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From Cell Biology to Tissue Engineering

In-situ analysis of mast cells and dendritic cells in coronary atherosclerosis in chronic kidney disease (CKD)

D.L. Wachter[1]*, D. Neureiter[5]*, V. Câmpean[4], K.F. Hilgers[3], M. Büttner-Herold[4], C. Daniel[4], K. Benz[2+] and K. Amann[1+]

[1]Department of Pathology, [2]Department of Pediatrics, [3]Department of Nephrology, [4]Department of Nephropathology, Friedrich-Alexander University (FAU) of Erlangen-Nürnberg, Germany and [5]Department of Pathology, University of Salzburg, Salzburg, Austria

*: shared first author

+: shared senior author

Summary. Aims. Mast cells (MC) and dendritic cells (DC) have immune modulatory function and can influence T-cell activity. Both cell types have been found in atherosclerotic plaques and are thought to play an important role for plaque stability. Compared to matched segments of the non-renal population, patients with chronic kidney disease (CKD) show a more pronounced and more aggressive course of atherosclerosis with higher plaque calcification and significantly higher complication rates. It was the aim of this study to analyze the number and localization of MCs and DCs, macrophages, T- and B-cells as well as the expression of markers of inflammation such as CRP and NFκB in calcified and non-calcified atherosclerotic plaques of patients with CKD and control patients. Methods. Fifty coronary atherosclerotic plaques from patients with endstage CKD (CKD, n=25) and control (n=25) patients were categorized according to the Stary classification and investigated using immunohistochemistry (markers for MC, DC, T, B, macrophage and NFκB). Expression was analyzed separately for the complete plaque area as well as for the different plaque subregions and correlations were analyzed. Results. We found only very few DCs and MCs per lesion area with slightly increased numbers in calcified plaques. MCs per plaque area were significantly more frequent in CKD than in control patients and this was independent of plaque calcification. MCs were most frequently found in the shoulder and basis of the plaque. DCs per plaque area were significantly less in calcified plaques of CKD compared to control patients. In control, but not in CKD patients, DCs were significantly more frequent in calcified than in non-calcified plaques. Within the plaques, DCs were similarly distributed between all 4 subregions. Conclusions. Coronary atherosclerotic plaques of CKD patients showed a significantly higher number of MCs whereas DCs were less frequent compared to control patients particularly if plaques were calcified. These findings might indicate a potential proinflammatory role of MCs, but not of DCs in atherosclerotic lesions of CKD patients, adding another characteristic of advanced atherosclerosis in these patients.

Key words: Atherosclerosis, Chronic kidney disease, Mast cells, Dendritic cells, Cardiovascular disease

Offprint requests to: Prof. Dr. Kerstin Amann, Department of Nephropathology, University of Erlangen-Nürnberg, Krankenhausstr. 8-10, 91054 Erlangen, Germany. e-mail: kerstin.amann@uk-erlangen.de

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Introduction

Patients with chronic kidney disease (CKD) very early in the course of the disease develop severe atherosclerosis of the aorta, coronary and peripheral arteries which is further characterized by a more aggressive course and in particular marked calcification of the arterial media and the atherosclerotic plaques (Schwarz et al., 2000; Amann, 2008). As a consequence, cardiovascular complications in these patients are

frequent and have a major impact on patients survival (Lindner et al., 1974; Braun et al., 1996; Goodman et al., 2000; Oh et al., 2002). Our previous analysis provided evidence that this aggressive course of atherosclerosis is associated with elevated pro-inflammatory markers (CRP, CD40, CD154) in the circulation as well as in the atherosclerotic lesions. In-situ a higher expression of the pro-inflammatory CD40-CD154 system (CD40-ligand, CD40L) was shown in coronary plaques of patients with chronic renal failure compared to non-renal control patients. It is widely accepted that atherosclerosis can be understood as a localized inflammatory disease (Ross, 1999; Hansson, 2002; Libby, 2002) and inflammatory cell infiltrates have been documented in atherosclerotic lesions of patients and animal models of CKD (Schwarz et al., 2000; Buzello et al., 2004) .

It has been shown that dendritic cells (DC) and recently also mast cells (MC) are involved in inflammation, immunity and T-cell-activation in atherosclerosis (Bobryshev and Lord, 1996, 1998a,b; Bobryshev et al., 1996; Bobryshev, 2010; Paulson et al., 2010; Swedenborg et al., 2011). As a consequence these cells may represent novel therapeutic targets (Bot and Biessen, 2011). Patients with CKD are known to show features of immunosuppression with decreased T-cell numbers, impaired T-cell activation and a lower number of circulating DCs (Girndt et al., 2001; Eleftheriadis et al., 2007; Lim et al., 2007a,b) most likely as a consequence of negative effects of CKD on the bone marrow and cell maturation. There is only limited information whether this is also true for the in-situ situation, i.e. whether the number and localization of DCs and MCs in plaques of CKD patients is different from that in non-renal control patients. When Hueso et al. (Hueso et al., 2015) investigated 3 different aortic lesions of CKD and control patients, i.e. adaptive as well as pathological intimal thickening of the aorta and fibroatheromas using immunohistochemistry, they found a significantly higher percentage of DCs in CKD patients compared to non-CKD controls with a wide range of values. There is no information, however, on whether this is also true for coronary plaques and whether there is correlation between DC or MC infiltration and plaque calcification.

Therefore, it was the aim of the present study to assess the number and localization of DCs and MCs in parallel with T-cell, B-cell and macrophage infiltrates and markers of inflammation such as NFκB in calcified and non-calcified atherosclerotic plaques of endstage CKD and control patients.

Materials and methods

Patient selection (Table 1)

At the time of autopsy coronary arteries of 25 patients with endstage CKD and of 25 non-renal control patients (including 10 similarly processed explanted hearts) were collected and examined at the Dept. of Pathology, Erlangen-Nürnberg according to a standard protocol (see below). The study was planned and conducted according to the general guidelines for studies using human material. The study protocol was approved by the local Ethics Committee (University of

Table. 1. Patient characteristics and laboratory findings.

	Non calcified control (n=13)	Non calcified CKD (n=12)	Calcified control (n=12)	Calcified CKD (n=13)	Kruskal-Wallis/ Chi-Square p-value
Age (years)	62.4±9.9	64.2±13.3	60.2±12.6	69.0 ± 11.7	ns
Gender (m:f in %)	69 : 31	83 : 17	67 : 33	77 : 23	ns
Body weight (kg)	84.5±10.9	61.3 ± 9.2**	129±50.7	78.2±24.7	0.002
Cardiac weight (g)	530±100	515±134	611±124	529±173	ns
eGFR MDRD (ml/min/1.73mm^2)	48.9±7.4	8.2±0.9**	61.3±17.7	11.4±2.4**	<0.0001
CKD stage (mean)	2.9±0.3	5±0**	2.7±0.3	4.7±0.2**	<0.0001
On dialysis (n, %)	0	12, (100%)**	0	4, (30.8%)*	<0.0001
Patients with RTx (%)	0	2, (16.7%)	0	1, (7.7%)	ns
Active smokers (n, %)	2, (15.4%)	0	2, (16.7%)	0	ns
Obesity (n, %)	1, (7.7%)	0	2, (16.7%)	0	ns
Sepsis (n, %)	1, (7.7%)	0	0	0	ns
Diabetes mellitus (n, %)	1, (7.7%)	2, (16.7%)	1, (8.3%)	6, (46.2%)*	0.04
Arterial hypertension (n, %)	6, (46.2%)	9, (75%)	6, (50%)	5, (38.5%)	ns
History of myocardial infarction (n, %)	6, (46.2%)	0**	4, (33.3%)	4, (30.8%)	ns
Serum creatinine (mg/dl)	1.7±0.8	7.4±2.6***	1.3±0.4	5.9±2.3**	<0.0001
CRP (mg/l)	89±88	22±7	109±118	159±78	ns
Calcium (Ca) (mmol/l)	2.0±0.2	2.4±1.7**	2.1±0.1	1.0±0.2###	0.0003
Inorganic phosphate (P) (mg/dl)	4.1±0.6	5.5±1.9*	4.1±0.7	6.9±2.5*	0.002
Cholesterol (mg/dl)	117±23	203±53*	164±131	124±51#	0.01

For the analysis of metric data Kruskal-Wallis test with Bonferroni post hoc test was used: ns=non-significant; for nominal data the Chi-Square test was used, *p<0.05 vs. control, **p<0.01 vs. control, ***p<0.001 vs. control; #p<0.05 non-calcified CKD vs. calcified CKD; ###p<0.001 non-calcified CKD vs. calcified CKD.

