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Final report

Exploring (patho)physiological role of anticholesterol antibodies and their diagnostic value in lipid raft-related diseases

Cholesterol is found in all mammalian cell membranes as a major structural component of so-called lipid rafts and caveolae. Cholesterol- and sphingolipid-rich membrane microdomains are capable of spatiotemporally organizing membrane receptors and other cell membrane components and thereby regulating many cellular processes, including signal transduction, membrane sorting and trafficking, cell polarization, immunological synapse formation, cell movement or pathogen entry (viruses, bacteria or protozoa). Pathological changes in size (in asthma, pore-forming toxins etc.) or content of lipid rafts, or particular proteins associated to them (in e.g. Alzheimer's disease (AD), viral infections) are involved in molecular mechanisms of several diseases.

Therefore, it is surprising that natural autoantibodies against cholesterol (ACHAs), with yet unknown functions, are found in sera of various mammalian species. We earlier developed monoclonal ACHAs and found that they could react with clustered cholesterol and structurally closely related sterols, but not with other lipids. They bound to lipoproteins as well as to locally clustered cholesterol (in lipid rafts and caveolae) in the cell membrane of various intact immunocytes. We found that our new anti-cholesterol antibodies have several intriguing biological activities as well, including inhibition of *in vitro* HIV-1 infection of human T- and macrophage cells, and elevation of the efficacy of yeast cells' phagocytosis and antigen presentation. The supposed mechanism behind these actions is crosslinking (clustering) of lipid rafts by these ACHA antibodies. Exploring the exact mechanism of action of ACHAs in immune cell functions and lipid raft-dependent diseases, however, requires further detailed *in vivo* studies to understand the complex role of ACHAs under normal and pathological conditions.

The aim of project was the detailed characterization of mouse monoclonal anti-cholesterol antibodies (ACHA clones AC1, AC8 and AC9) binding to antigens/cells/tissues in normal and pathological conditions (Alzheimer's disease) to improve their methodological utilization and to test their (cross-)reactivity (clones AC1, AC8 and AC9). Furthermore, the aim of using monoclonal ACHAs in *in vivo* studies was the modeling and better understanding (patho)physiological roles of natural anticholesterol antibodies present in the sera of all mammals.

Cloning of ACHAs and generation of scFv fragment

Cloning of the V_L and V_H regions of AC1, AC8, and AC9 showed that these antibodies may represent naturally occurring ACHAs. The V- and J-segments of V_L regions have 100% identity with germline genes. Furthermore, the identity of AC1 and AC8 V_H region V-segment and D-segment of all clones with germline genes is also 100%, while of AC9 is 97.92%. The least identity with germline genes is in the case of J-segments, but it did not go under 88% (*Table 1*).

As a next step, in collaboration with the Department of Biochemistry at ELTE, we planned to generate single chain variable fragment (scFv) fragment from the AC8 clone in order to map its antigen binding site by mutational analysis. Unfortunately, the purified a monobiotinylated scFv quickly aggregated and precipitated, which instability is a frequent problem with scFv's. Therefore, we moved forward to examine binding properties of ACHA clones by antigen microarray.

Vl analysis	AC1	AC8	AC9
V-gene and allele (identity: %)	IGKV8-30*01 (100%)	IGKV9-124*01 (100%)	IGKV16-104*01 (100%)
J-gene and allele (identity: %)	IGKJ2*01 (100%)	IGKJ1*01 (100%)	IGKJ5*01 (100%)
Vh analysis	**AC1**	**AC8**	**AC9**
V-gene and allele (identity: %)	IGHV2-9-1*01 (100%)	IGHV5-9-1*01 (100%)	IGHV1-4*01 (97.92%)
J-gene and allele (identity: %)	IGHJ4*01 (94.44%)	IGHJ4*01 (88.89%)	IGHJ1*02 (98.08%)
D-gene and allele (frame)	IGHD1-2*01 (2)	IGHD1-2*01 (2)	IGHD1-1*01 (3)

Table 1. Sequence analysis of ACHA antibody clones. The sequence of AC1, AC8, and AC9 monoclonal ACHAs was compared with germline VH genes by using the international ImMunoGeneTics information system/2QUEry and Standardization (IMGT/V-Quest).

