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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

Background: We investigated whether activation of circulating dendritic cells (DCs) or levels of FMS-like tyrosine kinase 3 ligand (Flt3L) and granulocyte-macrophage colony stimulating factor (GM-CSF), haematopoietic growth factors important for DC differentiation, could account for reduced blood DC numbers in coronary artery disease (CAD) patients.

Methods: Concentrations of Flt3L and GM-CSF were measured in plasma from CAD patients (n=15) and controls (n=12). Frequency and phenotype of myeloid (mDCs) and plasmacytoid (p)DCs were analysed by multicolour flow cytometry in fresh blood, and after overnight incubation with Toll-like receptor-4 or -7 ligands lipopolysaccharide or imiquimod. DC function was measured by interleukin (IL)-12 and interferon (IFN)-alpha secretion.

Results: Circulating numbers of CD11c⁺ mDCs and CD123⁺ pDCs and frequencies of CD86⁺ and CCR-7⁺ mDCs, but not pDCs, were declined in CAD. Also plasma Flt3L, but not GM-CSF, was lower in patients and positively correlated with blood DC counts. In response to lipopolysaccharide, mDCs upregulated CD83 and CD86, but CCR-7 expression and IL-12 secretion remained unchanged, similarly in patients and controls. Conversely, pDCs from patients displayed lower CD83 and CCR-7 expression after overnight incubation and showed a weaker imiquimod-induced upregulation of CD83 and IFN-alpha secretion.

Conclusions: Our data suggest that reduced blood DC counts in CAD are, at least partly, due to impaired DC differentiation from bone marrow progenitors. Decreased mDCs are presumably also explained by activation and subsequent migration to atherosclerotic plaques or lymph nodes. Although mDCs are functioning normally, pDCs from patients seemed to be both numerically and functionally impaired.

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 dendritic cells (DCs), 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 downregulation of endocytotic activity and upregulation of the immunoglobulin superfamily member CD83, of co-stimulatory molecules (CD40, CD80, CD86) and of antigen-presenting molecules (including class I and II major histocompatibility molecules), as well as production of cytokines [9].

There are two main DC subtypes with different functions: 1) myeloid (m)DCs, which are CD11c⁺ CD123⁻ and largely secrete interleukin-12 (IL-12) upon stimulation and 2) plasmacytoid (p)DCs, which are CD11c⁻ CD123^{bright} and produce mostly interferon [IFN]-alpha, 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 likely play an important role in the pathogenesis of atherosclerosis [8,11]. Many risk factors of atherosclerosis, like hyperlipidemia (high LDL levels) [12], oxidized (ox)LDL [13], free radicals [14], C-reactive protein (CRP) [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. Recently we and others demonstrated that blood DCs are numerically depleted in patients with coronary artery disease (CAD) [17-20]. The reasons for their decline are unclear, but it was not due to endothelial cell dysfunction or use of medication (β -blockers, statins) by CAD patients, which appeared to raise rather than suppress mDC counts. Moreover, neither changes in overall leukocyte counts, nor age could account for the thorough decreases in blood DCs [18].

Therefore, the present study investigated 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 and CD83. Indeed, *in vitro* studies, whereby DCs were generated from monocytes (moDCs) 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, CD86) and release cytokines (IL-12, IFN α) upon *in vitro* stimulation with lipopolysaccharide (LPS), an agonist of toll-like receptor (TLR)-4 expressed by mDCs and 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 FMS-like tyrosine kinase 3 Ligand (Flt3L) and granulocyte-macrophage colony-stimulating factor (GM-CSF) were determined, which are haematopoietic growth factors important for pDC and mDC differentiation and release from the bone marrow [25,26].

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 percutaneous transluminal coronary angioplasty (PTCA) with placement of a bare metal stent, and had $\geq 75\%$ 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 in 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, Erembodegem, Belgium) immediately before PTCA. Samples were analyzed within 4h after collection, or after overnight incubation with LPS or imiquimod (vide infra). 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 Iscove's Modified Dulbecco's Medium (IMDM) with L-glutamine, 25 mM HEPES (Invitrogen, Merelbeke, Belgium; v/v 1/1), and incubated in the absence or presence of either LPS (Escherichia coli 055:B5, 0.5 $\mu\text{g}/\text{mL}$, Sigma, Bornem, Belgium) or imiquimod (10 $\mu\text{g}/\text{mL}$, Invivogen, Toulouse, France) as described before [24]. The ligands were individually prepared according to the manufacturer's recommendations. Samples were incubated overnight at 37°C in a 5% CO_2 humidified atmosphere after addition of stimuli.

