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Diet-induced (epigenetic) changes in bone marrow augment atherosclerosis

Erik van Kampen,[1] *Armand Jaminon, Theo J. C. van Berkel, and Miranda Van Eck*

Division of Biopharmaceutics, Cluster of BioTherapeutics, Leiden Academic Centre for Drug Research, Leiden University, The Netherlands

RECEIVED JANUARY 14, 2014; REVISED MAY 22, 2014; ACCEPTED JUNE 24, 2014. DOI: 10.1189/jlb.1A0114-017R

ABSTRACT

Alterations in DNA methylation patterns in peripheral blood leukocytes precede atherosclerotic lesion development in mouse models of atherosclerosis and have been linked to cardiovascular death in patients. The aim of this study is to investigate the long-term changes induced by WTD feeding on BM cells and the consequences for atherosclerosis susceptibility. Hereto, WTD BM or Chow BM was transplanted into LDLR KO mice on chow. BM from WTD BM recipient mice exhibited hypomethylation of CpG regions in the genes encoding Pu.1 and IRF8, key regulators of monocyte proliferation and macrophage differentiation. In agreement, in blood, the numbers of leukocytes were 40% ($P<0.05$) higher as a result of an increase in F4/80$^+$ monocytes (3.4-fold; $P<0.01$). An increase of CD11c^{++} cells was also found (2.4-fold; $P<0.05$). Furthermore, spleens were enlarged, and the percentage of F4/80$^+$ cells expressing CD86 was induced (1.8-fold; $P<0.01$), indicating increased activation of splenic macrophages. Importantly, mice reconstituted with WTD BM showed a significant, 1.4-fold ($P<0.05$) increase in aortic root plaque size in the absence of changes in serum cholesterol. We conclude that WTD challenge induces transplantable epigenetic changes in BM, alterations in the hematopoietic system, and increased susceptibility to atherosclerosis. Manipulation of the epigenome, when used in conjunction with blood lipid reduction, could thus prove beneficial to treat cardiovascular disorders. ***J. Leukoc. Biol.*** **96: 833–841; 2014.**

Abbreviations: ABCA1=ATP-binding cassette transporter A1, APC=allophycocyanin, apoE=apolipoprotein E, BM=bone marrow, BMT=bone marrow transplantation, Chow BM=bone marrow from LDLR knockout mice fed a regular chow diet, ERY=erythrocyte, FC=free cholesterol, HSC=hematopoietic stem cell, IRF8=IFN regulatory factor 8, KO=knockout, Lin=lineage, LINE=long interspersed nuclear element, LSK=lineage-negative, Sca-1-positive, c-Kit-positive, LT-HSC=long-term hematopoietic stem cell, MK=megakaryocyte, PMN=polymorphonuclear neutrophilic leukocyte, Pu.1=spleen focus-forming virus proviral integration oncogene, Q-PCR=quantitative PCR, ST-HSC=short-term hematopoietic stem cell, Tal.1=T cell acute lymphocytic leukemia 1, TC=total cholesterol, WTD=Western-type diet, WTD BM=bone marrow from LDLR knockout mice fed a Western-type diet

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

Introduction

The increasing prevalence of cardiovascular disorders in Western societies is linked to several risk factors, such as smoking, diet, and genetic predisposition. Recently, epigenetic mechanisms, such as histone modification and DNA methylation, have been proposed as factors contributing to cardiovascular risk [1, 2].

DNA methylation is a key mechanism for epigenetic regulation of gene expression [3]. The presence of methyl groups on cytosine nucleotides in guanine and cytosine-rich gene regions, called CpG islands, can influence transcription of associated genes. Epigenetic mechanisms, such as DNA methylation, do not determine short-term changes in gene expression but rather, enable long-term changes in expression, as may occur when a cell differentiates [4]. In line, in utero exposure to nutritional factors has been shown to have lifelong implications for the epigenome, with associated changes in phenotype [5, 6]. Moreover, diet-induced changes to the epigenome are common in adult life and have been implicated in cancer and longevity [7–10].

Many common nutrients, such as folates or vitamin C, affect DNA methylation patterns when deficient in diet or taken in excess [9, 11]. In the context of atherosclerosis, this raises the possibility that dietary components known to increase cardiovascular risk, such as excess cholesterol or alcohol consumption, can influence the epigenome. There is increasing support in the literature for the hypothesis that changes in DNA methylation are associated with increased risk for atherosclerosis. In 2010, Alkemade et al. [5] described how diet-induced hypercholesterolemia and prenatal exposure to high serum lipid levels induce epigenetic histone modifications in vascular smooth muscle cells and endothelial cells, suggesting that altered epigenetic patterning in the vasculature might affect atherosclerosis susceptibility. In line, alterations in DNA methylation profiles of the arterial wall precede lesion development, as observed in aortas of 4-week-old apoE KO mice, a widely used model to study atherosclerosis [12].

In addition to vascular smooth muscle cells and endothelial cells, altered DNA methylation patterns of leukocytes have

1. Correspondence: Gorlaeus Laboratories HB839, Einsteinweg 55, 2333 CC Leiden, The Netherlands. E-mail: evankampen@lacdr.leidenuniv.nl

been implicated in atherosclerosis. PBMCs of 4-week old apoE KO mice show altered DNA methylation profiles even before the mice developed detectable atherosclerotic lesions [12]. Furthermore, increased global DNA methylation in peripheral blood leukocytes has been linked with increased inflammation and cardiovascular death in patients [13–17]. Exposure of differentiated human THP-1 cells to proatherogenic lipoproteins promotes global DNA hypermethylation [12, 18]. This suggests that hypercholesterolemia might form a link between altered DNA methylation patterning in blood leukocytes and increased susceptibility to atherosclerotic lesion development. In line, a study by Pearce et al. [19] demonstrated that human blood lipid profiles positively correlate with global DNA methylation, measured as LINE methylation. Interestingly, HDL cholesterol levels are negatively correlated with LINE methylation, whereas LDL cholesterol shows a strong, positive correlation. In a study in familiar hypercholesterolemia patients with elevated LDL cholesterol levels as a result of mutations in the LDLR, concentrations of circulating HDL cholesterol correlated with demethylation of the promoter for ABCA1, a cellular cholesterol transporter determining HDL levels in the circulation [20].

The aim of this study is to test whether dietary-induced hypercholesterolemia can lead to alterations in the epigenetic programming of BM DNA and consequently, lead to phenotypic alterations in HSCs, including monocytes and macrophages. Hereto, we fed LDLR KO mice a proatherogenic WTD and subsequently, used their BM for transplantation to LDLR KO recipients fed regular chow. We show that after transplantation, the recipients have altered BM methylation patterns when compared with controls transplanted with BM from chow-fed controls, profoundly altered leukocyte counts, and an increased susceptibility to atherosclerosis.

MATERIALS AND METHODS

Animals and BMT

LDLR KO mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and were bred at the Gorlaeus Laboratories in Leiden, The Netherlands. Male mice (age 12 weeks) were fed a high-fat, high-cholesterol WTD, containing 15% cacao butter and 0.25% cholesterol (WTD; Special Diets Services, Essex, UK) for 45 weeks or were kept on control chow diet (RM3; Special Diets Services) for the indicated periods. At 45 weeks, mice were killed, and BM was isolated for BMT to 12-week-old female LDLR KO recipient mice. In short, 3×10^6 BM cells were injected into the tail vein of lethally irradiated recipients [21]. During the whole experiment, the BM recipients were kept on a regular chow diet. At 16 weeks after transplantation, the mice were anesthetized using a mix of rompun, ketamine, and atropine at a lethal dose. Mice were then exsanguinated and perfused with PBS.

All animal work was approved by the regulatory authority of Leiden University and carried out in compliance with the Dutch government guidelines.

