Oxidized Low-Density Lipoprotein Induces Long-Term Proinflammatory Cytokine Production and Foam Cell Formation via Epigenetic Reprogramming of Monocytes

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Objective—Although the role of monocytes in the pathogenesis of atherosclerosis is well established, the persistent vascular inflammation remains largely unexplained. Recently, our group reported that stimulation of monocytes with various microbial products can induce a long-lasting proinflammatory phenotype via epigenetic reprogramming, a process termed trained immunity. We now hypothesize that oxidized low-density lipoprotein (oxLDL) also induces a long-lasting proinflammatory phenotype in monocytes, which accelerates atherosclerosis by proinflammatory cytokine production and foam cell formation.

Approach and Results—Isolated human monocytes were exposed for 24 hours to medium or oxLDL. After washing and resting for 6 days, the cells were exposed to toll-like receptor 2 and 4 agonists. Pre-exposure to oxLDL increased mRNA expression and protein formation on toll-like receptor 2 and 4 stimulation of several proatherogenic proteins, including interleukin-6, interleukin-18, interleukin-8, tumor necrosis factor-α, monocyte chemoattractant protein 1, and matrix metalloproteinase 2 and 9. In addition, foam cell formation was enhanced after oxLDL exposure, which was associated with an upregulation of scavenger receptors CD36 and scavenger receptor-A and downregulation of ATP-binding cassette transporters, ABCA1 and ABCG1. Chromatin immunoprecipitation performed 6 days after oxLDL stimulation demonstrated increased trimethylation of lysine 4 at histone 3 in promoter regions of *tnfα*, *il-6*, *il-18*, *mcp-1*, *mmp2*, *mmp9*, *cd36*, and *sr-a.* Finally, pretreatment of the monocytes with the histone methyltransferase inhibitor methylthioadenosine completely prevented the oxLDL-induced long-lasting proinflammatory phenotype.

Conclusions—Brief exposure of monocytes to a low concentration of oxLDL induces a long-lasting proatherogenic macrophage phenotype via epigenetic histone modifications, characterized by increased proinflammatory cytokine production and foam cell formation. **(*Arterioscler Thromb Vasc Biol*. 2014;34:1731-1738.)**

Key Words: atherosclerosis ■ epigenomics ■ inflammation ■ monocytes ■ oxidized low-density lipoprotein

Atherosclerosis is a chronic inflammatory disorder of the arterial vessel wall, which is initiated by an accumulation of lipoproteins in the intimal layer of the vascular wall. Subsequent activation of the overlying endothelium leads to the recruitment of circulating monocytes.[1] Monocyte entry into the vessel wall is crucial for the onset of the disease because mice lacking monocyte chemokines or their receptors have less atherosclerosis.[2–4] Within the intimal layer, monocytes differentiate into macrophages that subsequently engulf oxidized low-density lipoprotein (oxLDL) particles via scavenger receptors, mainly CD36 and scavenger receptor-A (SR-A),[5] leading to the formation of foam cells.[6] In addition, lesional macrophages can be activated by various stimuli, leading to the release of proinflammatory cytokines and matrix-degrading proteins, thereby promoting disease progression and plaque destabilization.[7,8] This activation of macrophages occurs via membrane-bound and intracellular pattern recognition receptors, including toll-like receptor (TLR)-2 and TLR4. Interestingly, the rate of progression of atherosclerosis is critically dependent on the characteristics of the circulating monocyte pool. It has been established in mouse models of atherosclerosis that hypercholesterolemia and a myocardial infarction can accelerate atherogenesis by changing the number and characteristics of circulating monocytes.[9,10] Although the role of monocytes in atherosclerosis has now been well established, it still remains elusive why the strong inflammatory response in the arterial wall persists in time.

Recently, our group reported that monocytes can build up an immunologic memory after microbial stimulation via epigenetic reprogramming of histone modifications, and this

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Nonstandard Abbreviations and Acronyms	
acLDL	acetylated LDL
DC	dendritic cells
H3K4me3	histone 3 Lysine 4 trimethylation
LDL	low-density lipoprotein
MCP-1	monocyte chemoattractant protein 1
MMP	matrix metalloproteinase
oxLDL	oxidized LDL
SR-A	scavenger receptor-A
TLR	toll-like receptor
TNF	tumor necrosis factor

mechanism has been termed trained immunity.[11,12] In humans, monocytes isolated from healthy volunteers after Bacillus Calmette–Guérin vaccination present an increased production of proinflammatory cytokines ex vivo after re-exposure to various unrelated pathogens.[13] This enhanced proinflammatory state of the monocytes could be detected ≤1 year after first exposure.[14] In addition to Bacillus Calmette–Guérin, trained immunity can also be induced by *Candida albicans* and its cell wall component β-glucan.[15] It has been shown that trained immunity is at least partly mediated by an increased trimethylation of histone 3 at lysine 4 (H3K4me3), which is associated with an increased expression of target genes on restimulation of the cells, including various proinflammatory cytokines. The kinetics of the epigenetic process leading to this sustained functional reprogramming of monocytes and macrophages has recently been described, and the epigenetic units responsible for this process have been termed latent enhancers.[16]

