Chapter 5

Disrupting myeloid IL-10R1 signalling impedes atherogenesis in hyperlipidaemic LDLR−/− mice

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In preparation
Abstract

Aims & Background

The anti-inflammatory cytokine IL-10 acts on various vascular (immune) cells to suppress atherosclerosis development. However, the exact contribution of specific cell types in this context remains unclear. Here, we investigated the functional involvement of macrophages and neutrophils in IL-10-mediated atheroprotection by abrogating IL-10R signalling in a myeloid-specific manner.

Methods & Results

We used LysMCre-IL-10R1\textsuperscript{floxed} mice to conditionally abrogate the IL-10R1 gene in myeloid cells. In cultured primary macrophages, IL-10R-deficiency evokes a pro-inflammatory phenotype characterized by high IL-12p40 and TNF production upon LPS challenge. After ten weeks of high cholesterol feeding, IL-10R1\textsuperscript{del}-transplanted LDLR\textsuperscript{-/-} mice revealed significantly decreased plaque size and severity compared to wildtype mice. We associated this surprising phenotype with diminished plaque neutral lipid content, lower myeloid cell accumulation and enhanced levels of apoptosis.

Conclusions

Since myeloid IL-10R1-deficiency attenuates plaque size and severity in LDLR\textsuperscript{-/-} mice, macrophages and neutrophils are less likely to contribute to IL-10-mediated atheroprotection.
Introduction

Atherosclerosis forms the pathological substrate for ischemic heart disease through a multifaceted interplay between inflammatory responses and hyperlipidaemia. Characteristically, monocyte-derived macrophages and other leukocytes accumulate in the arterial intima in response to local lipoprotein retention. Here, a vicious cycle of immune activation and sustained cell recruitment fuels an inflammatory state, in part by production of pro-inflammatory cytokines such as TNF, IL-1β, IL-6, IL-12 1-3. Opposing this inflammatory response are select anti-inflammatory cytokines (e.g. interleukin (IL-10)) that support a more balanced plaque microenvironment. Since its presence in human atherosclerosis was recognized 4-7, a large body of evidence has emerged endorsing IL-10 as a potent regulator of several cellular processes central to plaque development. In plaques, this pleiotropic cytokine is produced by leukocytes, most notably macrophages 8-10, and acts to (auto)regulate pro-inflammatory cytokine synthesis 11-14. Furthermore, IL-10 influences foam cell formation by stimulating both scavenger receptor-mediated uptake of modified lipoproteins by macrophages and subsequent cholesterol efflux 1,3,15. Additionally, IL-10 has been described to convey pro-survival signals to immune cells 4,6,16-21, limit breakdown of extracellular matrix 5,8,10,22-24 and reduce procoagulant factors in plaques 11,25.

As such, over the past 15 years, IL-10 signalling has provided researchers with a suitable target for selective intervention in experimental atherosclerosis. Indeed, a host of studies has employed either recombinant IL-10 administration 10,15,26,27, IL-10 gene therapy 16-21,28 or disruption of IL-10 5,22-24,29 and its downstream signalling components 25,30 to effectively consolidate its status as an atheroprotective cytokine. Accordingly, others have reported that IL-10 might serve as an important protective and prognostic factor in patients with unstable angina 10,26-28.

Yet, the ways by which IL-10 impacts the atherosclerotic process are incompletely understood. For instance, it remains uncertain which of IL-10’s many target cell types mediate its beneficial properties in atherosclerosis. Because myeloid responsiveness to IL-10 is required for appropriate immunoregulation in several inflammatory settings 28,31, we hypothesized that myeloid cells might similarly be critical to IL-10-mediated modulation of plaque inflammation. Hence, we assessed the functional involvement of macrophage and neutrophil IL-10R signalling in atherogenesis using hyperlipidaemic mice carrying a conditional deletion of the IL10R1 gene. Our results reveal an unexpected pro-atherogenic role for myeloid IL-10R1 signalling in LDLR⁻/⁻ mice.
Materials & Methods

Animals

Mice carrying floxed IL-10R1 alleles were crossed to LysMCre-mice to yield animals with a homozygously floxed IL-10R1 gene and either wildtype or heterozygous knock-in for LysMCre (IL-10R1\textsuperscript{wt} and IL-10R1\textsuperscript{del}, respectively). These animals were backcrossed to C57Bl6 eight times. LDLR\textsuperscript{-/-} mice were acquired from Jackson Laboratories (Bar Harbor, Maine, US) and had been backcrossed to C57Bl6 ten times. The Committee for Animal Welfare of Maastricht University Medical Center approved all study protocols (permit-nr. 2008-064 and 2010-167).

