Fluorescent activated cell sorting : an effective approach to study dendritic cell subsets in human atherosclerotic plaques

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Abbreviations used: DC: dendritic cell; FACS: fluorescence activated cell sorting; cDC: conventional dendritic cell; pDC: plasmacytoid dendritic cell.

Abstract

Different immune cell types are present within atherosclerotic plaques. Dendritic cells (DC) are of special interest, since they are considered as the 'center of the immuniverse'. Identifying inflammatory DC subtypes within plaques is important for a better understanding of the lesion pathogenesis and pinpoints their contribution to the atherosclerotic process. We have developed a flow cytometry-based method to characterize and isolate different DC subsets (i.e. $CD11b^+$, $Clec9A^+$ and $CD16^+$ conventional (c)DC and $CD123^+$ plasmacytoid (p)DC) in human atherosclerotic plaques. We revealed a predominance of pro-inflammatory $CD11b^+$ DC in advanced human lesions, whereas atheroprotective $Clec9A^+$ DC were almost absent. $CD123^+$ pDC and $CD16^+$ DC were also detectable in plaques. Remarkably, plaques from distinct anatomical locations exhibited different cellular compositions: femoral plaques contained less $CD11b^+$ and $Clec9A^+$ DC than carotid plaques. Twice as many monocytes/macrophages were observed compared to DC. Moreover, relative amounts of T cells/B cells/NK cells were 6 times as high as DC numbers.

For the first time, fluorescent activated cell sorting analysis of DC subsets in human plaques indicated a predominance of $CD11b^+$ cDC, in comparison with other DC subsets. Isolation of the different subsets will facilitate detailed functional analysis and may have significant implications for tailoring appropriate therapy.

Key words: dendritic cells, atherosclerosis, fluorescence activated cell sorting, plaques

1. Introduction

It has long been assumed that atherosclerosis could be simply explained by lipid accumulation in the vessel wall leading to endothelial dysfunction. However, atherosclerosis is now recognized as an inflammatory disease that stems from the involvement of both innate and adaptive immunity (Libby et al., 2002;Hansson et al., 2006;Hansson and Libby, 2006). Extensive evidence proves that different immune cell types are present within atherosclerotic plaques. Among them, dendritic cells (DC) are of special interest, since they are considered as 'the immuniverse center'. By presenting antigens, expressing costimulatory molecules and secreting cytokines, DC stimulate and activate other immune cells, including T cells, B cells and macrophages (Hansson et al., 2006).

Traditional approaches to study different immune cell types, their activation status and their cellular composition within atherosclerotic plaques are so far based on RT-PCR and immunohistochemistry. For example, immunohistochemistry has revealed that in non-diseased human arteries, only few DC ($S100^+$, $fascine^+$) are found in the subendothelial layer of the intima and in the adventitia (Van Vré et al., 2011b). Increased numbers are found in pathological intimal thickening. DC become even more numerous when the lesion progresses to a fibrous cap atheroma, and they further accumulate in complicated atherosclerotic plaques (Van Vré et al., 2011a). Both fully mature DC ($CD83^+$) and DC precursors ($BDCA\text{-}1^+$, $BDCA\text{-}2^+$) can be found in advanced lesions. Moreover, > 90% of the activated DC in plaques clusters with lesional T-cells (Van Vré et al., 2011a). Yet, immunohistochemistry does not allow functional studies (Grivel et al., 2011). This limitation can be overcome by isolating the cells from the tissue. The only technology that can accomplish the characterization and isolation of multiple immune cells simultaneously in individual plaques is multiparametric fluorescence activated cell sorting (FACS). However, only limited attempts were made to perform FACS on human

endarterectomy specimens, and a number of problems, such as compromised cell surface marker expression as well as intense autofluorescence resulting from plaque debris (extracellular lipids, dead cells) in the cell suspensions after enzymatic digestion, have been reported (Bonanno et al., 2000).

In this study, we have developed a new flow cytometry-based method to analyze immune cell composition and to characterize and isolate different DC subsets (i.e. $CD11b^+$, $Clec9A^+$ and $CD16^+$ conventional (c)DC and $CD123^+$ plasmacytoid (p)DC) in human plaques. DC isolation from human plaques has never been performed previously and was extremely challenging, given the low DC frequency and the strong interference of autofluorescence emitted by the cellular debris and lipids in the plaque. This method opens new possibilities to investigate immune aspects of the disease and to translate mechanistic insights from mouse studies into humans.

2. Materials and Methods

2.1 Patients

To develop the protocol for isolation of total DC and other immune cell populations from human plaques, 47 patients [39 men (83%) and 8 women (17%), age range: 49 to 89 (70 ± 1) years] were recruited from the clinical departments of Thoracic and Vascular surgery of the Antwerp University Hospital and ZNA Middelheim. Patients that were eligible for endarterectomy at the carotid (N=28; 60%) and femoro-popliteal level (N=19; 40%) were included.

For the study of DC subsets in human plaques, another 40 patients [25 men (63%) and 15 women (37%), age range: 55 to 88 years (71 ± 1)] were included. From these patients, 25 plaques (63%) were derived from the carotid artery and 15 from the femoral artery (37%). Baseline patient characteristics are shown in table 1.

Protocols were approved by the local Ethics Committee, and all research was based on written informed consent and proper arrangements for protecting the confidentiality of personal data of the individuals concerned.

2.2 Plaque handling after surgery

Atherosclerotic plaques were collected in RPMI 1640 (Life Technologies, Merelbeke, Belgium) and kept at room temperature until processing (1-2 hours after surgery). The endarterectomy specimens were dissected into small pieces depending on the sample size and digested by an optimized enzymatic mixture (vide results).