A genome-wide analysis of the epigenetic changes induced by the exposure of monocytes to β-glucan revealed that the top 500 genes with a clear increase of H3K4Me3 include many genes that are involved in the process of atherosclerosis. Therefore, we recently hypothesized that trained innate immunity contributes to atherosclerosis.[12] We now investigate whether sustained epigenetic remodeling of monocytes can also be induced by nonmicrobial stimuli that are relevant to atherosclerosis, such as oxLDL. In addition, we characterize the phenotype of the long-term activated monocyte-derived macrophage with regard to production of cytokines and chemokines that orchestrate the process of atherosclerosis and with regard to foam cell formation in detail.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

β-Glucan Training Induces Enrichment of H3K4me3 on Genes Relevant to Atherosclerosis

From our previous work on monocyte training with β-glucan, we analyzed the top 500 H3K4Me3 enriched genes for their involvement in the process of atherosclerosis.[15] These genes involved not only various cytokines and chemokines that are relevant to atherogenesis but also scavenger receptors and other proteins involved in foam cell formation and proteins involved in atherosclerotic plaque destabilization (Table I in the online-only Data Supplement).

OxLDL but Not LDL Induces Training of Monocytes In Vitro

Using the established in vitro model of human primary monocyte training, we explored the effect of LDL and oxLDL on training (Figure 1A). Incubation of monocytes with LDL and oxLDL did not induce cytokine production (interleukin [IL]-6, tumor necrosis factor [TNF]-α, or IL-1β) in the first 24-hour stimulation period (Figure IB–ID in the online-only Data Supplement). Moreover, incubation with oxLDL in a concentration ≤10 μg/mL and incubation with LDL (≤100 μg/mL) was not cytotoxic, as demonstrated by an lactate dehydrogenase cytotoxicity assay (Figure IA in the online-only Data Supplement). However, incubation of cells with oxLDL on day 1 induced a significant dose-dependent increase in IL-6 and TNFα production by monocytes that were restimulated with TLR ligands on day 6. A significantly increased cytokine production of both TLR4 (lipopolysaccharide; Figure 1B and 1C) and TLR2 (Pam3Cys; Figure IIA and IIB in the online-only Data Supplement) was observed for stimulation. Interestingly, preincubation with native LDL in a concentration ≤100 μg/mL did not have any effect on the capacity of monocytes to produce IL-6 and TNFα on restimulation, suggesting that the modification of LDL is required to induce training. In an additional set of experiments, oxLDL was kept

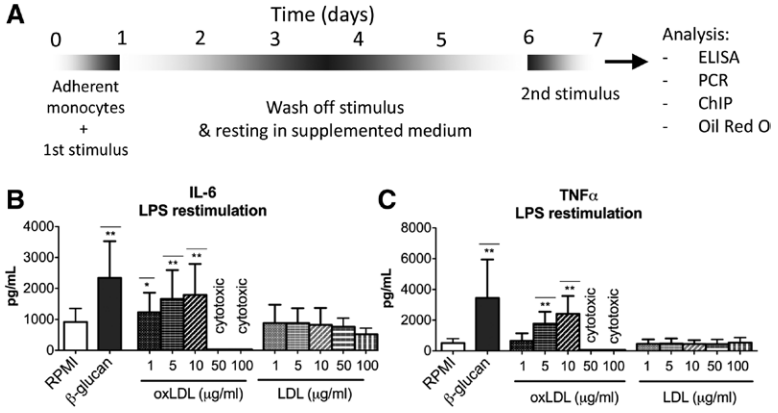

Figure 1. Priming with low concentrations of oxidized low-density lipoprotein (oxLDL) but not native LDL augments interleukin (IL)-6 and tumor necrosis factor (TNF)-α production on restimulation with lipopolysaccharide (LPS). **A**, Schematic representation of the in vitro experimental setup. **B**, IL-6 and (**C**) TNFα production were measured in the supernatants of adherent monocytes from healthy volunteers, exposed for 24 hours to medium alone, β-glucan as a positive control, or different concentrations of oxLDL or native LDL. After washing and 6 days of resting period, monocytes were restimulated with LPS (n=8; **P*<0.05, ***P*<0.01 compared with the RPMI control). ChIP indicates chromatin immunoprecipitation; and PCR, polymerase chain reaction.

in the medium for the entire 6-day resting period. Similar potentiating effects were obtained on the inflammatory profile of monocytes (Figure III in the online-only Data Supplement).

To assess whether training could also be induced by alternative modification of LDL, we repeated the experiments with acetylated LDL (acLDL), which also binds SR-A but not CD36. Indeed, restimulation of the cells on day 6 with either TLR4 or TLR2 agonists induced an increase in IL-6 and TNFα production, similar to oxLDL, albeit higher concentrations of acLDL were required (Figure IIC in the online-only Data Supplement).

