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Title: Novel immune cell-based therapies for atherosclerosis
Issue Date: 2015-05-27
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OxLDL-Induced Apoptotic Dendritic Cells as a Novel Therapy for Atherosclerosis

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Abstract

Objective Modulation of immune responses may form a powerful approach to treat atherosclerosis. It has been shown that clearance of apoptotic cells results in tolerance induction to cleared antigens by DCs; however, this seems impaired in atherosclerosis as antigen-specific tolerance is lacking. This could partially result from a reduced emigration of DCs from atherosclerotic lesions due to the high cholesterol environment. Nonetheless, local induction of anti-inflammatory responses by apoptotic cell clearance seems to dampen atherosclerosis, as inhibition of apoptotic cell clearance worsens atherosclerosis. In this study we assessed whether intravenous administration of oxLDL-induced apoptotic (apopox-DCs), and as a control unpulsed apoptotic DCs (apopctrl-DCs), could modulate atherosclerosis by inducing tolerance.

Methods and Results Adoptive transfer of apopox-DCs into LDLr−/− mice either before or during Western-type diet resulted in increased numbers of CD103+ tolerogenic splenic DCs, with a concomitant increase in regulatory T cells. Interestingly, both types of apoptotic DCs induced an immediate 40% decrease in Ly-6Chigh monocyte numbers and a 50% decrease in circulating CCL2 levels, but only apopox-DC treatment resulted in long-term effects on monocytes and CCL2 levels. While initial lesion development was reduced by 40% in both treatment groups, only apopox-DC treatment prevented lesion progression by 28%. Interestingly these lesions displayed enhanced stability, determined by a robust 45% increase in collagen content.

Conclusion Our findings clearly show that apoptotic DC treatment significantly decreases lesion development, but only apopox-DCs can positively modulate lesion progression and stability. These findings may translate into a safe treatment of patients with established cardiovascular diseases using patient-derived oxLDL-induced apoptotic DCs.
OxLDL-Induced Apoptotic Dendritic Cells

Introduction

Immune responses are, besides dyslipidemia, driving forces behind atherosclerotic disease development and progression\(^1\). Dendritic cells (DCs) have been shown to play a crucial role in atherosclerosis. They engulf autoantigens, such as oxidized low-density lipoprotein (oxLDL), in the vessel wall and induce differentiation and clonal expansion of antigen-specific T cells in secondary lymphoid organs. Subsequently, T cells migrate to the lesions and exacerbate local inflammatory responses. In more advanced atherosclerotic lesions, however, the cholesterol-rich environment may prevent DC migration\(^2\). DCs can then also activate T cells locally, which correlates with rupture-prone regions of the lesions\(^3\).

DCs play a critical role in maintaining homeostasis and self-tolerance. They are crucially involved in apoptotic cell clearance, termed efferocytosis, which does not result in maturation of the phagocytes\(^4\). While it was assumed that clearance of potentially harmful apoptotic cell content is essential to avoid induction of (auto)immune responses, it was soon discovered that apoptotic cells can actively suppress inflammatory responses. The production of pro-inflammatory cytokines, e.g. TNF-α and IL-6, in response to LPS is decreased in phagocytes upon efferocytosis, while the production of e.g. IL-10, prostaglandin E2, and TGF-β is increased\(^5\). Furthermore, DCs produce nitrite in response to apoptotic cell uptake, which can inhibit T cell responses\(^6\). Both early and late apoptotic cells\(^7\) of different origin, e.g. apoptotic peripheral blood lymphocytes\(^8\), neutrophils\(^9\), T cells\(^10\) and DCs\(^11\), can have anti-inflammatory effects. In vivo, apoptotic cells have been shown to downregulate the immunostimulatory capacity of both splenic marginal zone macrophages and DCs\(^12,13\).

By clearing apoptotic cells, DCs can present (auto)antigens derived from ingested apoptotic cells on MHC class I/II molecules, whereas macrophages fail to do so\(^14,15\). DCs can induce anergy in both CD4\(^+\) T cells\(^16\) and CD8\(^+\) T cells\(^13,15\), while potentiating Treg responses\(^11,17\), thereby inducing tolerance.

Antigen-presenting cells recognize apoptotic cells due to newly exposed or modified membrane molecules on their cell surface. Disruption of this recognition results in the onset of inflammation and autoimmunity. Mice lacking receptors (such as TIM receptors and TAM receptors, e.g. Mertk) or bridging molecules (e.g. milk fat globule-epidermal growth factor 8)) involved in the recognition of apoptotic cells show a similar phenotype: inefficient apoptotic cell clearance resulting in enhanced activation of CD4\(^+\) T cells and/ or B cells, elevated levels of autoantibodies and spontaneous systemic autoimmunity\(^18\). LDLr\(^{-/-}\) mice reconstituted with bone marrow lacking Mertk or milk fat globule-epidermal growth factor 8, ApoE\(^{-/-}\) mice with a defective Mertk, and C1qa-deficient LDLr\(^{-/-}\) all develop more atherosclerosis\(^19-22\). Moreover, we have recently shown that blockade of TIM-3 has the same effect\(^23\).

As apoptotic cell clearance is crucial for maintaining tolerance, apoptotic cell administration may be used for anti-inflammatory therapies. Indeed, apoptotic cells can protect mice against LPS-induced shock\(^24\) and myelin cross-linked apoptotic cells can reduce antigen-specific responses in experimental autoimmune encephalomyelitis\(^25\). In the present study we determined whether apoptotic DCs could be used to treat
Atherosclerosis. We induced apoptosis in DCs by oxLDL (apop\textsuperscript{ox}-DCs), which possibly also delivers an atherosclerosis-specific antigen in the context of regulatory signals to viable DCs, or by proteasomal inhibition (apop\textsuperscript{ctrl}-DC). Both apoptotic DCs were injected intravenously to enable their uptake by DCs to modulate T cell responses in developing and established atherosclerosis. We show here for the first time that apoptotic DC-treatment is highly effective in reducing initial atherosclerosis. Interestingly, the presence of oxLDL in apoptotic DCs has additional beneficial effects on lesion progression and stability.

