Innate immune cell activation and epigenetic remodeling in symptomatic and asymptomatic atherosclerosis in humans in vivo

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Abstract

Background and aims: We have recently reported that monocytes can undergo functional and transcriptional reprogramming towards a long-term pro-inflammatory phenotype after brief in vitro exposure to atherogenic stimuli such as oxidized LDL. This process is termed ‘trained immunity’, and is mediated by epigenetic remodeling and a metabolic switch towards increased aerobic glycolysis. We hypothesize that trained immunity contributes to atherogenesis. Therefore, we investigated the inflammatory phenotype and epigenetic remodeling of monocytes from patients with and without established atherosclerosis.

Methods: Monocytes were isolated from 20 patients with severe symptomatic coronary atherosclerosis (total plaque score > 4 on coronary computed tomography angiography) and 17 patients with asymptomatic carotid atherosclerosis and matched controls for both groups. Ex vivo stimulation, RNA analysis and chromatin immunoprecipitation were performed.

Results: Monocytes from patients with symptomatic atherosclerosis have a higher production of pro-inflammatory cytokines upon LPS stimulation than healthy controls (TNFα 499 ± 102 vs. 267 ± 45 pg/ml, p = 0.01). This was associated with lower histone 3 lysine 4 trimethylation (H3K4me3) (19% vs. 33%, p = 0.002), and lower H3K27me3 (0.005% vs. 0.08%, p < 0.0001) on the TNFα promoter. Furthermore, relative mRNA expression of the glycolytic rate limiting enzymes hexokinase 2 and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 was higher in patients (0.7 ± 0.2 vs. 0.3 ± 0.1 resp. 1.7 ± 0.2 vs. 1.0 ± 0.1, p = 0.007 resp. 0.003) compared to control individuals. Interestingly, this pro-inflammatory phenotype was only present in patients with symptomatic atherosclerosis, and not in patients with asymptomatic carotid atherosclerosis.

Conclusions: Circulating monocytes of patients with symptomatic, but not asymptomatic, atherosclerosis have a pro-inflammatory phenotype and increased expression of glycolytic enzymes, associated with epigenetic remodeling at the level of histone methylation.

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1. Introduction

Cardiovascular diseases (CVD) are the leading cause of death worldwide, projected to cause 30 million deaths per year in 2030 [1]. The main underlying pathological process of CVD is atherosclerosis, a chronic low-grade inflammatory disorder of the arterial wall. Macrophages are the most abundant cells found in plaques and play central roles in the various stages of atherosclerosis [2]. Plaque macrophages are both derived from circulating monocytes that bind to activated endothelial cells and enter the intimal layer as well as from local proliferating plaque macrophages [3]. The mechanism that explains the persistent non-resolving inflammation within atherosclerotic plaques remains to be elucidated.

We recently proposed that trained immunity contributes to the
persistent vascular inflammation that characterizes atherosclerotic plaques [4]. Trained immunity denotes the process through which innate immune cells can adopt a long-term pro-inflammatory phenotype after brief exposure to certain pathogen- and danger-associated molecular patterns, such as oxidized low-density lipoprotein (oxLDL) particles [5]. Brief in vitro exposure of human monocytes to oxLDL induces a long-lasting pro-inflammatory macrophage phenotype that is characterized by increased pro-atherogenic cytokine and chemokine production and increased foam cell formation. The key mechanisms underlying this phenotype are epigenetic reprogramming at the level of histone methylation and a shift in the intracellular metabolism from oxidative phosphorylation towards an increased aerobic glycolysis (i.e. the Warburg effect) [6].

To test the hypothesis that trained immunity contributes to the development of atherosclerosis, we now performed a first proof-of-concept study in patients with established atherosclerosis. We aimed to investigate whether circulating monocytes of patients with atherosclerosis have a pro-inflammatory phenotype, and whether this is associated with epigenetic marks and change in metabolism that are characteristic of trained immune cells. To explore whether the role of trained immunity differs in the various stages of atherosclerosis, we studied monocytes from patients with symptomatic coronary atherosclerosis and subjects with asymptomatic carotid atherosclerosis.

