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A peptidomimetic inhibitor suppresses the inducibility of β 1-adrenergic autoantibody-mediated cardiac arrhythmias in the rabbit

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Abstract

Purpose—Previous studies demonstrated that burst pacing and subthreshold infusion of acetylcholine in β 1-adrenergic receptor (β 1AR)-immunized rabbits induced sustained sinus tachycardia. The aim of this study was to examine the anti-arrhythmogenic effect of a newly designed retro-inverso (RI) peptidomimetic inhibitor that specifically targets the β 1AR antibodies in the rabbit.

Methods—Six New Zealand white rabbits were immunized with a β 1AR second extracellular loop peptide to produce sympathomimetic β 1AR antibodies. A catheter-based electrophysiological study was performed on anesthetized rabbits before and after immunization and subsequent treatment with the RI peptide inhibitor. Each rabbit served as its own control.

Results—No sustained arrhythmias were induced at preimmune baseline. At 6 weeks after immunization, there was a marked increase in induced sustained tachyarrhythmias, predominantly sinus tachycardia, which was largely suppressed by the RI peptide. The atrial effective refractory period was shortened significantly in immunized rabbits compared to their preimmune state. The RI peptide reversed and prolonged this shortening. β 1AR antibody levels were negatively correlated with the atrial effective refractory period. Postimmune sera-induced β 1AR activation in transfected cells *in vitro* was also blocked by the RI peptide.

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Conflict of interest: D.C.K. as inventor has submitted US Patent No. 7, 348,305, Application No. PCT/US14/28362 regarding the retro-inverso peptide used in this manuscript.

Conclusions— β 1AR-activating autoantibodies are associated with reduction of the atrial effective refractory period and facilitate arrhythmia induction in this model. The RI peptide reversal may have important therapeutic implications in subjects who harbor these autoantibodies.

Keywords

Acetylcholine; Activating autoantibodies; β 1-Adrenergic receptor; Rabbit; Retro-inverso peptide; Tachyarrhythmias

1 Introduction

There is increasing evidence supporting a pathophysiological role for functionally active autoantibodies directed to the β 1/2-adrenergic receptors (β 1/2AR) in cardiac arrhythmias [1, 2]. β -Adrenergic autoantibodies are frequently observed in several pathological conditions characterized by rhythm disturbances, including idiopathic dilated cardiomyopathy, Chagas' disease, and myocarditis [3–5]. These autoantibodies primarily target the second extracellular loop (ECL2) of the receptor to mediate receptor activation [6, 7]. The presence of anti- β 1-adrenergic receptor (β 1AR) ECL2 antibodies has been reported to be associated with a high incidence of ventricular tachycardia and sudden death in idiopathic dilated cardiomyopathy [8]. A high prevalence of anti- β 1/2AR ECL2 antibodies has also been demonstrated in primary atrial and ventricular arrhythmias [9, 10] as well as inappropriate sinus tachycardia [11]. Recent findings from our group suggest that β 1AR and M2 muscarinic receptor autoantibodies are also related to a propensity for atrial fibrillation (AF) in Graves' hyperthyroidism [12]. A similar observation has been reported for lone paroxysmal AF [13].

We have used a rabbit model to study the electrophysiological effects of β 1AR and β 2AR autoantibodies. After immunization with the β 1AR or β 2AR ECL2 peptide, all animals developed high titers of β 1AR- or β 2AR-activating autoantibodies. We then demonstrated that burst pacing in conjunction with intravenous infusion of acetylcholine (ACh), at dosages which were insufficient by themselves to induce sustained tachyarrhythmias, subsequently produced sustained atrial tachycardia in β 2AR-immunized animals [14]. The atrial tachyarrhythmias induced in similarly studied β 1AR-immunized animals were predominantly sustained sinus tachycardia [15]. These data support the concept that autoantibody activation of β 1AR in the sinus node and β 2AR in the atrial myocardium selectively predisposes the rabbit heart to increased induction of distinct atrial tachyarrhythmias [16].

