Modulation of leukocyte homeostasis in atherosclerosis

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Induction of Macrophage Apoptosis and Its Effect on Atherosclerosis

$CD169^{+}$ Macrophage Ablation Stabilizes Atherosclerotic Lesions in $LDLr^{-/-}$ Mice

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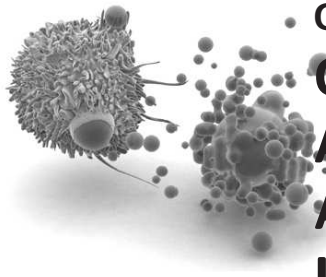

Chapter 4
CD169^{+} Macrophage Ablation Stabilizes Atherosclerotic Lesions in LDLr$^{-/-}$ Mice

"Abundant death, often cataclysmic in its onslaught, is just part of development in many animals..."
J. W. Saunders, Death in Embryonic Systems.

4.1 Abstract

Background: Previous studies have indicated that induction of macrophage apoptosis might lead to lesion destabilization in atherosclerosis. These studies however have caused simultaneous depletion of other myelocytes important for the development and progression of atherosclerosis, including monocytes and dendritic cells. In addition, no study has addressed the effect of recovery from induction of macrophage ablation in atherosclerosis, which is prime importance to assess the feasibility to translate preclinical results into clinical applications. In this study a more specific system allowed the induction of CD169^{+} macrophage apoptosis on advanced atherosclerosis for assessment of its effect both at baseline, and after recovery.

Methods and Results: Female LDLr$^{/}$ mice were lethally irradiated and reconstituted with bone marrow from syngeneic CD169–DTR donors expressing the human receptor for diphtheria toxin (DT), under CD169–promoter mediated transcriptional control. After recovery and 8 weeks of Western type diet, animals were injected with DT or inactive DT mutant (DTM) to ablate CD169^{+} macrophages during 2 weeks. Mice were sacrificed, either 3 days (baseline) or two weeks after the last DT–injection (recovery) and lesions were analyzed. No differences in plasma cholesterol levels were observed between treated and control groups neither at baseline nor after recovery. DT induction of CD169^{+} macrophage apoptosis caused increase in circulatory myelocytes and proinflammatory cytokines at baseline, but their levels recovered to normal after two weeks. CD169^{+} cell depletion induced plaque expansion and increased lesion apoptotic cell content with concomitant increase in relative necrotic core areas at baseline. However these variables normalized upon recovery. In addition, DT treatment led to a significant reduction in macrophage content at baseline and after recovery, illustrating effective ablation and turnover of lesion ma-

crophages. DT treatment had no effect on minimal fibrous cap thickness nor collagen and smooth muscle cell content at baseline. In sharp contrast, significant increases in collagen, fibrous cap thickness and SMC content were observed upon recovery from macrophage depletion.

Conclusions: Ablation of $CD169^+$ macrophages in advanced atherosclerosis leads to reduced macrophage contents with simultaneous collagen and smooth muscle cell accumulation and fibrous cap enlargement after recovery from acute induction of macrophage apoptosis, changes that are generally deemed beneficial for plaque stability.

4.2 Introduction

In developed countries, cardiovascular diseases have become an important problem, representing the principal cause of disability and death [1]. Atherosclerosis is the leading cause of cardiovascular diseases [2]. It is a chronic, inflammatory vasculopathy typified by the progressive accumulation of cholesterol and monocyte derived macrophages in the arterial wall [211, 212]. Lesion macrophages ingest oxidized cholesterol to become foam cells that undergo various forms of programmed cell death, including apoptosis and necroptosis [49], leading either to non inflammatory cell turnover upon efferocytosis by neighboring cells in early lesions [52], or to necrotic core formation in more advanced atherosclerosis [52, 213].

The transition of stable asymptomatic atherosclerotic lesions, to unstable ones, prone to rupture and clinical events such as acute myocardial and cerebral infarction and sudden death, is mediated by the formation of necrotic cores [52] and the erosion and rupture of lesion fibrous caps [214]. Necrotic cores form by accumulation of cells that committed to necroptosis [49] or when apoptotic macrophages were not removed before their membrane lost its integrity, leading to cell necrosis [213].

Necrotic cells leak lytic enzymes, oxidants, proinflammatory and thrombogenic intracellular contents in pools that enter the circulation upon fibrous cap fissure and rupture [45, 213]. Then, thrombogenic components of the lesion react with with blood clogging factors causing the formation of clots and resulting in vascular occlusion with subsequent hypoxia of downstream tissues [214], which in few minutes causes tissue infarction and death [215].

Current treatment strategies to control atherosclerosis include blood pressure stabilizers, anticoagulants, HMG–CoA reductase inhibitors and anti–inflammatory medicines. Cholesterol–lowering therapy leads to lesion regression and reduced macrophage content in human atherosclerosis [216]. Similarly, various methods of induction of advanced atherosclerotic lesion regression in mice and rabbits, including cholesterol withdrawal [217], phototherapy [218] and genetic complementation of mice with genetic deficiencies that cause atherosclerosis [69, 219], induce lesion stabilization.

The mechanisms behind those phenotypic changes can proceed independently of phagocyte efflux out of lesions, converging at reduced monocyte recruitment coupled to lesion macrophage turnover by apoptosis [69, 217, 218, 220–224]. In addition, induction of apoptosis with simultaneous induction of lesion regression by cholesterol withdrawal induces collagen deposition and higher contents of smooth muscle cells than those observed in atherosclerotic lesions of mice under cholesterol withdrawal treatment alone [217], which suggest the induction of macrophage apoptosis to in-

duce cell turnover and lesion regression, as promising mechanism to develop novel and improved therapies for atherosclerosis.

However, the long term effect of induction of macrophage apoptosis on atherosclerotic lesions has not been explored. In addition, it's short term effect has been difficult to elucidate because off target induction of apoptosis in other leukocytes. In fact, systemic induction of $CD11b^+$ myelocyte apoptosis has been reported to have no effect on atherosclerotic lesion phenotype, likely due to simultaneous ablation and reduced availability of circulatory monocytes [225]. Similarly, systemic induction of $CD11c^+$ dendritic cell and macrophage apoptosis has no effect on lesion macrophage contents, but results in advanced atherosclerotic lesion expansion, with simultaneous depletion of dendritic cells and increased plasma cholesterol levels, that might be responsive for the accelerated development of atherosclerotic lesions observed [23, 25].