**Material and Methods**

**Animals**

C57BL/6 and LDLr\textsuperscript{-/-} mice were originally obtained from the Jacksons Laboratory, kept under standard laboratory conditions, and administered food and water ad libitum. All animal work was approved by the Ethics Committee for Animal Experiments of Leiden University and conforms to Dutch government guidelines.

**DC cultures and stimulations**

Bone marrow cells were isolated from the tibias and femurs of C57BL/6 mice. The cells were cultured for ten days at 37°C and 5% CO\textsubscript{2} in 10 cm petri dishes (Greiner Bio-One) in IMDM supplemented with 10% FCS, 100 U/mL penicillin/streptomycin (all obtained from PAA), 2mM glutamax (Thermo Fisher Scientific) and 20 μM β-mercaptoethanol (Sigma Aldrich) in the presence of granulocyte-macrophage colony-stimulating factor. DC purity was assessed by CD11c expression (flow cytometry) and routinely found to be above 85%. Apoptosis for in vivo treatments was induced by 0.1 μM epoxomicin (apop\textsuperscript{ctrl}-DCs) or 50 μg/mL oxLDL (apop\textsuperscript{ox}-DCs). LDL was isolated from serum of a healthy volunteer after centrifugation of the serum according to Redgrave et al.\textsuperscript{26}. The isolated LDL was dialyzed against PBS with 10 μM EDTA (pH 7.4) for 24 hours at 4°C and oxidized by exposure to 10 μM CuSO\textsubscript{4} at 37°C for 20 hours as previously described\textsuperscript{27}.

For in vitro DC stimulations, 5 x 10\textsuperscript{5} DCs per well were plated in 24-well plates with indicated ratios of apop\textsuperscript{ox}-DCs or apop\textsuperscript{ctrl}-DCs for 24 hrs. After extensive washing with PBS, DCs were stimulated with 100 ng/mL LPS to determine cytokine responses.

**Co-cultures**

Single cell suspensions of spleens from LDLr\textsuperscript{-/-} mice were obtained by using a 70 μm cell strainer (Falcon). Red blood cells were lysed with erythrocyte lysis buffer (0.15 M NH\textsubscript{4}Cl, 10 mM NaHCO\textsubscript{3}, 0.1 mM EDTA, pH 7.3). CD4\textsuperscript{+} T cells (>95% purity) were isolated from splenocytes by using the BD IMagTM mouse CD4 T lymphocyte enrichment set according to manufacturer’s protocol (BD Biosciences). CD11c\textsuperscript{+} cells (>85% purity) were isolated from splenocytes by using CD11c MicroBeads according to manufacturer’s protocol (Miltenyi Biotec).

For DC-T cell co-cultures 0.1 x 10\textsuperscript{5} DCs were cultured with 1 x 10\textsuperscript{5} T cells per well, and for splenocyte proliferation 2 x 10\textsuperscript{5} splenocytes per well were cultured.
in quintuplicates in 96-well round-bottom plates (Greiner Bio-One) in the presence or absence of αCD3, or αCD3 and αCD28 (2 μg/mL, eBioscience) for 72 hours in complete RPMI 1640, supplemented with 10% FCS, 100 U/ml penicillin/streptomycin, 2mM L-glutamine (all obtained from PAA) and 20 μM β-mercaptoethanol (Sigma Aldrich). Proliferation was measured by Ki-67 expression. Relative Ki-67 expression was measured by flow cytometry and is expressed as mean Ki-67% with stimulation divided by mean Ki-67% without stimulation. T cell subsets in the culture were determined by flow cytometry.

**Atherosclerosis**

Atherosclerosis was induced in 10-12 weeks old male LDLr<sup>-/-</sup> mice by feeding a Western-type diet (WTD) (0.25% cholesterol and 15% cocoa butter; Special Diet Services). For the initial atherosclerosis study, mice were treated with 3 i.v. injections of PBS, 1.5 x 10<sup>6</sup> apop<sup>ox</sup>-DCs or 1.5 x 10<sup>6</sup> apop<sup>ctrl</sup>-DCs every other day prior to nine weeks WTD. For the progression study, a baseline group was sacrificed after ten weeks WTD, while the rest received 3 i.v. injections of PBS, 1.5 x 10<sup>6</sup> apop<sup>ox</sup>-DCs or 1.5 x 10<sup>6</sup> apop<sup>ctrl</sup>-DCs every other day and were put another nine weeks on WTD.

**Flow Cytometry**

At sacrifice, blood, spleen, bone marrow, and aorta were harvested. Single cell suspensions were obtained as described above. For aortic single cell suspensions, aortas were isolated and adventitial fat was removed. The aorta was then dissected and digested in a 20mM HEPES/ PBS buffer containing 125 U/ml Collagenase type XI, 60 U/ml hyaluronidase type 1-s, 60 U/ml DNase I, and 450 U/ml Collagenase type I at 37°C for 1 hour. Subsequently, 3 x 10<sup>5</sup> cells per sample were stained with the appropriate antibodies. To determine aortic Tregs, we pre-gated on CD3<sup>+</sup> cells within CD45<sup>+</sup> cells. The following antibodies were used: CCR2-purified (Abcam), anti-Rabbit-IgG-FITC (BD Biosciences), CD3-PerCP (BD Biosciences), CD4-PerCP (BD Biosciences), CD8-PerCP (BD Biosciences), CD11b-FITC, CD11c-APC, CD25-FITC, CD44-FITC, CD45- Pacific Blue, CD62L-Pacific Blue, CD103-FITC, FoxP3-APC, Gata-3-PE, Ki67-FITC, Ly-6C-PerCP, MHCII-Pacific Blue, RORyt-PE, and T-bet-Alexa Fluor 647. All antibodies were purchased from eBioscience, unless stated otherwise. For intracellular staining, cells were fixed and permeabilized according to the manufacturer’s protocol (eBioscience). Flow cytometry analysis was performed on the FACSCantoII and data were analyzed using FACSDiva software (BD Biosciences).