In the present study, we used this animal model to examine the therapeutic potential of a newly designed retro-inverso (RI) peptidomimetic inhibitor that specifically targets the β 1AR-activating autoantibodies. RI peptides, in which D-amino acids are substituted for L-amino acids in a reversed sequence, mimic the structure and antigenicity of the natural L-peptides but are resistant to protease degradation [17]. We have successfully used this approach recently to inhibit angiotensin receptor autoantibody-induced hypertension in the rabbit [18]. Here, we demonstrate that such an agent can also effectively block the arrhythmogenic effect of β 1AR autoantibodies in immunized rabbits. This decoy peptide

approach, if successful, would provide an important diagnostic and therapeutic tool for use with autoimmune-related tachyarrhythmias.

2 Methods

This study protocol was approved by the Institutional Animal Care and Use Committee of the Oklahoma City Veterans Affairs Medical Center and Oklahoma University Health Sciences Center and conforms to international standards for animal safety and comfort.

2.1 Animal immunization and pharmacological intervention

Six New Zealand white rabbits (2.5–3 kg) were immunized with 1 mg of the β 1AR ECL2 peptide (HWWRAESDEARRCYNDPKCCDFVTNR) [15] in 0.5 ml of complete Freund's adjuvant. The animals were boosted with the same peptide plus incomplete Freund's adjuvant (1 mg/0.5 ml) at 2 and 4 weeks. At 6 weeks, the rabbits were treated with an intravenous bolus injection (1 mg/kg) of a RI peptide d-DCCKPDNYCR, which mimics the functional epitope RCYNDPKCCD from the β 1AR ECL2 targeted by β 1AR-activating autoantibodies [19, 20]. These peptides were obtained from GenScript (Piscataway, NJ). Preimmune and postimmune sera were collected from all animals for ELISA and activity assays of the expected antibodies generated during immunization.

2.2 *In vivo* catheter electrophysiological study

Each animal was studied at baseline and restudied at the end of a 6-week immunization period. The animal was anesthetized with ketamine/xylazine (35 mg/5 mg/kg) and subjected to a catheter-based electrophysiological study [14–16]. Standard electrocardiograms (leads I–aVF) were continuously monitored. After shaving the neck area and application of betadine antiseptic, the right jugular vein was dissected and cannulated with a 4-French multi-electrode catheter. Under electrographic control, the catheter was passed into the right atrium to record atrial potentials in conjunction with the standard six-lead ECG. Atrial effective refractory period (ERP) was determined by programmed stimulation that consisted of eight basic stimuli (S1–S1=220 ms) followed by a premature stimulus (S2) using a Medtronic programmable stimulator (Model 5328; Medtronic Inc., Minneapolis, MN). The coupling interval of the S1–S2 was decreased from 150 ms initially by decrements of 10 ms and then 2 ms when approaching the ERP. Atrial tachyarrhythmia susceptibility was tested by bursts of stimuli (3–5-s duration) at a high frequency (20 Hz) and voltages that were at least twice the diastolic pacing threshold before and after the intravenous infusion of ACh in three incremental concentrations (10, 30, and 100 μ M) at a rate of 1 ml/min. These are equivalent to 0.6, 1.8, and 6 μ g/kg/min. The burst pacing was applied to a site in the right atrium at which a discrete atrial potential with little or no far-field ventricular potentials was recorded. Non-sustained (<10 s) and sustained ($\geq$ 10 s) arrhythmia occurrence was determined in response to burst pacing, at baseline, and then with each of the three concentrations of ACh infusion for 2 min before initiating burst pacing. The number of burst pacings ranged from 3 to 10. In the preimmune state, the number of bursts was most likely to be closer to 10 because it was more difficult to induce any non-sustained or sustained arrhythmia with or without ACh infusions, whereas after immunization, particularly with ACh infusion, two to three burst pacing events readily induced either non-sustained or

sustained arrhythmias. When this study was completed, the wound was closed and antibiotic treatment was instituted. The second and third electrophysiological studies were performed at the 6-week period before and 90 min after injection of the RI peptide. Therefore, each rabbit served as its own control. The various types of arrhythmias induced in the rabbit heart have been detailed previously [14, 15] and are defined as follows. These are based on the Lambeth Conventions for arrhythmia classification [21].

Sinus tachycardia: a regular, rapid heart rate 250 beats/min showing 1:1 atrioventricular conduction arising from the area of the sinus node with the intrinsic atrial electrogram occurring at the beginning of the P wave.