Erlangen-Nürnberg, Germany, no. 2684). Inclusion criteria for control patients was an estimated glomerular filtration rate (eGFR) >45 ml/min (for mean eGFR values see table 1). Exclusions criteria were metastasised tumors and autopsy persecution more than 2 days after death. In both groups clinical information provided at the time of autopsy was collected but detailed information on pre-existent coronary disease was not available. We tried to obtain every available information on the coronary risk profile, e.g. arterial hypertension (according to the prevailing WHO definition), smoking, diabetes mellitus, as well as relevant laboratory findings from the autopsy protocols and the available hospital records. The mean laboratory values during last hospitalisation were used for the statistical analysis. From the serum creatinine values eGFR and CKD stages were determined according to MDRD (Levey et al., 2007). Based on the calculated GFR-values, patient history and the absence or presence of calcified coronary lesions in histology all cases were subdivided into the following 4 groups:

1. non-calcified control
2. non-calcified CKD
3. calcified control
4. calcified CKD

Tissue preparation

At autopsy (n=40) or heart explantation (n=10) samples of all 3 coronary arteries were obtained and analyzed: ramus circumflexus of the left coronary artery (LCX), ramus interventricularis anterior of the left coronary artery (LAD), right coronary artery (RCA). As in a previous study (Schwarz et al., 2000) each artery was opened and carefully inspected by a pathologist. Upon inspection of every coronary artery the segment with the most severe looking atherosclerotic lesion which most likely indicates the clinically most relevant lesion was selected for further histological examination and dissected into 2-3 mm thick slices. These were then fixed instantly after removal with formaldehyde (8%), embedded in paraffin, sectioned (4 μm), stained using Haematoxylin-eosin (H&E), van Kossa technique (for demonstration of tissue calcification) and Elastica-van Gieson stain (for fibrous tissue). On histology the atherosclerotic plaques were first classified according to the well-established Stary (Stary, 1992) from type II to type VII lesion: type II lesion corresponds to early lesion (fatty streak), type III to intermediate lesion with small extra cellular lipid pools, type IV to atheroma lesion with calcium granules in the lipid core region, type V to fibroatheroma lesion, type VI to complicated lesion with haematoma and/or thrombosis, and type VII to calcified lesion which contain usually relatively few if any inflammatory cells. On the basis of our previous experience lesions were then regrouped as non-calcified (Stary type II-VI) and calcified (Stary type VII) and were further analysed as described below. Of note, the non-plaque bearing aspect of the coronary artery (intima, media, adventitia) was analysed separately.

Immunohistochemical analysis

Immunolabeling was performed on paraffin sections using the following antibodies: rabbit polyclonal antibody against S100 (Zytomed Systems GmbH, Berlin, Germany; 1:5000), mouse monoclonal antibody against mast cell tryptase (MT, Dako Deutschland GmbH, Hamburg, Germany; 1:1000), rabbit polyclonal antibody against CD117 (c-kit) (Epitomics, Burlingame, CA, USA; 1:100), mouse monoclonal antibody against CD21 (Dako; 1:100), mouse monoclonal antibody against CD1a (Epitomics; 1:200), mouse monoclonal antibody against BDCA-2 (blood dendritic cell antigen 2 protein; Merck Millipore, Darmstadt, Germany, clone 10E6.1; 1:100), rabbit polyclonal antibodies against CD3 (Zytomed; 1:400) and NF$\varkappa$B (Santa Cruz (1:200) mouse monoclonal antibodies against CD20 (Dako; 1:500), CD68 (Dako; 1:200), CD31 (Dako; 1:200) and smooth muscle actin (SMA, Dako; 1:300).

For antigen retrieval sections were incubated with a pressure cooker for 30-60 sec in preheated target retrieval solution (TRS) (catalyzed signal amplification kit, CSA, Dako Co, Hamburg, Germany), in citrate buffer (CD3, CD20, and BDCA-2), respectively. For S100, CD21 and CD68 we used pronase (2mg/ml) pre-treatment. The samples were then rinsed in Tris-buffered saline (pH 7.6), blocked for 30min with 100% FCS and subjected to the avidin-biotin system, or to Dako Envision polymer (Dako Co, Hamburg; Germany). Each incubation step was followed by a thorough rinse with TBS. Aminoethyl Carbazole (AEC) solution substrate (Dako Co, Hamburg, Germany) served as chromogen. All sections were counterstained with hematoxylin and examined using light microscopy. As negative control for immunohistochemical stainings, the primary antibody was replaced by non-immune mouse or rabbit serum (BioGenex; San Ramon, CA, USA) or Tris-buffered saline (pH 7.2).

Of note, when we used double staining of lymphoid tissue both MC markers (CD117 and MT) yielded comparable results albeit CD117 is not a specific mast cell marker but also known to be expressed by progenitor cells. However, double staining in coronary vessels was challenging most likely due to the decalcification process and unfortunately we were not succesful. In our hands S100 was the most reliable DC marker in the atherosclerotic plaques that was also used in the study of Hueso et al. (2015) whereas other markers that we had also tested i.e. CD1a, BDCA-2, CD21 failed to yield reliable results (despite positive controls which we had run in parallel). In order to exclude false positivity of S100 staining in macrophages or vascular smooth muscle cells serial sections were stained with S100, CD68 and SMA and staining results were compared.

Semiquantitative analysis of mast cells (MCs), dendritic cells (DCs), T and B-cells and macrophages

Since it became obvious that cell distribution within the plaque may matter particulary for plaque stability all plaques were subdivided into 4 anatomic regions as described before (Stintzing et al., 2005), i.e. fibrous cap, areas of the intima along the endothelium of plaque, shoulder region at the margin of the fibrous cap, basis at the base of plaque core and boundary region beside the core.

Due to the very low number of MCs and DCs within the plaques and the 4 regions, respectively, an automatic image analysis of the immunohistochemical positive cells was omitted in favour of a simplifying semi-quantitative scoring system according to the following criteria: 0= no positive cells, 1=1-2 cells per high power field (HPF), 2=2-5 positive cells per HPF, 3=5-10 positive cells per HPF, 4≥10 positive cells per HPF. The inflammatory infiltrates were also evaluated separately for each plaque subregion, i.e. cap, shoulder, basis and boundary in 5-10 HPF of the whole tissue sample according to following scoring system: CD3 and CD20: 0=no positive cells, 1=less than 5% positive cells (of all cells), 2=5-10% positive cells, 3≥10% positive cells; for CD68 quantifications 0=no positive cells, 1=less than 10% positive cells, 2=10-30% positive, 3=30-50 % positive cells, and 4≥50% positive cells.

Plaque neovascularisation as assessed by CD31 staining was rare and was therefore only qualitatively analysed as were changes of vascular smooth muscle cells visualized by SMA staining .

The extent and distribution of the immuno-histochemically positive cells were evaluated by 2 independent observers. The scores were then calculated as the mean score of these 2 observers.

Table 2. Correlation analysis of S100 and CD3 (overall and for the plaque subregions) showing the Kendall's rank-correlation coefficient τ, respectively.

CD3	Overall	Cap	Shoulder	Basis	Boundery
S100+DCs: Overall	0.379				
Cap		0.311	0.174	0.146	0.028
Shoulder		0.117	0.121	0.303	0.293
Basis		0.401*	0.165	0.224	0.093
Boundary		-0.043	0.179	0.045	0.174
CD117+MCs: Overall	0.153				
Cap		-0.167	0.044	0.232	0.262
Shoulder		0.263	0.211	0.348	0.189
Basis		-0.180	-0.133	-0.352	-0.139
Boundary		-0.244	0.033	-0.352	-0.139
Tryptase+MCs: Overall	0.127				
Cap		0.073	-0.025	0.122	0.087
Shoulder		-0.204	0.296	-0.275	0.346
Basis		0.000	0.255	0.267	0.213
Boundary		-0.010	-0.220	0.149	0.114

Table 3. Correlation analysis of S100 and CD68 (overall and for the plaque subregions) showing the Kendall's rank-correlation coefficient τ, respectively.

