Modulation of leukocyte homeostasis in atherosclerosis

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Systemic, but not Local Induction of CD115^{+} Macrophage Apoptosis Promotes Atherosclerotic Lesion Vulnerability in ApoE$^{-/-}$ Mice

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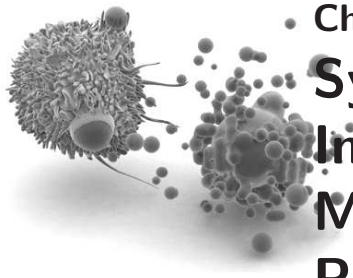

Chapter 5
Systemic, but not Local Induction of CD115$^+$ Macrophage Apoptosis Promotes Atherosclerotic Lesion Vulnerability in ApoE$^{-/-}$ Mice

"Almost everything, all external expectations, all pride, all fear of embarrassment or failure... these things just fall away in the face of death, leaving only what is truly important."
Steve Jobs

5.1 Abstract

Background: Although macrophage apoptosis is deemed to induce atherosclerotic lesion vulnerability, the actual impact of macrophage death on atherosclerosis remains unclear, because the experimental set–up used for its evaluation has had off–target effects on peripheral monocyte or myeloid populations. Therefore we here sought to compare spatially restricted local induction of macrophage apoptosis in atherosclerotic lesions versus systemic ablation, on advanced atherosclerosis.

Methods and Results: ApoE$^{-/-}$ mice, expressing a Macrophage Fas Induced Apoptosis (MaFIA) suicide gene in CD115$^+$ myelocytes were used to address the impact of conditional CD115$^+$ myelocyte ablation on advanced atherosclerotic lesions induced by perivascular collar placement. Systemic and local induction of CD115$^+$ myelocyte apoptosis was achieved respectively by intravenous injection or local delivery of AP20187 using micro–osmotic pumps connected to atherosclerotic lesions. As expected, local and systemic induction of CD115$^+$ myelocyte apoptosis caused accumulation of apoptotic cells in atherosclerotic lesions, and reduced their contents of macrophages. Local treatment caused lesion expansion, without detectable changes in necrotic core size and features of lesion stability such as VSMC accumulation, collagen deposition and fibrous cap thickness. In contrast systemic induction of CD115$^+$ myelocyte apoptosis caused marked lesion destabilization. Local CD115$^+$ myelocyte apoptosis had no significant effect on peripheral leukocyte

homeostasis. Systemic treatment in contrast caused extramedullary myelopoiesis and proinflammatory Ly6c^{hi} monocytosis, promoting lesion destabilization by multiple immune–mediated mechanisms.

Conclusions: This study is the first to segregate the impact of lesion restricted vs systemic induction of CD115^{+} myelocyte apoptosis in advanced atherosclerotic lesions. Divergent effects are observed using these strategies. Local induction of macrophage apoptosis causes lesion expansion but allows macrophage turnover without lesion vulnerability. In sharp contrast, systemic induction of macrophage apoptosis causes lesion vulnerability and disturbed leukocyte homeostasis.

5.2 Introduction

Atherosclerosis, characterized by the progressive accumulation of monocyte derived macrophages and oxidized cholesterol in the vasculature, is the underlaying cause of cardiovascular disease [2] and the single most important cause of death in the western world [1].

Atherosclerotic lesions transition from asymptomatic expansion to clinical manifestations such sudden death upon erosion and rupture of their fibrous caps [44]. This causes leakage of thrombogenic components of lesions into the circulation, and incites thrombus formation and infarction or death withing minutes of arterial occlusion.

Macrophage apoptosis coupled to impaired apoptotic cell clearance is believed to cause lesion destabilization and fibrous cap rupture in atherosclerosis [46], as the frequency of apoptotic macrophages and the volume occupied by necrotic cores are higher at sites of plaque rupture than in areas of intact fibrous caps [45, 244].

The percentage of apoptotic macrophages increases with the progression of atherosclerotic lesions towards destabilization and rupture [244, 245], which coupled to poor phagocytosis of apoptotic bodies fosters necrotic core formation and leads to release of pro–inflammatory cytokines with subsequent influx of circulatory monocytes, lesion expansion [46, 54] and thrombogenicity [246].

Genetic cell ablation models have indicated that higher susceptibility to apoptosis in macrophages delays early lesion formation [247–249], but causes lesion expansion [250–252] and instability [253, 254] in advanced atherosclerosis. Conversely, protection from apoptosis in atherosclerosis causes early lesion expansion [250, 251] but reduced atherosclerosis in advanced stages [255].

Collectively, these results have led to the generalized idea that macrophage apoptosis has a biphasic effect in atherosclerosis. However, pharmacological (reviewed by Martinet et al. [256]) and suicide gene mediated induction of cell ablation [25, 225] have afforded contradictory results regarding the impact of macrophage death on atherosclerosis.

In fact, systemic induction of CD11b^{+} myelocyte apoptosis has been reported to have no effect in atherosclerotic lesions, probably due to simultaneous ablation and reduced availability of circulatory monocytes [225]. In addition, combined induction of CD11c^{+} dendritic cell and macrophage apoptosis does not reduce lesion macrophage contents but results in advanced atherosclerotic lesion expansion, depletion of dendritic cells and increased plasma cholesterol levels [23, 25].

These inconsistent outcomes might be caused not only by intra–lesion induction of macrophage apoptosis, but also by peripheral induction of macrophage death and

off target ablation of other leukocytes, including monocytes and dendritic cells, which, we hypothesized, would directly or indirectly disturb intra–vascular and peripheral leukocyte homeostasis and activity, and thus complicate the interpretation of the results reported.

In this study we tested this hypothesis and assessed for the first time the effect of confined induction of lesion macrophage apoptosis on advanced atherosclerotic lesion phenotype. We used ApoE$^{-/-}$ MaFIA$^{+/+}$ mice prone to development of atherosclerosis and susceptible to induction of CD115^{+} cell apoptosis by administration of AP20187 [257,258]. Local ablation of macrophages was achieved by continuous release of AP20187 into atherosclerotic lesions that were evaluated and compared to those obtained upon systemic induction of macrophage apoptosis by intravenous injection of AP20187 in atherosclerotic mice.

Analysis of the data presented indicates that local induction of CD115^{+} myelocyte apoptosis in advanced atherosclerosis caused massive depletion of plaque macrophages and lesion expansion without changes in lesion composition and circulatory and spleen leukocyte homeostasis.

Systemic induction of CD115^{+} myelocyte apoptosis in contrast, led to features of lesion instability such as reduced collagen content and thinner fibrous caps, besides inducing lesion macrophage ablation. These effects proceeded with concurrent increase in proinflammatory resident and circulatory leukocytes, deemed to to promote atherosclerotic lesion vulnerability [7].

5.3 Methods

Mice C57BL/6J–Tg(Csf1r–EGFP–NGFR/FKBP1A/TNFRSF6)2Bck/J MaFIA$^{+/+}$ male mice obtained from the Jackson Laboratories, were back crossed to ApoE$^{-/-}$ mice for more than 12 generations to obtain homozygous, atherosclerotic prone, ApoE$^{-/-}$ MaFIA$^{+/+}$ mice. The MaFIA transgene was inherited according to Mendelian laws as demonstrated by qPCR analysis of gDNA (PureLink Genomic DNA Purification Kit, Invitrogen) performed in all experimental units [259].

Real time PCR reactions were performed in the 7500 Fast Real–Time PCR System (Applied Biosystems, Warrington, UK) using $10ng$ of gDNA plus 0.25μl of 20μM of EGFP forward and reverse primers and 5μl of Power SYBR Green PCR master mix (Applied Biosystems) in a total volume of 10μl. Primer sequences were: 5'-CCACATGAAGCAGCAGGACTT-3' forward, and 5'-GGTGCGCTCCTGGACGTA-3' reverse. PCR conditions were: 10 min 95^{o}C, 40 2-step cycles: 15 sec 95^{o}C, 1 min 60^{o}C and 1 min at 95^{o}C. Samples were run in triplicate and CT values compared to CT values obtained using gDNA of MaFIA$^{+/+}$ homozygous litter mates.

Animal Experiments All experiments with ApoE$^{-/-}$ MaFIA$^{+/+}$ mice, including characterization of the model, perivascular collar placement and induction of apoptosis of CD115^{+} cells *in-vivo* were performed in accordance with the Dutch Government guidelines for animal experiments and approved by the Institutional Animal Care and Use Committee of the University of Maastricht. Mice were housed in filter top cages in all experiments *in-vivo*. Food and water were provided *ad–libitum*. Drinking water with antibiotics (83 mg/l ciprofloxacin and 67 mg/l Polymixin plus 5g/l sucrose, 60,000 Units/l Polymixin B Sulfate and 100 mg/L Neomycin) was introduced two

weeks before the introduction of WTD and supplied until sacrifice. (All antibiotics were from Sigma–Aldrich, St. Louis, MO, USA).

Characterization of MaFIA Mice For assessment of the MaFIA model of induction of $CD115^+$ cell apoptosis, $ApoE^{-/-}$ $MaFIA^{+/+}$ mice (n=5 to 8) fed WTD (0.25% cholesterol and 15% cacao butter. Special Diet Services, Witham, Essex, UK) received injections of AP20187 (1 to 10 mg/kg) diluted in vehicle (4% ethanol, 10% PEG-400, 1.7% Tween, in water) before sacrifice and analysis of blood, peritoneal and spleen leukocyte counts. $ApoE^{-/-}$ $MaFIA^{+/+}$ receiving only vehicle were used as controls.

