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Associations between clinical features and therapy with macrophage subpopulations and T cells in inflammatory lesions in the aorta from patients with Takayasu arteritis

Short title: Macrophages and T cells in Takayasu arteritis.

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Abbreviations

ANOVA: Analysis of variance; CD: Cluster of differentiation; CRP: C-reactive protein; bDMARD: biological disease-modifying antirheumatic agents; csDMARD: conventional synthetic disease-modifying antirheumatic agents; DAB: 3'-diaminobenzidine tetrachloride; ESR: Erythrocyte sedimentation rate; GCA – Giant cell arteritis; H_2O_2: Hydrogen peroxide; HRP: Horse-radish peroxidase; IBM – International Business Machines; IFN – Interferon; IL: Interleukin; NIH: National Institutes of Health; NK: Natural killer; SPSS: Statistical Package for Social Sciences; TAK: Takayasu arteritis; Th – T helper; UK: United Kingdom; USA: United States of America.

Summary

Takayasu arteritis (TAK) is a large-vessel granulomatous vasculitis, the inflammatory infiltration in arteries comprises macrophages, multinucleated giant cells, $CD4^+$ and $CD8^+$ T cells, γδ T cells, NK cells, and neutrophils. However, it is unknown which subtype of macrophages predominates. This study aims to evaluate macrophages subpopulations in the aorta in TAK. Immunohistochemistry was performed in the aorta from TAK patients (n=22), patients with atherosclerotic disease (n=9), and heart transplant donors (n=8) using the markers: CD68, CD86, CD206, CD3, CD20, and CD56. Active disease was observed in 54.5% of patients and active histologic lesions were found in 40.9%. TAK patients presented atherosclerotic lesions in 27.3% of cases. The frequency of macrophages, M1 macrophages, T cells, B cells, and NK cells was higher in the aorta from TAK and atherosclerotic patients compared to heart transplant donors. In TAK, macrophages and T cells were the most abundant cells in the aorta, and the expression of CD206 was higher than CD86 (p=0.0007). No associations were found between the expression of cell markers and active disease, or with atherosclerotic lesions. In TAK patients, histological disease activity led to higher T cell counts than chronic fibrotic lesions (p=0.030), whereas prednisone use was associated with lower T cell counts (p=0.035). In conclusion, M1 macrophages were more frequent in TAK and atherosclerotic patients compared to heart transplant donors, while M2 macrophages dominated M1 macrophages in TAK. T cells were associated with histological disease activity and with prednisone use in TAK.

Introduction

Takayasu arteritis (TAK) is a systemic and granulomatous vasculitis that affects large vessels, usually in individuals younger than 40 years [1]. The inflammatory process in the arteries of TAK patients leads to concentric vessel wall thickening, and eventually, it evolves to segmental stenosis, occlusions, dilation, or aneurysm formation [2,3]. Adventitia and *vasa vasorum* are the primary sites where inflammatory lesions begin, and it extends to all layers of vessel walls. Although TAK is pan-arteritis, affected arteries in TAK patients can have non-affected areas so-called skip lesions [3].

The inflammatory infiltrates in TAK comprise dendritic cells, macrophages, multinucleated giant cells, neutrophils, γδ T cells, NK cells, B cells, and $CD4^+$ and $CD8^+$ T cells [4-6]. The granulomatous inflammation in large-vessel vasculitis may start with the arrival of interferon (IFN)γ-producing Th1 $CD4^+$ T cells [7]. In *vitro* studies have shown that stimulation of peripheral blood mononuclear cells from TAK patients not only drives the production of Th1 cytokines such as interleukin (IL)-12 and IFNγ but also the production of IL-6 and IL-17A [8]. Moreover, IFNγ, IL-6, and IL-17A are all present in arteries from TAK patients [8,9]. These findings indicate that both Th1 and Th17 cells play a role in the pathogenesis of TAK [10].