2.3 Optimization of the enzymatic digestion mixture

Fresh human peripheral blood mononuclear cells (PBMC) were digested for 2h or 24h at 37°C with collagenase IV (2.5 mg/mL or 1.25 mg/mL; Life Technologies) and DNase I (0.2 mg/mL; Roche, Mannheim, Germany), based on a recent paper in which activated T cells were isolated from atherosclerotic plaques (Grivel et al., 2011). PBMC were isolated from healthy volunteers by Ficoll density gradient centrifugation. After digestion, cells were blocked with mouse gamma globulins (BD Biosciences, Erembodegem, Belgium) and stained with mouse anti-human monoclonal antibodies: HLA-DR-PE, CD11c-PE, CD123-PE, CD11b-APC (all from BD Biosciences). Cell viability was assessed using propidium iodide.

2.4 Multiparametric FACS

DC were identified as negative for lineage (= a CD3, CD14, CD16, CD19, CD20, CD56 cocktail) and positive for the antigen presentation molecule HLA-DR and the β2-integrin CD11c (table 2). To eliminate the abundance of cell debris and extracellular lipids in the digested plaque suspensions, we used a gating strategy that allows separation of DC from nonspecific

fluorescent particles. In brief, antibodies were conjugated to FL-1 (FITC) and FL-2 (PE) specific fluorochromes, and FL-3 was used as a mock channel to identify nonspecific fluorescent particles. The totality of the staining tube was acquired and analyzed. Isotypically matched mouse immunoglobulins served as controls.

Next, FACS at 4°C allowed the physical separation of immune cell populations of interest from plaque tissue with a high degree of purity. Three populations were sorted: lineage^{neg} CD11c^{high} HLA-DR^{high} cells, lineage^{+} CD11c$^{neg/low}$ HLA-DR$^{neg/low}$ cells, and lineage^{+} CD11c^{+} HLA-DR^{+} cells.

For the DC subset analysis, cells were stained with an optimized 8-color mouse anti-human mAb panel, as described(Van Brussel et al., 2013) (table 2).

All measurements were performed on the FACSAria II (BD Biosciences). Data acquisition was done using FACSDiva 6.1.2.

2.5 Quantitative polymerase chain reaction (qPCR)

During FACS, isolated cells were collected in Qiazol. After chloroform addition, the homogenate was separated into aqueous and organic phases by centrifugation. The upper aqueous phase containing the RNA was extracted and ethanol was added. The sample was then applied to the RNeasy MinElute spin column from the miRNeasy Micro Kit (Qiagen, Venlo, The Netherlands). Finally, high-quality RNA was eluted in RNase-free water. Total RNA was reverse transcribed to first-strand complementary (c)DNA using SuperScript reverse transcriptase II (Life Technologies). Real-time PCR was performed using an ABI 7300 thermal cycler (Applied Biosystems, Halle, Belgium) using the SensiMix SYBR kit (GC Biotech, London, United Kingdom). The PCR conditions were: 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min, and 40 cycli of 95°C for 15 sec, 60°C for 1 min and 72°C for 30 sec. Nucleotide primers (Sigma, Diegem, Belgium) were used for detection and quantification of GAPDH and HPRT as reference genes, and resident plaque cell markers to investigate the purity of the sorted populations. Data

were analyzed using the ΔΔCT method. The lineage^{+} (CD11c HLA-DR)$^{+}$ group was used as the control group, the other populations as experimental groups. The fold change was calculated as 2-ΔΔCt.

2.6 Immunohistochemistry

To evaluate the efficiency of cell liberation from the plaques, we analyzed plaque tissue before and after enzymatic digestion. Endarterectomy specimens were cut into small pieces. One half was digested and subsequently formalin fixed and paraffin embedded, whereas the adjacent segments were directly formalin fixed and embedded in paraffin (without digestion) or OCT embedded for cryosections. Immunostaining was performed on 5 µm sections on all segments using markers listed in table 2. Images were analyzed using ImageJ.

2.7 Statistical analysis

Differences between plaques derived from the carotid and femoral artery were determined with unpaired Student's t-tests. Differences in epitope expression between digested and undigested samples were studied with the paired Student's t-test. Variables that failed normality were logarithmically transformed, or analyzed with the nonparametric Mann-Whitney U test or Wilcoxon matched pairs signed rank test. Data are shown as mean ± SEM. Statistical analysis was performed using Prism 5.0 (GraphPad, La Jolla, CA). $P < 0.05$ was considered statistically significant.

3. **Results and Discussion**

3.1 DC can be isolated from human plaques by enzymatic digestion.

To evaluate whether the digestion mixture preserves the cell surface marker expression, different collagenase IV concentrations (2.5 mg/mL, 1.25 mg/mL) were tested on PBMC from healthy volunteers for various incubation times (2h vs. 24h). The expression of CD11c, HLA-DR, CD11b and CD123 was analyzed by flow cytometry. The lowest collagenase IV concentration incubated for 24h resulted in significant reduction of CD11b, HLA-DR, CD11c and CD123 expression by 8.67±2.11%, 10.48±1.28%, 22.78±5.70% and 20.76±5.31%, respectively. Because of these rather strong effects, the digestion time was reduced to 2h. PBMC treatment with 2.5 mg/mL collagenase IV did not alter the marker expression compared to the untreated PBMC (2.79±0.79%, 3.04±0.72% and 6.17±2.76% reduction of CD11b, HLA-DR and CD123 expression, respectively). This was also true for 2h PBMC treatment with 1.25 mg/mL collagenase IV (reduction of CD11b and HLA-DR expression by 1.49±1.25% and 3.70±2.33%, respectively) (fig. 1).