Induction of $CD115^+$ Macrophage Apoptosis in Atherosclerotic Mice Bilateral perivascular collars were placed in male $ApoE^{/}$, $MaFIA^{+/+}$ mice three weeks after introduction of WTD (0.25% cholesterol and 15% cacao butter. W. Special Diet Services, Witham, Essex, UK) for induction of atherosclerosis [84]. $ApoE^{/}$, $MaFIA^{-/-}$ mice were used as control. $CD115^+$ cell apoptosis was induced *in-vivo* during 2 weeks starting 5 weeks after collar placement (Fig. 5.1A).

Systemic induction of apoptosis was achieved by 5 intravenous injections of 1mg/kg–body–weight of AP20187 diluted in vehicle (4% ethanol, 10% PEG-400, 1.7% Tween, in water), every 72 hours. Sacrifice of experimental mice (n=15) was done 16 hours after the last injection.

Local induction of apoptosis was achieved by controlled release of AP20187 diluted in vehicle (4% ethanol, 10% PEG-400, 1.7% Tween, in water), using ALZET osmotic pumps (model 2006) with micro–connector bifurcations (PlasticsOne Y Cannula Bifurcation 37200); connected via catheters (HelixMark®Standard Silicone Tubing 0.64 mm I.D.) to the adventitial side of perivascular collar induced atherosclerotic lesions (Fig. 5.1B).

The concentration of AP20187 in micro–osmotic pumps and therefore, the local concentration of AP20187 at the site of release was set at 100μM, which depending on body weight, led to a systemic dose of 0.018 ± 0.001 mg/kg–day. Sacrifice of experimental mice (n=15) was done 16 hours after the last injection. A baseline group of mice (n=8) was sacrificed 5 weeks after collar placement, without induction of apoptosis.

Plasma M-CSF, Cholesterol and Triglyceride Levels Blood samples were collected in tubes containing 5μl 0.5M EDTA and centrifuged for 10 minutes at 2100 rpm. Subsequently, plasma was isolated and used for M–CSF and total cholesterol measurement. M-CSF was determined using the mouse M-CSF Quantikine ELISA kit (RD Systems). Total plasma cholesterol levels were measured using a colorimetric assay (Roche Diagnostics, Mannheim, Germany).

Colony Forming Units Assays Spleen and bone marrow cells were isolated from respectively a fraction of the spleen, one tibia and one femur per mouse. Cells were counted twice using a Countess automated cell counter (Sigma–Aldrich) and the concentration was calculated for each sample. Respectively, 10,000 and 250,000 bone marrow and spleen cells were added separately to 2 ml methyl cellulose medium with recombinant cytokines (MethoCult Medium, StemCell Technologies, Grenoble, France). After incubation for 7 days (37^oC; 5% CO_2) the number of colonies was

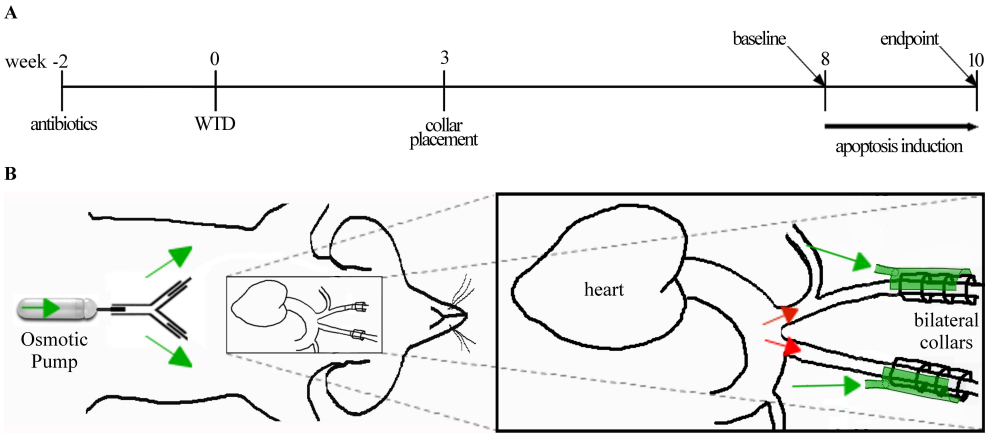

Figure. 5.1. *Experimental setup for systemic and local induction of CD115$^+$ cell apoptosis in atherosclerotic ApoE$^{-/-}$ MaFIA$^{+/+}$ mice. A. Advanced atherosclerotic lesions were induced to form by semi–constrictive perivascular collars placed around the common carotid arteries, three weeks after introduction of high cholesterol diet (WTD) [84]. CD115$^+$ cell apoptosis was triggered either by intravenous injection or local release of AP20187 during two weeks, starting five weeks after collar placement. B. Schematic representation of local induction of macrophage apoptosis in advanced atherosclerotic lesions using micro–osmotic pumps. Catheters (green) were used to connect osmotic pumps to the adventitial side of atherosclerotic lesions, for continuous release of apoptosis inducer AP20187.*

quantified by an independent operator. GM-CFU, E-CFU, G-CFU, and M-CFU colonies were specified based on morphology.

Flow Cytometry Blood and peritoneal cells, and spleen tissue were harvested at sacrifice and single cell suspensions were prepared. Lysis of erythrocytes was performed in ice cold NH_4Cl (8.4g/l) $NaHCO_3$ (1g/l) EDTA (37mg/l) during 3 minutes. Single cell suspensions were stained with anti -B220, CD3e, CD25, Ly6C, Ly6G, CD11b, CD11c, CD4, CD19, MHCII, CD86, CD40, F4/80, CD8a, FoxP3 following blocking of the Fc receptor with CD16/32 Fc blocking antibody. Antibodies were conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE),phycoerythrin–Cy7 (PE–C7), allophycocyanin (APC), peridinin cholorophyl protein (PerCP), peridinin cholorophyl protein–Cy5.5 (PerCP–Cy5.5) or eFluo450. A FACSCanto II (BD Biosciences) coupled to the FACSdiva^{TM} software was used for acquisition and analysis of data.

BD Trucount^{TM} tubes, were used for absolute cell counts in blood according to the protocol supplied by the manufacturer. In short, Fc–receptor blocking antibody was added to the Trucount tubes. Subsequently, 50μL anti–coagulated whole blood was added and the tube was gently vortexed. After incubation for ten minutes, the antibody cocktail was added and incubated at room temperature in the dark for an additional 20 minutes. Finally, hypotonic lysis buffer was added and samples were measured on a FACS Canto II flow cytometer (BD Biosciences).

Myeloid progenitors were measured in spleen and bone marrow. Cells were first

stained with biotin–conjugated antibodies against lineage markers (CD5, CD45RA (B220), CD11b, Gr-1 (Ly6G/G), 7-4, and Ter-119; Miltenyi Biotec). Next, lineage positive cells were depleted using streptavidin conjugated magnetic beads (Miltenyi Biotech) and LS columns (Miltenyi Biotech). Lineage negative fractions were subsequently stained with antibodies for Sca-1, c-Kit (CD117), CD34, CD16/32, CD115, and lineage markers (CD5, CD45RA (B220), CD11b, Ly6C, Ly6G, Ter-119) and measured on a FACS Canto II (BD Biosciences).

Isolation and Differentiation of Bone Marrow Derived and Peritoneal Macrophages: Bone marrow derived macrophages (BMDM) were obtained from non adherent macrophage precursors as previously described [85]. Femurs and tibia were flushed with phosphate buffered saline (PBS) and single cell suspensions were obtained by passing the bone marrow so obtained through a 70μm nylon cell strainer (BD Falcon, Breda, The Netherlands). Bone marrow precursors were differentiated into macrophages by culturing in 85% RPMI containing 10% FCS, 2mM glutamine, 1% pyruvate, 100U/ml penicillin, 100μg/ml streptomycin, 1% non essential amino acids and 15% L929 LCM medium for 7 days. All *in-vitro* experiments with BMDM were performed in RPMI containing 10% FCS, 2mM glutamine, 100U/ml penicillin, 100μg/ml streptomycin and 15% L929 LCM medium.

Peritoneal leukocytes were isolated from mice after cervical dislocation, by injecting 10 ml of sterile PBS into the caudal half of the peritoneal cavity using a 25-gauge and removing the saline containing peritoneal cells with a 19-gauge needle after shaking the entire body for some seconds. Cells were plated in RPMI 1640 supplemented with 10% FCS 50IU/ml penicillin, 50μg/ml streptomycin, 2mM glutamine, at 0.25x10^6 cell/well in 8-chambers culture slides (BD Falcon, Cat. No. 354108) and incubated for one hour at 37^{o}C. Non adherent cells were removed by washing 5 times with 500μl prewarmed PBS and adherent peritoneal macrophages (PEM) cells used for induction of apoptosis *in-vitro*.

Apoptosis Induction in Peritoneal Macrophages Peritoneal macrophages, obtained from MaFIA$^{+/+}$ ApoE$^{-/-}$ or MaFIA$^{-/-}$ ApoE$^{-/-}$ mice, were cultured with AP20187 (10nM) during 24 hours and stained for 1 hour with FLICA fluorescent polycaspase detection reagent, to label intracellular activated caspases. Cells were thereafter incubated with Hoechst (200μg/ml) to label cell nuclei, fixed with formaldehyde and mounted with Dako fluorescence mounting medium (S3023) for analysis by fluorescence microscopy.