This study, used a macrophage specific promoter to evaluate the effect of induction of $CD169^+$ macrophage apoptosis in atherosclerosis without off target ablation of monocytes and dendritic cells. Atherosclerotic lesion size and composition were evaluated at finalization and recovery from systemic induction of $CD169^+$ macrophage apoptosis. The results here presented indicate that despite signs of lesion vulnerability three days after induction of macrophage apoptosis, a short period of recovery allows macrophage turnover and lesion stabilization, which has tremendous implications for the development of treatments aiming at lesion stabilization involving macrophage turnover.

4.3 Methods

Bone Marrow Transplantation and Atherosclerosis Bone marrow transplantation experiments were approved by the regulatory authorities of the University of Leiden, and performed in accordance with the Dutch Government guidelines. Female $LDLr^{-/-}$ recipient mice were obtained from the local animal breeding facility at Leiden University. Animals were exposed to a single dose of 9Gy total body irradiation (0.19Gy/min, 200kV, 4mA) using an Andrex Smart 225 Rontgen Source (YXLON International, Copenhagen, Denmark) one day before reconstitution with bone marrow cells from CD169–DTR transgenic mice in which CD169 macrophages could be specifically ablated [226]. Drinking water with antibiotics (83 mg/l ciprofloxacin and 67 mg/l Polymixin) and 5g/l sucrose was introduced one week before irradiation and supplied during the experiment. Eight weeks after bone marrow transplantation mice were put on WTD composed of 0.25% cholesterol and 15% cacao butter (Special Diet Services, Witham, Essex, UK) fed during 11 weeks (baseline group) or 13 weeks (recovery group) until sacrifice. Diphteria toxin, and diphteria toxin mutant (MBL, International corporation RK-01-515 and RK-01-517) were injected intraperitoneally (1ng/μl to achieve a dose of 10ng/g body weight every 3^{rd} day).

Bone marrow transplantation efficiency was assessed as previously described [83] by qPCR of gDNA obtained from bone marrow cells extracted at sacrifice (PureLink Genomic DNA Purification Kit, Invitrogen). Real time PCR reactions (MyIQ Icycler, Biorad) were carried out using $25ng$ of gDNA plus 0.4μl of 15μM primers and 10μl of iQ SYBR®Green Supermix (Biorad) in a total volume of 20μl. Primer sequence sets were: LDLr-forward: 5'-GCTGCAACTCATCCATATGCA-3' and LDLr-reverse: 5'-GGAGTTGTTGACCTCGACTCTAGAG-3' or P50-forward: 5'-AACCTGGGAAT-

ACTTCATGTGACTAA-3' and P50-reverse: 5'-GCACCAGAAGTCCAGGATTAT-AGC-3'. PCR conditions were: 3 min 95^{o}C, 40 2-step cycles: 10 sec 95^{o}C, 45 sec 60^{o}C and 1 min at 95^{o}C. Samples were run in duplicate and CT values compared to CT values obtained from a standard curve of a dilution series mix of 100% : 0% to 0% : 100% of LDLr$^{-/-}$:LDLr$^{+/+}$ genomic DNA.

Cholesterol and Triglyceride Levels Blood samples were taken by tail bleeding one day before the introduction of WTD, five weeks after introduction of WTD and at sacrifice. Total cholesterol and triglyceride and phospholipid content was measured spectrophotometrically using enzymatic digestion of plasma (Roche Diagnostics, Almere, The Netherlands).

Blood Cell Analysis and Flow Cytometry: Whole blood samples were analyzed on a Sysmex blood cell analyzer (XT-2000i, Sysmex Europe GmbH, Norderstedt, Germany) for absolute quantification of leukocyte counts. Single cell suspensions of blood, bone marrow and peritoneal cells harvested at sacrifice were stained with fluorescent label conjugated antibodies after lysis of erythrocytes in ice cold NH_4Cl (8.4g/l) $NaHCO_3$ (1g/l) EDTA (37mg/l) during 3 minutes. Antibodies (eBioscience: B220 (25-0452-82, 1:150), CD11b (57-0112-82, 1:40), CD3e (45-0031-82, 1:50 or 11-0031-82, 1:300), CD19 (45-0193-82, 1:100 or 12-0193-83, 1:100), BD Pharmingen: Ly6G (551461, 1:100), CD11c (558079, 1:400), B220 (553089, 1:100), CD11b (552858, 1:300) and Miltenyi: Ly6c (130-093-134 or 130-093-136, 1:10)) were used after Fc receptor blockage with CD16/32 blocking antibody (eBioscience 14-0161-85, 1:100). A FACSCanto II (BD Biosciences) flow cytometer coupled to the FACSdiva^{TM} software was used for acquisition and analysis of data.

Analysis of Plasma Cytokine Levels The Luminex 100 Bio-Plex cytokine assay (Bio-Rad Laboratories, Inc; Hercules CA, USA) was used to determine cytokine expression in serum samples; cytokines investigated were: IL–1α, IL–1β, IL–2, IL–4, IL–5, IL–6, IL–10, IL–12(P40), IL–12(P70), IL–17, Eotaxin, Keratinocyte chemoattractant (KC), Monocyte chemoattractant protein–1 (MCP–1), Monocyte inflammatory protein–1α (MIP–1α) and Tumor Necrosis Factor–α (TNFα). Statistical analysis was performed for the cytokines that reached the limit of detection (IL–1β, Eotaxin, KC, MCP1 and MIP–1α)

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 (Tissue-Tek, Sakura Finetek). Immunohistochemistry of aortic roots was performed for Moma–2 (1;50, Serotec, Cat. no. MCA 519), activated Caspase-3 (1:100, Cell signaling Cat. nl. 9661L), anti–α-Smooth Muscle Actin (ASMA) fluorescein-isothyocyanate (FITC) conjugated antibody (1:3000, Sigma, Cat. F3777). HE and sirius red stainings were used for morphometric analysis and assessment of collagen content, respectively. Methyl green was used for

counterstain of activated Caspase-3 staining and hematoxilin for all other stainings. Sections were analyzed using a Leica Quantimet coupled to Qwin Image Analysis Software, or ImageJ.

