Chapter 5

Increased Foam Cell Formation and Atherosclerotic Plaque Apoptosis and Markedly Decreased Neutrophil Recruitment in LDLr−/− Mice Lacking Macrophage Mcl-1

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Manuscript in preparation

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Chapter 5

Abstract
The anti-apoptotic Bcl-1 family member myeloid cell leukemia 1 (Mcl-1) plays an important, in neutrophils essential, role in survival and differentiation of leukocyte subsets. Here, we investigated the impact of Mcl-1 deletion in neutrophils and macrophages on atherosclerotic plaque development and stability in Western type diet fed LDLr⁻/⁻ mice by a bone marrow (BM) transplantation strategy. First, Mcl-1 deficient peritoneal macrophages had an increased sensitivity to Ox-LDL induced cell death and showed altered expression of several pro-apoptotic Bcl-2 family members as compared to WT macrophages. In keeping apoptotic cell content in aortic root lesions of Mcl-1⁻/⁻ chimeras was elevated by 77% compared to WT controls. Second, lipid accumulation by peritoneal Mcl-1⁻/⁻ macrophages, induced by Ox-LDL and VLDL, was enhanced in vitro as well as in vivo. Third, Mcl-1⁻/⁻ macrophages showed a shift towards a pro-inflammatory M1 phenotype as apparent from their cytokine expression pattern and reduced phagocytic capacity. Despite these profound pro-atherogenic effects of Mcl-1 deficiency, the atherosclerotic plaque development and progression in LDLr⁻/⁻ mice on a Western-type diet did not differ between Mcl-1⁻/⁻ and WT BM recipients. This seeming paradox can be explained by the markedly lower neutrophil numbers and migratory capacity in Mcl-1 deficiency, which translates in impaired atherosclerotic lesional neutrophil infiltration thus corroborating the significance of neutrophils for atherogenesis. In conclusion, myeloid Mcl-1 deletion enhances Ox-LDL induced foam cell formation and cell death, favors a pro-inflammatory macrophage phenotype and markedly lowers neutrophil recruitment. The unaffected plaque initiation and growth indicates that neutrophil recruitment is essential to translate the pro-inflammatory phenotype into increased plaque formation.
Introduction

Macrophages are critical in the onset and progression of atherosclerotic lesion development. Monocyte infiltration with subsequent differentiation into macrophages, accumulation of lipids and secretion of various cytokines and growth factors all contribute to lesion progression¹. Plaque macrophages have been shown to undergo apoptosis at all stages², although apoptosis tends to increase with plaque progression³. Macrophages are also the predominant lesional phagocytes⁴. Defective macrophage apoptosis in early lesions has been shown to promote lesion development in different atherosclerotic mouse models⁵⁻⁷. In advanced lesions, macrophage apoptosis contributes to the formation of large necrotic cores consisting mostly of lipids and apoptotic cell debris². However, studies addressing effects of CD11b monocyte/macrophage apoptosis in advanced lesions did not reveal any differences in lesion size despite increased apoptotic cell content⁷,⁸. In human atherosclerotic lesions apoptosis of macrophages appeared to be enhanced only in more advanced stages⁹. This increase may at least in part be due to defective apoptotic cell phagocytosis in advanced lesions¹⁰.

Anti-apoptotic Mcl-1 is a member of the apoptosis regulating Bcl-2 family¹¹. It directly interacts with proapoptotic BH3-only proteins Bim and Bid and multidomain proapoptotic Bak¹²⁻¹⁴, thereby inhibiting apoptosis. Mcl-1 is expressed in various tissues including hematopoietic cells¹⁵, in which overexpression delays cell death in response to various stimuli¹⁶. Deletion of Mcl-1 in mice results in embryonic lethality¹⁷. A recent in vivo study in conditional knockout mice lacking Mcl-1 in neutrophils and macrophages demonstrated that Mcl-1 is necessary for neutrophil but not macrophage survival¹⁸,¹⁹. Mcl-1 induced neutropenia could be prevented by combined deletion of pro-apoptotic Bak and Bax¹⁹. In synovial fluid macrophages of rheumatoid arthritis patients Mcl-1 expression is enhanced and demonstrated to be essential for survival since Mcl-1 inhibition largely increased apoptosis²⁰. Mcl-1 has been implicated in macrophage lipid accumulation. Mcl-1 RNA expression was found to be increased in THP-1 macrophages following IL-10 exposure, which also augmented Ox-LDL induced foam cell formation²¹. Furthermore, silencing Mcl-1 and Bfl-1, another anti-apoptotic Bcl-2 family member, in these macrophages prevented IL-10 induced lipid accumulation²¹. The critical importance of macrophage apoptosis and foam cell formation in atherosclerosis points to a potential role of this anti-apoptotic factor in atherosclerosis.