Histological analysis of the aortic root

Serial sections (7 μm) of the aortic root were cut using a Leica CM3050S cryostat. The atherosclerotic lesion areas in Oil Red O-stained cryostat sections of the aortic root were quantified using the Leica image analysis system, consisting of a Leica DMRE microscope coupled to a video camera and Leica QWin imaging software (Leica, Cambridge, UK). The mean lesion area (in μm^2) was calculated from 10 consecutive Oil Red O-stained sections, starting at the appearance of the tricuspid valves.

To visualize plaque composition, a Masson Trichrome kit was used (HT15-1,4; Sigma Aldrich, St. Louis, MO, USA), consisting of Biebrich scarlet-acid fuchsin, phosphotungstic acid, phosphomolybdic acid, and aniline blue. The tissue was stained according to the manufacturer's instructions (procedure HT15).

Furthermore, aortic root sections were stained for the presence of T cells using an anti-CD3 antibody [RM-9107; Thermo Scientific (Waltham, MA, USA); dilution 1:150] after antigen retrieval by incubating the sections for 20 min in a Tris-EDTA buffer, pH 6. Leica QWin software was used to quantify adventitia size in the stained sections. $CD3^+$ cells were quantified and expressed relative to adventitia size.

Serum cholesterol and glucose measurements

Serum concentrations of FC were determined by enzymatic colorimetric assays with 0.048 U/mL cholesterol oxidase (228250; Calbiochem, Gibbstown, NJ USA) and 0.065 U/mL peroxidase (P8375; Sigma Aldrich) in reaction buffer (1.0 KPi buffer, pH 7.7, containing 0.01 M phenol, 1 mM 4-amino-antipyrine, 1% polyoxyethylene-9-laurylether, and 7.5% methanol). For the determination of TC, 0.03 U/mL cholesteryl esterase (228180; Calbiochem) was added to the reaction solution. Absorbance was read at 490 nm.

Glucose concentration was measured in plasma samples using an Accu-Chek compact system (3021548; Roche, South San Francisco, CA, USA).

DNA isolation and McrBc digestion

To quantify DNA methylation, DNA was isolated by column filtration (D3024; Zymo Research, Irvine, CA, USA) and incubated with McrBc enzyme (M0272; New England Biolabs, Ipswich, MA, USA) overnight, as described previously [6]. The enzyme cleaves methylated CpG sites, preventing amplification across these sites by RT-PCR. We designed primers to CpG regions in our genes of interest (**Table 1**). By calculating the Δ comparative threshold of DNA incubated with the McrBc enzyme compared with a non-McrBc-digested control, the level of DNA methylation for the genes of interest was determined.

Flow cytometry analysis and Sysmex

EDTA anti-coagulated blood samples and single-cell suspensions of BM and spleen, obtained using a 70-μm cell strainer (734-0003; VWR, Rad-

TABLE 1. Sequences of Primers Used in This Study

Gene	Forward	Reverse
Notch-1	GGTTTTGGTTTCCTGTTGCTTCTTGGG	ATCGTCTCTGCCCCGCTCTAAGT
Csf2ra	GGGGACTGGAGAAAAGGAAGTGGCT	CCTGTGGCCTGACTGTGTGTAGGT
Tal.1	GGGCTCTTTCCTTTTTCCGGCCTTGG	TTGGCTGCTTTTATTCGTGCCTGGAGC
Pu.1	TCTTCAACAGCTCAGGCTCGACACCTT	GGACCAGGTACTCACCGCTATGGCTT
IRF8	GGTGCTTGCTCTGGCTGCTCTC	CGCGCGGTGATTGGCAGATCTATT

nor, PA, USA), were used for FACS analysis. Red blood cells in the splenocyte preparations and blood were lysed with ERY lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3), and the cells were analyzed on a FACSCanto II (BD Biosciences, Mountain View, CA, USA), using the relevant FACS antibodies (all obtained from eBioscience, San Diego, CA, USA).

The amount of LSK progenitor cells in the isolated BM cells was evaluated by flow cytometry as follows: BM cells were stained with FITC-labeled anti-CD3, anti-CD4, anti-CD8, anti-CD11b, anti-CD19, anti-GR-1, anti-NK1.1, and anti-TER119 (all obtained from eBioscience) to discriminate between Lin^+ and Lin^- cells. Cells lacking these markers are designated further as Lin^-. The cells in the Lin^- population that stain double-positive for PE-labeled anti-LY6A/E ($Sca\text{-}1^+$) and APC-labeled anti-CD117 ($cKit^+$; both obtained from eBioscience), are defined as the LSK population. Leukocyte, red blood cell, and platelet counts were analyzed using an automated Sysmex XT-2000iV veterinary hematology analyzer (Sysmex, Kobe, Japan).

Statistical analysis

Statistically significant differences among the means of the different populations were tested using the unpaired Student's *t* test (GraphPad Prism software, GraphPad, La Jolla, CA, USA). A Welch correction was applied to the *t* test in the case of unequal variances in the dataset. The probability level (α) for statistical significance was set at 0.05. Results are expressed as an average ± SEM.

Online supplemental material

Time-dependent, diet-induced changes in DNA methylation of ABCA1, Pu.1, and Notch-1 were measured after periods of WTD feeding, from 8 up to 45 weeks. Hereto, male LDLR KO mice were fed WTD for 8, 20, 30, or 45 weeks. At the end of the dietary period, the mice were killed, and BM was isolated for DNA extraction, followed by DNA methylation analysis using McrBc (Supplemental Fig. 1).

RESULTS

Enhanced atherosclerotic plaque development in LDLR KO recipients transplanted with WTD BM

WTD BM or Chow BM, for 45 weeks, was transplanted into LDLR KO recipient mice. After the transplantation, the mice were kept on a regular chow diet for 16 weeks. To exclude possible confounding effects caused by differences in BM engraftment after the BMT, we performed a FACS analysis on BM cells isolated from the recipients at 16 weeks after transplantation. Both total Lin^- cells and LSK cells were counted, and no differences between the groups were found (**Fig. 1A** and **B**).

No differences were found in FC or TC levels in serum (**Fig. 2A**). Similarly, no differences in the distribution of cholesterol over the different lipoprotein fractions were found (data not shown). There was a 40% increase in blood glucose concentration in the animals receiving WTD BM at 16 weeks but not at 8 weeks after transplantation ($P<0.05$; Fig. 2B). Throughout the study, the mice were weighed, and a small dip in body weight, associated with the irradiation preceding the BMT, was observed 2 weeks after BMT and quickly recovered in the 3rd week. There was no difference in body weight between the treatment groups (Fig. 2C).

At 16 weeks after transplantation, the mice were killed, and plaque development was quantified at the tricuspid area in the aortic root. Both groups of mice had developed small fatty-streak lesions composed of macrophage foam cells (**Fig. 3A**). Interestingly, LDLR KO mice, reconstituted with WTD BM, displayed a 40% ($P<0.05$) increase in mean atherosclerotic lesion size when compared with LDLR KO mice that had received Chow BM (Fig. 3A and B).

Furthermore, reduced numbers of $CD3^+$ T cells (0.5-fold; $P<0.05$) were found in the adventitia underlying atherosclerotic plaques in the WTD BM recipients when compared with the Chow BM recipient mice ($P<0.05$; Fig. 3A and C).