2. Materials and methods

2.1. Study participants

Patients with symptomatic coronary atherosclerosis were selected from patients who had been referred to the cardiac emergency ward of the Canisius Wilhelmina Hospital in Nijmegen, from October 2013 to March 2014, because of chest pain, and who were evaluated with coronary computed tomography angiography (CCTA). We selected twenty patients with a total plaque score (TPS) > 4 (patients) and twenty control patients with a TPS = 0 (controls). Patients and controls were matched for age, gender and body mass index (BMI). Exclusion criteria were an acute coronary syndrome (at present or in the past), presence of a coronary stent, bypass surgery in the past, heart failure, liver or renal failure, as well as the use of anti-inflammatory medication other than statins, and thrombocyte aggregation inhibitors. Additional exclusion criteria were diabetes, rheumatoid arthritis, or other autoimmune diseases and signs/symptoms of acute infection.

Patients with asymptomatic carotid atherosclerosis were selected using carotid ultrasound from the participants of the population-based IN CONTROL study, from October 2013 to July 2015. A total of 39 asymptomatic subjects were included. Exclusion criteria for this study were a recent cardiovascular event, history of bariatric surgery or bowel resection, inflammatory bowel disease, renal dysfunction, increased bleeding tendency, use of oral subcutaneous anti-coagulant therapy, and use of thrombocyte aggregation inhibitors other than acetylsalicylic acid.

All laboratory analyses were performed blinded for group allocation. The study protocol was approved by the Institutional Review Board Arnhem/Nijmegen, The Netherlands, and prospectively registered at Clinicaltrials.gov (number NCT02393768).

2.2. Evaluation of atherosclerotic burden

Atherosclerotic burden was assessed as described in the Supplementary materials.

2.3. Blood sampling, isolation and stimulation of monocytes

EDTA plasma was obtained by venous blood sampling and was stored using standardized protocols. Plasma total cholesterol, HDL-c and LDL-c levels were analyzed using commercially available enzymatic methods. PAXgene tubes (Preanalytix, GmbH, Switzerland) were incubated for at least 2 h at room temperature before storage at −20 °C until RNA isolation.

Human peripheral blood mononuclear cells (PBMCs) were isolated from EDTA blood using Ficoll–Paque density gradient centrifugation (GE Healthcare, UK). Monocytes were isolated by magnetic activated cell sorting (MACS) according to manufacturer’s instructions (MACS, Miltenyi Biotec, Leiden, The Netherlands). Monocytes were diluted to 1 × 10^6/mL in RPMI 1640 Dutch-modified culture medium (Life Technologies). Invitrogen, Breda, The Netherlands) supplemented with 10 mM glutamine (Invitrogen), 10 μg/mL gentamicin (Centrafora), 10 mM pyruvate (Invitrogen). A total of 5 × 10^5 monocytes were seeded per well on flat-bottom 96-well plates (Corning, New York, USA) and stimulated for 24 h with RPMI only as a negative control, 10 ng/mL LPS (Sigma-Aldrich, St. Louis, MO; from E. coli serotype 055:B5), further purified as described [7] or 10 μg/mL Pam3Cys (EMC microcollections, Tübingen, Germany; L2000). After 24 h, plates were centrifuged and supernatants were stored at −20 °C until cytokine assessment.

2.4. Cytokine measurements

Cytokine production was determined in supernatants as described in Supplementary materials.

2.5. RNA isolation from PAXgenes

Total RNA purification was performed as described in Supplementary materials.

2.6. Chromatin immunoprecipitation

For the assessment of histone methylation, chromatin immunoprecipitation (ChIP) was performed as described previously [8]. In short, monocytes were cross-linked in methanol free 1% formaldehyde (28908, Thermo Scientific), followed by sonication to shear-DNA and immunoprecipitation using antibodies against H3K4me3, H3K27me3 and H3K9me3 (Diagenode, Seraing, Belgium). ChIPed DNA was processed further for qPCR analysis. Primers used in the reaction are listed in Supplementary Table 2. Samples were analyzed following a comparative Ct method, myoglobin was used as a negative control for H3K4me3, GAPDH for H3K9me3 and ELF4A2 for H3K27me3, H2B was used as a positive control for H3K4me3, ZNF UTR for H3K9me3 and MYT1 for H3K27me3 according to the manufacturer’s instructions.

