Prevention of oxLDL uptake leads to decreased atherosclerosis in hematopoietic NPC1-deficient Ldlr−/− mice

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A B S T R A C T

Background and aims: Atherosclerosis is a chronic inflammatory disease of medium and large vessels and is typically characterized by the predominant accumulation of low-density lipoprotein (LDL)-cholesterol inside macrophages that reside in the vessel walls. Previous studies clearly demonstrated an association specifically between the oxidized type of LDL (oxLDL) and atherosclerotic lesion formation. Further observations revealed that these atherosclerotic lesions displayed enlarged, lipid-loaded lysosomes. By increasing natural antibodies against oxLDL, pneumococcal vaccination has been shown to reduce atherosclerosis in LDL receptor knockout (Ldlr−/−) mice. Relevantly, loss of the lysosomal membrane protein Niemann-Pick Type C1 (NPC1) led to lysosomal accumulation of various lipids and promoted atherosclerosis. Yet, the importance of lysosomal oxLDL accumulation inside macrophages, compared to non-modified LDL, in atherosclerosis has never been established.

Methods: By transplanting NPC1 bone marrow into lethally irradiated Ldlr−/− mice, a hematopoietic mouse model for lysosomal cholesterol accumulation was created. Through injections with heat-inactivated pneumococci, we aimed to demonstrate the specifically contribution of lysosomal oxLDL accumulation inside macrophages in atherosclerosis development.

Results: While there were no differences in plaque morphology, a reduction in plaque size and plaque inflammation was found in immunized NPC1mut-transplanted mice, compared to non-immunized NPC1mut-transplanted mice.

Conclusions: Lysosomal oxLDL accumulation within macrophages contributes to murine atherosclerosis. Future intervention strategies should focus specifically on preventing oxLDL, unlike non-modified LDL, from being internalized into lysosomes. Such an intervention can have an additive effect to current existing treatments against atherosclerosis.

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1. Introduction

In 2013, cardiovascular disease (CVD) was a major cause of mortality worldwide causing approximately 17 million deaths [1]. One of the major underlying pathologies of CVD is atherosclerosis. Atherosclerosis is a chronic inflammatory disease of medium and large vessels, which is characterized by the accumulation of both lipid droplets and inflammatory cells in the vessel wall [2–4]. Macrophages are abundantly present in atherosclerotic lesions and play an important role in the clearance of modified, oxidized low-
density lipoproteins (oxLDL). These lipid-laden macrophages, also known as foam cells, are one of the main drivers of atherosclerosis development. Eventually, plaque rupture may occur, causing thrombosis and/or stroke, which are hallmarks of CVD [5–7,49].

Over the years, an increasing amount of evidence has showed the importance of immune activation in atherosclerotic lesions [4,8]. Previously, natural antibodies targeted at oxLDL (EO6) were shown to be abundantly present within atherosclerotic lesions [9,10]. In line with these observations, our group and others demonstrated that a pneumococcal-induced immune response against the phosphorylcholine (PC) epitope, present on oxLDL, leads to diminished atherosclerosis development and hepatic inflammation [11,12]. Moreover, an elegant study by Hörkkö et al. clearly demonstrated that these antibodies specifically prevent binding, uptake and degradation of oxLDL by macrophages [9]. These results demonstrate the importance of the uptake of oxLDL in macrophages in the context of atherosclerosis development. In general, after uptake by macrophages, cholesterol is initially directed to the lysosomes for hydrolysis. Inside these lysosomes, lysosomal enzymes mediate the hydrolysis of cholesteryl esters into free cholesterol that can be transported out of the lysosomes into the cytoplasm via Niemann-Pick Type C (NPC) proteins [13–16]. As trafficking of free cholesterol from the lysosome to the cytoplasm is mainly regulated by NPC proteins, the dysregulation of these NPC proteins plays an important role in atherosclerosis development. Indeed, dysfunctional NPC1 leads to excessive accumulation of various lipids, including both non-modified as well as modified oxLDL, inside lysosomes [17,18]. Despite the fact that several studies found that NPC1 plays an important role in atherosclerosis, and that a number of in vitro studies clearly demonstrated that it is specifically oxLDL that accumulates inside lysosomes of macrophages [19,20], the specific contribution of lysosomal oxLDL compared to non-modified LDL was neglected in these studies and has never been established in vivo [21–23]. In the current study, we aimed to demonstrate the contribution of lysosomal oxLDL accumulation to atherosclerosis, using a macrophage-specific NPC1 mutant (NPC1mut) mouse model in combination with pneumococcal immunization. In this study, we showed that pneumococcal immunization of NPC1mut-transplanted (-tp) mice led to a decrease in plaque lesion size, independently of cholesterol levels, and a decline in plaque inflammation. Together, our results indicate that lysosomal oxLDL accumulation inside macrophages contributes to atherosclerosis.