Statistical Analysis All data was analyzed by non-parametric Mann-Whitney U test or t-test, as appropriate, using MatLab Software. P values < 0.05, two sided, were considered significant. Data are presented as mean±S.E.M. Multiple comparisons were analyzed by one-way ANOVA, followed by Bonferroni multi-comparison test.

4.4 Results

Effects of Systemic Induction of CD169$^+$ Macrophage Apoptosis in Atherosclerosis CD169 (also known as Sialoadhesin or Siglec-1) is expressed by macrophages homing to inflammation loci in autoimmune diseases [227, 228] and regions of the arterial intima predisposed to atherosclerosis [229]. CD169 is not present in circulatory monocytes [230] although it is expressed by stromal macrophages present in the bone marrow, as well as by macrophages resident in the spleen marginal zone and lymph node sinus [231], where they trap blood and lymph-born antigens respectively, to induce T-cell mediated responses [232–234]. In addition, CD169 functions as an endocytosis receptor that binds and internalizes sialoadhesin carrying pathogens to increase the humoral response against internalized antigens [235]. However, CD169$^+$ macrophages mediate tolerance to apoptotic cell carried antigens upon phagocytosis [226].

CD169-DTR mice, expressing the human receptor for diphtheria toxin (hDTR) under regulation of the promoter for CD169, were previously developed for time controlled induction of CD169$^+$ macrophage apoptosis [226]. In these mice, administration of diphtheria toxin (DT) results in depletion of CD169$^+$ macrophages. CD169-DTR mice donor of bone marrow were used to reconstitute lethally irradiated LDLr$^{-/-}$ recipients, thereby generating chimeric CD169–DTR–LDLr$^{-/-}$ mice prone to development of atherosclerosis and susceptible to DT controlled induction of CD169$^+$ macrophage apoptosis. 92.2% of circulatory white blood cells were of donor origin after bone marrow transplantation, indicating successful engraftment.

Bone marrow transplantation using CD169-DTR donors and induction of macrophage apoptosis did not lead to any differences in total body weight along the experiment, nor did it affect total plasma cholesterol and triglyceride levels before and after introduction of WTD (Table. 4.1). Similarly, no differences were found in plasma cholesterol levels upon transient induction of macrophage apoptosis by administration of diphtheria toxin (DT) both at the end of the treatment and after two weeks of recovery (Table. 4.1).

Previous studies have assessed the effects of short term induction of macrophage apoptosis on atherosclerotic lesion size and apoptotic cell accumulation [25, 217, 225, 236], but the effect of recovery from induction of macrophage ablation on disease progression and phenotype, have not been reported. We therefore evaluated atherosclerotic lesions obtained from CD169–DTR–LDLr$^{-/-}$ mice at day 3 (baseline) and day 14 (recovery) after DT administration during two weeks to induce CD169$^+$ macrophage ablation.

At baseline, induction of CD169$^+$ macrophage apoptosis in advanced atheros-

Time	Group	TC (mg/dl)	TG (mg/dl)
Before WTD	DTM	225 ± 9	70 ± 8
	DT	250 ± 14	83 ± 10
After WTD	DTM	1328 ± 14	295 ± 12
	DT	1426 ± 15	276 ± 12
Baseline	DTM	1587 ± 14	286 ± 9
	DT	1625 ± 13	291 ± 7
Recovery	DTM	1735 ± 14	274 ± 8
	DT	1644 ± 13	269 ± 8

Table 4.1. *Plasma Cholesterol and Triglyceride concentration in CD169–DTR–LDLr$^{-/-}$ chimeric mice. Concentrations were measured one day before the introduction of WTD (Before WTD), five weeks after introduction of WTD (After WTD), at sacrifice (Baseline) and upon recovery from induction of apoptosis (Recovery), in DTM and DT treated in CD169–DTR–LDLr$^{-/-}$ chimeric mice.*

clerosis increased lesion burden (+26%, $P \prec 0.001$) and necrotic core area (+23%, $P \prec 0.01$), relative to total lesion area (Fig. 4.1A). Simultaneous increase in apoptotic cell density (4.5 fold, $P \prec 0.001$, Fig. 4.1B) without changes in lesion cellularity (Fig. 4.1B) and reduction in macrophage contents (−57%, $P \prec 0.01$, Fig. 4.1C) were observed, indicating successful induction of lesion macrophage apoptosis.

No differences were observed in plaque VSMC content (Fig. 4.2A), collagen deposition and fibrous cap area (Fig. 4.2B) upon DT treatment. Taken together, these results indicated that short term induction of CD169$^+$ macrophage apoptosis solely caused augmented macrophage turnover with increase in lesion burden and necrotic content, without signs of lesion vulnerability, nor reduced contents of VSMC or degradation of the extracellular matrix.

Macrophage apoptosis and the ability of phagocytes to clear apoptotic remnants are important processes that control the balance between lesion inflammation and necrotic core formation versus silent disposal of apoptotic corpses in advanced atherosclerosis. In fact, increased apoptosis of macrophages coupled to defective phagocytosis of death cells causes secondary necrosis leading to expansion of plaque necrotic cores, inflammation, and lesion susceptibility to rupture [54]. In addition, DT mediated induction of CD11c$^+$ macrophage and dendritic cell apoptosis causes increased levels of small inducible cytokines in atherosclerotic lesions [25]. We therefore hypothesized that treatment of CD169–DTR–LDLr$^{-/-}$ mice with DT besides causing macrophage apoptosis and expansion of necrotic cores might have triggered a pro-inflammatory response.