Antigen microarray

Protein arrays, including antigen microarrays, are emerging new tools not just in proteomics, but glycomics, lipidomics, and are also important for immunological research. We published a topical review on this subject to summarize applicability of (fluorescent) microarrays in immunoassays (Herbath et al., Methods Appl Fluoresc, 2014). In this project, antigen microarray analysis showed differential binding of ACHAs to cholesterol, LDL and HDL lipoproteins. While AC1 and AC8 are better in binding to crystalline cholesterol than AC9, AC1 and AC9 have higher affinity to LDL and HDL than AC8 (*Figure 1*). Similar results were obtained for lipoproteins by ELISA method. Furthermore, oxidation of LDL resulted in enhanced binding of AC1 and AC8 to the coated antigen (*Figure 2*). This finding raises the possibility that ACHAs may have a role in cholesterol metabolism and/or atherosclerosis *in vivo*.

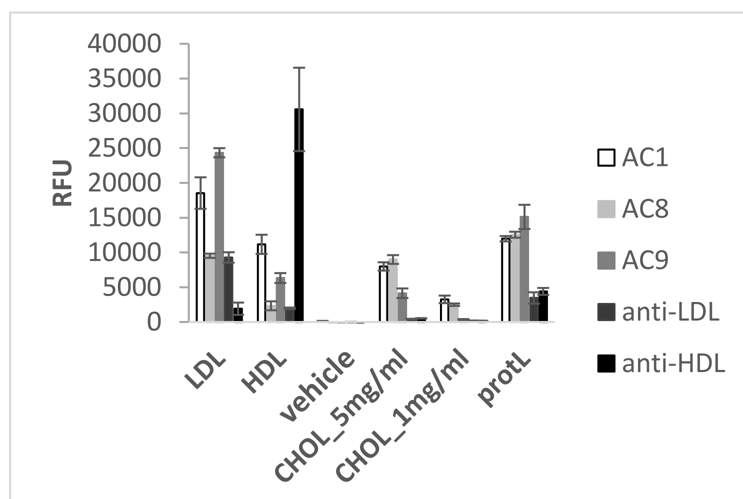

Figure 1. Differential binding of ACHAs to cholesterol and lipoproteins by antigen microarray. Cholesterol (Chol), LDL, HDL, vehicle, and proteinL (protL) were printed onto nitrocellulose membranes in triplicates and membranes were probed with ACHA monoclonal antibodies (AC1, AC8, AC9), monoclonal anti-LDL or anti-HDL. Their binding was detected by a goat-anti-mouse IgG+M-Alexa488 secondary antibody. Mean relative fluorescence units (RFU) are displayed. A representative result of three independent measurements is shown.

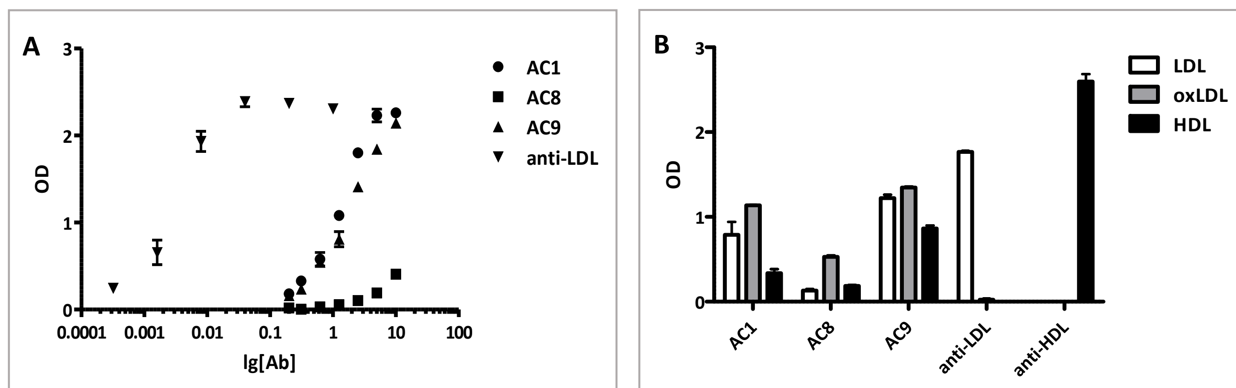

Figure 2. Differential binding of ACHAs to lipoproteins by ELISA. LDL (A, B), oxidized LDL (B), or HDL (B) was coated onto polystyrene plate in duplicates and then was probed with ACHA monoclonal antibodies (AC1, AC8, AC9), anti-LDL, or anti-HDL. Their binding was detected by a goat-anti-mouse IgG+M-HRP secondary antibody. Mean optical density (OD) values are displayed. A representative result of three independent measurements is shown.

