

Local and Systemic Cellular Immunity in Early Renal Artery Atherosclerosis

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Summary

Background and objectives Modern imaging techniques have increased the incidental detection of renal atherosclerotic disease (RAD). Because immune activation may hasten RAD progression, identifying cellular immune markers might provide clues to clinical activity. In this study, cellular immune markers were assessed in early RAD.

Design, setting, participants, & measurements Immune cell markers in peripheral blood of two groups of hypertensive patients with normal carotid and coronary arteries were evaluated: 28 patients had incidental RAD and 22 patients had normal renal arteries; 21 renal arteries obtained at necropsy from individuals with history of hypertension and tissue evidence of RAD were examined and matched with 21 individuals with normal renal arteries. Cell subpopulations were measured by flow cytometry in peripheral blood and direct cell count, respectively, using T and dendritic cells monoclonal antibodies.

Results Peripheral blood of RAD patients showed increased numbers of cells expressing CD3, CD4, CD83, and CD86. CD4 to CD8 ratio was 8.3 ± 1.4 (RAD) to 3.4 ± 0.9 (normal; $P < 0.001$). No differences were found in CD25, CD8, and S100 among groups. *Postmortem* samples from RAD showed increased CD3+, CD4+, CD86+, and S100+ cells, whereas CD25+ and CD8+ were unmodified between groups. CD4+ to CD8+ ratio was higher in the RAD_{PM} group.

Conclusions These results are consistent with an increased expression of immune cell markers in early RAD. Additional studies will explore if they may potentially turn into treatment targets to prevent disease progression.

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Introduction

Atherosclerosis is a chronic inflammatory disease of the arterial wall with a strong autoimmune component (1–7). Despite the lifestyle and genetic factors that contribute to the development of an atherosclerotic plaque, the mere presence of an atherosclerotic lesion seems to trigger alterations in the systemic immune response, which could play a substantial role in the disease progression (8). Indeed, many factors triggering T cell activation in an atheromatous plaque have been identified, including antigen-presenting dendritic cells (9,10), which seem to play a critical role in the initiation and development of the plaque. During the acute phase of endothelial cell injury, these dendritic cells act as members of the innate immune system through rapid response cytokine secretion that stimulates differentiation of naive T cells into effectors CD4+ T cells and also, activation of CD4 and CD8 T cells (11).

Until now, most of the data describing immune markers has been obtained from patients with symptomatic, well established carotid or coronary artery disease (12–14). However, little is known regarding immune cell markers in peripheral blood and within

injured vessels during the early stages of atherosclerosis. Although immune abnormalities have been proposed as potential therapeutic targets in established atheromatous disease, they have not been evaluated in asymptomatic incidentally detected renal atherosclerotic disease (RAD). Indeed, current knowledge recommends no specific action beyond antihypertensive treatment, unless severe refractory hypertension or renal disease progression ensues (15–17). Moreover, because current evidence shows little benefit from traditional interventions (surgery or angioplasty), the routine study of renal arteries has been discouraged in hypertensive patients with no clinical suspicion of RAD. This finding is particularly true when the atherosclerotic stenosis reduces vessel lumen in less than 50% (18,19).

Because autoimmune activity plays a pathogenic role in atherosclerotic disease, it seems reasonable to predict that early interventions to reduce inflammation could halt disease progression. However, although increased numbers of CD4+ cells have been described in discrete arteriosclerotic lesions in other vascular territories (20,21), we lack data on renal atherosclerotic lesions, particularly in their early stages. The presence of T cells

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in these early lesions would suggest that cell-mediated immune reactions are taking place during the disease process. For this reason, our aim in this study was to search for evidences of immune responses early in nonhemodynamically significant RAD. Evaluating levels of immune cell markers in peripheral blood could help detect early stages of RAD in asymptomatic hypertensive patients and design new strategies to prevent RAD progression.