**Histological analysis**

To determine plaque size, 10 μm cryosections of the aortic root were stained with Oil-Red-O and haematoxylin (Sigma Aldrich). Corresponding sections were stained for collagen fibers using the Masson’s Trichrome staining (Sigma Aldrich) or immunohistochemically with antibodies against a macrophage specific antigen (MOMA-2, polyclonal rat IgG2b, 1:1000, Serotec Ltd.). Goat anti-rat IgG alkaline phosphatase conjugate (dilution 1:100; Sigma Aldrich) was used as a secondary antibody and nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as
enzyme substrates. To determine the number of adventitial T cells, CD3 staining was performed using anti-mouse CD3 (clone SP7, 1:150, ThermoScientific). BrightVision Anti-rabbit-HRP was used as secondary antibody (Immunologic). The section with the largest lesion and four flanking sections were analyzed for lesion size and collagen content, two flanking sections were analyzed for macrophage and T cell content. All images were analyzed using the Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems). The percentage of collagen and macrophages in the lesions was determined by dividing the collagen- or MOMA-2-positive area by the total lesion surface area.

*Real-time PCR*

mRNA was isolated from the aortic arch using the guanidium isothiocyanate method and reverse transcribed (RevertAid Moloney murine leukemia virus reverse transcriptase). Quantitative gene expression analysis was performed on a 7500 Fast real-time PCR system (Applied Biosystems) using SYBR Green technology. The expression was determined relative to the average expression of three household genes: acidic ribosomal phosphoprotein PO (36B4), hypoxanthine phosphoribosyltransferase (HPRT), and 60S ribosomal protein L27 (Rpl27). For used primer pairs refer to Table 1.

<table>
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<tr>
<th>Gene</th>
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<th>Reverse</th>
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<tr>
<td>HPRT</td>
<td>TACAGCCCCCAATGGTAAAAGG</td>
<td>AGTCAGGGCAGCATATCACAAC</td>
</tr>
<tr>
<td>Rpl27</td>
<td>CGCCAAGCGATACCAAGATCAAGTCC</td>
<td>AGCTGGGTTCCCTCAGAAACATCCTGG</td>
</tr>
</tbody>
</table>

*Table 1. Primer Pairs used for qPCR analysis.* The relative expression of genes was determined relative to the average expression of the three household genes: acidic ribosomal protein 36B4, hypoxanthine phosphoribosyltransferase (HPRT), and 60S ribosomal protein L27 (Rpl27).

*Cytokine and nitrite analysis*

IL-10, TNF-α, IL-6 (BD Biosciences), and CCL2 (eBioscience) were determined by ELISA, according to manufacturer’s protocol. Nitrite production was assessed by mixing cell culture supernatant with an equal amount of Griess reagent (Sigma Aldrich) and measuring absorbance at 540 nm. Culture medium served as background control.

*Serum cholesterol levels*

Serum concentrations of total cholesterol were determined by enzymatic colorimetric assays (Roche Diagnostics). Absorbance was read at 490 nm. Precipath (standardized serum; Roche Diagnostics) was used as internal standard. The distribution of cholesterol over the different lipoproteins in serum was determined by fractionation of 30 µl of serum of each mouse using a Superose 6 column (3.2 x 300 mm, SmartSystem; Pharmacia). Total cholesterol content of the effluent was determined as described above.
**Statistical analysis**

Values are expressed as mean±SEM. Data of three groups were analyzed with one-way ANOVA and data of two or more groups with more than one variable were analyzed by two-way ANOVA, both followed by Bonferroni post-testing. Statistical analysis was performed using Prism (GraphPad). Probability values of P<0.05 were considered significant.

**Results**

**OxLDL-induced apoptotic DCs induce tolerogenic DCs and modulate T cell responses in vitro**

To induce DC apoptosis and simultaneously load DCs with an atherosclerosis-relevant antigen, we incubated bone marrow-derived DCs with increasing concentrations of oxLDL for 24 and 48 hours. After 48 hours, 50 μg/mL oxLDL was sufficient to induce late apoptosis in 97% of the DCs. We therefore chose this condition to generate oxLDL-induced apoptotic DCs (apopox-DCs) for further experiments. As a control, we generated apoptotic DCs by exposure to 0.1 μM epoxomicin (apopctrl-DCs), which induced late apoptosis in 95% of DCs (Figure 1).