To liberate cells from atherosclerotic plaques, we applied collagenase IV at concentrations (2.5 mg/mL and 1.25 mg/mL) and incubation times (2h) that did not significantly affect DC surface marker expression in PBMC. Collagenase IV at 2.5 mg/mL liberated more DC from human atherosclerotic tissue than did digestion with 1.25 mg/mL, as evaluated by the DC count in the lineage^{neg} CD11c^{high} HLA-DR^{high} gate. Therefore, treatment of plaques with 2.5 mg/mL collagenase IV and 0.2 mg/mL DNase I for 2h at 37°C was chosen as the most efficient digestion mixture with DC cell surface marker and viability preservation.

3.2 The optimized enzymatic digestion liberates more than 80% of plaque immune cells.

To verify whether or not the immune cells were liberated from the plaque after digestion and whether or not the digested plaque sample was contaminated by smooth muscle cells belonging to the inner layers of the tunica media, we studied the residual tissue left after enzymatic digestion immunohistochemically. 82.4±2.4% of immune and endothelial cells was liberated

from the plaque tissue using 2.5 mg/mL collagenase IV and 0.2 mg/mL DNase I for 2h at 37°C. In contrast, only 18.9±1.0% of smooth muscle cells was liberated (figure 2).

3.3 DC numbers/gr plaque tissue did not differ between plaques from the carotid and femoral artery.

The mock channel we used to gate out the nonspecific autofluorescent cellular debris in the plaque cell suspension allowed in depth analysis of the cellular components in the plaque. An average cell suspension from a digested plaque contained 20,908±2,712 DC, which were defined as negative for lineage markers, and highly positive for CD11c and HLA-DR.

We also evaluated the liberated DC number per gram of the plaque tissue's wet weight. On average, there were 8,257±1,022 DC per gram plaque tissue, and the average plaque weight was 2.29±0.36 gram.

The total plaque cell number from carotid plaques was significantly lower than that of femoral plaques (23±4 $x10^6$ vs. 37±7 $x10^6$; P=0.024). Also, carotid plaques contained significantly lower DC numbers than femoral plaques (18,063±3,441 vs. 29,176±4,643; P=0.023). This might be explained by the higher plaque weight from the femoral artery compared to carotid plaques (2.03±0.56g vs. 2.86±0.32g; P=0.003). After all, total cell numbers per gram (12.5±1.9 $x10^6$ vs. 13.0±2.4 $x10^6$; P=0.689) and DC numbers per gram (7,616±1,191 vs. 10,857±2,062; P=0.195) did not differ between plaques from the carotid and femoral artery.

We also investigated counts of other immune cell populations isolated from human plaques. From the liberated $lineage^+$ cells, 23.4±1.3% was also positive for CD11c and HLA-DR, most likely depicting monocytes and macrophages, whereas 76.6±1.3% was negative for or had low expression of CD11c and HLA-DR, presumably representing T cells, B cells and NK cells. For all those immune cells, no differences could be detected between plaques from the carotid and the femoral artery (table 3).

3.4 qPCR confirmed the purity of the immune populations sorted by FACS.

The FACS procedure of human plaques was validated by analyzing RNA expression in the three sorted populations: lineage^{neg} (CD11c HLA-DR)^{high} cells (~DC), lineage^{+} (CD11c HLA-DR)$^{+}$ cells (~monocytes, macrophages) and lineage^{+} (CD11c HLA-DR)$^{neg/low}$ cells (~T cells, B cells, NK cells).

Using the ΔΔCt method, the expression of genes of interest (GOI) was first normalized to endogenous references (GAPDH and HPRT). Then, the resulting ΔCt value for each GOI was normalized to that of the lineage^{+} (CD11c HLA-DR)$^{+}$ cells. qPCR confirmed the purity of the sorted populations. For example, the lineage^{neg} (CD11c HLA-DR)^{high} cells indeed showed the highest expression of typical markers expressed on DC (CD1a, S100B, CCR-5, CCR-7, CD11c, HLA-DR, CD11b) and very low expression of non-DC markers (e.g. CD14, CD20, CD31, vWF). Similar results were obtained for the other sorted populations (fig. 3). The high purity of the sorted cell populations indicate that these cells can be used for further functional analyses, by investigating the mRNA content or by cell culture studies.

3.5 Four different DC subsets can simultaneously be detected in human atherosclerotic lesions.

To analyze the four different DC subsets, ten-parameter flow cytometry(Van Brussel et al., 2013) was performed to identify pDC (CD123^{+}), Th1/Th17-inducing DC (CD11b^{+}), a specific CD11b^{+} DC subset (CD16^{+}) and crosspresenting DC (Clec9A^{+}) (Ganguly et al., 2013). The gating strategy is presented in figure 4. Recognizing the inflammatory DC types within plaques is important for a better understanding of the pathogenesis and may have significant implications for tailoring therapy. It is still unclear if all DC in human plaques serve as antigen-presenting cells, and very little is known about a preferential DC subset that is responsible for T-cell induced inflammation in the plaque.