Macrophages are heterogeneous innate immune cells present in all organs and tissues either as resident-cells or as migrating inflammatory cells derived from blood monocytes during inflammation or infectious states [11]. Macrophages play an important role in host defense against infectious agents and cancer cells, but they are also involved in tissue homeostasis by promoting the resolution of inflammatory processes and tissue repair [12]. Macrophages differentiate into different subpopulations depending on the features of the cytokines produced by other inflammatory cells in the environment [13]. Once differentiated, each macrophage subpopulation has a different pattern of gene expression and protein secretion, especially cytokines and chemokines. These subpopulations include the M1 and M2 macrophages that usually mirror Th1 and Th2 responses. M2 macrophages can further differentiate into the following subtypes: M2a, M2b, M2c, and M2d [14,15].

Although TAK is a granulomatous vasculitis with a central role of macrophages in the pathophysiology, it is unknown yet which macrophage phenotypes are predominant in arteries from TAK patients. Getting insight in these subpopulations could help in defining new biomarkers and the development of new treatment strategies.

Therefore, this study aims to evaluate M1 and M2 macrophage subpopulations in the aorta from TAK patients. Patients with atherosclerotic disease and heart transplant donors comprised the control groups. We also investigated associations between macrophage subpopulations and clinical and histology disease activity in TAK patients, as well as with other cell types present in the inflamed vessel wall such as T cells, B cells, and NK cells.

Patients and methods

Patients and controls

This study had a cross-sectional design. The inclusion criteria in the TAK group were the fulfillment of the American College of Rheumatology classification criteria or the Ishikawa diagnosis criteria for TAK modified by Sharma [16,17] and availability of aorta specimens after aortic surgery or autopsy. The control groups comprised patients who underwent aortic surgery for complicated atherosclerotic disease and from heart transplant donors. Atherosclerotic lesions in the aorta from the study's participants were defined as previously described [18]. The Institutional Review Board approved the research protocol on 17th December 2015, and this study complied with the Declaration of Helsinki (Comitê de Ética em Pesquisa da UNIFESP-EPM, nr. 1210/2015).

Disease-related variables

Information about demographics, disease features, and therapy was retrieved from medical records. Disease activity at the time of surgical intervention was ascertained by Kerr's criteria and by ITAS2010 (Indian Takayasu Clinical Activity Score) [19,20]. Disease activity was considered by the ITAS2010 if the patient scored ≥ 2 [20]. In TAK patients, disease activity based on histology was ascertained by a pathologist, as previously described [2,3]. The active histological disease was considered if mononuclear inflammatory infiltrates were detected in arterial layers such as media and adventitia with

or without multinucleated giant cells, edema, and intimal hyperplasia. On the other hand, the absence of histological disease activity in TAK was considered in the presence of prominent medial fibrosis, loss of smooth muscle cells, and vascularization within arterial wall layers without inflammatory mononuclear infiltration in the aorta [3]. Arteries from TAK patients were also assessed for concomitant atherosclerotic lesions.

Immunohistochemistry

Paraffin-embedded and formalin-fixed arterial specimens were processed for immunohistochemistry. Six 4μm sections were deparaffinized at 75-80ºC for 30 minutes, and slides went on consecutive baths with alcohol, distilled water, and Dako Buffer for 5 minutes each. Then all slides underwent antigen retrieval using the Dako Target Antigen Retrieval solution at the PT Link device (Agilent - Dako, Santa Clara, USA) with two baths of 20 minutes each at 90ºC followed by cooling of the slides at 65ºC. After this step, all slides were washed with EnVision^{TM}Flex buffer (Agilent - Dako, Santa Clara, USA) for 5 minutes. Then slides were placed at the Dako Autostainer 48^{TM} (Agilent - Dako, Santa Clara, USA) for automated immunohistochemistry. Endogenous peroxidase was blocked with a 10 volume concentration H_2O_2 solution for 10 minutes. The following primary antibodies were incubated for 30 minutes: mouse anti-human antibodies anti-CD3 (ref.# ab699) as T cell, anti-CD20 (ref.# ab9475) as B cell, anti-CD56 (ref.# ab8233) as NK cell markers while anti-CD86 (ref.# ab196564)and anti-CD206 (ref.# ab117644) used as M1 and M2 macrophage markers, respectively (Abcam, Cambridge, UK). Mouse anti-human anti-CD68 (ref.# IS613) antibodies were the pan-macrophage marker (Agilent – Dako, Santa Clara, USA). Antibody binding was detected with secondary rabbit anti-mouse antibodies labeled with horse-radish peroxidase (HRP) (ref.# ab97046, Abcam, Cambridge, UK). The peroxidase activity was developed with 3'-diaminobenzidine tetrachloride (DAB) for 10 minutes. Afterward, the nuclei were counterstained with hematoxylin for 5 seconds.