Six-colour flow cytometry

Table 2 represents staining procedures and monoclonal antibodies used in this study. Four colours were used to gate on 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 the C-C chemokine receptor type 7 (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.

200 μL blood was transported to each 5 mL FACS tube (BD Falcon, Erembodegem, Belgium). Erythrocytes were lysed by incubation with ammoniumchloride (Stemcell Technologies Inc., Grenoble, France) for 10 minutes at 4°C . Fc γ -receptors were blocked with mouse gamma globulins (BD Biosciences, Erembodegem, Belgium) for 10 minutes, 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, Erembodegem, Belgium) using automatic compensation settings, with further gating adjustments through the fluorescence minus one (FMO) method [30]. The strategy to select/gate DCs and to analyze their activation in whole blood samples is shown in figure 1. One million events were collected to visualize the total white blood cell population. Data-analysis was executed with the FacsDiva 6.1 software. The geometric mean fluorescence intensity (Geo-MFI) was used as a semiquantitative presentation of membrane molecule expression. DC numbers were

calculated as the proportion of DCs in the leukocyte gate (relative DC numbers) or those multiplied by the absolute white blood cell (WBC) count (absolute DC numbers), determined on an ABX Micros 60 (HORIBA, Deurne, Belgium).

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 IL-12 Quantikine ELISA Kits, R&D Systems, Bad Nauheim, Germany; VeriKine™ human IFN- α ELISA Kit, PBL Biomedical Laboratories, Boechout, Belgium), following the manufacturer's instructions. The assays employ the quantitative sandwich enzyme immunoassay technique. Each standard, blank and sample was run in duplicate on 96-well ELISA plates. Absorbance was read at 450 nm with reference absorbance of 540 nm. From the standard curve, samples with unknown concentration were interpolated. The detection limits were for GM-CSF 5.5 pg/mL, for Flt3L 22.7 pg/mL, for IL-12 2.6 pg/mL and for IFN- α 1.8 pg/mL.

Statistical analysis

Variables that failed normality were logarithmically transformed, or analyzed with the non parametric Mann-Whitney U test (IL-12 and IFN α). The latter data are shown as median, all others as mean \pm SEM. Differences between control subjects and CAD patients were determined with unpaired Student's t-tests. Antigen expression and cytokine release after overnight incubation with TLR ligands was analyzed by repeated measures ANOVA with activation (unstimulated versus LPS or imiquimod) as within subjects factor and group (control versus CAD) as between subjects factor. $P < 0.05$ was considered statistically significant. As data on circulating activated DCs were not available, we could not estimate sample sizes before starting the experiments. Yet, a post-hoc power analysis was performed [31].

Results

Characteristics of study populations

The age of both groups was not different, but male gender, hypertension and hyperlipidemia were more prominent in the CAD group (table 1). Other risk factors like smoking, low density lipoprotein (LDL) and the ratio LDL/high density lipoprotein (HDL) 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 white blood cells, a tendency toward increased numbers of monocytes and higher serum values of high sensitivity C-reactive protein (hsCRP). 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 (not shown) were lower in CAD patients compared to controls, strengthening previous investigations [17-20].

Basal frequencies of DCs expressing CD83 were very low (mDC $1.3 \pm 0.7\%$, pDC $0.2 \pm 0.1\%$), and not different between controls and CAD patients, whereas more DCs expressed CCR-7 (mDC $21.9 \pm 5.3\%$, pDC $13.7 \pm 2.9\%$) and CD86 (mDC $13.5 \pm 2.0\%$, pDC $19.7 \pm 2.8\%$). Interestingly, the frequency of mDCs expressing CCR-7 and CD86 was lower in the circulation of CAD patients (figure 2), whereas the percentage of pDCs expressing CCR-7 or CD86 remained unchanged. The expression level (estimated by means of 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 ($P = 0.24$), while plasma Flt3L was significantly lower in CAD compared to controls ($P = 0.005$) (figure 3A). Moreover, a positive correlation was found between plasma Flt3L and both pDC and mDC counts (figure 3B), whereas no significant correlations existed between plasma GM-CSF and mDC or pDC counts (data not shown).

Phenotypic changes upon *in vitro* stimulation.

