EL TRATAMIENTO CON EL ANTICUERPO ANTI-PROTEOGLICANO CHP3R99 NO AFECTA LA BIOENERGÉTICA MITOCONDRIAL EN RATONES

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Resumen

Los proteoglicanos son moléculas biológicas importantes que participan en la retención de las lipoproteínas pro-aterogénicas en la pared arterial y también son cruciales para la remoción hepática segura de las lipoproteínas aterogénicas remanentes circulantes. Recientemente obtuvimos un anticuerpo monoclonal quimérico nombrado chP3R99 que reacciona con moléculas sulfatadas, incluyendo los proteoglicanos. Aunque se conoce la química de la interacción proteoglicanos-lipoproteínas de baja densidad, las consecuencias de su inhibición por el anticuerpo chP3R99 sobre el metabolismo de lípidos y el estado redox mitocondrial no se han estudiado todavía. Es por eso que en el presente trabajo intentamos caracterizar la función de las mitocondrias hepáticas de ratones C57BL6 tratados subcutáneamente con 50 µg de chP3R99 (semanalmente durante 3 semanas). Los orgánulos se aislaron bajo condiciones estándares y se determinó el estado del NAD(P)H, la susceptibilidad a la ocurrencia del poro de permeabilidad mitocondrial y la producción de especies reactivas de oxígeno. Por otro lado, también se determinaron los niveles séricos de colesterol, triglicéridos, LDL-colesterol y HDL colesterol. Los resultados mostraron que no se produjeron cambios significativos en los niveles lipídicos entre los grupos. El estado mitocondrial del NAD(P)H no se afectó por el tratamiento con chP3R99 ni tampoco se indujo el poro de transición de permeabilidad mitocondrial ni incremento en la producción de especies reactivas de oxígeno. Los resultados demostraron que el tratamiento con cchP3R99 no interfiere con el metabolismo lipídico en ratones normo-lipémicos. Al mismo tiempo, la ausencia de efectos de este anticuerpo sobre los parámetros mitocondriales evaluados demostró que su tratamiento no afectó el estado redox mitocondrial hepático. Sin embargo, es obvio que se necesitan otros estudios toxicológicos para establecer el riesgo potencial del uso de esta inmunoterapia anti-proteoglicanos.

Palabras clave: Mitocondria, anticuerpo monoclonal, proteoglicano, NAD(P)H, especies reactivas de oxígeno

The anti-proteoglycan antibody chP3R99 treatment does not affect mitochondrial bioenergetic in mice

Abstract

The proteoglycans are important biological molecules that participate in the retention of proatherogenic lipoproteins in the arterial wall, but also they are crucial for safe hepatic removal of atherogenic remnant lipoproteins from the circulation. Recently we obtained a monoclonal chimeric antibody named chP3R99 that reacts with sulfated molecules, including proteoglycans. Although the chemistry of low-density lipoprotein-proteoglycans interaction has been explored, the consequences of its inhibition by chP3R99 antibody on lipid metabolism and mitochondrial redox status have not been studied yet. Therefore, in the present work we characterized the liver mitochondrial function from C57BL6 mice subcutaneously treated with 50 µg of chP3R99 (weekly during 3 weeks). Hepatic mitochondria were isolated under standard procedures and their NAD(P)H status, susceptibility to permeability transition pore occurrence, and reactive oxygen production were assessed. On the other hand, the serum cholesterol, triglycerides, LDL-cholesterol and HDL-cholesterol were also determined. The results showed no significant changes of serum lipids levels among groups. The mitochondrial NAD(P)H status was no affected by chP3R99 treatment, neither mitochondrial permeability transition pore nor reactive oxygen species production were induced. Our results demonstrated that chP3R99 treatment does not interfere on lipid metabolism in normolipemic mice. Also, the lack of chP3R99 effect on the evaluated mitochondrial parameters demonstrated that the treatment with this antibody did not affect the hepatic mitochondrial redox status. Nevertheless, further toxicological studies should be performed to address the potential risks associated with the use of anti-proteoglycans immunotherapy.