In the first cohort, we were able to identify DC as well as other immune cells. However, when all three characterized immune cell populations were taken together, they only consisted of 1.0±0.1% of total plaque suspension (range: 0.3 to 5.6%). To verify whether this was correct, we included the common leukocyte marker CD45. Indeed, the relative number of $CD45^+$ cells in the plaque was 1.0±0.1% (range: 0.2 to 3.9%). This is consistent with previous studies(Butcher et al., 2011). It is very likely that human plaques additionally contain large numbers of red blood cells, platelets, vascular smooth muscle cells and other cell types that lack CD45 expression. Within the $CD45^+$ population, we again defined general DC as $lineage^{neg}$ and $HLA\text{-}DR^+$, and we further classified DC subsets. pDC were identified as $lineage^{neg}$ $HLA\text{-}DR^+$ $CD123^+$ and consisted 19.5±2.1% of total DC. cDC were identified as $lineage^{neg}$ $HLA\text{-}DR^+$ $CD11c^+$ (50.6±2.9% of total DC) and they were subdivided into 1) $CD11b^+$ DC, which are described to promote atherosclerosis(Weber et al., 2011), and consisted 85.8±1.1% of cDC, 2) monocyte-derived $CD16^+$ DC, which consisted 8.8±1.3% of cDC, and 3) $Clec9A^+$ DC, which are described to be atheroprotective (Choi et al., 2011) and constituted 3.1±0.7% of the cDC. Weber et al. showed that mouse aortic tissue contained at least two DC types: $CD11b^+$ DC and $CD103^+$ DC (Weber et al., 2008;Bedoui and Heath, 2011). It has been reported that human tissues contain $Clec9A^+CD141^{high}$ cross-presenting DC with functional homology to mouse $CD103^+$ nonlymphoid tissue DC (Haniffa et al., 2012;Caminschi et al., 2008;Huysamen et al., 2008). Therefore, Clec9A was included in this study instead of CD103. A recent mice study showed that $CD11b^+$ DC characterized by CCL17 production, promote atherosclerosis and inhibit regulatory T cell (Treg) responses (Weber et al., 2011), whereas the presence of mouse $CD103^+$ DC is inversely correlated with aortic lesions size, suggesting an atheroprotective role (Choi et al., 2011) which might also be related to regulation of Treg homeostasis (Ait-Oufella et al., 2006). Since here, a $CD11b^+$ DC predominance was observed over $Clec9A^+$ DC in human plaques, it raises the possibility that local Treg cell responses during atherosclerosis are indeed

regulated through a balance between inhibitory effects by $CD11b^+$ DC and activating signals exerted by $CD103^+$ DC (Bedoui and Heath, 2011).

3.6 Plaques from distinct anatomical locations exhibit differences in their DC subsets.

$CD11b^+$ cDC were the most abundantly present DC subset in plaques derived from both anatomical locations (carotid: 85.5±1.7; femoral: 86.4±1.1%; P=0.832), whereas $Clec9A^+$ cDC were almost absent, although their relative numbers were significantly lower in femoral plaques (4.1±1.0 vs. 1.1±0.3%; P=0.013). On the contrary, $CD16^+$ cDC numbers were significantly higher in femoral plaques (6.3±0.9 vs. 13.5±2.8; P=0.004). $CD123^+$ pDC did not significantly differ between carotid and femoral plaques (22.2±2.9 vs. 14.5±2.5; P=0.14) (fig. 5). Since the weight of femoral plaques was again higher than the weight of carotid plaques (1.5±0.1 vs. 3.7±0.5 gr; P<0.001), we also calculated the numbers of cDC subsets and pDC per gram of plaque tissue. pDC and $CD16^+$ DC numbers did not significantly differ between plaques from the carotid and the femoral artery (pDC: 1802±570 vs. 981±460; P=0.07; $CD16^+$ cDC: 227±50 vs. 536±335; P=0.98). Remarkably, plaques from the femoral artery contained lower numbers of $CD11b^+$ cDC (3398±584 vs. 2158±757; P=0.04) and $Clec9A^+$ cDC (142±37 vs. 39±22; P=0.05) compared to carotid plaques. That plaques from distinct anatomical locations differ in their cellular composition is consistent with a recent study demonstrating more M1-like pro-inflammatory macrophages, and less atheroprotective M2-cells in carotid lesions compared to femoral lesions (Shaikh et al., 2012). However, based on our results, it cannot be stated that carotid lesions exhibit a more pro-inflammatory plaque phenotype, since atheroprotective $Clec9A^+$ DC numbers are also increased in carotid plaques. In future studies, it would be interesting to investigate a possible relationship between DC subset phenotype and the compositional markers of plaque (in)stability (e.g. calcium, fibroconnective tissue, lipid, lymphocytic cellular infiltrate) in the two distinct anatomical beds.

4. Conclusions

Here, we provided important information on how to process plaque material to generate reliable data on DC numbers in different conditions which enables a more precise interpretation of their immunological function. We developed a protocol and applied a gating technique in multiparameter flow cytometry to identify and isolate DC (including subsets) from human atherosclerotic lesions. We revealed a predominance of pro-inflammatory $CD11b^+$ DC, whereas atheroprotective $Clec9A^+$ DC are almost absent. $CD123^+$ pDC as well as $CD16^+$ DC are both detectable in plaques, however, their exact role in the disease process needs to be further studied.

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6. Disclosures

The authors declare no conflicts of interests.