In the present study we have investigated the role of myeloid Mcl-1 in atherosclerotic plaque development and progression using bone marrow transplantation of LDLr⁻/⁻ mice with Mcl-1⁺/⁻ LysMcre or control bone marrow, with a specific emphasis on neutrophil recruitment as pro-atherogenic factor.

Materials and Methods

Animals

All animal work was approved by regulatory authority of Leiden and performed in compliance with the Dutch government guidelines. LDLr⁻/⁻ mice were obtained from...
the local animal breeding facility. Mcl-1\(^{\text{fl/fl}}\) LysMcre mice were obtained from the Department of Immunology, Duke University Medical Center, Durham, NC, USA.

**Mcl-1 gene expression during atherogenesis**

To assess Mcl-1 gene expression during atherosclerotic lesion progression 20 male LDLr\(^{-/-}\) mice were fed a Western type diet (WTD) two weeks prior to surgery and throughout the experiment. Atherosclerotic carotid artery lesions were induced by perivascular collar placement as described by Von der Thüsen et al.\(^{22}\) and subsets of 4 mice were sacrificed at 0, 2, 4, 6 and 8 weeks after collar placement for expression analysis. The mice were anaesthetized and perfused with phosphate buffered saline (PBS, 150 mM NaCl, 1.5 mM Na\(_2\)HPO\(_4\), 8.6 mM Na\(_2\)HPO\(_4\), pH 7.4) after which both common carotid arteries were isolated, snap-frozen in liquid nitrogen and stored at -80\(^\circ\)C. Two to three carotid arteries were pooled per sample and total RNA was isolated using Trizol reagent (Invitrogen, Breda, The Netherlands). Gene expression was analyzed by real time PCR using ABI PRISM 7700 Sequence Detector (Applied Biosystems) with SYBR-Green technology. The primers used for expression of Mcl-1 and standard housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) are listed in table 1.

**Bone marrow transplantation and atherosclerosis induction**

Male LDLr\(^{-/-}\) mice (n=10) were housed in sterile ventilated cages with food (RM3, Special Diet Services, Witham, Essex, UK) and water ad libitum. The drinking water was supplied with antibiotics (83 mg/l ciprofloxacin and 67 mg/l Polymixin B) and 5 g/l sugar. The mice were exposed to a single dose of 9 Gy total body irradiation (0.19Gy/min, 200 kV, 4 mA,) using an Andrex Smart 225 Röntgen source (YXLON International, Copenhagen, Denmark) one day before transplantation. Bone marrow was extracted from femurs and tibia of male Mcl-1\(^{\text{fl/fl}}\) LysMcre (hereafter Mcl-1\(^{-/-}\)) and wild type (WT) littermates. Irradiated LDLr\(^{-/-}\) mice received either 2.5 x 10\(^{6}\) Mcl-1\(^{-/-}\) bone marrow cells (n=10) or 2.5 x 10\(^{6}\) WT bone marrow cells (n=10) via tail vein injection. After a recovery period of eight weeks mice were put on a WTD containing 0.25% cholesterol and 15% cacao butter (Diet W, Special Diet Services, Witham, Essex, UK) for an additional five (plaque initiation) or ten weeks (advanced plaque formation).

**Cholesterol and triglyceride assay**

Blood samples were taken by tail bleeding before bone marrow transplantation (BMT), before start of (week 0), after two and six weeks of WTD feeding (week 2 and week 6) and at the time of sacrifice (week 5 or week 10). Total cholesterol and triglyceride levels in serum were measured spectrophotometrically using enzymatic procedures (Roche Diagnostics, Almere, The Netherlands).

**Blood cell analysis and flow cytometry**

Blood samples were taken by tail bleeding immediately before BMT, before start of
(week 0) and after four weeks of WTD feeding (week 4) and at the time of sacrifice (week 5 or week 10). Peritoneal leukocytes were isolated at the time of sacrifice by peritoneal lavage with 10 ml PBS. Whole blood and peritoneal lavage samples were analyzed using a Sysmex blood cell analyzer (XT-2000i). White blood cells (WBC) were isolated by erythrocyte lysis of whole blood samples obtained by bleeding through the orbital sinus. For flow cytometry, WBC and peritoneal leukocytes were stained with fluorescently labeled antibodies against F4/80, CD19, CD4, CD71 and CD11b (eBioscience, Halle Zoersel, Belgium) and Gr1, CD8 and CXCR4 (BD Pharmingen, Breda, The Netherlands). Fluorescence-activated cell sorting (FACS) analysis was performed on FACSCalibur with CellQuest software (BD Biosciences).