CD68	Overall	Cap	Shoulder	Basis	Boundery
S100+DCs: Overall	0.085				
Cap		0.388*	0.105	-0.050	0.310
Shoulder		0.239	-0.153	-0.413	0.210
Basis		0.162	-0.083	-0.224	0.411
Boundary		0.199	0.176	0.104	0.268
CD117+MCs: Overall	0.190				
Cap		-0.100	0.112	0.088	-0.192
Shoulder		0.341*	0.187	0.012	-0.133
Basis		-0.229	-0.091	0.277	-0.100
Boundary		0.163	0.347	0.171	-0.019
Tryptase+MCs: Overall	0.097				
Cap		0.384*	-0.345	-0.408	0.271
Shoulder		0.282	0.231	0.015	-0.433
Basis		0.185	0.015	-0.070	0.107
Boundary		0.251	-0.213	-0.023	-0.281

DCs: dendritic cells, MCs: Mast cells. Asterik (*) indicates significant correlations ($p<0.05$, bold emphasized) between S100+DCs, CD117+MCs, Tryptase+MCs, CD3 positive T-lymphocytes and CD68 positive macrophages.

Statistics

Statistical analysis was performed using SPSS 11 software package (SPSS Inc., USA). Mann-Whitney U test was used to detect differences in ordinal variables, and the χ^2 test for those in proportions. Additionally, more than two groups of continuous data were analysed with Kruskal-Wallis test with Dunns multiple comparison as post-hoc test. Kendall's rank-correlation coefficient τ was used for correlation analysis (Tables 2, 3). Linear regression analysis was performed to evaluate independent factors. All tests were two-tailed, and statistical significance was determined at an α-level of 0.05.

Results

Patient characteristics and laboratory findings (Table 1)

According to the available creatinine and GFR values almost all CKD patients were in CKD stage 5. Endstage CKD patients (independent of the group) were slightly, but not significantly older and had significantly lower body weight than control patients. Gender distribution was comparable in all 4 groups; however, in total there were more male (37) than female patients (13). As expected mean eGFR was significantly but comparably lower in both CKD groups than in controls. In the non-calcified CKD group all 12 patients were on long-term hemodialysis whereas in the calcified group there were only 4 patients on dialysis. Analysis of the coronary risk profile showed a significantly ($p=0.035$) higher incidence of diabetes mellitus in patients of the calcified CKD group (46.2%) compared to the calcified control group (8.3%). The percentage of diabetes mellitus was markedly lower

in the non-calcified group (7.7% in non-renal vs. 16.7% in CKD patients). Of note, in the non-calcified groups significantly more control patients died of myocardial infarction than in the non-calcified CKD group (61.5% versus 0%, p=0.007). The percentage of myocardial infarction was comparable, however, in the two calcified groups (33.3% in non-renal vs. 30.8% in CKD patients). As expected, CKD patients had higher serum levels of creatinine and inorganic phosphate compared to controls. In addition, in the non-calcified CKD group S-calcium and S-cholesterol were also significantly higher than in control patients whereas values for LDL- and HDL cholesterol were not significantly different between the groups (data not shown).

Classification of coronary atherosclerotic lesions, calcification pattern and plaque morphology (Fig. 1)

Of 50 coronary lesions that were classified according to Stary (1992) most lesions were categorized as type III (65%) and IV (35%) in the non-calcified groups and per definition as type VII lesions (100%) in the calcified groups independent of the renal status. Detailed analysis of calcified areas using the Kossa stain showed that calcification of the coronary lesions was predominantly located in the intima, i.e. the arterial plaque, whereas we could hardly find any media calcification in the coronary arteries (Fig. 1).

Distribution of dendritic cells (DC) and mast cells (MC) in the non-plaque bearing part of the coronary artery (Fig. 2A-D)

Analysis of the non-plaque bearing aspect of the coronary artery showed that both DCs (A,B) and MCs (C,D) were predominantly located within the adventitia of the vessel with no significant difference between control and advanced CKD patients or non-calcified and

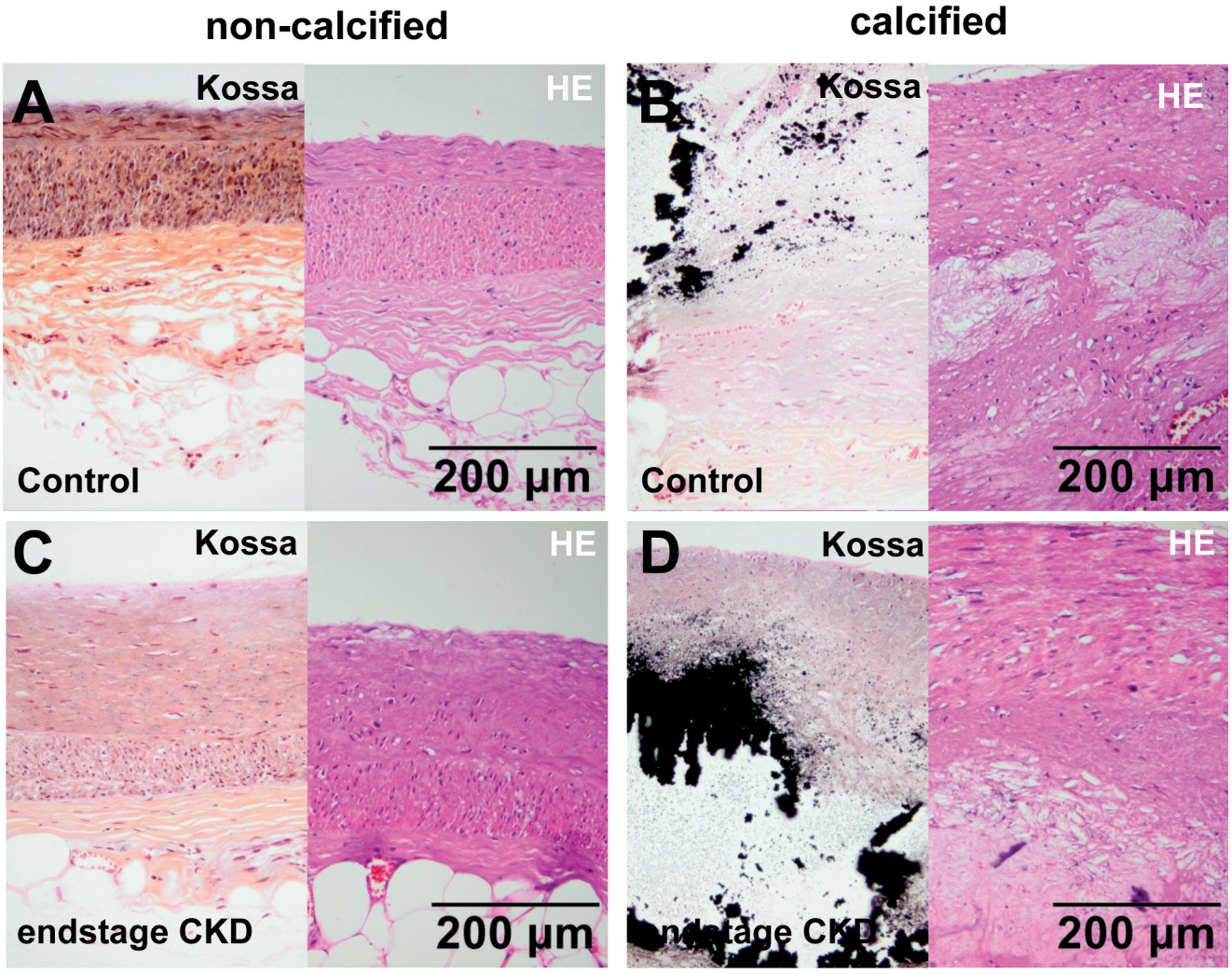

Fig. 1. Coronary atherosclerotic lesions were graded in non-calcified **(A, C)** and calcified **(B, D)** from non-renal control patients **(A, B)** and endstage CKD patients **(C, D)**. Sections were stained for calcification by Kossa (left picture, black stain) and Hematoxilin Eosin (HE, right picture).

calcified vessels, respectively (Fig. 2A-D). Interestingly, significant MC numbers were only seen in the adventitia but not within the media or intima of the coronary arteries with a tendency to higher values in non-calcified and calcified arteries of endstage CKD patients. DCs were also significantly higher in the adventitia, but in contrast to MCs they were also present in the intima and media of coronary arteries with a tendency to higher values in arteries with calcified plaques and in control compared to CKD patients.