![Graph](image1.png)

**Figure 1. OxLDL and epoxomicin induce apoptosis in DCs.** Quantification of apoptosis in DCs by flow cytometry. DCs were stimulated with indicated amounts of oxLDL. Early apoptosis was determined as AnnexinV+PI and late apoptosis as AnnexinV+PI by flow cytometry. Values are expressed as mean±SEM and are representative of three independent experiments. Representative dot plots of DCs treated with either medium, 50 μg/mL oxLDL or 0.1 μM epoxomicin for 48 hours are shown. Control indicates DCs without staining. Values are expressed as mean±SEM.
To assess whether these apoptotic DCs could modulate inflammatory responses by viable responder DCs, we added different amounts of apoptotic DCs to immature respDCs for 24 hours and subsequently stimulated them with 100 ng/mL LPS for another 24 hours. Both apoptotic DCs decreased the release of inflammatory cytokines from respDCs: Pre-exposure to apoptotic DCs resulted in a significant decrease of 40% in IL-6 and 80% in TNF-α responses, while

![Figure 2. Apoptotic DC-exposure modulates cytokine responses by DCs in vitro.](image)

Apoptotic DC-exposure prevents LPS-induced inflammatory responses by DCs. OxLDL-induced apoptotic DCs (apopox-DCs; black bars) and control apoptotic DCs (apopctrl-DCs; white bars) were added to DCs at indicated ratios for 24 hours; viable DCs remained constant. After extensive washing, 100 ng/mL LPS was added for another 24 hours. IL-6, TNF-α, and IL-10 responses were determined by ELISA and nitrite by Griess reagent. All values are expressed as mean±SEM and are representative of three independent experiments. *P<0.05, **P<0.01, ***P<0.001 compared to DCs without apoptotic DCs or to apopox-DCs as indicated.

![Figure 3. Apoptotic DC-exposure modulates T cell responses.](image)

Splenocytes from an LDLr−/− were co-cultured with increasing amounts of apoptotic DCs (solid lines) and control DCs (dotted lines) in the presence of oCD3/28 for 48 hours to induce T cell proliferation; splenocytes remained constant. Proliferation was assessed by Ki-67+ cells of CD4+ and CD8+ T cells. Tregs (FoxP3+CD25+), Th1 (T-bet+), Th2 (Gata-3+), and Th17 (ROeyt+) cells were determined within CD4+ T cells by flow cytometry. All values are expressed as mean±SEM and are representative of three independent experiments. *P<0.05, **P<0.01, ***P<0.001 compared to apoptotic DCs.
pre-exposure to apop\textsuperscript{ctrl}-DCs resulted in a decrease of 26% in IL-6 and 77% in TNF-\(\alpha\) responses of respDCs. The anti-inflammatory cytokine IL-10 was specifically increased, by 1.3 fold, when respDCs were pre-exposed to apop\textsuperscript{ox}-DCs, but not after pre-exposure to apop\textsuperscript{ctrl}-DCs. Nitrite production was robustly increased, by 26.7-fold, upon pre-exposure to apop\textsuperscript{ox}-DC, while pre-exposure to apop\textsuperscript{ctrl}-DCs only increased nitrite production by 4.5-fold (Figure 2).

We further established the immunomodulatory potential of apoptotic DCs by incubating them ex vivo with splenocytes from an LDLr\textsuperscript{-/-} mouse. Increasing numbers of apop\textsuperscript{ox}-DCs induced a significant 74% reduction in CD4\(^+\) T cell proliferation, as assessed by the expression of the cell cycle marker Ki-67, while apop\textsuperscript{ctrl}-DCs had no effect. This was in line with a robust 41% increase in Tregs and a 70% reduction in Th1 cells by apop\textsuperscript{ox}-DCs. In contrast, apop\textsuperscript{ctrl}-DCs exposure resulted in a significant 51% reduction in Treg, while Th1 cells were unaffected. Th17 cells were reduced in both apop\textsuperscript{ox}-DC and apop\textsuperscript{ctrl}-DC-treated splenocytes cultures by 63% and 57%, respectively, while Th2 responses were not affected. Interestingly, CD8\(^+\) T cell proliferation was reduced by about 65% by both types of apoptotic DCs. Consistent with effects observed on T cell responses, IL-2 and IFN-\(\gamma\) production were significantly reduced by both apoptotic DCs (Figure 3).

Exposure to apop\textsuperscript{ox}-DCs more effectively induced IL-10 responses in DCs and induced Treg responses compared to apop\textsuperscript{ctrl}-DCs. Moreover, an increased nitrite production and a stronger inhibition of T cell responses were observed by apop\textsuperscript{ox}-DCs compared to apop\textsuperscript{ctrl}-DCs. To assess the different potential of apop\textsuperscript{ox}-DCs more closely, we checked differences in viable DCs that had been exposed to these apoptotic DCs. We found that apop\textsuperscript{ox}-DCs potently induced expression of ABCA1 and ABCG1, two main target genes of liver X receptor (LXR). In addition to an upregulation of IL-10 and a downregulation of IL-6, we also found a significant upregulation of the co-

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\caption{Increased LXR activation in viable DCs exposed to oxLDL-induced apoptotic DCs. OxLDL-induced apoptotic DCs (apop\textsuperscript{ox}-DCs; black bars) and control apoptotic DCs (apop\textsuperscript{ctrl}-DCs; white bars) were added to DCs at indicated ratios for 24 hours; viable DCs remained constant. After extensive washing, 100 ng/mL LPS was added for another 24 hours. Relative mRNA expression was determined by qPCR. All values are expressed as mean±SEM. *\(P<0.05\), **\(P<0.01\), ***\(P<0.001\).}
\end{figure}
inhibitory molecule PD-L1, indicating a more potent anti-inflammatory capacity of apopox-DC-exposed DCs (Figure 4).

**OxLDL-presence in apoptotic DCs is needed to induce tolerogenic DCs and Treg responses in vivo**

To assess whether apopox-DC treatment is beneficial for atherosclerosis, we administered apopox-DCs and apopctrl-DCs three times *intravenous* every other day prior to nine weeks WTD-feeding of LDLr-/- mice. One hour after the first injection, the majority of apoptotic DCs were cleared by marginal zone DCs and macrophages, while some apoptotic DCs might have been cleared by red pulp macrophages (Figure 5).