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Table 1: Patient characteristics

	plaques from carotid artery	**plaques from femoral artery**	**Significance[#]**
First part of the study (immune cell populations) N = 47			
% of total plaques	60	40	
Age (years)	72 ± 2	68 ± 2	NS
Male gender (%)	75	95	NS
Degree of carotid artery stenosis (%)	84 ± 3	77 ± 4	NS
Risk factors (%)			
hypercholesterolemia	72	73	NS
diabetes mellitus	14	17	NS
hypertension	80	67	P = 0.054
smoking	52	82	P < 0.001***
family history	25	10	P < 0.01**
previous ischemic intervention	32	420	NS
Medications (%)			
aspirin	95	100	P = 0.059
statins	67	61	NS
beta-blockers	62	33	P < 0.001***
calcium channel blockers	33	39	NS
ACE-inhibitors	38	39	NS
Second part of the study (DC subsets) N = 40			
% of total plaques	63	37	
Age (years)	73 ± 2	70 ± 2	NS
Male gender (%)	64	67	NS
Degree of artery stenosis (%)	81 ± 2	84 ± 4	NS
Risk factors (%)			
hypercholesterolemia	83	93	P < 0.05*
diabetes mellitus	32	33	NS
hypertension	100	87	P < 0.001***
smoking	61	64	NS
family history	12	31	P < 0.01**
previous ischemic intervention	30	53	P < 0.01**
Medications (%)			
aspirin	91	93	NS
statins	74	87	P < 0.05*
beta-blockers	57	50	NS
calcium channel blockers	23	50	P < 0.001***
ACE-inhibitors	41	60	P < 0.05*

#: significance indicates possible significant differences between plaques derived from the carotid and plaques derived from the femoral artery.

Table 2 - *Monoclonal antibodies used for the immunophenotyping of cell subsets assessed in this study.*

Marker	Conjugate	clone	subsets	source	dilution
Flow Cytometry					
CD45	APC-H7	2D1	leukocytes	BD	
lineage 1	FITC	SK7 (CD3), SJ25C1 (CD19), L27 (CD20), MΦP9 (CD14), 3G8 (CD16), NCAM16.2 (CD56)	T lymphocytes, monocytes, macrophages, neutrophils, eosinophils, B lymphocytes, NK cells	BD	
lineage 2	FITC	SK7 (CD3), SJ25C1 (CD19), L27 (CD20), MΦP9 (CD14), NCAM16.2 (CD56)	T lymphocytes, monocytes, macrophages, neutrophils, eosinophils, B lymphocytes, NK cells	BD	
HLA-DR	PE	G46-6	DC, monocytes, macrophages	BD	
HLA-DR	V500	G46-6	total DC	BD	
CD11c	PE	B-ly6	DC, monocytes, macrophages	BD	
CD11c	PE-CF594	B-ly6	cDC	BD	
CD11b	BV421	ICRF44	BDCA-1^{+} cDC	BD	
CD16	PerCP-Cy5.5	3G8	moDC	BD	
Clec9A	APC	483409	BDCA-3^{+} cDC	R&D Systems	
CD123	PE	9F5	pDC	BD	
Immunohistochemistry					
CD45	purified	HI30	leukocytes	BD	1/5000
CD11b	purified	ICRF44	cDC	BD	1/300
CD11c	purified	B-ly6	DC, monocytes, macrophages	BD	1/3000
S100B	purified	4C4.9	DC	DAKO	1/1000
CD3	purified	N/A	T cells	Spring Bioscience	1/300
CD7	purified	15	NK cells	Immunosource	1/300
CD68	purified	PG-M1	macrophages	DAKO	1/3000
CD31	purified	JC70A	Endothelial cells	DAKO	1/2000
α-actin	purified	1A4	Smooth muscle cells	Sigma	1/5000

Abbreviations: NK cells: natural killer cells, DC: dendritic cells, cDC: conventional dendritic cells; pDC: plasmacytoid dendritic cells; moDC: monocyte-derived dendritic cells

Table 3: *Immune cell populations present in plaques from the carotid and femoral artery*

		carotid	femoral	P-value
total number of plaque cells (x 10^6)		23 ± 4	37 ± 7	P = 0.024*
plaque weight (gram)		2.03 ± 0.56	2.86 ± 0.32	P = 0.003**
% $Lineage^{neg}$ ($CD11c^{high}$ $HLA\text{-}DR^{high}$ #)	total DC population	0.14 ± 0.04	0.10 ± 0.02	P = 0.813
% $Lineage^{+}$ ($CD11c^{-}$ $HLA\text{-}DR^{+}$ #)	monocytes, macrophages	0.20 ± 0.03	0.24 ± 0.09	P = 0.813
% $Lineage^{+}$ ($CD11c^{neg/mid}$ $HLA\text{-}DR^{neg/mid}$ #)	T cells, B cells, NK cells	0.64 ± 0.07	0.73 ± 0.17	P = 0.964
% $Lineage^{-}$ ($CD11c^{mid}$ $HLA\text{-}DR^{mid}$ #)	basophils, mast cells, others	0.46 ± 0.07	0.54 ± 0.14	P = 0.768

% of non-autofluorescent plaque cells;
**P < 0.05; **P < 0.01*

Abbreviations: DC: dendritic cell; NK cells: natural killer cells

Figure legends:

Figure 1: *Evaluation of the effect of digestion mixture on the expression of cell surface markers*
PBMC isolated from healthy volunteers were incubated with different collagenase IV concentrations (2.5 mg/mL and 1.25 mg/mL) and various incubation times (2h vs. 24h). The expression of cell surface markers CD11c, HLA-DR, CD11b and CD123 was analyzed by flow cytometry. Data are shown as mean fluorescence intensity (MFI), n=5 ** $p<0.01$, *** $p< 0.001$ vs. control.