Characteristics of oxLDL-Induced Trained Monocyte-Derived Macrophages

In our experimental design, after 24-hour exposure to oxLDL, the monocytes were kept in RPMI (Roswell Park Memorial Institute) culture medium supplemented with 10% serum for 6 additional days until restimulation on day 6. During this period, the monocytes differentiate into monocyte-derived macrophages. We have previously reported that training with β-glucan did not induce skewing of the macrophages into type M1 or M2 macrophages.[15] In addition, monocytes can also differentiate into dendritic cells (DCs), such as TNF and nitric oxide synthase–producing DCs.[17] It should be realized that this categorization into distinct macrophage phenotypes likely represents a simplified model because recently a spectrum of macrophage activation states was revealed on stimulation with various stimuli, extending the current M1 versus M2-polarization model.[18] To characterize the phenotype of the monocytes-derived macrophages on day 7 after stimulation with oxLDL better, we measured the expression of several markers of classically activated and alternatively activated macrophages and of inflammatory Tip-DCs on day 7 (Figure IV in the online-only Data Supplement). We observed no significant differences in expression of TLR4, TLR2, and nitric oxide synthase (M1 markers); arginase1 and CD163 (M2 markers); and HLA-DR (human leukocyte antigen-DR), DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin), and CD83 expression (DC markers).

Training of Monocytes Induces an Atherogenic Cytokine and Chemokine Response on Secondary Stimulation

A broad spectrum of cytokines and chemokines has been implicated to play a role in atherogenesis, including IL-6, IL-18, TNFα, monocyte chemoattractant protein 1 (MCP-1), and IL-8.[19] To study the effect of brief exposure of oxLDL on the production of these cytokines and chemokines, monocytes were trained for 24 hours by medium alone as a negative control, β-glucan as a positive control, or oxLDL. After washing on day 1 and the additional 6-day resting period, cells were restimulated by lipopolysaccharide and Pam3Cys for 4 or 24 hours to study the induction of mRNA production and cytokine or chemokine production, respectively. Both β-glucan and oxLDL training of monocytes augmented the production of IL-6, TNFα, IL-8, and MCP-1 (Figure 2). We subsequently assessed whether the potentiation of cytokine production is exerted at the gene transcription level. On

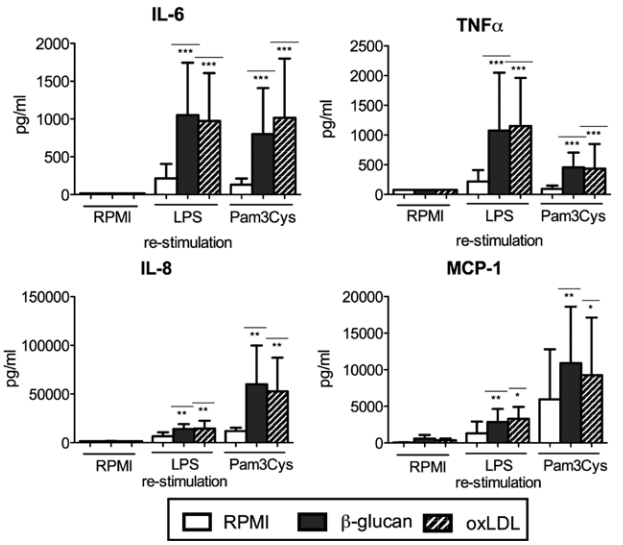

Figure 2. Primed monocytes have a more atherogenic cytokine and chemokine profile on restimulation than nonprimed monocytes. β-glucan and oxidized low-density lipoprotein (oxLDL) primed monocytes produce more interleukin (IL)-6, tumor necrosis factor (TNF)-α, IL-8, and monocyte chemoattractant protein 1 (MCP-1) on restimulation than untrained monocytes. Cytokine levels are measured in supernatants of cultured adherent monocytes from healthy volunteers, trained by β-glucan, oxLDL or medium alone for 24 hours and restimulated after 6 days with toll-like receptor (TLR) 2 or TLR4 agonists (n≥10 per group; **P*<0.05, ***P*<0.01, ****P*<0.005 compared with the RPMI [Roswell Park Memorial Institute] control). LPS indicates lipopolysaccharide.

restimulation of trained monocytes, *il-6*, *tnfα, il-8*, *mcp-1*, and also *il-18* mRNA expression showed a significant upregulation when compared with the untrained control (Figure 3). Interestingly, whereas the basal levels of *il-6, tnfα*, and *il-8* showed no difference on training, the baseline expression of *il-18* was significantly upregulated (Figure V in the online-only Data Supplement) and the baseline expression of *mcp-1* shows a similar effect, albeit not significantly, suggesting enhanced constitutive expression of these genes.

In addition to enhanced cytokine production, we also observed an enhanced baseline mRNA expression of matrix metalloproteinase 9 (*mmp9*) and increased expression of *mmp2* and *mmp9* on lipopolysaccharide restimulation, 2 of the most important matrix-degrading proteins, responsible for plaque destabilization (Figure 3). In contrast, *mmp8* was not upregulated at baseline or on restimulation (Figure 3; Figure V in the online-only Data Supplement). The upregulation of *mmp2* and *mmp9* and not *mmp8* mRNA is in accordance with our previous findings on H3K4Me3 enrichment in the promoter regions of *mmp2* and *mmp9* but not *mmp8* after training with β-glucan (Table I in the online-only Data Supplement).