Bone marrow transplantation

SPF-housed female LDLR\textsuperscript{-/-} mice were provided with water supplemented with neomycin (100mg/l; GIBCO, Breda, The Netherlands) and polymyxin B sulphate (6 x 10\textsuperscript{4} U/l; GIBCO, Breda, The Netherlands) one week prior to transplantation. Mice were subsequently subjected to medullar aplasia through 6 Gy total body irradiation on two consecutive days. LDLR\textsuperscript{-/-} recipients received intravenous injection of 5 x 10\textsuperscript{6} pooled bone marrow cells from either IL-10R1\textsuperscript{wt} mice or IL-10R1\textsuperscript{del} donors (all littermates). Bone marrow engraftment was evaluated ten weeks post-transplantation by isolating genomic DNA from blood leucocytes using the GFX Genomic Blood DNA purification kit (Amersham Pharmacia Biotech Inc., Arlington Heights, Illinois, USA) and assessing the quantity of native (LDLR\textsuperscript{-/-}) DNA using RT-qPCR, as previously described\textsuperscript{28,29}.

Atherosclerosis assessment

Following a six-week recovery period, LDLR\textsuperscript{-/-} recipients were provided a high-cholesterol diet (HCD) containing 16% fat, 0,15% cholesterol and no cholate (Western type diet 4021.13, Hope Farms, The Netherlands) for 6 or 10 weeks to induce either early or more advanced plaque formation. At indicated time points over the course of each experiment fasting blood samples were collected for analyses as described below. Upon sacrifice, hearts were isolated, dissected and frozen in Tissue-Tec (Shandon, Veldhoven, the Netherlands). Subsequently, aortic roots were cut into 7µm sections, of which serial cryosections were routinely stained with toluidine blue for plaque quantification using Adobe Photoshop software. Moreover, morphometric analysis for lesion composition (i.e. collagen content, necrosis, foam cell content and inflammatory burden) was performed. On the basis of their morphology, plaques were classified as early, moderate or advanced as described before\textsuperscript{29}. 

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Briefly, early lesions were considered to consist of foam cells, but lacked a necrotic core. Moderate lesions contained a fibrous cap and may or may not present with a necrotic core. However, no macrophage infiltration into the tunica media must be apparent. Advanced lesions did show convincing macrophage infiltration into the tunica media and may display elastic lamina degradation as well as more severe necrosis and fibrosis.

**Immunohistochemistry**

Murine tissue cryosections were fixed in acetone prior to incubation with antibodies against monocytes and macrophages (MOMA-2, a gift from G. Kraal, VUmc, Amsterdam); newly recruited myeloid cells (ERMP-58, a gift from P. Leenen, Erasmus MC, Rotterdam); infiltrated macrophages (Mac-1 directed against CD11b, a gift from G. Kraal); granulocytes (NIMP1 directed against Ly6G, a gift from P. Heeringa, UMCG, Groningen) and T-cells (KT3 directed against CD3, a gift from G. Kraal). Subsequently, staining was visualized using a biotin-labelled rabbit-α-rat secondary antibody and ABC kit (Vector Labs, Burlingame, CA, USA). Oil Red O staining enabled visualization of neutral lipids in plaques. TUNEL staining (Roche Diagnostics, Mannheim, Germany) was used for detection of apoptotic cells.

**Lipid analysis**

Total cholesterol and triglyceride levels were determined on plasma and homogenized liver samples using standard enzymatic kits according to manufacturer’s protocols (Sigma-Aldrich, Zwijndrecht, NL).

**Flow cytometry analysis**

Plasma cytokine levels were measured by flow cytometry using a Cytometric Bead Array (BD Biosciences, CA, USA) according to manufacturer’s instructions. Absolute numbers of circulating leukocytes were determined by FACS in combination with Trucount Beads (BD Biosciences, CA, USA). Leukocytes are defined as CD45+, T-cells as CD45+CD3+NK1.1+, NK-cells as CD45+CD3+NK1.1+, NKT-cells as CD45+CD3+lowNK1.1+low, B-cells as CD45+CD3+NK1.1+220+, granulocytes as CD45+CD3+NK1.1+220+CD11b+Ly6G+ and monocytes as CD45+CD3+NK1.1+220+CD11b+Ly6G+. Monocytes were further divided in Ly6Chigh, Ly6Clow and Ly6C- populations. Th-cells were defined as CD45+CD3+CD4+, while cytotoxic T-cells were CD45+CD3+CD8+. An overview of antibodies is presented in supplementary Table SI. All FACS measurements were performed on a FACSCanto II with FACSDiva software (both BD Biosciences, CA, USA).
Primary macrophage culture

Bone marrow cells were isolated from the hind limbs of wildtype and IL-10R1-deficient mice and cultured in RPMI-1640 (Life Technologies, Bleiswijk, the Netherlands) with addition of 10% heat-inactivated foetal calf serum (Bodinco, Alkmaar, the Netherlands), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (all from GIBCO Invitrogen, Breda, the Netherlands) and 15% L929-conditioned medium (LCM) for 8–9 days to generate bone marrow-derived macrophages (BMM), as described previously \(^1,^6,^30\). BMM were seeded at 3.5 x 10^5 cells/well in bacteriologic plastic 24-well plates (Greiner Bio-One, Alphen a/d Rijn, NL) and incubated with 10ng/ml of recombinant murine IL-10 (R&D Systems Inc., Abingdon, UK) as indicated. BMM were activated with 10 ng/ml LPS for 3, 8 or 24 hours where designated.