2.7. Statistics

Data are presented as mean (standard deviation) for continuous variables and as number (percentage) for categorical variables. To examine the difference in clinical characteristics between the different patient groups, we performed unpaired student t-tests or Mann-Whitney U tests for normal and non-normal distributed variables, respectively. Ex vivo monocyte experiments were analyzed using the Mann-Whitney U test. In vitro experiments were analyzed using the Wilcoxon signed rank test. A two-sided p-value below 0.05 was considered statistically significant. All data were analyzed using GraphPad Prism 6.
analyzed using Prism version 5.0 (GraphPad software, La Jolla, California) or SPSS version 21.0 (SPSS Inc., Chicago, Illinois).

3. Results

3.1. Baseline characteristics

Baseline characteristics of patients with symptomatic and subjects with asymptomatic atherosclerosis, and controls for both groups without atherosclerosis are listed in Table 1. The average total plaque score (TPS) derived from the Coronary Computed Tomography Angiography (CCTA) was 6.3 in patients (A), indicating severe coronary atherosclerosis, and 0 in controls (B), indicating no coronary atherosclerosis (Table 1). Patients with symptomatic coronary atherosclerosis (group A) and controls (group B) were matched for age, gender and body mass index (BMI). There were no significant differences between group A and B in blood pressure, total cholesterol, lipoprotein(a), LDL cholesterol, glucose or plasma creatinine. High density lipoprotein (HDL) cholesterol was slightly lower in patients with coronary artery disease (CAD) (1.45 mmol/L vs. 1.26 mmol/L, p = 0.04). Statin use was higher in patients than controls (75% vs. 15%, p < 0.001).

Similarly, there were no significant differences between subjects with (C) and without (D) asymptomatic carotid plaques, besides the presence of plaques measured by carotid ultrasound.

3.2. Increased systemic inflammation in patients with symptomatic atherosclerosis

We measured baseline circulating high-sensitive C-reactive protein (hsCRP), cytokines and chemokines in patients with symptomatic CAD and controls. Plasma samples of patients with symptomatic CAD did not show significantly higher concentrations of IL-6 and TNFα (Fig. 1A), although this may be due to the lack of power. There was no difference in MCP-1 concentration. hsCRP was significantly higher in patients compared to controls (0.01). Similarly, there were no significant differences between subjects with (C) and without (D) asymptomatic carotid plaques, besides the presence of plaques measured by carotid ultrasound.

3.3. Increased cytokine and chemokine production by monocytes from subjects with symptomatic, but not asymptomatic atherosclerosis

After overnight stimulation with the Toll-like receptor (TLR) 4 ligand lipopolysaccharide (LPS), monocytes isolated from patients with symptomatic CAD showed an increased production of interleukin-6 (IL-6), tumor necrosis factor-α (TNFα), IL-1β, IL-8 and monocyte chemotactracting protein-1 (MCP-1; Fig. 2A) compared to controls. Similar results were observed upon stimulation with the TLR2 ligand Pam3Cys (Supplementary Fig. 1). No differences were observed in production of the anti-inflammatory cytokine IL-10, since levels were below detection level (data not shown).

In contrast, monocytes from subjects with asymptomatic carotid atherosclerosis did not show increased production of IL-6, TNFα, IL-1β, IL-8 and MCP-1, upon TLR4 stimulation, compared to control subjects without carotid atherosclerosis (Fig. 2B). Similar results were obtained after stimulation of leukocytes by the TLR2 ligand Pam3Cys (Supplementary Fig. 2).