Materials and Methods

Patients

Asymptomatic stage 1 hypertensive patients scheduled for both coronary and carotid angiography were invited to participate in this prospective controlled pilot study that was performed at the Hospital Universitario Austral. The study volunteers signed an informed consent, and the research protocol was approved by the Institutional Clinical Research Board. Patients agreeing to take part in the study underwent coronary and carotid angiography as planned, and then, both renal arteries were assessed by standard angiographic techniques; 28 patients with asymptomatic RAD and less than 50% lumen reduction were then matched with 22 patients with normal renal arteries (NA group). According to the American Heart Association criteria (22), asymptomatic patients were defined as those patients with renal artery stenosis not accompanied by severe, malignant, or refractory hypertension and/or renal function impairment (glomerular filtration rate >60 ml/min per 1.73 m² and microalbuminuria <30 mg/24 h). Basal population characteristics are shown in Table 1. We excluded patients showing evidence of carotid and/or coronary atherosclerotic disease, acute pulmonary edema, myocardial infarction, heart failure (microalbuminuria >30 mg/24 h, glomerular filtration rate <60 ml/min per 1.73 m², or serum creatinine >1.2 mg), history of immunologic or autoimmune disease, use of direct immunomodulatory agents or corticoids, and acute or chronic infectious diseases and renal artery fibromuscular dysplasia. To avoid drug-induced immune modulatory effects, all antihypertensive medications were discontinued for 21 days before drawing blood samples. Blood pressure was controlled with doxazocine or thiazide diuretics in five patients from the RAD group and three patients from the NA group. These drugs were chosen, because they have no direct immunomodulatory effects. A 30-day washout period was applied for all other medications. Nine patients from the RAD group and six patients from the NA group were treated with hydroxymethyl glutaryl CoA inhibitors before inclusion.

Cell Counts. Whole blood (2 ml) was collected into heparin-treated tubes. PBMCs were isolated by Ficoll–Hypaque density gradient centrifugation. Residual red blood cells were removed by flash lysis with Milli-Q water at 4°C, and then, sample analysis was performed on a FACScan flow cytometer (Becton Dickinson, Los Angeles, CA) using the Cell Quest (Becton Dickinson Immunocytometry Systems, Los Angeles, CA) software.

Immunophenotyping. Immune cell markers were analyzed by immunohistochemistry (single and double staining) and flow cytometry using the following monoclonal antibodies: anti-CD4 (FL CD4; Serotec), anti-CD3 (FL CD3; Serotec), anti-CD83 (1:50, MCA1582; Serotec), anti-CD86

(1:100, MS296; Nemarkers), anti-S-100 (MCA2769; Serotec), anti-CD8 (1:50, DK25; DAKO), anti-CD25 (1:50, MCA2127; Serotec) and the appropriate isotype controls using the manufacturers' protocols.

Postmortem Studies

RAD is often associated with arteriosclerotic lesions in other vascular territories. Because this association could make the source of circulating immune markers uncertain, we then examined renal artery samples from necropsies done at the Buenos Aires Department of Forensic Medicine on subjects with no history of cardiovascular disease and whose cause of death was suicide, homicide, or accident. By these means, we matched (by sex and age) 21 individuals showing renal atherosclerotic plaques at autopsy [RAD postmortem (RAD_{PM})] with 21 individuals with normal renal arteries [normal arteries postmortem (NA_{PM})]. Legal authorization to use these tissue samples was obtained in each case.

After dissecting the renal arteries, samples were fixed by immersion in an immunohistochemical-Zinc fixative solution (BD Biosciences Pharmingen) at room temperature for 24–48 hours before being embedded in paraffin. Formaldehyde was not used because of its potential interference with immunohistochemical techniques. Finally, all the specimens were cut into 10- μ m sections and stained with hematoxylin-eosin. Immunolabeled sections were examined by light microscopy at 10 \times magnification.

Statistical Analyses

All values are expressed as mean \pm SD. For statistical comparisons, we used unpaired two-tailed *t* test and Fisher test for categorical data. A $P \leq 0.05$ was considered statistically significant.