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**Figure 5. Uptake of apoptotic DCs in the spleen.** OxLDL-induced apoptotic DCs were stained with CFSE. **A.** One hour after i.v. injection of 5 million apoptotic DCs, CFSE-positive cells were determined by flow cytometry within CD11b+ cells, Marginal zone macrophages (MZM), CD19+ cells (B cells), Marginal Zone (MZ) B cells, Follicular (Foli) B cells, Transitional (Trans) B cells and different subsets of DCs. Values are expressed as mean±SEM. **B.** Representative spleen sections stained with DAPI are shown. **C.** Gating strategies for different cell populations. **D.** Representative dot plots of macrophages and DCs that have taken up CFSE+ apoptotic DCs.
As splenic DCs efficiently clear apoptotic cells from the circulation\textsuperscript{13}, we analyzed these splenic DCs one week after apoptotic DC treatment. While absolute DC numbers remained unchanged, myeloid immunogenic CD8\textalpha\textsuperscript{+}CD11b\textsuperscript{+} DCs were significantly decreased by 10% and 12% in apop\textsuperscript{ox}-DC- and apop\textsuperscript{ctrl}-DC-treated mice, respectively. No significant changes in lymphoid tolerogenic CD8\textalpha\textsuperscript{+}CD11b DCs were observed. Interestingly, a significant 50% increase in anti-atherogenic CD103\textsuperscript{+}CD11b DCs was determined in the apop\textsuperscript{ox}-DC-treated-mice only (Figure 6A). To determine whether these DCs were tolerogenic, we isolated splenic CD11c\textsuperscript{+} cells and co-cultured them with CD4\textsuperscript{+} T cells. Additionally, we observed a significant 78% increase in Tregs and a 2.5-fold increase in IL-10 production in co-cultures with DCs from apop\textsuperscript{ox}-DC-treated mice only. Nitrile production was low but increased by 1.7-fold in DCs from apop\textsuperscript{ox}-DC- and apop\textsuperscript{ctrl}-DC-treated-mice, respectively. Th1, Th2, and Th17 cells did not significantly differ (Figure 6B). Overall DCs from apop\textsuperscript{ctrl}-DC-treated mice only mildly affected T cell proliferation, indicating that oxLDL-presence in apoptotic DCs is necessary to potently modulate immune responses by increasing Tregs.

As efferocytosis can modulate T cell responses induced by DCs in vivo\textsuperscript{28}, we monitored CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells in the circulation. While we did not observe effects on total CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells (data not shown), apop\textsuperscript{ox}-DC-treatment potently induced

\begin{figure}[h]
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\caption{Apop\textsuperscript{ox}-DC treatment results in tolerogenic DCs and increased Tregs in initial atherosclerosis. A. The effect of apoptotic DC-treatment on splenic DC phenotype. After one week, CD11c\textsuperscript{hi}MHCII\textsuperscript{+} DCs within Ly-6G- cells in the spleen and the percentage of myeloid CD8\textalpha\textsuperscript{+}DC11b\textsuperscript{+} DCs, lymphoid CD8\textalpha\textsuperscript{+}CD11b-DCs, and CD103\textsuperscript{+}CD11b DCs within these DCs were determined by flow cytometry. B. Splenic DCs of apop\textsuperscript{ox}-DC-treated mice induce Tregs and decrease T cell proliferation. We isolated splenic CD11c\textsuperscript{+} cells and co-cultured them with CD4\textsuperscript{+} T cells from LDLr\textsuperscript{-/-} mice one week on WTD in a ratio of 1:10 in the presence of cCD3 for 72 hours. Specific T cell proliferation was determined by %Ki-67 expression divided by background. Tregs were determined as FoxP3\textsuperscript{+}CD25\textsuperscript{+}CD4\textsuperscript{+} by flow cytometry. IL-10 production was determined by ELISA and nitrile production by Griess reagent. All values are expressed as mean±SEM and are representative of at least six mice.*P<0.05, **P<0.01, ***P<0.001.}
\end{figure}
Tregs in the circulation. A significant 24% increase was observed one week after apopox-DC treatment and remained mildly increased (10%, n.s.) nine weeks after treatment (Figure 7A). A similar pattern was observed in the spleen, where Tregs were robustly induced after one week (35%) and remained mildly increased (9%, n.s.) nine weeks after apopox-DC treatment (Figure 7B and data not shown). One week after treatment, the increase in circulating Tregs translated into significantly increased aortic Tregs only in the apopox-DC-treated group (Figure 7C). Apopctrl-DC treatment did not induce circulating or aortic Tregs (Figure 7A and 7C), while a similar increase of splenic Treg percentages was observed after one week WTD (Figure 7B). However, this appeared to be related to an overall decrease of CD4+ T cells and reduced subsets, resulting in an increased Treg percentage as absolute Treg numbers in the spleen were not changed and significantly less than in apopox-DC-treated mice (Figure 7D). After nine weeks WTD, apopctrl-DC treatment had no effect on T cell subsets (data not shown). Additionally, we determined T cell numbers in the aortic root after nine weeks and found a significant 68% and 52% reduction in apopox-DC-treated mice and apopctrl-DC-treated-mice, respectively (Figure 7E).

In line with the described effect of apoptotic cells on inflammation, we saw a dramatic 7-fold and 6.2-fold reduction of IL-6 expression in the aortic arch in apopox-DC-

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**Figure 7. Apoptotic DC treatment reduces the inflammatory status in initial atherosclerosis.**

A. Treatment with apopox-DCs increases circulating Tregs, while treatment with apopctrl-DCs has no effect. During the experiment Tregs (FoxP3+CD25+ within CD4+ T cells) were monitored in circulation. Representative dot plots are shown.