Quantification of cellular markers

All slides were scanned and digitally stored using the Nanozoomer Digital Pathology Scanner (NDP Scan U10074-01; Hamamatsu Photonics K.K., Hamamatsu, Japan), and the Aperio ImageScope Viewer software (Leica Biosystems, Wetzlar, Germany) was used for quantification of the expression of each marker in arteries. A

semi-quantitative score was used for quantification as follows: 0 (absence of expression), 1 (≤1%), 2 (2-20%), 3 (20-50%) and 4 (>50%), according to the frequency of positive cells in 10x magnification fields [21] throughout the whole artery by two raters (AWSS and JPS) who were blinded for the patient data. A mean score of each rater was calculated by summing up the quantification of each cell marker in all fields of the aorta and by dividing it for the total number of fields. Then, the mean score from both raters was calculated and used for statistical analysis. Seven aorta specimens presenting the best tissue integrity and clear distinction between layers throughout the arteries were chosen for the quantification of macrophage markers in each layer (i.e., intima, media, and adventitia).

Statistical analysis

Data were analyzed by the IBM SPSS Statistics for Windows version 21.0, and graphs were built using GraphPad Prism software version 5.0. The median and interquartile range (IQR) were used to present continuous variables, while categorical variables were presented as the percentage and absolute number. Comparisons between two groups for continuous variables were performed by the Mann-Whitney's U test, while for comparisons among three groups, the Kruskal-Wallis test was used. The Mann-Whitney's U test was used as a post hoc test. Chi-square test or Fisher's exact test analyzed categorical variables. Spearman's correlation coefficient analyzed correlations. The significance level considered was 5% ($p < 0.05$).

Results

Features of patients with Takayasu arteritis

The study groups comprised TAK patients (n = 22), patients with atherosclerotic disease in the aorta (n = 9), and heart transplant donors (n = 8). TAK patients were younger than those with atherosclerotic disease and heart transplant donors [26.0 (19.0-31.8) vs. 69.0 (61.5-75.0) vs. 64.5 (50.0-70.5) years; $p < 0.0001$]. The frequency of females was also higher in TAK patients than in patients with atherosclerotic disease but not between TAK and heart transplant donors (72.7% vs. 22.2% vs. 42.9%; $p = 0.028$). All enrolled TA patients had aortic aneurysms, and the thoracic aorta was the most

frequent artery studied in all groups (86.4% vs. 77.8% vs. 100.0%), followed by the abdominal aorta.

Twelve (54.5%) TAK patients had active disease based on the clinical evaluation by Kerr's criteria, while nine (40.9%) patients were considered to present active disease based on ITAS2010. Twelve (54.5%) of all TAK patients underwent vascular/heart surgery at disease presentation due to aortic root dilation and aortic insufficiency. The median time since the diagnosis of TAK was 6.5 (1.0-48.0) months when aorta specimens were obtained. Twelve (54.5%) TAK patients were not under glucocorticoid or immunosuppressive therapy at the time of the vascular procedure (Supplementary material, Table S1).

Nine TAK patients (40.9%) presented active histological lesions in the aorta. Seven (31.8%) of TAK patients had both clinical and histological disease activity, whereas 20.0% of TAK patients considered in remission had active inflammation in the aorta. Six TAK patients (27.3%) presented concomitant atherosclerotic disease in aorta specimens. Nine (40.9%) TAK patients received prednisone therapy at a median daily dose of 17.5mg (5.0-20.0mg), and three patients (13.6%) received conventional synthetic disease-modifying antirheumatic agents (csDMARDs) or biological bDMARDs (i.e., two patients on methotrexate, one on mycophenolate mofetil, and one on tocilizumab) (Supplementary material, Table S1).

