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An *in vitro* model mimicking the complement system to favor directed phagocytosis of unwanted cells

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ABSTRACT

Background: Opsonization, is the molecular mechanism by which target molecules promote interactions with phagocyte cell surface receptors to remove unwanted cells by induced phagocytosis. We designed an *in vitro* system to demonstrate that this procedure could be driven to eliminate adipocytes, using peptides mimicking regions of the complement protein C3b to promote opsonization and enhance phagocytosis. Two cell lines were used: (1) THP-1 monocytes differentiated to macrophages, expressing the C3b opsonin receptor CR1 in charge of the removal of unwanted coated complexes; (2) 3T3-L1 fibroblasts differentiated to adipocytes, expressing AQP7, to evaluate the potential of peptides to stimulate opsonization. (3) A co-culture of the two cell lines to demonstrate that phagocytosis could be driven to cell withdrawal with high efficiency and specificity.

Results: An array of peptides were designed and chemically synthesized p3691 and p3931 joined bound to the CR1 receptor activating phagocytosis ($p < 0.033$) while p3727 joined the AQP7 protein ($p < 0.001$) suggesting that opsonization of adipocytes could occur. In the co-culture system p3980 and p3981 increased lipid uptake to 91.2% and 89.0%, respectively, as an indicator of potential adipocyte phagocytosis.

Conclusions: This *in vitro* model could help understand the receptor–ligand interaction in the withdrawal of unwanted macromolecules *in vivo*. The adipocyte-phagocytosis discussed may help to control obesity, since peptides of C3b stimulated the CR1 receptor, promoting opsonisation and phagocytosis of lipid-containing structures, and recognition of AQP7 in the differentiated adipocytes, favored the phagocytic activity of macrophages, robustly supported by the co-culture strategy.

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1. Introduction

Phagocytosis is a process utilized by mononuclear phagocytes and neutrophils to ingest and clear large particles (>0.5~mm), including infectious agents, senescent cells, and cellular debris. Particle internalization is initiated by the interaction of specific receptors on the surface of the phagocyte with ligands on the surface of the particle [1].

The complement cascade is a collective term used to designate a group of plasma and cell membrane proteins that play a key role in the host defense process and it has more than sixty components and activation fragments. The alternative pathway is an antibody-independent [2] complement activation pathway that uses recognition molecules like C3b to identify and coat the host or targets [3]. This phenomenon, known as *opsonization*, leads to immune adherence followed by internalization and phagocytosis in a non-inflammatory manner [4].

Complement receptor type 1 (CR1), also called CD35, is a type I membrane-bound glycoprotein expressed on erythrocytes, monocytes, neutrophils, B cells, some T cells, and glomerular podocytes [5]; it has an important role in removing immune complexes (ICs)

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from circulation. ICs opsonized by C3b can bind and activate CR1 on the cell surface. While transiting through the liver and spleen, these ICs are removed by macrophages [6].

Macrophages in white adipose tissue of lean and obese mice and humans are selectively localized to dead adipocytes forming a so-called crown-like structure. In lean adipose tissue, the ratio of the macrophages over adipocytes is 5%, whereas during obesity it rises to 50% [7,8,9].

Adipocytes can be hundreds of times larger than macrophages, so they are too large to be digested by conventional phagocytic processes. The nature of the interaction between macrophages and apoptotic adipocytes has not been studied in detail, but it has been described in the different cellular processes, like *exophagy* [10] in which large moieties or species tightly bound to the extracellular matrix are initially digested by macrophages in an extracellular acidic lytic compartment, and macrophages engulf pieces of living adipocytes through *trogocytosis* [7].

The purpose of the present work is to build up a mechanism created with mimicking peptides based on the effect of C3b, which can opsonize unwanted cells and also activate CR1. In this way, we are looking to direct phagocytosis of adipocytes that are not in the apoptotic process (Fig. S1).

2. Materials and methods

2.1. Peptide design

Crystallographic structures of CR1 and C3b (PDB ID code:5fo9) [11] are available, which were used to locate interaction epitopes between the opsonin and receptor, and selected peptide sequences of C3b capable of bind and activate CR1. The structures were analyzed through Chimera software and PDBsum [12]. Aquaglicerol protein 7 (AQP7) was selected, because of its specificity [13], to evaluate the potential effect of the mimicking peptides to opsonize adipocytes, attract macrophages and mimic the mechanism performed by C3b. Human and mouse AQP7 were aligned with NCBI blast protein tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and then were modeled with I-Tasser [14,15], and refined with ModFOLD6 [16,17]. Firedock and Patchdock servers [18] were used to establish docking of AQP7 and different sequences of insulin peptide. All peptide structures were predicted using PEP-FOLD3 server [19].

Hybrid peptides were designed by selecting those with phagocytic activity by triggering CR1, and peptides demonstrating binding to AQP7, both with a hinge of two prolines to improve the flexibility of the synthezided molecule and, in this way, also the contact with the desired proteins. Proline and pseudoproline have shown to be superior than other alternatives, such as glycine, since they not only generate greater angles of rotation, but it is also a recurring strategy to facilitate the synthesis of long peptides by preventing aggregation, self-association and β-sheet formation [20,21,22].

2.2. Peptide synthesis

Selected peptides were synthesized by solid-phase multiple-peptide system using Fmoc amino acids (Iris Biotech) and D-biotin (Merck) to label them (0.4 meq/g of Rink Resin, Iris Biotech). Peptides were cleaved using trifluoroacetic acid–triisopropylsilane–1.2-ethandithiol–ultrapure water (TFA/TIS/EDT/H_2O), in volume proportions of 92.5, 2.5, 2.5, and 2.5, (Novabiochem, Merck Millipore). Peptides were purified by reverse-phase column chromatography C18 (Merck, Darmstad, Germany) with a 0–100% acetonitrile–water mixture gradient. Peptides were then lyophilized and analyzed using RP-HPLC (JASCO Corp., Tokyo, Japan) on a XBridge™ BEH C18 column (100 × 4.6 mm, 3.5 μm) (Water Corp., Milford, MA, USA) with a solvent B (acetonitrile with 0.05% TFA) versus solvent A (water with 0.05% TFA) 0–70% gradient, at a flow rate of 1 mL/min for 8 min at 214 nm. Chromatograms were obtained using ChromPass Chromatography Data System software (Version 1.7.403.1, JASCO Corp., Tokyo, Japan) [23].

Mass spectra of crude synthetic peptides and purified fractions were first obtained in a LCMS-2020 ESI-MS (Shimadzu Corp., Kyoto, Japan), loading 10 μL of 0.1 μg/μL peptide under positive ion mode at 4.5 kV and 350°C for 15 min. Spectra were recorded and the resulting data analyzed using the Lab Solutions 5.42 SP3 software (Shimadzu Corp.). ESI-MS was performed for monitoring the correct synthesis by identifying various positive m/z ions corresponding to the product (z = +1, +2, +3, etc.) [24].