Foam Cell Formation Is Enhanced in Trained Monocytes-Derived Macrophages

In the vessel wall, the uptake of oxLDL by macrophages leads to the generation of foam cells, thereby contributing to the formation of atherosclerotic plaques.[6] Therefore, we explored foam cell formation of trained monocyte-derived macrophages in our in vitro model. For this purpose, we trained adherent

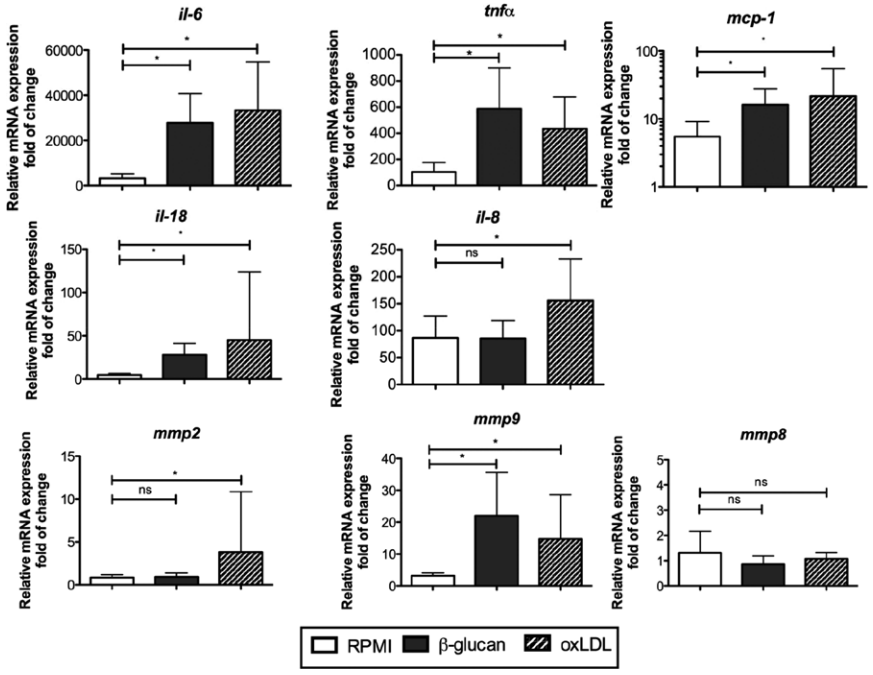

Figure 3. mRNA expression of pro-atherogenic cytokines is enhanced in trained monocytes. Adherent monocytes of healthy volunteers were exposed to β-glucan, oxidized low-density lipoprotein, or medium alone for 24 hours and restimulated after 6 days with lipopolysaccharide for 4 hours. Relative mRNA expression values are shown when compared with the untrained control. Expression of *il-6*, *tnfα, mcp-1, il-8*, *il-18, mmp2*, *mmp9* but not *mmp8* in trained monocytes is significantly higher on restimulation than in untrained monocytes (n=6 per group; **P*<0.05).

monocytes and subsequently induced foam cell formation on day 6 by incubation with a high dose of oxLDL. Oil Red O staining revealed an increased intracellular accumulation of lipid droplets in cells stimulated with oxLDL and β-glucan when compared with control cells (Figure 4A–4C). The first 24-hour priming of monocytes with a low dose of oxLDL did not induce foam cell formation (data not shown). To quantify the uptake of oxLDL by foam cells, intracellular apolipoprotein B levels were measured and compared with the medium control. Both β-glucan and oxLDL exposure significantly increased the intracellular apolipoprotein B concentration on day 6 (Figure 4D).

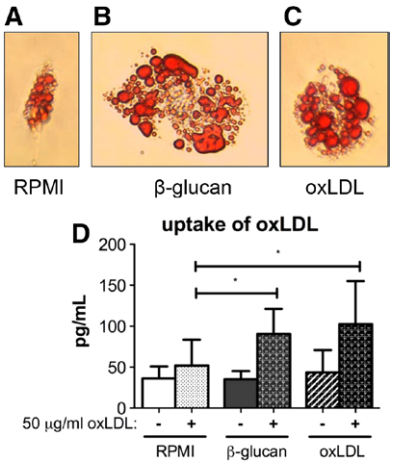

Figure 4. Foam cell formation is significantly enhanced in trained monocytes in vitro. Adherent monocytes of healthy volunteers were exposed to β-glucan, oxidized low-density lipoprotein (oxLDL), or medium alone for 24 hours and rested for 6 days in supplemented medium. Four hours before foam cell induction, the cells were starved from serum and were subsequently incubated with a high amount of oxLDL (50 μg/mL) for 24 hours. Intracellular apolipoprotein B levels were measured by ELISA (**D**) n=6; **P*<0.05, and foam cell morphology of trained monocytes was compared with untrained monocytes by staining with Oil Red O (**A**–**C**, single cell, ×40; [**A**] RPMI [Roswell Park Memorial Institute], [**B**] β-glucan, [**C**] oxLDL; representative pictures).