To test this hypothesis, we evaluated whether the induction of CD169$^+$ macrophage apoptosis elicited systemic inflammation in DT treated CD169–DTR–LDLr$^{-/-}$ mice. As seen in Figure 4.3A, plasma levels of MCP-1 and IL–1β were increased three fold at baseline although no significant differences in Eotaxin, KC and MIP–1α were observed (Fig. 4.3A). Interestingly, plasma cytokine levels returned to normal upon recovery (Fig. 4.3B). Similarly, absolute levels of circulatory monocytes and neutrophils were 4 and 3.5 fold increased at baseline ($P \prec 0.001$, Fig. 4.4A-B). No

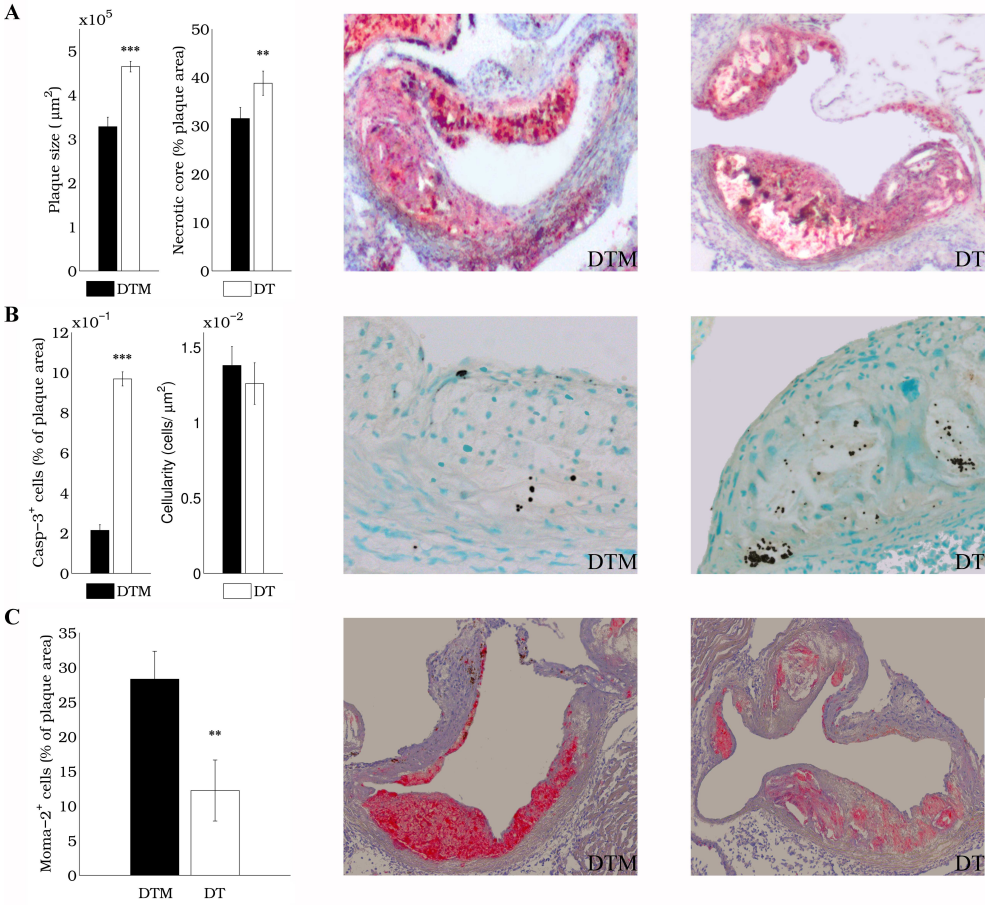

Figure. 4.1. *Lesion expansion, higher necrosis and macrophage apoptosis, upon induction of CD169$^+$ macrophage apoptosis in atherosclerotic mice. CD169–DTR–LDLr$^{-/-}$ chimeric mice fed WTD during eight weeks were treated with diphtheria toxin (DT) for two weeks, to induce CD169$^+$ cell apoptosis. Diphtheria toxin mutant (DTM) treated mice were used as control. Three days after the last injection of DT, animals were sacrificed and aortic root sections analyzed for lesion size (A) and necrotic core area using HE stained sections. B. Activated Caspase-3 staining was used for quantification of apoptotic cells and cell density. C. Moma–2 staining was used for quantification of macrophage contents. The induction of CD169$^+$ cell apoptosis leads to increased lesion burden, expansion of necrotic cores (A) and increase in apoptotic cell content (B), with concomitant reduction in macrophage area (C). No difference was observed in lesion cellularity (B). Central and right panels in A-C, display pictures of sections obtained from DTM (central panel) and DT (right panel) treated mice and stained with HE (A) or antibodies against activated Caspase–3 (B) and Moma–2 (C).* $**P \leq 0.01$, $***P \leq 0.001$.

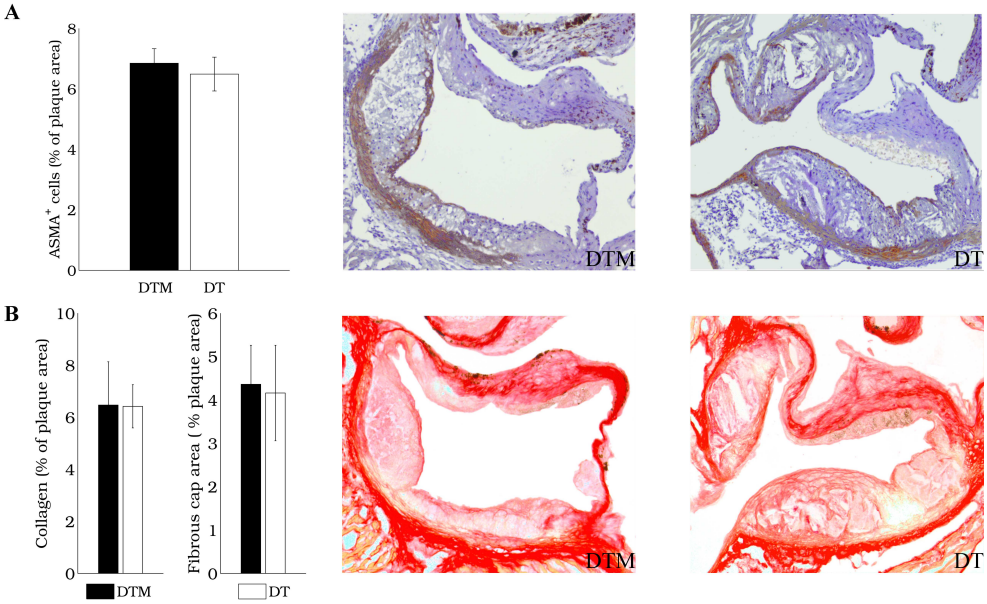

Figure. 4.2. *Lesion phenotype at baseline upon induction of CD169$^+$ macrophage apoptosis. CD169–DTR–LDLr$^{-/-}$ chimeric mice fed WTD during eight weeks were treated with diphtheria toxin (DT) for two weeks, to induce CD169$^+$ cell apoptosis. Diphtheria toxin mutant (DTM) treated mice were used as control. No differences were observed in SMC VSMC (A) and collagen (B) contents three days after the last injection of DT. Fibrous cap areas were similar in DT treated and control mice (B). Central and right panels in A-B, display pictures of $\alpha - SMC$ (A) and Sirius Red (B) staining in sections of DTM (central panel) and DT (right panel) treated mice.*

changes in lymphocyte levels were observed at this time point (Fig. 4.4C). However, circulatory monocyte and neutrophil percentages returned to basal levels after recovery (Fig. 4.4D-E), indicating that the induction of CD169$^+$ cell apoptosis caused transient elevation of inflammatory cytokines and expansion of circulatory myelocytes. Lymphocyte levels remained not altered at recovery (Fig. 4.4F). No changes in absolute circulatory Ly6c monocyte subsets were observed, both at baseline and upon recovery (data not shown).