**Tissue harvesting and analysis**
Two hours before sacrifice five mice from each group, Mcl-1−/− or WT, received intraperitoneal injections of the chemokine KC (200 ng/ml in 1 ml PBS) or PBS control. The mice were anesthetized and perfused with PBS. Heart, spleen and liver were harvested and stored in 4% formaldehyde solution. Cryosections were prepared of the aortic root and of spleen tissue and stained with hematoxylin and eosin (HE) or Oil Red O. Lesion size was quantified using Leica image analysis system, consisting of a Leica DMRE microscope with camera and Leica Qwin Imaging software (Leica Ltd, Cambridge, UK). Immunohistochemical stainings were performed for macrophage (MOMA-2, Sigma, Zwijndrecht, The Netherlands) and vSMC (α-smooth muscle actin, Sigma) content. Apoptotic cell content was quantified using terminal deoxytransferase dUTP nick-end labeling (TUNEL) kit (Roche Diagnostics).

**LDL and VLDL isolation**
LDL and VLDL were obtained from human plasma by density gradient ultracentrifugation for 20h at 4ºC. Oxidation of LDL was performed by incubation with CuSO₄ for 24 hours at 37ºC after which the oxidation reaction was terminated by addition of EDTA (final concentration 200 µM).

**Lipid loading of peritoneal macrophages**
Peritoneal leukocytes were isolated from mice that received an i.p. injection of PBS before sacrifice and subsequently combined resulting in 3 pools of Mcl-1−/− and of WT peritoneal leukocytes. Cells were plated at 0.25 x 10⁶ cells/well in 8 chamber culture slides (BD Falcon, Breda, The Netherlands). Non-adherent cells were removed and adherent macrophages were stimulated with 20 μg/ml oxidized LDL (Ox-LDL) or 50 ug/ml VLDL for 24 hours after which slides were washed with PBS and stained with Oil Red O. Lipid loading was quantified as the ratio between the Oil Red O stained cell area and total cell surface.

**Apoptosis of peritoneal macrophages**
Mcl-1−/− and WT peritoneal leukocyte pools (n=3 each) were plated at a density of 0.5 x 10⁶ cells/well in a 24 well plate. Non-adherent cells were removed and adherent
macrophages were stimulated with 40 ug/ml Ox-LDL for 24 hours. The macrophages were detached with Accutase (PAA Laboratories GmbH, Cölbe, Germany), stained with FITC labeled Annexin V (ImmunoTools, Friesoythe, Germany) and propidium iodide (Sigma) and subsequently analyzed by flow cytometry (FACSCalibur, BD Biosciences, Breda, The Netherlands).

**Isolation and differentiation of bone marrow derived macrophages**
Bone marrow cells were isolated by flushing femurs and tibia with PBS and single cell suspensions were obtained by passing the suspension through a 70 µm nylon cell strainer (BD Falcon). Bone marrow cells were differentiated into macrophages by culturing in 70% RPMI, supplemented with 20% FCS, glutamine (2 mM), sodium pyruvate (1%), penicillin (100 U/ml), streptomycin (100 μg/ml) and non-essential amino acids (1%), and 30% L929 conditioned DMEM for 7 days. Bone marrow derived macrophages (BMDM) were used for apoptosis assay. BMDM experiments were performed in RPMI containing FCS (10%), glutamine (2 mM) and penicillin (100 U/ml) and streptomycin (100 µg/ml).

**Phagocytosis assay**
Jurkat cells were labeled with CellTracker Orange CMTMR fluorescent dye (Molecular Probes, Merelbeke, Belgium). Apoptosis was induced by incubation with 1 µM staurosporine (Sigma) for 2 hours. Apoptosis was assessed by flow cytometry after FITC labbeled Annexin V and propidium iodide staining of the cells. 70% of Jurkat cells were found to undergo apoptosis after incubation with staurosporine. BMDM were incubated for 1 hour with apoptotic Jurkat cells or red fluorescent beads (2 µm Fluospheres, Molecular Probes) at 37ºC. After thorough washing with PBS (5 times) phagocytosis was analyzed on a FACSCanto with FACSDiva software (BD Biosciences).