DNA methylation of key transcription factors for hematopoiesis is reduced in WTD BM-transplanted mice

DNA methylation in the promoter regions of the Pu.1, IRF8, and GM-CSFR genes, important to monocyte and macrophage hematopoiesis, was measured using Q-PCR on McrBc-digested DNA. Furthermore, methylation of Tal.1 and Notch-1 genes, important to T cell, MK, and ERY hematopoiesis, was measured in the same manner (**Fig. 4A**). Interestingly, Pu.1 was demethylated significantly in BM cells of the WTD BM recipi-

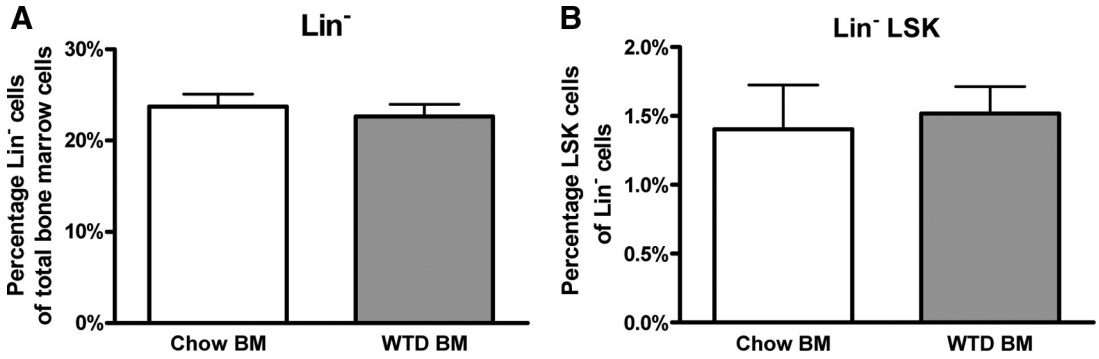

Figure 1. No difference in amounts of BM progenitor cells between LDLR KO recipients transplanted with WTD BM or Chow BM. (A) BM cells were stained for myriad differentiation markers (see Materials and Methods). The cells that were negative for any of these markers were designated Lin^-. Flow cytometry (FACS) analysis of BM samples did not reveal a difference in the amount of Lin^- BM cells between the groups. (B) The amount of LSK cells in the Lin^- population of BM cells was determined, but there was no detectable difference between the groups of BM recipients. Results are expressed as mean ± SEM. Significance was assessed by unpaired *t* test.

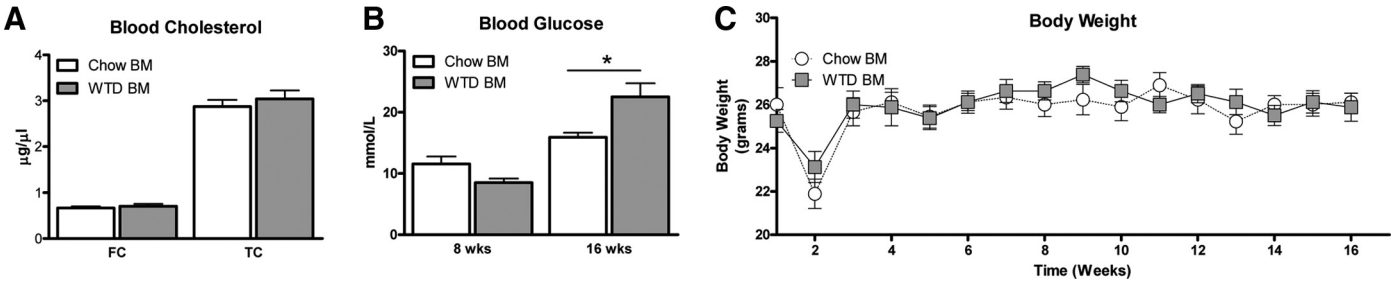

Figure 2. No difference in blood cholesterol content or body weight but increased blood glucose in mice receiving WTD BM. (A) Plasma samples of BM recipient mice isolated 16 weeks after transplantation were analyzed for FC and TC content. No difference between treatment groups was detected by unpaired *t* test, $n = 8$. (B) At 16 weeks after transplantation, blood glucose levels in the WTD BM recipients were increased compared with Chow BM control, $*P < 0.05$, $n = 5$. (C) The body weight of the BM recipients was measured weekly throughout the study. No difference between the groups was found (n=9). Results are expressed as mean ± SEM. Significance was assessed by unpaired *t* test.

ents, as was IRF8 (twofold, $P<0.05$; and fourfold, $P<0.05$, respectively; Fig. 4B and C). We did not observe any differences in DNA methylation of the genes for GM-CSFR, Tal.1, and Notch-1 (Fig. 4D–F).

Effect of transplantation of WTD BM on leukocyte counts in LDLR KO recipients but not on red blood cell and platelet counts

WTD BM recipient mice displayed a 40% increase in leukocytes in the blood compartment at 8 weeks and 16 weeks after transplantation ($P<0.001$, $P<0.05$; **Fig. 5A**). There was no difference in red blood cells or platelets at 8 weeks or 16 weeks after transplantation (Fig. 5B and C).

Next, FACS analysis was performed to analyze the effects on leukocyte subsets at 16 weeks after transplantation. Despite the observed increase in total blood leukocytes, we did not detect changes in the amounts of circulating $CD3^+$ cells (**Fig. 6A**). A trend to increased $NK1.1^+$ cells was observed, but this failed to reach statistical significance (Fig. 6B). There was also no difference in the number of circulating $CD19^+$ cells (Fig. 6C). Ultimately, the increase in total blood leukocyte counts was driven by strikingly augmented numbers of circulating $F4/80^+$ monocytes (3.4-fold, $P<0.01$; Fig. 6D) [22, 23]. There was also a significant increase in circulating $CD11c^{++}$ dendritic cells, but the contribution to the absolute increase in total leukocytes was minimal (2.4-fold, $P<0.05$; Fig. 6E) [24].

In line with the finding that the blood leukocyte counts of WTD BM recipients were elevated, spleen weight was increased in the WTD BM recipient mice ($P<0.01$; **Fig. 7A**). Moreover,