Interestingly, induction of CD169$^+$ macrophage apoptosis has been reported to induce increased egress of bone marrow stem cell progenitors to the circulation [237]. In addition, monocytes infiltrating atherosclerotic lesions were recently shown to arise also from bone marrow stem cell progenitors that egress from the medullar niche to accumulate in the splenic red pulp, where they expand and differentiate before intravasation and recruitment from the circulation into crescent lesions [66]; which suggested that increased spleen and/or medullar hematopoiesis might have given rise to the increase amount of circulatory myelocytes observed in DT treated atherosclerotic mice at baseline (Fig. 4.4A-B). In agreement with this hypothesis, increased levels of spleen monocytes (2.5 fold, $P \prec 0.001$) and granulocytes (three

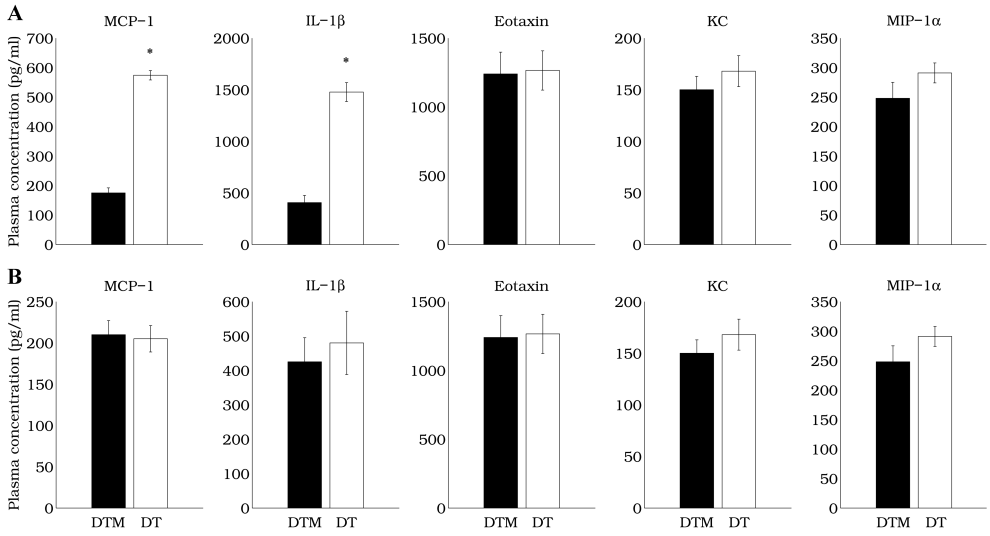

Figure. 4.3. *Plasma cytokine levels at baseline and after recovery from induction of CD169$^+$ Macrophage apoptosis in atherosclerotic mice. A. Induction of CD169$^+$ macrophage apoptosis increases plasma levels of MCP–1 and IL–1β but not of Eotaxin, KC and MIP–1α at baseline. B. Plasma concentration of these cytokines returns to normal 2 weeks after recovery from apoptosis induction treatment. IL–1α, IL–2, IL–4, IL–5, IL–6, IL–10, IL–12(P40), IL–12(P70), IL–17 and TNFα did not reach the threshold of detection, neither in DT, nor in DTM treated controls.* $*P \leq 0.05$.

fold, $P \prec 0.001$) were observed (Fig. 4.5A-B) with reciprocal reduction in spleen lymphocytes (Fig. 4.5C).

Reduced levels of spleen plasmacytoid dendritic cells were also observed (pDC, −35%, $P \prec 0.01$; Fig. 4.5D) upon induction of CD169$^+$ macrophage apoptosis, with simultaneous increase in resident dendritic cells (rDCs, two fold, $P \prec 0.01$; Fig. 4.5E). No differences were found in CD8$^+$ and CD8$^-$ rDCs in the spleen (Fig. 4.5F). Similarly, no differences were observed in differenciated bone marrow leukocyte levels (Appendix C. Fig. C1).

Lesions from CD169–DTR–LDLr$^{-/-}$ mice that were allowed to recover for two weeks upon DT treatment had similar size and necrotic content as those observed in DTM treated controls (Fig. 4.6A), but strikingly transitioned towards lesion stability. In fact, while apoptotic cell contents and cell densities were similar in DT and DTM treated mice (Fig. 4.6B), their content of macrophages was remarkably reduced upon recovery from induction of CD169$^+$ cell ablation (−50%, Fig. 4.6C). Simultaneously, a two fold increase in VSMC VSMC content and collagen deposition was observed ($P \prec 0.01$, Fig. 4.7A-B) with concomitant expansion of fibrous cap areas (Fig. 4.7B), that indicated that recovery from induction of CD169$^+$ cell ablation favoured lesion fibrosis and stabilization.