Compared to basal measurements, overnight incubation of whole blood samples without any stimulus raised the expression (MFI) of CCR-7 and CD86 in mDCs without changing MFI of CD83 (data 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 (data not shown). Nevertheless, imiquimod stimulation resulted in a significant upregulation of all three activation markers ($P < 0.001$), both in pDCs from controls and patients. CAD seemed 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 imiquimod with respect to upregulation of CCR-7 and CD86 (figure 4). In contrast, the upregulation 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 5A). In contrast, IFN-alpha levels were increased after IQ-incubation, both in patients and controls. Interestingly, the IFN-alpha concentration in imiquimod-treated samples tended to be smaller in CAD patients ($P=0.085$, figure 5B). Indeed, the net rise induced by imiquimod (control 45 ± 10 pg/ml, CAD 22 ± 8 pg/ml, $P=0.036$, 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. Here 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, blood dendritic cell antigen (BDCA)-1 and BDCA-2 to identify mDCs and pDCs respectively [17-20]. Previously we demonstrated that the

decline of 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. Previously, we assessed the impact of endothelial cell function by measuring relationships between flow-mediated dilatation (FMD) 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 subsets, the current 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 oxidized LDL, heat shock protein, CRP, hypoxia, and altered NO synthase activity of the endothelium promote DC maturation, and raise CCR-7 expression and subsequent DC migration into lymph nodes [12,32]. Moreover, *in vitro* studies showed an increased activation status of monocyte-derived DCs 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 mentioning a weak expression of CD40 and CD86 on circulating BDCA-1⁺ mDCs and BDCA-2⁺ pDCs [19]. Since the expression level (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 to earlier *in vitro* findings [21-23]. Yet, less circulating mDCs expressing CCR-7 and CD86, but not pDCs, were noticed in CAD patients. This points to increased recruitment of mDC 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 did not yet reach sufficient maturation.

Apart from accumulation 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 recent study showed that in the absence of GM-CSF murine atherosclerotic lesions were significantly decreased in size and contained dramatically less DCs [37]. However, in our 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

homeostasis [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 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 lower in CAD patients and positively correlated with pDC and mDC counts. Although Kingston et al recently described that Flt3L deficiency primarily affects DC progenitors and pDC numbers [25], it has also been demonstrated that Flt3L treatment leads to an increase of both CD11c⁺ CD123⁻ mDC and CD11c⁻ CD123⁺ pDC numbers [43,44]. So 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 showed 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 (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 Della Bella's findings, who also demonstrated 61.5% CD86⁺ mDCs in the unstimulated tube (versus 92.0% in the LPS-stimulated tube) [24]. In response to LPS, mDCs vigorously upregulated expression (assessed by MFI) of CD83 and CD86, without significant rise of CCR-7. However, the lack of interaction in the repeated measurements ANOVA indicated that mDCs of control and CAD groups showed 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 of Tavakoli et al, who compared IL-12 secretion between healthy controls and patients with Hepatitis B virus using the same IL-12 ELISA kit [45]. Taken together, these data indicate, that the peripheral blood mDCs in CAD patients are numerically depleted, but functionally unaltered as indicated by the *in vitro* maturation test.

Also pDCs significantly raised expression of all maturation markers in response to *in vitro* stimulation with imiquimod, and this was accompanied by increased biosynthesis of IFN-alpha, the prototypic cytokine of pDCs. In contrast to 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 imiquimod, suggesting that CAD affects the upregulation of these markers during *in vitro* culture of pDCs. Even more importantly, patients showed a significantly weaker upregulation of the maturation marker CD83 after *in vitro* stimulation with imiquimod, as shown by the interaction in the repeated measures ANOVA. This was further corroborated by the attenuated secretion of IFN-alpha 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 to pDCs from control subjects.

Study limitations

The *in vitro* activation of DCs by imiquimod or LPS, as indicated by upregulation of CD83, CCR-7 and CD86, is presumably partly due to activation of other leukocytes 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, though bystander cells may have

activated the DCs, the flow cytometric analysis of the activation markers was strictly focused on DCs.

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

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

The current study was not designed to study the impact of medication. Previously, we showed that use of statins or beta-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 seen in pDCs [18]. So it seems unlikely that medication affected DC enumeration in the current 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 has to be noticed that both study groups were age-matched ($P=0.345$), but not gender-matched ($P=0.038$). However, previous studies by Pérez-Cabezas et al and Yilmaz et al showed no significant differences in the levels of blood DC precursors with regard to gender [20,51]. CAD patients also had increased numbers of white blood cells, 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 for 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 earlier studies of CAD patients [17,19].

