Decreased numbers of peripheral blood dendritic cells in patients with coronary artery disease are associated with diminished plasma Flt3 ligand levels and impaired plasmacytoid dendritic cell function

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ABSTRACT

We investigated whether activation of circulating DCs (dendritic cells) or levels of Flt3L (FMS-like tyrosine kinase 3 ligand) and GM-CSF (granulocyte/macrophage colony-stimulating factor), haematopoietic growth factors important for DC differentiation, could account for reduced blood DC numbers in CAD (coronary artery disease) patients. Concentrations of Flt3L and GM-CSF were measured in plasma from CAD patients (n = 15) and controls (n = 12). Frequency and phenotype of mDCs (myeloid dendritic cells) and pDCs (plasmacytoid dendritic cells) were analysed by multicolour flow cytometry in fresh blood, and after overnight incubation with TLR (Toll-like receptor)-4 or -7 ligands LPS (lipopolysaccharide) or IQ (imiquimod). DC function was measured by IL (interleukin)-12 and IFN (interferon)-α secretion. Circulating numbers of CD11c+ mDCs and CD123+ pDCs and frequencies of CD86+ and CCR-7+ mDCs, but not pDCs, were declined in CAD. In addition, plasma Flt3L, but not GM-CSF, was lower in patients and positively correlated with blood DC counts. In response to LPS, mDCs up-regulated CD83 and CD86, but CCR-7 expression and IL-12 secretion remained unchanged, similarly in patients and controls. Conversely, pDCs from patients had lower CD83 and CCR-7 expression after overnight incubation and had a weaker IQ-induced up-regulation of CD83 and IFN-α secretion. In conclusion, our results suggest that reduced blood DC counts in CAD are, at least partly, due to impaired DC differentiation from bone marrow progenitors. Decreased levels of mDCs are presumably also explained by activation and subsequent migration to atherosclerotic plaques or lymph nodes. Although mDCs are functioning normally, pDCs from patients appeared to be both numerically and functionally impaired.

Key words: coronary artery disease, dendritic cell activation, dendritic cell differentiation, haematopoietic growth factor, Toll-like receptor.

Abbreviations: BDCA, blood dendritic cell antigen; CAD, coronary artery disease; CCR-7, CC chemokine receptor type 7; CRP, C-reactive protein; DC, dendritic cell; Flt3L, FMS-like tyrosine kinase 3 ligand; FMD, flow-mediated dilatation; GM-CSF, granulocyte/macrophage colony-stimulating factor; (Geo-)MFI, (geometric) mean fluorescence intensity; HDL, high-density lipoprotein; hsCRP, high-sensitivity CRP; IFN, interferon; IL, interleukin; IQ, imiquimod; LDL, low-density lipoprotein; LPS, lipopolysaccharide; mDC, myeloid dendritic cell; mAb, monoclonal antibody; moDC, monocyte-derived dendritic cell; oxLDL, oxidized LDL; pDC, plasmacytoid dendritic cell; PTCA, percutaneous transluminal coronary angioplasty; WBC, white blood cell.

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INTRODUCTION

Atherosclerosis is a chronic disease of the arterial vasculature, triggered by various factors [1] and leading to an inflammatory response. The progressive accumulation of immune cells, such as DCs (dendritic cells), as well as their retention in the plaque [2], are important hallmarks in atherogenesis. DCs are professional antigen-presenting cells and participate both in innate and adaptive immune reactions [3,4]. DCs are heterogeneous and encompass populations with different phenotypes and properties [5]. They exist in tissues and blood in an immature state, but upon encounter with microbial antigens or upon exposure to pro-inflammatory cytokines they undergo a highly regulated maturation process [6–8]. Maturation involves down-regulation of endocytotic activity and up-regulation of the immunoglobulin superfamily member CD83, of co-stimulatory molecules (CD40, CD80 and CD86) and of antigen-presenting molecules (including MHC class I and II molecules), as well as production of cytokines [9].