Tissue Harvesting, Immunohistochemistry and Plaque Morphometry Mice were anesthetized, sacrificed and exsanguinated. Samples of organs were snap–frozen in liquid nitrogen and stored at -80^{o}C. Animals were perfused with PBS followed by 4% formaldehyde before dissection of heart, aorta, common carotid arteries, spleen, thymus, omentum and liver. Organs were stored in 4% formaldhehide overnight (4.5 times diluted Zinc Formal–Fix, Thermo Electron Corporation, Breda, The Netherlands) before being embedded in paraffin. Immunohistochemistry of carotid arteries was performed for Mac3 (1:30, BD Pharmigen Cat. 553322), anti–α–smooth–muscle–actin (ASMA) fluorescein–isothyocyanate (FITC) conjugated antibody (1:3000, Sigma, Cat. F3777) and apoptotic cells (TUNEL TMR–red staining, Roche). HE and sirius red

stainings were used for morphometric analysis and assessment of collagen contents respectively. Sections were analyzed using a Leica Quantimet coupled to Qwin Image Analysis Software, or ImageJ.

Bones were analyzed for the effects of dimerizer treatment on hematopoiesis. Contralateral tibiae were fixated for 24 hours and decalcified for 2 weeks in decalcification buffer (PBS with 0.05 mM EDTA). Buffer was replaced every 3 days to obtain sufficient level of decalcification. Tibiae were subsequently embedded in paraffin and 4μm thick sections were cut for RUNX2 and TRAP histological analysis. All histological analyses were performed, blinded, by two independent operators using Quantimet (Leica) with Qwin3 Image Analysis Software (Leica), or ImageJ.

Statistical Analysis All data were tested for normality and analyzed using t–tests or non–parametric Mann–Whitney U test, as appropriated, with MatLab Software. P values < 0.05, two sided, were considered significant. Data are presented as mean$\pm$ S.E.M. Multiple comparisons were analyzed by one–way ANOVA, followed by Bonferroni multi–comparison test.

5.4 Results

Macrophage Fas Induced Apoptosis (MaFIA) transgenic mice, designed for inducible depletion of CD115$^+$ cells were generated by Burnett and colleagues [257, 258]. In those mice, a suicide protein is co–expressed with EGFP, which serves as reporter gene. The suicide protein comprises a extracellular AP20183 binding protein motif, a transmembrane region of the human low affinity nerve growth factor receptor and the cytoplasmic domain of FAS. The MaFIA transgene uses for transcriptional regulation the promoter of CSF1R, also known as CD115 or M-CSFR; which is expressed in myeloid phagocytes, including neutrophils, monocytes and macrophages.

The MaFIA mechanism of induction of CD115$^+$ cell apoptosis relies on administration of AP20187 to mice expressing MaFIA [257, 258]. Binding to AP20187, cross links the extracellular domain of neighboring MaFIA transgene proteins bringing this way their cytoplasmic FAS domains in close contact. This triggers activation of the FAS–Caspase-8 extrinsic pathway of apoptosis without off target effects due to the high specificity provided by the system [260]. MaFIA mice on C57BL/6J background were crossed to C57BL/6J–ApoE$^{-/-}$ mice to generate homozygous ApoE$^{-/-}$, MaFIA$^{+/+}$, atherosclerotic prone mice.

A detailed characterization of ApoE$^{-/-}$ MaFIA$^{+/+}$ mice as a model for induction of CD115$^+$ macrophage apoptosis in atherosclerosis is described in the data supplement (Appendix D. Table D.1 and Appendix D. Fig. D1 –D5). Assessment of expression of MaFIA in various leukocyte populations, including myelocytes and leukocytes is included, as well as a description of cells susceptible to induction of apoptosis by administration of AP20187.

Advanced atherosclerotic lesions were induced by bilateral perivascular collar placement on common carotid arteries of WTD fed ApoE$^{-/-}$ MaFIA$^{+/+}$ mice. ApoE$^{-/-}$ MaFIA$^{-/-}$ mice were used as control. The effect of induction of CD115$^+$ cell apoptosis on advanced atherosclerotic lesions was assessed using two approaches. In the first approach, only lesion CD115$^+$ cells were targeted for ablation by local release of AP20187. In the second approach, systemic apoptosis of CD115$^+$ cells was

induced by intravenous injection of AP20187.

$CD115^+$ cell apoptosis was induced during 2 weeks starting 5 weeks after perivascular collar placement and eight weeks after introduction of WTD. At this time point (baseline) no significant differences in total body weight (30.27 ± 0.82 vs. $31.89 \pm .91$ gr, $Pval > 0.05$) and plasma cholesterol levels ($Pval > 0.05$. Appendix E. Fig. E1A) were observed between $MaFIA^{+/+}$ and $MaFIA^{-/-}$ control mice.

Similarly, no significant differences were observed in plaque burden, lipid core, lesion cellularity and relative necrotic core content in $MaFIA^{+/+}$ mice, compared to $MaFIA^{-/-}$ controls (Appendix E. Fig. E2A-E). Furthermore, no differences were found in cell composition ($TUNEL^+$ apoptotic cells, $MAC3^+$ macrophages and $ASMA^+$ VSMC), collagen content and minimal fibrous cap thickness, as assessed by histological quantifications (Appendix E. Fig. E3A-D). Therefore the presence of MaFIA did not influence the development of atherosclerotic lesions, as expected.

Systemic, but Not Local Induction of $CD115^+$ Myelocyte Apoptosis Leads to Atherosclerotic Lesion Vulnerability Local induction of $CD115^+$ cell apoptosis was achieved using osmotic micropumps containing AP20187. Osmotic pumps were connected to catheters whose outlets were attached to perivascular collars for topical release of dimerizer on the adventitia of carotid artery atherosclerotic lesions (Fig. 5.1).

The concentration of AP20187 in osmotic pumps at the site of release was set at 100μM (0.142mg/ml). The osmotic pump model with the slowest release rate (0.15μl/hour) was chosen to minimize systemic effects due to leakage into the periphery, bringing about pharmaceutically inactive systemic doses of 0.018 ± 0.001 mg/kg–day. No significant differences in body weight (29.45 ± 0.97 vs. 28.93 ± 1.02 gr, $Pval > 0.05$) and plasma cholesterol levels (Appendix E. Fig. E1B) were observed between $ApoE^{-/-}$ $MaFIA^{+/+}$ and $ApoE^{-/-}$ $MaFIA^{-/-}$ controls.

Local ablation of $CD115^+$ macrophages using the above experimental setup led to a marked accumulation of apoptotic cells in atherosclerotic lesions (Fig. 5.2A). Furthermore, the amount of lesion macrophages relative to lesion area was 57% reduced (Fig. 5.2B), which indicated succesful induction of lesion macrophage apoptosis. Minimal fibrous cap thickness and relative contents of VSMC and collagen were not altered (Fig. 5.2C-D); indicating that local induction of $CD115^+$ cell apoptosis had no apparent effects on lesion stability. However, a two fold increase in lesion size (Fig. 5.3A) was observed, with no differences in lipid core, cell density and necrotic core area, relative to total lesion area (Fig. 5.3B-E).

Taken together, these results provided a signature of the effect of confined macrophage apoptosis on lesion progression, thus allowing us to segregate local from systemic effects by comparison to mice receiving intravenous injection of AP20187(1 mg/kg body weight, every 72 hours, for two weeks) as next detailed.

Systemic induction of $CD115^+$ cell apoptosis caused a significant reduction in body weight in $MaFIA^{+/+}$ mice (-15.6%, $Pval \prec 0.01$), not seen in $MaFIA^{-/-}$ controls. Nevertheless, plasma cholesterol levels were not affected by intravenous administration of dimerizer (Appendix E. Fig. E1C), indicating that induction of $CD115^+$ myelocyte apoptosis had no influence on cholesterol metabolism.

Systemic induction of $CD115^+$ myelocyte apoptosis led to a significant accumulation of apoptotic cells, (4.5 fold, $P \prec 0.001$; Fig. 5.4A) in atherosclerotic lesions