Immunohistochemistry in the aorta

The markers CD68, CD86, CD3, CD20, and CD56 had a significantly increased expression in the aorta from TAK patients and patients with atherosclerotic disease in comparison to heart transplant donors. The only expression of M2 macrophages was similar in all groups (Table 1). CD68, CD86, CD206, CD3, CD20, and CD56 are all expressed in the aorta of an active TAK patient (Figure 1). Figure 2 illustrates the expression of CD68 in multinucleated giant cells and the surroundings of *vasa vasorum* in the aorta from a TAK patient. The expression of CD68, CD86, CD206, CD3, CD20, and CD56 is shown in the aorta from patients with atherosclerotic disease and in heart transplant donors (Supplementary Figures S1 and S2).

Macrophages are the most abundant cells in arteries of TAK patients compared to T cells ($p = 0.0008$), B cells ($p < 0.0001$) and NK cells ($p < 0.0001$). On the other hand, T cells were more frequent than B cells ($p = 0.0002$) and NK cells ($p = 0.0001$). No significant differences were observed between B cells and NK cells in TAK patients ($p = 0.404$) (Figure 3). M2 macrophages were more frequent in the aorta from TAK patients compared to M1 macrophages (Figure 4). The M1/M2 ratio in the aorta from TAK patients was 0.420 (IQR: 0.289-0.756).

Correlations between the expression of cell markers in the aorta from TAK patients are described in Table 2. The expression of the pan-macrophage marker (CD68) was significantly correlated with the expression of CD3 and of CD20, but not with CD56. CD68 was more strongly correlated with the M2 marker (CD206) than with the M1 marker (CD86). However, the expression of the M1 macrophage marker (CD86) was correlated with CD3 and with CD20, while no other correlations were found with CD206 expression, including CD3, CD20 and CD86.

The distribution of macrophages was analyzed in the three layers of the aorta from seven TAK patients. Although the median expression of all macrophage markers was higher in the adventitia compared to the media and the intima, these differences were not statistically significant (Figure 5).

Disease-related variables and cellular infiltration in the aorta of TAK patients

The expression of each cell marker was analyzed regarding disease activity based on clinical assessment, histologic disease activity, concomitant atherosclerotic lesions in the aorta, and prednisone use. There were no significant differences between TAK patients presenting with active disease and those considered in remission using Kerr's criteria and the definition of disease activity by the ITAS2010 (Supplementary material, Table S2). Nevertheless, when histologic disease activity was analyzed, the T cell marker CD3 was higher expressed in TAK patients with active inflammatory infiltrate compared to those presenting chronic fibrotic lesions [1.540 (0.808-2.483) vs. 0.764 (0.392-1.255); $p = 0.030$]. The expression of macrophage markers in the aorta was similar between TAK patients with and without histologic disease activity (Supplementary material, Table S3).

The impact of atherosclerotic lesions on cellular infiltration in the artery evaluated in TAK patients was also analyzed. A tendency for a higher frequency of M2 macrophages was observed in TAK patients with atherosclerotic lesions compared to those without atherosclerosis [1.922 (1.845-2.146) vs. 1.081 (0.536-2.116); p = 0.070]. No other differences regarding the pan-macrophage marker CD68, M1 macrophages, T cells, B cells, and NK cells were found between TAK patients with and without atherosclerotic lesions in the aorta (Supplementary material, Table S4). The majority of TAK patients included in this study were not treated with csDMARDs or bDMARDs. Thus, current prednisone use was the only variable related to therapy assessed. The expression of the T cell marker CD3 was significantly lower in TAK patients who used prednisone compared to patients without prednisone [0.470 (0.303-1.306) vs. 1.211 (0.714-2.483); p = 0.035]. The expression of macrophages markers, B cells, or NK cells markers was similar in the aorta from TAK patients using prednisone or not (Supplementary material, Table S5).