Circular dichroism (CD) spectra were obtained at 25°C over 190–250 nm, sweep speed of 100 nm/min and optical path of 0.1 cm, using a Jasco J-815 CD Spectrometer coupled with a Jasco CDF4265/15 Peltier controller (Jasco Corp., Japan) for temperature control. In 0.1 cm cuvettes the peptide solutions were prepared at 1.0 ± 0.5 mM concentration in 30% trifluoroethanol (TFE). Spectra Measurement® and Spectra Analysis® were used to obtain spectra and data analysis [25].

2.3. Cell culture

2.3.1. Macrophage differentiation of monocytes

THP-1 human acute monocyte leukemia cells were obtained from the (American Type Culture Collection, Manassas, Virginia, USA). The cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (Dulbecco's) in 5% CO_2 at 37°C. Macrophage differentiation of THP-1 cells was induced by adding 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma, USA) or 1 μg/mL retinoic acid (RA, Merck) [26].

2.3.2. Adipocyte differentiation of preadipocytes

3T3-L1 preadipocytes (American Type Culture Collection, Manassas, Virginia, USA) were grown to confluence in DMEM–FBS (ThermoFisher) and incubated at 37°C in a 5% CO_2 atmosphere. Two days after reaching confluence (day 0), the culture medium was changed to DMEM–FBS supplemented with insulin (50 μg/mL), 3-isobutyl-1-methylxanthine (0.4 mM), and dexamethasone (1 μM) (Sigma). After two days, the medium was again changed to DMEM–FBS supplemented with insulin (50 μg/mL). Finally, three days later, the medium was changed to DMEM–FBS. Medium was replaced with fresh DMEM–FBS every three days thereafter [10,27,28].

2.4. Cell labeling and microscopy

Cells were fixed in 3% paraformaldehyde (PFA, Sigma) for 15 min, permeabilized with triton X-100 0.1% containing phosphate buffer saline (PBS) and blocked for 1 h with 4% albumin containing PBS. Then, the cells were incubated with biotin labeled peptides in PBS pH 7.2 for 12 h. For cytometry detection, Streptavidin-Alexa 647 (Thermo) was added for 1 h at room temperature (RT) and washed two times again. Nuclei were labeled with 1 μM TO-PRO-3 (Thermo) for 5 min at RT.

Samples were analyzed in a Leica TCS SP5 II spectral confocal microscope (Leica Microsystems Inc.), and the images were obtained using a Leica 40×/1.25 Oil HCX PL APO CS lens (Leica Microsystems Inc.).

2.5. Phagocytosis test

Red fluorescent latex beads (LB), polystyrene carboxylated, at 2.5% p/p (Sigma Aldrich) were coupled with streptavidin

(ProspecBio) in a one step process, by incubating 500 μL of LB 0.125 mg of Streptavidin with 1.25 mg of EDC (Thermo) in acetate buffer solution pH 5.0, 0.2 M for 2 h under rotary agitation.

Beads were subsequently spun down and washed twice in PBS in order to remove the excess of unbound streptavidin, and then resuspended at a final dilution of 0.5% in PBS. Streptavidin-coated beads were stored for up to one week at 4°C prior to use.

Two hundred microliters of streptavidin-coated beads was then conjugated with 1 mg of biotinylated peptide by incubation in phosphate PBS pH 5.5 for 30 min. Beads were subsequently spun down and washed twice in PBS in order to remove excess unbound peptide, and then resuspended at a final dilution of 0.5% in PBS.

Finally, the product latex beads–streptavidin–biotin–peptide was used to incubate them in a 96-wells plate containing macrophages THP-1 derived 4×10^4 cells/well for 1 h at 37°C with a MOI (multiplicity of infection) of 100/cell and determinate by flow cytometry the score of LB internalized and demonstrate the phagocytic activity this conjugate [29].

2.6. Co-culture

3T3-L1-GPF fibroblasts or adipocytes differentiated for ten days were stained with Bodipy 493/503 1 μg/mL for 10 min. Macrophages were layered on the top of adipocytes in a of 5:1 ratio and were cocultured for 24 h at 37°C in a 5% CO_2 atmosphere and 25 μM of hybrid peptides [30].

Phagocytic ratio was determined as foam cell formation by counting macrophages containing Bodipy labeled lipid droplets, analyzed with a Cytoflex flow cytometer (Beckman Coulter). Data were analyzed using the CytExpert 1.2 software recording at least 10,000 events for each sample.

2.7. Statistical analysis

Statistical analysis was done using R software. In spite of meeting statistical assumptions (normality and homoscedastic of samples), non-parametric hypothesis testing was performed due to the inherent small sample size of the experiments (although same conclusions resulted from parametric test). For comparisons involving more than two samples, Kruskal Wallis test was performed and if the null hypothesis (H_0) was rejected (i.e. significative difference between samples or groups), a non-parametric post-hoc analysis was performed to find the samples with differences among them [31].

3. Results

3.1. Peptide design

– CR1

Three peptides 15–18 residues long were designed, namely peptides 3687, 3688 and 3691, which belong to the MG6, MG2 and CUB C3b regions, respectively [32] (See chrystallographic structure worked in Fig. 1). Also, peptide 3931, a short sequence derivative of 3691 with 13 amino acids, was synthetized to improve the efficiency of synthesis. This consideration is relevant for the next step in the hybrid construction, in order to obtain a smaller number of epitopes able to interact with proteins in the co-culture system and lower cost of synthesis.

– AQP7

Two peptides 30 residues long from the B chain of insulin, 3689 and 3690, were defined as possible binders to AQP7. Derivative peptides: 3722 and 3727, of 15 and 24 amino acid residues each, were synthetized. See all peptide sequences in Table 1.

Predicted sequences of peptides based on C3b opsonin 3687 and 3688 resulted in a random coil with one α-helix trend of one turn. Peptides 3691 and 3931 were α-helical trend. Peptides 3722 and 3727 with AQP7 affinity were α-helical and α-helical with one β-sheet trend, respectively (Fig. S2). CD performed on the hybrid peptides confirmed their α-helical trend structure in both cases.

3.2. Cell labeling

3.2.1. Biotinylated peptide binding to CR1 receptor on THP-1

Peptide binding was demonstrated by fluorescence microscopy and flow cytometry. Only peptide 3691 showed a significant and optical difference in its fluorescence when compared with control ($p = 0.033$) because it adhered to CR1. For efficiency purposes, a small peptide of 13 residues: 3931, derived from 3691, was designed. Fluorescence intensity was similar with 3691 ($p = 0.99$) and different to control ($p = 0.47$) (Fig. 2).