There are two main DC subtypes with different functions: (i) mDCs (myeloid dendritic cells), which are CD11c+/CD123− and largely secrete IL (interleukin)-12 upon stimulation and (ii) pDCs (plasmacytoid dendritic cells), which are CD11c−/CD123bright and produce mostly IFN (interferon)-α, particularly in response to viral infections [10]. Given their role in bridging innate and adaptive immunity, and in priming antigen-specific T-cell responses, DCs probably play an important role in the pathogenesis of atherosclerosis [8,11]. Many risk factors of atherosclerosis, such as hyperlipidaemia [high LDL (low-density lipoprotein) levels] [12], oxLDL (oxidized LDL) [13], free radicals [14], CRP (C-reactive protein) [15] and nicotine [16], also lead to the maturation of DCs. The migratory nature of activated DCs might then allow them to interact closely with T-cells in lymphoid tissue, thereby inducing T-cell activation and aggravating inflammation.

We [17,18] and others [19,20] have demonstrated that blood DCs are numerically depleted in patients with CAD (coronary artery disease). The reasons for their decline are unclear, but it was not due to endothelial cell dysfunction or use of medication (β-blockers and statins) by CAD patients, which appeared to raise rather than suppress mDC counts. Moreover, neither changes in overall leucocyte counts nor age could account for the thorough decreases in blood DCs [18].

Therefore the aim of the present study was to investigate whether increased activation of pDCs and mDCs in CAD patients was responsible for the numerical decline, by measuring the expression of different maturation markers: CD86, CCR-7 (CC chemokine receptor type 7) and CD83. Indeed, in vitro studies, whereby DCs were generated from moDCs (monocyte-derived dendritic cells) demonstrated more CD40+ [21], CD80+ [21] or CD86+ [21–23] moDCs and a greater immunostimulatory capacity [22,23] in DCs derived from monocytes of CAD patients. Consequently, we also investigated the functional capabilities of mDCs and pDCs from CAD patients to mature (CD83, CCR-7 and CD86) and release cytokines (IL-12 and IFN-α) upon in vitro stimulation with LPS (lipopolysaccharide), an agonist of TLR (Toll-like receptor)-4 expressed by mDCs and IQ (imiquimod), an agonist of TLR-7 expressed by pDCs, which are both known to induce DC maturation in vitro [24]. Alternatively, impaired differentiation or reduced release from the bone marrow could also account for the lower counts in CAD patients. To address that issue, plasma concentrations of Flt3L (FMS-like tyrosine kinase 3 ligand) and GM-CSF (granulocyte/macrophage colony-stimulating factor) were determined, which are haematopoietic growth factors important for pDC and mDC differentiation and release from the bone marrow [25,26].

MATERIALS AND METHODS

Subjects

The study was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association and has been approved by the Ethical Committee of the University Hospital of Antwerp. Informed consent was obtained from all patients. Fresh whole peripheral blood samples were obtained from 15 patients who underwent PTCA (percutaneous transluminal coronary angioplasty) with placement of a bare metal stent and had ≥75% stenosis in one coronary segment (27% in the left anterior descending coronary artery, 20% in the circumflex artery and 53% in the right coronary artery) and from 12 age-matched healthy volunteers. Study population characteristics are summarized (Table 1). The control group had no known pre-existing cardiovascular or other inflammatory or immune diseases and was completely free of symptoms and medication intake. Patients with acute or recent (less than 3 months) myocardial infarction, acute or chronic infections, malignancies or autoimmune diseases and immunosuppressive drugs were excluded from the study. Blood was collected in vacuum tubes containing K2EDTA (BD Vacutainers) immediately before PTCA. Samples were analysed within 4 h after collection or after overnight incubation with LPS or IQ (see below). Plasma samples were frozen at −80°C for analysis of cytokines and haematopoietic factors.

In vitro stimulation with TLR ligands

Whole blood (1 ml) was diluted in IMDM (Iscove’s modified Dulbecco’s medium) with l-glutamine, 25 mM Hepes (Invitrogen; 1:1, v/v), and incubated in the absence
Table 1  Characteristics of the control and CAD populations