Keywords: Mitochondria, monoclonal antibody, proteoglycans, NAD(P)H, reactive oxygen species

Introduction

Mitochondria are usually described as the “powerhouse unit” of the cell, because they contain the molecular machinery that governs many distinct metabolic pathways by which chemical energy (coming from lipids, carbohydrates, and proteins) is converted into ATP. Indeed, it is in this organelle that pyruvate oxidation, fatty acid β-oxidation, the tricarboxylic acid cycle, and oxidative phosphorylation, take place (Goldstein and Marin-García, 2004). The affection of any component of mitochondrial machinery may results in a bioenergetic dysfunction and cellular damage (De Pauw et al., 2009).

Proteoglycans (PGs) are important macromolecules composed of a protein core and complex, linear, long-chain carbohydrates, called glycosaminoglycans (GAGs). GAGs consist of repeating disaccharide units bearing negatively charged sulfate and carboxyl groups (Kahlil et al., 2004). GAGs are responsible for low-density lipoprotein (LDL) retention in the subendothelial space of major arteries; and also are crucial in hepatic removal of atherogenic remnant lipoproteins from the circulation (Nakashima et al., 2008).

When affinities of LDL for pericellular PGs of fibroblasts and those for the LDL receptor in the same cells were compared, LDL affinity for the receptor was at least 2 orders of magnitude higher than that for the cell surface PGs. However, the capacity of the PGs for LDL binding was much higher (Olsson et al., 1997). A similar observation was made by assessing LDL association with the cell surface; LDL interaction with PGs was a lower-affinity but higher-capacity process (Galeano et al., 1998). These results indicate that PGs by themselves are sufficient for the association of relatively large amounts of LDL in the extracellular space (Kahlil et al., 2004). They also predict that such compounds capable to interfere with PGs-LDL interaction could be valuable for atherogenesis prevention, since LDL retention by PGs represents an important condition for LDL oxidation, their internalization by macrophages and the subsequent foam cells formation (Williams and Tabas, 1995; Tabas et al., 2007).

In the Antibody Engineering Department of the Center of Molecular Immunology (Havana, Cuba) it was obtained a human IgG1 monoclonal chimeric antibody named chP3R99, which strongly reacts with sulfated molecules, such as GAGs (Fernández-Marrero et al., 2011). Recently, our group demonstrated that chP3R99 inhibited the 80% of LDL-PGs binding *in vitro* and induced a cascade of idiotypic antibodies which are able to inhibit LDL-PGs binding and LDL oxidation. Moreover, *in vivo* studies showed that low doses of chP3R99 inhibited atherosclerotic lesions formation in rabbits (Soto et al., 2012) and mice (Brito et al., 2012).

Nevertheless, such therapeutic benefits could have a noxious implication for cells function. It is well known that syndecan-1, a member of heparan sulfate proteoglycans (HS-PGs), is a key molecular mediator for the hepatic clearance of atherogenic remnant lipoproteins from the circulation (Stanford et al., 2009; Williams and Chen, 2010). Also, HS-PGs are important for the cellular uptake and turnover of lipoproteins, in part by enhancing the accessibility of these macromolecules to lipoprotein receptors and lipases (Kolset and Salmivirta, 1999). It has also been demonstrated that HDL may be taken up by hepatocytes through mechanisms involving cell surface HS-PGs (Kolset et al., 1999). Therefore, an important limitation of anti-PG-based antiatherogenic therapies is the potential interference with this physiological mechanism. This might result in an altered lipid metabolism, triggering a mitochondrial oxidative stress.

In this line, we sought to address the potential risk of the inhibition of LDL-PGs binding by the treatment with chP3R99. Thus, we aimed to evaluate the effects of chP3R99 treatment on serum lipids level and mitochondrial redox status. As we had thought, the immunization with chP3R99 did not interfere with lipid metabolism, neither with normal mitochondrial function.

Materials and methods

Reagents

Bovine serum albumin (BSA), ADP, cyclosporin A, EGTA, succinate, rotenone, safranine, N-(2-hydroxyethyl) piperazine-N`-2-ethanesulfonic acid (HEPES), and carbonyl cyanide p-trifluoromethoxyphenylhydrazone (CCCP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were commercially products of the highest purity grade available.

Monoclonal antibodies

chP3R99 and hR3 monoclonal antibodies were obtained, purified and conjugated to biotin as described previously (Mateo de Acosta et al., 1997; López-Requena et al., 2003). Specificity was confirmed by enzyme-linked immunoadsorbent assay (ELISA). hR3 recognized the human epidermal growth factor receptor; it was used as isotype-matched control.