**Table 1.** Primer sequences

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<th>Gene</th>
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<td>iNOS</td>
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**RNA isolation and realtime PCR**
Murine RAW 264.7 cells or BMDM were incubated for 24 h with or without Ox-
Increased Foam Cell Formation and Plaque Apoptosis in Macrophage Mcl-1 Deficiency

LDL (20 or 40 μg/ml). Total RNA was extracted using guanidine thiocyanate (GTC, Sigma, according to Chomczynski24). After RT-PCR, gene expression was analyzed by quantitative real-time PCR using ABI PRISM 7700 Sequence Detector (Applied Biosystems) using SYBR-Green technology and the primers listed in table 1. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) and acidic ribosomal phosphoprotein P0 (36B4) were used as standard housekeeping genes.

Statistic analysis
Values are expressed as mean ± SEM or presented as mean + upper limit of the SEM. Analysis to compare two groups was performed by two-tailed Student’s t-test. For analysis of relative mRNA expression data t-test was performed on ΔCt values. Statistical significance was set at p<0.05.

Results
Expression of Mcl-1 in murine macrophages and during atherogenesis
We first examined whether Mcl-1 has a potential role in atherosclerotic lesion development by assessing Mcl-1 gene expression in murine RAW 264.7 macrophages and the influence of oxidized LDL (Ox-LDL) on Mcl-1 expression. Mcl-1 is expressed in RAW 264.7 cells (fig. 1A). RAW 264.7 derived foam cells, generated by exposure to 20 or 40 μg/ml OxLDL for 24h displayed decreased mcl-1 expression (p≤0.05, fig. 1A).

Next, we monitored Mcl-1 gene expression in LDLr⁻/⁻ mice during atherogenesis. Mcl-1 gene expression during atherogenesis was measured in carotid artery lesions of western type diet fed LDLr⁻/⁻ mice equipped with semi-constrictive collars to accelerate atherosclerotic lesion formation. Mcl-1 gene expression was gradually increased during lesion development and in particular advanced plaques (> six weeks after collar induction of atherosclerotic lesion development) displayed a significant upregulation of increased Mcl-1 (p<0.05, fig. 1B). These results suggest that Mcl-1 is regulated during lesion progression which could contribute to atherosclerotic lesion

Figure 1. Regulation of Mcl-1 expression in vitro in RAW 264.7 macrophages and in vivo during atherogenesis. A. Mcl-1 was abundantly expressed in non-stimulated RAW 264.7 macrophages but downregulated upon exposure to 20 μg/ml or 40 μg/ml Ox-LDL for 24h. B. Vascular Mcl-1 expression gradually increased during lesion progression in a model of collar induced carotid artery atherogenesis in LDLr⁻/⁻ mice (*p<0.05).
development or lesion stability.

**Total cholesterol levels and body weight**

Next we generated LDLr<sup>−/−</sup> mice with myeloid Mcl-1 deletion by reconstituting lethally irradiated LDLr<sup>−/−</sup> mice with Mcl-1<sup>fl/fl</sup> LysMCre (hereafter Mcl-1<sup>−/−</sup>) or wild type (WT) bone marrow cells. After a recovery of eight weeks mice were put on a Western type diet (WTD) and effects of Mcl1<sup>−/−</sup> deficiency on lipid levels were monitored by measuring serum total cholesterol (TC) and triglyceride (TG) levels throughout the experiment. Average TC and TG levels did not differ between Mcl-1<sup>−/−</sup> and WT transplanted animals. Mcl-1 deletion did not affect total body weight.

Myeloid Mcl-1 deletion increases lesional apoptotic cell content but does not affect atherosclerotic lesion size

Five (early plaque formation) or ten weeks (advanced plaque formation) after WTD feeding aortic roots were isolated, sectioned and analyzed. Apoptotic cells content appeared to be increased by 71% (NS) and 77% (p=0.002) in atherosclerotic lesions of Mcl-1<sup>−/−</sup> fed a WTD for 5 and 10 weeks, respectively, compared to WT transplanted mice (Fig. 2A,B). However, despite its profound impact on apoptosis, myeloid Mcl-1 deletion did neither alter early atherogenesis (5.97 ± 2.62 *10<sup>4</sup> μm<sup>2</sup> and 4.73 ±
Increased Foam Cell Formation and Plaque Apoptosis in Macrophage Mcl-1 Deficiency

2.29 * 10^4 μm² for Mcl-1^-/- and WT reconstituted mice respectively, 5 weeks of WTD) nor advanced lesion formation (3.31 ± 1.66 * 10^5 μm² and 3.44 ± 1.38 *10^5 μm², 10 weeks of WTD) (Fig. 2D) Necrotic core size and lesion macrophage (Moma-2) and collagen content were unaffected by myeloid Mcl-1 deletion as well (fig. 2C,E,F).