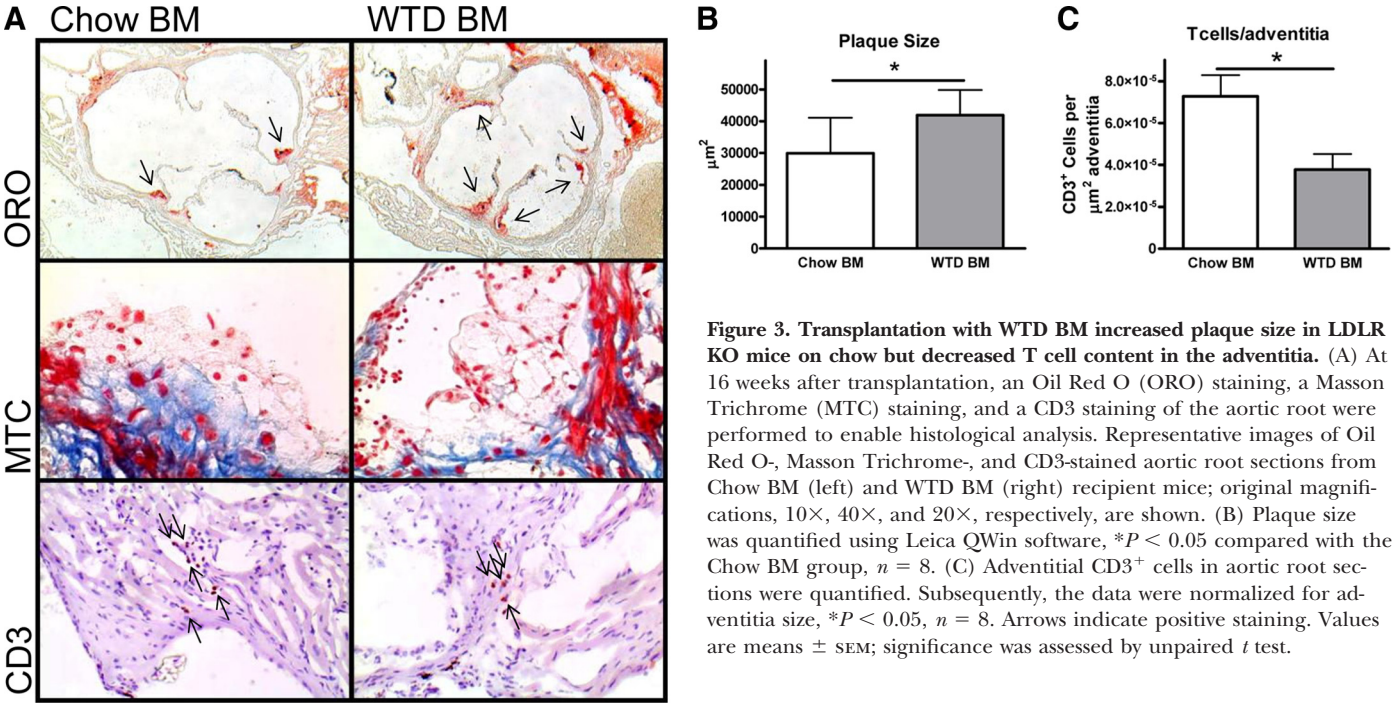

Figure 3. Transplantation with WTD BM increased plaque size in LDLR KO mice on chow but decreased T cell content in the adventitia. (A) At 16 weeks after transplantation, an Oil Red O (ORO) staining, a Masson Trichrome (MTC) staining, and a CD3 staining of the aortic root were performed to enable histological analysis. Representative images of Oil Red O-, Masson Trichrome-, and CD3-stained aortic root sections from Chow BM (left) and WTD BM (right) recipient mice; original magnifications, 10×, 40×, and 20×, respectively, are shown. (B) Plaque size was quantified using Leica QWin software, $*P < 0.05$ compared with the Chow BM group, $n = 8$. (C) Adventitial $CD3^+$ cells in aortic root sections were quantified. Subsequently, the data were normalized for adventitia size, $*P < 0.05$, $n = 8$. Arrows indicate positive staining. Values are means ± SEM; significance was assessed by unpaired *t* test.

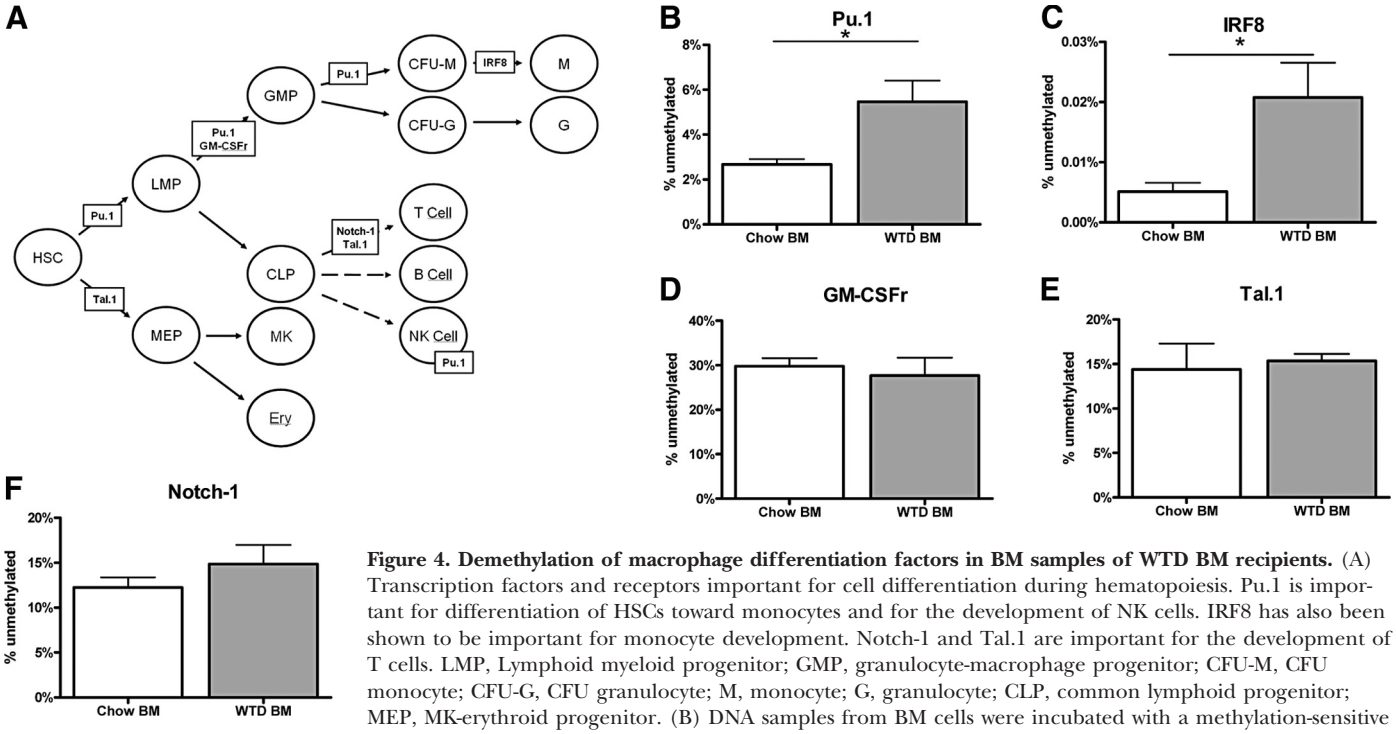

Figure 4. Demethylation of macrophage differentiation factors in BM samples of WTD BM recipients. (A) Transcription factors and receptors important for cell differentiation during hematopoiesis. Pu.1 is important for differentiation of HSCs toward monocytes and for the development of NK cells. IRF8 has also been shown to be important for monocyte development. Notch-1 and Tal.1 are important for the development of T cells. LMP, Lymphoid myeloid progenitor; GMP, granulocyte-macrophage progenitor; CFU-M, CFU monocyte; CFU-G, CFU granulocyte; M, monocyte; G, granulocyte; CLP, common lymphoid progenitor; MEP, MK-erythroid progenitor. (B) DNA samples from BM cells were incubated with a methylation-sensitive restriction enzyme, McrBc. Primers were designed for a CpG region in the start of the Pu.1 gene. Loss of PCR amplification as a result of enzymatic cleavage of the DNA was quantified using Q-PCR and comparing the signal with undigested, control DNA. Data are expressed as percentage of unmethylated DNA, $*P < 0.05$, $n = 7$. (C) DNA methylation of IRF8 was quantified as for Pu.1, $*P < 0.05$, $n = 7$. (D–F) Similarly, DNA methylation was quantified for GM-CSFR, Tal.1, and Notch-1; no significant differences were found. Results are expressed as mean ± SEM, $n = 7$. Significance was assessed by unpaired t test with Welch correction if required.

on average, 2.5-fold more splenocytes could be isolated from spleens of WTD BM recipients compared with spleens from the Chow BM group ($P<0.05$; Fig. 7B). No significant changes could be detected in the relative size of T cell, B cell, macrophage, and dendritic cell populations in the spleen by FACS (data not shown). However, we did observe differences in immune cell activation. The amounts of $CD14^+$ monocytes (1.5-fold, $P<0.05$; Fig. 7C) and $CD86^+$ $F4/80^+$ macrophages (1.8-fold, $P<0.01$; Fig. 7D) were increased. This indicates a strong increase in macrophage costimulatory potential and a decrease in T cell activation.