Animals

Male C57BL6 mice, 4 month old and weighing 20-26 g, were used in the present study. All procedures were approved by the Faculty of Pharmacy Institutional Animal Ethics Committee (Instituto de

Farmacia y Alimentos, Universidad de La Habana, Cuba) and in accordance with the Guidelines on the Handling and Training of Laboratory Animals published by the Universities Federation for Animals Welfare, Brazil, 1992. The mice had free access to standard laboratory rodent chow diet (CENPALAB, La Habana, Cuba), water *ad libitum* and were housed at 22 ± 2 ºC in a 12 h light-dark cycle.

Experimental design

Animals were divided in two groups of 5 animals each: control (treated with the isotype-matched control hR3) and the group treated with chP3R99 antibody. A dose of 50 µg of monoclonal antibodies in 250 µL of PBS was subcutaneously administered once a week during three weeks. At day 22, the end of the experiment, overnight fasted animals were killed by cervical dislocation. Blood samples were withdrawn by cardiac punction, meanwhile livers were rapidly extracted and processed.

Serum lipid assay

Serum total cholesterol (TC), triglycerides (TG), LDL cholesterol (LDLc) and high density lipoprotein cholesterol (HDLc) were determined in mice´s serum samples collected on day 22 (end of the experiment), using commercial enzymatic kits (MBL International Corporation, MA, USA).

Isolation of mice liver mitochondria and standard incubation procedure

Mitochondria were isolated by conventional differential centrifugation from the liver of adult mice fasted overnight as previously described (Kaplan and Pedersen, 1998; Pardo Andreu et al., 2011a). The livers were homogenized in 250 mM sucrose, 1 mM EGTA, and 10 mM HEPES buffer (pH 7.2). The mitochondrial suspension was washed twice in the same medium containing 0.1 mM EGTA and the final pellet was resuspended in 250 mM sucrose. The protein content was measured by the method of Biuret with BSA as protein standard (Gornall et al., 1949).

The experiments were carried out in standard medium containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES buffer (pH 7.2), 2 µM rotenone and 5 mM succinate as a FAD-linked respiratory substrate. Other additions are indicated in each technique and in figure legends.

Determination of NAD(P) redox state

The oxidation or reduction of pyridine nucleotides in the mitochondrial suspension was followed in a Hitachi F-4010 spectrofluorimeter operating at 366 nm excitation and 450nm emission (Fagian et al., 1990; Pardo Andreu et al., 2011b). All incubations were conducted in the presence of cyclosporine A, an inhibitor of mitochondrial permeability transition, in order to avoid fluorescence changes secondary

to mitochondrial swelling, release of matrix NAD(P)H, or inhibition of NAD(P)H transhydrogenase activity due to increased membrane proton leakage.

Measurement of mitochondrial transmembrane electrical potential (ΔΨ)

The mitochondrial membrane potential was estimated as fluorescence changes of safranin (Akerman and Wikstrom, 1976), recorded in a model Hitachi F-4010 spectrofluorimeter operating at excitation and emission wavelengths of 495 and 586 nm, respectively, with slit widths of 5 nm. Mitochondria (0.5 mg/mL) were incubated in 2 mL of standard medium supplemented with 5 µM safranine, 2 µM rotenone and 5 mM succinate. Relative changes in membrane potential were expressed in arbitrary fluorescence units.

Reactive oxygen species production

Production of reactive oxygen species (ROS) by liver mitochondria was followed by measuring the conversion of Amplex Red (Molecular Probes, Eugene, OR) in the presence of extra-mitochondrial horseradish peroxidase, to highly fluorescent Resorufin, by H_2O_2 (Votyakova and Reynolds, 2001; Nuñez Figueredo et al., 2014). Mitochondria (0.5 mg/mL) were incubated in standard incubation medium supplemented with 10 µM Amplex Red and 1 U/mL horseradish peroxidase at 30ºC with continuous stirring. Resorufin fluorescence was measured in a Hitachi F-4010 spectrofluorimeter at 563 nm for excitation and 587 nm for emission.

Statistical analyses

Results are presented as mean ± standard deviation of 5 independent experiments. Statistical analysis was performed using two-way ANOVA, assuming equality of variance with Student-Newman-Keuls post-hoc test for pairwise comparisons. Results with $P<0.05$ were considered to be statistically significant.