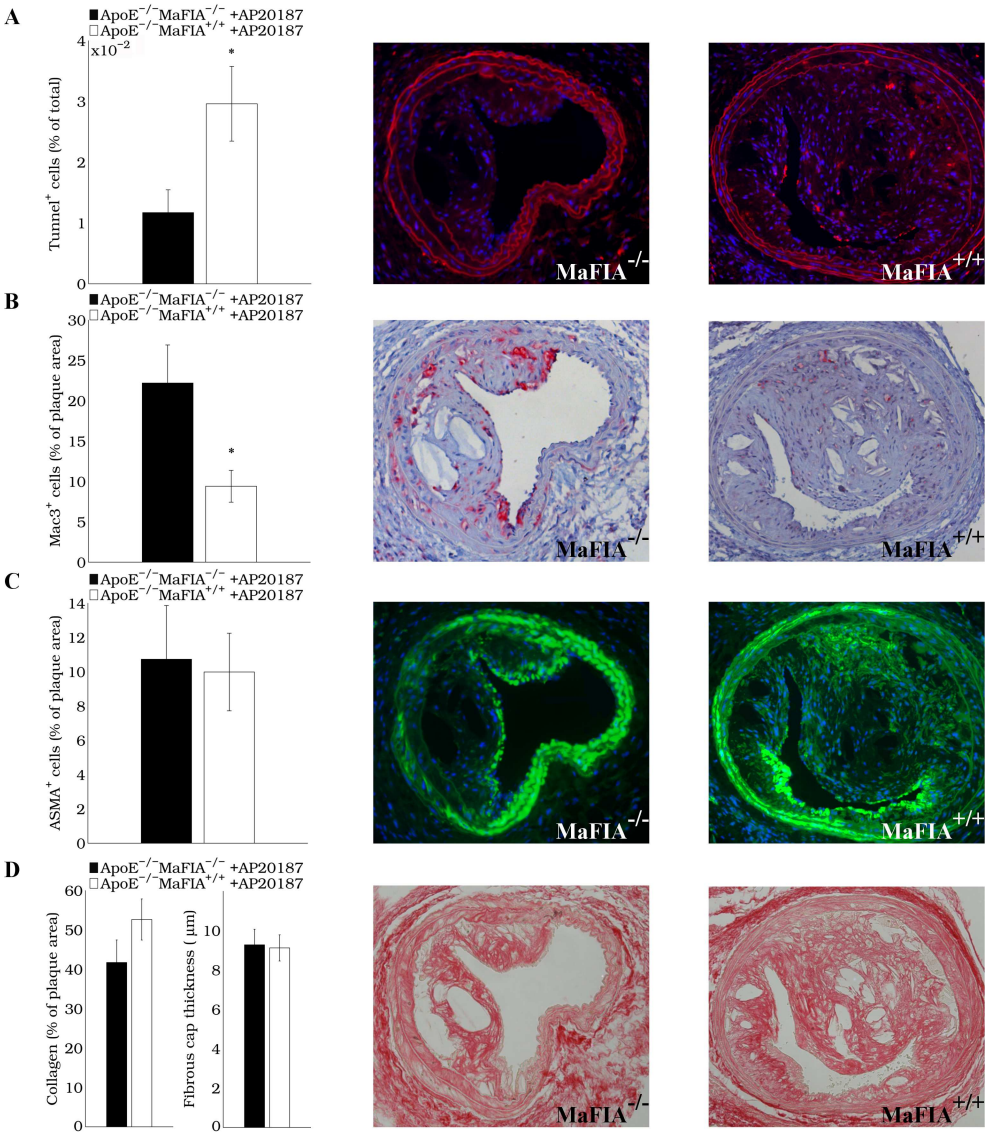

Figure. 5.2. *Local induction of CD115$^+$ macrophage apoptosis causes advanced atherosclerotic lesion expansion. A-D. Apoptotic cells (A), macrophages (B), VSMC (C), collagen contents (D, left panel) and minimal fibrous cap thickness (D, right panel) upon local induction of CD115$^+$ myelocyte apoptosis in MaFIA$^{+/+}$ ApoE$^{-/-}$ mice. MaFIA$^{-/-}$ ApoE$^{-/-}$ mice were used as control. 2.5 fold increased accumulation of apoptotic cells (A), with concomitant 58% reduction in lesion macrophages (B) was observed. The total amount of VSMC (C) and collagen (D, left panel) contents was not altered, nor was the minimal fibrous cap thickness changed (D, right panel). Middle and right panels in A-D. Representative pictures of TUNEL (A), Mac3 (B), ASMA (C) and collagen (D) stainings, used for quantification.* $*P \leq 0.05$.

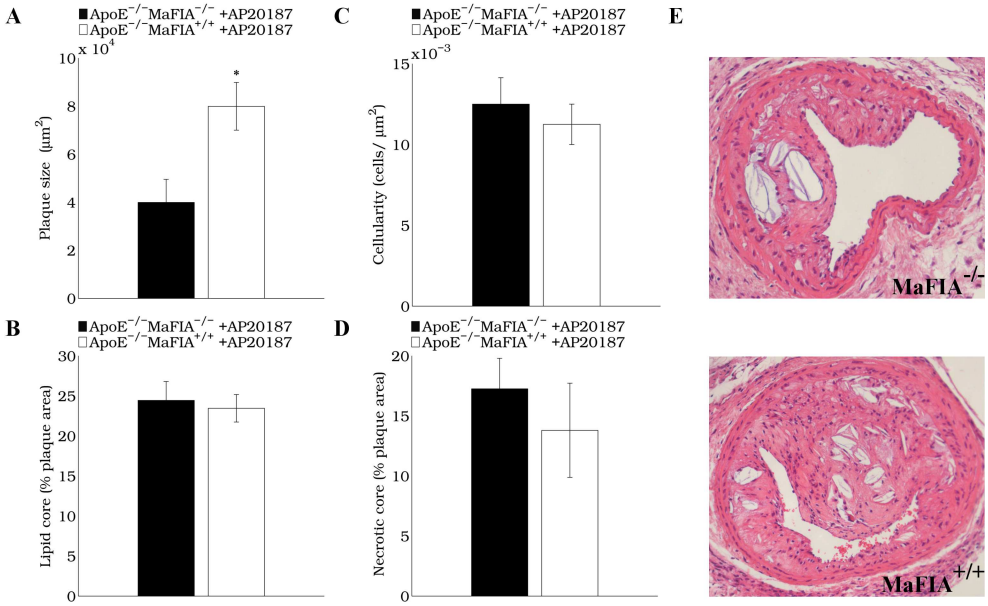

Figure. 5.3. *Effects of local induction of CD115$^+$ macrophage apoptosis on advanced atherosclerotic lesion phenotype. A-D. Lesion size (A) relative lipid core area (B), cellularity (C) and necrotic core composition (D) in MaFIA$^{-/-}$ ApoE$^{-/-}$ (control) and MaFIA$^{+/+}$ ApoE$^{-/-}$ (treated) atherosclerotic mice after local release of AP20187 (0.01mg/Kg–day) during two weeks, starting eight weeks after introduction of WTD. Local induction of macrophage apoptosis leads to two fold lesion expansion (A). No other differences were observed. E. Representative pictures of HE staining, used for quantification.* $*P \leq 0.05$.

that displayed reduced macrophage contents indicating intralesion induction of macrophage apoptosis, as expected.

Strikingly, atherosclerotic lesions from MaFIA$^{+/+}$ atherosclerotic mice receiving intravenous injections of AP20187 displayed reduced VSMC contents (Fig. 5.4B-C), with concomitant reduction in collagen accumulation (Fig. 5.4D) and thinning out of the fibrous cap (Fig. 5.4D). However, no significant differences were observed on lesion size, relative lipid core content, cellularity and necrotic core area percentage (Fig. 5.5A-E).

Taken together, these results indicated that systemic induction of CD115$^+$ myelocyte apoptosis caused lesion vulnerability in advanced atherosclerosis, without affecting plaque burden. Thus while increased plaque apoptosis and reduced macrophage contents were observed upon systemic and local AP20187 treatment; systemic ablation of CD115$^+$ myelocytes clearly resulted in a distinct lesion phenotype. Correspondingly, a sharp contrast was observed between the effects that local and systemic induction of CD115$^+$ monocyte/macrophage apoptosis had on blood and spleen myelocyte homeostasis. In fact, local release of AP20187 on atherosclerotic lesions had no effect on blood granulocytes, monocytes and Ly6C monocyte subsets, and only

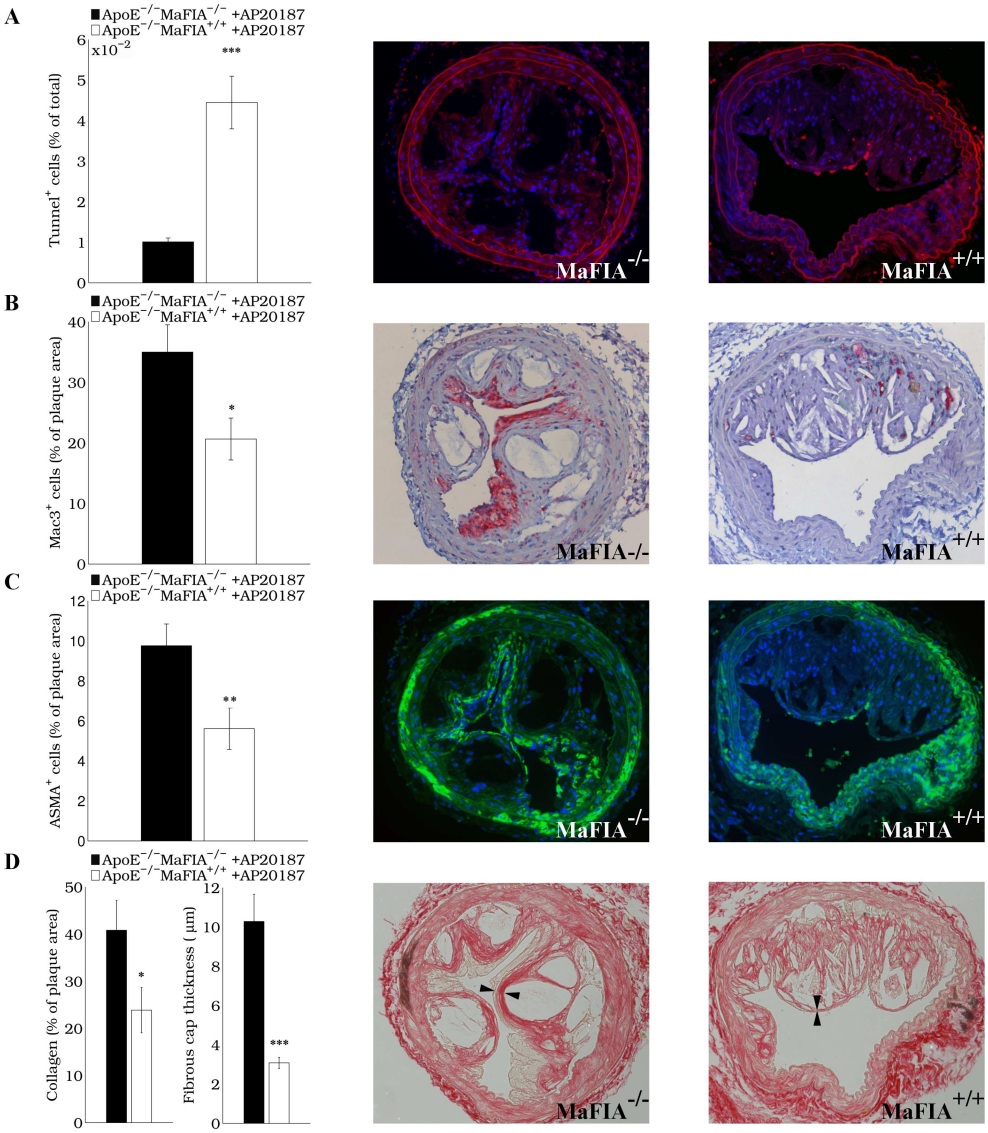

Figure. 5.4. *Effects of systemic induction of CD115$^+$ macrophage apoptosis on advanced atherosclerotic lesion composition. A-D. ApoE$^{-/-}$ MaFIA$^{-/-}$ and ApoE$^{-/-}$ MaFIA$^{+/+}$ mice, received intravenous injections of AP20187 (1mg/Kg every 72 hours) during two weeks, starting eight weeks after introduction of WTD. 4.5 fold increased apoptotic cells (A), with concomitant 41% reduction in macrophages (B) was observed. The total amount of VSMC (C) and collagen (D, left panel) contents was reduced by 42.5% and 41.5%; respectively. Minimal fibrous cap thickness was reduced by 70% (D, right panel). Middle and right panels in A-D are representative pictures of TUNEL (A), Mac3 (B), ASMA (C) and collagen (D) stainings, used for quantification.* $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