3.2.2. Biotinylated peptide binding to AQP7 protein on 3T3-L1 cells

As in the previous experiment, peptide binding was demonstrated by fluorescence microscopy and flow cytometry (Fig. 3). Two peptides and the shorter sequences derived from them were tested. For efficiency purpose, shorter peptides were considered to evaluate their fluorescence. When comparing fluorescence intensity of peptides in adipocytes by flow cytometry, only peptide 3722 had statistically significant differences with respect to the control ($p < 0.01$). Peptide 3727 showed small fluorescence by microscopy but statistically was not significant with respect to the control.

3.3. Phagocytosis test

Undifferentiated cells and two conditions of differentiation were considered: THP-1 monocytes, PMA differentiated monocytes and RA differentiated monocytes in order to demonstrate phagocytosis effect in the three peptides evaluated before and each one conjugated with fluorescent LB.

RA treatment induced the highest phagocytosis level of LB by cells in 335% with the LB-3931 peptide conjugation compared with phagocytosis of control (LB without conjugated peptide). Peptide 3691 also increased phagocytosis level by 147% in this condition when compared with control. Similar results were found on THP-1 cells, which has 314% and 249% of increase when compared with control, respectively. However, when monocytes were differentiated with PMA, cells are much more activated and phagocytosis increasing is lower (27% and less). These data (Fig. 4) show that phagocytosis was highly increased when the streptavidin-LB were conjugated with biotinylated peptide 3691 and 3931 in THP-1 cells without a differentiator inductor and with RA.

3.4. Phagocytosis (foam cell formation test)

Following co-incubation of adipocytes and macrophages, Bodipy signal of lipids internalized by macrophages was quantified (Fig. 5). While foam cell formation at 2 h was equally prevalent in macrophages without peptide (control) and both hybrid peptides selected (3980 and 3981), at 24 h of incubation, there was an increase in lipid content in macrophages of 48.8% in peptide 3980 and 39.4% in peptide 3981 when compared with control. Phagocytosis increase with peptide 3980 and 3981 represents a statistically significant difference when compared with control (p-value<0.023 and p-value <0.012 respectively).

When adrenaline was added in the co-culture system, the signal was significantly higher when compared with control with both peptides: 91.2% and 89.0%, respectively when compared with con-

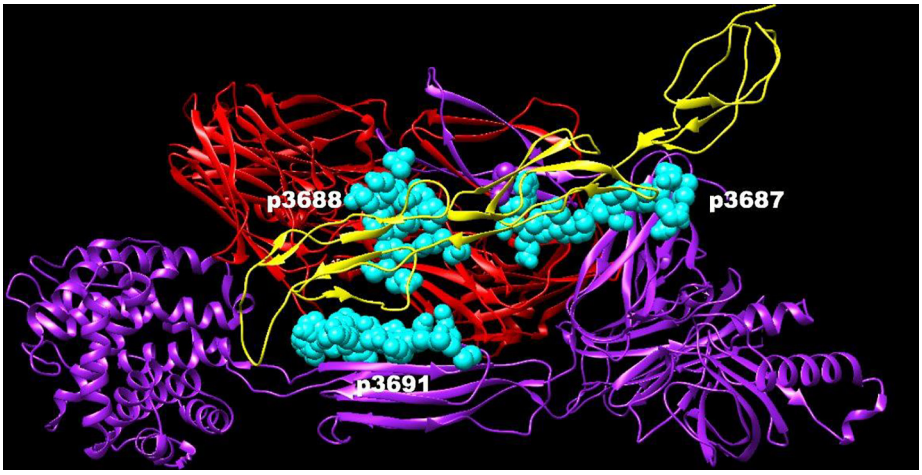

Fig. 1. Crystallographic structure of CR1 (yellow) and opsonin C3b (red: chain A, purple: chain B). Interaction sequences of C3b with CR1, and selected as peptides to be synthesized are labeled in magenta.

Table 1
Synthezised peptide sequences and molecular weights (MW).

Peptide Code	Sequence	Large	MW (g/mol)	MW (g/mol) + biotin
3687	IAEENIVSRSEFPESWL	17	2006	2232
3688	DSLSSQNQLGVLPLS	15	1558	1784
3691	RSSKITHRIHWESASLLR	18	2177	2404
3931	RSSKITHRIHWES	13	1637	1863
3689	FVNQHLTGSHLVEALYLVTGERGFFYTPKA	30	3396	3622
3690	AKPTYFFGREGTVLYLAEVLHSGTLHQNVF	30	3396	3622
3722	LTGSHLVEALYLVTG	15	1573	1799
3727	AKPTYFFGREGTVLYLAEVLHSGT	24	2657	2883
3980	RSSKITHRIHWESPPLTGSHLVEALYLVTG	30	3386	–
3981	RSSKITHRIHWESPPAKPTYFFGREGTVLYLAEVLHSGT	39	4470	–

trol, verifying the effect of adrenaline on AQP7 migration and bigger percentage of opsonization with both peptides compared with no adrenaline addition.

This cell co-culture results are consistent with the observation that designed peptides through bioinformatic tools are effective for phagocytosis cell activation not only with the standardized LB test, but with living cells too. Adrenaline effect on AQP7 can improve this effect. However, the effect caused by designed hybrid peptides is time dependent.

To determine whether peptide mediated phagocytosis is specific for adipocytes, THP-1 monocytes were co-incubated for 24 h with 3T3-L1 (GPF) cells, pre-treated with 1 μM of adrenaline for 1 h, and with 3980 or 3981 hybrid peptides (Fig. 6). Following co-incubation, green fluorescence corresponding to fibroblasts and internalized by macrophages was measured by flow cytometry. Phagocytosis mediated by peptides 3980 and 3981 did not produce a significant increase in the fibroblast phagocytosis by macrophages (Chi-square: 1.86, *p*-value: 0.39, df: 2).

These data demonstrate that cell phagocytosis could be a peptide mediated process with high efficiency and specificity for adipocytes, which does not occur when macrophages are exposed to other cell types.

4. Discussion

The approach of a model capable of compiling physiological processes in humans can be modeled *in silico* with simple bioinformatics tools.

When crystallographic structures are reported, the pathway to obtain the protein sequences with bounds is shorter. Databases allow us to evaluate both the amino acids involved in these links and the nature of them: salt bridges, hydrogen bonds or unlinked contacts. There are different effects associated with the role of C3b and CR1, so this study allowed to deduce which of the C3b and CR1 domains are involved in the activation of the phagocytic process.

Peptides 3691 and 3931, which generated favorable results in the binding and activity tests, allowed to infer that the CUB subunit of C3b is the one involved in the activation process, by connecting to the B chain of the active crystallographic structure, corresponding to the sushi 8 and sushi 9 domains from CR1 [32].

When crystallographic structures such as AQP7 do not exist, modeling and validation tools give quite estimated insights into the tertiary structure of proteins. Docking of modeled structures with proteins that have some indication of interaction, such as insulin and IgG, [3], provides an approximation of the intensity of binding. These tools allow to decide with high certainty which proteins and sequences are best candidates to be designed and synthesized.