**Macrophage sensitivity to Ox-LDL induced apoptosis is increased**

Incubation of peritoneal macrophages with 40 μg/ml Ox-LDL for 24 hours showed that Mcl-1^-/- macrophages have an increased sensitivity towards Ox-LDL induced cell death compared to WT macrophages (Fig. 3A). Mcl-1 deletion also appeared to decrease cell survival in non-stimulated macrophages. Gene expression of several BH3-only proapoptotic Bcl-2 family proteins was assessed in Mcl-1^-/- and WT bone marrow derived macrophages (BMDM). The expression of Bim, PUMA (p<0.01) and NOXA (n.s.), proapoptotic proteins that all were seen to interact with Mcl-1,25-27, appeared to be decreased in non-stimulated Mcl-1^-/- macrophages (fig. 3B-D). Exposure to Ox-LDL decreased gene expression of PUMA and NOXA but did not change that of Bim in WT macrophages. Conversely, in Mcl-1^-/- macrophages Ox-LDL incubation resulted in elevated Bim and PUMA expression.

![Figure 3](image.png)

*Figure 3. Cell death and expression of Bcl-2 family members in peritoneal and bone marrow derived macrophages. A. Mcl-1 deficiency leads to enhanced cell death of unstimulated and Ox-LDL (40 μg/ml) stimulated peritoneal macrophages. B-D. Bim, PUMA and NOXA gene expression was affected by Mcl-1 deletion (black bars) in both unstimulated and Ox-LDL (40 μg/ml) stimulated BMDM. (*p<0.05, **p<0.01)*

**Macrophage lipid loading is enhanced**

Peritoneal foam cell numbers were increased by 2.5 fold (p<0.05) in Mcl-1^-/- compared
to WT transplanted mice (Fig. 4A). Total peritoneal macrophage numbers were unchanged. This finding led us to examine the lipid loading capacity of peritoneal macrophages in vitro. Macrophages were incubated for 24h with 20 µg/ml Ox-LDL or 50 µg/ml VLDL and stained with Oil Red O. In agreement with the elevated peritoneal foam cell counts in vivo, lipid accumulation in non-stimulated Mcl-1\(^{-/-}\) macrophages was markedly increased (fig. 4B,C). Lipid loading in WT macrophages remained unchanged after incubation with Ox-LDL for 24h, but was substantially enhanced in Mcl-1\(^{-/-}\) macrophages (p<0.01). While incubation with VLDL increased lipid content in both WT and Mcl-1\(^{-/-}\) macrophages, this increase was considerably (3,6 fold) higher in the latter cells (p=0.01).

In addition to enhancing the lipid loading capacity, Mcl-1 deficiency resulted in an increased presence of multinucleated giant cells (MGC) within the peritoneal

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**Figure 4. Mcl-1 deletion enhances lipid loading of peritoneal macrophages.** A. Elevated macrophage derived foam cell numbers in peritoneum of Mcl-1\(^{-/-}\) BM (black bars) compared to WT BM transplanted mice (white bars). B. Lipid loading of Mcl-1\(^{-/-}\) peritoneal macrophages (black bars) was dramatically increased after incubation with Ox-LDL (20 μg/ml) or VLDL (20 μg/ml), whereas WT macrophages (white bars) only showed a moderate increase in lipid loading after incubation with VLDL. C. Representative micrographs of Oil Red O stained unstimulated and Ox-LDL or VLDL stimulated peritoneal macrophages. Multinucleated giant cells are indicated by an arrow. (*p<0.05, **p<0.01) D. Multinucleated giant cell levels were largely increased among Mcl-1\(^{-/-}\) peritoneal macrophages compared to WT macrophages.
macrophage population both at baseline and after incubation with Ox-LDL or VLDL (p<0.05, Fig. 4C and 4D, MGC are indicated by arrows).