3.4. Circulating cells of patients with symptomatic CAD have a pro-inflammatory phenotype associated with metabolic reprogramming

3.4.1. Increased pro-inflammatory cytokine expression

To further characterize the pro-inflammatory phenotype in patients with symptomatic CAD, we measured baseline RNA expression of pro-inflammatory cytokines and other inflammatory genes. Baseline expression of TNFα, IL-1β and IL-1RA was significantly higher in patients with symptomatic CAD compared to controls (Fig. 3A and Supplementary Fig. 3B), expression of MCP-1, IL-8, IL-6 as well as expression of HIF-1α, c-MYC and STAT3 showed no difference.

3.4.2. Increased expression of glycolytic enzymes

We have recently reported that the persistent pro-inflammatory phenotype of monocytes after induction of trained immunity by β-glucan is critically dependent on epigenetic reprogramming and a metabolic shift towards increased aerobic glycolysis [6]. We measured mRNA expression of hexokinase 2 (HK2) and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3),

Table 1

Baseline characteristics of the participants.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>(A) Symptomatic coronary atherosclerosis (n = 20)</th>
<th>p value</th>
<th>(B) Controls (n = 20)</th>
<th>p value</th>
<th>(C) Asymptomatic carotid atherosclerosis (n = 17)</th>
<th>p value</th>
<th>(D) Controls (n = 22)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>52 ± 11</td>
<td>0.78</td>
<td>53 ± 12</td>
<td>0.07</td>
<td>67 ± 6</td>
<td>0.03</td>
<td>66 ± 4</td>
<td>0.43</td>
</tr>
<tr>
<td>Gender, % male (n)</td>
<td>75 (15)</td>
<td>0.19</td>
<td>50 (10)</td>
<td>0.08</td>
<td>29 (5)</td>
<td>0.45</td>
<td>45 (10)</td>
<td>0.32</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.6 ± 4.1</td>
<td>0.08</td>
<td>26.5 ± 3.5</td>
<td>0.08</td>
<td>29.6 ± 2.3</td>
<td>0.24</td>
<td>31.1 ± 4.7</td>
<td>0.95</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>145 ± 21</td>
<td>0.63</td>
<td>142 ± 17</td>
<td>0.54</td>
<td>130 ± 10</td>
<td>0.95</td>
<td>130 ± 14</td>
<td>0.95</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>80 ± 14</td>
<td>0.54</td>
<td>83 ± 11</td>
<td>0.72</td>
<td>79 ± 6</td>
<td>0.92</td>
<td>82 ± 9</td>
<td>0.22</td>
</tr>
<tr>
<td>Family history, % yes (n)</td>
<td>60 (12)</td>
<td>0.21</td>
<td>35 (7)</td>
<td>0.21</td>
<td>47 (8)</td>
<td>0.24</td>
<td>37 (8)</td>
<td>0.24</td>
</tr>
<tr>
<td>Smoking, % active (n)</td>
<td>20 (4)</td>
<td>0.00</td>
<td>20 (4)</td>
<td>0.00</td>
<td>6 (1)</td>
<td>0.72</td>
<td>9 (2)</td>
<td>0.72</td>
</tr>
<tr>
<td>Smoking (pack years)</td>
<td>18 ± 12</td>
<td>0.47</td>
<td>18 ± 12</td>
<td>0.47</td>
<td>22 ± 0</td>
<td>1.0</td>
<td>22 ± 0</td>
<td>1.0</td>
</tr>
<tr>
<td>Statin use, % yes (n)</td>
<td>75 (15)</td>
<td>0.00</td>
<td>75 (15)</td>
<td>0.00</td>
<td>0 (0)</td>
<td>0.00</td>
<td>0 (0)</td>
<td>0.00</td>
</tr>
<tr>
<td>TChol, mmol/L</td>
<td>5.1 ± 1.2</td>
<td>0.92</td>
<td>4.9 ± 1.7</td>
<td>0.92</td>
<td>6.2 ± 0.9</td>
<td>0.55</td>
<td>6.0 ± 1.07</td>
<td>0.55</td>
</tr>
<tr>
<td>LDLc, mmol/L</td>
<td>3.0 ± 1.0</td>
<td>0.93</td>
<td>2.9 ± 1.2</td>
<td>0.93</td>
<td>4.12 ± 0.8</td>
<td>0.47</td>
<td>4.09 ± 0.9</td>
<td>0.47</td>
</tr>
<tr>
<td>HDLc, mmol/L</td>
<td>1.3 ± 0.3</td>
<td>0.04</td>
<td>1.5 ± 0.6</td>
<td>0.04</td>
<td>1.4 ± 0.3</td>
<td>0.70</td>
<td>1.4 ± 0.2</td>
<td>0.70</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>6.3 ± 1.8</td>