Cross-reactivity of ACHAs to antigens selected by the BLAST analysis or some lipid components was also examined by antigen microarray. AC9 and AC1 have higher cross-reactivity to the printed lipid antigens. The cross-reactivity of all ACHAs to ssDNA may highlight that cholesterol and ssDNA share structurally common epitopes for anticholesterol antibodies.

ACHAs could bind to cell lysates printed onto nitrocellulose membranes as well, but when cholesterol was extracted by lipid extraction method, ACHA binding was decreased to the background level. This might be due to improper clustering of cholesterol, which is an important factor in recognition by the antibody. Another possibility is that cholesterol, in the lipid extract, is hindered by other lipids interfering with the accessibility of the small epitope. Nevertheless, since sensitive cholesterol measuring methods became available on the market (Amplex Red Cholesterol Assay Kit, Competitive Cholesterol ELISA kit), we concentrated on the detection of clustered cholesterol found in various biological samples.

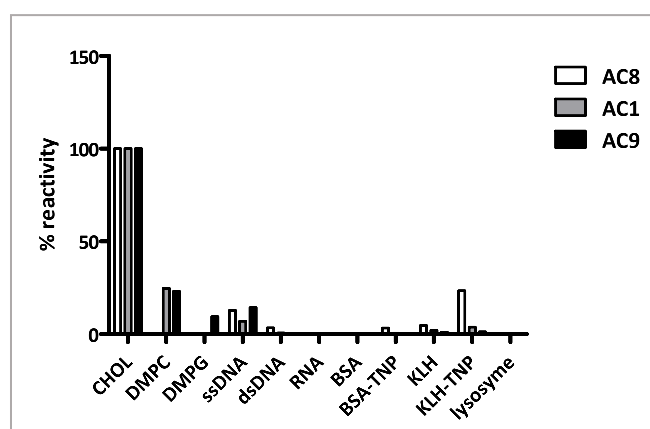

Figure 3. Cross-reactivity of ACHAs to various antigens by antigen microarray. Cholesterol (Chol), dimyristoyl-phosphocholine (DMPC), dimyristoyl-phosphoglycerol (DMPG), single and double-stranded DNA (ssDNA, dsDNA), RNA, BSA, BSA-trinitrophenyl (BSA-TNP), keyhole limpet hemocyanin (KLH), KLH-TNP, and lysozyme were printed onto nitrocellulose membranes in triplicates and membranes were probed with ACHA monoclonal antibodies (AC1, AC8, AC9). Their binding was detected by a goat-anti-mouse IgG+M-Alexa488 secondary antibody. Percent of reactivity (cholesterol was 100 %) is displayed. A representative result of two independent measurements is shown.

Binding of ACHAs to tissues, cells and extracellular vesicles

Fluorescence immunohistochemistry experiments show that ACHA antibodies bind to murine skeletal muscle and to the brain. In case of skeletal muscle, AC8 (mouse IgG3) binding pattern is similar to what was observed previously for an IgM monoclonal ACHA by another group. Ethanol or acetone fixation reduced AC8 binding since these solvents extract cholesterol from the tissue (*Figure 4*). It was published that cholesterol recognition by filipin III or perfringolysin O derivative (theta-toxin) in the brain is very weak normally, but cholesterol accumulation/deposition (e.g. Nieman-Pick disease, AD) can be observed by staining of brain slices with this cholesterol-binding molecules. No data is reported for ACHA staining of the brain, so far. We observed AC8 binding to normal murine brain and showed a differential binding pattern for different brain regions. In addition, enhanced AC8 staining was detected in amyloid plaques of APP/PS1 mouse, a model for AD. AC8 staining partially overlapped with Iba1 or GFAP staining (*Figure 5*). Iba1 is a microglia marker, while GFAP is an astrocyte marker; and both cell types are enriched in amyloid plaques. Therefore, the enhanced AC8 binding might be partly due to the accumulation of these cells around plaques, and partly because of the lipid accumulation in plaques derived from the necrotic/apoptotic cells. We collaborated with Dr. Zsolt Borhegyi in the AD experiment, who gave the mice and his assistance.

