Atheroprotective Krüppel-like factor 4 is downregulated in monocyte subsets of patients with coronary artery disease

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Dear Sirs,

The zinc finger transcription factor Krüppel-like factor 4 (KLF4) plays an important role in the regulation of cellular functions, including proliferation, differentiation or activation (1). Circulating monocytes, which give rise to tissue-resident macrophages, are essential cellular mediators of atherosclerosis (2). Recently, it could be demonstrated that KLF4-deficient macrophages adopt a proinflammatory phenotype (3, 4). Moreover, myeloid KLF4 deficiency in mice was associated with increased vascular inflammation and atherosclerotic lesion formation (4). Human monocytes can be divided into so-called classical CD14⁺CD16⁻ and CD16⁺ monocytes, the latter consisting of intermediate CD14⁺CD16⁻ and non-classical CD14⁺CD16⁺ monocytes (2). Pro-atherogenic properties are mostly attributed to CD16⁺ monocytes (5), however, CD14⁺CD16⁻ monocytes also were found to be increased in individuals with cardiovascular risk factors in some studies (2). So far, the expression of KLF4 in human monocyte subsets and its relation to atherosclerosis has not been investigated.

Therefore, we recruited 52 patients with stable, angiographically confirmed coronary artery disease (CAD; 77% male; median age, 69.5 years; median body mass index 27.1 kg/m²; median blood leukocyte count, 6.7x10⁹/µl; median high-sensitivity C-reactive protein blood level, 2.60 mg/l) at the Department of Cardiology and Pulmonary Medicine and analysed KLF4 expression in their monocyte subsets. Their results were compared to healthy controls without any cardiovascular risk factors (CONTR; n=18; 78% male, p=not significant; 57.5 years, p<0.001; body-mass-index 25.2 kg/m², p=not significant; leukocyte count, 5.6x10⁹/µl, p<0.05; high-sensitivity C-reactive protein, 0.77 mg/l, p<0.01) which were recruited at the Department of Transfusion Medicine. As these individuals did not undergo cardiac catheterisation, we cannot exclude the presence of clinically silent cardiovascular disease in CONTR subjects. Persons with an active infection, malignancy, nephropathy or acute (within the last 2 months) coronary syndrome were excluded. Informed consent was obtained according to the requirements of the local ethics committee. Mononuclear cells were isolated from heparinised peripheral venous blood by Histopaque® (Sigma-Aldrich, St. Louis, MO; USA) density gradient centrifugation. Subsequently, cells were stained with fluorescein isothiocyanate (FITC)-conjugated human CD14 (clone MqP9; BD) and phycoerythrin (PE)-conjugated CD16 (clone 3G8; BD) antibodies, or their respective isotype controls. Next, cells were permeabilised by Flow Cytometry Permeabilization Buffer/Wash Buffer I (R&D Systems, Indianapolis, IN; USA) and then stained with an allophycocyanin (APC)-conjugated human KLF4 antibody (R&D Systems), or its respective isotype control. Cells were investigated on a BD FACS Canto® II flow cytometer at three wavelengths and analysed using BD FACS Diva® software. After gating monocytes according to their forward and sideward scatter profiles, cells were subdivided into CD14⁺CD16⁻, CD14⁺CD16⁺ and CD14⁺CD16⁻ monocytes and further examined with regard to their KLF4 expression. Moreover, tumour necrosis factor (TNF)-α plasma levels were measured in duplicate using a commercial high sensitivity enzyme-linked immunosorbent assay (eBioscience, San Diego, CA, USA), according to the manufacturer’s instructions. Comparisons were performed using the non-parametric Mann-Whitney U or Kruskal-Wallis tests, the latter followed by Dunn’s multiple comparison test. All statistical tests were two-sided and a probability (p) value of <0.05 was considered significant.

The median percentage of circulating CD14⁺CD16⁻ monocytes was 84%, of CD14⁺CD16⁺ monocytes 11% and of CD14⁺CD16⁻ monocytes 5.4% in the CONTR subjects, and 84%, 11% and 4.7%,

Figure 1: KLF4 expression in monocytes of CAD patients and healthy controls. In 52 patients with coronary artery disease (CAD) and 18 healthy controls, the relative percentage of Krüppel-like factor 4 (KLF4⁺) cells (A) as well as KLF4 mean fluorescence intensity minus the respective isotype control (MFI-FMO) in KLF4⁺ cells (B) was examined in monocyte subsets by flow cytometry. CAD patients were stratified according to the presence of arterial hypertension and/or diabetes mellitus (i.e. 10 patients without both risk factors, 31 with arterial hypertension, 10 with both risk factors) and the KLF4⁺ monocyte percentage (C) was analysed. Total monocytes of healthy individuals (n=4) incubated in medium supplemented with plasma of either CONTR subjects or CAD patients were evaluated regarding their KLF4⁺ expression (D). TNF-α levels were measured in the plasma of study participants (E) and correlated with the relative KLF4⁺ monocyte percentage (F). Data in panel A-E are presented as column diagrams displaying the median as well as their upper and lower quartiles. For intra-group comparisons (i.e. within each monocyte subset), the level of significance is shown as follows: +++ indicates p<0.001, ++ p<0.01, and + p<0.05 vs. CD14⁺CD16⁻; ### indicates p<0.001 vs. CD14⁺CD16⁻.