Atrial tachycardia: a regular, rapid heart rate 250 beats/min showing a constant A-A interval with the intrinsic atrial electrogram occurring after the beginning (usually around the peak) of the P wave.

Atrial flutter: a regular, rapid atrial rate 250 beats/min showing distinct P waves between variable QRS cycles.

Junctional tachycardia: a regular, rapid heart rate 200 beats/min showing 1:1 atrioventricular conduction with the intrinsic atrial electrogram occurring just prior to or during the QRS complex of the associated ECG lead.

2.3 Enzyme-linked immunosorbent assay

Antibodies produced in the sera were detected by ELISA [15]. Briefly, microtiter plates were coated with the $\beta 1AR$ ECL2 peptide at 10 $\mu\text{g/ml}$ in coating buffer. Sera were diluted 1:10, 000, and goat anti-rabbit IgG conjugated with alkaline phosphatase and its substrate para-nitrophenylphosphate 104 were used to detect antibody binding. The optical density (OD) values were read at 405 nm at 60 min.

2.4 cAMP assay

Rabbit sera were tested for $\beta 1AR$ activation using the cAMP Hunter eXpress GPCR Assay kit (DiscoverX, Fremont, CA) [15]. Briefly, 30,000 $\beta 1AR$ -transfected Chinese hamster ovary cells were dispensed into each well of 96-well culture plate and incubated overnight. The medium was removed, and assay buffer containing the cAMP antibody and rabbit sera (1:100) in the presence and absence of βAR blocker propranolol (1 μM) were sequentially added and incubated for 30 min. Preincubation of sera with an excess of the RI peptide was also tested for neutralization studies. cAMP standard and negative (buffer) and positive (isoproterenol 100 nM) controls were included in each assay. All samples were tested in triplicate. Following sample treatment, cAMP detection reagent and solution were added, and luminescent signal was read on a TD-20/20 Luminometer (Turner BioSystems, Sunnyvale, CA). The cAMP values are expressed as percentage of buffer baseline to normalize the individual data.

2.5 Statistical analysis

Results are expressed as mean $\pm$ SEM. Differences in occurrence of arrhythmias was assessed using generalized estimating equations to account for the repeated measures on each rabbit. ACh was included as a covariate. The correlation matrix for the repeated measures was

initially set as unstructured, but the final estimated matrix reflected the observed high correlation in response within each rabbit across ACh levels and lower correlation from, for example, preimmunization to post-immunization. Differences in atrial ERP and cAMP production were assessed by a paired Student's *t* test or one-way ANOVA with Newman-Keuls post hoc test as appropriate. A Pearson correlation test also was performed to examine the relationship between antibody values and atrial ERP. Data analyses were conducted using IBM SPSS Statistics (IBM SPSS Statistics v. 20.0, IBM Corp., Armonk, NY). A *P* value of <0.05 was considered statistically significant.

3 Results

3.1 Development of an epitope-mimicking peptidomimetic

We designed a RI peptide based on the $\beta 1$ AR ECL2 epitope sequence RCYNDPKCCD. This enantiomer peptide, made of all D-amino acids in a reversed sequence (d-DCCKPDNYCR), maintains a side chain topology similar to that of the original L-amino acid peptide, thus mimicking the molecular structure and antigenicity of the peptide RCYNDPKCCD (Fig. 1a). A schematic depiction of autoantibody activation of $\beta 1$ AR and autoantibody neutralization by the RI peptide is shown in Fig. 1b.

3.2 *In vivo* inhibition of arrhythmia induction in $\beta 1$ -adrenergic receptor-immunized animals by the retro-inverso peptide

The right atrial ERP was measured in each rabbit before and after immunization and subsequent treatment with the RI peptide. Each animal served as its own control. At 6 weeks after immunization, the ERP shortened significantly from 85 ± 2 to 74 ± 2 ms ($P < 0.01$). Injection of the RI peptide reversed this shortening followed by a significant prolongation over 90 min (115 ± 4 ms, $P < 0.001$ vs. postimmune; $P < 0.01$ vs. preimmune) (Fig. 2). A significant negative correlation was found between the $\beta 1$ AR antibody levels and ERP ($r = -0.89$, $P = 0.02$) (Fig. 3).