Figure 2: *Evaluation of the efficiency of cell liberation from plaques.* Pieces of plaque tissue before and after digestion with a mixture of 2.5 mg/mL collagenase IV and 0.2 mg/mL DNase I for 2h at 37°C. A. Immunohistochemical staining of immune cells. Figures are representative for 3 independent samples. Magnification 10x. B. Quantification of immunohistochemistry using Image J.

Figure 3: *qPCR validation of the purity of the sorted immune populations.*
The validity of the FACS procedure of human atherosclerotic plaques was evaluated by analyzing RNA expression in the DC and other immune cell populations. A. In total, three cell populations were sorted out of plaques by FACS: lin^{neg} (CD11c HLA-DR)^{high}, lin^{+} (CD11c HLA-DR)^{high} and lin^{+} (CD11c HLA-DR)$^{neg/mid}$ cells. B. RNA expression analysis in the DC and other cell populations confirmed the validity of the FACS procedure of human plaques.

Figure 4: *Gating strategy for DC subset analysis.*
A. Haematopoietic cells were defined as brightly stained with CD45-APC-H7. B. Among haematopoietic cells, lineage negative cells were selected to identify the total DC population. C. Lineage-negative haematopoietic cells were further divided into $CD11c^{neg}$ HLA-DR^{+} pDC and $CD11c^{+}$ HLA-DR^{+} cDC (D.). Based on their expression of CD123, pDC were identified (E.), whereas cDC were further subdivided based on their expression of Clec9A (G.), CD11b (F.) or CD16 (H.) to differentiate between $CD1c^{+}$ cDC, $CD141^{+}$ cDC and $CD16^{+}$ DC respectively.

Figure 5: *$CD11b^{+}$ DC are the most dominant DC subset in human plaques*. Also, DC subset composition differs in plaques of the carotid artery and the femoral artery. Absolute numbers of $CD123^{+}$ pDC and $CD16^{+}$ DC did not significantly differ between plaques from the carotid and the femoral artery, however, plaques from the femoral artery contained lower numbers of $CD11b^{+}$ mDC and Clec9A^{+} mDC. Cell numbers were counted per gram plaque tissue. Data were analyzed with the nonparametric Mann-Whitney U test and shown as mean ± SEM. N = 14 for carotid artery, N = 10 for femoral artery. *$P < 0.05$.

Figure 1 (single column fitting image):

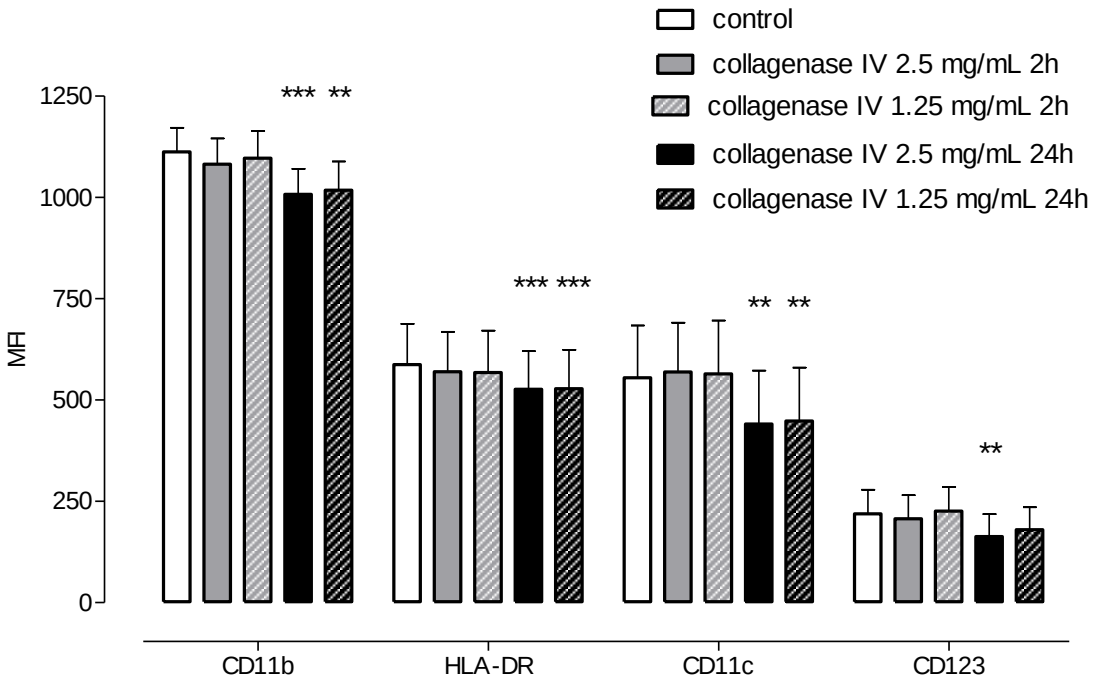

Figure 2 (2 column fitting image):