Serum lipids	chP3R99-LALA	hR3

Results

The serum levels of TC, TG, LDLc and HDLc were not modified by the treatment with chP3R99 (Table 1) suggesting that the anti-PG-based immunization did not affect lipid metabolism under our experimental conditions.

TC (mg/dL)	156.03 ± 3.01	160.17 ± 4.46
LDLc (mg/dL)	78.27 ± 0.79	78.12 ± 1.01
HDLc (mg/dL)	27.97 ± 0.64	28.74 ± 0.89
TG (mmol/L)	2.35 ± 0.19	2.36 ± 0.51

Table 1. Effect of chP3R99-LALA mAb on serum lipid profile.

Values are means ± SEM. TC indicates total cholesterol; TG, triglycerides; HDLc, high-density lipoprotein cholesterol; LDLc, low-density lipoprotein cholesterol.

The Panel A of Figure 1 shows that the treatment with chP3R99 anti-PG antibody did not affected the mitochondrial NAD(P)H status. The fluorescence units (mean ± SD at 400 s) were 29.51 ± 3.27 (line a) and 27.2 ± 4.15 (line b). In fact, the addition of *t*-butylhydroperoxide (TBOOH), a well-known NAD(P)H oxidant, showed similar pattern for the reduced nicotinamide depletion in chP3R99 or the isotype treated groups (Figure 1, panel B). The fluorescence units after *t*-butylhydroperoxide addition (mean ± SD at 250 s) were 14.20 ± 2.68 (line a) and 14.74 ± 2.19 (line b). Isocitrate was added to restore NAD(P)H, and the fluorescence units (mean ± SD at 400 s) were 28.24 ± 1.93 (line a) and 29.66 ± 3.84 (line b). No statistical differences were found when comparing line a versus line b in the above mentioned conditions.

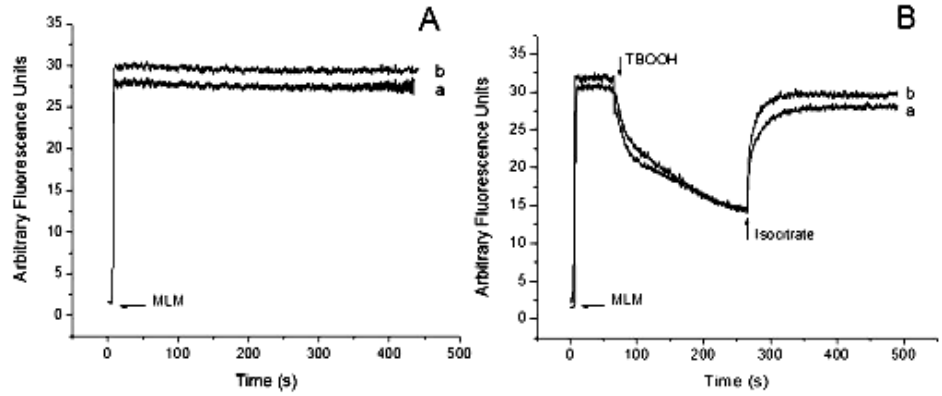

Figure 1. The reduced state of mitochondrial pyridine nucleotides was not disturbed by chP3R99 *in vivo* treatment (A). Similar sensitivity of mitochondrial reduced pyridine nucleotides from isotype or chP3R99-treated mice to *t*-butylhydroperoxide (TBOOH) induced oxidation (B). Mice liver mitochondria (MLM, 1 mg/ml) from isotype (line a) or chP3R99-treated mice (line b) were added to standard reaction medium in the presence of 100 µM EGTA. Isocitrate (1 mM) or *t*-butylhydroperoxide (50 µM) were added as indicated. Results are representative of 5 experiments conducted with independent mitochondrial preparations. There were no statistical differences ($p>0.05$).

Since this assay was conducted in the presence of EGTA, a classical mitochondrial permeability transition (MPT) inhibitor, we also addressed the sensibility of mitochondria from chP3R99-treated mice to undergo this process in the presence of Ca^{2+} (absence of EGTA). Figure 2 shows that energized mitochondria from chP3R99-treated mice built and sustained a transmembrane electrical potential (ΔΨ), evidenced by a diminution in safranine fluorescence, similarly as the isotype-treated mice mitochondria. The fluorescence units (mean ± SD at 400 s) were 40.59 ± 4.63 (line a) and 42.08 ± 5.26 (line b). The addition of 20 µM Ca^{2+} was unable to dissipate the membrane potential, indicating the non occurrence of MPT in mitochondria from chP3R99-treated mice. The slight dissipation of ΔΨ elicited by calcium followed similar patterns in both mitochondrial groups. The fluorescence units after Ca^{2+} addition (mean ± SD at 400 s) were 47.77 ± 6.26 (line c) and 52.36 ± 5.17 (line d). The protonophore CCCP was added at the end of the experiments (Figure 2) to disrupt ΔΨ.