Intra-plaque distribution of dendritic cells (DC) (Fig. 3A-F)

Qualitative inspection of coronary plaques, however, documented a relatively higher in-situ expression of S100+ DCs in calcified (Fig. 3B,D) compared to non-calcified lesions (Fig. 3A,C) with a tendency to lower numbers in endstage CKD patients. In fact, semiquantitative scoring of S-100+ DCs per plaque area and per plaque subregion (Fig. 3E,F) revealed a significantly ($p<0.05$) lower DC score in endstage CKD compared to control patients. The total number of DCs per coronary atherosclerotic lesion area, was significantly ($p<0.05$) higher in calcified (Fig. 3F) vs. non-calcified (Fig. 3E) lesions and this was particularly the case for control patients. In line with the overall lower number of DCs in coronary lesions of endstage CKD compared to control patients detailed distribution

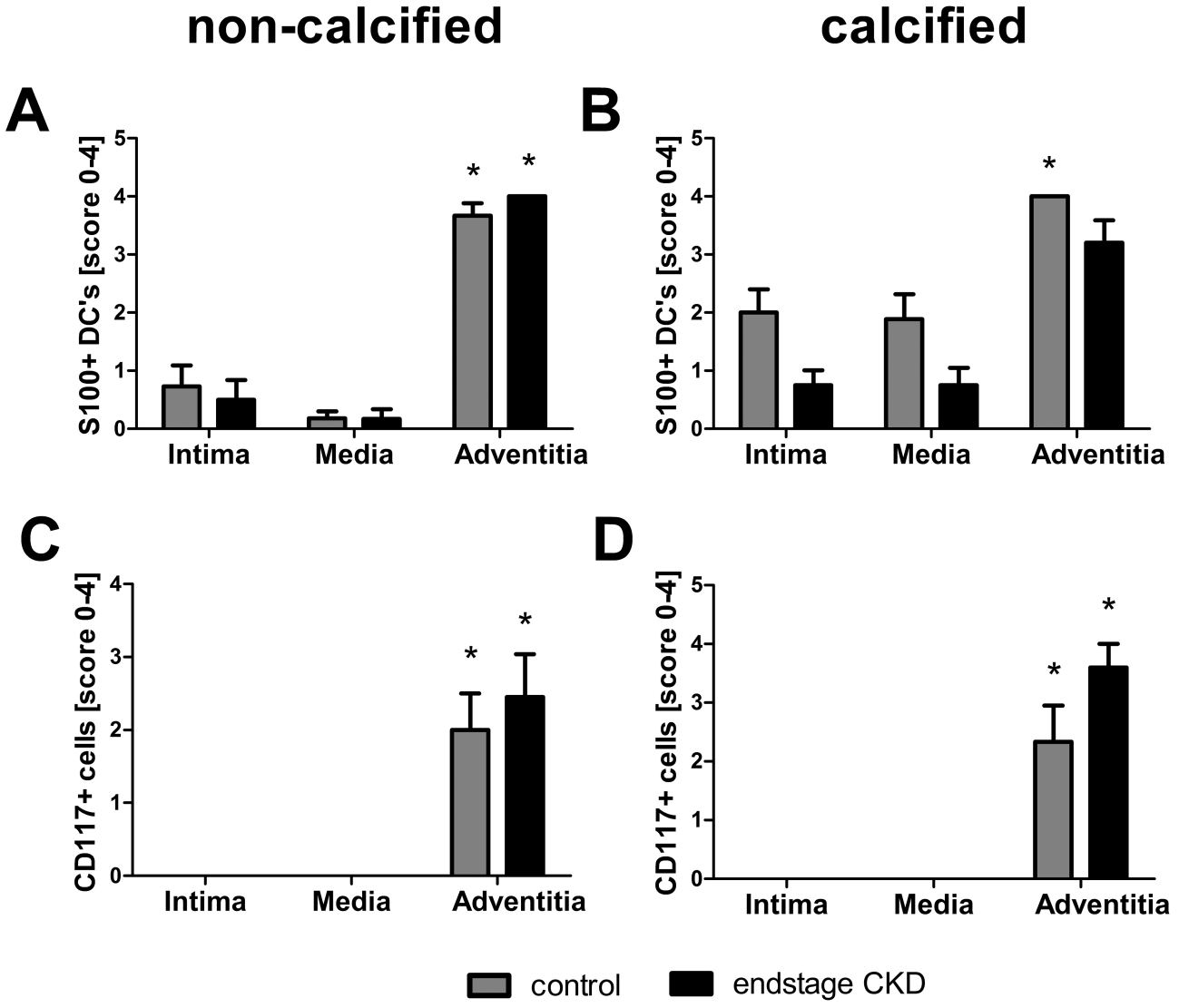

Fig. 2. Comparison of presence and distribution of S-100+ dendritic cells (DC, **A, B**) and CD117+ cells (**C, D**) within the 3 compartments of the non-plaque bearing aspect of the coronary artery of control (gray bars) and endstage CKD patients (black bars) with non-calcified (**A, C**) and calcified (**B, D**) plaques using semiquantitative immunohistological scores. Non-calcified control n=11; calcified control n=9; non-calcified CKD n=12; calcified CKD n=13; *: $p<0.05$ vs media and intima.