<td>0.37</td>
<td>5.8 ± 1.6</td>
<td>0.37</td>
<td>5.4 ± 0.8</td>
<td>0.71</td>
<td>5.3 ± 0.7</td>
<td>0.71</td>
</tr>
<tr>
<td>Creatinine, mmol/L</td>
<td>86.7 ± 13.8</td>
<td>0.19</td>
<td>80.3 ± 16.5</td>
<td>0.19</td>
<td>82.2 ± 12.5</td>
<td>0.67</td>
<td>80.1 ± 17.2</td>
<td>0.67</td>
</tr>
<tr>
<td>% Monocytes</td>
<td>12.3 ± 3.5</td>
<td>0.34</td>
<td>13.5 ± 4.3</td>
<td>0.34</td>
<td>8.6 ± 1.3</td>
<td>0.65</td>
<td>8.9 ± 2.1</td>
<td>0.65</td>
</tr>
<tr>
<td>Total Plaque Score (TPS)</td>
<td>6.3 [4–12]</td>
<td>&lt;0.0001</td>
<td>0 &lt;0.0001</td>
<td>&lt;0.0001</td>
<td>100 (17)</td>
<td>&lt;0.0001</td>
<td>0 (22)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Plaque on carotid ultrasound % (n)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20 (22)</td>
<td></td>
<td>0 (22)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD or n (%). BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TChol, total cholesterol; LDLc, low density lipoprotein cholesterol; HDLc, high density lipoprotein cholesterol; Lp(a), lipoprotein (a).
the two rate-limiting enzymes in the glycolysis pathway, as well as the expression of other important metabolic enzymes in patients with symptomatic CAD and control patients. Patients with symptomatic CAD showed a higher baseline expression of HK2, PFKFB3, pyruvate kinase 1 (PKM1), argininosuccinate lyase (ASL), malate dehydrogenase 1 (MDH1), pyruvate dehydrogenase alpha 1 (PDHA1) and transaldolase 1 (TALDO1) (Fig. 3A and Supplementary Fig. 3B). This is consistent with an upregulation of genes of the glycolytic pathway, but also enzymes in the pentose-phosphate pathway and Krebs cycle (Fig. 3B). Expression of HK2 and IL-1b (R = 0.41, p = 0.01), PFKFB3 and TNFa (R = 0.45, p = 0.006) and PFKFB3 and IL-1b (R = 0.78, p < 0.001) are significantly correlated, corroborating an association between upregulated glycolysis and an increased baseline inflammatory state (Supplementary Fig. 3A).

### 3.5. Increased cytokine production is associated with lower repressive epigenetic marks in patients with symptomatic CAD

Trained immunity is mediated by epigenetic reprogramming at the level of histone methylation [9]. Previously, we showed that increased cytokine production after brief exposure to oxLDL or β-glucan was associated with an enrichment of the activating histone mark H3K4me3, and training was prevented by nonselective inhibitors of methyltransferases [5,10]. Since patients with symptomatic CAD have elevated pro-inflammatory cytokine production on baseline, as well as increased pro-inflammatory cytokine production upon ex vivo stimulation, we hypothesized that this is due to epigenetic reprogramming of the monocyte population. We assessed H3K4me3 levels on the promoters of IL-6, IL-1β and TNFa, since these were significantly upregulated upon ex vivo stimulation. Surprisingly, and in contrast to what we observed in our in vitro experiments with β-glucan and oxLDL-induced training [5], the promoters of these pro-inflammatory genes had lower H3K4me3 in patients with symptomatic CAD than controls (Fig. 4A). Since TNFa showed the most significant increase in both plasma levels, baseline RNA expression as well as ex vivo production in patients vs. controls, we investigated this cytokine in more detail. Using six primers covering most part of the promoter (Supplementary Fig. 4A), we confirmed consistent downregulation of H3K4me3 throughout the promoter (Fig. 4B).