DISCUSSION

A growing body of evidence indicates an important role for DNA methylation alterations in determining atherosclerosis susceptibility via a causal link among blood lipoproteins, DNA

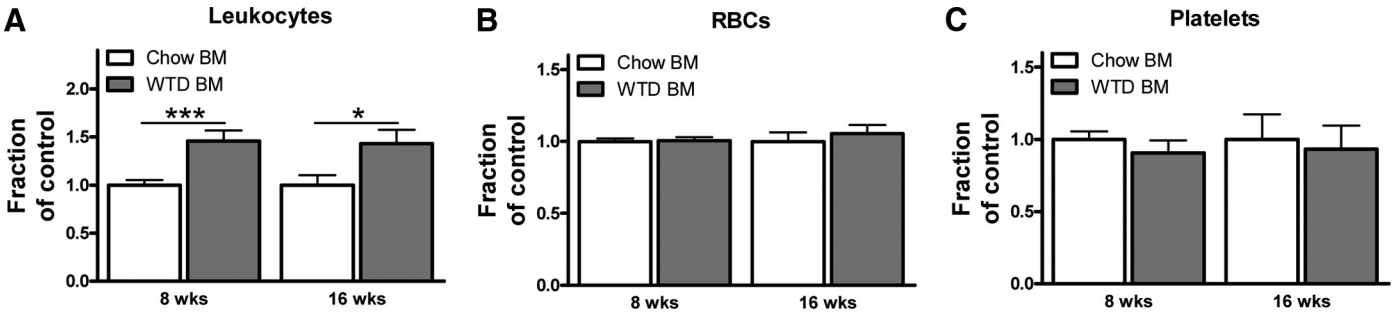

Figure 5. Increase in circulating leukocyte counts but no difference in red blood cell and platelet counts at 8 weeks and 16 weeks after BMT with WTD BM or Chow BM. (A) Leukocyte counts were analyzed by a hematology analyzer at 8 weeks and 16 weeks after BMT. At both time-points, an increase in circulating leukocytes in the WTD BM recipient mice was detected. $***P < 0.001$, $*P < 0.05$ at 8 weeks and 16 weeks, respectively. (B and C) Similarly, red blood cells (RBCs) and platelets were quantified using a hematology analyzer. No differences between the groups at either time-point could be found; results are expressed as mean ± SEM, $n = 10$. Significance was assessed by unpaired t test.

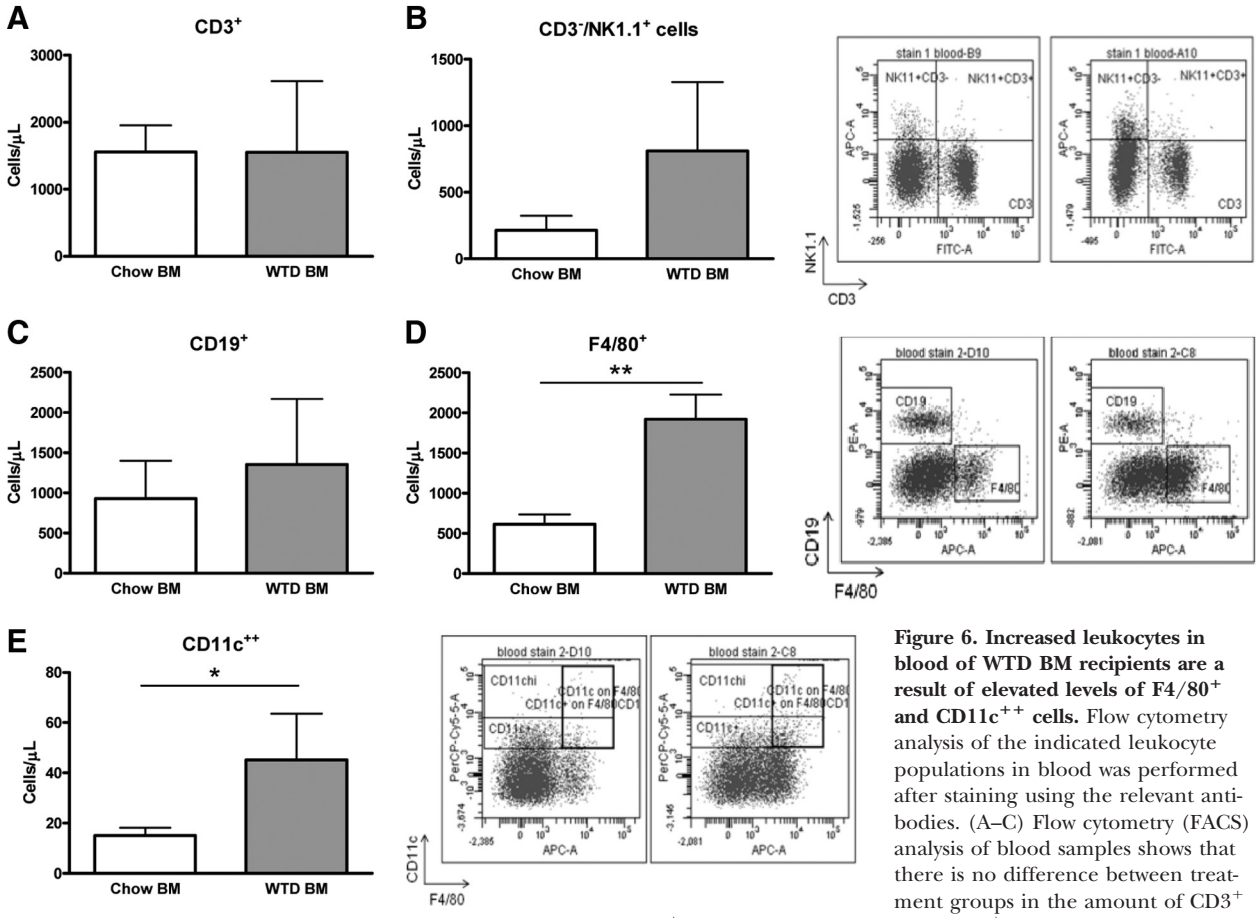

Figure 6. Increased leukocytes in blood of WTD BM recipients are a result of elevated levels of F4/80$^+$ and CD11c^{++} cells. Flow cytometry analysis of the indicated leukocyte populations in blood was performed after staining using the relevant antibodies. (A–C) Flow cytometry (FACS) analysis of blood samples shows that there is no difference between treatment groups in the amount of CD3$^+$ cells, cells stained positive for NK1.1 and negative for CD3, and CD19$^+$ cells. (D) The amount of F4/80$^+$ leukocytes in blood, when corrected for total blood leukocytes, is elevated significantly in the WTD BM recipients, $^{**}P < 0.01$. (E) The amount of leukocytes with high CD11c expression is increased in mice that received WTD BM, $^{*}P < 0.05$. Representative scatter plots are included to the right of the corresponding graphs (left panel: Chow BM; right panel: WTDBM). Results are expressed as mean ± SEM, $n = 5$. Significance is assessed by unpaired t test with Welch correction if required. (Right) APC-A, FITC-A, and PE-A, APC-area, FITC-area, and PE-area.

methylation, and leukocyte function. In this study, we investigated if long-term WTD feeding can lead to changes in BM, causing transplantable long-term changes in leukocytes and atherosclerosis susceptibility. Hereto, a BMT was performed with WTD BM or Chow BM. The results show that changes in the BM epigenome induced by WTD feeding promote a transplantable macrophage-driven leukocytosis phenotype and increased atherosclerosis. Exposure to a high-fat, high-cholesterol diet thus induces long-term changes in BM, leading to an increased susceptibility to atherosclerosis later in life.

The challenging of LDLR KO mice with WTD induces an increase in circulating levels of proatherogenic lipoproteins, such as very LDL and LDL, and a decrease in HDL [25]. The current study, for the first time, made it possible to discriminate between the acute, direct effects of these alterations in lipoprotein concentrations on circulating leukocytes and chronic effects on progenitor cells in the BM. Different populations of progenitor cells can be distinguished in whole BM samples, including ST-HSCs and LT-HSCs. ST-HSCs have very limited potential for self-renewal and are typically exhausted at 8–10 weeks after transplantation [26, 27]. To ensure that the effects of BMT observed in the current study are fully determined by the LT-HSC population, the effects of the transplantable changes of WTD feeding on BM were measured at 16 weeks after transplantation.