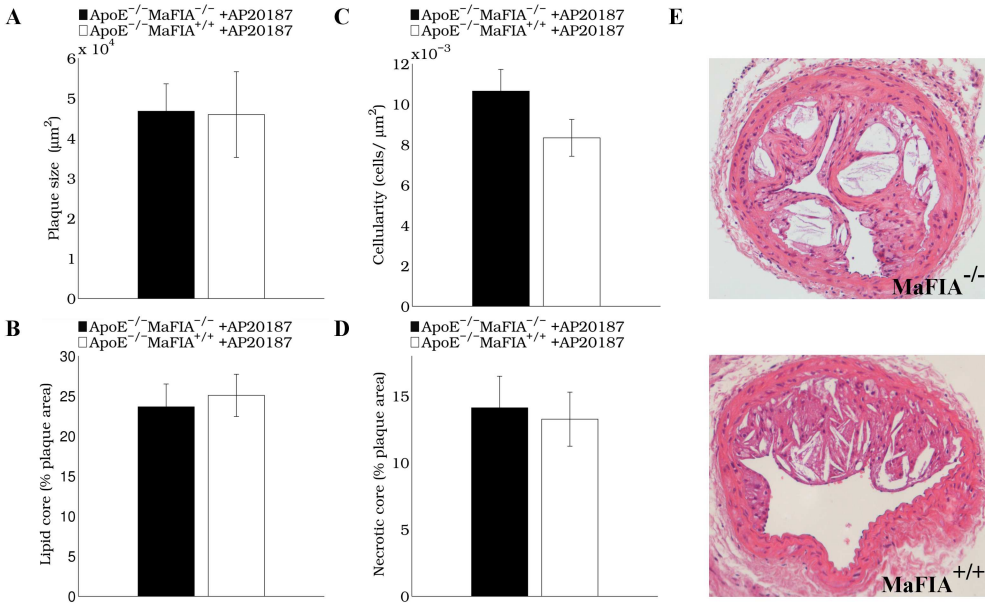

Figure. 5.5. *Effects of systemic induction of CD115$^+$ macrophage apoptosis on advanced atherosclerotic lesion phenotype. A-D. Lesion size (A) relative lipid core area (B), cellularity (C) and necrotic core composition (D) in MaFIA$^{-/-}$ ApoE$^{-/-}$ (control) and MaFIA$^{+/+}$ ApoE$^{-/-}$ (treated) atherosclerotic mice receiving intravenous injections of AP20187 (1mg/Kg every 72 hours) during two weeks, starting eight weeks after introduction of WTD. No significant differences were observed. E. Representative pictures of HE staining, used for quantification.*

caused a slight but significant increase in CD11b$^+$Ly6G^{int} myelocytes with simultaneous reduction in pro–atherosclerotic B220$^+$ B2 B lymphocytes (Fig. 5.6A-C).

Intravenous injections of dimerizer during two weeks on the other hand, unexpectedly caused blood monocytosis (Fig. 5.6D) and expansion of the pro–inflammatory Ly6c^{hi} subset (Fig. 5.6E) as observed at the time of sacrifice. No differences were observed in circulatory granulocyte levels, despite depletion of bone marrow macrophages which, as occurs upon systemic induction of CD115$^+$ myelocyte apoptosis in MaFIA mice [237, 261, 262], has been shown to induce neutrophilia [238]. This, was likely caused by counteracting depletion of CD11b$^+$Ly6G$^+$ granulocytes, which were demonstrated to express MaFIA (see Appendix D. Table D.1, Appendix D. Fig. D1 and discussion in Page 139). CD11b$^+$Ly6G^{int} myelocytes displayed a similar increase as that observed upon local induction of CD115$^+$ cell apoptosis (Compare Fig. 5.6 A and D). No significant differences were seen on blood lymphocyte levels (Fig. 5.6F).

Local release of dimerizer had no major effects on spleen size (Fig. 5.7A) while systemic administration of dimerizer in contrast, caused pronounced splenomegaly (Fig. 5.7B). Similarly, local administration of dimerizer had no effect on spleen dendritic cell and lymphocyte contents (Fig. 5.7C-E), while intravenous injection of dimerizer caused reduced percentages of dendritic cells and T lymphocytes (Fig. 5.7F-H) likely reciprocal to the expansion in myelocytes observed. Both, systemic and

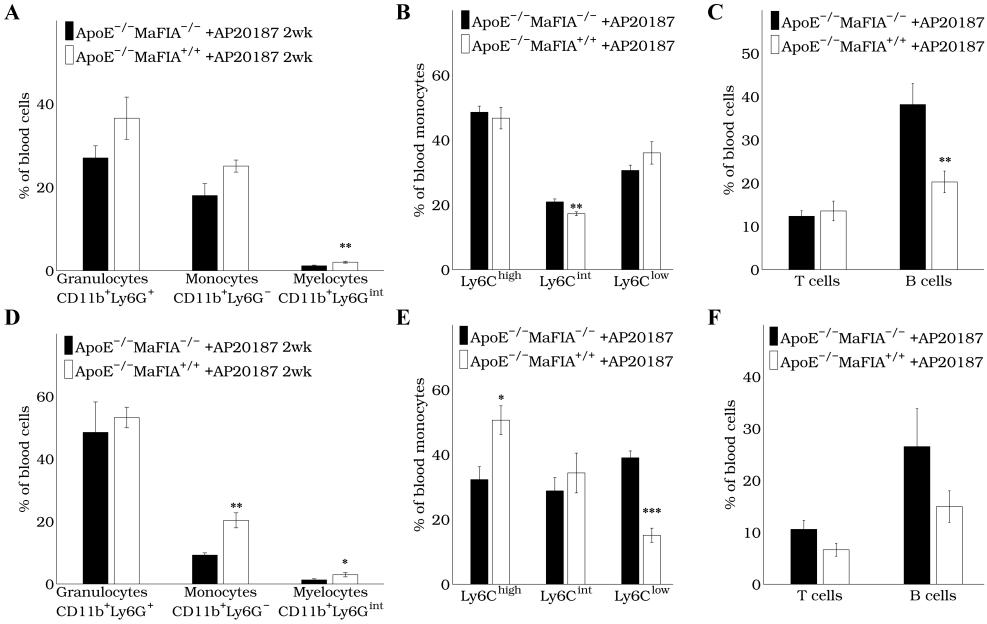

Figure. 5.6. *Blood Leukocyte homeostasis upon local and systemic induction of CD115$^+$ myelocyte apoptosis in ApoE$^{-/-}$ MaFIA$^{+/+}$ mice. Advanced atherosclerosis was induced by WTD during 10 weeks and perivascular collar placement. Blood leukocyte composition was analyzed by FACS upon local (A-C) and systemic (D-F) induction of CD115$^+$ myelocyte apoptosis. No differences in granulocytes, monocytes (A) and Ly6c^{hi} myelocytes (B) were observed upon local treatment, which however induced ablation of pro–atherosclerotic B220$^+$ B2 B lymphocytes (C). In sharp contrast, systemic administration of AP20187 caused expansion of blood CD11b$^+$ Ly6G$^-$ Monocytes (D) and Ly6c^{hi} monocytosis (E). The reduction of B220$^+$ B2 lymphocytes did not reach statistic significance in the systemic application (F). Importantly, local and systemic induction of CD115$^+$ myelocyte apoptosis caused significant increase in CD11b$^+$ Ly6G^{int} myelocytes.* $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

local treatment caused reductions in pro–atherosclerotic B220$^+$ B2 B spleen lymphocytes in agreement with their expression of CD115 (Appendix D. Table D.1, Page 141. See also References [263,264]). These results therefore indicated that besides causing lesion vulnerability, systemic induction of CD115$^+$ cell apoptosis had profound effects on peripheral homeostasis.

Induction of CD115$^+$ Myelocyte Apoptosis Causes Extra–Medullary Myelopoiesis
Extravascular monocyte homeostasis upon systemic induction of CD115$^+$ myelocyte apoptosis was next assessed considering that this treatment caused profound disturbances in peripheral leukocyte homeostasis and lesion phenotype, not observed in atherosclerotic mice upon local administration of AP20187.

