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CARDIOVASCULAR RESEARCH Onlife

LDL's unexpected travel partners in the road to atherosclerosis

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Commentary on 'LDL delivery of microbial small RNAs drives atherosclerosis through macrophage TLR8' by R.M. Allen *et al.*, *Nature Cell Biology*, https://doi.org/10.1038/s41556-022-01030-7.[1]

Atherosclerosis is a complex disease that is characterized by the accumulation of fatty deposits in arteries. Atherosclerosis is the pathophysiological base for most forms of cardiovascular disease (CVD).[2] The disease is driven by a large number of known factors, including inflammation, oxidative stress, and metabolic dysfunction, and many more to be unravelled. In recent years, there has been a growing interest in developing new therapeutics for atherosclerosis that targets some of the underlying molecular mechanisms of the disease. One promising approach involves targeting the RNA cargo of lipoproteins such as the HDLs[3,4] and LDL,[5] the latter being identified to play a role in promoting inflammation and immune cell activation in atherosclerosis. Of note, inflammation plays a key role in the initiation and progression of atherosclerosis[6] partly, driven by immune cells including monocyte-derived plaque resident macrophages.[6,7] In response to the accumulation of lipoproteins within the arterial wall, macrophages become activated and secrete inflammatory cytokines that promote the recruitment of additional immune cells to the site of injury. However, thanks to the sequencing technology advances, subpopulations of macrophages have been identified, and the picture is not black or white anymore. Depending on the environment, tissue, cavity, or fluid, macrophages can perform a specific function, regardless of the pre-established anti- or pro- inflammatory markers.[8] Recent research has focused on understanding the profiling of macrophages and the molecular mechanisms that trigger the activation of macrophages in atherosclerosis, with the aim of identifying new targets for the treatment of the disease. A comprehensive study by Allen *et al.*[1] investigated the role of microbial small RNAs (msRNAs) on LDL in promoting macrophage inflammatory activation through the Toll-like receptor 8 (TLR8) pathway and promoting the development of atherosclerosis.

In atherosclerosis, the accumulation of small, dense, and oxidized LDL particles in atherosclerotic plaques is known to promote the formation of foam cells through fluid-phase pinocytosis.[4,7] However, the impact of the native LDL (nLDL) cargo on macrophages populations phenotype and its role in inflammation remain unclear. In the research article, the authors aimed to understand the impact of nLDLs on macrophages. To this end, human THP-1 macrophages were exposed to nLDL, and the resulting changes in gene expression were analysed using high-throughput RNA-sequencing (RNA-seq). The results showed that nLDL exposure up-regulated 365 genes and down-regulated 141 genes, and many of these genes were linked to the transcription factors NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and C/EBPβ (CCAAT/enhancer-binding protein beta), known regulators of inflammation and atherosclerosis. Furthermore, nLDL incubation resulted in the activation of macrophage cytokine expression and secretion through NF-κB apart from the already widely reported apolipoprotein B accumulation. The study's findings also highlight the importance of macrophage polarization in atherosclerosis. Intriguingly, primary monocytes differentiated to alternatively activated macrophages [macrophage colony-stimulating factor, interleukin (IL)-4, and IL-13] did not induce IL-1β or IL-6 expression in response to nLDL exposure but repressed tumour necrosis factor-α. Noteworthy, this investigation used high-throughput RNA-seq and network analysis to understand the impact of nLDL on *in vitro* macrophages allowing a comprehensive examination of gene expression changes and the identification of potential regulatory pathways.

Mechanistically, TLRs are part of the innate immune system, which recognizes pathogen-associated molecular patterns (PAMPs). The recognition of PAMPs in atherosclerosis has been extensively studied, and it is known that TLR2, TLR4, and TLR6 are involved in the recognition of modified LDL particles, such as oxidized LDL.[6,9] However, the involvement of TLR8 in nLDL-induced polarization has not been fully understood. The authors discovered that nLDL activates TLR8 *in vitro*, resulting in the activation of NF-κB signalling pathway, which leads to the secretion of cytokines by macrophages. The activation of TLR8 was shown to be necessary and sufficient for the nLDL-induced activation of NF-κB in human macrophages. The study's findings suggest that targeting TLR8 may hold potential for therapeutic development in the treatment and prevention of CVD and other diseases featuring hyperlipidaemia and chronic inflammation.