2. Materials and methods

2.1. Mice, bone marrow transplantation, immunization, and diet

All animals were housed under standard conditions and had access to food and water ad libitum. All animal experiments were approved by the committee for Animal Welfare of Maastricht University and were performed according to the Dutch regulations. The immunogen, Streptococcus pneumoniae (heat-inactivated pneumococci, R36A strain, Birmingham, AL) was prepared as described previously [24]. Niemann-Pick type C1m1N mutant (NPC1mut) mice on a C57BL/6 background were a kind gift from Prof. Dr. Lieberman from University of Michigan Medical School. Ldlr−/− mice were obtained from our in-house breeding. Bone
marrow transplantation and pneumococcal immunizations were performed as described previously [11,25]. A detailed Materials and Methods section, concerning the methodology for the histological analysis, immunohistochemistry, RNA isolation and qPCR, is available in the Supplemental Materials and Methods. The primer sequences can be found in Table 1. Groups were compared with the two-way ANOVA for repeated measurements or by two-tailed unpaired t-test using GraphPad Prism (Version 5.03). Outliers were determined using the Grubbs’ Test.

3. Results

3.1. Plaque formation is reduced in NPC1mut-tp mice after immunization, independently of plasma lipid levels

We used Ldlr<sup>−/−</sup> mice, lethally irradiated and subsequently

![Fig. 2. IgM auto-antibodies in plasma of NPC1mut-tp mice.](image)

Mice were immunized with Streptococcus pneumoniae strain R36A or treated with PBS (control). IgM auto-antibodies targeted at oxLDL (EO6 and copper (Cu)-oxLDL) were measured in the plasma of immunized and non-immunized NPC1mut-tp mice. All data are represented as mean ± SEM. Data are significant at * p < 0.05, **p < 0.01, ***p < 0.001.

![Fig. 3. Histological analysis of plaque area in (non)-immunized NPC1mut-tp mice.](image)

(A) Representative pictures of the toluidine staining and (B) quantification of the total plaque area of the aortic root in (non)-immunized NPC1mut-tp mice. (C) Gene expression of Cd68 in the aortic arch corrected for cyclophilin A expression. All data are represented as mean ± SEM. Data are considered to be significant at * p < 0.05, **p < 0.01, ***p < 0.001.

Table 2

Plasma lipid levels of NPC1mut-tp mice without immunization (control) or after immunization with Streptococcus pneumoniae, strain R36A was determined at the end of the experiment after sacrifice (21 weeks). No significant changes were observed.

<table>
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<tr>
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<th>NPC1mut-tp Control</th>
<th>NPC1mut-tp R36A</th>
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<tbody>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>22.10 (±1.10)</td>
<td>20.81 (±0.78)</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.42 (±0.15)</td>
<td>1.42 (±0.13)</td>
</tr>
<tr>
<td>Free fatty acids (mmol/l)</td>
<td>0.96 (±0.05)</td>
<td>0.98 (±0.03)</td>
</tr>
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transplanted with bone marrow from NPC1mut mice, as a tool to induce lysosomal cholesterol accumulation in hematopoietic cells. To provoke an immune response to oxLDL, mice were immunized with heat-inactivated Streptococcus pneumoniae (see Fig. 1 for the experimental set-up). To confirm previous studies that successfully demonstrated elevated anti-oxLDL IgM titers after pneumococcal immunization [11,12], we analyzed the IgM antibody titers in the plasma of non-immunized and immunized NPC1mut-tp mice, at different time points. Immunization with heat-inactivated pneumococci led to a strong elevation of IgM autoantibodies directed against oxLDL (EO6) compared to non-immunized mice (Fig. 2). IgM antibodies directed against copper-oxLDL (Cu-oxLDL) were also increased in immunized mice compared to control, at different time points (Fig. 2). Moreover, the anti-oxLDL IgM responses were sustained over time until the end of the experiment. Plasma lipid levels were not significantly different between immunized and non-immunized mice (Table 2). Immunization of NPC1mut-tp mice resulted in a trend \( p = 0.055 \) towards a reduced plaque area (17%) in the aortic root (Fig. 3A and B). In the aortic arch, gene expression of Ccl68, a macrophage marker often used as surrogate marker for plaque size [26,27], was significantly reduced (43%) (Fig. 3C), independently of plasma lipid levels.