Only a few non-sustained arrhythmias were induced by burst pacing at baseline and at each of the three incremental concentrations of infused ACh. If no arrhythmia could be elicited, no response was registered. From a pathophysiological standpoint, we compared the induction of sustained (> 10 s) arrhythmias in each rabbit before and after antibody expression and the subsequent RI peptide treatment. Table 1 summarizes the effects of $\beta 1$ AR antibodies and the RI peptide on arrhythmia induction. In the preimmune state, no induced sustained arrhythmias were observed out of 24 induction attempts (four in each of the six animals). In the postimmune state, there were 16 episodes of sustained arrhythmias (67 vs. 0 % preimmune, $P < 0.0001$) including ten sinus tachycardias (42 vs. 0 % preimmune), three junctional tachycardias, two atrial tachycardias, and one atrial flutter out of the total of 24 induction attempts. The induced sustained arrhythmias were significantly reduced by the RI peptide. Only three sustained arrhythmias (13 vs. 67 % postimmune, $P = 0.0022$) including two sinus tachycardias (8 vs. 42 % postimmune) and one junctional tachycardia were induced out of 24 induction attempts following RI peptide injection. Induced sustained arrhythmias after RI peptide were not significantly different from the preimmune state ($P = 0.61$). The baseline heart rate after immunization was significantly

increased (preimmune: 150 ± 4 vs. postimmune: 177 ± 8 beats/min, $P < 0.05$), which was also suppressed by the RI peptide (158 ± 6 beats/min, $P < 0.05$ vs. postimmune).

Examples of atrial burst pacing-induced sinus tachycardia, atrial tachycardia, atrial flutter, and junctional tachycardia are shown in Fig. 4.

3.3 *In vitro* inhibition of $\beta 1$ -adrenergic receptor antibody activity by the retro-inverso peptide

All six rabbits developed high antibody levels to $\beta 1$ AR after immunization. Rabbit sera were tested for their activation potential using a cell-based cAMP assay. The postimmune sera stimulated significant cAMP production in $\beta 1$ AR-transfected Chinese hamster ovary cells (Fig. 5). Sera-induced $\beta 1$ AR activation was abolished by the non-selective β AR blocker propranolol or by preincubation with the RI peptide. No significant increase in cAMP production was found with the preimmune sera compared to buffer baseline.

4 Discussion

Therapeutic strategies targeting pathological $\beta 1$ AR autoantibodies in dilated cardiomyopathy have been investigated in several studies. These include antibody elimination or neutralization by immunoadsorption [22, 23], specific peptides, and aptamers [24, 25]. Orthosteric receptor antagonists such as β -blockers produce variable outcomes since they also block the normal functional ligand and impair concurrent normal physiological responses. The decoy peptides also have the potential to induce antigen-specific immune tolerance leading to suppression of autoantibody production which is not the case with orthosteric blocking agents [26]. Small peptide inhibitors have several advantages over other small molecules, including high specificity and low levels of toxicity and immunogenicity [27]. A modified $\beta 1$ AR ECL2 cyclic peptide has been used to neutralize $\beta 1$ AR autoantibodies [26] and is under limited trial in Europe [28]. In this study, we tested a novel epitope-mimicking RI peptide that specifically targets the arrhythmogenic $\beta 1$ AR autoantibodies produced in the rabbit. RI peptides offer the advantage of increased biological half-life and bioactivity over the natural L-peptides. Compared to the cyclic peptide approach, this construct is relatively simple and can be easily adapted to targets of other autoantibodies and administered orally.

We used subthreshold dosages of ACh along with burst atrial pacing as a standardized means of facilitating our study of atrial tachyarrhythmias. These dosages have previously been shown in our laboratory to not induce sustained atrial tachyarrhythmias in our rabbit model. The muscarinic effects of ACh are known to directly shorten the action potential and also to affect pericardiac tissues such as pulmonary vein myocardium via ganglionic nerve activation. Many investigators have used ACh to directly produce AF in rabbits as well as animals with a larger heart that is more susceptible for AF induction. We have observed that the dosages of ACh used in our younger rabbits are subthreshold for induction of sustained tachyarrhythmias and only occasionally induce non-sustained arrhythmias. This supports the concept that even when the burst pacing is applied, changes in the atrial response are subthreshold for induction of a sustained arrhythmia. Other investigators have observed that ACh induces AF even in control experiments [29, 30]. There are a number of features that