**Mcl-1** transplanted **LDLr** mice display splenomegaly and altered macrophage phenotype.

Relative spleen weight was increased by nearly two-fold (p=0.006) in Mcl-1/− compared to WT transplanted mice (fig. 5B), which in Mcl-1 LysMCre deleted mice has been shown to result from increased myeloid cell content in splenic red pulp. BM derived macrophages with MCL1 deletion showed features of a pro-inflammatory M1 phenotype (fig 5D-H). IL10 expression in OxLDL stimulated Mcl-1−/− macrophages was reduced while that of the M2 marker arginase I was unaltered. Conversely, expression of proinflammatory M1 cytokines TNFα, and IL12 subunit p35 was upregulated after Ox-LDL stimulation, an effect absent in WT macrophages. In agreement with the M1 shift, macrophages exhibited reduced phagocytosis of early apoptotic Jurkat cells (fig. 5C), a phenomenon that was reported to be more pronounced in M2 than in M1 macrophages.

![Graphs and figures](image-url)
Mcl-1 deletion alters neutrophil levels and characteristics

Mcl-1 deletion affects neutrophil levels and phenotype. A. Circulating neutrophil levels were profoundly reduced in Mcl-1<sup>-/-</sup> compared to WT transplanted mice and even more so after Western type diet feeding. B. Aortic root atherosclerotic lesions of Mcl-1<sup>-/-</sup> BM transplanted LDLr<sup>-/-</sup> mice contained fewer neutrophils as can also be seen in representative micrographs (right panel, arrows indicate neutrophils). C,D. CXCR4 expression on Mcl-1<sup>-/-</sup> neutrophils is elevated compared to WT neutrophils. E. WT BM transplanted mice displayed a massive neutrophil influx into the peritoneal cavity in response to i.p. KC injection (white bars) which is almost completely prevented in Mcl-1<sup>-/-</sup> BM transplanted mice (black bars). F. The KC induced increase in circulating neutrophil numbers observed in WT chimeras (white bars) was absent in Mcl-1<sup>-/-</sup> BM transplanted mice as well (black bars). (*p<0.05, **p<0.01, ***p<0.001)

Mcl-1 deletion alters neutrophil levels and characteristics

Mcl-1 was shown to be essential for neutrophil survival and as a result circulating and splenic neutrophils numbers were reduced by 80 and 86%, respectively, in Mcl-1<sup>fl/fl</sup>LysMcre mice. Indeed Mcl1<sup>-/-</sup>-chimeras were almost depleted in circulating neutrophils both before WTD feeding and under hyperlipidemic conditions (four and ten weeks after WTD feeding) (-82% to 91%, p<0.001, Fig. 6A). Neutrophil
Increased Foam Cell Formation and Plaque Apoptosis in Macrophage Mcl-1 Deficiency

content in Mcl-1−/− atherosclerotic lesions was decreased by 72% (0.63 ± 0.31 *10^{-5} per μm^2 lesion) compared to WT lesions (2.28 ± 0.63 *10^{-5} neutrophils per μm^2 lesion, p<0.05, Fig. 6B), which might point to an enhanced adhesive capacity of residual neutrophils in circulation.

We therefore examined neutrophil phenotype and function in both groups of animals. CXCR4 expression was increased on circulating (2.2 fold, p<0.001) as well as on peritoneal (1.7 fold, p<0.05) Mcl-1−/− neutrophils (Fig. 6C,D), suggesting altered migratory capacity. Remarkably, Mcl-1−/− neutrophils showed a decreased responsiveness to KC, a potent neutrophil chemoattractant. Neutrophil content in the peritoneal cavity and circulation was measured 2 hours after i.p. injection of KC or PBS. Peritoneal neutrophil influx in response to KC was prominent in WT transplanted mice whereas Mcl-1−/− transplanted mice only showed a minor, non-significant, increase in peritoneal neutrophils (Fig. 6E,F). Of note, neutrophil recruitment was paralleled by a moderate increase in circulating neutrophils in WT but not Mcl1−/− mice.

Discussion

In this study we determined the role of Mcl-1 in atherosclerotic plaque initiation and progression. Mcl-1 is an anti-apoptotic Bcl-2 family member^19^ and although several members of this family have been investigated in the context of atherosclerosis^6,8,29^, the role of Mcl-1 in disease progression has not been assessed thus far. Here, we studied effects of specific deletion of Mcl-1 in lysozyme M expressing myeloid subsets such as neutrophils and macrophages on early and advanced atherosclerosis.