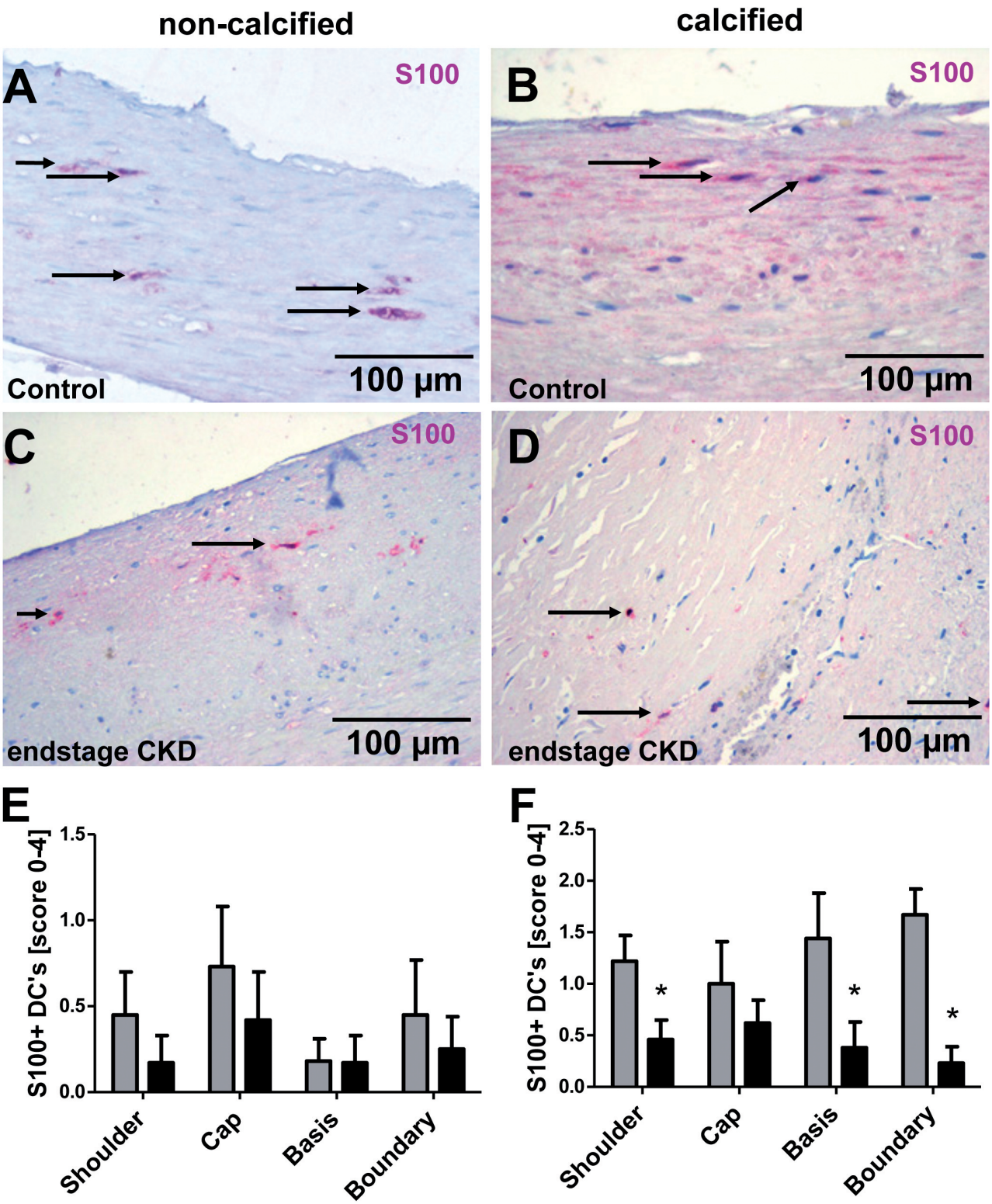

Fig. 3. Qualitative **(A-D)** and semiquantitative **(E, F)** analysis of intra-plaque distribution of dendritic cells (DCs) by S-100 immunohistochemistry in control (**A, B,** gray bars) and endstage CKD patients (**C, D**, black bars). Of note, on inspection a higher in-situ expression of S100+ DCs (arrow) was seen in calcified (**B, D, F**) compared to non-calcified (**A, C, E**) lesions of both control (**A, B**) and endstage CKD (**C, D**) patients. Examples of S100-+ cells are marked by arrows. Using the semiquantitative scoring system DC number per coronary atherosclerotic lesion area was indeed significantly ($p<0.05$) higher in calcified **(F)** vs. non-calcified **(E)** lesions of both endstage CKD (black bars) and particularly control patients (gray bars) with an overall tendency to higher numbers in control patients. Inside calcified plaques **(F)** a significantly ($p<0.05$) lower DC score was seen in shoulder, basis and boundary regions of endstage CKD patients (black bars) compared to controls (gray bars). Non-calcified control n=11; calcified control n=9; non-calcified CKD n=12; calcified CKD n=13; Mean± SEM. *: $p<0.05$ vs. control patients.

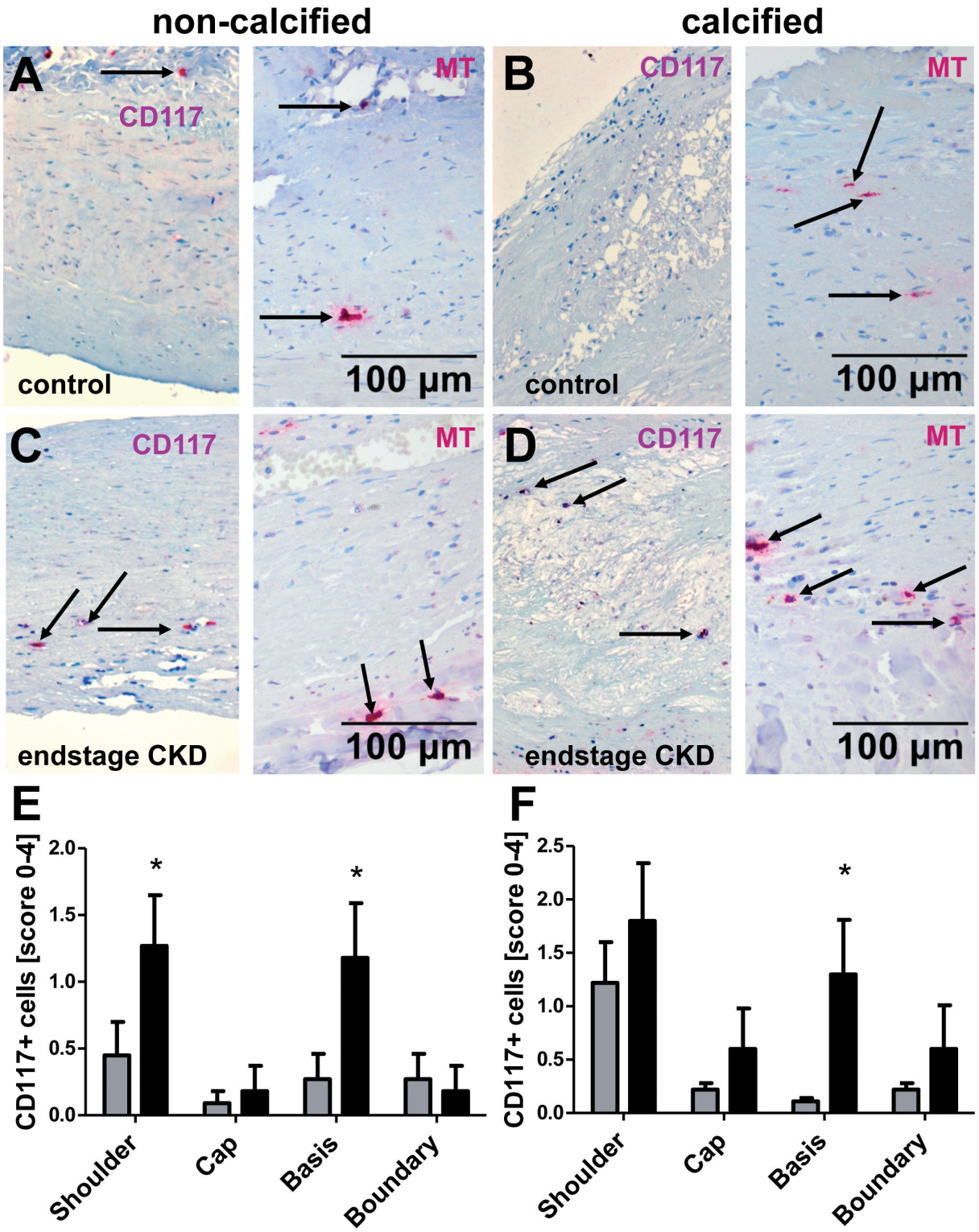

Fig. 4. Qualitative **(A-D)** and semiquantitative **(E, F)** analysis of intra-plaque distribution of mast cell tryptase (MT) + and CD117+ mast cells (MCs) by immunohistochemistry in control patients (**A, B,** gray bars) and endstage CKD patients (**C, D**, black bars). Qualitative inspection documented more mast cell tryptase (MT)+ and CD117+ cells (arrow) in calcified (**B, D**) compared to non-calcified (**A, C**) lesions with a tendency to higher numbers in endstage CKD (**C, D**) than in control (**A, B**) patients. Examples of MT+ and CD117+ cells are marked by arrows. In fact, semiquantitative analysis found the CD117+ score per coronary atherosclerotic lesion significantly ($p<0.05$) higher in non-calcified **(E)** and calcified **(F)** lesions of endstage CKD (black bars) vs. control patients (gray bars). Within the plaque the CD117+ score was significantly ($p<0.05$) higher in the basis and shoulder region of non-calcified **(E)** and the basis region of calcified **(F)** lesions of endstage CKD (black bars) compared to control patients (gray bars). Non-calcified control n=11; calcified control n=9; non-calcified CKD n=11; calcified CKD n=11; Mean± SEM. *: $p<0.05$ vs. control patients.