3.2. The inflammatory response is dampened in atherosclerotic lesions in immunized NPC1mut-tp mice

To investigate whether immunization with heat-inactivated pneumococci led to alterations in plaque morphology, we analyzed monocyte/macrophage and collagen content, as well as necrosis and apoptosis in the aortic root plaques of (non)-immunized NPC1mut-tp mice. Immunohistological staining for monocyte/macrophage content revealed no significant differences between immunized and non-immunized mice (Fig. 4A). Similarly, collagen content assessed by Sirius Red staining, as well as the amount of necrosis and apoptosis, was not different between the two groups (Fig. 4B–D). To examine whether immunization with heat-inactivated pneumococci led to reduced plaque inflammation, inflammatory gene expression levels were determined in the aortic arch of NPC1mut-tp mice. Gene expression levels of the inflammatory markers tumor necrosis factor-alpha (Tnfa), C–C chemokine receptor type 2 (Ccr2) and integrin Alpha M (Itgam) showed a trend towards reduced expression in immunized NPC1mut-tp mice but was not considered significant when compared to control (Fig. 5A–C). Inflammamson activation, triggered by oxLDL crystals, is involved in atherosclerosis development and can be measured by one of its downstream targets, namely caspase-1 that in turn activates interleukin-18 (IL-18) [27]. Caspase-1 gene expression was
significantly reduced in the aortic arch of immunized NPC1mut-tp mice, whereas no changes in gene expression of Il-18 were observed (Fig. 5D and E). Despite a clear reduction in Caspase-1 mRNA expression in the aortic arch, we did not observe any changes in caspase-1 activity in the aortic root between immunized and non-immunized NPC1mut-tp mice, most likely due to the presence of large necrotic areas (Supplementary Fig. 1).

To expand our analyses of inflammation, we performed additional staining for macrophages (MAC-1), neutrophils (NIMP) and T-cells (CD3+). No differences were observed in the number of MAC-1 positive cells between the two experimental groups and showed that MAC-1 is mainly expressed on foam cells, which is in line with our staining of MOMA, which is also expressed on macrophages (Supplementary Fig. 2). Next, immunohistological staining for neutrophils and T-cells was performed on frozen tissue sections of the aortic root of immunized and non-immunized NPC1mut-tp mice. No differences in the amount of neutrophils between immunized and non-immunized NPC1mut-tp mice were observed. In contrast, a significant increase in the amount of T-cells was observed in immunized NPC1mut-tp mice (Supplementary Fig. 3). However, it is important to note that increased T-cell amounts could constitute a reaction to dampen inflammasome activation [28], which could explain these findings.

4. Discussion

Our results demonstrate that pneumococcal immunization can modulate atherogenesis in hematopoietic NPC1-deficient Ldr−/− mice. While previous studies showed that pneumococcal immunization led to reduced lysosomal cholesterol accumulation [12], the current findings demonstrate the specific importance of oxLDL within lysosomes in atherosclerosis development. The aim of this study was to demonstrate the contribution of lysosomal oxLDL accumulation, rather than non-modified LDL, to atherosclerosis, by exclusively preventing the macrophage-mediated uptake and subsequent storage of oxLDL in NPC1mut-tp mice. We show that upon induction of anti-oxLDL antibodies, atherosclerosis development is reduced in NPC1mut-tp mice compared to non-immunized mice. In vitro, it has been shown that, unlike LDL, oxLDL is retained in the lysosomes and is resistant to efflux into the cytoplasm [29–31]. Stimulation of murine macrophages with oxLDL led to increased production of TNF and IL-6 in response to LPS stimuli [32]. Studies also demonstrated that oxLDL uptake by macrophages led to a release of pro-inflammatory cytokines via reactive oxidant species (ROS)-dependent inflammasome activation, thereby promoting foam cell formation [33,34]. These in vitro findings clearly demonstrated the major contribution of oxLDL to foam cell formation and inflammation, thereby influencing atherosclerosis development. In line with this observation, despite a similar relative monocyte/macrophage content, gene expression analysis of the aortic arch revealed that caspase-1 expression was significantly reduced in immunized NPC1mut-tp mice in the current study. Indeed, numerous studies have shown that caspase-1 plays an important role in atherosclerosis [27,35,36]. In previous studies, we already demonstrated that pneumococcal immunization resulted in reduced lysosomal cholesterol accumulation and cholesterol crystal formation [12]. In line with these findings, others observed that cholesterol crystal-induced inflammasome activation contributes to the development of atherosclerosis via caspase-1-mediated activation [37]. These observations indicate that blocking oxLDL uptake in macrophages of immunized NPC1mut-tp mice results in a reduced caspase-1-mediated inflammatory response.