For the evaluation of the ACHA binding to neurons, we examined its binding to primary mouse hippocampal neuron cultures unfixed or fixed with 4% paraformaldehyde. We found staining of dendrites and axons and the soma plasma membrane, which is in accordance with the literature claiming that lipid rafts are important platforms of neuron signaling. It is worth to note that paraformaldehyde fixation led to increased staining by ACHA of these cells, probably due to the clustering effect of the used fixation method.

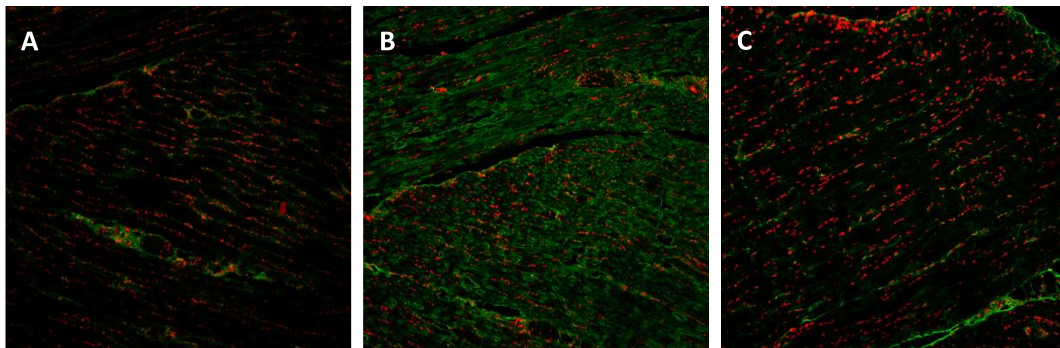

Figure 4. Fluorescent micrograph of AC8 staining in a frozen cross-section of murine skeletal muscle. 8 μm frozen sections were fixed with 4% paraformaldehyde (A, B) or ethanol (C), then probed with mIgG3 isotype control (A) or AC8 (B, C). Antibody binding was detected by a goat-anti-mouse IgG+M-Alexa488 secondary antibody (green). Nuclei were counterstained with propidium iodide (red) (20x objective, representative images).

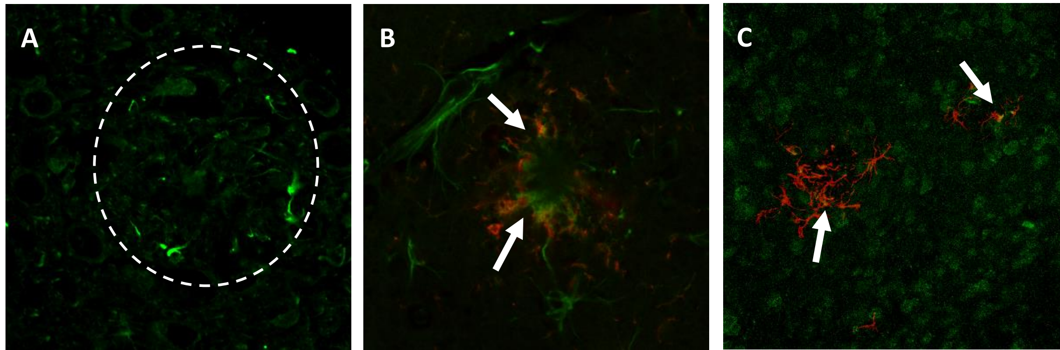

Figure 5. Fluorescent micrograph of AC8 staining in a vibrosections of mouse APP/PS1 brain. 30 μm vibrosections were probed with AC8 (green, A-C), Iba1 (red, B), or GFAP (red, C). Antibody binding was detected by a goat-anti-mouse IgG3-Alexa488 secondary antibody for AC8, a goat-anti-rabbit IgG-Alexa594 secondary antibody for Iba1, and a goat-anti-mouse IgG1-Alexa647 secondary antibody for GFAP. Dotted line delimits an amyloid plaque. White arrows indicate spatial overlapping of fluorescent signals in the double-labeled samples (40x objective, representative images).