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Controls (n = 12)</th>
<th>CAD patients (n = 15)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>56.3 ± 1.6</td>
<td>59.1 ± 2.2</td>
<td>0.345</td>
</tr>
<tr>
<td>WBCs (×10⁶/ml)</td>
<td>6.2 ± 0.3</td>
<td>8.2 ± 0.9</td>
<td>0.050</td>
</tr>
<tr>
<td>Monocytes (×10⁶/ml)</td>
<td>0.4 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>0.088</td>
</tr>
<tr>
<td>Risk factors (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>50.0</td>
<td>86.7</td>
<td>0.038</td>
</tr>
<tr>
<td>Smoking</td>
<td>33.3</td>
<td>30.0</td>
<td>1.000</td>
</tr>
<tr>
<td>Diabetes</td>
<td>0</td>
<td>6.7</td>
<td>0.362</td>
</tr>
<tr>
<td>Hypertension</td>
<td>8.3</td>
<td>46.7</td>
<td>0.030</td>
</tr>
<tr>
<td>Hyperlipidaemia</td>
<td>8.3</td>
<td>60.0</td>
<td>0.006</td>
</tr>
<tr>
<td>Lipids (mg/dl)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL</td>
<td>47 ± 4</td>
<td>42 ± 4</td>
<td>0.378</td>
</tr>
<tr>
<td>LDL</td>
<td>102 ± 8</td>
<td>120 ± 11</td>
<td>0.222</td>
</tr>
<tr>
<td>LDL/HDL</td>
<td>2.3 ± 0.3</td>
<td>3.0 ± 0.3</td>
<td>0.169</td>
</tr>
<tr>
<td>Inflammation markers (pg/ml)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>hsCRP</td>
<td>0.11 ± 0.03</td>
<td>0.53 ± 0.3</td>
<td>0.010</td>
</tr>
<tr>
<td>IL-12</td>
<td>3.2 ± 0.4</td>
<td>2.9 ± 0.5</td>
<td>0.404</td>
</tr>
<tr>
<td>IFN-α</td>
<td>9.5 ± 1.4</td>
<td>16.2 ± 7.5</td>
<td>0.159</td>
</tr>
<tr>
<td>Current medication (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid-lowering therapy</td>
<td>—</td>
<td>67</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Aspirin</td>
<td>—</td>
<td>87</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>β-Blockers</td>
<td>—</td>
<td>40</td>
<td>0.020</td>
</tr>
<tr>
<td>ACE inhibitors</td>
<td>—</td>
<td>20</td>
<td>0.231</td>
</tr>
<tr>
<td>Proton pump inhibitor</td>
<td>—</td>
<td>20</td>
<td>0.231</td>
</tr>
</tbody>
</table>

or presence of either LPS (Escherichia coli 055:B5, 0.5 μg/ml; Sigma) or IQ (10 μg/ml; Invivogen) as described previously [24]. The ligands were individually prepared according to the manufacturer’s recommendations. Samples were incubated overnight at 37°C in a 5% CO₂ humidified atmosphere after addition of stimuli.

Six-colour flow cytometry

Staining procedures and mAbs (monoclonal antibodies) used in the present study are shown in Table 2. Four colours were used to gate mDCs and pDCs, which were identified as lineage⁻ /HLA-DR⁺/CD11c⁺ and lineage⁻ /HLA-DR⁺/CD123⁺ respectively. Two more colours were used to analyse the expression of CD83, a member of the immunoglobulin superfamily with unknown functions [27], and CCR-7, which is required for the migration of DCs to lymph nodes [28], in the first tube or CD86, which is required for T-cell activation [29], in the second tube.

Blood (200 μl) was transferred to each 5 ml FACS tube (BD Falcon). Erythrocytes were lysed by incubation with ammonium chloride (Stemcell Technologies) for 10 min at 4°C. Fcy receptors were blocked with mouse γ globulins (BD Biosciences) for 10 min, before samples were stained with fluorochrome-conjugated mouse anti-human mAbs (Table 2). Staining conditions for each mAb were preliminarily determined in titration assays.

Data acquisition was performed on a FacsCanto II flow cytometer (BD Biosciences) using automatic compensation settings, with further gating adjustments through the FMO (fluorescence minus one) method [30]. The strategy to select/gate DCs and to analyse their activation in whole-blood samples is shown (Figure 1). A total of 1 × 10⁶ events were collected to visualize the total WBC (white blood cell) population. Data analysis was executed with the FacsDiva 6.1 software. The Geo-MFI (geometric mean fluorescence intensity) was used as a semi-quantitative presentation of membrane molecule expression. DC numbers were calculated as the proportion of DCs in the leucocyte gate (relative DC numbers) or those multiplied by the absolute WBC count (absolute DC numbers), determined on an ABX Micros 60 (HORIBA).