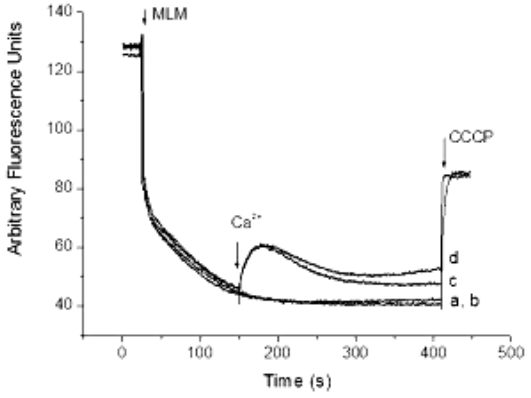

Figure 2. chP3R99 treatment did not affect mitochondrial membrane potential. (MLM, 0.5 mg/mL) from isotype (lines a, d) or chP3R99-treated mice (line b, c) were added to standard reaction medium supplemented with 10 µM safranine. Mitochondria, Ca^{2+} (20 µM) or CCCP (1 µM) were added as indicated. Results are representative of 4 experiments conducted with independent mitochondrial preparations. There were no statistical differences ($p>0.05$).

The figure 3 shows that mitochondria isolated from both, hR3 and chP3R99 antibodies-treated mice generated ROS with similar patterns. The fluorescence units (mean ± SD at 400 s) were 297.82 ± 16.05 (line a) and 313.38 ± 19.14 (line b). The addition of *t*- butylhydroperoxide (100 µM final concentration) into de incubation medium induced an increase in ROS generation, as expected. As in the absence of the oxidant, the patterns of ROS induction were comparable.

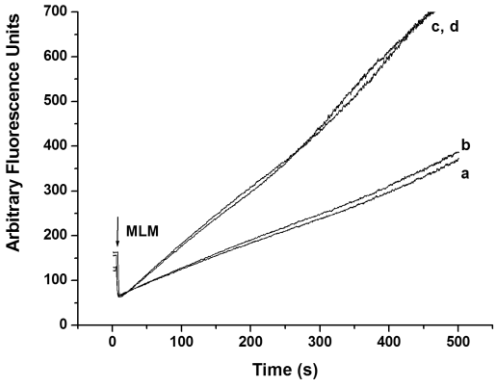

Figure 3. Effects of chP3R99 mice treatment on ROS generation by mitochondria energized with succinate. MLM (0.5 mg protein) were incubated in the standard medium supplemented with 0.1 mM EGTA, 1 μM Amplex Red and 1 IU/mL horseradish peroxidase, at a final volume of 2 ml as described in Materials and methods. Experimental conditions were: MLM isolated from isotype-treated mice in the absence (line a) or in the presence of 100 μM *t*-butylhydroperoxide (line c), MLM isolated from chP3R99-treated mice in the absence (line b) or in the presence of 100 μM *t*-butylhydroperoxide (line d). Results are representative of 5 experiments conducted with independent mitochondrial preparations.

Discussion

It was previously demonstrated that chP3R99 monoclonal antibody reacts with GAGs, including heparin, heparan sulfate (HS), and dermatán sulfate (DS) in a similar way despite differences in sulfation pattern among these GAGs. The highest reactivity of chP3R99 was observed for chondroitin sulfate (CS). This antibody also recognized human aortic PGs and decorin, a PG made up of CS and DS. In contrast, chP3R99 showed a lower reactivity with hyaluronic acid a non-sulfate-containing GAG (Soto et al., 2012).

In apolipoprotein E-deficient (apoE$^{-/-}$) mice fed a high-fat, high cholesterol diet, chP3R99 showed a high reactivity against its antigens. This chimeric antibody elicited an immunodominat anti-idiotypic response in the absence of adjuvant. The antiatherosclerotic effect of this antibody was associated with increased mice sera reactivity against heparin and sulfated GAGs, including CS and DS. In addition, purified IgG from chP3R99-immunized mice blocked the retention of apolipoprotein B-containing lipoproteins within the arterial wall of apoE$^{-/-}$ mice (Brito et al., 2012).