may explain our use of ACh to produce subthreshold changes. These include use in the whole animal rather than *in vitro* as occurs with Langendorff preparations, the younger age of our rabbits, and using systemic infusion rates that are associated with diminished tissue responses. Using each rabbit as its own control, we were able to compare their responses to ACh infusion and burst pacing before and after immunization and pharmacological intervention. We found no instance of an induced sustained arrhythmia in the preimmune studies, whereas sinus tachycardia was observed in 10 of 16 induced sustained arrhythmias in the postimmune studies, which was consistent with our previous findings that the majority of the induced tachyarrhythmias in β 1AR-immunized rabbits were of sinus origin. Sustained junctional tachycardia was also induced but at a lower frequency (3 of 16). Intravenous injection of the RI peptide after β 1AR antibody production significantly reduced the incidences of these induced sustained arrhythmias. It was not surprising that the RI peptide did not completely block the induction of sustained arrhythmias. We were unable to justify a dosage response owing to limitations on time of anesthesia in these sensitive animals, and there might be an incomplete neutralization of the autoantibodies with the dosage employed. In addition, several weeks of autoantibody exposure may create subtle but real non-visible changes in the atrial structure that would make the tissues still susceptible to induction of tachyarrhythmias. These might well be suppressed by longer-term RI peptide therapy.

There is emerging evidence suggesting that the autonomic nervous system plays an important role in the initiation and maintenance of atrial tachyarrhythmias [31]. Both sympathetic and vagal stimulation can shorten atrial refractoriness. In human atrium, calcium influx and potassium repolarizing currents carried by the ultra-rapid and slow components are under strong adrenergic control. Their activation by β 1AR stimulation can lead to shortening of atrial ERP and action potential duration [32]. In addition, several studies have shown that β blockade lengthens atrial ERP and action potential duration and has a potential anti-arrhythmic effect [33–35]. We have recently reported that a high proportion of Graves' patients with AF have activating autoantibodies to the β 1AR and M2 cholinergic receptors, and their serum IgG induced hyperpolarization, decreased action potential duration, enhanced early afterdepolarization formation, and facilitated triggered firing in isolated canine pulmonary veins [12].

We have demonstrated that expression of sympathomimetic β AR-activating antibodies in our rabbit model led to a significant reduction of atrial ERP. This effect was rapidly and completely reversed by the RI peptide. The post RI peptide period was accompanied by a significant overshoot of the ERP. β 1AR-activating antibodies do not induce significant receptor desensitization [5, 36]. This lack of desensitization probably was quickly replaced as the β 1AR antibody activity was rapidly removed by the RI decoy peptide. It is likely that a compensatory increase in circulating norepinephrine and epinephrine rapidly induced receptor desensitization and led to the observed marked increase in ERP. We have infused a non- β 1AR RI peptide into non-immune rabbits in separate experiments without evidence for a direct cardiac effect of the RI molecule.

It is of interest that the β 1AR antibody levels were negatively correlated with the atrial ERP. This is compatible with the possibility that the antibodies altered a measureable parameter of electrophysiological remodeling. The agonist activity of the rabbit antibodies was confirmed

by their activation of β 1AR-mediated cAMP production in cultured cells, which was also blocked by the RI peptide. The specificity of these antibodies makes them potentially important tools for study of receptor specificity for various cardiac tachyarrhythmias.

5 Limitations

The rabbit model provides a convenient means for induction of autoantibodies as opposed to larger animals. We have found that the immunized rabbit is particularly useful for examination of a variety of tachyarrhythmias associated with adrenergic autoantibodies. However, a disadvantage of the rabbit is the relative resistance to sustaining tachyarrhythmias owing to the relatively small size of the heart. We have incorporated the addition of graded concentrations of ACh along with burst pacing to initiate arrhythmias that allows us to use this otherwise convenient model to investigate the effect of autoantibodies on arrhythmogenesis. The present study demonstrated significant reduction in induced arrhythmias in immunized rabbits with acute treatment with the RI peptide. Future studies will need to examine the long-term effect of RI peptides on suppression of antibody-mediated arrhythmias. Such studies will require intensive study of the pharmacodynamics of such peptides, their safety, and ability to suppress autoantibody production as well as activity. We have performed limited studies on animals with repeated RI peptide injections for 3 months, which showed no anti-RI peptide antibody production or organ toxicity.