The overall activation state of the chromatin, however, is dictated not only by the activating mark H3K4me3, but also by alternative activating marks and repressive marks. Two important repressive histone marks are trimethylation of H3K9 and trimethylation of H3K27 [11]. H3K27me3, in particular, has been associated with control of gene expression upon ex vivo LPS stimulation [12,13]. Using our in vitro model of oxLDL training, we observed long-term lower repressive marks on the promoter regions of pro-inflammatory genes. Both H3K9me3 as well as H3K27me3 in vitro are lower in trained than untrained monocytes for IL-6 and TNFa (Supplementary Fig. 4B). Following these observations, we were interested in the repressive marks in our patients. For H3K9me3, no significant differences were observed on the TNFa promoter region (Fig. 4C). With regard to H3K27me3, the TNFa promoter showed a lower occupancy throughout the whole promoter (p < 0.0001, Fig. 4D). Interestingly, levels of H3K27me3 were correlated with levels of H3K4me3 on TNFa promoter (R = 0.37, p = 0.03, Fig. 4E) and negatively correlated with the expression of TNFa itself (R = –0.41, p = 0.02, Fig. 4E). Expression of EZH2, the only methyltransferase known to induce H3K27me3 was not different between patients and controls (Fig. 4D). H3K9me3 and H3K27me3 showed no differences for IL-6 and IL-1β (Supplementary Fig. 4C).

### 4. Discussion

The results of this study add additional evidence in favor of the hypothesis that trained innate immunity contributes to the process of atherosclerosis [4]. We demonstrated that circulating monocytes of patients with established atherosclerosis have a pro-inflammatory phenotype, associated with epigenetic remodeling at the level of histone methylation and increased expression of rate limiting enzymes of the glycolysis pathway and the pentose phosphate pathway. Interestingly, this pro-inflammatory phenotype was present only in patients with severe symptomatic coronary atherosclerosis, and not in patients with mild asymptomatic carotid atherosclerosis.
The underlying molecular basis of trained immunity is only partially deciphered and comprises changes in chromatin organization and intracellular metabolism. In human monocytes trained with β-glucan, a shift from oxidative phosphorylation towards aerobic glycolysis through the AKT-mTOR-HIF-1α pathway was observed, accompanied by changes in histone marks such as H3K4me1, H3K4me3 and H3K27Ac. Both pathways are non-redundant, since pharmacological inhibitors of glycolysis, such as 2-deoxy-D-glucose and inhibitors of the mTOR pathway, such as metformin, as well as non-specific inhibitors of histone methyltransferases, prevented β-glucan-induced trained immunity [6,10].

Here we show that in patients with symptomatic CAD, monocytes display an increased pro-inflammatory cytokine response upon ex vivo stimulation of TLR4. Monocytes from subjects with carotid atherosclerosis and controls were stimulated with TLR4 ligand LPS for 24 h and IL-6, TNFα, IL-1β, IL-8 and MCP-1 were measured in the supernatant after 24 h of incubation at 37 °C, 5% CO2.