Results

Patients Basal Features

Baseline characteristics in the two groups, including sex, age, BP, and cardiovascular risk factors, were similar. The mean number and distributions of antihypertensive drugs used were similar between groups (Table 1).

PBMCs were isolated from asymptomatic patients with and without renal artery stenosis. As shown in Table 2, the number of CD3+ cells, reflecting total T cell population, was 2.2 higher in RAD compared with NA patients ($P < 0.001$). Similarly, CD4+ cells, denoting T helper cells maturity, were significantly increased ($\times 2.9$) in the RAD group ($P < 0.001$). CD86+ cells, a marker of mature antigen-presenting cells, rose by a factor of 5.1 in RAD patients ($P < 0.001$). Consistent with these findings, the number of cells expressing CD83, a marker of activated dendritic cells, increased by a factor of 1.4 times in RAD patients compared with the NA group ($P < 0.001$) (Table 2). Moreover, the CD4+/CD8+ ratio was 2.4 times higher in the RAD group as a result of the increased number of CD4+ T lymphocytes and the lack of difference in the number of CD8+ cells between groups ($P < 0.001$) (Table 2). No statistical differences were found between the RAD and NA groups in the number of cells expressing CD25+, a marker of regulatory T cells, and the number of cells expressing S100+, a marker of dendritic cells and macrophages also

Table 1. Basal population characteristics

	Patients Group			Postmortem Group			P	P ^a Patients Versus Postmortem Groups
	RAD	NA	P	RAD	NA	P		
N	28	22		21	21			
Female n (%)	13 (46.4)	11 (52)	1.00	9 (42.8)	11 (47.6)	0.75	1.00 (RAD); 0.41 (NA)	
Age (yr)	55 ± 9	54 ± 8	0.68	56 ± 7.2	55.5 ± 6.8	0.81	0.21 (RAD); 0.51 (NA)	
SBP/DBP (mmHg)	145.5 ± 6.4/93 ± 4.6	144.2 ± 4.8/93.6 ± 3.0	0.43/0.59	144 ± 3.2/95 ± 5.1	145 ± 3.8/95 ± 4.4	0.36/1.00	0.33/0.15 (RAD); 0.54/0.22 (NA)	
Smokers n (%)	10 (35%)	8 (32%)	1.00	7 (30%)	7 (30%)	1.00	1.00 (RAD); 1.00 (NA)	
Dyslipemia n (%)	7 (25)	6 (27.7)	1.00	7 (33.3)	7 (33.3)	1.00	0.54 (RAD); 0.74 (NA)	
BMI (kg/m ²)	28 ± 7	28.5 ± 5.2	0.78	27.8 ± 4.9	28.2 ± 5	0.79	0.91 (RAD); 0.84 (NA)	
Number of antihypertensive drugs ^a	2.5 ± 1.5	2.0 ± 1	0.18	2.0 ± 1.0	1.5 ± 1.0	0.11	0.19 (RAD); 0.11 (NA)	

Values given as mean ± SD unless noted otherwise. Hypertension was defined according to VII JNC guidelines and/or current antihypertensive drug treatment. Smokers are individuals with ≥5 pack-years smoking history. Dyslipidemia is defined as LDL cholesterol >160 mg/dl and/or fasting triglycerides >200 mg/dl in untreated subjects and hypertensive patients with normal renal arteries. The last column represents differences in basal characteristics between alive and postmortem groups. P significance was established as <0.05. RAD, renal arteriosclerotic disease; NA, normal renal arteries; SBP, systolic BP; DBP, diastolic BP; BMI, body mass index; JNC VII, Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure. ^aDistribution of the different drugs type showed no significant difference among groups.

described in some endothelial and glial cells. Results from individual samples for the above-mentioned markers are depicted in Figure 1.