Determination of Flt3L, GM-CSF, IL-12 and IFN-α

Flt3L, GM-CSF, IL-12 and IFN-α were measured in plasma with commercially available ELISA kits [human Flt-3 Ligand, human GM-CSF and human...
Table 2 Staining strategies and list of mAbs used
PerCP-Cy, peridinin—chlorophyll protein complex—cyanine; APC, allophycocyanin; PE, phycoerythrin; NK, natural killer. All antibodies were from BD Biosciences.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Description</th>
<th>Conjugate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staining 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3</td>
<td>Lineage marker for T-cells</td>
<td>PerCP—Cy5.5</td>
</tr>
<tr>
<td>CD14</td>
<td>Lineage marker for monocytes</td>
<td>PerCP—Cy5.5</td>
</tr>
<tr>
<td>CD16</td>
<td>Lineage marker for NK cells and granulocytes</td>
<td>PerCP—Cy5.5</td>
</tr>
<tr>
<td>CD19</td>
<td>Lineage marker for B-cells</td>
<td>PerCP—Cy5.5</td>
</tr>
<tr>
<td>CD20</td>
<td>Lineage marker for B-cells</td>
<td>PerCP—Cy5.5</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>MHC class molecule</td>
<td>APC—Cy7</td>
</tr>
<tr>
<td>CD11c</td>
<td>mDC marker β2 integrin</td>
<td>APC</td>
</tr>
<tr>
<td>CD123</td>
<td>pDC marker IL3kr</td>
<td>PE</td>
</tr>
<tr>
<td>CD83</td>
<td>Maturation marker on DCs</td>
<td>FITC</td>
</tr>
<tr>
<td>CD197</td>
<td>Migration to lymphoid organs</td>
<td>PE—Cy7</td>
</tr>
<tr>
<td>Staining 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lineage</td>
<td>Stains T-, B- and NK cells, monocytes and granulocytes</td>
<td>FITC</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>MHC class molecule</td>
<td>PerCP</td>
</tr>
<tr>
<td>CD11c</td>
<td>mDC marker β2 integrin</td>
<td>APC</td>
</tr>
<tr>
<td>CD86</td>
<td>Co-stimulatory molecule</td>
<td>PE</td>
</tr>
</tbody>
</table>

RESULTS

Characteristics of study populations
The age of both groups was not different, but male gender, hypertension and hyperlipidaemia were more prominent in the CAD group (Table 1). Other risk factors such as smoking, LDL and the ratio LDL/HDL (high-density lipoprotein) were not different, but the CAD group contained one patient with diabetes. Since the control group consisted of volunteers without known diseases, medication intake differed between both study groups.

CAD patients also had increased numbers of WBCs, a tendency towards increased numbers of monocytes and higher serum values of hsCRP (high-sensitivity CRP). IL-12 and IFN-α serum levels were similar in both study groups.

Numbers and functional characteristics of circulating blood DCs
Absolute and relative numbers of mDCs and pDCs (Table 3) and of total DCs (results not shown) were lower in CAD patients compared with controls, strengthening previous findings [17–20]. Basal frequencies of DCs expressing CD83 were very low (1.3 ± 0.7% for mDCs and 0.2 ± 0.1% for pDCs) and were not different between controls and CAD patients, whereas more DCs expressed CCR-7 (21.9 ± 5.3% for mDCs and 13.7 ± 2.9% for pDCs) and CD86 (13.5 ± 2.0% for mDCs and 19.7 ± 2.8% for pDCs). Interestingly, the frequency of mDCs expressing CCR-7 and CD86 was lower in the circulation of
Figure 1 Gating strategy of DCs with activation markers in whole-blood samples
(A) The scatter density plot with the three obvious WBC populations (lymphocytes, monocytes and granulocytes) with the gate on the PBMCs (peripheral blood mononuclear cells). (B) The total DC population, which is lineage−/HLA-DR+. (C) Staining 1: mDCs on the left-hand plot, defined as CD11c+ (C1); pDCs on the middle plot, defined as CD123+ (C2). In this staining tube, expression of CD83 (D1) and CCR-7 (D2) were also measured. (E) Staining 2, where mDCs are defined as CD11c+ and pDCs as CD11c−. The third activation marker, CD86, was measured in D0 (F).