Trained Monocytes Have an Increased Expression of Scavenger Receptors and Reduced Expression of Cholesterol Efflux Transporters

OxLDL is taken up by macrophages via scavenger receptors, of which CD36 and SR-A are considered the most important.[5] Interestingly, the genes of these receptors showed enriched H3K4me3 in our previous study of training with β-glucan (Table I in the online-only Data Supplement). To study the expression of these scavenger receptors, we trained adherent monocytes with oxLDL and on day 6 the cells were processed for Western blot analysis. We found that both CD36 and SR-A were strongly upregulated in trained monocytes in comparison with untrained controls (Figure 5A). RNA expression analysis also showed upregulation of gene expression *cd*36 and *sr-a* (Figure 5B and 5C). Because foam cell formation represents the net effect of cholesterol influx and efflux, we also studied the expression of the cholesterol efflux transporters ATP binding cassette transporters, *abca1* and *abcg1*. For both transporters, there was a significant downregulation of mRNA expression (Figure 5D and 5E).

Enriched H3K4me3 on the Promoters of Proatherogenic Genes by oxLDL Exposure

Our previous reports showed that training of monocytes by β-glucan increased H3K4me3 at the level of the promoters of proinflammatory cytokine genes. Here, we investigated whether oxLDL training also induced H3K4 trimethylation on genes encoding for proinflammatory cytokines, chemokines, MMPs, or scavenger receptors. Therefore, we trained adherent monocytes as described above and performed chromatin immunoprecipitation on H3K4me3 on day 6, to assess

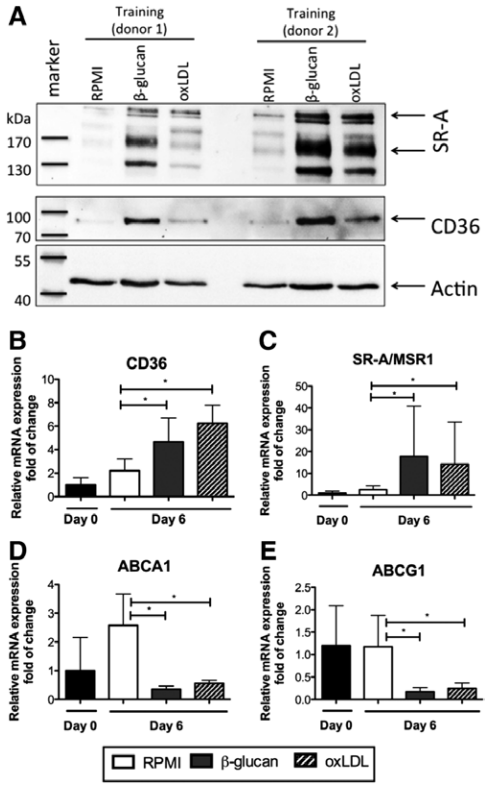

Figure 5. Expression of scavenger receptors CD36 and scavenger receptor-A (SR-A) is increased on trained monocytes. Adherent monocytes of healthy volunteers were exposed to medium, β-glucan, or oxidized low-density lipoprotein (oxLDL) for 24 hours. After 6 days, cells were lysed and equal protein concentrations were put on SDS-PAGE. Arrows indicate the proteins CD36 (≈86 kDa), SR-A (≈220–250 kDa, depending on dimerization/trimerization), and actin as control. Two different healthy volunteers are shown; pictures are representative of ≥6 donors. Protein expression for both receptors was enhanced for β-glucan training and oxLDL training when compared with the untrained control (**A**). Subsequently, mRNA expression of CD36 (**B**) and SR-A (**C**) was measured in cells on day 0 and day 6 and shows increased mRNA expression of the scavenger receptors. Cholesterol efflux transporters ABCA1 (**D**) and ABCG1 (**E**) show downregulated mRNA expression on training (n=6; **P*<0.05).

sustained epigenetic changes. The percentage input H3K4me3 on the promoters of *tnfα*, *il-6*, *mcp-1*, *il-18*, *mmp2*, *mmp8*, *mmp9*, *cd36*, and *sr-a* was measured and compared with the untrained control. We confirmed enrichment of H3K4me3 on the promoters of *tnfα*, *il-6*, *mcp-1*, *il-18*, *cd36*, *sr-a*, *mmp2*, and *mmp9* but not on the promoter of *mmp8*, which correlates with the mRNA expression data (Figure 6).

OxLDL Training of Monocytes Is Dependent on Methyltransferases

Finally, to show that training by oxLDL is indeed dependent on histone methylation, we used the nonselective pharmacological blocker of histone methyltransferases methylthioadenosine. We preincubated adherent monocytes with either culture medium or 1 mmol/L of methylthioadenosine, 1 hour before adding culture medium, β-glucan, or oxLDL. After a 24-hour incubation period, washing, and 6-day resting, the cells were restimulated on day 6 with lipopolysaccharide and Pam3Cys. As a control, cells were also preincubated with the nonselective demethylase inhibitor Pargyline (3 μmol/L). IL-6 and TNFα concentrations were measured in the supernatant and compared with the untrained control (Figure 7A and 7B; Figure VIA and VIB in the online-only Data Supplement). Indeed, pretreatment with methylthioadenosine completely abolished the training induced by oxLDL, whereas pargyline did not affect the training. Similarly, the augmented foam cell formation on day 6 after training with oxLDL was completely abolished by pretreatment with methylthioadenosine (Figure 7C).