B. Splenic Tregs and C. Aortic Tregs were determined by flow cytometry after one week WTD. We pre-gated on CD3+ T cells within CD45+ cells.

D. CD4+ T cell responses in the spleen as determined by flow cytometry and absolute amounts of Tregs after one week WTD.

E. Quantification of CD3+ T cells in the aortic root after nine weeks WTD. F. IL-6 expression in the aortic arch after nine weeks WTD was determined by qPCR.

G. IL-10 in the serum was determined by ELISA. All values are expressed as mean±SEM and representative of at least six mice.*P<0.05, **P<0.01, ***P<0.001.
DC- and apop\textsuperscript{ctrl}-DC-treated mice, respectively (Figure 7F). We also observe an initial significant increase in IL-10 in the serum, corresponding to an induction of tolerance (Figure 7G).

**OxLDL-preservation in apoptotic DCs enhances beneficial effects on monocytes**

Interestingly, while we aimed to modify DCs and subsequent T cell responses, major effects on circulating monocytes were observed by the apoptotic DC treatments. One week after treatment, a significant 40% and 36% reduction in circulating inflammatory Ly-6C\textsuperscript{hi} monocytes in apop\textsuperscript{ox}-DC-treated and apop\textsuperscript{ctrl}-DC-treated mice was determined, respectively. In the apop\textsuperscript{ox}-DC-treated mice also CCR2 expression of monocytes was significantly reduced one week after treatment. Nine weeks after treatment with apop\textsuperscript{ox}-DCs a 30% decrease in total monocyte numbers was observed, whereas apop\textsuperscript{ctrl}-DC-treated mice did not show a significant decrease in monocytes (Figure 8A). In line with these observations, the apop\textsuperscript{ctrl}-DC-treated mice only showed an initial decrease in CCL2 serum levels, while in apop\textsuperscript{ox}-DC-treated mice CCL2 levels were reduced by about half during the entire length of the experiment (Figure 8B).

![Figure 8. Apoptotic DC treatment reduces monocyte responses in initial atherosclerosis. A.](image)

These results clearly indicate that oxLDL presence in apoptotic DCs provides a long-term anti-inflammatory effect on monocytes. This was not a result of exposure to oxLDL by itself as treatment with oxLDL mildly increased CCL2 levels and significantly increased monocytes, as well as Ly-6C\textsuperscript{hi} monocytes, compared to apop\textsuperscript{ox}-DC-treated.
mice (data not shown). Interestingly, CCL2 expression in the aortic arch was still 2-fold decreased in both apoptotic DC-treated groups at sacrifice (Figure 8C). The effects of apoptotic DCs on monocytes were not due to a difference in the number of progenitors in the bone marrow, determined as Lin−Sca-1+c-Kit+ (LSK) cells (data not shown). Moreover no differences in monocyte proliferation, determined by Ki-67 expression, in bone marrow or spleen were observed; also no differences in the numbers of (Ly-6C+) monocytes, or levels of CCR2 expression on monocytes were observed (data not shown).

Apoptotic DC treatment reduces initial atherosclerotic lesion

Nine weeks after the apoptotic DC treatment, we analyzed aortic root lesion sizes and found a significant reduction of 39% in apopox-DC-treated-mice and 43% in apopctrl-DC-treated mice (Figure 9A). Lesion stability was not different between the groups as collagen content was on average 20% (Figure 9B). In line with the observed effects on monocyte numbers, a significant 24% decrease in the relative macrophage content was determined in the apopox-DC-treated-mice, whereas this was not affected in the apopctrl-DC-treated-mice (Figure 9C). Consistent with this, a significant 51% decrease in CD68 mRNA expression in the aortic arch of apopox-DC-treated-mice was observed. Also apopctrl-DC-treated mice showed a 55% reduction, in line with initial decreases of inflammatory monocytes (Figure 9D). The effects on lesion formation were not due to effects of apoptotic DCs on bodyweight or serum cholesterol levels as these did not differ between the groups (data not shown).
OxLDL-presence in apoptotic DCs reduces inflammatory responses in established atherosclerosis

As the apoptotic DC treatment proved highly effective in reducing initial atherosclerosis, we next determined whether it could reduce inflammation in pre-established atherosclerotic lesions. To this end, LDLr−/− mice that had been kept on a WTD for ten weeks, were treated three times with apoptotic DCs every other day and left for another nine weeks on WTD to assess lesion progression. We again did not observe any effects on bodyweight or serum cholesterol levels (data not shown). Similar to earlier observations, apopox−DC treatment was effective in modifying splenic DCs one week after apopox−DC-treatment: myeloid CD8α−CD11b+DCs were significantly decreased by 8% and CD103+CD11b−DCs were significantly increased by 17%. No significant effects were observed on lymphoid CD8α+CD11b−DCs or by the treatment with apopctrl−DCs (Figure 10A). Moreover, we again only observed a significant 22% increase in circulating Tregs in apopox−DC-treated mice one week after treatment (Figure 10B).

Interestingly, again only the apopox−DC-treatment resulted in a 35% reduction in monocytes and a 19% reduction in Ly-6C+ monocytes nine weeks after treatment. In line with earlier observations, a significant 41% decrease in CCL2 serum concentrations in apopox−DC-treated mice was observed. No significant effects of apopctrl−DC treatment on immune responses in established atherosclerosis were found (Figure 10C).