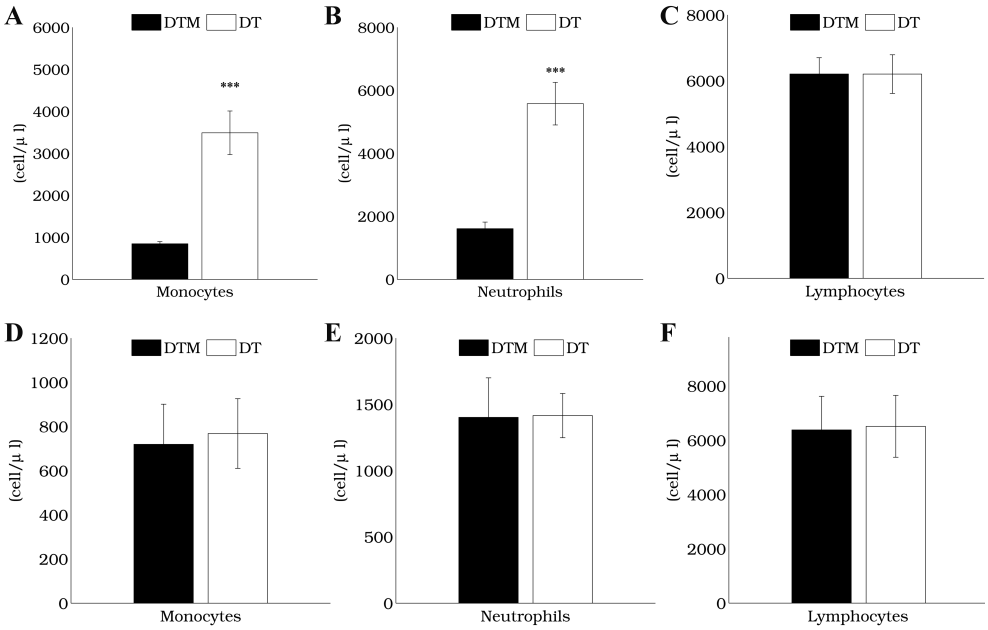

Figure. 4.4. *Effect of induction of CD169$^+$ cell apoptosis on blood leukocyte homeostasis. CD169–DTR–LDLr$^{-/-}$ chimeric mice fed WTD during eight weeks were treated with diphtheria toxin (DT) for induction of CD169$^+$ cell apoptosis during two weeks. Diphtheria toxin mutant (DTM) treated mice were used as control. A-C. Induction of CD169$^+$ macrophage apoptosis leads to increase levels of monocytes (A) and neutrophils (B) three days after the last injection of DT, when no differences are observed in circulatory lymphocyte counts (C). D-F. The increase in absolute levels of circulatory myelocytes observed, is normalized upon recovery during two weeks (D-E). No further changes in circulatory lymphocytes are observed upon recovery (F).* $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

4.5 Discussion

We evaluated the effects of systemic induction of CD169$^+$ macrophage apoptosis in atherosclerosis at baseline and following recovery and stabilization. CD169, which is not present by circulatory monocytes [230] is expressed by macrophages in regions of the arterial intima predisposed to atherosclerosis [229], stromal macrophages in the bone marrow, marginal zone macrophages and macrophages resident in the lymph node sinus.

Transient induction of macrophage apoptosis was achieved by intravenous injections of diphtheria toxin to CD169–DTR–LDLr$^{-/-}$ chimeric mice obtained upon transplantation of bone marrow cells from CD169-DTR mice [226] into LDLr$^{-/-}$ atherosclerotic prone recipients.

Induction of macrophage apoptosis in CD169–DTR–LDLr$^{-/-}$ mice by administration of DT during two weeks caused significant expansion of atherosclerotic lesions and increase in lesion apoptotic cells and necrotic core areas, with concomitant reduc-

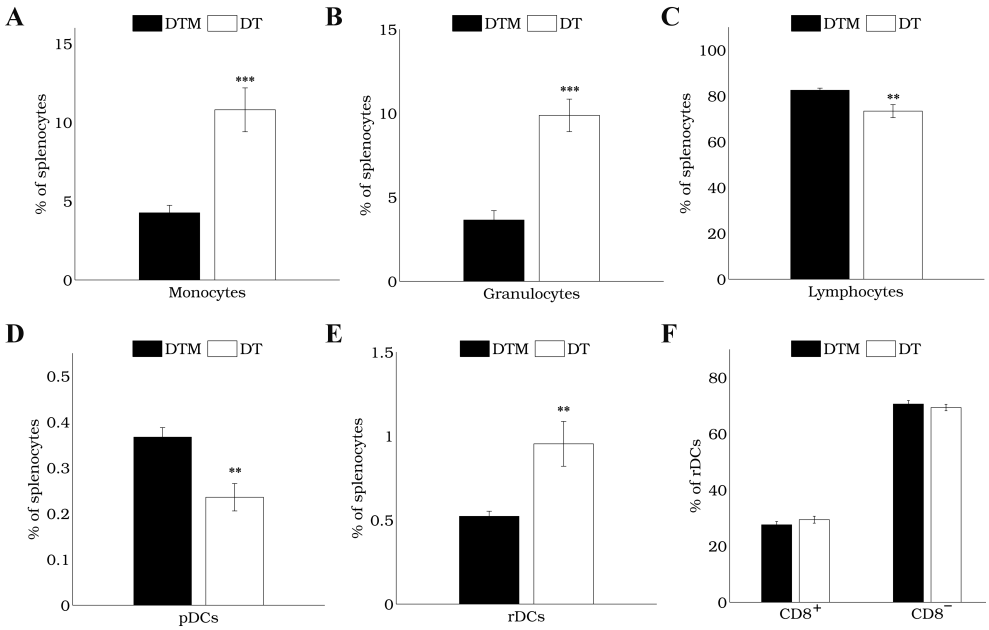

Figure. 4.5. *Effect of induction of CD169$^+$ cell apoptosis on spleen leukocyte homeostasis. CD169–DTR–LDLr$^{-/-}$ chimeric mice fed WTD during eight weeks were treated with diphtheria toxin (DT) for induction of CD169$^+$ cell apoptosis during two weeks. Diphtheria toxin mutant (DTM) treated mice were used as control. A-C. Induction of CD169$^+$ macrophage apoptosis leads to increase levels of monocytes (A) and neutrophils (B) three days after the last injection of DT, when no differences are observed in spleen lymphocyte percentage (C). D-F. Reduced contents of spleen pDC are observed (D) with concomitant increase in rDC (E). No changes in rDC subsets are observed (F).* $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

tion in macrophage contents, that indicated successful induction of lesion macrophage apoptosis and overloading of the efferocytosis capacity of remaining lesion phagocytes.

In addition, induction of CD169$^+$ cell apoptosis led to a sharp increase in absolute circulatory myelocytes levels at baseline that rapidly normalized upon recovery, reaching similar levels to those observed in DTM control mice. No differences in absolute lymphocyte levels were observed both at baseline and after recovery, in agreement with previous characterization of CD169-DTR mice that demonstrated successful depletion of CD169$^+$ macrophages without induction of apoptosis in monocytes, T and B leukocytes and DC [226].