Finally, the CAD group contained a patient with diabetes, who was treated orally with a sulphonylurea 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

This study demonstrates for the first time that the decrease of 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 *in vitro* activation, indicating that they are not only numerically, but also functionally impaired in CAD. In contrast, the decrease of 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 to 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 *in vitro* stimulation with LPS. These important changes indicate that both subsets of DCs are involved in CAD. Though it remains to be established whether DCs exert pro- or anti-atherogenic activities, the current data showed once more [18] that pDCs and mDCs behave very differently in CAD.

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Table 1: Characteristics of the control and coronary artery disease (CAD) populations.

	Controls	CAD patients	p-value
	N = 12	N = 15	
<i>Age (years ± SEM)</i>	56.3 ± 1.6	59.1 ± 2.2	0.345
<i>White blood cells (*10⁶/mL)</i>	6.2 ± 0.3	8.2 ± 0.9	0.050
<i>Monocytes (*10⁶/mL)</i>	0.4 ± 0.1	0.6 ± 0.1	0.088
<i>Risk Factors (%)</i>			
Gender	50.0	86.7	0.038
Smoking	33.3	30.0	1.000
Diabetes	0	6.7	0.362
Hypertension	8.3	46.7	0.030
Hyperlipidemia	8.3	60.0	0.006
<i>Lipids (mg/dL)</i>			
HDL	47 ± 4	42 ± 4	0.378
LDL	102 ± 8	120 ± 11	0.222
LDL/HDL	2.3 ± 0.3	3.0 ± 0.3	0.169
<i>Inflammation markers (pg/mL)</i>			
hsCRP	0.11 ± 0.03	0.53 ± 0.3	0.010
IL-12	3.2 ± 0.4	2.9 ± 0.5	0.404
IFNα	9.5 ± 1.4	16.2 ± 7.5	0.159
<i>Current medication (%)</i>			
Lipid-lowering therapy	0	67	<0.001
Aspirin	0	87	<0.001
Beta-blockers	0	40	0.020
ACE-inhibitors	0	20	0.231
Proton pump inhibitor	0	20	0.231

P values of unpaired Student's t-test (age, white blood cells, lipids, inflammation markers) and Chi Square (categorical variables). ACE = angiotensin converting enzyme

Table 2
Staining strategies and list of monoclonal antibodies used.

marker	Description	conjugate
<i>Staining 1</i>	<i>lineage, HLA-DR, CD11c, CD123, CD83, CCR-7</i>	
CD3	lineage marker - T-cells	PerCP-Cy5.5
CD14	lineage marker – monocytes	PerCP-Cy5.5
CD16	lineage marker - NK cells, granulocytin	PerCP-Cy5.5
CD19	lineage marker - B-cells	PerCP-Cy5.5
CD20	lineage marker - B-cells	PerCP-Cy5.5
HLA-DR	Major Histocompatibility Class molecule	APC-Cy7
CD11c	MDC marker - β 2 integrin	APC
CD123	PDC marker IL3R α	PE
CD83	maturation marker on DCs	FITC
CD197 (CCR-7)	migration to lymphoid organs	PE-Cy7
<i>Staining 2</i>	<i>lineage, HLA-DR, CD11c, CD86</i>	
lineage	stains T-,B- and NK-cells, monocytes, granulocytes	FITC
HLA-DR	Major Histocompatibility Class molecule	PerCP
CD11c	MDC marker - β 2 integrin	APC
CD86	co-stimulatory molecule	PE

Abbreviation: PerCP-Cy peridinin–chlorophyll–protein complex–cyanine; APC allophycocyanin; PE phycoerythrin; FITC fluorescein isothiocyanate
All antibodies were from BD Biosciences (Erembodegem, Belgium).

Table 3

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

	Controls N = 12	CAD patients N = 15	p-value
<i>Relative numbers (% of WBC)</i>			
MDC (CD11c ⁺)	0.31 ± 0.07	0.14 ± 0.01	< 0.001
PDC (CD123 ⁺)	0.18 ± 0.02	0.08 ± 0.01	< 0.001
<i>Absolute numbers (cells/mL)</i>			
MDC (CD11c ⁺)	19871 ± 5350	11462 ± 1033	0.023
PDC (CD123 ⁺)	10710 ± 900	6715 ± 657	0.001

Results of unpaired Student's t-tests.

Table 4

Basal expression (geometric MFI) of activation markers on circulating DC subsets from CAD patients and controls.