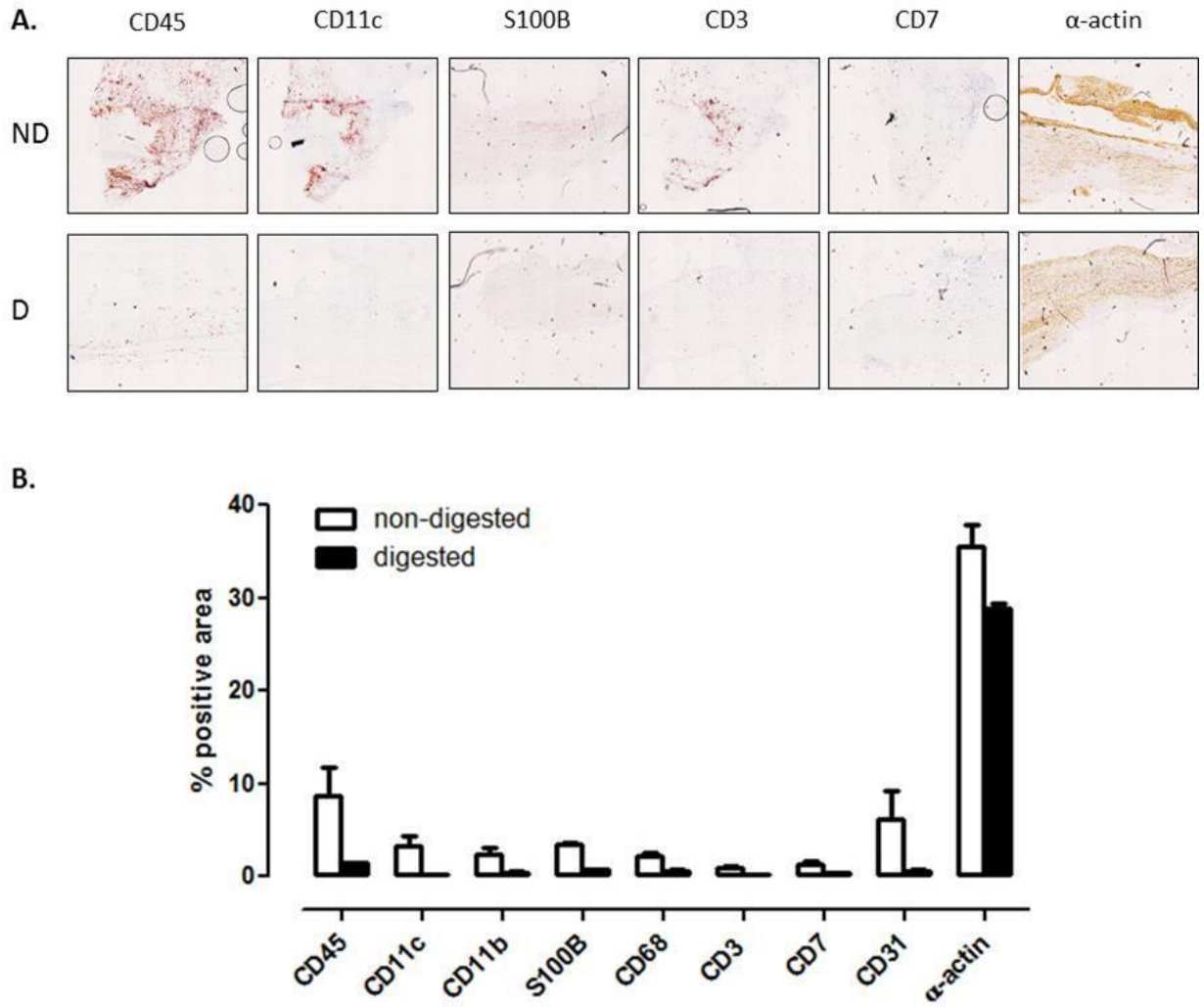

Figure 3 (2 column fitting image):