Analysis of DNA methylation of key transcription factors for hematopoiesis in the BM of the recipient mice at 16 weeks after transplantation showed a striking decrease in Pu.1 and IRF8 methylation, enabling increased transcription of these genes, whereas GM-CSFR, Tal.1, and Notch-1 were unaffected. Tal.1 is crucial for development of a wide range of HSCs, including lymphoid and erythroid Lin [28], GM-CSFR is implicated in the development of myeloid cells [29], and Notch-1 signaling is important for T cell development [30]. In agreement with the unaffected methylation status of Notch-1 and Tal.1, FACS analysis revealed no difference in absolute amounts of circulating T cells. Furthermore, red blood cells and platelets were unaltered. The observed decrease in Pu.1 and IRF8 methylation points toward involvement of myeloid and NK cells. The concentration of Pu.1 in the nucleus of progenitor cells determines myeloid cell fate, with higher concentrations of Pu.1 guiding cells to differentiate into macrophages, whereas low concentrations result in increased differentiation toward lymphocytes (Fig. 4A) [31, 32]. In line,

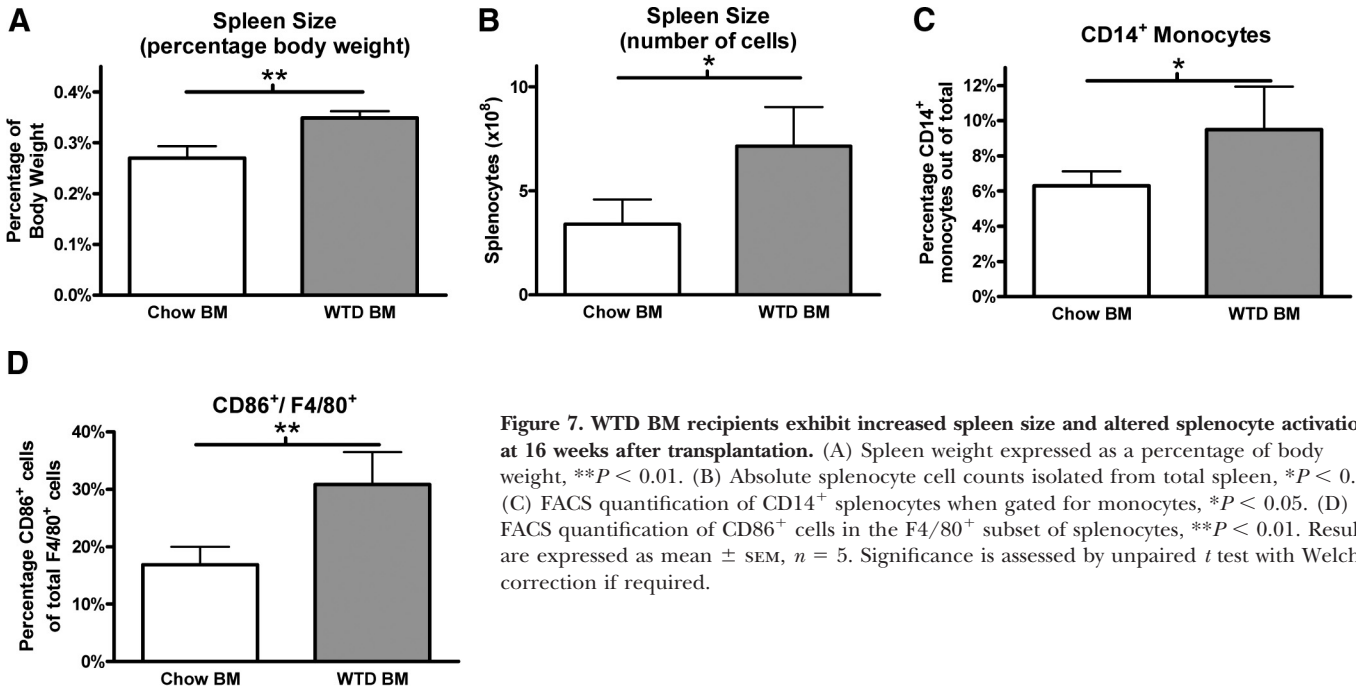

Figure 7. WTD BM recipients exhibit increased spleen size and altered splenocyte activation at 16 weeks after transplantation. (A) Spleen weight expressed as a percentage of body weight, $^{**}P < 0.01$. (B) Absolute splenocyte cell counts isolated from total spleen, $^{*}P < 0.05$. (C) FACS quantification of $CD14^+$ splenocytes when gated for monocytes, $^{*}P < 0.05$. (D) FACS quantification of $CD86^+$ cells in the $F4/80^+$ subset of splenocytes, $^{**}P < 0.01$. Results are expressed as mean ± SEM, $n = 5$. Significance is assessed by unpaired t test with Welch correction if required.

profoundly augmented amounts of $F4/80^+$ cells were found in the circulation of LDLR KO mice transplanted with WTD BM. Pu.1 is also important for NK cell function, with abrogated Pu.1 levels resulting in defective proliferative responses [33]. Furthermore, Colucci et al. [33] showed that Pu.1-deficient NK cells display reduced expression of c-kit and IL-7R, which are both important for NK cell generation. Decreased methylation of Pu.1, as observed in the current study in WTD BM recipients, is thus anticipated to lead to increased NK cell production. In agreement, there is a trend toward increased counts of NK cells in blood of LDLR KO mice transplanted with WTD BM. NK cells are capable of producing proinflammatory macrophage-activating factors, such as TNF-α and IFN-γ, and are thus anticipated to have a proatherogenic function [34]. The exact role of NK cells in atherosclerosis, however, is still under debate [35–37].

IRF8 performs a function similar to that of Pu.1 and has been shown to cobind with Pu.1 in gene promoter regions to induce expression of genes, such as Kruppel-like factor 4 and arginase 1 [38–40]. In the current study, augmented amounts of $F4/80^+$ cells were found in the circulation of LDLR KO mice transplanted with WTD BM. Doring et al. [29] showed that mice receiving IRF8 KO BM display increased atherosclerosis associated with higher counts of PMNs in the circulation and in the atherosclerotic plaque. Importantly, depletion of PMNs abrogated the proatherosclerotic effect, leading the authors to conclude that this cell type played an essential role in the increased plaque development in the absence of BM IRF8. In our study, we did not observe any differences in blood neutrophil populations between WTD BM- and Chow BM-transplanted mice. It is therefore unlikely that the effects of WTD feeding on BM IRF8 methylation influence atherosclerosis susceptibility via an effect on neutrophils.

Consecutive FACS analysis of spleen tissue revealed higher levels of CD14 expression by monocytes in the spleen, as well as induced expression of CD86 in the F4/80-positive splenocytes. CD14 is known to play an important role in M1 macrophage responses, and reduction of CD14 expression results in decreased production of proinflammatory factors, such as TNF-α, iNOS, and IFN-γ [41–43]. CD86 is a marker for macrophage activation [44, 45]. Increased expression of CD86 on F4/80 cells thus indicates an increased costimulatory potential of splenic macrophages and monocytes committed to macrophage differentiation in WTD BM recipients.

Although there was no difference in blood cholesterol or body weight between the treatment groups, the WTD BM recipient mice were found to be hyperglycemic. The WTD BM mice display an altered myeloid cell phenotype, with more $F4/80^+$ cells in the circulation and increased activation of monocytes and macrophages in the spleen. The activation status of resident macrophages in adipose tissue plays a crucial role in regulating glucose uptake by adipocytes [46, 47]. Therefore, it is possible that the observed hyperglycemia at 16 weeks after transplantation is a consequence of the WTD BM-induced changes in hematopoiesis and macrophage activation.