Postmortem Studies

Because immune processes could also stem from incidental arteriosclerotic lesions in other nonrenal vascular territories, we then assessed the presence of immune markers in renal arteries obtained at necropsy from cadavers showing renal arteriosclerotic disease and renal arteries obtained in the same manner from subjects without RAD.

No differences were found between groups in sex, age, and cardiovascular risk factors. Postmortem samples were matched to the age, sex, medical disease, and hypertensive record of patients from the live group (Table 1). Atherosclerotic plaques, when present, were assessed according to the morphologic classification proposed by Virmani *et al.* (23); 19 plaques showed fibrotic tissue, 11 of which presented small necrotic cores and 2 of which showed lipid content with microhemorrhages.

Table 3 shows the distribution of immune markers in tissue samples obtained from renal arteries postmortem. CD3⁺ cells content was five times higher in renal atheromas from the RAD_{PM} group compared with arteries from the NA_{PM} group ($P < 0.001$). The CD4⁺ cell content in renal artery plaques from RAD_{PM} group was four times higher than in renal arteries from the NA_{PM} group ($P < 0.001$). Both CD3⁺ and CD4⁺ T cells were predominantly found in intensely neovascularized adventitial layers (vasa vasorum) (Figure 2). The number of infiltrating CD8⁺ T lymphocytes (*i.e.*, cytotoxic T cells) was slightly but not significantly higher in RAD_{PM} compared with NA_{PM}. In these comparisons, the CD4⁺/CD8⁺ ratio within renal artery plaques was 2.4 times higher in samples taken from RAD patients, which was a result of a marked increase in CD4⁺ cell content ($P < 0.001$). No significant differences were found in CD25⁺ expression among groups. The S-100⁺ cell content was 4.6 times higher in RAD_{PM} plaques compared with NA_{PM} renal artery samples ($P < 0.001$), suggesting that dendritic cells were markedly abundant in the former group. Consistently, in RAD_{PM} lesions, the number of CD86⁺ cells was 9.6 times higher ($P < 0.001$) and the number of CD83⁺ cells was 5.6 times higher ($P < 0.001$) compared with similar counts in samples from the NA_{PM} group.

Discussion

Recent research supports a role for immunologic events in arteriosclerotic disease (24–28). In agreement with this notion, we found evidence suggesting an increased immune expression in asymptomatic early RAD patients that could entail a potential target for therapeutic intervention.

In this study, the expression of immune cell markers in the peripheral blood of hypertensive patients with early RAD was compared with hypertensive patients with normal renal arteries. Because these studies showed evidences of immune responses that could, in fact, stem from incidental arteriosclerotic lesions in other nonrenal vascular territories, the presence of immune markers was then assessed in renal arteries obtained at necropsy from cadavers showing renal arteriosclerotic disease and compared with

Table 2. Distribution of immune markers in peripheral blood cells from asymptomatic hypertensive patients with and without renal atherosclerotic disease

Immune Marker	RAD ($n=28$; $\times 10^6$ cells/L)	NA ($n=22$; $\times 10^6$ cells/L)	P
CD3	1317.9 \pm 29.2	599.2 \pm 11.8	<0.001
CD4	911.6 \pm 77.6	309.7 \pm 28.9	<0.001
CD83	131.4 \pm 12.8	79.2 \pm 10.4	<0.001
CD86	401.7 \pm 47.1	78.0 \pm 9.8	<0.001
S-100	102.3 \pm 17.9	109.4 \pm 16.3	0.15
CD8	109.0 \pm 37.9	119.3 \pm 13.0	0.22
CD25	86.9 \pm 5.4	90.2 \pm 9.6	0.13
CD4+/CD8+ ratio	8.3 \pm 1.4	3.4 \pm 0.9	<0.001
CD25+/CD4+ ratio	0.09 \pm 0.02	0.29 \pm 0.09	<0.001

Values are given as mean \pm SD. RAD, renal atherosclerotic disease; NA, normal arteries.

renal arteries obtained at necropsy from subjects without renal artery disease.