Table 3 Basal absolute and relative numbers of circulating DCs in CAD patients and controls

<table>
<thead>
<tr>
<th>Circulating DCs</th>
<th>Controls (n = 12)</th>
<th>CAD patients (n = 15)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative numbers (% of WBC)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mDC (CD11c+)</td>
<td>0.31 ± 0.07</td>
<td>0.14 ± 0.01</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>pDC (CD123+)</td>
<td>0.18 ± 0.02</td>
<td>0.08 ± 0.01</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Absolute numbers (cells/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mDC (CD11c+)</td>
<td>19871 ± 5350</td>
<td>11462 ± 1033</td>
<td>0.023</td>
</tr>
<tr>
<td>pDC (CD123+)</td>
<td>10710 ± 900</td>
<td>6715 ± 657</td>
<td>0.001</td>
</tr>
</tbody>
</table>
CAD patients (Figure 2), whereas the percentage of pDCs expressing CCR-7 or CD86 remained unchanged. The expression level (estimated by means of Geo-MFI) of CD83, CCR-7 and CD86 by mDCs and pDCs was comparable for CAD patients and controls (Table 4).

**Plasma concentration of GM-CSF and Flt3L**

Plasma concentrations of GM-CSF did not differ between controls and CAD patients ($P = 0.24$), whereas plasma Flt3L was significantly lower in CAD compared with controls ($P = 0.005$) (Figure 3, upper panels). Moreover, a positive correlation was found between plasma Flt3L and both pDC and mDC counts (Figure 3, lower panels), whereas no significant correlations existed between plasma GM-CSF and mDC or pDC counts (results not shown).

**Phenotypic changes upon in vitro stimulation**

Compared with basal measurements, overnight incubation of whole-blood samples without any stimulus increased the expression (MFI) of CCR-7 and CD86 in mDCs without changing MFI of CD83 (results not shown). In mDCs, LPS increased the expression of CD83 and CD86 ($P < 0.001$), but not of CCR-7 ($P = 0.154$). Although there was a tendency to lower CD83 expression in CAD patients (both in unstimulated and in LPS-stimulated samples), the mDC responses to LPS were not different between controls and CAD patients, as indicated by the lack of significant interaction in the repeated-measures ANOVA (Figure 4).

In pDCs, overnight incubation doubled the MFI of CCR-7$^+$, without changing CD83 and CD86 expression (results not shown). Nevertheless, IQ stimulation resulted in a significant up-regulation of all three activation markers ($P < 0.001$) in both pDCs from controls and patients. CAD appeared to affect CD83 ($P = 0.012$) and CCR-7 ($P = 0.006$) expression, since these were significantly lower in patients. pDCs from CAD patients and controls responded equally to IQ with respect to the up-regulation of CCR-7 and CD86 (Figure 4). In contrast, the up-regulation of CD83 upon IQ stimulation was significantly lower in pDCs from patients, as indicated by the significant interaction in the repeated-measures ANOVA ($P = 0.013$).

**Functional DC characteristics upon in vitro stimulation**

To further unravel immunostimulatory capacity of both subsets, we measured cytokines associated with mDCs (IL-12) and pDCs (IFN-$
\alpha$). IL-12 levels did not change...
after incubation with LPS (Figure 5, left-hand panel). In contrast, IFN-α levels were increased after IQ incubation in both patients and controls. Interestingly, the IFN-α concentration in IQ-treated samples tended to be smaller in CAD patients \( (P = 0.085) \) (Figure 5, right-hand panel). Indeed, the net increase induced by IQ \( (45 \pm 10 \text{ pg/ml in controls and } 22 \pm 8 \text{ pg/ml in CAD patients}; P = 0.036, \) as determined using a Mann–Whitney \( U \) test) was 50% smaller in samples from CAD patients.

**DISCUSSION**

Circulating DCs originate from bone marrow and home to tissues, and their number and function reflect the state of host immune responses. Limited information has been reported, so far, about functional features of circulating DCs from CAD patients. In the present study, we show the presence of numerical abnormalities of both DC subsets, and functional impairment of pDCs, but no phenotypical or functional abnormalities in mDCs of patients with CAD.