The increased numbers of circulating $F4/80^+$ cells, combined with augmented activation status of splenic monocytes, indicate a more proinflammatory immune cell profile in mice transplanted with WTD BM. We therefore hypothesize that the observed increase in atherosclerosis susceptibility in LDLR KO mice transplanted with WTD BM is mainly monocyte and macrophage driven. Atherosclerotic plaques in our study are relatively small. At this early stage of atherosclerotic plaque development, monocyte infiltration into the vessel wall is a determining factor, and in agreement, the plaques are mainly composed of foam cells. Additionally, we stained for CD3+

cells in the adventitia surrounding the plaques, as these can contribute to plaque formation [48]. The amount of adventitial CD3+ cells at the site of the atherosclerotic plaque was decreased, indicating that CD3+ cells probably did not contribute to the observed increase in plaque size.

To conclude, in this study, we show that long-term WTD feeding results in altered DNA methylation patterns in the BM. These changes in methylation can be detected even after transplantation of the BM to an atherosclerosis-prone mouse model on a chow-diet regime. We have described that mice receiving WTD BM display increased activation and circulating numbers of F4/80$^+$ cells in blood. As a possible mechanism, we present an increased production of Pu.1 and IRF8 based on the observed decrease in DNA methylation of these transcription factors. These findings shed new light on the causality between dietary factors DNA methylation and atherosclerosis. We show that although differences in diet result in altered DNA methylation patterns, these changes in methylation can contribute independently to atherosclerotic lesion development. We suggest that manipulation of the epigenome, when used in conjunction with blood lipid reduction, could prove a promising target strategy to reduce atherosclerosis.

AUTHORSHIP

E.v.K. contributed to execution of experiments, design of the experiments, and writing of the manuscript. A.J. contributed to the execution of experiments and experimental design. T.J.C.v.B. and M.V.E. contributed significantly to conception of the study, experimental design, and manuscript revision.

ACKNOWLEDGMENTS

This work was supported by The Netherlands Heart Foundation (grant 2009B075 to E.v.K.) and The Netherlands Organization for Scientific Research (Vici grant 91813603 to M.V.E.). M.V.E. is an Established Investigator of The Netherlands Heart Foundation (grant 2007T056).

DISCLOSURES

The authors declare no conflicts of interest.

REFERENCES

1. Napoli, C., Crudele, V., Soricelli, A., Al-Omran, M., Vitale, N., Infante, T., Mancini, F. P. (2012) Primary prevention of atherosclerosis: a clinical challenge for the reversal of epigenetic mechanisms? *Circulation* **125,** 2363–2373.
2. Lund, G., Zaina, S. (2009) Epigenetics, transgenerational effects and risk factors for atherosclerosis. *Curr. Opin. Lipidol.* **20,** 150–151.
3. Matouk, C. C., Marsden, P. A. (2008) Epigenetic regulation of vascular endothelial gene expression. *Circ. Res.* **102,** 873–887.
4. Sorensen, A. L., Jacobsen, B. M., Reiner, A. H., Andersen, I. S., Collas, P. (2010) Promoter DNA methylation patterns of differentiated cells are largely programmed at the progenitor stage. *Mol. Biol. Cell* **21,** 2066–2077.
5. Alkemade, F. E., van Vliet, P., Henneman, P., van Dijk, K. W., Hierck, B. P., van Munsteren, J. C., Scheerman, J. A., Goeman, J. J., Havekes, L. M., Gittenberger-de Groot, A. C., et al. (2010) Prenatal exposure to apoE deficiency and postnatal hypercholesterolemia are associated with altered cell-specific lysine methyltransferase and histone methylation patterns in the vasculature. *Am. J. Pathol.* **176,** 542–548.
6. Vanhees, K., Coort, S., Ruijters, E. J., Godschalk, R. W., van Schooten, F. J., Barjesteh van Waalwijk van Doorn-Khosrovani, S. (2011) Epigenetics: prenatal exposure to genistein leaves a permanent signature on the hematopoietic lineage. *FASEB J.* **25,** 797–807.
7. Jacob, R. A., Gretz, D. M., Taylor, P. C., James, S. J., Pogribny, I. P., Miller, B. J., Henning, S. M., Swendseid, M. E. (1998) Moderate folate depletion increases plasma homocysteine and decreases lymphocyte DNA methylation in postmenopausal women. *J. Nutr.* **128,** 1204–1212.
8. Pufulete, M., Al-Ghnaniem, R., Khushal, A., Appleby, P., Harris, N., Gout, S., Emery, P. W., Sanders, T. A. (2005) Effect of folic acid supplementation on genomic DNA methylation in patients with colorectal adenoma. *Gut* **54,** 648–653.
9. Davis, C. D., Uthus, E. O. (2004) DNA methylation, cancer susceptibility, and nutrient interactions. *Exp. Biol. Med. (Maywood)* **229,** 988–995.
10. Ford, D., Ions, L. J., Alatawi, F., Wakeling, L. A. (2011) The potential role of epigenetic responses to diet in ageing. *Proc. Nutr. Soc.* **70,** 374–384.
11. Chung, T. L., Brena, R. M., Kolle, G., Grimmond, S. M., Berman, B. P., Laird, P. W., Pera, M. F., Wolvetang, E. J. (2010) Vitamin C promotes widespread yet specific DNA demethylation of the epigenome in human embryonic stem cells. *Stem Cells* **28,** 1848–1855.
12. Lund, G., Andersson, L., Lauria, M., Lindholm, M., Fraga, M. F., Villar-Garea, A., Ballestar, E., Esteller, M., Zaina, S. (2004) DNA methylation polymorphisms precede any histological sign of atherosclerosis in mice lacking apolipoprotein E. *J. Biol. Chem.* **279,** 29147–29154.
13. Stenvinkel, P., Karimi, M., Johansson, S., Axelsson, J., Suliman, M., Lindholm, B., Heimburger, O., Barany, P., Alvestrand, A., Nordfors, L., et al. (2007) Impact of inflammation on epigenetic DNA methylation—a novel risk factor for cardiovascular disease? *J. Intern. Med.* **261,** 488–499.
14. Sharma, P., Kumar, J., Garg, G., Kumar, A., Patowary, A., Karthikeyan, G., Ramakrishnan, L., Brahmachari, V., Sengupta, S. (2008) Detection of altered global DNA methylation in coronary artery disease patients. *DNA Cell Biol.* **27,** 357–365.
15. Castillo-Diaz, S. A., Garay-Sevilla, M. E., Hernandez-Gonzalez, M. A., Solis-Martinez, M. O., Zaina, S. (2010) Extensive demethylation of normally hypermethylated CpG islands occurs in human atherosclerotic arteries. *Int. J. Mol. Med.* **26,** 691–700.
16. Post, W. S., Goldschmidt-Clermont, P. J., Wilhide, C. C., Heldman, A. W., Sussman, M. S., Ouyang, P., Milliken, E. E., Issa, J. P. (1999) Methylation of the estrogen receptor gene is associated with aging and atherosclerosis in the cardiovascular system. *Cardiovasc. Res.* **43,** 985–991.
17. Hiltunen, M. O., Turunen, M. P., Hakkinen, T. P., Rutanen, J., Hedman, M., Makinen, K., Turunen, A. M., Aalto-Setala, K., Yla-Herttuala, S. (2002) DNA hypomethylation and methyltransferase expression in atherosclerotic lesions. *Vasc. Med.* **7,** 5–11.
18. Rangel-Salazar, R., Wickstrom-Lindholm, M., Aguilar-Salinas, C. A., Alvarado-Caudillo, Y., Dossing, K. B., Esteller, M., Labourier, E., Lund, G., Nielsen, F. C., Rodriguez-Rios, D., et al. (2011) Human native lipoprotein-induced de novo DNA methylation is associated with repression of inflammatory genes in THP-1 macrophages. *BMC Genomics* **12,** 582.
19. Pearce, M. S., McConnell, J. C., Potter, C., Barrett, L. M., Parker, L., Mathers, J. C., Relton, C. L. (2012) Global LINE-1 DNA methylation is associated with blood glycaemic and lipid profiles. *Int. J. Epidemiol.* **41,** 210–217.
20. Guay, S. P., Brisson, D., Munger, J., Lamarche, B., Gaudet, D., Bouchard, L. (2012) ABCA1 gene promoter DNA methylation is associated with HDL particle profile and coronary artery disease in familial hypercholesterolemia. *Epigenetics* **7,** 464–472.
21. Van Eck, M., Bos, I. S., Hildebrand, R. B., Van Rij, B. T., Van Berkel, T. J. (2004) Dual role for scavenger receptor class B, type I on bone marrow-derived cells in atherosclerotic lesion development. *Am. J. Pathol.* **165,** 785–794.
22. Qu, C., Edwards, E. W., Tacke, F., Angeli, V., Llodra, J., Sanchez-Schmitz, G., Garin, A., Haque, N. S., Peters, W., van Rooijen, N., et al. (2004) Role of CCR8 and other chemokine pathways in the migration of monocyte-derived dendritic cells to lymph nodes. *J. Exp. Med.* **200,** 1231–1241.
23. Tacke, F., Alvarez, D., Kaplan, T. J., Jakubzick, C., Spanbroek, R., Llodra, J., Garin, A., Liu, J., Mack, M., van Rooijen, N., (2007) Monocyte subsets differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques. *J. Clin. Invest.* **117,** 185–194.
24. Vremec, D., Pooley, J., Hochrein, H., Wu, L., Shortman, K. (2000) CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. *J. Immunol.* **164,** 2978–2986.
25. Ishibashi, S., Brown, M. S., Goldstein, J. L., Gerard, R. D., Hammer, R. E., Herz, J. (1993) Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. *J. Clin. Invest.* **92,** 883–893.
26. Smith, L. G., Weissman, I. L., Heimfeld, S. (1991) Clonal analysis of hematopoietic stem-cell differentiation in vivo. *Proc. Natl. Acad. Sci. USA* **88,** 2788–2792.
27. Yang, L., Bryder, D., Adolfsson, J., Nygren, J., Mansson, R., Sigvardsson, M., Jacobsen, S. E. (2005) Identification of Lin(−)Sca1(+)kit(+)CD34(+)Flt3− short-term hematopoietic stem cells capable of rapidly reconstituting and rescuing myeloablated transplant recipients. *Blood* **105,** 2717–2723.