Training of oxLDL Is Induced via a TLR Pathway

oxLDL can activate innate immune cells via TLR4, TLR2, TLR6, and CD36 heterodimerization.[20] To assess the mechanisms of oxLDL training, we preincubated the cells with TLR4 and TLR2 blockers before exposure to oxLDL on day 1. Pretreatment of the cells with both TLR4 and TLR2 blockers showed a partial reduction of the training effect (Figure 8A and 8B). Next, we examined which intracellular pathways are responsible for the training effect. Therefore, we pretreated the cells with pharmacological inhibitors of several intracellular pathway proteins, which are known to be involved in the TLR pathways, including extracellular regulated kinase, phosphoinositide 3 kinase, spleen tyrosine kinase, and c-Jun N-terminal kinase. Pretreatment of the cells with inhibitors of extracellular regulated kinase and phosphoinositide 3 kinase significantly reduced the cytokine production on day 6, whereas inhibition of spleen tyrosine kinase and c-Jun N-terminal kinase showed no effect. (Figure 8D–8G). Finally, we excluded that CD36-mediated endocytosis of oxLDL is involved, by demonstrating that pretreatment of the cells with cytochalasin B, an inhibitor of actin polymerization, did not inhibit the training (Figure 8C).

Discussion

In this study, we demonstrate that macrophages derived from monocytes that have been exposed to modified forms of LDL, including oxLDL, show a long-term proinflammatory phenotype because of epigenetic reprogramming of histones. These findings provide a novel mechanism that can contribute to the persistent vessel wall inflammation that is characteristic of atherosclerosis and can provide novel pharmacological targets to treat patients with atherosclerosis.

It is generally accepted that mononuclear phagocytes play a central role in the various stages of atherosclerosis.[8] The prevailing paradigm has long dictated that monocytes and macrophages do not have any immunologic memory, in contrast to cells of the adaptive immune system. This assumption was recently challenged by the observation that 24-hour exposure of human monocytes to *C albicans*, β-glucan, or Bacillus Calmette–Guérin induced long-term activated macrophages by epigenetic reprogramming of their transcriptional programs.[13–15] Analysis of markers for M1 and M2 macrophages suggested global pan-activation of these cells rather than simple skewing into M1 or M2 subtypes.[15] Because an analysis of the top 500 regions of enriched H3K4me3 after stimulation with these microbial products revealed many genes that are of importance in the initiation and progression of atherosclerosis (Table I in the online-only Data Supplement), we recently

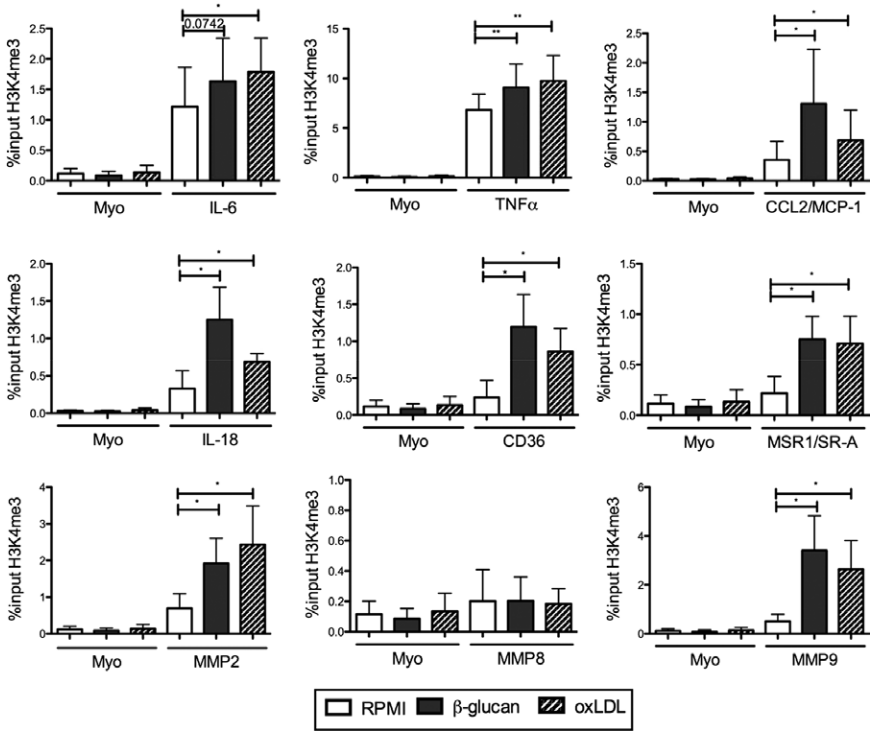

Figure 6. Training induces enriched histone 3 lysine 4 trimethylation (H3K4me3) on the promotors of proatherogenic genes. Chromatin immunoprecipitation of H3K4me3 was performed on chromatin from adherent monocytes from healthy volunteers, which were trained as described previously. Quantification of H3K4me3 was performed by quantitative polymerase chain reaction analysis of the promotors of *tnf*-α, *il-6*, *il-18*, *mcp*-1, *mmp-2*, *mmp8*, *mmp9*, *cd36*, and *sr-a*. Results are shown as percentage input, and myoglobulin was used as a negative control. Significant upregulation of H3K4me3 on the promotors of TNFα, IL-6, IL-18, MCP-1, MMP2, MMP9, CD36, and SR-A but not MMP8 was shown for β-glucan training as well as oxidized low-density lipoprotein (oxLDL) training (n=6; **P*<0.05, ***P*<0.01 compared with the untrained RPMI [Roswell Park Memorial Institute] control). MSR indicates macrophage scavenger receptor.

