II</sub> loop of the  $\beta_2$ -AR) sequentially replaced by alanine: human anti- $\beta_1$ -aabs (IgG fractions) were pre-absorbed with the indicated cyclopeptide mutants (40 mol/mol IgG, 4°C, 16 h). Anti- $\beta_1$ -aab-induced cAMP production was measured in HEK293 cells expressing the native  $\beta_1$ -AR functionally coupled to a cAMP FRET sensor. (A) Representative recordings of FRET ratios obtained upon addition of anti- $\beta_1$ -aabs prepared from a male DCM patient followed by the maximal signal achieved with 1.0  $\mu$ M of (-)-isoproterenol (Iso). (B) Prevention of cAMP stimulation after pre-absorption of patient anti- $\beta_1$ -aabs. Results are normalized to the values without pre-absorption. Columns represent mean  $\pm$  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- $\beta_1$  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- $\beta_2$  (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- $\beta_1$ EC<sub>II</sub>-induced activation of native human  $\beta_1$ -AR (Figure

5). Consequently, cyclopeptides that faithfully mimic the auto-epitope conformation relevant for allosteric  $\beta_1$ -AR activation should also efficiently block the effects of stimulating anti- $\beta_1$ EC<sub>II</sub>-abs.

**Figure 5** Stimulation of mutant  $\beta_1$ -ARs bearing point mutations within or flanking the presumed auto-epitope of functional anti- $\beta_1$ EC<sub>II</sub> antibodies: to ensure reproducibility of the test agent anti- $\beta_1$ EC<sub>II</sub> antibody, HEK293 cells expressing human  $\beta_1$ -AR with the indicated point mutations were exposed to monoclonal anti- $\beta_1$ EC<sub>II</sub>-abs raised in mice (mouse Mab 23-6-7,<sup>26</sup> white columns) or rats (rat Mab 13F6,<sup>26</sup> 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  $\mu$ M of the full agonist (–)-isoproterenol. Data are given as mean  $\pm$  SEM of at least six independent experiments per column. Differences between the wild-type  $\beta_1$ -AR and the indicated mutants were analysed by one-way ANOVA with subsequent Dunnett's post-hoc test for multiple comparisons; \*\* $P < 0.01$ ; † $P < 0.0001$ .



## Discussion

We here provide a set of direct and indirect evidence delineating the minimal structure and conformation of the  $\beta_1$ -AR epitope targeted by anti- $\beta_1$ -aabs supposed to play an important role in various cardiovascular disorders.<sup>9</sup> Allosteric  $\beta_1$ -AR activation by these autoantibodies is considered a severe risk factor if not a cause of CHF.<sup>10,11</sup> It is long known that such stimulating aabs mostly target the  $\beta_1$ -EC<sub>II</sub>.<sup>23</sup> However, attempts at further narrowing the target epitope have yielded ambiguous results: autoantibodies involved in Chagas cardiomyopathy are thought to target residues 201–205<sup>33</sup>; 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<sup>22,23,33</sup>), encompassing almost the entire  $\beta_1$ -EC<sub>II</sub> 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.<sup>34</sup> By now, it seems quite clear that disease-relevant anti- $\beta_1$ -aabs are only those that stimulate the  $\beta_1$ -AR<sup>21</sup> and that such aabs bind to a conformational epitope, thereby stabilizing an active conformation and prolonging the active state of the  $\beta_1$ -AR.<sup>17</sup> Consequently,

stimulating anti- $\beta_1$ -aabs poorly cross-react with linear peptides,<sup>12,13,24</sup> which probably explains why epitope mapping with linear peptides has not yielded a clearer picture of the relevant  $\beta_1$ -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- $\beta_1$ -aabs in an experimental approach using cells expressing functionally coupled human  $\beta_1$ -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.<sup>19,20</sup> This therapeutic effect is conveyed not only by scavenging (and thus neutralizing) cardio-pathogenic anti- $\beta_1$ EC<sub>II</sub>-abs but possibly also by induction of B-cell tolerance.<sup>35</sup> 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- $\beta_1$ EC<sub>II</sub>-abs but also a reduction of anti- $\beta_1$ EC<sub>II</sub>-secreting B cells.<sup>19</sup> While long-lived plasma cells express very little or no immunoglobulins on the cell surface,<sup>36</sup> B cells do and could thus also serve as targets of  $\beta_1$ EC<sub>II</sub>-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  $\beta_1\text{EC}_{\text{II}}$ /GST-FPs 3 days prior to the analysis of the spleens. Whereas long-lived plasma cells were not targeted by  $\beta_1\text{EC}_{\text{II}}$ -CPs, preventive as well as therapeutic application of the same CPs resulted in a  $\sim 80\%$  reduction of the frequencies of splenocytes secreting anti- $\beta_1\text{EC}_{\text{II}}$ -abs.<sup>19</sup> This finding indicates that in immunized anti- $\beta_1\text{EC}_{\text{II}}$ -positive rats, repeated injections of  $\beta_1\text{EC}_{\text{II}}$ -CPs may lead to impaired B-cell receptor (BCR)-mediated  $\beta_1\text{EC}_{\text{II}}$ -specific memory B-cell expansion (and differentiation into anti- $\beta_1\text{EC}_{\text{II}}$ -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<sup>37</sup> or the systemic application of aptamers.<sup>38</sup>