Activation marker	DC subset	Controls	CAD patients	p-value
CD83 ⁺	CD11c ⁺ mDC	271 ± 11	273 ± 4	0.691
	CD123 ⁺ pDC	261 ± 4	262 ± 8	0.906
CCR-7 ⁺	CD11c ⁺ mDC	3461 ± 684	2640 ± 162	0.246
	CD123 ⁺ pDC	2464 ± 185	2192 ± 118	0.213
CD86 ⁺	CD11c ⁺ mDC	683 ± 36	630 ± 26	0.263
	CD123 ⁺ pDC	821 ± 102	1053 ± 120	0.181

P-values of unpaired Student's t-test. *MFI = Mean Fluorescence Intensity*

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Figure legends

Figure 1 - Gating strategy of DCs with activation markers in whole blood samples. Panel A demonstrates the scatter density plot with the three obvious white blood cell populations (lymphocytes, monocytes and granulocytes) with the gate on the peripheral blood mononuclear cells (PBMCs). **Panel B** defines the total DC population, which is lineage⁻ HLA-DR⁺. **Panel C** represents staining 1: mDCs on the left 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. **Panel E** represents staining 2, where mDCs are defined as CD11c⁺ and pDCs as CD11c⁻. The third activation marker, CD86, was measured here on D0 (**F**).

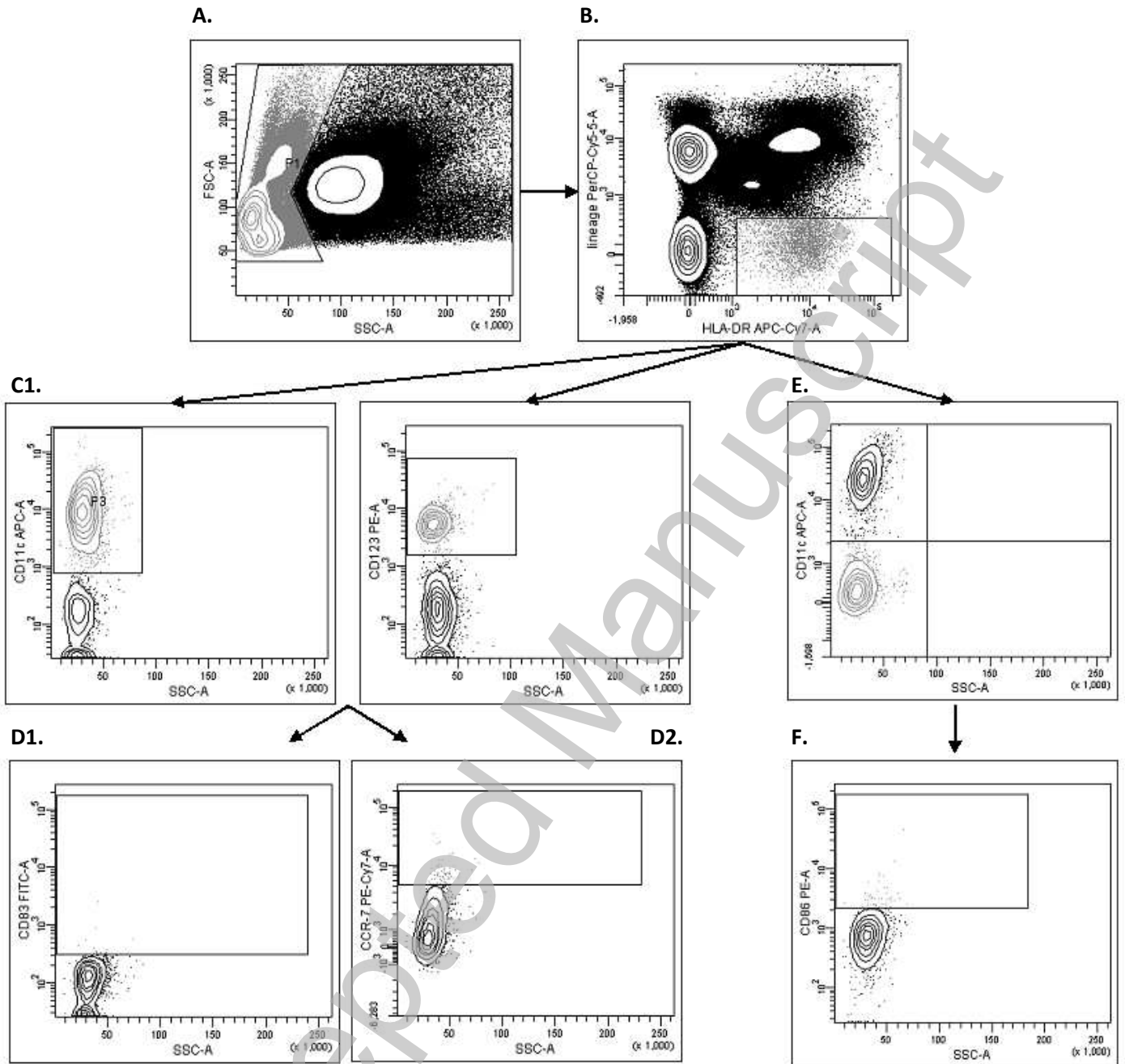
Figure 2 - Relative numbers of circulating activated DC-subtypes in CAD patients and controls. CAD patients have significantly lower numbers of CCR-7⁺ mDCs and CD86⁺ mDC compared to healthy individuals. Mean and SEM shown by bars. *P < 0.05, unpaired Student's t-test.