No differences in differentiated leukocytes were observed in the bone marrow of DT treated CD169–DTR–LDLr$^{-/-}$ chimeras, but the percentage of monocytes, granulocytes and dendritic cells was increased in the spleen at baseline, which considering (i) the increased amount of circulatory myelocytes observed, (ii) the increased egress of hematopoietic stem cell progenitors (HSCP) to the circulation observed in CD169–DTR mice upon DT treatment [237] (iii) the enhanced extramedullary

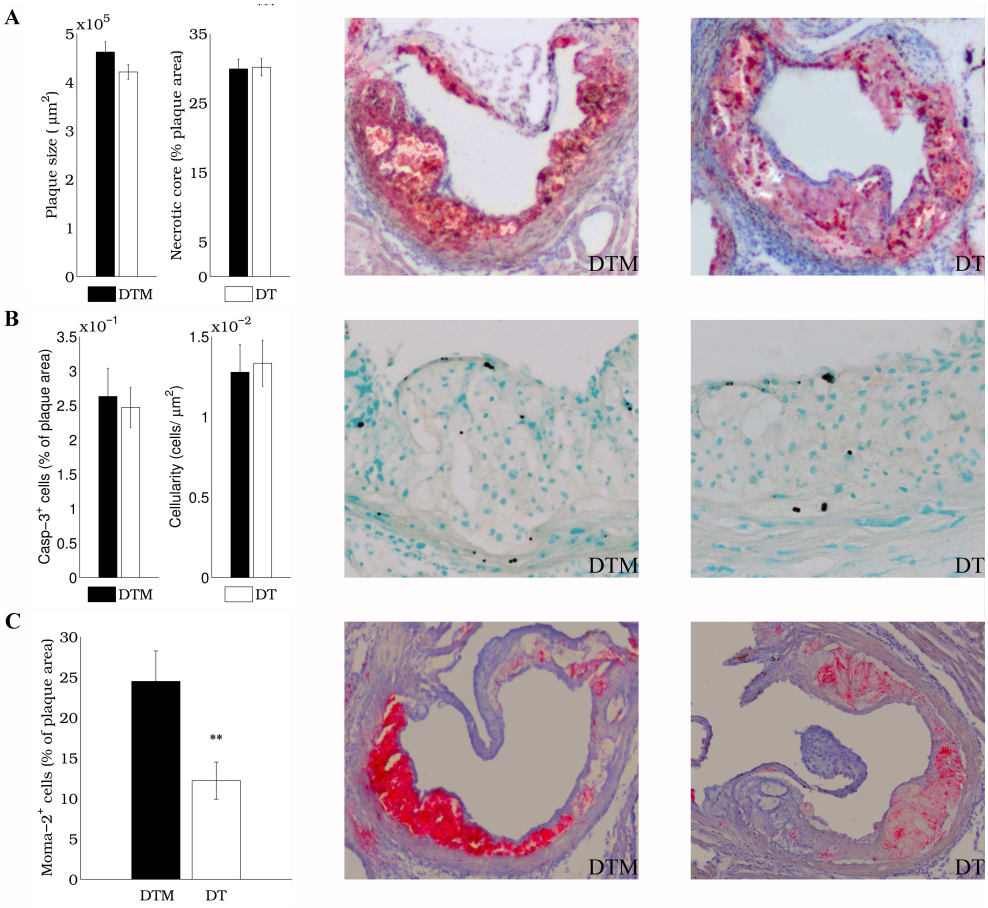

Figure. 4.6. *Lesion size, necrotic core and macrophage apoptosis, upon recovery from induction of CD169$^+$ macrophage apoptosis in atherosclerotic mice. CD169–DTR–LDLr$^{-/-}$ chimeric mice fed WTD during eight weeks were treated with diphtheria toxin for two weeks (DT) to induce of CD169$^+$ cell apoptosis. Diphtheria toxin mutant (DTM) was used as control. Mice were allowed to recover for two more weeks before sacrifice and analysis of lesion phenotype. A. HE stained aortic root sections were analyzed for lesion size and necrotic core area. B. Activated Caspase-3 staining was used for quantification of apoptotic cell and overall cell density. C. Moma − 2 staining was used for quantification of macrophage content. Recovery from induction of CD169$^+$ cell apoptosis caused previously expanded necrotic cores to normalize (A) (Compare with Fig. 4.1A). Similarly, the levels of apoptotic cells were reduced in DT treated mice, reaching the same level found in DTM treated, control mice (B) (Compare with Fig. 4.1B). The content of macrophages remained reduced in DT treated mice (C) (Compare with Fig. 4.1C). No difference was observed in lesion size (A) and cellularity (B).* $**$ $P \leq 0.01$.