The pro-inflammatory phenotype was associated with changes in the epigenetic landscape in the monocytes. First, we studied the level of H3K4me3, a long-term activating histone mark. In contrast to our in vitro observations, however, H3K4me3 occupancy on TNFα promoter was lower in patients with CAD. The accessibility of the chromatin is determined by activating epigenetic marks as well as repressive epigenetic marks. The repressive mark H3K27me3 importantly contributes to the LPS-induced pro-inflammatory cytokine production [12]. Previously, Wierda et al. used immunohistochemistry to show a

Fig. 2. Ex vivo stimulation of monocytes. (A) Monocytes from patients with symptomatic CAD have a pro-inflammatory phenotype upon ex vivo stimulation of TLR4 compared to matched controls. Monocytes from patients and controls were stimulated with TLR4 ligand LPS for 24 h and IL-6, TNFα, IL-1β, IL-8 and MCP-1 were measured in the supernatant after 24 h of incubation at 37 °C, 5% CO2 (n = 20 vs. 20, *p < 0.05, **p < 0.01). (B) Monocytes from patients with asymptomatic carotid atherosclerosis do not show an increased pro-inflammatory phenotype upon ex vivo stimulation of TLR4. Monocytes from subjects with carotid atherosclerosis and controls were stimulated with TLR4 ligand LPS for 24 h and IL-6, TNFα, IL-1β, IL-8 and MCP-1 were measured in the supernatant after 24 h of incubation at 37 °C, 5% CO2.

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reduction of global levels of H3K27me3 modification in vessels with advanced plaques, which was not accompanied by alterations in global levels of the corresponding histone methyltransferase EZH2 [17], corresponding to our current findings. Greissel et al. showed a significant reduction of repressive marks H3K9me2 and H3K27me2 with increasing plaque severity, in plaque macrophages [18]. Although measured in plaque macrophages and not in circulating cells, these studies indicate that repressive marks are strongly associated with the severity of atherosclerotic disease. In our study, we also determined H3K9me3 and H3K27me3 on the TNFα promoter. Although H3K9me3 was not significantly different between patients with and without atherosclerosis, H3K27me3 was much lower and correlated with cytokine RNA expression levels. Recently, Schmidt et al. studied the transcriptional regulator network of human inflammatory macrophages by histone marks. They showed that when promoter regions only have H3K4me3 marks, they are in an open chromatin state. When promoter regions are additionally trimethylated on H3K27me3, the promoter region is ‘poised’ and transcription is prevented [19]. Furthermore, Kruidenier et al. showed that H3K27 demethylation is a critical determinant for LPS induced TNFα production, and higher baseline levels of H3K27me3 can result in lower TNFα production [12]. Baseline RNA expression analysis showed increased expression of the rate-limiting enzymes of the glycolytic pathway as well as
other important rate-limiting metabolic enzymes in patients with severe atherosclerosis. Previously, we have shown that trained immunity depends on a metabolic shift, resulting in increased glycolysis [6,10]. An increase in gene expression of the two rate-limiting glycolytic genes is compatible with this metabolic shift that characterizes trained immunity [10]. The upregulation of other rate-limiting metabolic enzymes involved in the pentose phosphate pathway and Krebs cycle indicates a more general activation of intracellular metabolism, as shown in Fig. 4B. Recently, Tawakol et al. showed that in plaque macrophages there is a relationship between PFKFB3 expression and pro-inflammatory activation, via hypoxia-induced upregulation of macrophage glycolysis. The authors showed a linear relationship between macrophage energetics on the one hand and inflammatory activation on the other hand, regulated via PFKFB3 expression [20]. Furthermore, Shirai et al. showed that circulating monocytes and monocyte-derived macrophages from patients with established atherosclerosis have increased inflammation and glycolysis [21]. This study, however, differs markedly from our study, since these patients already suffered from an acute myocardial infarction, which in itself is a well-known trigger of innate immune activation [22]. In our study, all subjects with coronary atherosclerosis were free from previous cardiovascular events. Here we show, for the first time, that baseline gene expression of glycolysis and intracellular metabolism...
genes in circulating cells is also increased in event-free patients with established atherosclerosis, which is also associated with changes in the epigenetic landscape.