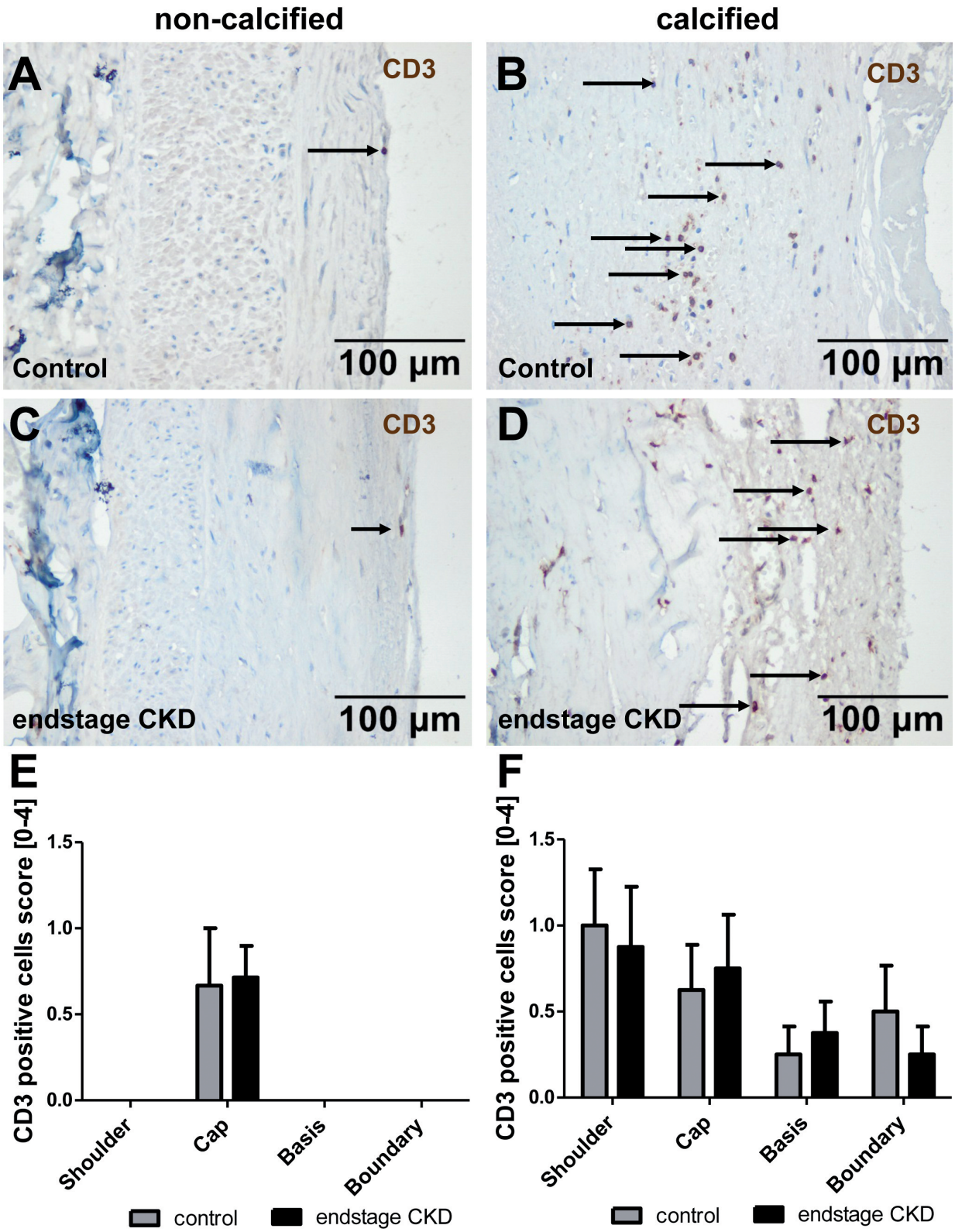

Fig. 5. Qualitative (**A-D)** and semiquantitative (**E, F**) analysis of intra-plaque distribution of CD3 positive T-cells by immunohistochemistry in control patients (**A, B**, gray bars) and endstage CKD patients (**C, D**, black bars). Qualitative inspection documented higher numbers of CD3-positive T-cells in calcified plaques being more scattered (**B, D**) compared to non-calcified (**A, C**) lesions. Examples of CD3+ cells are marked by arrows. Semiquantitative analysis found higher values in calcified **(F)** versus non-calcified lesions **(E)** but CD3 scores were comparable in endstage CKD (black bars) compared to control patients (gray bars). Non-calcified control n=8; calcified control n=8; non-calcified CKD n=7; calcified CKD n=8; Mean± SEM.

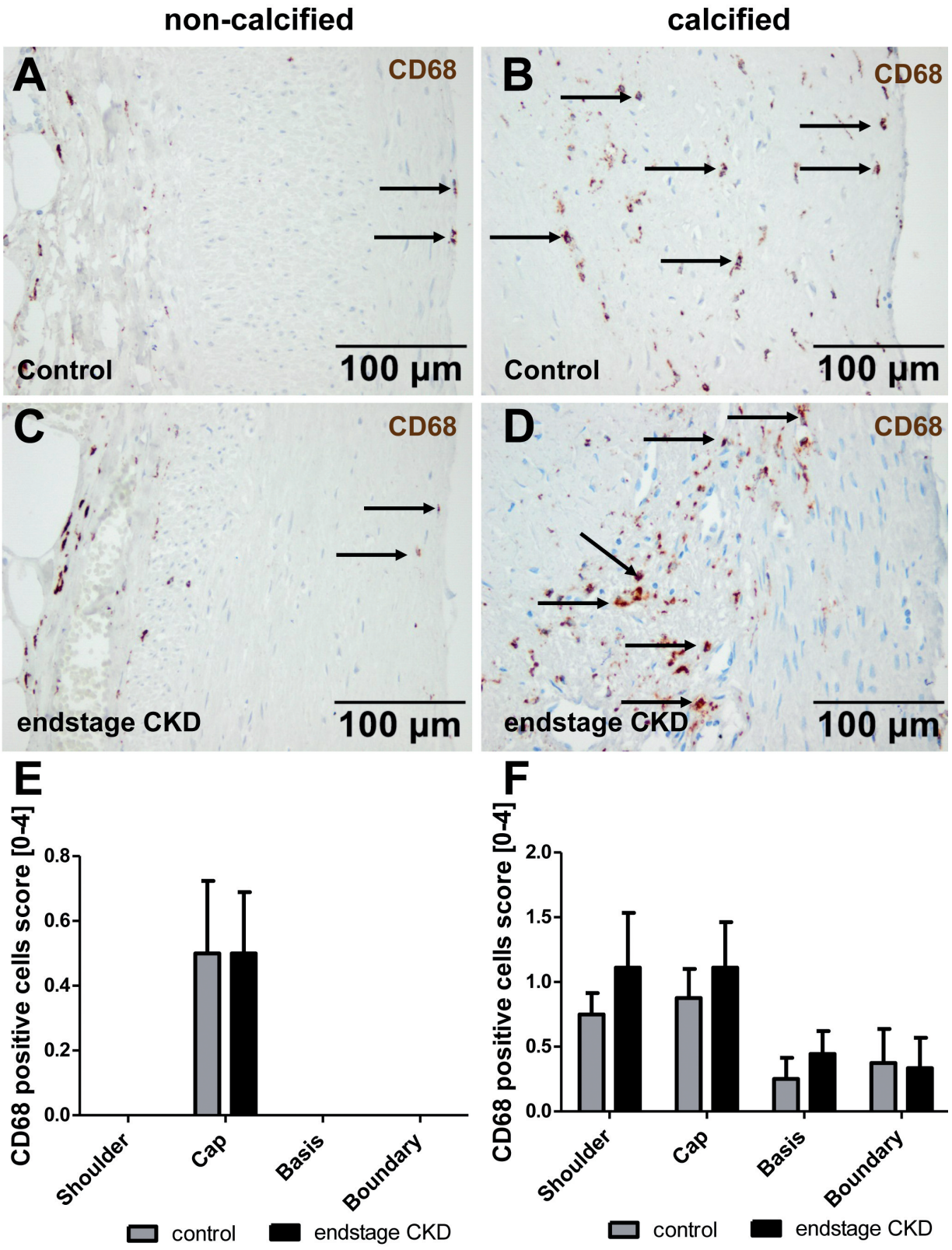

Fig. 6. Qualitative **(A-D)** and semiquantitative **(E, F)** analysis of intra-plaque distribution of CD68 positive macrophages by immunohistochemistry in control patients (**A, B,** gray bars) and endstage CKD patients (**C, D,** black bars). Qualitative inspection documented higher numbers of CD68+ macrophages in calcified plaques (**B, D**) compared to non-calcified (**A, C**) lesions. Examples of CD68+ cells are marked by arrows. Semiquantitative analysis found higher values in calcified **(F)** versus non-calcified lesions **(E)** but CD68 scores were comparable in endstage CKD (black bars) compared to control patients (gray bars). Non-calcified control n=8; calcified control n=8; non-calcified CKD n=8; calcified CKD n=9; Mean± SEM.