Cholesterol is found not just in plasma membranes of various cells and in lipoproteins, but in membrane bilayer of extracellular vesicles (EVs) (apoptotic bodies, microvesicles, and exosomes) as well. EVs are important in intercellular communication and they are increasingly explored in various fields of biology, including tumor biology or immunology. The research group of Prof. Edit Buzás (Semmelweis University), searching for markers of extracellular vesicles, characterized the lipid composition and lipid order of cell-derived vesicles with our assistance and antibody. We found that AC8 antibody that recognizes clustered cholesterol, enriched in lipid rafts, showed relatively weak binding to all EV subpopulations with exosomes showing the strongest staining (Osteikoetxea et al., PLos One, 2015). Furthermore, microvesicles and apoptotic bodies were found to be more sensitive to detergent lysis than exosomes, which is due probably due to their differential lipid composition (Osteikoetxea et al., Organic & Biomolecular Chemistry, 2015). A workgroup from Dublin also asked for our antibody AC8, in order to stain microvesicles that have been produced after LPS stimulation of human macrophages. They have assessed this single staining by flow cytometry and reported to us that the staining worked well. Exosomes and microvesicles contain various biomolecules, including galectins, in order to function as carriers and intercellular messengers. Earlier, we collaborated with Dr. Gabor Nandor (MTA-TTK) and showed that placental galectin-13 (also known as placental protein 13/PP13) may also translocate with different EVs through the syncytiotrophoblast membrane in a lipid raft-dependent manner. As a continuation, in this project we published a review on the important immunoregulatory role of PP13, protecting pregnancy (Than et al., Front Immunol, 2014)

Role of anticholesterol antibodies in cholesterol metabolism

Based on the differential binding of ACHAs to lipoproteins it is an interesting question whether they could have a role in cholesterol metabolism. Indeed, several workgroups suggested this hypothesis, but no direct evidence has been found yet. We first studied the influence of ACHAs on the uptake of LDL and oxLDL. Interestingly, we found that AC1 and AC8, but not AC9 or a murine anti-LDL antibody could inhibit uptake of fluorescent LDL and oxLDL by macrophages (*Figure 6*). Interestingly, although AC8 has the lowest affinity to LDL, in overall, it has the highest capacity to inhibit LDL internalization. This may reflect the capability of ACHAs to influence/control cholesterol uptake of peripheral cells.

We also studied whether ACHAs can influence serum level of LDL *in vivo*. Therefore, mice were intraperitoneally injected with AC8 (75μg/dose) or PBS for 4 weeks and, after serum collection, ACHA and lipoprotein levels were measured. Serum IgG ACHA level was significantly elevated in AC8-treated mice, compared to controls. Furthermore, VLDL/LDL but not HDL concentration was significantly decreased upon AC8 treatment (PBS-treated: 39.24±8.91 μg/ml, AC8-treated: 23.36±3.69 μg/ml, $p<0.05$). A negative correlation was observed between ACHA level and VLDL/LDL concentration (*Figure 7*). In contrast, when mice were kept previously on high cholesterol diet for 4 weeks before injection of ACHAs (200μg/dose), AC1 treatment elevated serum level of VLDL/LDL compared to isotype control treatment. AC8 also elevated, but this did not reach statistical significance (*Figure 8*). Therefore, under physiological cholesterol levels, enhanced clearance of LDL with the help of ACHAs may explain the observed decrease in VLDL/LDL. However, under pathological conditions, ACHAs may further worsen the disease, since they can bind to atherosclerotic plaques, activate the complement system, and induce inflammation.