Figure 3 - Plasma concentration of haematopoietic growth factors GM-CSF and Flt3L. A. Plasma concentrations of GM-CSF were not significantly different between patients and controls, in contrast to plasma Flt3L, which was significantly lower in CAD. **B.** A positive correlation between plasma Flt3L concentrations and both mDC and pDC counts was found. Mean and SEM shown by bars. **P < 0.01, unpaired Student's t-test.

Figure 4 - DC activation upon incubation of whole blood with TLR-4 or -7 ligands. Incubation with LPS or imiquimod (IQ) resulted in a clear upregulation of CD83 and CD86 on mDCs and pDCs respectively. Imiquimod resulted also in significant upregulation of CCR-7 on pDCs. In addition to the effect of incubation, there was also an effect of CAD: patients' pDCs 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 upregulation of CD83 was less pronounced in pDCs from patients. Horizontal bars show mean and SEM. ***P < 0.001 for activation; ^xP < 0.05 and ^{xx}P < 0.01 for CAD effect.

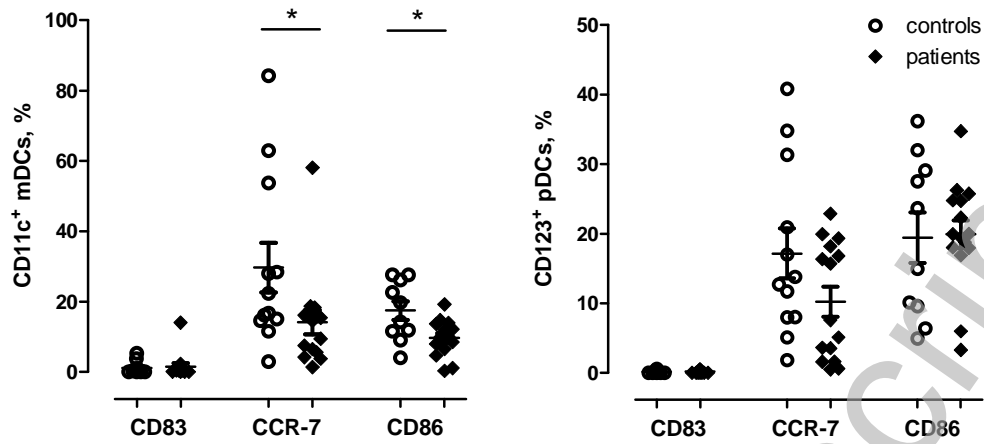
Figure 5 - Cytokine secretion after incubation with TLR-4 or -7 ligands. LPS failed to stimulate IL-12 production (**5A**), while imiquimod (IQ) induced a significantly higher INF-alpha production compared to unstimulated (---) samples (**5B**), but without significant (P=0.085) difference between controls and CAD patients. Horizontal bars show medians. Data analyzed with Wilcoxon matched pairs signed rank test (unstimulated versus LPS or imiquimod) or Mann Whitney U-test (differences between patients and controls). **P < 0.01