In follow up studies, systemic induction of CD115$^+$ cell apoptosis in ApoE$^{-/-}$ MaFIA$^{+/+}$ mice fed WTD during three weeks, caused similar disturbances in blood

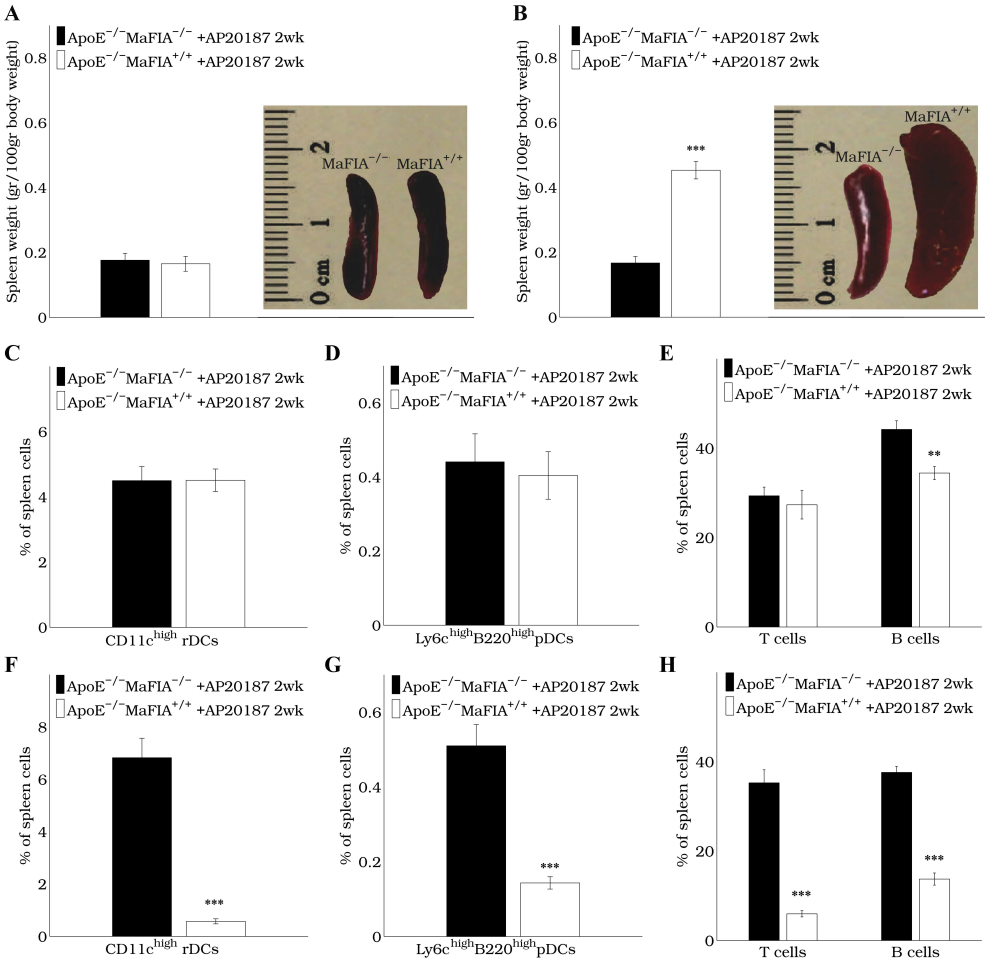

Figure. 5.7. *Spleen leukocyte homeostasis upon local and systemic induction of CD115$^+$ myelocyte apoptosis in ApoE$^{-/-}$ MaFIA$^{+/+}$ mice. Advanced atherosclerosis was induced by WTD during 10 weeks and perivascular collar placement. A-B. Measurement of spleen weight demonstrated that local induction of CD115$^+$ myelocyte apoptosis had no significant effect on spleen size, relative to body weight (A). Systemic administration of AP20187 in contrast, caused pronounced splenomegaly (B). Similarly, local delivery of AP20187 had no significant effects on spleen dendritic cells (C-D) and T lymphocytes (E). Systemic induction of CD115$^+$ myelocyte apoptosis in contrast caused massive depletion of rDC (F), pDC (G) and T lymphocytes (H). Depletion of B220$^+$ B2 B lymphocytes was observed upon both, local and systemic induction of CD115$^+$ myelocytes apoptosis (E, H).* $**P \leq 0.01$, $***P \leq 0.001$.

and spleen myelocytes (data not shown) as those observed before, in atherosclerotic mice fed WTD during ten weeks (Fig. 5.6D-F and 5.7B, F-H). Importantly, analysis of blood myelocytes one week before finalization of AP20187 treatment demon-

strated that almost total ablation of circulatory monocytes and granulocytes was followed by recovery (Appendix D. Fig. D5) indicating, a compensatory expansion of blood myelocytes in response to induction of apoptosis. Indeed, increased levels of spleen $CD11b^{+}Ly6G^{-}$ monocytes and $CD11b^{+}Ly6G^{hi}$ granulocytes were observed in $ApoE^{-/-}$ $MaFIA^{+/+}$ following systemic administration of AP20187 (Fig. 5.8A-B). However, fewer spleen $CD11b^{+}Ly6G^{-}$ monocytes were $CD115^{+}$ (Fig. 5.8C).

Depletion of bone marrow stromal macrophages, as occurs in AP20187 treated MaFIA mice [237, 261, 262], causes G-CSF mediated mobilization of bone marrow hematopoietic stem and progenitor cells (HSPC) into the circulation, leading to splenomegaly, extramedullary myelopoieisis and severe neutrophilia [238]. In agreement with this, the concentration of G-CSF has been reported to increase in AP20187 treated MaFIA mice [261]. Furthermore, depletion of $CD115^{+}$ myelocytes in AP20187 treated MaFIA mice has been reported to cause mobilization of HSPC into the circulation and spleen [237, 261]. This, together with the expansion of circulatory monocytes and normal levels of blood granulocytes observed upon systemic administration of AP20187 in $ApoE^{-/-}$ $MaFIA^{+/+}$ atherosclerotic mice, made us hypothesize that enhanced extramedullary myelopoiesis might have counteracted the depletion of $CD115^{+}$ myelocytes expected.

Consistently, severe splenomegaly was observed in in atherosclerotic mice used for assessment of lesion phenotype upon systemic ablation of $CD115^{+}$ myelocytes (Fig. 5.6H) as well as in follow up studies (Fig. 5.8D). Furthermore, FACS analysis of spleen hematopoietic progenitors and colony forming units (CFU) assays indeed indicated that systemic induction of $CD115^{+}$ cell apoptosis in $ApoE^{-/-}$ $MaFIA^{+/+}$ mice, caused enhanced spleen granulopoiesis and monocytopoiesis. Increased levels of primordial spleen LSK progenitors were observed one week after initiation of treatment (Fig. 5.8E). This expansion was followed by normalization at the second week, when granulocyte–macrophage progenitor cell (GMP) levels, relative to total LK spleen cells, were seven fold increased (Fig. 5.8F).

A slight but not significant elevation relative to the control group was observed for common myeloid progenitors (CMP) (Fig. 5.8F), suggesting that systemic induction of $CD115^{+}$ cell apoptosis, caused increased amounts of spleen granulocytes and monocytes downstream of GMP and not at the CMP commitment stage. In agreement with these results, a four fold increase in total spleen colony forming units (CFU) was observed after one and two weeks of systemic administration of dimerizer (Fig. 5.8G). In addition, increased counts of macrophage colony forming units, (GM–CFU), granulocyte CFU (G–CFU) and macrophage CFU (M–CFU) were observed at both time points (Fig. 5.8G).

Reduced levels of bone marrow osteoclasts were seen in $ApoE^{-/-}$ $MaFIA^{+/+}$ mice, fed WTD during three weeks before systemic administration of AP20187 (Fig. 5.9A), in agreement with previously published data [261]. This, would have been expected to cause accumulation of bone marrow hematopoietic stem and progenitor cells (HSPC), considering that osteoclasts facilitate the mobilization of immature hematological progenitors into the circulation [265–267]. However, systemic administration of AP20187 to $MaFIA^{+/+}$ mice causes (i) downregulation of cytokines important for HSPC retention, (ii) indirect loss of endosteal osteoblasts by impairment of endosteal niches and (iii) HSC mobilization into the blood and spleen, [237, 261, 262] suggesting that the levels of bone marrow HSPC in treated $MaFIA^{+/+}$ mice were dependent on