Gene expression

RNA was isolated from BMM and liver samples using the High Pure RNA Isolation Kit (Roche, Woerden, the Netherlands). With regard to RNA isolation from aortic arches we used the RNeasy mini column kit (Qiagen, Venlo, the Netherlands). 500ng total RNA was reverse transcribed using iScript cDNA Synthesis Kit (BioRad, Veenendaal, the Netherlands). Quantitative PCR (qPCR) was performed using 10ng cDNA, 300nM of each primer, and sensiMix SYBR Hi-ROX (Bioline, Brussels, Belgium) in a total volume of 20μl. Gene expression levels were corrected for cyclophilin A as reference gene. Primer sequences are available upon request.
Cytokine secretion

TNF and IL-12 secretion by wt and IL-10R1\textsuperscript{del} BMM was quantified in supernatants after LPS exposure using corresponding ELISA kits (Invitrogen, Bleiswijk, NL) according to manufacturer’s protocols.

Macrophage oxLDL loading

BMM, left untreated or treated for 24 hours with 10ng/ml rIL-10 were incubated for 3 hours in Optimem-1 with 12.5 mg/ml DiI-labelled oxLDL (SanBio, Uden, NL). Uptake was assessed by flow cytometry after residual oxLDL was washed away.

Statistics

Data are presented as mean ± the standard error of the mean (SEM). All statistical analyses were performed using GraphPad Prism (GraphPad Software Inc.). Groups were compared using (Welch-corrected) two-tailed, non-paired t-tests. Significance was accepted at the level of \( p < 0.05 \). *, ** and *** indicate \( p < 0.05, \) 0.01 and 0.001, respectively.

Results

\textbf{IL-10R1\textsuperscript{del} primary macrophages are refractory to IL-10 and hyperresponsive to LPS}

To assess macrophage function in absence of IL-10R signalling, we specifically deleted IL-10R1 in the myeloid lineage by crossing IL-10R1\textsubscript{floxed} mice \cite{1,28} with LysMCre mice \cite{31-33}. The resulting lysMCre-IL-10R1\textsuperscript{floxed/floxed} mice were compared to IL-10R1\textsuperscript{floxed/floxed} mice as wildtype controls and will be referred to as IL-10R1\textsuperscript{del} and IL-10R1\textsuperscript{wt}, respectively. Deletion in bone marrow-derived macrophages (BMM) resulted in an approximate 80% decrease in \textit{IL10R1} gene expression (Fig.1A, further characterization has been described in \cite{28,34}). This significantly impaired induction of established IL-10 target genes (e.g. suppressor of cytokine signalling 3, SOCS3; IL-4Rα) upon IL-10 treatment (Fig.1B, 1C), indicating that genetic ablation of IL-10R1 was both efficient and effective. As IL-10 is an important regulator of innate pathogen-associated inflammation, we tested the response to lipopolysaccharide (LPS). After 24 hours of LPS stimulation, IL-10R1\textsuperscript{del} BMM showed enhanced secretion of pro-inflammatory cytokines TNF and IL-12p40 in comparison to wt cells (Fig. 1D, 1E).
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Figure 1. IL-10R1-deficiency in primary macrophages yields a pro-inflammatory phenotype in vitro. (A-C) Abrogating myeloid IL-10R1 signalling is efficient and effective. Bone marrow-derived macrophages were cultured as described in the Materials & methods section. Gene expression analysis shows significantly lower transcription of the gene encoding IL-10R1 in IL-10R1<sup>del</sup>-cells (grey bars) versus IL-10R1<sup>wt</sup>-cells (white bars). Upon 24 hours incubation with 10ng/ml recombinant IL-10 these cells display reduced expression of known IL-10-inducible genes SOCS3 and IL-4Rα. (D, E) Upon incubation with 10ng/ml LPS for 8 or 24 hours, IL-10R1<sup>del</sup> BMM reveal increased secretion of pro-inflammatory cytokines TNF and IL-12p40 as measured by ELISA. (F, G) Opposed to incubation with 10ng/ml IL-10 alone for 24 hours, sequential incubation of IL-10R1<sup>del</sup> BMM with 24h 10ng/ml LPS impairs inhibition of LPS-induced pro-inflammatory cytokine expression by IL-10. Data represent means ± SEM.
Targeted disruption of IL-10R signalling in myeloid cells attenuates atherosclerotic plaque size and severity in LDLR\(^{-/-}\) mice

Next, we examined the involvement of myeloid IL-10R1 signalling in atherosclerosis development. To this end, we reconstituted lethally irradiated low-density lipoprotein receptor-deficient (LDLR\(^{-/-}\)) mice with bone marrow from either IL-10R1\(^{wt}\) or IL-10R1\(^{del}\) mice. Repopulation of the bone marrow compartment was found to be similar for both groups (97.6\(\%\) ± 0.1 vs. 97.1\(\%\) ± 0.3, ns). After a six-week recovery period, mice were fed a high
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cholesterol diet (HCD) for 10 weeks. Subsequent quantification of atherosclerosis in the aortic sinus revealed a dramatic 70% reduction in lesion area in IL-10R1\textsuperscript{del} mice vs. wildtype-transplanted animals (57.3x10\textsuperscript{3} \(\mu m^2\) ± 10.1 vs. 192.5x10\textsuperscript{3} \(\mu m^2\) ± 16.7, \(p<0.0001\)) (Fig.2A, 2B). Moreover, classification of plaque severity showed that lesions from IL-10R1\textsuperscript{del-tp} LDLR\textsuperscript{-/-} mice were less advanced in comparison to their wildtype counterparts (Fig.2C, Chi-square test \(p<0.0001\)). In line with these findings, routine pathological assessment of plaque composition showed lower levels of collagen, necrosis, monocyte adhesion and adventitial influx in IL-10R1-deficient LDLR\textsuperscript{-/-} mice (Fig. 2D). Additional quantification of plaque collagen area using Sirius Red staining confirmed that lesions from IL-10R1-deficient animals have 34% less fibrous tissue in relation to lesion size (Fig.2E, \(p = 0.007\)).