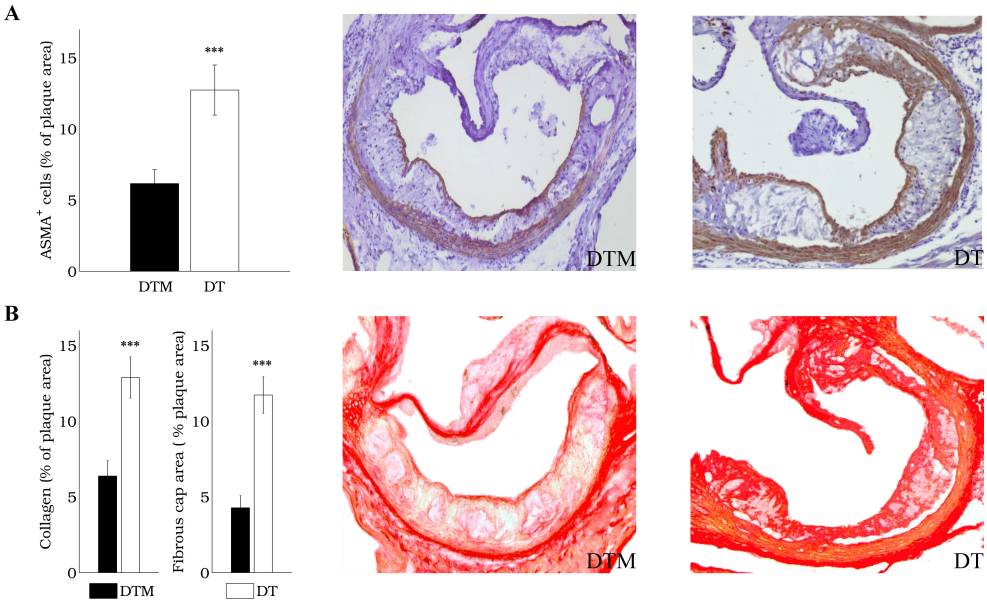

Figure. 4.7. *Features of lesion stability are observed after recovery from induction of CD169$^+$ macrophage apoptosis in atherosclerotic mice. CD169–DTR–LDLr$^{-/-}$ chimeric mice fed WTD during eight weeks were treated with diphtheria toxin (DT) for induction of CD169$^+$ cell apoptosis during two weeks. Diphtheria toxin mutant (DTM) treated mice were used as control. Mice allowed to recover from induction of apoptosis displayed increased lesion SMC (A) and collagen (B) contents, and thickening of fibrous caps (C). Central and right panels in A-B, display pictures of ASMA (A) and Sirius Red (B) staining in sections of DTM (central panel) and DT (right panel) treated mice.* $***P \leq 0.001$

hematopoiesis observed upon depletion of CD169$^+$ macrophages [238] and the accumulation of HSC in the spleen where they expand and differentiate before intravasation and accumulation in crescent atherosclerotic lesions [66], suggest that increased extramedullary spleen myelopoiesis might have taken place upon DT mediated induction of CD169$^+$ cell apoptosis in LDLr$^{-/-}$ atherosclerotic chimeras.

In addition, analysis of plasma cytokines indicated that induction of CD169$^+$ macrophage apoptosis caused a temporal increase in MCP1, which is known to mediate the accumulation of leukocytes in arterial lesions [239, 240] and IL–1β, which expression induces secretion of other proatherogenic cytokines and correlates with the severity of atherosclerosis [241].

Strikingly however, the transient increase in proinflammatory cytokines in the circulation, and the expansion of blood and spleen myelocytes, although expected to cause accelerated atherosclerosis [242], caused transient expansion of atherosclerotic lesions that rapidly normalized upon recovery. Treatment with DT had no effect on lesion cellularity both at baseline and after recovery, excluding cell density bias in the interpretation of results.

The expansion of lesion burden with concomitant increase in lesion apoptosis and reduction in macrophage contents observed upon induction of $CD169^+$ cell apoptosis contrasts to the results reported by Stoneman et al. [225] who demonstrated that acute induction of $CD11b^+$ cell apoptosis had no effect on advanced atherosclerotic lesion size despite increased apoptotic cell densities and reduction in macrophage contents. However, induction of $CD11b^+$ myelocyte apoptosis led to massive depletion of circulatory $CD11b^+$ myelocytes and thus reduced availability of circulatory monocytes to extravasate into atherosclerotic lesions; making difficult to discern the true effect of macrophage apoptosis in atherosclerosis. With our studies in contrast no reduction in absolute circulatory monocyte counts was observed, but transient monocytosis and increase in plasma pro-inflammatory cytokines.

Our results agree with those obtained by Gautier et al. [25], who reported that systemic induction of $CD11c^+$ cell apoptosis caused lesion expansion, accumulation of apoptotic cells and increased levels of newly recruited $CD11b^+$ macrophages in advanced atherosclerosis. However, we observed reduction in lesion macrophages upon DT administration to CD169–DTR–$LDLr^{-/-}$ mice. In sharp contrast, CD11c-DTR mice treated with DT had similar contents of lesion macrophages, compared to controls; which was suggested by the authors to be caused by progressive recruitment of circulatory monocytes masking the lost of macrophages [25]. Although a non–significant ablation of lesion $CD68^+$ macrophages could not be discarded.

Induction of $CD11c^+$ cell apoptosis as reported by Gautier et al. [25], caused massive depletion of dendritic cells and increased plasma cholesterol levels that might be responsive for the accelerated development of atherosclerotic lesions observed [23, 25]. With our experiments in contrast we did not observe ablation but expansion of spleen resident dendritic cells. In addition, no differences were observed in plasma cholesterol levels upon induction of $CD169^+$ macrophage apoptosis in atherosclerotic mice.

Regarding the effect of induction of $CD169^+$ macrophage apoptosis on atherosclerotic lesion stability, our results implicate that lesions from DT treated mice, which exhibited features of instability such as increased necrotic core and apoptotic cell densities at baseline, transitioned into a more stable phenotype characterized by increased contents of collagen and VSMC and expansion of fibrous caps after recovery. Furthermore, necrotic cores and apoptotic cell contents where normalized during recovery, reaching similar levels as those found in control DTM treated mice.

Using different strategies, Waksman et al. [218], and Secchiero et al. [243], reported that induction of cell apoptosis in advanced atherosclerosis resulted in progressive reduction and stabilization of atherosclerotic lesions by sustained removal of macropha- ges and progressive repopulation with smooth muscle cells. These results, although dependent on a different mechanism, agree with our results that indicate that induction of $CD169^+$ macrophage apoptosis in atherosclerotic lesions might trigger lesion stabilization.

The mechanism responsible for induction of lesion stabilization after recovery from induction of $CD169^+$ macrophage apoptosis remains to be fully elucidated, although it is conceivable that induction of $CD169^+$ macrophage apoptosis caused preferential depletion of classically activated macrophages (CAM) skewing the balance towards a higher M2 to M1 intra-lesion macrophage ratio, which is deemed to induce lesion stabilization . The pattern of expression of CD169, which is upregulated upon

pro-inflammatory and tissue degradative stimulus such as IFNγ, LPS and TNFα while being downregulated by IL–4 (CD169 belongs to Cluster C-IV Fig. 2.9A-C and 2.11, Pages 36 and 38, in Chapter 2) agrees with this hypothesis.

In conclusion, this study demonstrates for the first time that advanced atherosclerotic lesions follow a biphasic phenotype that transients from reduced contents of macrophages coupled to lesion expansion and increased apoptosis and necrosis to a phenotype with traits of improved lesion stability upon selective induction of CD169$^+$ macrophage ablation. This opens many opportunities for future research and development of treatment strategies to induce macrophage turnover and lesion stabilization by induction of macrophage apoptosis, in advanced atherosclerosis.