OxLDL-presence in apoptotic DCs is needed for stabilization and reduction of lesion progression

Nine weeks after the last treatment, lesion sizes were compared to the baseline group, which was sacrificed at the time of apoptotic DC treatment. Atherosclerotic lesion progression in the aortic root was significantly attenuated by 38% in apopox−DC-treated
mice, whereas apop\textsuperscript{ctrl}-DC treatment had no significant effect on lesion progression (Figure 11A). Furthermore, we now observed a more stable lesion phenotype in apop\textsuperscript{ox}-DC-treated-mice: aortic root lesions were composed of 44.0±3.2% collagen, while those of control mice were composed of 33.2±3.9% and of apop\textsuperscript{ctrl}-DC-mice of 30.3±2.1% collagen (Figure 11B). Although inflammatory monocytes were again reduced, no effect on lesional macrophage content was observed (Figure 11C). The reduced T cell responses again resulted in a 64% decrease in T cells in the aortic root of apop\textsuperscript{ox}-DC-treated-mice. Interestingly also a 53% decrease of T cells was observed in apop\textsuperscript{ctrl}-DC-treated-mice, compared to control mice (Figure 11D).

**Figure 11.** Apop\textsuperscript{ox}-DC-treatment reduces lesion progression and increases stability. A. At sacrifice, lesion size in the three valve area of the aortic root was determined; representative cross-sections stained with Oil-Red-O and hematoxylin are shown. B. Collagen content was determined by Massons’ Trichrome staining. C. Macrophage content was determined by MOMA-2 staining. D. T cell numbers were determined by αCD3 staining. The dotted line indicates baseline values. All values are expressed as mean±SEM and are representative of all mice. Scale bar, 300 µm. *P<0.05, **P<0.01.
Discussion

Tolerogenic DC therapies have been investigated in autoimmune diseases for their potential to induce tolerance. In atherosclerosis, a study by Hermansson et al. demonstrated that adoptive transfer of apoB100-pulsed tolerogenic DCs, which induced Treg responses in vitro, could attenuate lesion development, by reducing CD4+ T cell responses in vivo. In this study we show that apoptotic DCs and in particular oxLDL-induced apoptotic-DCs may form an effective treatment for atherosclerosis by inducing tolerogenic DC responses in vivo. In addition, treatment with apoptotic DCs also dramatically modifies monocyte responses, which to our knowledge has not previously been described for apoptotic cell treatment. As oxLDL-induced apoptotic DCs contain oxLDL, the main antigen implicated in atherosclerosis, they are capable of transferring atherosclerosis-relevant antigenic peptides in the context of tolerogenic signals. While it needs to be established to what extent antigen-specific tolerance was induced, our results clearly show that oxLDL-induced apoptotic DCs are needed to modulate ongoing immune responses in established atherosclerosis.

We first established in vitro that uptake of apoptotic DCs modulates LPS responses by viable DCs and modulates T cell responses. However, only oxLDL-induced apoptotic DCs were able to increase IL-10 by viable DCs in response to LPS and induce Treg responses. While both oxLDL-induced and unpulsed apoptotic DCs reduced CD8+ T cell proliferation and Th17 responses, only oxLDL-induced apoptotic DCs were able to decrease CD4+ T cell proliferation and Th1 responses, indicating that the presence of oxLDL in apoptotic cells could more potently modulate T cell responses.

Next the effect of apoptotic DC-treatment on initial and advanced atherosclerotic lesions was assessed. In both initiation and progression of atherosclerosis, only apo\textsuperscript{ox}-DC-treated mice showed an induction of a tolerogenic phenotype in splenic DCs, resulting in an increase in Tregs and a decrease in T cell proliferation, likely via IL-10 and nitrite production. Similarly, we found increased Tregs in the circulation and the aorta of apo\textsuperscript{ox}-DC-treated-mice only. This translated into reduced numbers of T cells in the aortic root adventitia, indicating that inflammatory T cell responses and recruitment in apo\textsuperscript{ox}-DC-treated mice were attenuated. In apo\textsuperscript{ctrl}-DC-treated mice, a mild decrease in inflammatory splenic DCs was seen in initial atherosclerosis. This resulted in an overall reduced splenic T cell response as well as reduced adventitial T cells. However, the effects of apo\textsuperscript{ctrl}-DCs were milder compared to the apo\textsuperscript{ox}-DC-treated mice and an induction of Tregs was absent, indicating that presence of oxLDL potentiates the effect of apoptotic DCs on T cell responses and its presence in apoptotic DCs is needed to promote Treg responses.