6 Conclusions

This study supports a pathophysiological role of β 1AR-activating autoantibodies in cardiac arrhythmias. Pharmacological blockade of pathological autoantibodies with peptide inhibitors may open a new avenue for developing novel therapeutic approaches for patients who harbor these autoantibodies.

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Fig. 1.

a Chemical structure of the $\beta 1$ -adrenergic receptor ($\beta 1\text{AR}$) epitope peptide RCYNDPKCCD and epitope-mimicking retro-inverse (RI) peptide d-DCCKPDNYCR. **b** Schematic diagram depicting norepinephrine (NE) and autoantibody (AAb) activation of $\beta 1\text{AR}$ and the blocking effect of RI peptide on $\beta 1\text{AR}$ AAb. The orthosteric ligand NE binds to a hydrophobic pocket formed by the transmembrane helices, while the allosteric antibodies bind to the second extracellular loop of the receptor, both of which lead to sequential activation of stimulatory G protein (Gs), adenylyl cyclase (AC), and cAMP-dependent protein kinase A (PKA). The RI peptide binds to the antibodies and prevents their binding to and activation of the membrane receptor

Fig. 2.

Effective refractory period (ERP) measured in the right atrium (RA) before and after immunization and subsequent retro-inverso (RI) peptide injection in the rabbit. Compared to the preimmune state, the postimmune state demonstrated a significant shortening of ERP, which was reversed and prolonged by the RI peptide treatment. * $P < 0.01$ vs. preimmune; # $P < 0.001$ vs. postimmune, $n=6$

Fig. 3.

Correlation analysis between the β 1-adrenergic antibody optical density (OD) values and the right atrial effective refractory period (RA ERP) in immunized rabbits. There was a significant negative correlation between the antibody levels and ERP

Fig. 4.

Examples of atrial burst pacing-induced sinus tachycardia, atrial tachycardia, atrial flutter, and junctional tachycardia observed in the present study. **a** Burst pacing-induced sinus tachycardia. The atrial electrogram (RA3) was recorded at the high right atrium, and the intrinsic deflection aligned with the beginning of the upright P wave of ECG lead II (see magnified *insert* at the end of the traces). **b** Burst pacing-induced atrial tachycardia with the intrinsic deflection of RA3 aligning just prior to the peak of the upright P wave in ECG lead II (see magnified *insert* at the end of the traces). **c** Burst pacing-induced atrial flutter. Note the regular morphology of the P waves in ECG lead I which is characteristic for atrial flutter. The very rapid rate causes varying degrees of conduction and block in the atrioventricular node leading to the irregular ventricular rate. **d** Burst pacing-induced junctional tachycardia with 1:1 atrioventricular conduction. The atrial electrogram (RA4) was recorded from a site close to the atrioventricular junction and occurs immediately preceding each ventricular complex

Fig. 5.

Retro-inverso (RI) peptide inhibition of rabbit antisera-induced cAMP production in Chinese hamster ovary cells transfected with β_1 -adrenergic receptor. Compared to the preimmune sera, the postimmune sera induced a significant increase in cAMP production, which was effectively blocked by the RI peptide and by the non-selective β blocker propranolol. Isoproterenol (100 nM) was used as a positive control. * $P < 0.001$ vs. preimmune; # $P < 0.001$ vs. postimmune, $n = 6$. Data are expressed as percent of buffer baseline

Table 1

Retro-inverso (RI) peptide inhibition of induced sustained tachyarrhythmias in β 1-adrenergic receptor-immunized rabbits ($n=6$)

Induced arrhythmias	Preimmune	Postimmune	Postimmune + RI peptide
Sinus tachycardia	0/24	10/24	2/24
Junctional tachycardia	0/24	3/24	1/24
Atrial tachycardia	0/24	2/24	0/24
Atrial flutter	0/24	1/24	0/24
Total	0/24	16/24	3/24

For comparison of number of sustained tachyarrhythmias: preimmune vs. postimmune, $P<0.0001$; postimmune vs. postimmune + RI peptide, $P=0.0022$; preimmune vs. postimmune + RI peptide, $P=0.61$