argued that this mechanism of trained innate immunity could explain the persistent vessel wall activation in atherosclerosis.[12]

OxLDL is crucial for the development of atherosclerosis by inducing cytokine and chemokine production in macrophages by stimulation of membrane-bound CD36 and TLR2, TLR4, and TLR6.[20,21] In addition, in isolated endothelial cells, oxLDL promotes IL-8 and MCP-1 secretion by increased acetylation of histone H3 and H4.[22] On the basis of these findings, we have now demonstrated that 24-hour exposure of isolated human monocytes to a low concentration of oxLDL induces the formation of macrophages with a long-lasting proinflammatory phenotype. These cells have several characteristics that can contribute to the formation of atherosclerotic plaques. By increased production of IL-6, TNFα, IL-8, IL-18, and MCP-1, these cells can promote and maintain inflammation in the vessel wall. Moreover, by upregulation of scavenger receptors, CD36 and SR-A, and reduced expression of cholesterol export transporters, ABCA1 and ABCG1, foam cell formation is augmented in these cells. Finally, increased production of MMP2 and MMP9 can contribute to destabilization of the atherosclerotic plaque. This long-lasting proatherogenic phenotype induced by oxLDL resulted from enrichment of H3K4Me3 in the promoter regions of the various cytokines, chemokines, and transporters and was prevented by pharmacological inhibition of histone methyltransferases.

mRNA analysis of oxLDL-stimulated monocyte-derived macrophages revealed no significant changes in expression of M1, M2, or DC markers. These findings argue against simple skewing of these cells into proinflammatory classically activated macrophages or proinflammatory DCs, as previously also described for stimulation of monocytes with β-glucan.[15] Pathway analyses with pharmacological inhibitors revealed that the effects of oxLDL are dependent on stimulation of TLR2 and TLR4. Experiments with the inhibitor of actin polymerization cytochalasin B demonstrated that CD36-mediated endocytosis of oxLDL is not required for the training of monocytes to occur. In addition, the observation that acLDL, which also binds SR-A but not CD36, suggests that CD36 is not needed to induce this biological effect.[23] Further characterization of the signaling pathways revealed that oxLDL-induced training is critically dependent on the activation of the MAP kinases extracellular regulated kinase 1/2 and

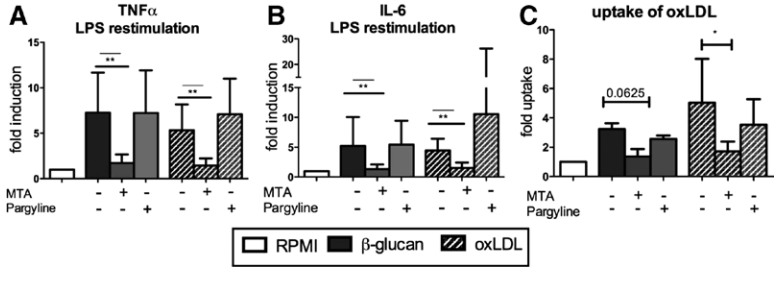

Figure 7. Methyltransferase inhibition prevents training by oxidized low-density lipoprotein (oxLDL). Tumor necrosis factor (TNF)-α (**A**) and interleukin (IL)-6 (**B**) levels were measured in supernatants of adherent monocytes primed for 24 hours with β-glucan as a positive control, oxLDL, or medium alone in the absence or presence of the histone methyltransferase inhibitor methylthioadenosine (MTA) and the histone demethylase inhibitor Pargyline. At day 6, cells were restimulated with lipopolysaccharide (LPS). Next, foam cell formation was measured after pretreatment of the cells with histone methyltransferase inhibitor MTA (**C**). Results are shown as fold induction when compared with the untrained control group (n=8 per group; **P*<0.05, ***P*<0.01).