On the other hand, Brito and coworkers (2012) demonstrated that chP3R99 did not interfere the binding between VLDL and syndecan-1, a sulfated PG expressed on human Huh7 cell membranes

(Sutton et al., 2007). Here we showed that chP3R99 did not interfere with serum lipids metabolism. In accordance, it was previously reported that the antiatherogenic effect of chP3R99 was exerted by mechanisms other than serum lipid modulation (Soto et al., 2012; Brito et al; 2012).

Considering that in cells, fatty acids are essential for mitochondrial energy generation, we evaluated the potential risk of chP3R99 reactivity against PGs on mitochondrial function. The MPT pore, a non-specific channel originally thought to span both mitochondrial membranes, mediates the increases in mitochondrial permeability associated with cell death (Kroemer et al., 2007). The pore itself is permeable to solutes up to 1.5 kDa. This causes equilibration of H^+ across the inner membrane, which dissipates $\Delta\Psi m$ and inhibits ATP production. A concomitant influx of water causes swelling of the mitochondria, which stretches the membranes to the point where the outer membrane fails. The mitochondrial pore is redox, Ca^{2+}, voltage, adenine nucleotide, and pH sensitive (Di Lisa et al., 2007). In our experimental schedule, the chP3R99 treatment did not induce this process, which is very positive because it is known that apoptosis contributes with plaque instability and cardiovascular complications (Murphy and Steenbergen, 2008). Also, during atherogenic development there is a trigger of apoptotic pathways, including the mitochondrial route (Baines, 2009).

Nowadays, mitochondrial energy metabolism is recognized as the most quantitatively important source of ROS in the majority of eukaryotic cell types. The bulk of mitochondrial ROS generation occurs at the electron transport chain, as a by-product of respiration (Pittaluga et al., 2006). As a result, this generation can occur at relatively high rates compared to cytosolic ROS production and it is primarily determined by metabolic conditions (Murphy and Steenbergen, 2008). It is important to highlight that ROS production rate observed in the liver mitochondria from chP3R99-treated animals was similar to controls. Mitochondrial ROS are known to be important determinants in cell function, participating in many signaling networks and also in a variety of degenerative processes, including atherosclerosis-associated cardiovascular diseases (Kowaltowski et al., 2001).

The treatment with chP3R99 mAb did not affect the redox state of NAD(P)H, which is the major source of reducing equivalents for the antioxidant systems glutathione peroxidase/reductase and thioredoxine peroxidase/reductase. The reduced state of NAD(P) in mitochondrial matrix is controlled by the membrane potential-sensitive NADP+ transhydrogenase (Hoek and Rydstrom, 1988). This nucleotide plays a key role in cellular defense against ROS, inhibiting biomolecules damages and oxidative stress (Jo et al., 2001). Low content of NAD(P) have been associated with an increase of oxidative stress in hypercholesterolemic LDL-receptor knockout mice (Paim et al., 2008). Using the same animal model, Pardo-Andreu and coworkers demonstrated that Vimang® extract, an antioxidant

supplement, prevents mitochondrial dysfunction by a preservation of NAD(P) redox state without lowering cholesterol (Pardo-Andreu et al., 2008).

Antiatherosclerotic drug discovery is an active research field. Nowadays, only hypolipidemic treatment is accepted for coronary artery disease therapy. But the principal disadvantage of statins is their capacity to act directly on mitochondria, either *in vitro* or *in vivo*, inducing MPT (Velho et al., 2006).

In our previous studies, no evident toxicity was observed in the animals treated with chP3R99 mAb. Here we also demonstrated that chP3R99 therapy have no noxious effects on lipid metabolism nor mitochondrial function. Nevertheless, additional toxicological studies are needed to address other potential risks associated with the generation of antibodies against self-antigens such as PGs, which are involved in many physiological processes (Tabas et al., 2007).

Taking together, our results showed that the treatment with the anti-PGs chP3R99 mAb does not affect the lipid metabolism neither mitochondrial function, having important toxicological implications. Others experiments for further toxicology examination of chP3R99 mAb therapy should be addressed, but our findings represent the first line of evidence on the safety of this immunotherapeutic approach.

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