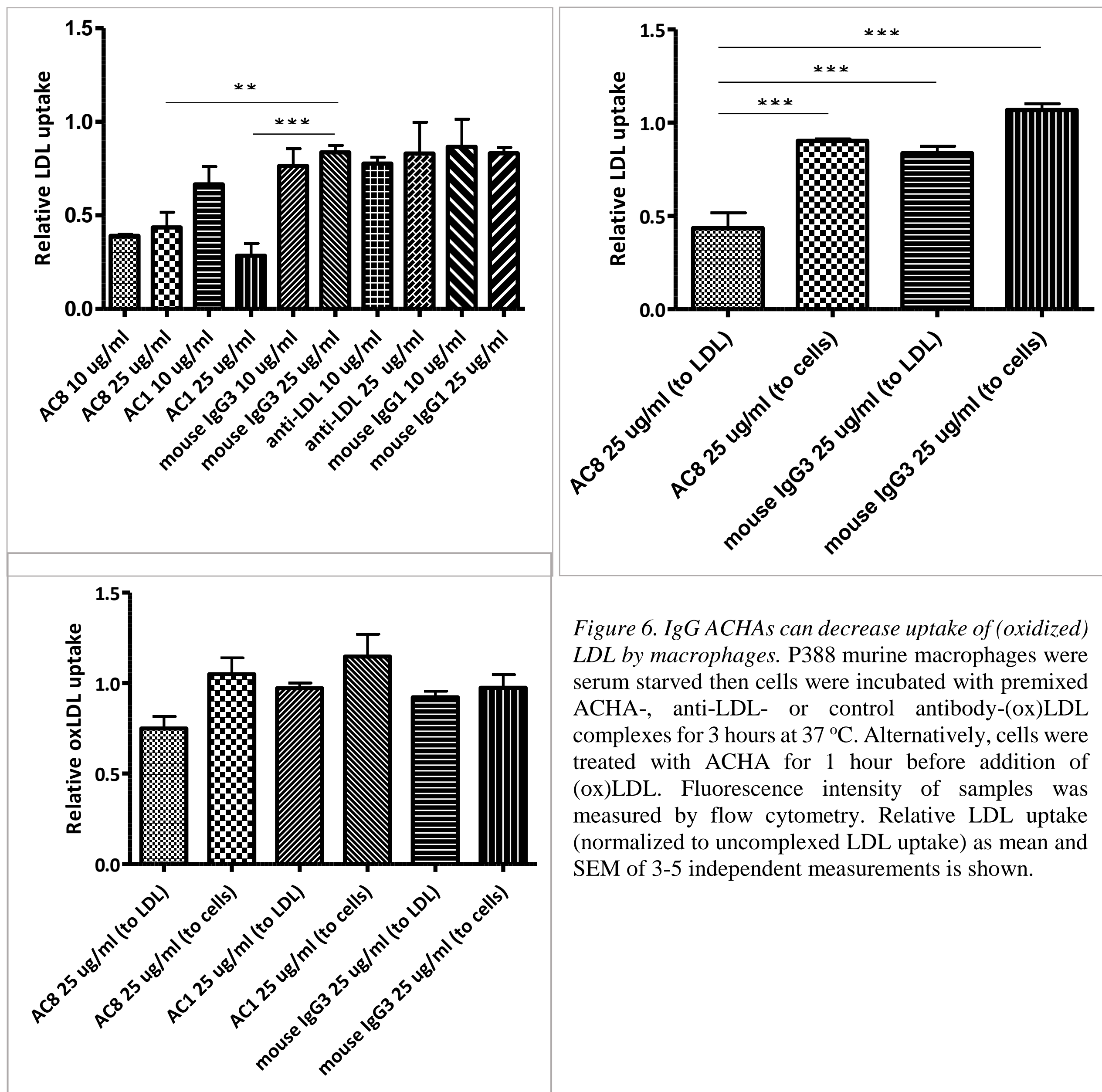

Figure 6. IgG ACHAs can decrease uptake of (oxidized) LDL by macrophages. P388 murine macrophages were serum starved then cells were incubated with premixed ACHA-, anti-LDL- or control antibody-(ox)LDL complexes for 3 hours at 37 °C. Alternatively, cells were treated with ACHA for 1 hour before addition of (ox)LDL. Fluorescence intensity of samples was measured by flow cytometry. Relative LDL uptake (normalized to uncomplexed LDL uptake) as mean and SEM of 3-5 independent measurements is shown.

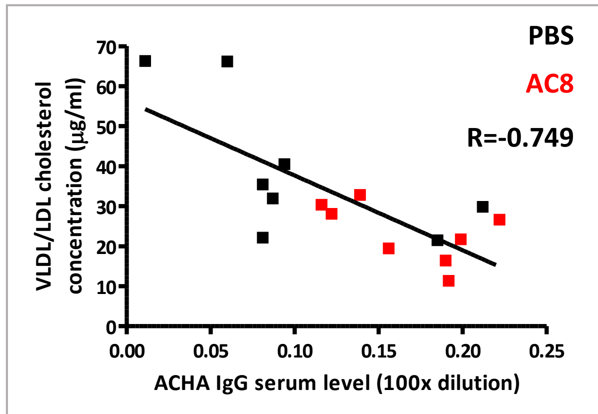

Figure 7. AC8 can decrease VLDL/LDL level in vivo in correlation with serum ACHA level. Balb/c mice were intraperitoneally injected with AC8 or PBS for 4 weeks and, after serum collection, ACHA level mas measured by ELISA and lipoprotein level was measured by a fluorimetric kit (Amplex Red).