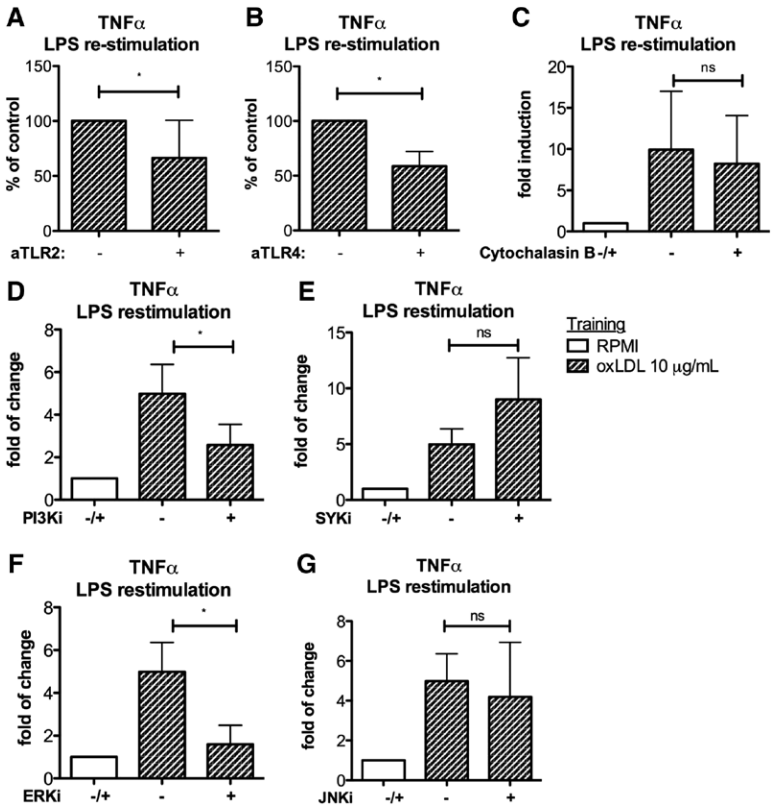

Figure 8. Pathway analysis of oxidized low-density lipoprotein (oxLDL) training. At day 6, cells were restimulated with lipopolysaccharide (LPS). Results are shown as percentage change in training or fold of change and when compared with the untrained control group (n=5 per group; *P<0.05). ERK indicates extracellular regulated kinase; JNK, c-Jun N-terminal kinase; PI3K, phosphoinositide 3 kinase; and SYK, spleen tyrosine kinase.

phosphoinositide 3 kinase but not spleen tyrosine kinase and c-Jun N-terminal kinase.

Our findings have 3 important implications. First, our results increase our understanding of atherogenesis by revealing a novel mechanism that can contribute to the persistent inflammation of the vessel wall that is characteristic of atherosclerosis. Second, this mechanism might offer a novel explanation for the association between infections and atherosclerosis.[24] Interestingly, administration of Bacillus Calmette–Guérin, which has been shown to induce training of monocytes,[13] to rabbits fed a cholesterol-enriched diet augments the formation of atherosclerosis.[25] On the basis of these findings, it is tempting to speculate that exposure to microorganism, such as *C pneumonia*, induces histone modifications in circulating monocytes, which subsequently home to the arterial endothelial lining and promote atherogenesis. In endothelial cells, exposure to *C pneumoniae* induces inflammatory gene expression via acetylation of histone H4 and phosphorylation and acetylation of histone H3.[26] Finally, our results provide novel pharmacological targets to prevent or slow down the process of atherosclerosis: histone modifications are not static but amenable to (pharmacological) modulation by interfering with histone methyltransferases and histone demethylases. Particularly in the field of hematology and oncology, nonspecific methyltransferase inhibitors and histone deacetylase inhibitors are already used in clinical trials.[27] As a proof-of-concept Choi et al[28] showed that pharmacological modulation of histone acetylation can indeed modulate the development of atherosclerosis. Treatment of LDL-R$^{-/-}$ mice with the histone deacetylase inhibitor trichostatin A increased atherosclerotic lesion size and increased macrophage accumulation in aortic sinus.

A few limitations of our study need to be discussed. First, to prevent H3K4Me3 in our experiments, we used methylthioadenosine, which is a nonselective histone methyltransferase inhibitor. More specific inhibitors of H3K4Me3 are currently not available. To exploit our findings to develop novel pharmacological strategies in the treatment of atherosclerosis, the development of these more specific inhibitors is critical. Second, we only studied H3K4Me3, which is only one of the epigenetic marks associated with an open transcriptionally active chromatin. Additional experiments are required to understand the epigenetic pathways involved in oxLDL-induced training of monocytes fully. Finally, our study only involves in vitro stimulation of isolated human monocytes. It needs to be emphasized that we used LDL that was oxidized in vitro using copper sulfate, which probably does not represent fully the in vivo modified LDL in the vessel wall. In addition, acLDL does not occur in vivo and we cannot exclude additional oxidation of acLDL during the 24-hour incubation period. Therefore, to translate our findings to clinical practice, it is critical to perform future studies to investigate whether the epigenetic marks can be identified in patients with risk factors for atherosclerosis or patients with established atherosclerosis.

In conclusion, we provide the first in vitro evidence that oxLDL exposure of human monocytes induces epigenetic histone modifications that result in a long-lasting proinflammatory phenotype with increased production of proinflammatory cytokines and chemokines, augmented foam cell formation, and increased production of MMPs. Additional studies both in animal models and in humans are necessary to provide definitive evidence that this mechanism affects the development of atherosclerosis and is amenable to pharmacological modulation.

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Disclosures

Dr Riksen served on a Scientific Advisory Board of AstraZeneca, which is unrelated to the current article. The other authors report no conflicts.

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Significance

This study provides a novel concept of how oxidized low-density lipoprotein can induce long-lasting proinflammatory and proatherogenic phenotypic changes in monocytes via epigenetic histone modifications. These findings do not only increase our understanding of the process of atherosclerosis but also provide potential novel pharmacological targets to prevent or slow atherogenesis.