First we established Mcl-1 expression in RAW 264.7 macrophages and showed that Mcl-1 expression is decreased in response to Ox-LDL. In addition vascular Mcl-1 expression steadily increases with lesion progression in LDLr−/− mice. Given that macrophage content and apoptosis gradually increases with plaque progression^3^ and that Mcl-1 was seen to affect macrophage lipid accumulation^21^ these findings suggest that Mcl-1 could be a critical player in atherosclerosis. Therefore we investigated the role of Mcl-1 atherosclerotic lesion development and stability, in LDLr−/− chimeras with Mcl-1 deficiency in macrophages and neutrophils generated by transplantation of Mcl-1^{fl/fl} LysMcre or wildtype (WT) bone marrow. The use Mcl-1 deficiency in lysozyme M expressing cells enabled us to specifically study effects of Mcl-1 deficiency in macrophage and neutrophil differentiation and apoptosis and their contribution to disease. Mcl-1 was previously demonstrated to be involved in cell death of various leukocyte subsets^16,18, 30-33^.

Overexpression of Mcl-1 in hematopoietic cells protected from cell death in response to various stimuli^16^ and inhibition of Mcl-1 expression in human neutrophils and macrophages resulted in increased apoptosis^31,32^. In vivo overexpression of Mcl-1 promoted survival of lymphocytes and myeloid cells^18,30,33^. Our data largely correspond with these findings in that Mcl-1−/− macrophages displayed decreased survival compared to WT macrophages. Additionally, we show that Mcl-1 participates in Ox-LDL induced cell death as Mcl-1−/− macrophages were more susceptible to cell death.
after Ox-LDL incubation. Mcl-1 deficiency was seen to influence the expression of other apoptosis regulating Bcl-2 family members, such as Bim, PUMA and NOXA, which all were decreased in unstimulated Mcl-1<sup>−/−</sup> compared to WT macrophages. Whereas Ox-LDL stimulation resulted in decreased PUMA and NOXA expression in WT macrophages, in Mcl-1<sup>−/−</sup> macrophages this led to an increase in PUMA expression together with increased Bim. Bim, PUMA and NOXA are BH3-only pro-apoptotic Bcl-2 family members involved in the initiation of apoptosis<sup>14</sup>, which exhibit reduced pro-apoptotic activity upon interaction with Mcl1 and all other pro-survival Bcl-2 family members (Bim and PUMA) or Mcl-1 and A1 only (NOXA)<sup>12,25-27</sup>. The dysregulated expression of BH3-only proteins in Mcl-1<sup>−/−</sup> macrophages suggests that apart from inhibiting apoptosis by interaction with BH3-only proteins, Mcl-1 might also exert anti-apoptotic effects at the transcriptional level in response to an apoptotic stimulus.

In line with the in vitro findings apoptotic cell content in advanced aortic root lesions (10 weeks of WTD) was increased in mice with myeloid Mcl-1 deficiency, indicating that Mcl-1 is a major survival protein in atherosclerotic lesions. However despite a 77% increase in apoptosis, atherosclerotic lesion burden was unaltered in Mcl-1<sup>−/−</sup> BM recipients, as were necrotic core size and macrophage and collagen content. Similar findings were obtained when studying plaque initiation (five weeks of WTD feeding). Our results correspond with those from Thorp et al.<sup>8</sup>, who showed increased macrophage apoptosis but unchanged lesion burden in Bcl-2<sub>−/−</sub>-LysMCre ApoE<sup>−/−</sup> mice that are deficient in macrophage and neutrophil Bcl-2<sup>8</sup>. However macrophage apoptosis in early atherogenesis was demonstrated to be beneficial in several studies in LDLr<sup>−/−</sup> and ApoE<sup>−/−</sup> mice with deleted expression of pro-apoptotic or survival factors<sup>5,6,35,36</sup>. Two studies assessing apoptosis in advanced atherosclerosis presented contradictory results. Stoneman et al.<sup>7</sup> observed in CD11b<sub>−</sub>-hDTR / ApoE<sup>−/−</sup> mouse that diphtheria toxin induced ablation of CD11b<sup>+</sup> monocytes and macrophages did not result in any effects on lesion initiation or progression although lesion apoptotic cell content was largely increased. Conversely, in a recent study by Gautier et al.<sup>37</sup> apoptosis in advanced lesions in ApoE<sup>−/−</sup> mice was seen to aggravate disease progression. Here, we found that macrophage and neutrophil specific deletion of Mcl-1 in LDLr<sup>−/−</sup> mice had additional consequences, both at a systemic level and within the lesion itself, which may have masked effects of the observed increased lesion apoptosis.