The finding that both blood CD11c+ mDCs and CD123+ pDCs show a decline in the circulation of CAD patients is new and strongly corroborated previous results that were based on enumeration of another set of markers, BDCA (blood dendritic cell antigen)-1 and BDCA-2 to identify mDCs and pDCs, respectively \[17–20\]. Previously, we have demonstrated that the decline in blood DCs was not due to (tendencies towards) increased numbers of monocytes or total WBCs in CAD patients \[18\].

A first explanation for the decline could be increased recruitment of circulating DCs into atherosclerotic lesions, either due to endothelial cell activation or due to activation of circulating DCs by pro-atherogenic factors in CAD patients. Recently, we \[18\] assessed the impact of endothelial cell function by measuring relationships between FMD (flow-mediated dilatation) and DC numbers in groups of patients with different types of CAD. A poor FMD response was indeed associated with changes in mDC numbers, but, unexpectedly, endothelial dysfunction was associated with increased, rather than decreased, mDC counts without affecting pDC numbers \[18\].

Since we could not obtain direct evidence for the role of activated endothelial cells in the decline of both...
Incubation with LPS or IQ resulted in a clear up-regulation of CD83 and CD86 on mDCs and pDCs respectively. IQ also resulted in the significant up-regulation of CCR-7 on pDCs. In addition to the effect of incubation, there was also an effect of CAD: pDCs from patients had significantly lower CD83 and CCR-7 expression. Finally, mDCs of patients and controls always responded equally to stimulation, as indicated by the lack of interaction, but up-regulation of CD83 was less pronounced in pDCs from patients. Means and S.E.M. are shown by the bars. ***P < 0.001 for activation; *P < 0.05 and **P < 0.01 for a CAD effect.
subsets, the present study was focused on the activation status of circulating blood DCs. Efficient DC migration requires maturation and, indeed, it has been described that atherosclerosis favouring factors, including oxLDL, heat-shock protein, CRP, hypoxia and altered NOS (NO synthase) activity of the endothelium promote DC maturation and increase CCR-7 expression and subsequent DC migration into lymph nodes [12,32]. Moreover, in vitro studies have shown an increased activation status of moDCs in CAD patients [21–23]. Since moDCs may not reflect the natural biology of circulating blood DCs, we directly measured expression of maturation markers in circulating blood DCs by means of flow cytometry. As expected, very few blood DCs displayed a mature phenotype, as indicated by the restricted expression of CD83, without differences between controls and CAD patients. A minority of the circulating pDCs (14–20 %) and mDCs (14–22 %) had a more mature phenotype and expressed low levels of CD86 or CCR-7. This limited activation of blood DCs corroborates a report briefly describing a weak expression of CD40 and CD86 on circulating BDCA-1+ mDCs and BDCA-2+ pDCs [19]. Since the expression level (Geo-MFI) of the three maturation markers on circulating mDCs and pDCs was similar in CAD patients and controls, we conclude that there was no indication of increased DC maturation in CAD patients, in contrast with previous in vitro findings [21–23]. However, less circulating mDCs expressing CCR-7 and CD86, but not pDCs, were observed in CAD patients. This points to increased recruitment of mDCs expressing CCR-7 into lymph nodes or atherosclerotic plaques. The latter hypothesis is supported by the demonstration of CCR-7 ligands CCL19 and CCL21 in human atherosclerotic lesions, which may attract cells expressing CCR-7 from the circulation [33]. The decline of CD86+ mDCs could possibly be explained by double positivity of CD86 with CCR-7 on mDCs, which leads to filtering out of the circulation into the plaque [34]. The mDCs that remain in the blood in this setting could be those that have not yet reached sufficient maturation.