The prediction of peptides' secondary structures gave clear indications about their nature since when checking them by CD (Figs. S3a, S3b) it was confirmed that peptides used for the generation of the hybrids, were α-helix trend as in the prediction. Both peptides 3687 and 3688 gave poor fluorescence and phagocytic activity, which correlates with the higher proportion of random coil structure they have. α-Helix secondary structures could be more effective because the C3B domains, MG2 and MG6, are also helical, so the interaction between peptides and CR1 occurs, as with opsonin, in terms of sequences and structure.

The adhesion test to target proteins was performed by labeling peptides during chemical synthesis. Use of biotin-labeled peptides

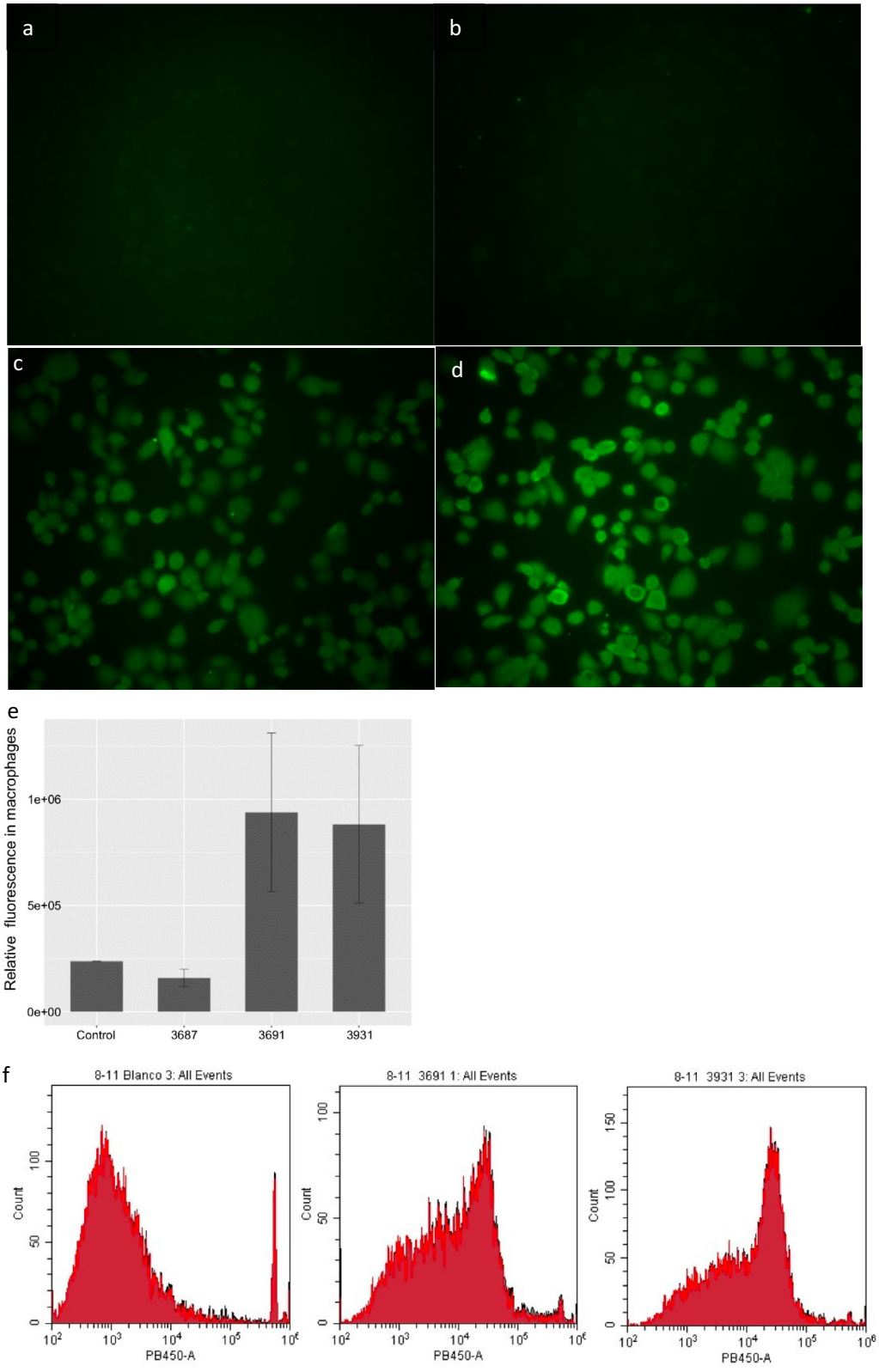

Fig. 2. Peptide fluorescence of THP-1 cells. Cells were incubated with biotin labeled peptides for 12 h. Streptavidin-Alexa405 was added for 1 h and cells were washed. (a) Control: peptide without affinity to CR1 receptor, (b–d) designed peptides 3687, 3931 and 3691. (e) Quantitative analysis of peptide binding to macrophages by flow cytometry. Data are expressed as mean ± SD of Alexa405 fluorescence intensity in macrophages of three different wells with 8000 cells counted each. When comparing samples, a statistical difference was found between all samples (F: 15.21, p-value: <0.01**, df: 3) When performing post-hoc analysis, statistical differences were found between all samples, except for control, and peptides 3687 (p value = 0.19), 3691 and 3931 (p value = 0.99). (f) flow cytometry plot depicting profile of adipocytes fluorescence (red) v/s cells count of control, peptide 3931 and peptide 3691.