CD4+ lymphocytes and mature dendritic cells were significantly increased in peripheral blood from individuals with renal artery disease, suggesting the involvement of immune mechanisms and inflammation. These findings in patients were sustained by a fivefold increase in the number of cells expressing S-100, a dendritic cell marker, in arteriosclerotic arteries obtained at necropsy. Moreover, peripheral blood from patients with renal atherosclerotic disease showed an increased number of cells expressing CD83, one of the best known maturation markers for human dendritic cells, which has been found elevated in advanced stages of atherosclerotic plaques (28). However, the increased number of CD4+ cells in our study could be better explained by the microenvironment of the arteriosclerotic lesion, a source of chemokines capable of recruiting them (29,30). Interestingly, these CD4+ cells did not seem to be active, because CD25 expression did not rise in RAD patients compared with individuals with normal renal arteries.

It is worth noting that CD25 is also a marker of activation of CD4+ regulatory T cells, a phenotype that mediates a tolerogenic state. In RAD patients, CD25 remained unchanged, suggesting that the increased number of CD4+ does not correspond to the suppressive phenotype, which further implicates an immune effect during the disease early stages. In this respect, an indirect estimate of a tolerogenic or pro-inflammatory microenvironment could be the CD25/CD4 ratio. Indeed, we found this ratio to be four times lower in renal artery specimens obtained at necropsy from individuals with RAD compared with samples from NA subjects, meaning that these microenvironments were liable to prolong pro-inflammatory processes. This notion is also supported by the greater number of cells expressing the costimulatory ligand CD86 in RAD_{PM} samples, suggesting that T cell stimuli might be enhanced in the microenvironment of the renal vascular plaque.

The findings in patients with and without RAD are supported by the results obtained in renal arteries specimens taken at necropsy. Indeed, the association of increased CD4+ T cells with CD83+ cells in the RAD_{PM} group but not the NA_{PM} group suggests T cell differentiation as well as dendritic cell maturation in early RAD. Again, in this case, the greater number of mature dendritic and CD4 T cells was not

accompanied by an increase in CD25+ T cell expression, suggesting that these cells might not have an activated status in early RAD disease. Nevertheless, both cell types are likely relevant in disease development, because CD4+ cells have been shown to be involved in the atherosclerotic process (25,31). Certainly, T cells are the predominant immune cells within human arteriosclerotic lesions (32). In addition, increased numbers of dendritic cells have been identified in the arterial intima and adventitia of atherosclerotic susceptible regions (33). In contrast, a reduction in dendritic cell accumulation has also been described within the intima, which could protect it from the development of arteriosclerotic lesions (34). Indeed, dendritic cells may likely mature only after they are lodged in the plaque. Thus, we believe that the recruitment of dendritic cells in renal arteriosclerotic plaques may play an important immunologic role in the early stages of plaque formation. In fact, our findings suggest an early response in renal arteriosclerotic disease that is manifested by an active dendritic cell network acting locally and systemically through T cell-attracting chemokines.

It is worth noting that Millionig *et al.* (10), while studying vascular beds of healthy young individuals, found higher density of dendritic cells in areas of vessel bifurcation where turbulent flows predominate. There, turbulent flow might lead to an increased contact time between leukocytes and the injured endothelial surface, resulting in recruitment and transmigration of circulating blood cells through the endothelial wall. In this respect, Weis *et al.* (9) described endothelial determinants of dendritic cell adhesion and migration into the vessel wall at the onset of plaque development, suggesting that endothelial activation is a promoter of dendritic cell-mediated immune activation.

Although dendritic cell identification was not a specific aim of this study, our data strongly suggest their involvement in plaque progression and inflammation. In this particular case and bearing in mind the natural history of the atherosclerotic lesion, inflammatory changes are predictable. Although intensity is low during the initial stages of initial thickening, inflammation increases, and recurring episodes of immune responses accompany the progression to a fully developed atherosclerotic plaque (35).