In order to confirm the consistency of this phenotype, we performed an analogous experiment with a six-week period of high cholesterol feeding. Notably, while plaque size and severity were reduced in comparison to 10 weeks HCD, IL-10R1\textsuperscript{del-tp} LDLR\textsuperscript{-/-} mice demonstrated an identical 70% decrease in plaque area (44.2x10\textsuperscript{3} \(\mu m^2\) ± 6.4 vs. 153.3 x10\textsuperscript{3} \(\mu m^2\) ± 20.7, \(p = 0.001\)) (Fig.S1A, S1B). Together, these findings signify that disruption of myeloid IL-10R signalling strongly inhibits atherosclerosis development.

**Figure S1.** Plaque size and severity are reduced upon 6 weeks HCD in IL-10R1\textsuperscript{del-tp} LDLR\textsuperscript{-/-} mice. (A) Quantification of lesion area in aortic roots from IL-10R1\textsuperscript{wt} and IL-10R1\textsuperscript{del-tp} LDLR\textsuperscript{-/-} mice upon 6 weeks HCD, showing a consistent phenotype with reduced atherogenesis. (B) Plaque progression was reduced in myeloid IL-10R1-deficiency. Data represents means ± SEM.

**Myeloid IL-10R1-deficiency attenuates hypercholesterolaemia, plaque neutral lipid content and macrophage cholesterol loading.**

Because hyperlipidaemia is a driving force of atherosclerosis, we explored circulating lipid levels in IL-10R1\textsuperscript{del-tp} LDLR\textsuperscript{-/-} mice. Whilst bodyweight and plasma triglyceride content were equal between genotypes, plasma cholesterol was markedly reduced in IL-10R1-deficient animals (Table I). This effect was greatest after 10 weeks HCD, but already apparent on chow
diet. Next, we questioned whether this resistance to diet-induced hypercholesterolaemia in IL-10R1-del-tp LDLR−/− mice affected plaque size and morphology. Through linear regression analysis of plasma cholesterol levels and plaque size we observed an equal slope for both genotypes, suggesting a similar contribution of cholesterol to lesion development in IL-10R1wt-tp and IL-10R1del-tp LDLR−/− mice (Fig.S2A). However, the generated curves were different with regard to their slope, signifying that for given cholesterol levels IL-10R1-deficient plaques were smaller. To confirm this, we selected mice with comparable cholesterol exposure over time (n=6/genotype) and found that in absence of myeloid IL-10R1-signalling plaque burden was still significantly decreased (4.98x10³ µm² ± 13.9 vs. 159.3x10³ µm² ± 21.1, p = 0.001) (Fig.S2B, S2C). Thus, reduced blood cholesterol levels could not fully account for altered lesion size in these animals. However, as IL-10 is known to influence macrophage cholesterol handling in both in vitro and in vivo settings 1,4-6, we quantified plaque neutral lipid accumulation by Oil Red O (ORO) staining. This revealed significantly reduced ORO+ area in relation to plaque size in IL-10R1-del-tp LDLR−/− mice (Fig.3A, 3B), suggesting diminished foam cell formation in vivo. We subsequently validated this finding in vitro, where IL-10R1del BMM displayed significantly less oxLDL uptake compared to IL-10R1wt cells upon 3 hours of oxLDL-incubation (Fig.3C).

This effect could be emulated in wt cells through treatment with a neutralizing αIL-10 antibody, whereas uptake by IL-10R1del BMM was not affected (Fig. 3D). This effect was associated with decreased transcription of the scavenger receptors CD36 and SR-A, as well as efflux transporters ABCA1 and ABCG, which were shown previously to be IL-10-inducible genes. (Fig.3E, 3F) 1,14,35. Consequently, although additional factors beyond cholesterol are likely causal to reduced atherogenesis in myeloid IL-10R1 deficiency, resistance to macrophage cholesterol loading might certainly contribute to the altered lesion size and composition in these mice.