Figure 1



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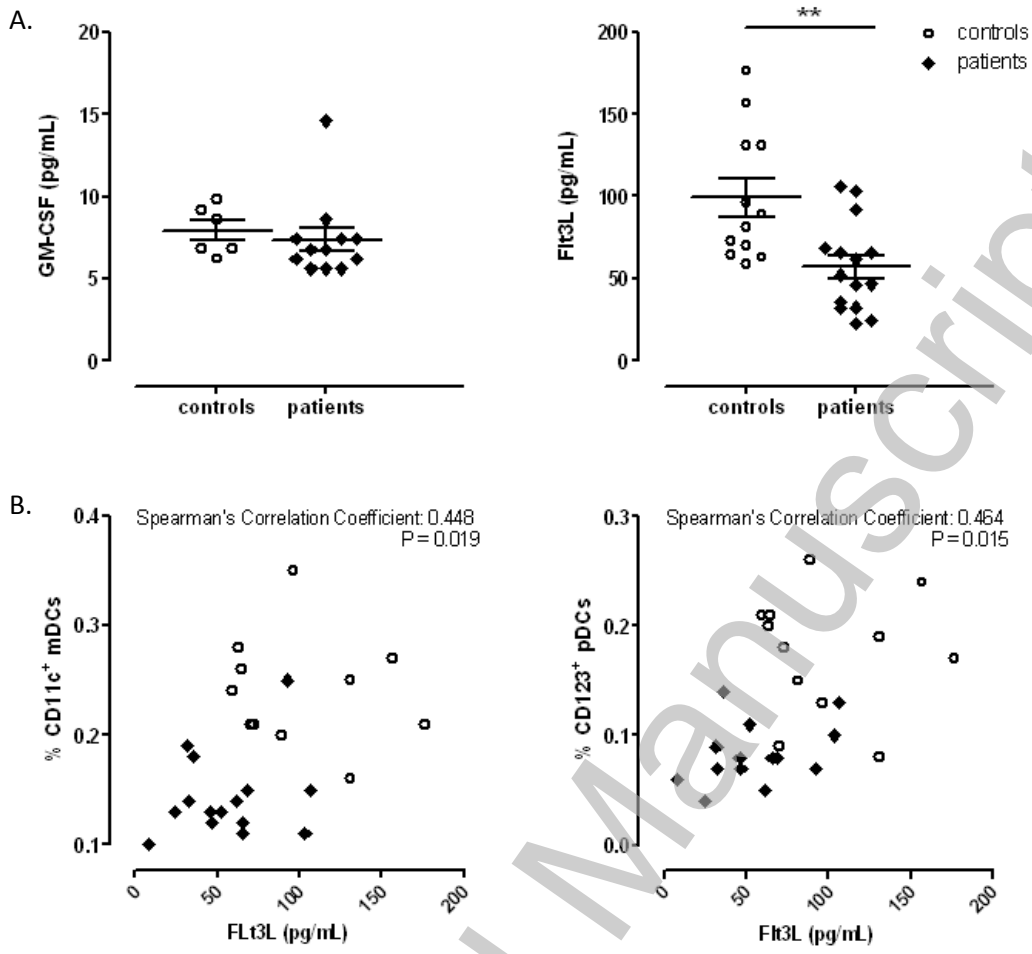
Figure 2



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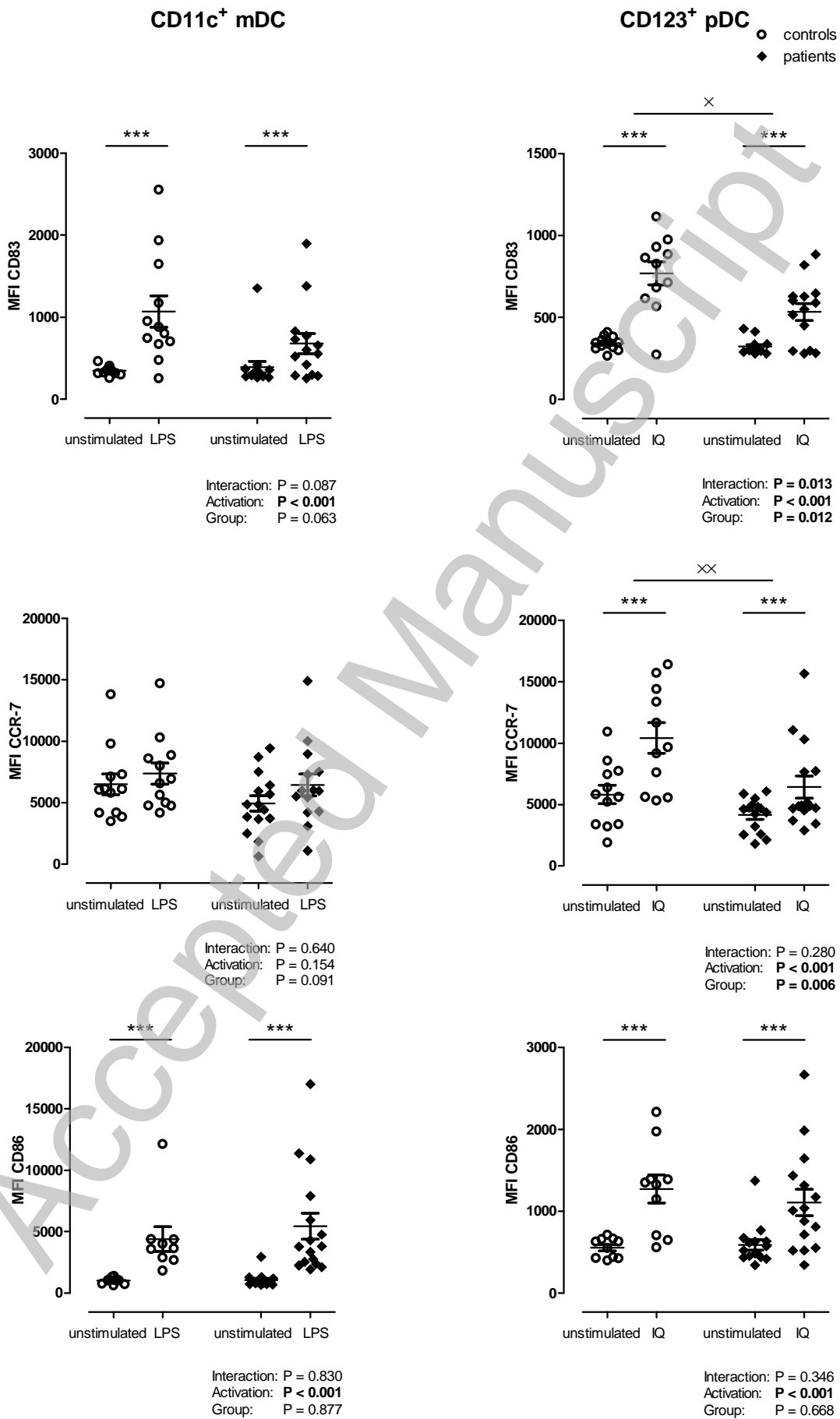
Figure 3