Apart from accumulating in atherosclerotic plaques or lymphoid tissues, the decline of circulating DCs in CAD patients could also be due to reduced differentiation in or release from the bone marrow. Therefore we determined plasma levels of haematopoietic cytokines involved in DC generation and expansion. GM-CSF is the primary growth factor used to generate DCs in vitro from early bone-marrow-derived myeloid progenitors [35] and to generate moDCs [15], whereas Flt3L has been used in vitro and in vivo to generate DCs [36]. Interestingly, a study has shown that, in the absence of GM-CSF, murine atherosclerotic lesions were significantly decreased in size and contained dramatically less DCs [37]. However, in our present study, plasma concentrations of GM-CSF in CAD patients were similar to those of controls, indicating that it is unlikely that this growth factor accounts for the decreased numbers of blood DCs in human CAD. Moreover, no significant correlations were found between plasma GM-CSF levels and mDC or pDC counts. With regard to the control population, the absence of correlations between GM-CSF and DC counts strengthens the notion that GM-CSF-mediated DC differentiation is less important during normal homoeostasis [25,38]. In contrast, GM-CSF-mediated DC differentiation is described to be more important in vivo during inflammation [39]. Although inflammation plays an important role in CAD, our present findings suggest that GM-CSF is not a primary factor for in vivo DC differentiation in CAD.

Flt3L is a major cytokine involved in both pDC and mDC development from haematopoietic stem cells and their release from the bone marrow [40–42]. Interestingly, plasma concentrations of Flt3L were significantly

Figure 5 Cytokine secretion after incubation with TLR-4 or -7 ligands
LPS failed to stimulate IL-12 production (left-hand panel), whereas IQ induced a significantly higher IFN-α production compared with unstimulated (---) samples (right-hand panel), but without a significant (P = 0.085) difference between controls and CAD patients. Horizontal bars show medians. **P < 0.01, as determined using a Wilcoxon-matched pairs signed rank test (unstimulated compared with LPS or IQ) or Mann–Whitney U test (differences between patients and controls).
lower in CAD patients and positively correlated with pDC and mDC counts. Although Kingston et al. [25] recently described that Flt3L deficiency primarily affects DC progenitors and pDC numbers, it has also been demonstrated that Flt3L treatment leads to an increase in both CD11c+/CD123− mDC and CD11c−/CD123+ pDC numbers [43,44]. Thus the reduced Flt3L levels suggest that the lower blood DC numbers in CAD are, at least partly, due to reduced differentiation or release of DC precursors from the bone marrow.

Since circulating DCs from CAD patients were numerically depleted and had an immature expression pattern, we wondered whether CAD might alter their ability to achieve maturation. To this end, whole-blood samples were incubated overnight with ligands specific for TLR-4 and -7 and subsequently evaluated for expression (Geo-MFI) of the three activation markers on both DC subsets. DCs that were incubated overnight in the absence of additional stimuli became slightly activated. This is consistent with the findings of Della Bella et al. [24], who also demonstrated 61.5% CD86+ mDCs in the unstimulated tube (compared with 92.0% in the LPS-stimulated tube). In response to LPS, mDCs vigorously up-regulated expression (assessed by Geo-MFI) of CD83 and CD86, without a significant increase in CCR-7. However, the lack in interaction in the repeated-measures ANOVA indicated that mDCs from the control and CAD groups had a similar phenotypic maturation of all markers after exposure to LPS. In addition, we investigated cytokine production by mDCs, but secretion of IL-12 remained low, even after LPS stimulation, and was not influenced by CAD. This was also the case in a study by Tavakoli et al. [45], who compared IL-12 secretion between healthy controls and patients with hepatitis B virus using the same IL-12 ELISA kit. Taken together, these results indicate that the peripheral blood mDCs in CAD patients are numerically depleted but functionally unaltered, as indicated by the in vitro maturation test.

In addition, pDCs significantly increased the expression of all maturation markers in response to in vitro stimulation with IQ, and this was accompanied by increased biosynthesis of IFN-α, the prototypic cytokine of pDCs. In contrast with mDCs, the pDCs of patients and controls behaved differently during this in vitro challenge. CD83 and CCR-7 expression on pDCs of CAD patients was lower, both with and without IQ, suggesting that CAD affects the up-regulation of these markers during in vitro culture of pDCs. Even more importantly, patients had a significantly weaker up-regulation of the maturation marker CD83 after in vitro stimulation with IQ, as shown by the interaction in the repeated-measures ANOVA. This was corroborated further by the attenuated secretion of IFN-α in samples from CAD patients. This clearly indicates that pDCs from CAD patients are not only numerically, but also functionally, impaired, since they were more resistant to activation compared with pDCs from control subjects.