Interestingly, apo\textsuperscript{ox}-DC-treatment affected both monocyte counts and inflammatory monocytes in initial and advanced atherosclerosis, while treatment with apo\textsuperscript{ctrl}-DCs only affected inflammatory monocytes in initial atherosclerosis. One week after treatment, Ly-6C\textsuperscript{hi} monocytes were reduced, which was in line with a significant drop in CCL2 serum levels. However, only the apo\textsuperscript{ox}-DC-treatment resulted in a long-term decrease of Ly-6C\textsuperscript{hi} and CCR2\textsuperscript{+} monocytes, CCL2 serum levels, and decreased circulating monocytes. A study by Nahrendorf et al. has shown that
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both Ly-6C hi and Ly-6C low monocytes can ingest fluorescent nanoparticles, while a later study by Peng et al. 31 found that Ly-6C low monocytes preferentially ingest apoptotic cells, although also Ly-6C hi monocytes were found to ingest apoptotic cells. Ly-6C low monocytes were found to migrate to the spleen where they differentiated into DCs, which were able to inhibit T cell responses 31. However, it has been shown that Ly-6C hi monocytes downregulate Ly-6C upon uptake of latex beads 32 and therefore also Ly-6C hi monocytes could have substantially taken up apoptotic cells. Moreover, Ly-6C hi and CCR2+ monocytes were found to be directly linked to the generation of CD103+ DCs in the lung 33. This indicates that Ly-6C hi and CCR2+ monocytes could also engulf apoptotic cells, migrate to the spleen and locally differentiate into CD103+ DCs, which seems to be augmented by oxLDL presence in apoptotic DCs. These studies indicate that apoptotic DC exposure and their uptake by monocytes may have directly affected Ly-6C expression. Indeed, our lab shows that monocytes co-cultured in vitro with apoptotic DCs downregulate Ly-6C (V. Frodermann and J. Kuiper, unpublished data). This, however, does not explain long-term effects on monocytes in apop ox-DC-treated mice. Swirski et al. 34 and Tsou et al. 35 previously described the critical role of Ly-6C hi and CCR2+ monocytes in atherosclerosis and their direct correlation with lesional macrophages. They show that the inflammatory environment prevents the production of Ly-6C low monocytes 34. On the other hand, CCL2−/− mice have decreased Ly-6C hi monocytes 36. Since we observed a decrease in the levels of inflammatory cytokines IL-6 and CCL2, this may explain the long-term reduction in circulating inflammatory Ly-6C hi and CCR2+ monocytes in apop ox-DC-treated mice. This long-term effect could also explain why we only see decreased lesional macrophages in apop ox-DC-treated mice. The initial drop in inflammatory monocytes and CCL2 serum levels is sufficient to reduce initial lesion sizes in both apoptotic-DC-treated groups, which is in line with reduced lesions in CCL2−/− and CCR2−/− mice 36,37. Apop ctrl-DC treatment did not affect monocyte homeostasis in advanced atherosclerosis, again indicating that the presence of oxLDL is required to potentiate the effect on monocytes and to affect them in the advanced stages of disease.

However, reduced CCL2 serum levels and reduced monocyte responses do not affect macrophage content of pre-established lesions. Therefore, in contrast to early lesions, advanced lesions seem to be more affected by a general decrease in the inflammatory status and a decrease in T cell responses, while early lesions are more modulated by effects on monocyte responses. This is in line with earlier work from our group showing that CCR2 deficiency does not affect established lesions 38 and a recent study showing that macrophage content of established lesions is determined by local macrophage proliferation rather than influx of monocytes 39. The pronounced effects of apop ox-DCs on T cell responses and inflammation most likely contribute to the clear beneficial effects on pre-established lesions. Only apop ox-DC-treatment increased lesion stability. This treatment induced an increased production of IL-10 by splenic DCs and initial increases of serum IL-10 levels were found upon treatment. IL-10 has been shown to promote lesion stability as both C57BL/6 40 and LDLr−/− mice deficient in IL-10 exhibit reduced collagen and lesion stability.

From our experiments, it is clear that oxLDL-induced apoptotic DCs are more
efficient in inducing anti-inflammatory responses than non-antigen-pulsed apoptotic DCs. Although our laboratory indicates that antigen processing and presentation by apoptotic DCs themselves are not required to reduce T cell responses, viable DCs could present antigens derived from oxLDL, transferred via apoptotic DCs, in a tolerogenic context, making these DCs more potent in inducing tolerance (V. Frodermann and J. Kuiper, unpublished data). Furthermore, it was found that both uptake of cholesterol and apoptotic cells results in a strong activation of LXR\textsuperscript{42-44}. Indeed, we found that DC exposure to apop\textsuperscript{ox}-DCs resulted in a much stronger upregulation of ABCA1 and ABCG1, two target genes of LXR, than exposure to apop\textsuperscript{ctrl}-DCs. The activation of LXR has been shown to induce a tolerogenic phenotype in DCs, with decreased pro-inflammatory IL-12p70 and increased IL-10 production. Moreover, LXR-activated DCs were found to inhibit T cell activation and reduce IFN-γ production by T cells\textsuperscript{45}. Interestingly, also ApoA-I activation of ABCA1 has been shown to reduce production of TNF-α and IL-6\textsuperscript{46}, which may further promote an anti-inflammatory phenotype of DCs. In addition to the increased IL-10 production by apop\textsuperscript{ox}-DC-exposed DCs, we also observed an upregulation of PD-L1 on these DCs. PD-L1 was shown to promote Treg development and maintenance\textsuperscript{47} and this could contribute to the observed induction of Tregs preferentially by apop\textsuperscript{ox}-DC-treatment. Therefore, overall enhanced LXR activation through excess cholesterol might contribute to the enhanced anti-inflammatory effect of oxLDL-induced apoptotic DCs.

Apoptotic cell treatment was shown to be effective and safe in patients. In graft-versus-host disease, extracorporeal photopheresis has been employed to reduce immune responses. Up to 10% of the patient’s circulating leukocytes are irradiated, resulting in apoptosis, and re-infused, which leads to Treg induction and disease amelioration, without inducing generalized immunosuppression\textsuperscript{48}. Moreover, a recent phase I clinical trial in multiple sclerosis patients showed that treatment with myelin peptide cross-linked apoptotic splenocytes decreases antigen-specific T cell responses\textsuperscript{49}. For atherosclerosis, we prove in this study that apoptotic DC treatment can significantly reduce lesion development, but that the presence of oxLDL in apoptotic DCs is needed to make them potent modulators of established atherosclerosis. Treatment with oxLDL-induced apoptotic DCs would offer a major advantage: LDL can be isolated from the patients, oxidized, and added to patient-derived DCs, providing tolerance induction by patient-specific LDL-derived peptides. This circumvents the search for relevant antigenic-peptides and ensures a patient-tailored treatment. Apop\textsuperscript{ox}-DC-treatment should therefore prove to be a very specific and efficient treatment of atherosclerosis.
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References