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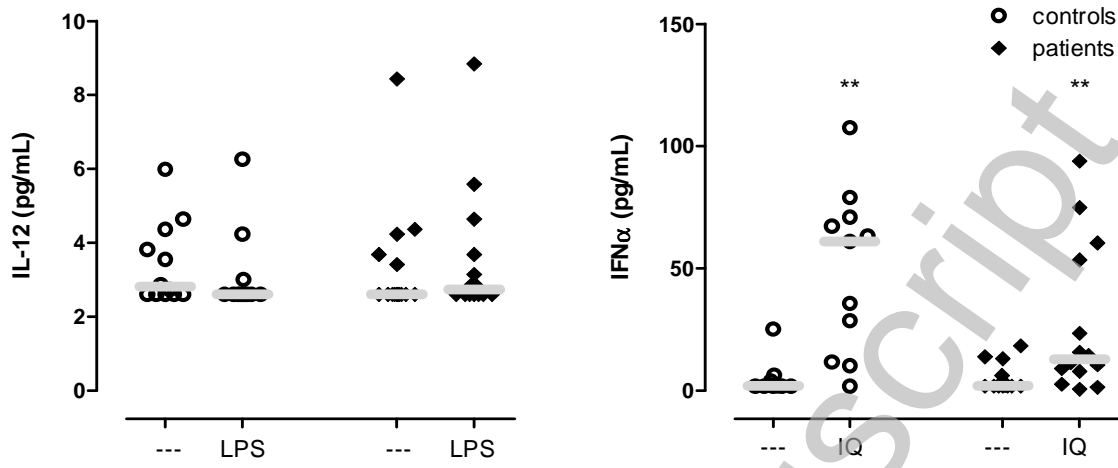
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Figure 4



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Figure 5



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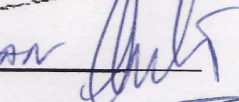



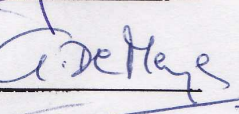
Title of manuscript: Decreased numbers of peripheral blood dendritic cells in patients with coronary artery disease are associated with diminished plasma Flt3 ligand levels and impaired plasmacytoid dendritic cell function

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- Statistical Analysis: IVB, HB
- Report Writing: IVB, EVV, JB, HB
- Obtained funding: CV, JB, HB
- Critical revision of the article: IVB, EVV, JB, HB, CV, GDM
- Final approval of the article: IVB, EVV, JB, HB, CV, GDM

The undersigned hereby accept responsibility for the conduct of this study and the interpretation and analysis of the data. By signing, the author(s) certify that standards for authorship have been met, the final manuscript has been read and approved, and that neither the submitted manuscript nor any significant part has been submitted for publication elsewhere or previously published.

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