Uptake of oxLDL is mainly facilitated by CD36 and macrophage

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**Fig. 5. Quantification of plaque inflammation in (non)-immunized NPC1mut-tp mice in the aortic arch.** (A–E) Relative gene expression of Tnfa, Caspase-1, Ccr2, Itgam and Il-18 in NPC1mut-tp mice immunized with R36A or control injections with PBS. All data are represented as mean ± SEM. Data are significant at *p < 0.05, **p < 0.01, ***p < 0.001.
scavenger receptor 1 (MSR1) and contributes to foam cell formation [38]. Previous research of our group demonstrated that while there was a clear reduction in inflammation, foam cell formation was unaltered in Cd36−/−-tp, Msr1−/−-tp and Cd36−/−/Msr1−/−-tp mice compared to WT-tp mice [25,39]. Further detailed analysis revealed that specifically oxLDL is internalized by macrophages, and this has been shown to be associated with lysosomal cholesterol accumulation and increased inflammation. Prevention of oxLDL uptake led to a reduction of lysosomal cholesterol accumulation and a decrease in inflammation in vivo [39,40]. Overall, these data indicate that it is not the total amount of cellular cholesterol, but rather the specific accumulation of lysosomal oxLDL that contributes to atherosclerosis development and inflammation.

Although lipid-lowering agents are the primary treatment for atherosclerosis and other CVDs, the residual risk for a cardiovascular event remains present in these patients [41–43]. As not total cholesterol, but rather the uptake of oxLDL is contributing to the development of atherosclerosis, lowering LDL alone may not be efficient. In the past, numerous in vitro and in vivo studies have shown that anti-oxidants could be beneficial in reducing atherosclerotic lesions [44]. Surprisingly, despite some observational studies that showed a correlation between anti-oxidants and atherosclerosis, primary and secondary intervention in clinical randomized studies failed in humans [44]. The fact that cholesterol can undergo oxidation within lysosomes could be important to explain why these studies failed [45] and why we have observed only a moderate reduction in atherosclerotic lesion size within this study. Besides oxLDL, other cholesterol products such as aggregated LDL, sphingomyelin and cholesteryl ester-rich lipid dispersions can also accumulate inside lysosomes [29,46,47]. This could also explain the moderate reduction in plaque size in the current study when compared to previous findings by Binder et al. who performed similar pneumococcal immunizations in hyperlipidemic mice without an NPC1 mutation [11]. Taking into account the negligible plasma oxLDL concentrations relative to LDL [48], the reduction in plaque size and inflammation exceeded our expectations and underlines the importance of oxLDL in atherosclerosis development. Future intervention strategies should focus specifically on preventing oxLDL, rather than non-modified LDL, from being internalized into lysosomes. Such an intervention can have an additive effect to current existing treatments against atherosclerosis.

Conflict of interest

The authors declared that they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author contributions

MLJJ, RSS: planned experiments; MLJJ, SMAW, Tho, JL, The, Yo, PJG; performed the experiments; MLJJ, SMAW, Tho, MJG, JLJ, The, Yo, PJG, MMPC, RSS; analyzed and interpreted data; MLJJ, SMAW, MMPC, RSS; wrote the manuscript; MLJJ, SMAW, Tho, MJG, JL, The, Yo, PJG, CJB, MMPC, RSS; critical revised the manuscript; RSS obtained funding.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2016.10.038.

References