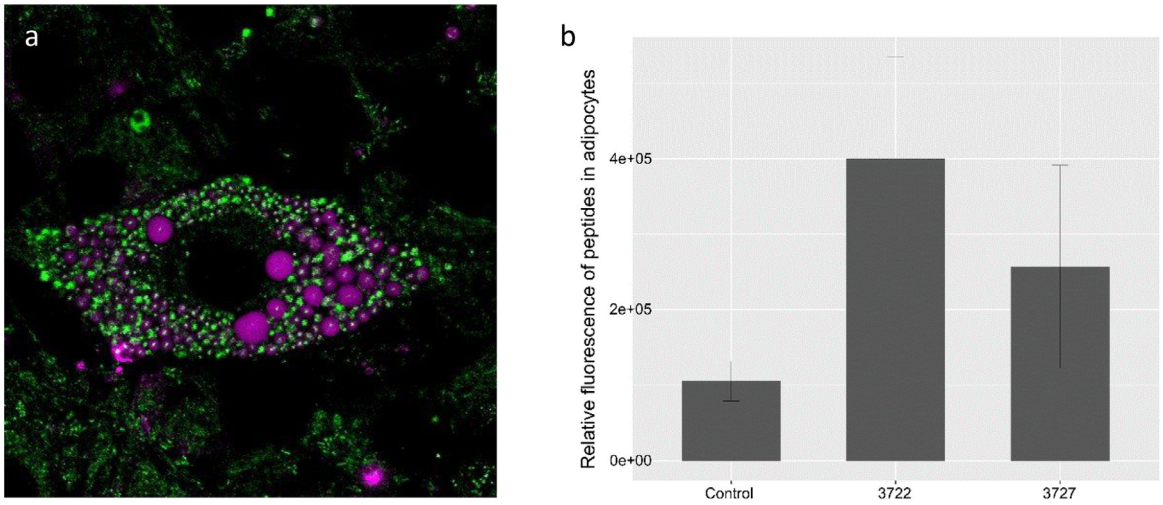

Fig. 3. Fluorescence of 3T3-L1 cells incubated with biotin labeled peptides and Streptavidin-Alexa405. (a) Confocal image of biotinylated peptide 3722 and Streptavidin-Alexa405 (green). Lipid droplets were labeled with Bodipy 493/503 (purple); (b) quantitative analysis through flow cytometry of binding of fluorescently labeled peptides to adipocytes following incubation for 12 h. Data were derived from three different wells, with more than 3000 cells each. A statistically significant difference was found between all samples (F: 12.88, p-value: <0.01**, df: 2) When performing post-tests, statistical differences were found significant only between control and 3722 (p-value <0.01). No statistically significant differences were found between other samples (Control-3727: p-value = 0.056;3727–3722: p-value = 0.17).

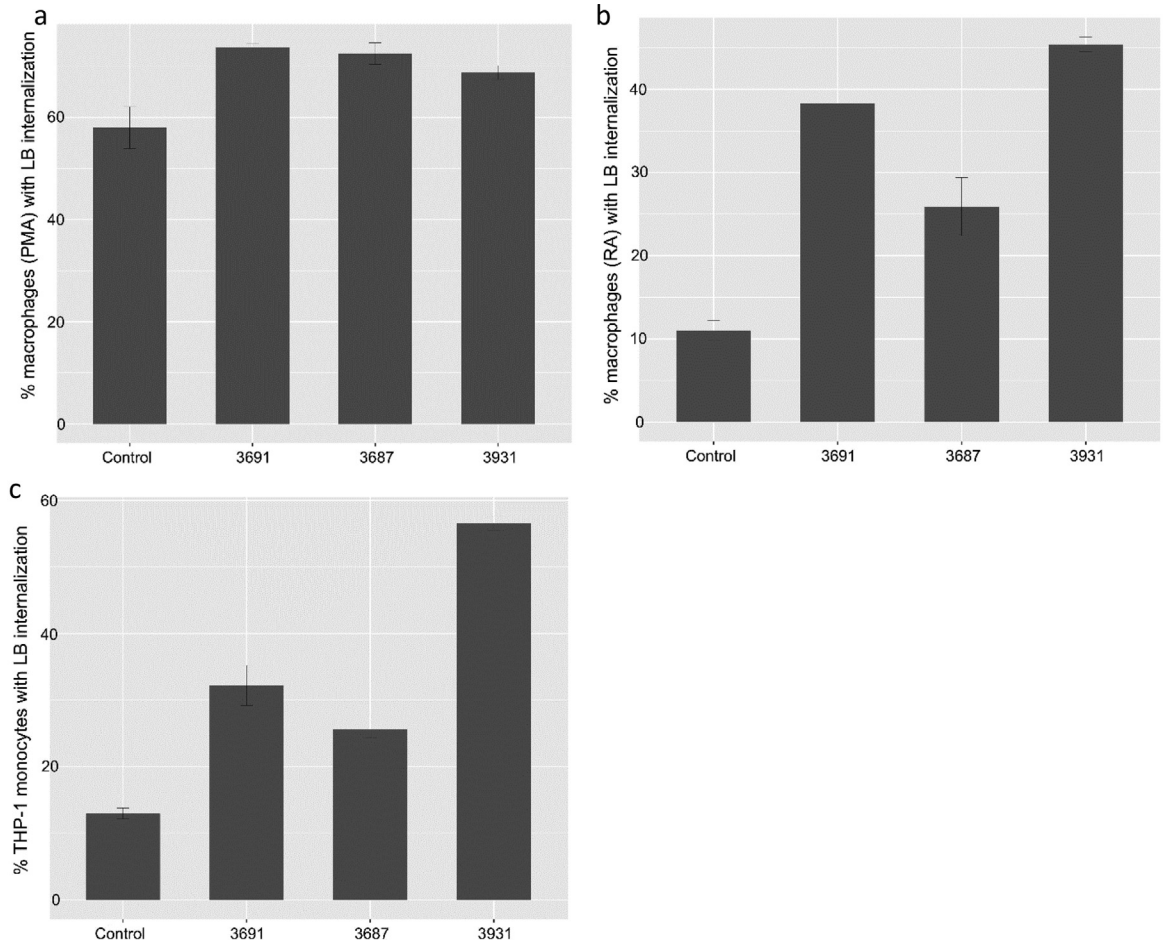

Fig. 4. Quantification of the fluorescent LB internalized after incubation for 30 minutes and MOI 100 of control: LB, LB-3691, LB-3687 and LB-3931. (a) Phagocytosis test with THP-1 monocytes in suspension, (b) phagocytosis test with PMA macrophages differentiated from THP-1 (c) phagocytosis test with RA macrophages differentiated from THP-1. Fluorescence data are from two different experiments on three plates each. Statistically significant differences between control and peptide 3691 were found (p value <0.01) in all three cell types: Monocytes, PMA and RA. Same results were found with peptide 3931 (p-value <0.01). When peptide 3687 was analyzed, smaller or no differences were found (p-value = 0.07 with PMA, 0.038 with RA and 0.05 in monocytes).