Given that CD8+ cells were increased in neither peripheral blood of asymptomatic patients with RAD nor post-mortem samples with RAD, we can conclude that a specific

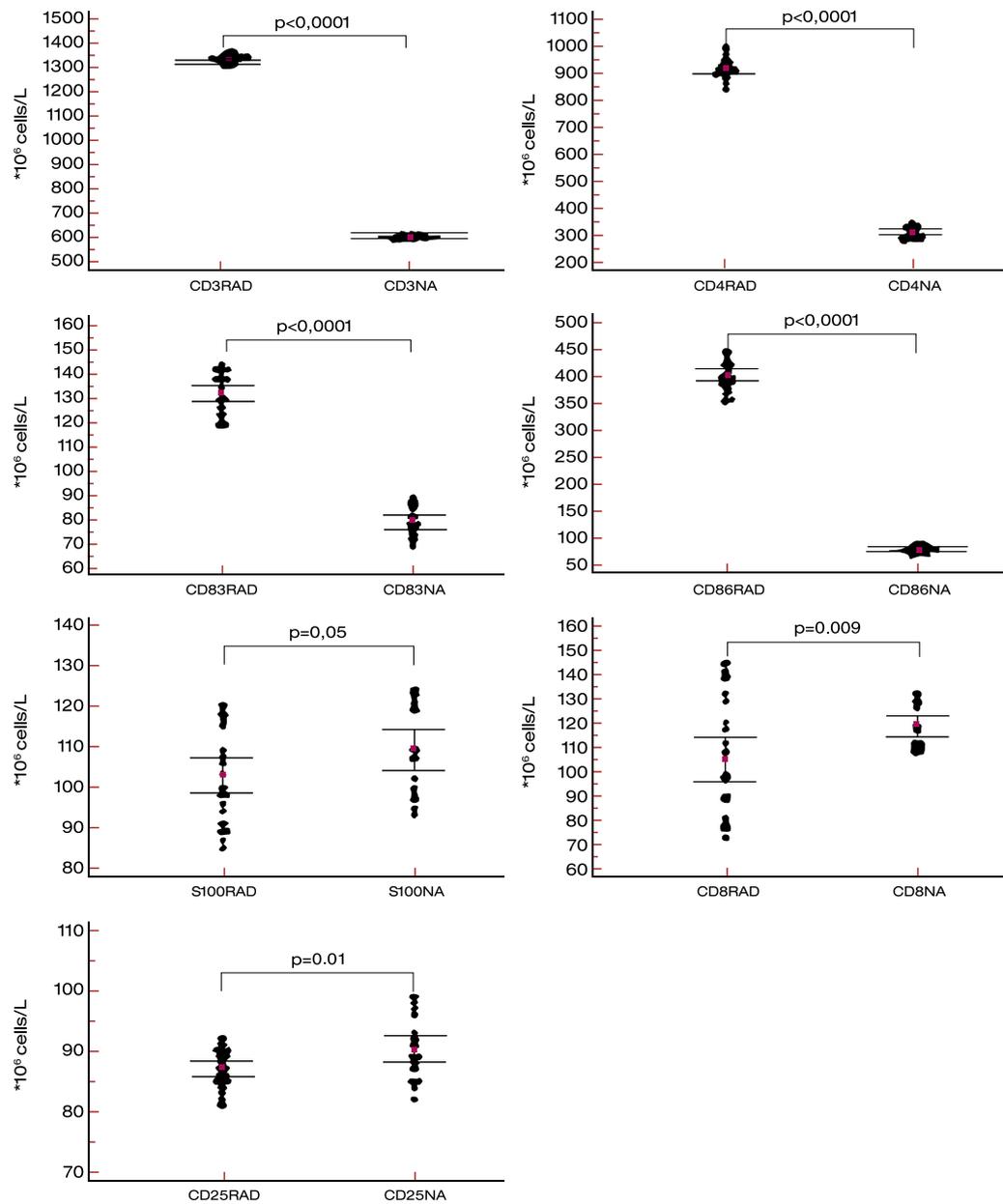


Figure 1. | Cellular immune markers. Plot of each immune marker evaluated in peripheral blood samples from patients with renal atherosclerotic disease (RAD) and normal arteries (NA). Mean values are shown as red dots.