28. Porcher, C., Swat, W., Rockwell, K., Fujiwara, Y., Alt, F. W., Orkin, S. H. (1996) The T cell leukemia oncoprotein SCL/Tal-1 is essential for development of all hematopoietic lineages. *Cell* **86,** 47–57.
29. Doring, Y., Soehnlein, O., Drechsler, M., Shagdarsuren, E., Chaudhari, S. M., Meiler, S., Hartwig, H., Hristov, M., Koenen, R. R., Hieronymus, T., et al. (2012) Hematopoietic interferon regulatory factor 8-deficiency accelerates atherosclerosis in mice. *Arterioscler. Thromb. Vasc. Biol.* **32,** 1613–1623.
30. Radtke, F., Wilson, A., Stark, G., Bauer, M., van Meerwijk, J., MacDonald, H. R., Aguet, M. (1999) Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity* **10,** 547–558.
31. Dahl, R., Simon, M. C. (2003) The importance of PU. 1 concentration in hematopoietic lineage commitment and maturation. *Blood Cells Mol. Dis.* **31,** 229–233.
32. DeKoter, R. P., Singh, H. (2000) Regulation of B lymphocyte and macrophage development by graded expression of PU. 1. *Science* **288,** 1439–1441.
33. Colucci, F., Samson, S. I., DeKoter, R. P., Lantz, O., Singh, H., Di Santo, J. P. (2001) Differential requirement for the transcription factor PU. 1 in the generation of natural killer cells versus B and T cells. *Blood* **97,** 2625–2632.
34. Getz, G. S. (2002) Do natural killer cells participate in a killer vascular disease? *Arterioscl. Thromb. Vasc. Biol.* **22,** 1251–1253.
35. Linton, M. F., Major, A. S., Fazio, S. (2004) Proatherogenic role for NK cells revealed. *Arterioscl. Thromb. Vasc. Biol.* **24,** 992–994.
36. Schiller, N. K., Boisvert, W. A., Curtiss, L. K. (2002) Inflammation in atherosclerosis: lesion formation in LDL receptor-deficient mice with perforin and Lyst(beige) mutations. *Arterioscl. Thromb. Vasc. Biol.* **22,** 1341–1346.
37. Whitman, S. C., Rateri, D. L., Szilvassy, S. J., Yokoyama, W., Daugherty, A. (2004) Depletion of natural killer cell function decreases atherosclerosis in low-density lipoprotein receptor null mice. *Arterioscl. Thromb. Vasc. Biol.* **24,** 1049–1054.
38. Kurotaki, D., Osato, N., Nishiyama, A., Yamamoto, M., Ban, T., Sato, H., Nakabayashi, J., Umehara, M., Miyake, N., Matsumoto, N., et al. (2013) Essential role of the IRF8-KLF4 transcription factor cascade in murine monocyte differentiation. *Blood* **121,** 1839–1849.
39. Tsujimura, H., Nagamura-Inoue, T., Tamura, T., Ozato, K. (2002) IFN consensus sequence binding protein/IFN regulatory factor-8 guides bone marrow progenitor cells toward the macrophage lineage. *J. Immunol.* **169,** 1261–1269.
40. Pourcet, B., Feig, J. E., Vengrenyuk, Y., Hobbs, A. J., Kepka-Lenhart, D., Garabedian, M. J., Morris S. M., Jr., Fisher, E. A., Pineda-Torra, I. (2011) LXRα regulates macrophage arginase 1 through PU. 1 and interferon regulatory factor 8. *Circ. Res.* **109,** 492–501.
41. Knuefermann, P., Nemoto, S., Misra, A., Nozaki, N., Defreitas, G., Goyert, S. M., Carabello, B. A., Mann, D. L., Vallejo, J. G. (2002) CD14-deficient mice are protected against lipopolysaccharide-induced cardiac inflammation and left ventricular dysfunction. *Circulation* **106,** 2608–2615.
42. Merlin, T., Woelky-Bruggmann, R., Fearns, C., Freudenberg, M., Landmann, R. (2002) Expression and role of CD14 in mice sensitized to lipopolysaccharide by *Propionibacterium acnes*. *Eur. J. Immunol.* **32,** 761–772.
43. Zanoni, I., Ostuni, R., Marek, L. R., Barresi, S., Barbalat, R., Barton, G. M., Granucci, F., Kagan, J. C. (2011) CD14 controls the LPS-induced endocytosis of Toll-like receptor 4. *Cell* **147,** 868–880.
44. Mosser, D. M. (2003) The many faces of macrophage activation. *J. Leukoc. Biol.* **73,** 209–212.
45. Xia, W., Hilgenbrink, A. R., Matteson, E. L., Lockwood, M. B., Cheng, J. X., Low, P. S. (2009) A functional folate receptor is induced during macrophage activation and can be used to target drugs to activated macrophages. *Blood* **113,** 438–446.
46. Lumeng, C. N., Bodzin, J. L., Saltiel, A. R. (2007) Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J. Clin. Invest.* **117,** 175–184.
47. Lumeng, C. N., Deyoung, S. M., Saltiel, A. R. (2007) Macrophages block insulin action in adipocytes by altering expression of signaling and glucose transport proteins. *Am. J. Physiol. Endocrinol. Metab.* **292,** E166–E174.
48. Kyaw, T., Winship, A., Tay, C., Kanellakis, P., Hosseini, H., Cao, A., Li, P., Tipping, P., Bobik, A., Toh, B. H. (2013) Cytotoxic and proinflammatory CD8+ T lymphocytes promote development of vulnerable atherosclerotic plaques in apoE-deficient mice. *Circulation* **127,** 1028–1039.

KEY WORDS:
DNA methylation · Pu.1 · IRF8 · hematopoiesis · macrophage · CpG island