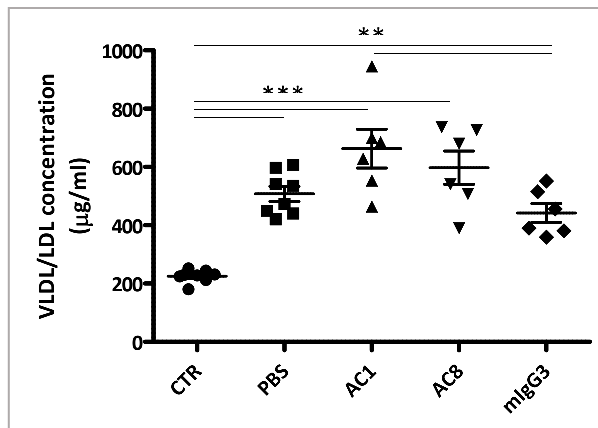

Figure 8. ACHAs increase VLDL/LDL level in vivo in mice on high cholesterol diet. C57BL/6 mice were kept on high cholesterol diet for 8 weeks. AC1, AC8, IgG3 or PBS was injected intraperitoneally in the second half of the diet (4 weeks). After serum collection, lipoprotein level was measured by a fluorimetric kit (Amplex Red).

Novel entry mechanism of estradiol, a sex steroid derived from cholesterol, into leukocytes

Cholesterol is an important precursor for sex steroids, including estradiol. There is a growing body of evidence that estradiol can act on various immune cells and regulate their development, activation, and apoptosis. However, the role of sex hormone binding globulin (SHBG) in the regulation of these actions of estradiol is controversial. Moreover, the expression of SHBG and its internalization by potential receptors, as well as the influence of SHBG on estradiol uptake and signaling in leukocytes has remained unexplored. We found that human and murine lymphocytes and monocytes bound and internalized external SHBG (*Figure 8*). Furthermore, cell surface-bound SHBG was detected in close proximity to membrane ERs while highly colocalizing with lipid rafts. The SHBG-membrane ER interaction was found functional since SHBG promoted estradiol uptake (*Figure 9*) by lymphocytes and subsequently influenced Akt phosphorylation, included in the rapid non-genomic estradiol signaling pathway (Balogh et al., Sci Rep, submitted). Our results clearly show that cell surface binding and internalization of SHBG-E2 into leukocytes may modulate E2-mediated signaling, highlighting a novel, SHBG/R_{SHBG}-dependent entry pathway of E2 in these cells. We suggest that this novel pathway and the already described free and mER-mediated entry of E2 into target cells are not mutually exclusive. Cooperation of various mERs with each other and with classical ERs may result in a fine balancing of entry modes and subsequent immunomodulatory effects.