We propose that the pro-inflammatory phenotype of circulating monocytes in patients with severe coronary atherosclerosis reflects a trained immune phenotype, but we can only speculate about the stimulus that has instigated this immunological program. We have previously shown that several PAMPs and DAMPs can induce trained immunity, including *Bacillus Calmette-Guerin*, *Candida albicans*, β-glucan, and pro-atherogenic stimuli such as modified LDL and Lp(a) [5,8,9,23]. Our patients and controls did not show any differences in total cholesterol levels, LDL cholesterol or Lp(a), but the majority of the patients were being treated with statins. It is probable that before the initiation of statin treatment, LDL was higher in patients with CAD. Furthermore, the effects of statins on epigenetic modifications are still unknown. HDL cholesterol was lower in patients, but we do not yet know the effects of HDL cholesterol on trained immunity. Interestingly, the trained immune phenotype of circulation monocytes was only present in patients with severe symptomatic coronary atherosclerosis, and not in patients with asymptomatic carotid atherosclerosis, which was diagnosed by screening of healthy subjects. A potential explanation for this finding is that trained immunity contributes to the progression of complex atherosclerotic plaques and not to the initial formation of early lesions, which are observed in the majority of subjects. This finding is consistent with previous findings in patients with elevated levels of lipoprotein(a), which can also induce trained immunity *in vitro* [23]. Interestingly, most studies report that elevated lipoprotein(a) is not associated with early atherosclerotic lesions (e.g. carotid IMT) whereas elevated lipoprotein(a) is consistently associated with cardiovascular events, which mostly result from vulnerable atherosclerotic plaques [24–26]. Obviously, this finding requires confirmation in a larger cohort of patients.

Our study has several potential limitations. First of all, our results only indicate an association between atherosclerosis and trained immunity, and do not provide proof of causality. In theory, effects on the immune system could be caused by the atherosclerosis itself. Intervention studies in animal models of atherosclerosis will be required to provide final proof that trained immunity contributes to the development/progression of atherosclerosis. Secondly, the majority of patients with CAD used statins, in contrast to the control group. However, since statins are known to have potent anti-inflammatory effects [27], it is very likely that they have contributed to the pro-inflammatory monocyte phenotype, although they may have partially masked its full effects. Thirdly, it is important to realize that RNA expression was studied in whole blood using PAXgene tubes, to prevent changes in the RNA expression levels by the isolation of monocytes. However, it is likely that differences in RNA expression that we observed between patients with and without atherosclerosis are relevant for the monocyte fraction: first, we measured expression of various cytokines that are predominantly derived from monocytes, including TNF, IL1b, and IL6. Secondly, there is a strong correlation between the expression of enzymes of the glycolytic pathway (HK2/PPFB3) and these cytokines, suggesting that this increased glycolysis is relevant for monocytes. Finally, in a previous study in patients with atherosclerosis, an increased expression of glycolytic enzymes was also observed in isolated monocytes after differentiation into M1 macrophages, compared to monocytes from patients without atherosclerosis [21].

Evidently, with regard to the epigenetic reprogramming, we have only focused on a few epigenetic marks on the promoters of the various cytokines. We studied the ChiPseq promoters of genes that were upregulated in protein and RNA expression and searched for association, whereas a more unbiased approach (e.g. genome wide ChIP seq) could reveal new pathways and models for explaining differences.

Here we show that monocytes of patients with symptomatic CAD but not patients with asymptomatic carotid atherosclerosis have a pro-inflammatory phenotype, which is associated with metabolic reprogramming on the level of RNA expression towards increased glycolysis, and with epigenetic remodeling of pro-inflammatory genes. These findings suggest that trained immunity could play a role in the progression of mild and asymptomatic atherosclerotic plaques towards symptomatic disease.

Conflicts of interest

NPR served on a Scientific Advisory Board of AstraZeneca, which is unrelated to the current paper. NPR and MERG received non-restricted research grants from AstraZeneca, unrelated to the current paper.

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Author contributions

SB, MERG and NPR designed the study. IvDM, JR, JdG, TN and MERG included all the patients and controls, EJL analyzed the atherosclerosis burden. SB performed all the blood isolation and analysis. CD supplied the materials for high sensitivity ELISA on plasma. SB and NPR wrote the article. LABJ, MGN, MERG, IvDM, JR and JdG revised the article.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2016.10.019.

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