**Table I.** Basal characteristics of wt and IL-10R1-deficient LDLR−/− mice on chow and high-cholesterol diet

<table>
<thead>
<tr>
<th>Basal characteristics</th>
<th>chow diet</th>
<th>4w HCD</th>
<th>10w HCD</th>
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<tbody>
<tr>
<td>IL-10R1wt</td>
<td>IL-10R1del</td>
<td>IL-10R1wt</td>
<td>IL-10R1del</td>
</tr>
<tr>
<td>Bodyweight (g)</td>
<td>17.9 ± 0.2</td>
<td>18.3 ± 0.3</td>
<td>20.4 ± 0.1</td>
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<tr>
<td>Cholesterol (mM)</td>
<td>8.60 ± 0.30</td>
<td>7.62 ± 0.27a</td>
<td>30.68 ± 1.58</td>
</tr>
<tr>
<td>Triglycerides (mM)</td>
<td>0.49 ± 0.03</td>
<td>0.57 ± 0.04</td>
<td>1.02 ± 0.08</td>
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a indicates p < 0.01, b indicates p > 0.0001.
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Figure S2. Reduced plasma cholesterol levels do not fully account for attenuated atherogenesis in IL-10R1del-tp LDLR\(^{-/-}\) mice. (A) Linear regression analysis of plasma cholesterol and plaque size showed a significant difference in the elevation of regression lines. (B) After selecting mice with similar cholesterol exposure in both groups, plaque size (C) was still significantly reduced in IL-10R1del-tp vs. wildtype-tp mice. AUC: area under the curve. Bars represent means ± SEM.

Figure 3. Altered cholesterol handling in IL-10R1\(^{-/-}\) LDLR\(^{-/-}\) mice. (A) Representative micrographs of Oil red O-stained aortic roots of wt and IL-10R1\(^{-/-}\) LDLR\(^{-/-}\) mice. (B) Neutral lipid accumulation was measured in plaques from both genotypes. IL-10R1-deficiency significantly reduces ORO\(^{+}\) area. (C, D) oxLDL uptake by wildtype and IL-10R1\(^{-/-}\) BMM was assessed upon 3 hours incubation with 12,5 mg/ml Dil-labelled oxLDL in OPTIMEM-medium. Bars represent GeoMean ± SEM after culture with oxLDL alone or oxLDL and 10ng/ml IL-10. Histogram of positive counts in both genotypes. (E, F) Gene expression analysis of oxLDL-loaded IL-10R1wt and IL-10R1del BMM for scavenger receptors (CD36, SR-A) and efflux transporters (ABCA1, ABCG1) reveals reduced mRNA levels in IL-10R1 deficiency. Data represents means ± SEM.

<table>
<thead>
<tr>
<th>Table SII. Blood leukocyte profile in wildtype and IL-10R1-deficient LDLR(^{-/-}) mice</th>
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<tr>
<td><strong>IL-10R1wt</strong></td>
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<tr>
<td><strong>Total Leukocytes</strong> (CD45+)</td>
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<tr>
<td><strong>Myeloid cells</strong></td>
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<tr>
<td><strong>Granulocytes</strong> (CD11b+ Ly6G+)</td>
</tr>
<tr>
<td><strong>Monocytes</strong> (CD11b+ Ly6C(^{-/-}))</td>
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<tr>
<td><strong>Lymphoid cells</strong></td>
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<tr>
<td><strong>T-cells</strong> (CD3+ NK1.1(^{-/-}))</td>
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<td></td>
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<td></td>
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<tr>
<td><strong>NKT-cells</strong> (CD3(^{-/-}) NK1.1(^{-/-}))</td>
</tr>
<tr>
<td><strong>NK-cells</strong> (CD3(^{-/-}) NK1.1(^{+}))</td>
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<tr>
<td><strong>B-cells</strong> (B220+)</td>
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<tr>
<td></td>
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<tr>
<td><strong>B1</strong> (CD11b(^{+}))</td>
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Absolute numbers of circulating leukocytes in IL-10R1del-tp LDLR\(^{-/-}\) are similar to wildtype-tp animals. All samples were measured by flow cytometry (n=8/group).
**Myeloid IL-10R1 deficiency limits plaque macrophage and neutrophil content, but does not affect systemic inflammation.**

Since IL-10 impedes the inflammatory responses that drive plaque progression, we tested whether myeloid IL-10R1-deficiency affected systemic and intimal inflammation in our atherosclerosis model. Upon quantification of absolute blood leukocytes numbers after 6 weeks HCD we found no differences in myeloid subsets, while in the lymphoid populations only a mild increase in NKT-cells was observed (Table SII). Additionally, plasma levels of inflammatory cytokines (e.g. IL-10, TNF, IL-12p70) were also comparable between genotypes (Fig.4A).