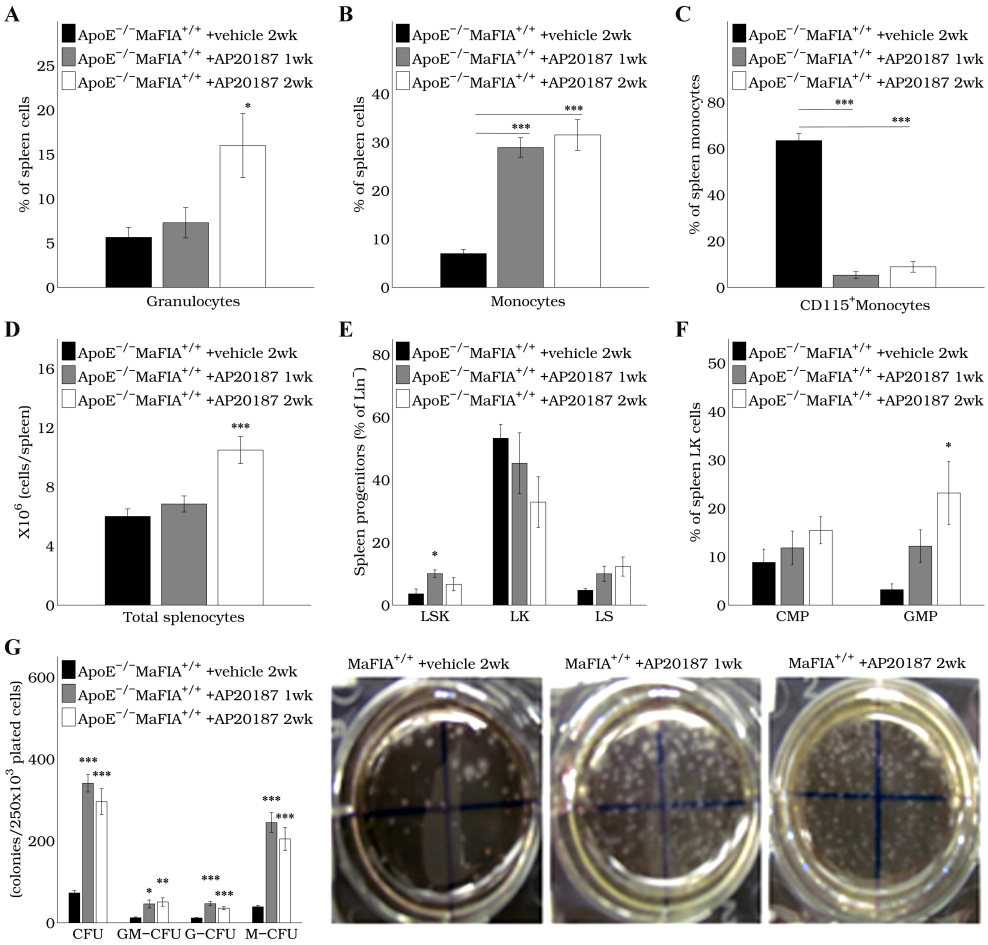

Figure. 5.8. *Spleen myelocytosis is observed upon systemic induction of CD115$^+$ myelocyte apoptosis in ApoE$^{-/-}$ MaFIA$^{+/+}$ mice fed WTD during three weeks. Systemic administration of AP20187 during two weeks, led to increased percentages of spleen granulocytes two weeks after initialization of treatment (A, white bar). Similarly, increased monocyte levels were observed, one week after initializing treatment (B, gray bar) and upon its completion (B, white bar). Downregulation of CD115 was observed in spleen CD11b$^+$ Ly6G$^-$ monocytes one and two weeks upon systemic induction of CD115$^+$ myelocyte apoptosis (C). In addition, total splenocyte levels were increased after two weeks of treatment, indicating induction of splenomegaly (D). Systemic induction of CD115$^+$ cell apoptosis led to increased levels of LSK progenitors one week upon treatment (E). This was followed by expansion of GMP by the second week (F). Increased counts of total CFU and myeloid CFU were observed one and two weeks upon treatment (G), indicating enhanced spleen myelopoiesis. Pictures representative of CFU assays are included (G, right panel).* $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

indirect effects on osteoblasts counteracting the ablation of osteoclasts, and leading to HSPC mobilization.

As net result, no significant differences were found in total bone marrow cells, both after one and two weeks of treatment (Fig. 5.9B). In addition, bone marrow LSK, LK and LS progenitors, as well as GMP and CMP progenitors, total CFU, multipotent GEMM–CFU (data not shown) and committed CFU (GM–CFU, G–CFU, M–CFU) precursor cell counts were not disturbed by the first and second weeks upon intravenous injection of AP20187 (Fig. 5.9C-D).

Collectively, these results therefore indicated that systemic induction of CD115^{+} myelocyte apoptosis causes extramedullary myelopoiesis, likely as result of increased mobilization of bone marrow HSPC.

Systemic administration of AP20187 (1mg/kg, every 72 hours during two weeks) to ApoE$^{-/-}$ MaFIA$^{+/+}$ mice fed WTD during three weeks had no impact in plasma M–CSF (CSF–1) concentration (Control ApoE$^{-/-}$ MaFIA$^{+/+}$ mice receiving vehicle: 1481 pg/ml, treated during one week: 1535 pg/ml, treated during 2 weeks 1475 pg/ml. ANOVA $Pval = 0.8092$). Similarly, the amount of peritoneal granulocytes, monocytes, atheroprotective CD19^{+} B1a lymphocytes and macrophages (Appendix E. Fig. E4A-D) was not altered upon systemic administration of AP20187. However, a sharp increase in Ly6c^{hi} M1 macrophages with concomitant reduction in their Ly6c^{lo} M2 counterparts was observed (Appendix E. Fig. E4E-F) in agreement with the positive regulation of the M2 transcriptome exerted by M–CSF on macrophages [268]. The later result suggests therefore that preferential depletion of tissue repairing macrophages upon systemic induction of CD115^{+} cell apoptosis, might have contributed to the features of lesion vulnerability observed.

Taken together, these results therefore indicated that systemic administration of AP20187 (1mg/kg, every 72 hours) allowed us the induction of lesion macrophage apoptosis with continuous supply of circulatory monocytes provided by mobilization of HSC progenitors into the circulation, extravasation into the spleen and induction of spleen monocytopoiesis. Higher doses of AP20187 in contrast, caused massive ablation of both, circulatory and tissue myelocytes (Appendix D. Fig. D3. See also discussion of the MaFIA model and description of pilot experiments performed to chose the dose regimen used 142).

5.5 Discussion

Although genetic [25, 225] and pharmacological [222, 224, 236, 269] ablation strategies have helped to pinpoint the contribution of plaque macrophage apoptosis to the development and progression of atherosclerosis, and have even been proposed as a the- rapeutic option to stabilize atherosclerotic lesions [256, 270], the outcomes of these studies are discrepant, possibly due to lack of site–and/or cell–specificity of the intervention.

We therefore evaluated the impact of local and systemic induction of CD115^{+} myelocyte apoptosis, in advanced atherosclerotic lesions previously induced by perivascular collar placement. Using this approach, we were able to segregate plaque–confined from peripheral effects. Local and systemic administration of AP20187 caused effective lesion macrophage ablation, as illustrated by significant accumulation of TUNEL positive apoptotic cells inside atherosclerotic lesions that displayed reduced con-

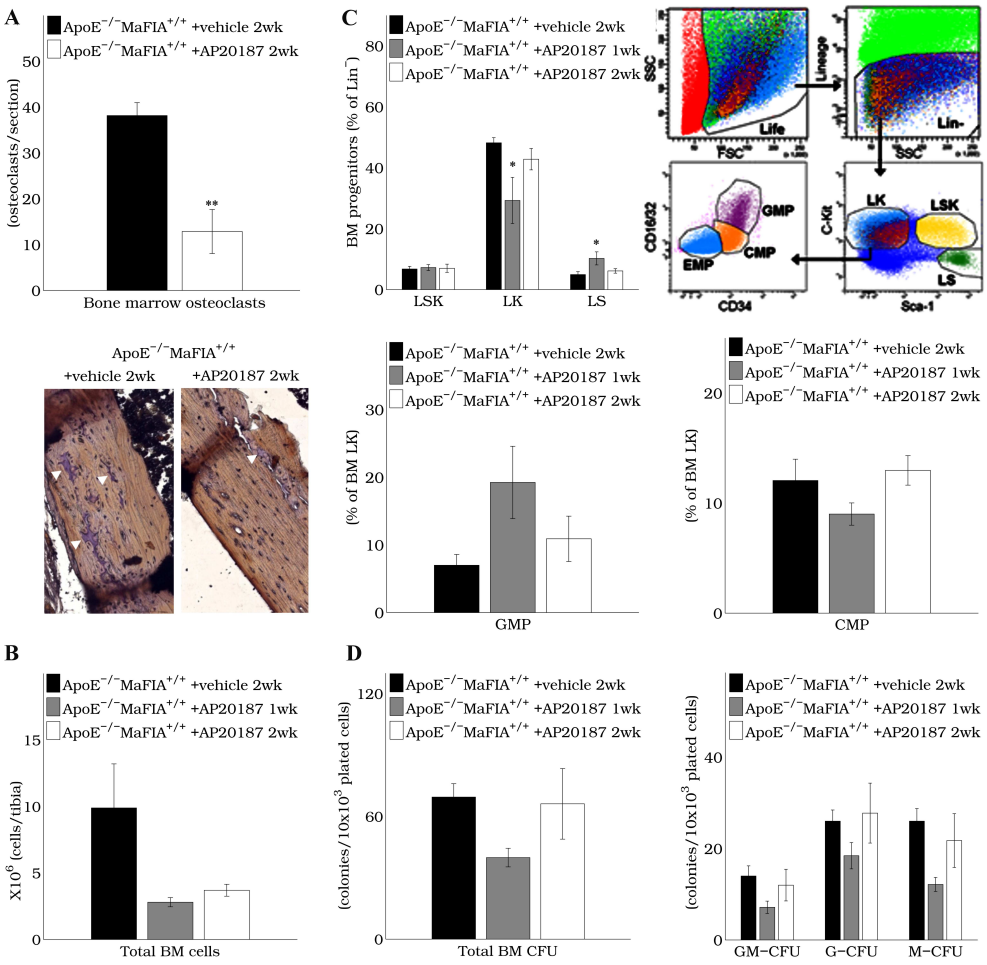

Figure. 5.9. *Bone marrow leukocyte homeostasis upon systemic induction of CD115$^+$ myelocyte apoptosis in ApoE$^{-/-}$ MaFIA$^{+/+}$ mice fed WTD during three weeks. Except for osteoclasts (A), systemic induction of CD115$^+$ myelocyte apoptosis during two weeks had no significant effect on bone marrow cellularity (B) leukocyte progenitors (C, top–left panel) GMP and CMP cells (C, bottom panel), total colony forming units (CFU) (D, left panel) and myeloid CFU (D, right panel). Representative pictures of immunohistochemistry for quantification of bone marrow osteoclasts are included (A, bottom panel) as well as dot plots illustrative of FACS analysis of bone marrow progenitors (C, top–right panel).* $*P \leq 0.05$, $**P \leq 0.01$.

tents of Mac3$^+$ macrophages. We show that local induction of CD115$^+$ monocyte/macrophage apoptosis causes plaque expansion, without changes in fibrous cap thickness, collagen deposition and smooth muscle cell contents. Systemic induction of CD115$^+$ cell apoptosis causes, in sharp contrast, features of lesion vulnerability such as reduced contents of VSMC and collagen and thinner fibrous caps.