immune response should be involved in this disease. Whether the described features are linked to an autoimmune response remains to be elucidated (36,37). In this respect, the increase CD4/CD8 ratio may resemble the ratio of other autoimmune disorders such as multiple sclerosis (38). Therefore, an epitope-spreading mechanism triggered by a limited antigen exposure initially causes a moderate immune response. This response is followed by more antigen exposure, which further expands the effect. Such a mechanism is compatible with local events happening in discrete areas without systemic involvement (39,40).

Although consistent with the natural history of the atherosclerotic plaque, this observational study did not address mechanisms, which is a limitation; thus, we can

only suggest a scenario of adaptive immune mechanisms in a clinically silent setting. Another study limitation resides in the inability to define whether the increased CD4+ found in RAD patients is responsible for the tissue injury. Thus, we have not included a specific methodology to identify the antigen or antigens that could be triggering the immune reaction.

At any rate, this finding is of particular interest, keeping in mind that no specific treatment has been recommended for this disease stage. Potential inferences include interventions to prevent renal disease progression. However, more research is needed to understand the mechanisms involved, including the possibility of concomitant immunologic responses at other vascular beds (coronary and carotids) and the eventual involvement of tissue-specific antigens.

Table 3. Immune markers expression in renal arteries obtained *postmortem* from asymptomatic hypertensive subjects

Immune Marker	RA _{PM} (n=21)	NA _{PM} (n=21)	P
CD3	91.8 ± 10.2	18.9 ± 2.6	<0.001
CD4	81.4 ± 8.6	19.1 ± 2.8	<0.001
CD8	16.6 ± 3.4	15.5 ± 5.7	0.45
CD25	12.7 ± 4.5	11.7 ± 6.0	0.54
S-100	7.9 ± 1.2	1.7 ± 0.4	<0.001
CD86	11.5 ± 3.7	1.2 ± 0.5	<0.001
CD83	34.2 ± 6.3	6.1 ± 2.1	<0.01
CD4+/CD8+ ratio	3.6 ± 0.7	1.5 ± 0.3	<0.001
CD25+/CD4+	0.15 ± 0.08	0.61 ± 0.02	<0.001

Values are expressed as mean ± SD. RA_{PM}, atherosclerotic renal arteries *postmortem* samples; NA_{PM}, normal renal arteries *postmortem* samples.

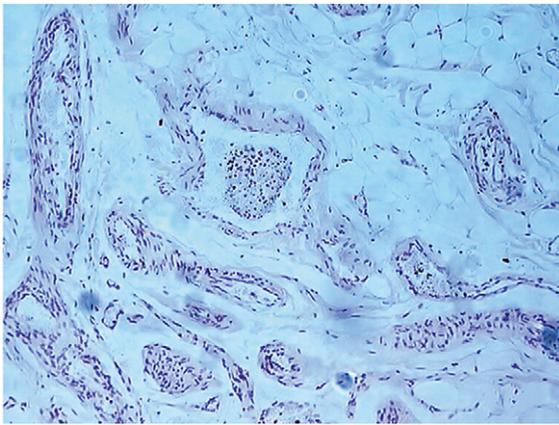


Figure 2. | CD4+ T cells. CD4+ T cells in atherosclerotic tissue from the right renal artery (*postmortem* sample). Cumules of CD4+ T cells were intensively associated with highly vascularized areas.

In summary, our study shows evidence of adaptive immune cell mechanisms in hypertensive patients with early asymptomatic renal artery disease, which could eventually be monitored by analyzing immune cell markers in the peripheral blood. These findings would encourage additional research to examine potential diagnostic and preventive interventions early in the development of RAD, thus averting the management of the more advanced disease, which still remains controversial.

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Disclosures

None.

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