**Table SII.** Blood leukocyte profile in wildtype and IL-10R1-deficient LDLR<sup>−/−</sup> mice

<table>
<thead>
<tr>
<th></th>
<th>IL-10R1&lt;sup&gt;wt&lt;/sup&gt;</th>
<th>IL-10R1&lt;sup&gt;del&lt;/sup&gt;</th>
<th>p-value</th>
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<tbody>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Leukocytes (CD45&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>11745 ± 882</td>
<td>14490 ± 1634</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Myeloid cells</strong></td>
<td></td>
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<tr>
<td>Granulocytes (CD11b&lt;sup&gt;+&lt;/sup&gt;Ly6G&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>2108 ± 376</td>
<td>3469 ± 772</td>
<td>ns</td>
</tr>
<tr>
<td>Monocytes (CD11b&lt;sup&gt;+&lt;/sup&gt;Ly6G&lt;sup&gt;−&lt;/sup&gt;)</td>
<td>1066 ± 112</td>
<td>1216 ± 117</td>
<td>ns</td>
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<tr>
<td>- Ly6C&lt;sup&gt;high&lt;/sup&gt;</td>
<td>382 ± 55</td>
<td>418 ± 47</td>
<td>ns</td>
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<tr>
<td>- Ly6C&lt;sup&gt;low&lt;/sup&gt;</td>
<td>53 ± 8</td>
<td>73 ± 15</td>
<td>ns</td>
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<tr>
<td>- Ly6C&lt;sup&gt;−&lt;/sup&gt;</td>
<td>637 ± 62</td>
<td>734 ± 80</td>
<td>ns</td>
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<tr>
<td><strong>Lymphoid cells</strong></td>
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<tr>
<td>T-cells (CD3&lt;sup&gt;+&lt;/sup&gt;NK1.1&lt;sup&gt;−&lt;/sup&gt;)</td>
<td>1827 ± 155</td>
<td>2138 ± 250</td>
<td>ns</td>
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<tr>
<td>- Th-cells (CD4&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>1216 ± 98</td>
<td>1418 ± 178</td>
<td>ns</td>
</tr>
<tr>
<td>- Cytotoxic T-cells (CD8&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>568 ± 48</td>
<td>672 ± 68</td>
<td>ns</td>
</tr>
<tr>
<td>- Memory T-cells (Ly6C&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>160 ± 17</td>
<td>197 ± 29</td>
<td>ns</td>
</tr>
<tr>
<td>NKT-cells (CD3&lt;sup&gt;low&lt;/sup&gt;NK1.1&lt;sup&gt;low&lt;/sup&gt;)</td>
<td>21 ± 3</td>
<td>27 ± 3</td>
<td>0.03</td>
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<tr>
<td>NK-cells (CD3&lt;sup&gt;+&lt;/sup&gt;NK1.1&lt;sup&gt;−&lt;/sup&gt;)</td>
<td>125 ± 16</td>
<td>91 ± 16</td>
<td>ns</td>
</tr>
<tr>
<td>B-cells (B220&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>6331 ± 468</td>
<td>6053 ± 1125</td>
<td>ns</td>
</tr>
<tr>
<td>- B1 (CD11b&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>70 ± 5</td>
<td>62 ± 12</td>
<td>ns</td>
</tr>
<tr>
<td>- B2 (CD11b&lt;sup&gt;−&lt;/sup&gt;)</td>
<td>6262 ± 465</td>
<td>5991 ± 1116</td>
<td>ns</td>
</tr>
</tbody>
</table>

Absolute numbers of circulating leukocytes in IL-10R1<sup>del</sup>-tp LDLR<sup>−/−</sup> are similar to wildtype-tp animals. All samples were measured by flow cytometry (n=8/group).
Figure 4. Myeloid IL-10R1-deficiency in LDLR−/− does not affect systemic inflammation, but limits macrophage and neutrophil accumulation in plaques. (A) Circulating plasma levels of mouse inflammatory cytokines were determined through Cytometric Bead Array (CBA) and flow cytometry, revealing no significant differences between genotypes. (B, C) To assess inflammatory mediators in the vessel wall, RNA isolation from aortic arches was performed as described in the Methods & Materials section. Normalized expression of CD68 indicated reduced macrophage content and thus reduced plaque formation in aortic arches of IL-10R1wt-tp and IL-10R1del-tp LDLR−/− animals. Inflammatory gene expression analysis for demonstrates equal mRNA levels for these genes in the aortic arch, except for reduced expression of IL-12p40. (D-I) Aortic root plaques were evaluated for the presence of macrophages recently infiltrated myeloid cells and neutrophils through staining for MOMA-2, ER-MPS8 and NIMP1. Lesions consisted mainly of macrophages, accordingly showing a significant reduction of MOMA-2+ area in IL-10R1del-tp LDLR−/− mice (D,E). Likewise, numbers ER-MPS8+ cells were significantly lowered in myeloid IL-10R1-deficiency upon 6 and 10 weeks of HCD (F, G). Plaque neutrophil content was transiently decreased in IL-10R1del-tp LDLR−/− after 6 weeks, but not 10 weeks of HCD. Data represents means ± SEM.
Figure 5. Liver inflammation in IL-10R1<sup>wt</sup>-tp and IL-10R1<sup>del</sup>-tp LDLR<sup>−/−</sup> mice. (A) Inflammatory gene expression is unaltered in livers of wildtype- and IL-10R1<sup>del</sup>-transplanted recipients. Liver RNA was isolated as described in the Methods & Materials section. (B-G) Liver cryosections were assessed for leukocyte content through immunohistochemistry for Mac-1 (B, C), NIMP1 (D, E) and CD3 (F,G), targeting infiltrated macrophages, neutrophils and T-cells, respectively. Only neutrophil content was significantly decreased in myeloid IL-10R1-deficiency. Arrows indicate stained cells. Data represents means ± SEM.
To further assess the inflammatory status of the vessel wall, we first assessed inflammatory gene expression in aortic arches of wt and IL-10R1-deficient recipients. Although limited aortic expression of the ubiquitous macrophage marker CD68 reflected impaired atherogenesis in the latter (Fig.4B), myeloid IL-10R1 disruption did not elicit a significantly skewed inflammatory profile. Paradoxically, only IL-12p40 showed a reduced expression level (Fig.4C). Subsequently, we measured plaque leukocyte content. As shown by MOMA-2 staining, lesions consisted mostly of macrophages, which corresponded to a predictable decrease in total plaque macrophage area in IL-10R1del-tp mice (Fig.4D, 4E, p = 0.02). Accordingly, the number of newly recruited myeloid cells (as measured by ER-MP58 staining) was smaller in IL-10R1del lesions after 6 weeks and 10 weeks of HCD (Fig.4F, 4G). Neutrophil counts were only significantly decreased in IL-10R1del-tp LDLR-/- mice at 6 week HCD, but not after 10 weeks high cholesterol feeding (Fig.4H–I). Equal amounts of CD3+ T-cells were observed in both groups (data not shown).