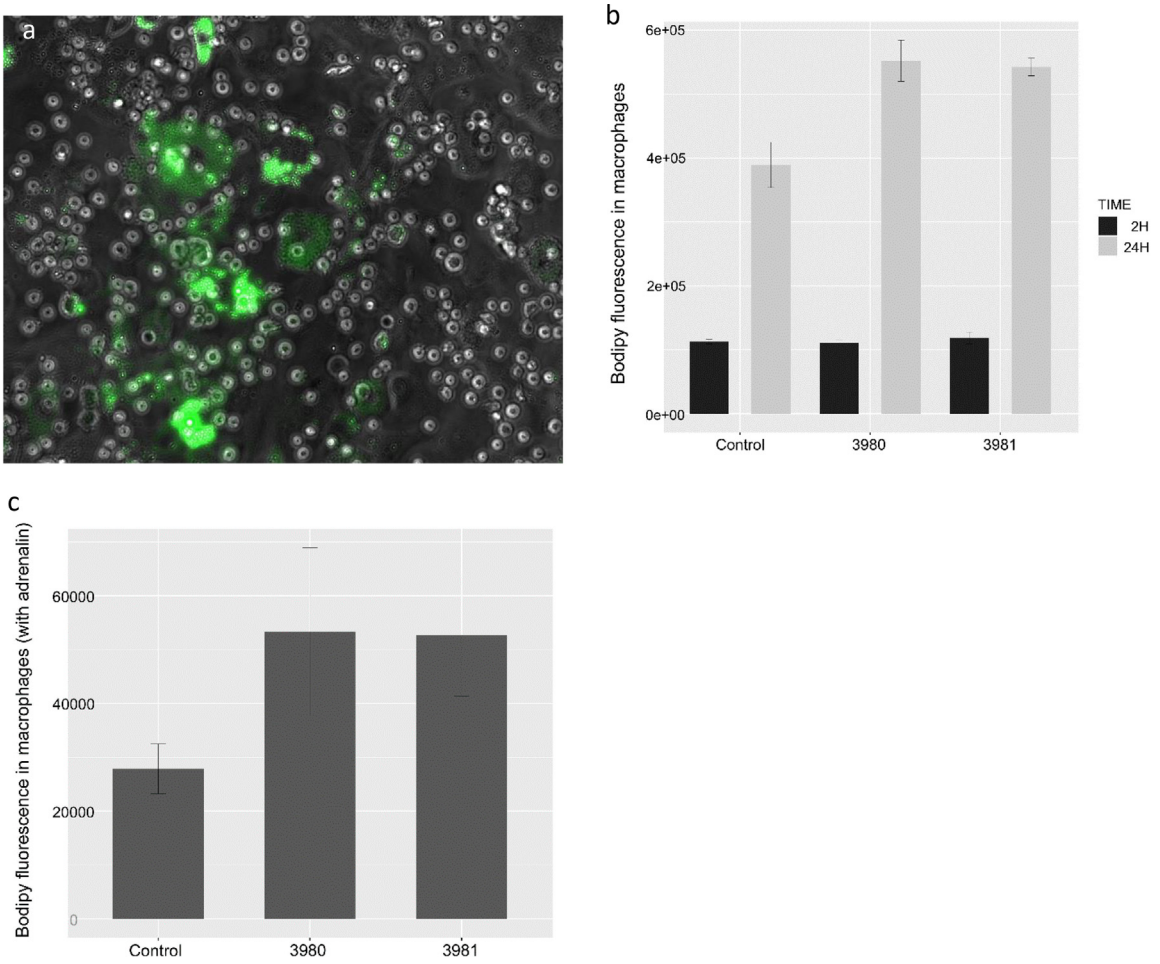

Fig. 5. (a) Fluorescence microscopy imaging of adipocytes-macrophages co-culture. Lipid droplets were stained with Bodipy (green); (b) Lipids uptake by macrophages at 2 and 24 h after co-incubation with 25 μM of hybrid peptides. When comparing samples at 2 h, no differences were founded between control and both peptides (*p*-value = 0.9046). Statistically significant differences were found between control and peptide 3980 (*p*-value: 0.023), and between control and peptide 3981 (*p*-value 0.012); (c) Bodipy signal at 24 h of co-incubation with 25 μM of hybrid peptides and 1 μM of adrenaline. A statistically significant difference was found between the samples (Chi-square: 8.72, *p*-value: 0.01, *df: 2). When performing post-hoc Kruskal Nemenyi test, statistical differences were found between Control and 3980 groups (p-value: 0.02*) and between control and 3981 peptide (*p*-value: 0.04). No statistically significant differences were found between 3980 and 3981 peptides (*p*-value: 0.957).

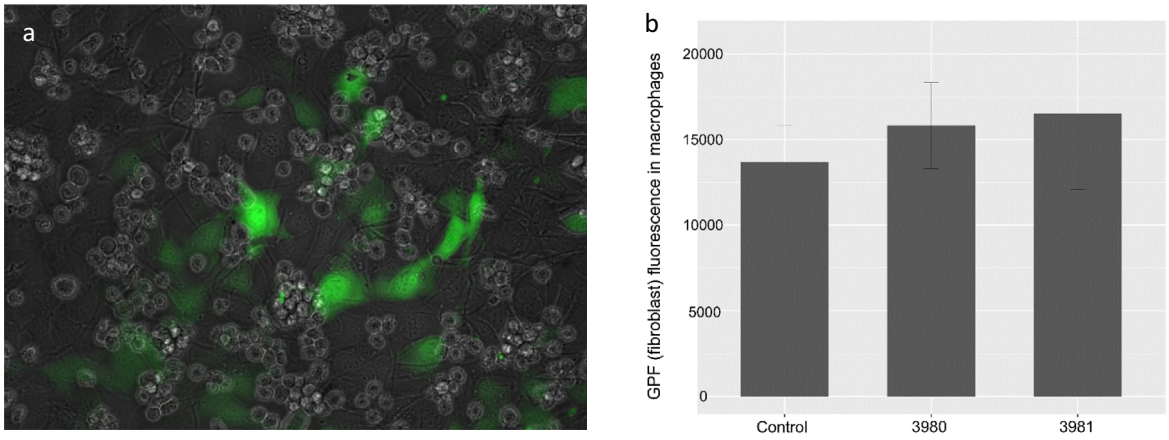

Fig. 6. (a) Fluorescence microscopy imaging of green 3T3-L1 and macrophages co-culture. (b) GPF fibroblast signal of macrophages uptake at 24 h after co-incubation with 25 μM of hybrid peptides and 1 μM of adrenalin. No statistically significant difference was found between the samples (Chi-square: 1.86, *p*-value: 0.39, df: 2).

to later incorporate streptavidin-labeled with stable fluorophores such as Alexa405 or Alexa647 allowed to obtain more robust results than those obtained with rhodamine-labeled peptides, which are less time-stable and possibly with nonspecific bindings to the ligand and binding to wells of the plates. In addition to the robustness of the technique and its greater specificity can be ascertained since the peptide's sandwich system rules out the possibility of nonspecific binding.

One of the three initial peptides designed from the C3b sequence, demonstrated binding in THP-1 cells. Peptide 3691,

which corresponded to an 18 amino acid sequence, was shortened to 13 (peptide 3931) to improve efficiency. Adhesion was also observed. However, previous structural tests are insufficient to ensure that the peptides can have the desired effect since the mechanism of activation of CR1 is unknown. On the other hand, peptides that did not adhere to cells, could activate the receptor without necessarily binding to it. That is why it is necessary to carry out activity tests.

The phagocytosis test with LB [26,33,34,35] is quite versatile and incorporates different phagocytosis mechanisms in which different receptors involved have been described: FcR, ManR, CR [26], with different activation times. In our test, LB were conjugated with peptides. Fluorescent beads were chosen to be analyzed by flow cytometry over microscopy because in this way, it is possible to obtain robust results considering a cell number greater than 5000 in each analysis, parameterizing fluorescence by number of cells.