First, apart from an increased sensitivity to Ox-LDL induced cell death Mcl-1<sup>−/−</sup> macrophages showed augmented lipid accumulation after incubation with Ox-LDL and VLDL. In keeping we observed elevated foam cell levels in vivo in the peritoneal cavity of Mcl-1<sup>−/−</sup> BM compared to WT BM recipients. These findings seems to contrast with those of Halvorsen et al.<sup>21</sup>, who reported reduced IL-10 induced OxLDL loading by THP-1 macrophages in vitro after siRNA mediated silencing of Mcl-1 and Bfl-1 expression. The authors did not assess effects of Mcl-1 inhibition alone, without IL10 stimulation. Based on our data we hypothesize that the apoptosis-prone phenotype of Mcl-1<sup>−/−</sup> macrophages facilitates uptake of lipids.
Another remarkable characteristic was the high propensity of Mcl-1−/− cells to form multinucleated giant cells (MGC). MGC originate from monocyte-macrophage lineage and result from aberrant cell fusion. Although the actual mechanism of MGC formation is hitherto unclear, their presence is thought to reflect an elevated inflammatory status with a crucial role for the cytokine environment in which they are formed. The latter notion is corroborated by the splenomegaly in Mcl-1−/− chimeras and a shifted polarization of Mcl-1−/− macrophages towards a pro-inflammatory M1 macrophage phenotype as judged from their cytokine expression pattern and reduced phagocytic capacity.

Previously Mcl-1 has been proven to be essential for appropriate differentiation of hematopoietic stem cells and development of lymphocytes and neutrophils, whereas monocytes and macrophages display normal development in the absence of Mcl-1. Lately, neutrophils are increasingly perceived as important players in atherogenesis. Increased neutrophil count is positively correlated with coronary artery disease and in mouse models neutrophils have been demonstrated to be present in atherosclerotic lesions and adventitia. Depletion of neutrophils in ApoE−/− mice results in reduced atherosclerotic lesion formation. In our study, Mcl-1−/− BM transplanted LDLr−/− mice were displaying overt neutropenia, consistent with previous findings that Mcl-1 is essential for neutrophil survival. Impaired neutrophil survival translated in a reduced atherosclerotic lesion neutrophil content although the decrease was less pronounced than in circulation. The latter finding may be explained by an altered migratory capacity of residual neutrophils. Dzhagalov et al. showed that residual neutrophils in plain Mcl-1fl/fl LysMcre mice were shown to express Mcl-1 and apparently had escaped Cre-mediated Mcl-1 deletion. In our study, both in circulation and in the peritoneal cavity CXCR4+ neutrophil numbers were increased in Mcl-1 deficiency, indicative of increased stromal retention and decreased release of neutrophils from the bone marrow. Administration of CXCR4 blocking antibody to mice was reported to reduce retention of circulating neutrophils in while increasing their release from bone marrow. In addition it was shown that CXCR2 ligand KC induces desensitization to effects of CXCR4 ligand SDF-1α. Vice versa, SDF-1α is able to attenuate neutrophil response to KC. In our study, circulating neutrophils in Mcl-1−/− BM recipients had a reduced migratory response to chemoattractant KC in vivo. This reduced response might be partly attributable to enhanced SDF-1α /CXCR4 signaling and partly be a result of the substantially reduced neutrophil pool.

In summary, myeloid Mcl-1 deficiency enhanced Ox-LDL induced cell death of macrophages ex vivo as well as atherosclerotic lesion apoptosis in BM transplanted LDLr−/− mice. Furthermore, mcl-1 deficiency was seen to reduce phagocytic capacity and to promote macrophage lipid uptake and macrophage polarization towards a pro-inflammatory M1 phenotype. Despite these supposedly pro-atherogenic effects of Mcl-1 deficiency, it did not aggravate lesion development or progression. This apparent paradox indicates that the markedly lower neutrophil numbers in circulation and plaque, contravene the pro-atherogenic capacity of Mcl-1 deficiency.
Chapter 5

illustrating that neutrophil recruitment is essential to translate a pro-inflammatory phenotype into increased atherosclerotic plaque formation.
References

20. Liu H, Huang Q, Shi B, Eksarko P, Temkin V, Pope RM. Regulation of Mcl-1 expression in rheumatoid
Chapter 5

Increased Foam Cell Formation and Plaque Apoptosis in Macrophage Mcl-1 Deficiency