These findings could be partly extrapolated to the liver by showing that hepatic expression of inflammatory regulators was similar for both groups (Fig.5A). Whereas we did observe a decreased hepatic neutrophil presence in myeloid IL-10R1-deficiency, infiltrated macrophage and T-cell counts proved comparable for both IL-10R1wt and IL-10R1del mice (Fig.5B-G). Hence, in contrast to our in vitro data, loss of myeloid IL-10 responsiveness does not lead to systemically enhanced inflammation in an in vivo atherosclerotic setting. Rather, our findings reveal IL-10 affects the amount of macrophages and neutrophils present in atherosclerotic lesions.

**Figure 6.** Myeloid IL-10R1 deficiency enhances plaque apoptotic cell count. (A) Quantification of TUNEL+ cells in atherosclerotic lesions of IL-10R1wt-tp and IL-10R1del-tp LDLR-/- mice reveals higher apoptotic cell count/plaque area in myeloid IL-10R1-deficiency. (B) Linear regression analysis shows regression lines with equal slopes, but significantly different elevations, indicating IL-10R1del-plaques have a similar degree of apoptosis than larger wt lesions. Data represents means ± SEM.
Myeloid IL-10R1 deficiency enhances plaque apoptotic cell count.

Intraplaque apoptosis is an important determinant of plaque progression and IL-10 has been shown previously to protect both myeloid and lymphoid cells from apoptotic cell death. Therefore, we investigated the effect of myeloid IL-10R1 deficiency on cell survival in murine plaques. Apoptosis quantification using TUNEL staining revealed a significantly higher incidence of apoptotic cells/mm² plaque in lesions from IL-10R1del-tp animals (Fig.6A). Linear regression analysis reveals that while the increment in apoptotic cell count with increasing lesion area is similar for both genotypes, IL-10R1del plaques accumulate apoptotic cells at an earlier time point in lesion development than their wt counterparts (Fig.6B). As such, altered susceptibility to apoptotic cell death in earlier stages of plaque development could contribute to reduced lesion size in IL-10R1del-tp LDLR⁻/⁻ mice.

Discussion

Despite previous reports attesting to the anti-atherogenic properties of IL-10, the specific cell types responsible for IL-10’s protective effects in atherosclerosis remain to be defined. We used hyperlipidaemic mice carrying a conditional deletion of the ligand-binding domain of the IL-10 receptor complex (i.e. IL-10R1) in their myeloid lineage to assess whether macrophages and neutrophils form the basis of IL-10-mediated atheroprotection.

IL-10’s ability to negatively regulate macrophage activation was recognized soon after its discovery in 1989. Since then, others have strengthened this premise under varying circumstances. Comparably, our current in vitro findings demonstrate that primary macrophages deficient for IL-10R1 lose most of their responsiveness to IL-10 and consequently are less well equipped to dampen TLR4-mediated inflammation in vitro. Thereby, we reinforce the observations by Pils et al., who demonstrated the pro-inflammatory nature of the IL-10R1del phenotype in an experimental LPS endotoxaemia model. Whereas prior evidence demonstrates a robust and consistent decrease in atherosclerosis development and associated inflammation by enhancing local or systemic IL-10 levels, haematopoietic or systemic deficiency for this cytokine typically yields increased plaque formation. In this light, we expected IL-10R1del-tp LDLR⁻/⁻ mice to display increased inflammation and atherogenesis. Instead, we observed that loss of IL-10R signalling in myeloid cells attenuates atherosclerotic lesion size and severity in HCD-fed LDLR⁻/⁻ mice through altered susceptibility to diet-induced hypercholesterolaemia, leukocyte accumulation and apoptosis. This not only shows that the involvement of IL-10 in atherosclerosis development is more complex than thought before, but also suggests IL-10 exerts diverging cell-specific effects that act as important determinants of disease outcome.
Lipid metabolism has a central role in the pathogenesis of atherosclerosis. Previous reports concerning IL-10 overexpression in experimental atherosclerosis have reported an average decrease in plasma cholesterol levels of approximately 50% \cite{18,20,24,44}. Contrasting, our data reveal a substantial reduction in plasma cholesterol levels in mice with abrogated myeloid IL-10R signalling, which was also reflected in the amount of neutral lipids present in murine atherosclerotic lesions. The mechanisms responsible for this robust phenotype will be discussed in detail in the next chapter.