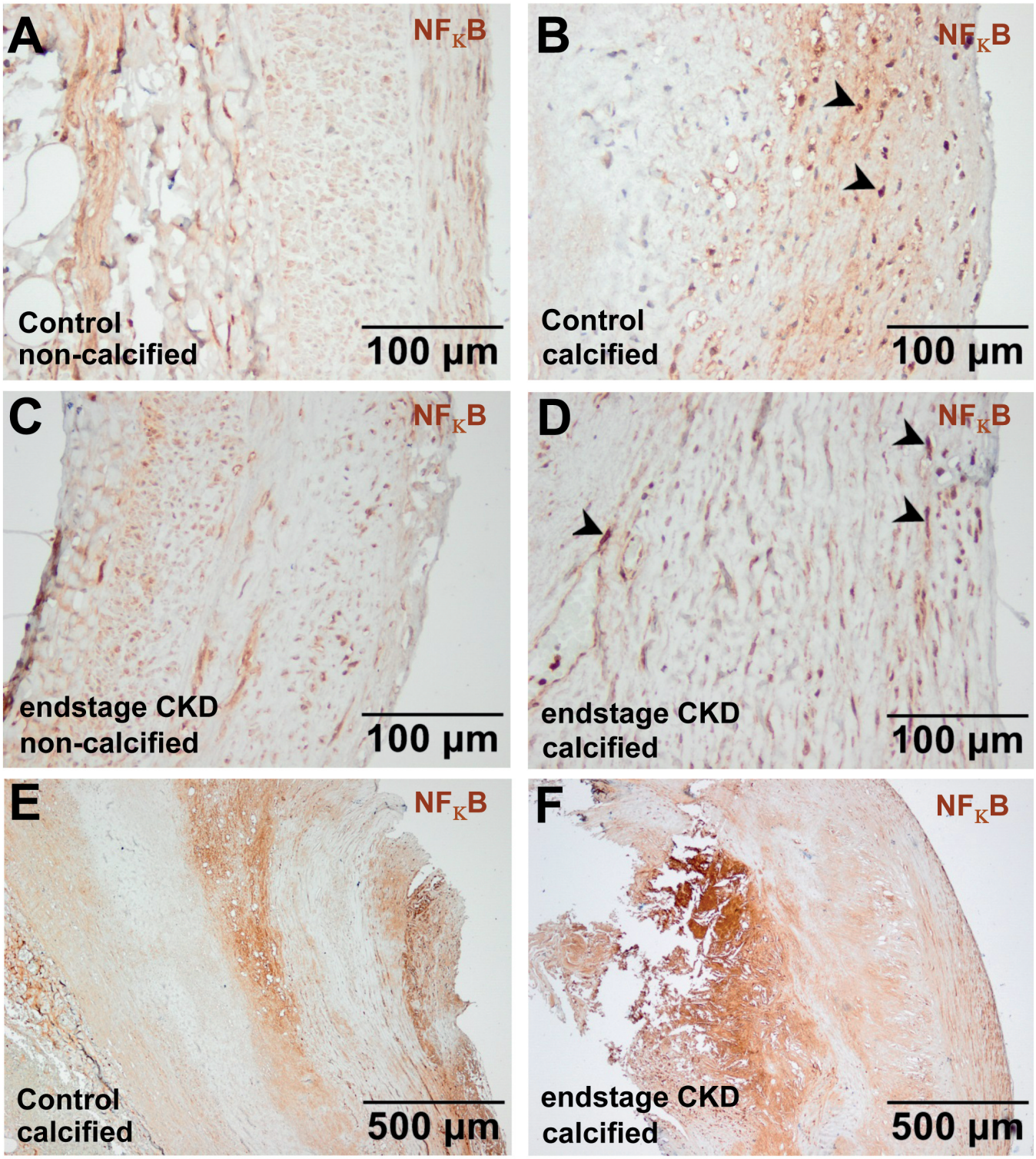

Fig. 7. Qualitative **(A-F)** analysis of intra-plaque distribution of NFκB by immunohistochemistry in control patients **(A, B, E)** and endstage CKD patients **(C, D, F)**. Qualitative inspection documented weak staining for NFκB in non-calcified plaques **(A, C)** and slightly enhanced staining in calcified plaques **(B, D**; arrowheads) and locally restricted strong staining for NFkB in calcified lesions of control **(E)** and endstage CKD groups **(F).**

analysis inside calcified plaques revealed a significantly ($p<0.05$) lower DC score in all but the cap subregion of the coronary plaques in endstage CKD patients compared to controls. Of note, expression analyses inside non-calcified plaques showed a similar tendency, but no significant differences between control and endstage CKD patients. The DC scores in endstage CKD patients and controls correlated positively overall and in all plaque subregions ($p<0.05$). A positive correlation was also observed between the Stary grading and the DC score. However, we observed a negative correlation of boundary S100+ DC's with CKD stages ($r=-0.389$; $p=0.012$).

As briefly mentioned before, staining with other DC antibodies than S100, i.e. BDCA-2, CD1a and CD21 was completely negative in our specimens so that we did not further pursue these analyses.

Intra-plaque distribution of mast cells (MC) (Fig. 4A-F)

Of note, both MC markers that were used, i.e. CD117 and mast cell tryptase (MT) showed comparable staining results. Qualitative inspection documented a higher expression of CD117+ cells in calcified (Fig. 4B,D) compared to non-calcified (Fig. 4A,C) lesions with a tendency to slightly more positive cells in endstage CKD (Fig. 4C,D) than in control (Fig. 4A,B) patients. On a semiquantitative analysis, the CD117+ cell score per coronary atherosclerotic lesion area was in fact significantly ($p<0.05$) higher in non-calcified (Fig. 4E) and calcified (Fig. 4F) lesions of endstage CKD vs. control patients. Detailed analysis of the intraplaque distribution of CD117+ cells revealed a significantly ($p<0.05$) higher score in the basis and shoulder region of non-calcified and in the basis region of calcified lesions of endstage CKD compared to control patients. Interestingly, basis CD117+ cell infiltration correlated significantly with CKD stage ($r=0.464$, $p=0.003$). Expression analyses in the boundary and cap subregions showed no differences between either calcified or non-calcified lesions of both groups.

Analysis of intraplaque distribution of inflammatory cells (T-cells, B-cells and macrophages), NFκB, neovascularization and vascular smooth muscle cell changes (Figs. 5-7)

In order to further analyse the nature of the inflammatory cell infiltrate inside the plaque as well as plaque neovascularisation and potential changes of vascular smooth muscle cells (VSMC), specific markers for macrophages (CD68), T- (CD3) and B-lymphocytes (CD20), endothelial cells (CD31) and VSMC (SMA) were used on consecutive paraffin sections. In 6 out of 50 coronary samples (12%) inflammatory cells were completely absent in advanced CKD and control specimen (n.s.). In non-calcified vessels of both endstage CKD and control patients CD3+ cells were only rarely found (Fig. 5A,C,E). In contrast, in both calcified groups CD3+ T-cells were more frequently detected and predominantly seen in the shoulder and cap subregions (Fig. 5B,D,F). The scores for CD68+ macrophages were relatively low and in non-calcified plaques restricted to Cap region (Fig. 6A,C,E). In calcified plaques the number of CD68+ macrophages tended to be higher and were distributed in different plaque regions with no significant difference between endstage CKD and control patients (Fig. 6B,D,F). B-lymphocytes were seen only very rarely so that scoring did not make sense. Staining for the inflammatory marker NFκB showed weak staining in non-calcified groups (Fig. 7A,C). Within calcified plaques NFκB was more frequently expressed in single cells (Fig. 7B,D; arrowheads). In addition, NFκB was also detected in calcified plaques showing locally high expression in a diffuse staining pattern but was lacking in other plaque regions (Fig. 7E,F). By inspection plaque neovascularisation as assessed by CD31 staining was comparably rare in all 4 groups and did not colocalize consistently with immune cells. SMA expression within the plaque was not different between endstage CKD patients and controls nor within the plaque subregions.

Correlation of MC and DC infiltration with CD3+ T-cells and CD68+ macrophages (Tables 2, 3)

Overall, both MC and DC scores correlated positively, but not significantly with CD3+ T-cells and CD68+ macrophages. Detailed correlation analysis of MCs and DCs with inflammatory cells (CD3+, CD68+) (Tables 2, 3) inside plaques subregions revealed (i) a positive association of both cell types with T-cells in endstage CKD specimens and (ii) a significant linkage between CD3+ T-lymphocytes and CD68+ macrophages with DCs in the shoulder and particularly in the cap region of all investigated cases ($p<0.01$, $r>0.311$).