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respectively, in the CAD group (p<0.01 vs CONTR). For inter-group comparisons. In CONTR individuals, the lowest percentage of KLF4+ cells was detected in CD14+CD16 monocytes (51%) compared to CD14+CD16+ (73%; p<0.01) and CD14+CD16- (72%; p<0.01) cells (Figure 1A). Although a similar distribution could be observed in CAD patients, the percentage of KLF4+ monocytes was markedly reduced in all monocyte subsets compared to CONTR (CD14+CD16+ 20%; p<0.01; CD14+CD16- 36%, p<0.001; CD14+CD16- 31%, p<0.001). The median of the KLF4 mean fluorescence intensity minus the respective isotype control (MFI-FMO) in KLF4+ cells (Figure 1B) was also lowest in CD14+CD16+ monocytes compared to CD14+CD16+ (p<0.001 for CONTR and CAD) and CD14+CD16- (p<0.05 for CONTR) cells, and significantly reduced KLF4 levels were detected in CD14+CD16+ monocytes of CAD patients (p<0.01 vs CONTR). Stratification of CAD patients in those with and without cardiovascular risk factors revealed that individuals without arterial hypertension and diabetes mellitus (n=10) exhibited a higher percentage of KLF4+ monocytes compared to patients with arterial hypertension, but without diabetes mellitus (n=31; p<0.01) or those with both risk factors (n=10; p<0.001; Figure 1C). Of note, a similar distribution pattern was observed, if monocyte subsets were examined separately (not shown). In order to investigate the influence of circulating factors on KLF4 expression, mononuclear cells obtained from healthy persons were incubated in cell medium (Opti-MEM; Life Technologies) containing 10% heparinised plasma of representative CONTR individuals or CAD patients, respectively, for 4 hours. Interestingly, subsequent flow cytometry analysis of monocyte KLF4 expression revealed that the number of KLF4+ monocytes was reduced in cells treated with CAD plasma (p<0.05 vs CONTR plasma; Figure 1D).

The differential role of monocyte subsets in atherosclerosis is currently under intensive investigation (2). Mouse studies revealed different functional properties of monocyte subpopulations during vascular healing processes. For example, Ly-6C<sup>high</sup>, i.e. the presumed mouse analogue of CD16 monocytes, were found to be phagocytic, proteolytic and proinflammatory, whereas Ly-6C<sup>low</sup>, i.e. the presumed mouse analogue of CD16 monocytes, exhibited reparative and angiogenic properties (6). In humans, CD14+CD16+ monocytes are suggested to be linked to angiogenic processes (7, 8). Consistent with the recently suggested atheroprotective role of KLF4 (3, 4), in the current study, the percentage of KLF4+ cells and its expression strength per individual cell were significantly elevated in CD14+CD16+and CD14+CD16- compared to CD14+CD16+ monocytes.

Furthermore, we could demonstrate that coronary atherosclerosis was associated with reduced circulating KLF4+ monocyte numbers, regardless of the monocyte subtype. Potentially, and analogous to findings in murine macrophages in atherosclerosis (3), downregulation of KLF4 expression might favour the adoption of a proinflammatory phenotype and thus the formation and progression of atherosclerotic lesions. Interestingly, when comparing peripheral blood monocytes from individuals with or without CAD, previous gene expression analyses also revealed differences at the transcriptional level (9, 10), suggesting that distinct (gene) expression patterns may contribute to coronary atherosclerosis progression.

The reproducibility of our findings by <i>ex vivo</i> incubation of total monocytes, obtained from healthy subjects, with plasma from CAD patients indicates that circulating factors might be causally involved in the observed downregulation of monocyte KLF4 expression in patients with coronary atherosclerosis, possibly as consequence of elevated circulating cytokine levels in this proinflammatory condition (3). Previously, it was demonstrated that inflammatory cytokines such as TNF-α are able to reduce KLF4 expression in macrophages (4), and elevated TNF-α levels have been reported in the blood of patients with CAD (11). We also observed increased TNF-α plasma levels in the CAD population compared to the CONTR group (0.238 vs 0.036 pg/ml; p<0.01; Figure 1E). Moreover, the relative percentage of KLF4+ monocytes was found to be inversely associated with TNF-α blood levels (r=-0.275; p<0.05; Figure 1F). It therefore may be speculated that elevated circulating inflammatory factors (including TNF-α, but also others) may have contributed to the observed reduction of KLF4+ monocyte subsets in our CAD patient population.

In summary, our study demonstrates that expression of the atheroprotective transcription factor KLF4 in human monocyte subsets is downregulated in patients with established coronary atherosclerosis, possibly in response to circulating factors. Future studies will have to examine in more detail the potential of KLF4 as biomarker and whether (therapeutic) modulation of KLF4 may be useful in regulating monocyte function in atherosclerosis.

Conflicts of interest
None declared.

References