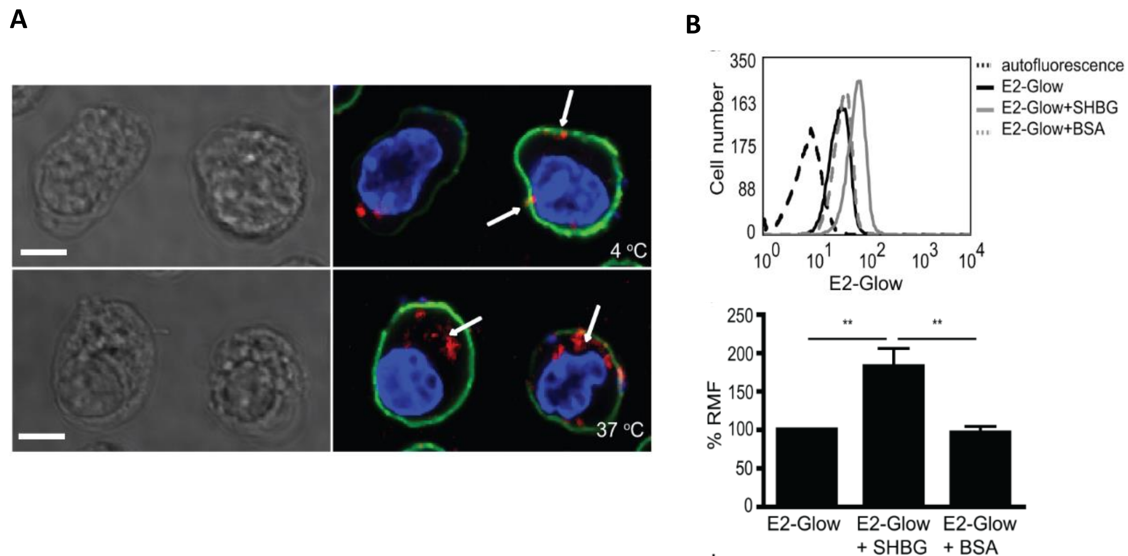

Figure 9. SHBG is internalized into lymphocytes and promotes uptake of estradiol (A) Binding (upper panel) and internalization (lower panel) of SHBG-CF633 into A20 murine B cells were visualized by confocal microscopy. Images demonstrate mainly plasma membrane localization (white arrows) of SHBG-CF633 (red) at 4 °C (top), while its internalization (white arrows) occurred at 37 °C (bottom). Cholera toxin-B-Alexa488 (green) and Hoechst 33342 (blue) served for counterstaining plasma membranes and nuclei, respectively. Scale bars: 5 µm. Data are from three independent measurements. (B) A20 cells were incubated with 10 nM E2-Glow previously mixed or not with 50 nM SHBG or BSA, and fluorescence was detected by flow cytometry. Representative flow cytometric histograms are displayed (upper panel). Overall flow cytometric data on cellular uptake of E2-Glow in the presence or absence of SHBG or BSA is represented as mean and standard error of mean (SEM) values (lower panel). RMF: ratio of the mean fluorescences of E2-Glow and autofluorescence.

Summary

In summary, we were able to improve the methodological utilization of our monoclonal ACHAs since we could use them in antigen microarray, we could detect cholesterol in various tissues, accumulation of cholesterol in amyloid plaques in the brain of mice with Alzheimer’s disease, as well as detected clustered cholesterol in the membrane of extracellular vesicle populations. Furthermore, our *in vitro* and *in vivo* findings show that ACHAs may influence cholesterol metabolism by modulating VLDL/LDL level under physiological and pathological conditions.

Scientific articles that are already published as a result of the project:

*1: Herbáth M, Papp K, **Balogh A**, Matkó J, Prechl J. Exploiting fluorescence for multiplex immunoassays on protein microarrays. Methods Appl Fluoresc. 2014, 2(3):032001.*

*2: Than NG, **Balogh A**, Romero R, Kárpáti E, Erez O, Szilágyi A, Kovalszky I, Sammar M, Gizurarson S, Matkó J, Závodszky P, Papp Z, Meiri H. Placental Protein 13 (PP13) - A Placental Immunoregulatory Galectin Protecting Pregnancy. Front Immunol. 2014, 5:348*

*3: Osteikoetxea X, **Balogh A**, Szabó-Taylor K, Németh A, Szabó TG, Pálóczi K, Sódar B, Kittel, Á, György B, Pállinger É, Matkó J, Buzás, EI. Improved characterization of EV preparations based on protein to lipid ratio and lipid properties. PLoS One. 2015, 10(3):e0121184. (OTKA-PD grant number has not been indicated)*

4: Osteikoetxea X, Sódar B, Németh A, Szabó-Taylor K, Pálóczi K, Vukman KV, Tamási V, ***Balogh A****, Kittel, Á, Pállinger É, Buzás, EI. Differential detergent sensitivity of extracellular vesicle subpopulations. Organic & Biomolecular Chemistry. 2015, 13(38):9775-82.*

Manuscripts just submitted or in preparation:

1: ***Balogh A****, Kárpáti E, Schneider AE, Hetey Sz, Szilágyi A, Juhász K, László G, Hupuczi P, Závodszky P, Papp Z, Matkó J, Than NG. Sex hormone binding globulin provides a novel entry pathway for estradiol and influences subsequent signaling in leukocytes via membrane receptor. Scientific Reports. 2018 (submitted).*

2: Szenes É, László G, Tóvári J, Matkó J, ***Balogh A****. Exploring the role of anticholesterol antibodies in cholesterol metabolism (2018, in preparation).*

3: Szenes É, Borhegyi Zs, László G, Matkó J, ***Balogh A****. Detection of cholesterol in the murine brain in Alzheimer's disease (2018, in preparation).*