Correlation of MC and DC number with calcification, clinical or laboratory findings

Of note, the DC score but not the MC score in the intima and media as well as in plaque subregions (overall, shoulder and basis) correlated positively with the Stary classification (types II to VII) of atherosclerotic lesions ($r=0.281$ and $p<0.05$). The dialysis status ($r=-0.282$ ($p=0.041$)) as well as the inorganic phosphate levels ($r=-0.269$ ($p=0.039$)) were significantly negatively associated with the DC score (media and plaque subregions (overall)) in contrast to the MC score (inorganic phosphate levels $r=0.256$ ($p=0.044$), plaque subregions (overall)). In addition, inorganic phosphate levels, but not dialysis status correlated significantly with T-lymphocytes in the cap and basis region. Furthermore, myocardial infarction and diabetes mellitus status were significantly positively linked to the MCs and DCs scores, respectively ($r=0.321$, $p<0.05$, intima and plaque subregions (overall)). Finally, the serum C-reactive protein (CRP)

levels were significantly positively associated with the MC score in the atherosclerotic plaques subregions (overall) whereas this was not the case for the DC score.

Discussion

Patients with chronic kidney disease (CKD) develop early and severe atherosclerosis which has a major impact on cardiovascular complications and on patients survival (Lindner et al., 1974; Braun et al., 1996; Goodman et al., 2000; Oh et al., 2002). Among others chronic systemic and local microinflammation, through activation of T-lymphocytes, macrophages, mast cell (MCs) and dendritic cells (DCs) which accumulate in atherosclerotic plaques, plays a central pro-atherosclerotic role in this particular population (Kaysen, 2004). It is not known, however, whether these immune cells play a particular role in advanced atherosclerotic coronary lesions in endstage CKD patients. Therefore, we investigated the in-situ expression and distribution of markers for MCs, DCs, T- and B-cells and macrophages as well as of markers of inflammation such as NFκB in different stages of atherosclerotic lesions from calcified and non-calcified coronary plaques of endstage CKD and control patients.

In contrast to MCs where there is not much data available a role of DCs in atherosclerosis has already been discussed for quite some time. DCs are the major antigen-presenting cells that bridge the innate and adaptive immune responses. DCs activation by pathogens results in cytokine secretion, up-regulation of co-stimulatory molecules and priming naïve T and B cells to the captured antigens (Agarwal et al., 2010). In vivo, DCs have also been found in atherosclerotic plaques in rodents (mice) (Bobryshev et al., 2001; Ozmen et al., 2002) and humans (Zernecke, 2015). CKD is regarded as an immunocompromised state most likely due to negative effects of uremic toxins on the bone marrow and the lymphoid tissue. In CKD patients disturbances of the immune system with lower T-cell number and impaired T-cell activation as well as a lower number of circulating DCs have been described (Lim et al., 2007a,b; Verkade et al., 2007). Of note, these findings in the circulation of CKD patients are paralled by our in-situ finding of significantly lower DC numbers in the coronary plaques of endstage CKD patients compared to non-renal control patients. In our study dialysis status as well as the inorganic phosphate levels were significantly negatively associated with the DC score. In the vasculature DCs were found in the subendothelial space as well as at the media-adventitia junction in the proximity of the vasa vasorum (Bobryshev and Lord, 1996; Ma-Krupa et al., 2004) (Pryshchep et al., 2008) which was also the primary site where we found DCs in our study. DCs may thus play an important role for plaque stability since they were associated with rupture-prone areas (Bobryshev and Lord, 1995a,b; Yilmaz et al., 2004, 2006; Niessner et al., 2006). In a previous study a highly irregular distribution of DCs in advanced atherosclerotic plaques was found with a higher presence in the shoulder region of the plaque where the neovascularization was particularly prominent (Bobryshev and Lord, 1998b).

Our findings in coronary arteries of CKD and control patients are different from that of Hueso et al. (2015) who investigated 3 different aortic lesions also using S100 as DC marker and found a significantly higher percentage of DCs in CKD patients compared to non-CKD controls albeit with a wide range of values. This different findings in different sites of the vascular tree are not a discrepancy per se but may point to local differences in the vascular response to atherosclerotic stimuli i.e. due to differences in shear stress, pressure, etc.

Also using S100 as the most reliable in-situ marker we found increased DC numbers in the shoulder, boundary and basis subregions of plaques in good correlation with the Stary classification of atherosclerotic lesions. In addition, in the shoulder and cap region S100 positivity correlated with CD3 and CD68 infiltrates which may indicate a potential proinflammatory role of DCs that might be important in terms of plaque instability. It is also speculated that DCs activation by nicotine, stress, hypoxia or CRP may stimulate the local immune response (Van Vre et al., 2008). This might be of interest in view of our previous finding of an increased in-situ expression of CRP in atherosclerotic plaques of CKD patients compared to controls (Campean et al., 2007).

Of note and of potential limitation of our study is the fact that we were only able to use S100 as a reliable marker for DCs whereas all the other DC markers that we tested did not stain any cell (despite positive controls which we had run in parallel). This could be explained by the fact that CD1a is known to be expressed by just a small population of DCs and the intensity seems to be lower. Negativity for BDCA-2 which is a marker of plasmacytoid DC indicates that no such DC subtypes were present in the atherosclerotic plaques. Since S100 can also be positive in macrophages and vascular smooth muscle cells we used serial CD68 and SMA staining to exclude unspecificity.

Another limitation of our study might be the fact that we used autopsy material for our analysis which limits the amount of clinical information that was available for analysis.

The knowledge and information on a potential role of MCs in atherosclerosis is relatively scarce compared to DCs. As in our study MCs have been shown to accumulate in the adventitia of human atherosclerotic plaques. Recently, it was demonstrated that MC accumulation may contribute to plaque progression and instability (Kovanen, 2007; Bot et al., 2015). MCs regulate inflammation and immunity and their granules contain heparin, histamine, and several other enzymes i.e. tryptase, chymase, carboxypeptidase, and several cytokines and chemokines which may all affect plaque composition and stability (Kovanen, 1995; Kovanen et

al., 1995; Swedenborg et al., 2011). Since chymase, which among others, is known to affect the renin angiotensins system (RAS) by conversion of AngI to AngII one may speculate that MCs thereby also activate the proatherosclerotic RAS pathway in CKD patients (Miyazaki et al., 2006; Nehme and Zibara, 2017). In addition, in atherosclerosis MCs have been described to be involved in medial and adventitial neovascularisation, regulation of T cell responses and endothelial cell adhesion molecules (Mayranpaa et al., 2009; Zhang et al., 2011). Of note, in our study MC scores correlated positively, but not significantly with CD3+ T-cells and CD68+ macrophages whereas neovascularization of the plaque was only a very rare event which did not correlate to inflammation

Within human coronary atherosclerotic plaques of non-renal patients activated MCs have been found in the shoulder region which is commonly regarded as the predilection site of atheromatous rupture (Kaartinen et al., 1994). To our knowledge, there is no data on the distribution or a possible role of MCs in coronary artery plaques of patients with any stage of CKD artery .

With respect to the effect of CKD there was a tendency for more MCs in vessels from endstage CKD patients particularly in calcified ones which may point to a more important and possibly specific role of MCs compared to DCs under the condition of renal failure and particularly in calcification. Detailed correlation analysis of MCs with inflammatory cells (CD3+, CD68+) (Tables 2, 3) inside plaques subregions revealed a positive association with T-cells in endstage CKD specimens and a significant correlation between the MC score within the plaque and the serum C-reactive protein (CRP) levels suggesting a potential link of MCs to local T- and macrophage activation and systemic inflammation.

If this might be transferred to the human situation a recent experimental study using the animal model of the ApoE knockout mouse (Bot et al., 2011) may be of particular interest since pharmacological MC chymase inhibition using the protease inhibitor RO5066852 was shown to reduce atherosclerotic plaque progression and to improve plaque stability. If this finding could be translated into the human condition pharmacological MC inhibition might possibly evolve as a new therapeutic target in the treatment of atherosclerosis particularly in the advanced condition of chronic renal failure.

In conclusion, coronary artery plaques of patients with endstage CKD are more advanced and are characterized by a significantly lower number of DCs and even more importantly a significantly higher number of MCs which correlated nicely with intraplaque T-cell distribution compared to non-renal control patients with coronary atherosclerosis. These data may suggest that MCs rather than DCs may be potentially involved in T-cell activation and plaque instability in advanced atherosclerotic lesions of endstage CKD patients. Thus, MC accumulation in atherosclerotic and particularly calcified plaques may be another specific characteristic of more advanced atherosclerosis in the setting of CKD which may potentially open the door for new treatment strategies, i.e. pharmacologic MC chymase inhibition.

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