Furthermore, the very same peptide core conveying to cyclopeptides the potency to prevent allosteric  $\beta_1$ -AR activation by anti- $\beta_1\text{EC}_{\text{II}}$ -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<sup>211–214</sup> framed by the equally essential disulfide bridge between cysteines C<sup>209</sup>↔C<sup>215</sup>. In the intact receptor, this structure is constrained by another adjacent disulfide bridge linking C<sup>216</sup> to the transmembrane region next to EC<sub>I</sub>. Both pairs of disulfide bridges are relevant for receptor function.<sup>39,40</sup> According to the 3D structure of the turkey  $\beta_1$ -AR,<sup>41</sup> the assumed auto-epitope is located at the C-terminal end of the backward-oriented  $\alpha$ -helix constituting  $\beta_1\text{-EC}_{\text{II}}$ . Several features pre-dispose the NDPK<sup>211–214</sup> motif as an autoantigen and allosteric trigger: N<sup>211</sup>, D<sup>212</sup>, and K<sup>214</sup> are prone to bond with the binding surface of the antibody, while P<sup>213</sup> is needed to interfere with the H-bond formation to neighbouring helix residues, thereby bending the local  $\alpha$ -helix<sup>42</sup> and creating a unique structure for antibody recognition. The allosteric activation trigger is most probably provided by D<sup>212</sup>, the exchange of which to asparagine is known to cause paradoxical receptor activation by antagonistic ligands.<sup>43</sup>

## Limitations

The fact that human anti- $\beta_1$ -aabs are polyclonal and that the concentration of specific anti- $\beta_1$ -aabs in the circulation of a patient is probably much lower than in specifically immunized anti- $\beta_1\text{EC}_{\text{II}}$ -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.<sup>28,34</sup> The EC<sub>II</sub> loop of the  $\beta_1$ -AR is thought to be composed of 30 AAs (AA 195–225 of the receptor protein), and most functionally active human anti- $\beta_1$ -aabs were shown to be directed against this segment

of the  $\beta_1$ -AR membrane protein, representing a readily accessible target on the cell surface. Thus, it seems conceivable that *polyclonal* human anti- $\beta_1$ -aabs may target different epitopes (stretches of five to eight AAs) even within the 30 AAs that constitute  $\beta_1\text{EC}_{\text{II}}$ , which might result in diverging functional effects and/or downstream effects.<sup>12</sup> 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  $\beta_1$ -AR,  $\beta_2$ -AR,  $\beta_3$ -AR, M2-muscarinergic, and/or angiotensin II AT<sub>1</sub> receptors), or *cross-reactions* (or *molecular mimicry*)<sup>44</sup> 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  $\beta_1$ -AR by human anti-myosin aabs,<sup>45</sup>  $\beta_1$ -derived cyclopeptides would probably fail to hinder anti-myosin-aab-induced stimulation of cardiac  $\beta_1$ -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- $\beta_1$ -aabs were shown to increase cAMP production, whereas human anti-M2-aabs inhibit cAMP production,<sup>46</sup> or myocardial damage induced by anti- $\beta_1$ -aabs in heart failure was found to be alleviated by human anti- $\beta_2$ -aabs<sup>47</sup>).

## 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- $\beta_1\text{EC}_{\text{II}}$ -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- $\beta_1$ -aabs are polyclonal, their target epitope(s) might encompass not only the here unmasked structural motif in  $\beta_1\text{-EC}_{\text{II}}$ , but also structural motifs in other extracellular domains of the human  $\beta_1$ -AR, for example, in  $\beta_1\text{-EC}_I$ ,<sup>10,22</sup> 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  $\beta_1$ EC<sub>II</sub>-CP treatment of anti- $\beta_1$ EC<sub>II</sub> antibody-positive rats<sup>19</sup>—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,<sup>48</sup> 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  $\beta_1$ EC<sub>II</sub>-CPs (used in our previous therapy study in anti- $\beta_1$ EC<sub>II</sub> antibody-positive rats<sup>19</sup>). 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 HEK $\beta_1$ E<sub>1</sub> 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 CorImmune GmbH), München-

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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  $\beta_1$ AR-mutants expressed in HEK293-cells

Figure S1 - Neutralization of a monoclonal mouse anti- $\beta_1$ EC<sub>II</sub>-antibody by 22mer-cyclopeptides corresponding to the human  $\beta_1$ -EC<sub>II</sub>, each having a different non-conserved amino-acid (compared to the amino-acids constituting the EC<sub>II</sub>-loop of the  $\beta_2$ -AR) sequentially replaced with alanine.

Figure S2 - Time course of left ventricular diameters from rats immunized against  $\beta_1$ EC<sub>II</sub> or receiving a monoclonal rat anti- $\beta_1$ EC<sub>II</sub> and corresponding control animals, determined by echocardiography.

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