Initially, the work was done with macrophages differentiated with PMA, however, since these cells are basally very active, the increase in phagocytosis is statistically significant even with two peptides (p-value <0.01) although difference in percentages is not considerable (19–27%) when compared with control. Since THP-1 monocytes also express CR1 and their differentiation to macrophages with RA has been reported to induce CR1 expression, the activity tests were also evaluated on both cell types: with and without RA. In these samples, more promising results were obtained because cells were less active, and it was possible to observe an increase in phagocytosis. With peptide 3691, phagocytosis ratio increases by 147% in THP-1 cells and 249% on monocytes stimulated with RA when compared with control. Peptide 3931 activate phagocytosis up to 335% on THP-1 monocytes and 314% in monocytes stimulated with RA.

Our co-culture system with THP-1 and 3T3-L1 cell lines do not belong to the same species (human and mouse respectivelly) and each one of them has different requirements for culture media, so the fact of using the RMPI medium in the co-culture may have affected in some way the behavior of adipocytes.

Since we are evaluating the phagocytosis activating effect of a peptide compared with a control, any bias introduced by the culture medium affects both conditions equally, So any significant difference in the results of both conditions cannot be attributed to the culture medium. However, this is only a model that needs to be validated *in vivo* and remove this variable.

In the case of the alignments of the murine and human AQP7 proteins, were shown to have a homology greater than 83% (Fig. S4), so we consider that this test can be extrapolated to a human system. Considerable increase in the phagocytic activity of the system with the hybrid peptides was observed in the co-culture test. Stimulation of adipocytes with adrenaline, which allowed translocation of AQP7 to the extracellular membrane [36], increased two times the phagocytic ratio. Adrenaline would allow our model to be more efficient in terms of phagocytosis activity. Adrenaline also has an important effect on macrophages by decreasing inflammatory cytokine MIP-1α (macrophage inflammatory protein 1-α) levels and increasing anti-inflammatory IL-10 citokine levels in THP-1 monocytes differentiated with LPS. This would contribute to improve the inflammatory state of fat tissue; however, there are no reports about monocyte differentiation with retinoic acid [37,38].

Co-culture performed with 3T3-L1-GPF fibroblasts and macrophages, allowed to verify the specificity of hybrid peptides, since either of them showed a significant increase in macrophage uptake fluorescence and therefore of its phagocytic activity (p-value 0.39).

These cells, even when they belong to the same line, should not express AQP7 [39], so the fact that phagocytosis is not increased, allows to consider it not only an effective system, but also specific for the designed hybrid peptides.

Future studies should focus on evaluating the model in a murine *in vivo* system initially to provide a platform for possible therapeutic action in humans.

Generation of peptides designed from bioinformatics modeling and tools are an important approach for creating biotechnological treatments, allowing new therapeutic proposals to be realized. The proposed model is capable of generating the expected effect on adipocytes and could be considered for other therapeutic purposes; however, the molecular mechanism must be studied in greater detail.

Conflicts of Interest

The authors declare no conflict of interest.

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Supplementary material

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References

[1] Allen LH, Aderem A. Mechanisms of phagocytosis. Curr Opin Immunol 1996;8:36–40. https://doi.org/10.1016/S0952-7915(96)80102-6.
[2] Ueno N, Wilson M. Receptor-mediated phagocytosis of *Leishmania*: Implications for intracellular survival. Trends Parasitol 2012;28:335–44. https://doi.org/10.1016/j.pt.2012.05.002. PMid: 22726697.
[3] Holers VM. Complement and its receptors: New insights into human disease. Annu Rev Immunol 2014;32:433–59. https://doi.org/10.1146/annurev-immunol-032713-120154. PMid: 24499275.
[4] Atkinson JP, Du Clos TW, Mold C, et al. 21 The human complement system: Basic concepts and clinical relevance. 5th ed. Elsevier Ltd; 2018;21:299–317. https://doi.org/10.1016/B978-0-7020-6896-6.00021-1.
[5] Krych M, Hourcade D, Atkinson JP. Sites within the complement C3b/C4b receptor important for the specificity of ligand binding. Proc Natl Acad Sci 1991;88:4353–7. https://doi.org/10.1073/pnas.88.10.4353. PMid: 1827918.
[6] Stoute JA. Complement receptor 1 and malaria. Cell Microbiol 2011;13:1441–50. https://doi.org/10.1111/j.1462-5822.2011.01648.x. PMid: 21790941.
[7] Sárvári AK, Doan-Xuan Q-M, Bacsó Z, et al. Interaction of differentiated human adipocytes with macrophages leads to trogocytosis and selective IL-6 secretion. Cell Death Dis 2015;6. https://doi.org/10.1038/cddis.2014.579. PMid: 25611388.
[8] Shapiro H, Pecht T, Shaco-Levy R, et al. Adipose tissue foam cells are present in human obesity. J Clin Endocrinol Metab 2013;98:1173–81. https://doi.org/10.1210/jc.2012-2745. PMid: 23372170.
[9] Lumeng CN. Adipose tissue macrophages: a piece of the PAI of metabolic syndrome. Sci Transl Med 2010;2:20ps7. https://doi.org/10.1126/scitranslmed.3000850. PMid: 20371488.
[10] Haka AS, Barbosa-Lorenzi VC, Lee HJ, et al. Exocytosis of macrophage lysosomes leads to digestion of apoptotic adipocytes and foam cell formation. J Lipid Res 2016;57:980–92. https://doi.org/10.1194/jlr.M064089. PMid: 27044658.
[11] Forneris F, Wu J, Xue X, et al. Regulators of complement activity mediate inhibitory mechanisms through a common C3b-binding mode. EMBO J 2016;35:1133–49. https://doi.org/10.15252/embj.201593673. PMid: 27013439.
[12] Laskowski RA, Jabłońska J, Pravda L, et al. PDBsum: Structural summaries of PDB entries. Protein Sci 2018;27:129–34. https://doi.org/10.1002/pro.3289. PMid: 28875543.
[13] Hansen JS, Krintel C, Hernebring M, et al. Perilipin 1 binds to aquaporin 7 in human adipocytes and controls its mobility via protein kinase A mediated