In line with the authors previous findings,[10] they propose LDL-msRNA cargo as a plausible explanation of TLR8 activation in macrophages. The authors previously reported that that lipoproteins of mice are enriched with msRNAs that are probably derived from microbes of internal and external environment,[10] and they have proved that nLDL contain high levels of msRNA responsible for activating macrophages in atherosclerosis.

As a potential therapeutic solution, the authors focus on the small RNA (sRNA) mechanisms as a new way of blocking inflammation in macrophages implicated in atherosclerosis. Until now, efforts have been focused in designing lipidic nanoparticles enriched with sRNAs;[11] however, the authors used non-targeting (scrambled) locked nucleic acid (LNA) oligonucleotides (nt-LNA) to antagonize TLR8 signalling induced by nLDL. The authors first screened commercially available LNA and identified the nt-LNA as a molecule effective in inhibiting the TLR8 pathway. Additionally, nt-LNA treatment was able to reduce inflammatory responses in macrophages and inhibit nLDL-induced cytokine production in primary human macrophages. Finally, the study investigated the effects of nt-LNA on atherosclerosis in mice, validating a reduction in the development of atherosclerosis in mice fed with Western diet. They performed scRNA-seq from atherosclerotic plaque samples to identify specific macrophage phenotypes, including

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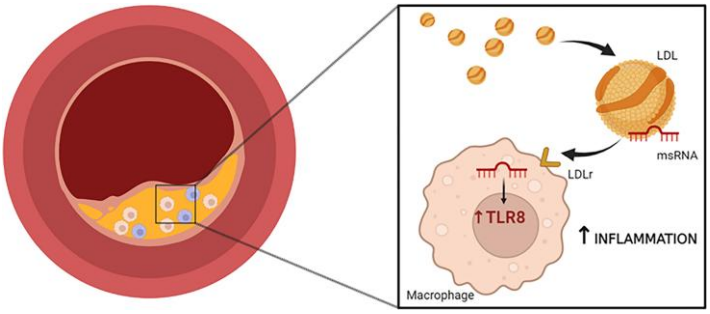

Figure 1 Depicts the interaction and exchange of signalling between various components within the atherosclerotic plaque, including LDL and macrophages. Researchers have discovered that msRNAs are abundant on LDL and can activate TLR8, leading to pro-inflammatory macrophage polarization and cytokine secretion. The use of non-targeting locked nucleic acids to competitively antagonize TLR8 prevented macrophage polarization induced by native LDL *in vitro* and re-organized lesion macrophage phenotypes *in vivo*, as identified by single-cell RNA sequencing. These results suggest that LDL-msRNA instigates inflammation associated with atherosclerosis and supports alternative functions of LDL beyond cholesterol transport.

inflammatory, foamy, cavity, proliferating, repair, and two clusters of resident-like macrophages. Supporting the presence of both pro- and anti-inflammatory macrophages, but with different functions because of the environment. Their results suggest that nt-LNA treatments promote the reorganization of macrophage phenotype landscape from foes classified as lipid-storage macrophages towards expansion of friends in the form of resident and repair macrophages that correlate with reduced disease burden. However, this hypothesis needs further exploration in future studies to shed more light on the potential beneficial effects of targeting LDL-msRNA in maintaining overall health and the relationship between the microbiome and atherosclerosis.