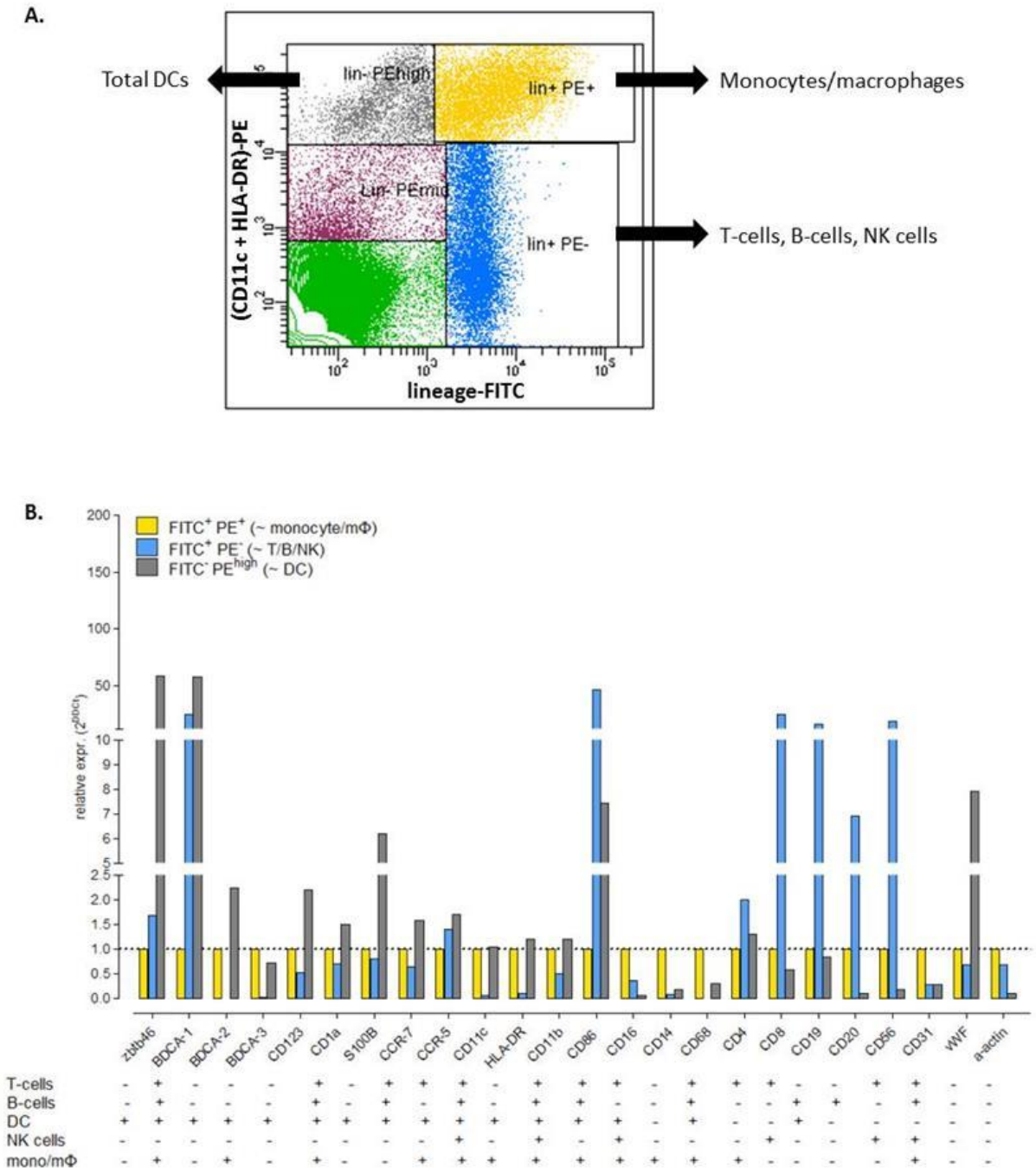

Figure 4 (2 column fitting image):

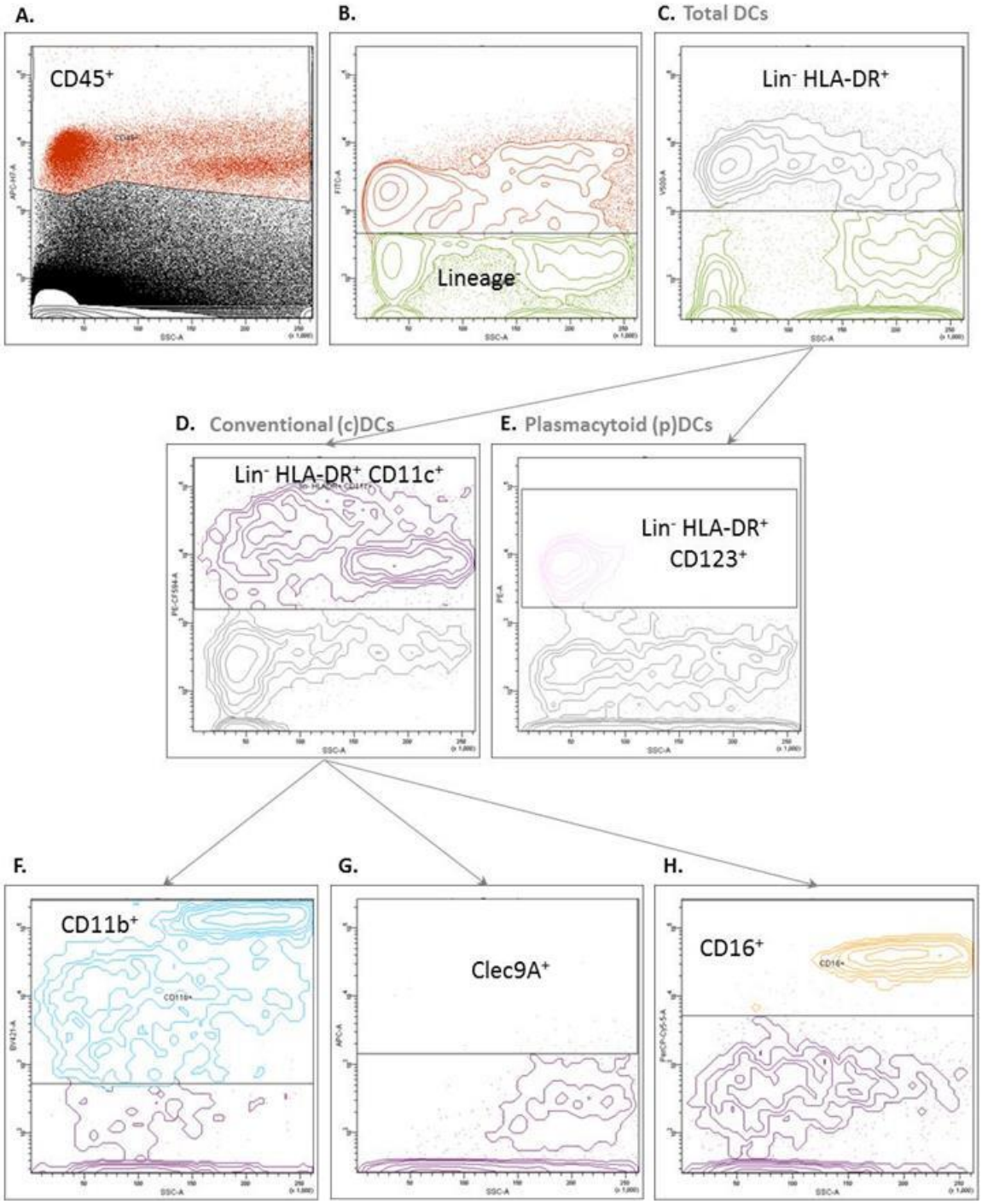

Figure 5 (single column fitting image):