At plaque level, macrophages take up extravasated lipids through scavenger receptors CD36 and SR-A \cite{1,3,5,6,42}, while facilitating cellular cholesterol efflux through expression of ATP binding cassette (ABC) transporters ABCA1 and ABCG1 \cite{43,45}. Convincing \textit{in vitro} and \textit{in vivo} evidence exist on the influence of IL-10 on these cellular processes. Halvorsen et al. were the first to show that IL-10 enhances foam cell formation \cite{4,46,47,48}. Whereas some conflicting reports exist \cite{4,44,50}, these findings were soon corroborated and expanded by others, revealing IL-10 induces both uptake and efflux of modified LDL particles through stimulation of scavenger receptors and ABC transporters \cite{1,3,5,6,17}. Accordingly, coronary lesions from a Japanese study population showed co-localisation of IL-10 expressing macrophages and oxLDL \cite{34,45}. Our data are in line with these reports by showing that IL-10R1\textsuperscript{del} macrophages display impaired oxLDL uptake due to lowered expression of scavenger receptors, while IL-10R1-deficient bone marrow recipients have reduced plaque lipid content.

Macrophage apoptosis is widely recognized as an important phenomenon in atherosclerotic disease \cite{46,47,51-53}. Prior evidence suggests that IL-10 modulates cell survival under hyperlipidemic conditions. In oxLDL-loaded monocytic THP1 cells, IL-10 treatment protected from ER-stress-induced apoptosis through induction of pro-survival factors \cite{4,29,50,54,55}. Additionally, the presence of IL-10 in plaque tissue has been associated to reduced levels of apoptosis in both experimental and human settings \cite{5,6,17,56}. According to our current understanding, the functional implications of apoptosis in plaque progression are diverging based on the stage of lesion development \cite{34,57,58}. Whereas apoptotic cell death in early lesions can reduce the intimal accumulation of foam cells and thus limit its growth, in more advanced stages apoptosis contributes to necrotic core development and thereby negatively affect plaque stability. In LDLR\textsuperscript{−/−} mice, we found that myeloid IL-10R1 deficiency confers a higher incidence of apoptotic cells per mm\textsuperscript{2} of plaque area as measured by TUNEL staining. Thus, reduced lesion size in IL-10R1\textsuperscript{del}-tp LDLR\textsuperscript{−/−} mice is likely in part attributable to enhanced levels of apoptosis.

Even though IL-10R1\textsuperscript{del} BMM display greater inflammatory potential \textit{in vitro}, we consider the lack of amplified systemic inflammation in IL-10R1\textsuperscript{del}-tp LDLR\textsuperscript{−/−} mice a reflection of the mechanical intricacies that come with cell-cell interactions under hyperlipidaemic conditions. As myeloid IL-10R signalling is dispensable with regard to countering pro-inflammatory mediators in the atherosclerotic vessel wall, other cell types are likely to
facilitate IL-10-based atheroprotection. Although it is possible that lymphoid cells such as T-cells are important in this respect, circulating T- and B-cell numbers were similar between genotypes in our experiments. Alternatively, endothelial cells could act as an influential player in IL-10-mediated immunomodulation. Aside from the fact that IL-10 can favourably affect endothelial cell function \(^{51-53,59-62}\), work by us and others has revealed contrasting outcomes when using macrophage and endothelial cell-specific approaches to interfere with inflammatory signalling pathways \(^{29,54,55}\).

In this context, some have even argued that a dichotomy between pro- and anti-inflammatory cytokines is too simplistic and that a given cytokine may behave in either way depending on multiple variables (e.g. its tissue levels, target cell type, timing, and experimental model) \(^{56}\). In support of this notion, several publications have described uncharacteristic roles for IL-10 in stimulating CD8\(^+\) T-cells \(^{57,58}\) and NK-cells \(^{59-62}\), boosting IFN\(\gamma\) production and IFN\(\gamma\)-dependent gene expression \(^{63}\) and decreasing survival in graft-versus-host disease. Thus, aside from the present study, there have been several reports demonstrating an unfavourable role for IL-10 in inflammatory disease. Moreover, this might help explain why IL-10 treatment regimes in inflammatory bowel disease and rheumatoid arthritis have yielded somewhat disappointing results \(^{64,65}\).

In summary, abrogating myeloid responsiveness to IL-10 strongly reduces plaque development in atherogenic LDLR\(^{-/-}\) mice by mediating resistance to diet-induced hyperlipidaemia and compromising recruitment and survival of myeloid cells in atherosclerotic lesions. Hence, myeloid cells are not responsible for IL-10 mediated atheroprotection. These findings challenge us to re-evaluate our understanding of IL-10 as a favourable influence on atherosclerosis development. Rather, our studies reveal that IL-10 can induce opposing effects in its target cell types, indicating its functional involvement in atherosclerosis is more complex than previously appreciated. Continued investigation directed at dissecting the cell-specific responses to IL-10 is warranted and might help us to more fully comprehend IL-10’s therapeutic potential in chronic inflammatory disease.
References