This study highlights that: (i) LDL contain non-host sRNAs involved in the pro-inflammatory effects in macrophages, suggesting that reducing levels of LDL-msRNA *in vivo* may confer protection against atherosclerotic CVD. (ii) LDL-msRNA signalling mitigation could be achieved by TLR8 regulation. Consequently, targeting TLR8 appears as a potential therapeutic approach in the prevention of atherosclerosis progression and eventually its transition to clinical events (albeit the latter remains unexplored). (iii) The use of nt-LNA to inhibit TLR8 activation represents a novel strategy that has not been widely explored. The study also shows that chemically modified nt-LNA can be effective in blocking TLR8 activation. The study's strength lies in its use of a comprehensive approach that integrates *in vitro* and *in vivo* experiments with single-cell RNA-seq, providing information about the macrophage populations found in the atherosclerotic plaque to explore the mechanisms by which LDL-msRNA drives atherosclerosis. Additionally, this study highlights the importance of understanding the origin and effects of sRNA in lipoproteins, particularly in relation to inflammatory signalling in macrophages. This suggests that off-target effects of nt-LNA can be exploited for therapeutic purposes. The results of this study are particularly exciting because they demonstrate that this approach can prevent atherosclerosis progression in mice. Whether these principles will apply in the much more complex human atherosclerotic setting remains unknown but deserves to be explored. More research is needed to confirm the safety and efficacy of this approach before it can be tested in humans. Hence, the results of this study might not lead to immediate applications, but in the long-term, those findings can lead to personalized medicine treatments using designed nt-LNAs carried by lipidic particles to modulate macrophage immune response such as TLR8 in a low invasive delivery method. Similarly, this study opens new avenues to identify the mechanism of action for nLDL-induced cytokine expression and the mechanisms and route by which LDL acquires msRNA. Further studies are also needed to investigate the impact of genetic, environmental, lifestyle, and socio-economic factors on nLDL-msRNA content.

Overall, this study makes an important contribution to our understanding of the molecular mechanisms of atherosclerosis and provides valuable insights into the impact of nLDL on macrophages profiling. The authors' use of high-throughput RNA-seq and network analysis allows for a comprehensive examination of gene expression changes and the identification of potential regulatory pathways, which could inform future research on the role of macrophages in atherosclerosis. Without doubt, this study provides a promising new avenue for developing treatments for atherosclerosis and other inflammatory diseases.

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Authors

Biography: Dr Soumaya Ben-Aicha earned her PhD at the Hospital Santa Creu i Sant Pau, University of Barcelona, Barcelona, Spain. During her PhD programme, she studied the impact of hypercholesterolaemia over the HDLs on coronary artery disease and consequent myocardial infarction in large animal models. Moreover, her intense contribution to further projects, based on the pleiotropic effects of statin treatment, resulted in intellectual property and collaboration from the private sector as well as numerous publications as first and co-author. Dr Ben-Aicha did a stay at the Universitatklinikum-Hamburg-Eppendorf, Germany, to analyse in parallel the impact of statins on the cardiovascular rhythm. She later moved to London, UK, as a post-doctoral research associate at the Imperial College London (ICL), founded by the British Heart Foundation (BHF). Later, she was awarded with the prestigious Centre of Excellence Fellowship by the BHF and become a transition research fellow. Her passion is focused on the prevention of atherosclerosis disease while being involved in university teaching work. In detail, her research is focused on the impact of human-derived nanoparticles on macrophages and immune cells in the pre-clinical and clinical arena with therapeutic focus. As a proof of that, Dr Ben-Aicha published and presented those studies at different journals and congresses, receiving prestigious honours and awards at the national and international level (ESC, ESCI, SEC, FCBV…).

Biography: Prof. Borja Ibáñez holds a degree in medicine from the Universidad Complutense de Madrid and PhD from the Universidad Autónoma de Madrid. He completed his clinical fellowship in cardiology at the Fundacion Jimenez Diaz Hospital in Madrid, during which he became interested in clinical research, working mainly with invasive imaging techniques for the study of the atherothrombotic disease. After completing his training in clinical cardiology, he made a training period of 3 years in basic research at Mount Sinai in New York. His doctoral thesis focused on the study of the ability of HDL-cholesterol to stabilize atheroma plaques and their assessment using non-invasive imaging tools. Since returning to Spain, he combines his scientific activity in the CNIC with clinical activity in the Fundación Jimenez Diaz University hospital. His passion is the study of myocardial diseases, with a clear translational vocation. His research ranges from the study of the mechanisms responsible for the development of myocardial diseases to clinical trials to test therapies identified by his group in pre-clinical studies. His clinical activity consists mainly in coronary interventions of patients suffering an acute myocardial infarction. To perform this translational research, he uses non-invasive imaging technology, mainly magnetic resonance, also including the development of new imaging algorithms to improve the use both on research and clinical levels.

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