**Study limitations**

The in vitro activation of DCs by IQ or LPS, as indicated by the up-regulation of CD83, CCR-7 and CD86, is presumably partly due to activation of other leucocytes in the blood sample, which then release cytokines that caused paracrine activation of pDCs and mDCs [46,47]. However, we chose to reflect the in vivo situation by incubating whole-blood samples, rather than isolated blood DCs. More importantly, although bystander cells may have activated the DCs, the flow cytometric analysis of the activation markers was strictly focused on DCs.

This cannot be said for the secretion of IL-12 and IFN-α. After overnight incubation of whole blood with TLR ligands, both cytokines could be produced by leucocytes other than DCs. IL-12 is rather specific for mDCs [48], but can also be released by monocytes [49]. However, it is important to note that the cellular source was not very relevant in our present study, since IL-12 release was hardly raised by LPS. In contrast, IFN-α secretion was clearly stimulated by IQ, and pDCs are the principal source of type I IFNs [50]. Hence the reduced IFN-α biosynthesis in blood of CAD patients points to a functional deficit in this subset of DCs.

Furthermore, sample sizes were small (n = 12 compared with n = 15), but post hoc analyses revealed that the power in the present study was sufficient (0.7–0.9) to detect 40% changes in activation status of circulating blood DCs, or after in vitro stimulation between both groups.

The present study was not designed to investigate the impact of medication. Recently, we [18] have shown that the use of statins or β-blockers was associated with changes in circulating blood mDCs. However, intake of either medication led to increased, rather than decreased, mDC counts and their effects occurred independently of the presence of CAD and were not observed in pDCs [18]. Thus it appears unlikely that medication affected DC enumeration in the present study, but the impact on activation of DCs remains to be established. For that purpose, patients with chest pain, but without angiographic evidence of significant (i.e. less than 75%) coronary stenosis [18], would be a more appropriate control group.

It should be mentioned that both study groups were age-matched (P = 0.345), but not gender-matched (P = 0.038). However, previous studies by Pérez-Cabezés et al. [51] and Yilmaz et al. [20] showed no significant differences in the levels of blood DC precursors with regard to gender. CAD patients also had increased numbers of WBCs, a tendency towards increased numbers of monocytes and higher serum values of hsCRP, which are characteristics of atherosclerosis. Acute infection was excluded by absence of clinical symptoms or signs suggestive of local infectious disease. Results of...
the chest X-ray and urine sediment analysis were normal in all patients as well. Inverse associations between DC numbers and hsCRP levels were reported in previous studies of CAD patients [17,19].

Finally, the CAD group contained a patient with diabetes, who was treated orally with a sulfonylurea derivate. However, when the diabetic patient was excluded from the statistical analysis (resulting in \( n = 14 \) for the CAD group), all significant effects and conclusions were retained.

Conclusions

The present study demonstrates, for the first time, that the decrease in blood DCs in CAD is, at least partly, due to a defect in Flt3L levels, which regulate DC generation from the bone marrow. Moreover, pDCs of CAD patients were more resistant to \textit{in vitro} activation, indicating that they are not only numerically, but also functionally, impaired in CAD. In contrast, the decrease in circulating mDCs in CAD was presumably also partly explained by activation and subsequent migration to atherosclerotic plaques or lymphoid organs, as blood mDC numbers expressing CCR-7 and CD86 were lower in CAD patients compared with controls. However, the function of the mDCs that remained circulating in CAD patients appeared to be preserved and normal, since they responded in exactly the same way as control mDCs to \textit{in vitro} stimulation with LPS. These important changes indicate that both subsets of DCs are involved in CAD. Although it remains to be established whether DCs exert pro- or anti-atherogenic activities, the results of the present study have shown once more [18] that pDCs and mDCs behave very differently in CAD.

AUTHOR CONTRIBUTION

Ilse Van Brussel, Emily Van Vrė, Johan Bosmans and Hidde Bult were responsible for the concept and research question, study design and writing of the paper. Ilse Van Brussel, Emily Van Vrė and Hidde Bult were responsible for data collection/processing. Ilse Van Brussel and Hidde Bult were responsible for the statistical analysis. Christiaan Vrints, Johan Bosmans and Hidde Bult obtained the funding. Ilse Van Brussel, Emily Van Vrė, Johan Bosmans, Hidde Bult, Christiaan Vrints and Guido De Meyer were responsible for the critical revision and the final approval of the manuscript.

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