phosphorylation. Metabolism 2016;65:1731–42. https://doi.org/10.1016/j.metabol.2016.09.004. PMid: 27832861.
[14] Zhang C, Freddolino PL, Zhang Y. COFACTOR: improved protein function prediction by combining structure, sequence and protein – protein interaction information. Nucleic Acids Res 2017;45:291–9. https://doi.org/10.1093/nar/gkx366. PMid: 28472402.
[15] Yang J, Zhang Y. I-TASSER server: New development for protein structure and function predictions. Nucleic Acids Res 2015;43:174–81. https://doi.org/10.1093/nar/gkv342. PMid: 2588314.
[16] Maghrabi AHA, Mcguffin LJ. ModFOLD6: An accurate web server for the global and local quality estimation of 3D protein models. Nucleic Acids Res 2017;45:W416–21. https://doi.org/10.1093/nar/gkx332. PMid: 28460136.
[17] McGuffin LJ, Shuid AN, Kempster R, et al. Accurate template-based modeling in CASP12 using the IntFOLD4-TS, ModFOLD6, and ReFOLD methods. Proteins Struct Funct Bioinf 2018;86:335–44. https://doi.org/10.1002/prot.25360. PMid: 28748648.
[18] Schneidman-duhovny D, Inbar Y, Nussinov R, et al. PatchDock and SymmDock: Servers for rigid and symmetric docking. Nucleic Acids Res 2005;33:W363–7. https://doi.org/10.1093/nar/gki481. PMid: 15980490.
[19] Lamiable A, Thévenet P, Rey J, et al. PEP-FOLD3: Faster de novo structure prediction for linear peptides in solution and in complex. Nucleic Acids Res 2016;44:W449–54. https://doi.org/10.1093/nar/gkw329. PMid: 27131374.
[20] Jacob J, Duclohier H, Cafiso DS. The role of proline and glycine in determining the backbone flexibility of a channel-forming peptide. Biophys J 1999;76:1367–76. https://doi.org/10.1016/S0006-3495(99)77298-X.
[21] Mueller LK, Baumruck AC, Zhdanova H, et al. Challenges and perspectives in chemical synthesis of highly hydrophobic peptides. Front Bioeng Biotechnol 2020;8:1–17. https://doi.org/10.3389/fbioe.2020.00162. PMid: 32195241.
[22] Abedini A, Raleigh DP. Incorporation of pseudoproline derivatives allows the facile synthesis of human IAPP, a highly amyloidogenic and aggregation-prone polypeptide. Org Lett 2005;7:693–6. https://doi.org/10.1021/ol047480. PMid: 15704927.
[23] Ojeda N, Cárdenas C, Guzmán F, Marshall SH, Elkins CA. Chemical synthesis and *in vitro* evaluation of a phage display-derived peptide active against infectious Salmon anemia virus. Appl Environ Microbiol 2016;82:2563–71. https://doi.org/10.1128/AEM.00184-16. PMid: 26896129.
[24] Luna OF, Gomez J, Cárdenas C, et al. Deprotection reagents in fmoc solid phase peptide synthesis: Moving away from piperidine?. Molecules 2016;21:1–12. https://doi.org/10.3390/molecules21111542. PMid: 27854291.
[25] Cárdenas C, Guzmán F, Carmona M, et al. Synthetic peptides as a promising alternative to control viral infections in Atlantic Salmon. Pathogens 2020;9:1–17. https://doi.org/10.3390/pathogens9080600. PMid: 32717804.
[26] Kurynina AV, Erokhina MV, Makarevich OA, et al. Plasticity of human THP 1 cell phagocytic activity during macrophagic differentiation. Biochemistry 2018;83:200–14. https://doi.org/10.1134/S0006297918030021. PMid: 29625541.
[27] Martini CN, Brandani JN, Gabrielli M, et al. Effect of hexavalent chromium on proliferation and differentiation to adipocytes of 3T3-L1 fibroblasts. Toxicol In Vitro 2014;28:700–6. https://doi.org/10.1016/j.tiv.2014.02.003. PMid: 24576443.
[28] Kramer AH, Joos-Vandewalle J, Edkins AL, et al. Real-time monitoring of 3T3-L1 preadipocyte differentiation using a commercially available electric cell-substrate impedance sensor system. Biochem Biophys Res Commun 2014;443:1245–50. https://doi.org/10.1016/j.bbrc.2013.12.123. PMid: 24388983.
[29] Lloyd YM, Ngati EP, Salanti A, et al. A versatile, high through-put, bead-based phagocytosis assay for *Plasmodium falciparum*. Sci Rep 2017;7:14705. https://doi.org/10.1038/s41598-017-13900-4. PMid: 29089635.
[30] Harris LALS, Skinner JR, Wolins NE. Imaging of neutral lipids and neutral lipid associated proteins. Methods Cell Biol 2013;116:213–26. https://doi.org/10.1016/B978-0-12-408051-5.00011-5. PMid: 24099295.
[31] Wilcox R. Introduction to robust estimation and hypothesis testing. 3rd ed. Elsevier; 2012. p. 379–440. , https://doi.org/10.1016/B978-0-12-386983-8.00008-1.
[32] Krych-Goldberg M, Atkinson JP. Structure-function relationships of complement receptor type 1. Immunol Rev 2001;180:112–22. https://doi.org/10.1034/j.1600-065X.2001.1800110.x. PMid: 11414353.
[33] Ackerman ME, Moldt B, Wyatt RT, et al. A robust, high-throughput assay to determine the phagocytic activity of clinical antibody samples. J Immunol Methods 2012;366:8–19. https://doi.org/10.1016/j.jim.2010.12.016. PMid: 21192942.
[34] Tedesco S, De Majo F, Kim J, et al. Convenience versus biological significance: Are PMA-differentiated THP-1 cells a reliable substitute for blood-derived macrophages when studying in vitro polarization?. Front Pharmacol 2018;9:1–13. https://doi.org/10.3389/fphar.2018.00071. PMid: 29520230.
[35] Ishikawa M, Mashiba R, Kawakatsu K, et al. A high-throughput quantitative assay system for macrophage phagocytic activity. Macrophage 2018;5:1–7.
[36] Kishida K, Kuriyama H, Funahashi T, et al. Aquaporin adipose, a putative glycerol channel in adipocytes. J Biol Chem 2000;275:20896–902. https://doi.org/10.1074/jbc.M001119200. PMid: 10777495.
[37] Li CY, Chou TC, Lee CH, et al. Adrenaline inhibits lipopolysaccharide-induced macrophage inflammatory protein-1α in human monocytes: The role of β-adrenergic receptors. Anesth Analg 2003;96:518–23. https://doi.org/10.1097/00000539-200302000-00040. PMid: 12538206.
[38] Zinyama RB, Bancroft GJ, Sigola LB. Adrenaline suppression of the macrophage nitric oxide response to lipopolysaccharide is associated with differential regulation of tumour necrosis factor-α and interleukin-10. Immunology 2001;104:439–46. https://doi.org/10.1046/j.1365-2567.2001.01332.x. PMid: 11899430.
[39] Madeira A, Camps M, Zorzano A, et al. Biophysical assessment of human aquaporin-7 as a water and glycerol channel in 3T3-L1 adipocytes. PLoS One 2013;8. https://doi.org/10.1371/journal.pone.0083442. PMid: 24376702.
[40] Miyauchi T, Yamamoto H, Abe Y, et al. Dynamic subcellular localization of aquaporin-7 in white adipocytes. FEBS Lett 2015;589:608–14. https://doi.org/10.1016/j.febslet.2015.01.025. PMid: 25643985.
[41] Morgat A, Lombardot T, Coudert E, et al. Enzyme annotation in UniProtKB using Rhea. Bioinformatics 2019;36:1896–901. https://doi.org/10.1093/bioinformatics/btz817. PMid: 31688925.