Peripheral myelocyte homeostasis, in particular circulatory and spleen monocyte and neutrophil counts and dendritic cell percentages, although unchanged in the local application were altered upon systemic administration of AP20187, indicating that the differences in lesion phenotype observed could be at least in part attributed to peripheral effects of systemic $CD115^+$ cell ablation; with extramedullary myelopoiesis and $Ly6c^{hi}$ skewed monocyte differentiation as main culprits.

No differences in necrotic core size were observed upon both, systemic and local treatment, indicating that the level of apoptosis reached was not high enough to overload the efferocytosis capacity of remaining lesion phagocytes. Similarly, no effect on lesion cellularity and total plasma cholesterol levels were observed, excluding cell density and cholesterol metabolism bias in the interpretation of results.

Our study is thus the first to segregate the impact of lesion–restricted versus systemic $CD115^+$ monocyte/macrophage ablation on advanced atherosclerosis, exposing the true therapeutic and experimental perspective of plaque macrophage depletion strategies; and raising several fundamental questions. The first of which is how local induction of $CD115^+$ myelocyte apoptosis induced plaque expansion.

The only off target effect observed upon local delivery of AP20187 on atherosclerotic lesions was the ablation of proatherogenic $B220^+$ B2 lymphocytes, which according to Ait–Oufella and colleagues [271] and contrary to our results, should have caused reduced atherosclerotic lesion burden. This suggest that any anti–inflammatory effect induced by ablation of B2 lymphocytes, if present, was overruled by other counteracting mechanism.

M–CSF, the ligand for CD115, favors the differentiation of macrophages towards an anti–inflammatory and wound healing M2 phenotype [268, 272]. It is therefore conceivable that systemic induction of $CD115^+$ myelocyte apoptosis might have preferentially depleted M2 macrophages in atherosclerotic lesions; shifting their balance towards higher contents of pro–inflammatory and proteolytic M1 macrophages. However, preferential ablation of alternatively activated M2 macrophages, leading to higher contents of pro–inflammatory M1 macrophages in atherosclerotic lesions, is unlikely to have caused lesion expansion, because besides inflammation, higher contents of M1 macrophages would have induced lesion vulnerability, which was not observed.

More likely, the expansion of atherosclerotic lesions observed upon local treatment might be related to increased inflammation under conditions of augmented intra–lesion $CD115^+$ macrophage apoptosis [52] or local proliferation, which has recently been postulated as the predominant process responsible for the replenishment of macrophages in experimental mouse atherosclerosis [273].

A second question concerns the mechanisms responsible for inducing lesion vulnerability upon systemic, but not local administration of AP20187. Systemic administration of AP20187 caused splenomegaly, induced ablation of spleen dendritic cells and exerted a profound impact in circulatory leukocyte homeostasis, characterized by blood monocytosis and expansion of proinflammatory blood $Ly6c^{hi}$ monocytes. This likely predisposed the immune response towards atherosclerotic lesion vulnerability already in the circulation; considering that $Ly6c^{hi}$ monocytes are prompt to extravasation into atherosclerotic lesions, and differentiation into pro–inflammatory and proteolytic macrophages [61, 274, 275].

In addition, systemic depletion of $CD115^+$ macrophages might have caused a higher M1/M2 macrophage ratio in atherosclerotic lesions leading to thinner fibrous

caps, lessened accumulation of VSMC and reduced deposition of collagen, as observed. In agreement with this notion, increased levels of Ly6c^{hi} macrophages, known to display an M1 phenotype [276], were observed in the peritoneum of mice receiving systemic treatment, suggesting that a similar shift might have taken place in atherosclerotic lesions; yet further experiments using more markers of macrophage differentiation would be needed to reach definitive conclusions on this point.

A fundamental question is why, despite the expression of MaFIA, systemic induction of CD115^{+} myelocyte apoptosis during two weeks had no effect in blood granulocytes and caused increase, not depletion of circulatory monocytes in ApoE$^{-/-}$ MaFIA$^{+/+}$ mice fed WTD during 10 weeks. As to the first, depletion of bone marrow stromal macrophages, as occurs in AP20187 treated MaFIA mice [237, 261, 262], causes severe neutrophilia [238], which would have counteracted the CD115^{+} dependent depletion of neutrophils induced by AP2018 administration.

Regarding circulatory monocytes and Ly6c^{hi} monocytes, their increase at the second week of systemic induction of CD115^{+} cell apoptosis, in advanced atherosclerosis, was rather surprising, considering their expression of MaFIA (as detailed in the data supplement, page 139). However, systemic induction of CD115^{+} myelocyte apoptosis led to almost total ablation of blood monocytes by the first week of treatment; followed by recovery and normalization of blood monocyte counts by the second week, indicating that the dose given although effectively inducing monocyte apoptosis, was not lethal, but rather induced a myelopoietic boost, which, as we next explain, was mediated by mobilization of bone marrow hematopoietic stem and progenitor cells, coupled to extramedullary myelopoiesis.

Induction of CD115^{+} cell apoptosis led to reduced levels of osteoclasts in agreement with previously published results [261]. This would have been expected to cause reduced intravasation and accumulation of hematopoietic progenitors in the osteal niche. Considering that osteoclasts facilitate their mobilization into the circulation [265–267]. However bone marrow progenitor cells and total bone marrow cell counts were not altered, likely because induction of CD115^{+} myelocyte apoptosis in MaFIA mice causes increased levels of G–CSF in the circulation, impairment of endosteal niches, loss of endosteal osteoblasts, downregulation of cytokines required for HSC retention, including CXCL12, Ang–1 and KL, and mobilization of HSC into the circulation and spleen, leading to increased spleen myelopoiesis [237, 261, 262].

In agreement with these data, increased levels of spleen granulocytes and monocytes were observed one and two weeks after initiation of systemic treatment, as result of primordial LSK progenitor expansion by the first week of treatment, followed by granulocyte–macrophage progenitor expansion and enhanced myelopoiesis by the second week.

Therefore, spleen monocytopoiesis was likely responsible for the expansion in pro–inflammatory Ly6c^{hi} lesion infiltrating monocytes observed upon systemic induction of CD115^{+} macrophage apoptosis in advanced atherosclerosis. This is consistent with recently published studies that have identified the spleen as the major source of inflammatory monocytes in hyperlipidemic and normolipidemic mice [66, 277–279]. In addition, extramedullary produced mononuclear phagocytes have been suggested to display a pro–inflammatory phenotype because of their profile of expression of cytokines, reactive oxygen species and proteases [66]. It is therefore conceivable, that the induction of extramedullary myelopoiesis by systemic administration of AP20187

not only provided a source of circulatory monocytes, but also promoted lesion vulnerability.

Downregulation of CD115 as observed in differentiated monocytes might have contributed to their survival under conditions of $CD115^+$ cell ablation, triggered by systemic administration of AP20187. In fact, Robbins et al. have shown that GM–CSF and IL–3 are sufficient to drive myelopoiesis of spleen progenitors [66]. In addition, Leuschner and colleagues reported that IL–1β, but not M–CSF (the ligand for CD115), regulates spleen monocytopoiesis, and is upregulated in spleens of mice induced to develop extramedullary myelopoiesis [278]. It is therefore conceivable that the increased myelopoiesis observed upon systemic induction of $CD115^+$ cell ablation proceeded independent of M–CSF and CD115. Down–regulation of CD115 in myelocytes arise however questions, as to what extent $CD115^-$ phagocytes displayed the complete repertoire of immune functions exhibited by their $CD115^+$ counterparts; which falls beyond the scope of this study and requires further investigation.

Collectively, our results indicate that systemic induction of $CD115^+$ myelocyte apoptosis in $ApoE^{-/-}$ $MaFIA^{+/+}$ atherosclerotic mice allowed us the ablation of lesion macrophages with continuous supply of circulatory monocytes provided by mobilization of HSC progenitors into the circulation and induction of spleen monocytopoiesis.

Extramedullary myelopoiesis, proinflammatory $Ly6c^{hi}$ monocytosis, depletion of spleen dendritic cells and increased M1/M2 tissue macrophage levels were all effects of systemic induction of $CD115^+$ myelocyte apoptosis not observed upon local treatment, that likely contributed to the features of lesion vulnerability observed.

In conclusion, this study is the first to address the impact of spatially confined ablation of $CD115^+$ monocytes and macrophages in advanced atherosclerotic lesion phenotype. We show that systemic and local induction of $CD115^+$ cell ablation led to buildup of apoptotic cells and reduced contents of macrophages in advanced atherosclerosis. In addition, we demonstrated that local induction of $CD115^+$ myelocytes causes lesion expansion, with no major changes in plaque composition. In contrast, systemic (but not local) induction of $CD115^+$ myelocyte apoptosis causes lesion destabilization, possibly owing to augmented extramedullary myelopoiesis and ensuing $Ly6c^{hi}$ monocytosis.

Simultaneous manipulation of systemic leukocyte homeostasis and local induction of macrophage apoptosis offer therefore new opportunities for research and development of improved treatments for atherosclerosis.