No significant correlations were found between prednisone daily dose and the expression of CD68 (rho = 0.026; p = 0.951), CD86 (rho = -0.039; p = 0.927), CD206 (rho = 0.013; p = 0.976), CD3 (rho = 0.196; p = 0.642), CD20 (rho = 0.143; p = 0.735) or CD56 (rho = 0.665; p = 0.072) in the aorta from TAK patients.

Discussion

In this study, TAK patients and patients with atherosclerotic disease had a higher expression of macrophages markers, especially markers of M1 macrophages, as well as T cell, B cell and NK cell markers in the aorta compared to heart transplant donors, whereas M2 macrophages were found to be equally present in TAK and control groups. Macrophages are the most frequent cell type in the aorta from TAK patients, followed by T cells, B cells, and NK cells. Conversely, the expression of the macrophage M2 marker CD206 is higher than the M1 marker CD86 in the aorta from TAK patients. Macrophages and their subpopulations were found in all layers of the aorta from TAK patients. Active disease based on histology findings and prednisone use had a positive and a negative association with CD3 expression in the aorta, respectively. No macrophage marker had significant associations with histologic or clinical parameters in TAK patients.

Due to its inflammatory nature, patients with atherosclerosis [22] were included in this study as a positive control group, while heart transplant donors were included as negative controls, which is healthy individuals with a lower chance of presenting any disease of the aorta. Besides, we decided to assess only aorta specimens from TAK patients and from control groups to avoid the influence of vascular-bed specific phenotypes, since vascular responses to the same stimulus may vary in different vascular trees [23]. As expected, expression of all but one cell marker was higher in the aorta from both TAK patients and patients with atherosclerotic disease. Only the expression of the M2 marker CD206 was similar in all groups. This finding may reflect the predominance of M2 macrophages in the aorta from healthy individuals. These M2 macrophages may be either tissue-resident macrophages or the response to stressful stimuli elsewhere in individuals with brain death due to other causes.

In this study, macrophages and T cells were the most frequent cells in the aorta from TAK patients. This observation highlights the importance of these two cell types in the pathogenesis of TAK, in which chronic granulomatous inflammation of the artery is a hallmark of the disease [6].

In TAK, the prevailing pathogenic model is that $CD4^+$ T cells are stimulated by dendritic cells in the adventitia towards a Th1 and Th17 responses with the production of cytokines that lead to activation of macrophages [6-8]. In line with the Th1 response in the pathophysiology of TAK, M1 macrophages were more frequent in the aorta from TAK patients compared to heart transplant donors as it is well-known that M1 and M2 macrophages phenotypes mirror the Th1 and Th2 responses [24], respectively. However, IL-17A induces a heterogeneous profile of macrophages with M1/M2 features, also known as atypical M2-like macrophages that have increased expression of inducible nitric oxide synthase (iNOS) and CD206 in mouse studies [25,26]. Thus, the higher expression of CD206 than CD86 in the aorta from TAK patients may not be only related to M2a or M2c macrophages, the subtypes that mostly express CD206 [27], but a reflex of the effect of Th17 response on macrophages leading to the atypical M2-like macrophages in the aorta from TAK patients. Further studies are necessary to clarify this issue.

In line with our results, the investigation of macrophage markers in systemic vasculitides such as giant cell arteritis (GCA), IgA vasculitis and in granulomatosis with

polyangiitis (GPA) demonstrated predominantly M2 macrophages in affected tissues despite significant differences in the pathophysiology of these diseases [28-33]. In GPA patients, the granulomatous inflammation of the upper airways is associated with a higher frequency of M2 macrophages and Th2 $CD4^+$ T cells compared to M1 macrophages and Th1 $CD4^+$ cells [29]. In addition, soluble CD163, a scavenger receptor used as an M2 marker, served as a surrogate marker of disease activity in GCA and glomerulonephritis in ANCA-associated vasculitis [32,33]. This evidence highlights the role of M2 macrophages in the pathophysiology of systemic vasculitides.

In TAK, the inflammatory process is believed to start in the adventitia and to evolve to all arterial layers as the disease progresses [3]. Thus, we assessed the expression of macrophage markers in the three layers of the arterial wall (i.e., intima, media, and adventitia) to evaluate whether macrophages would cluster in a predominant layer of the aorta in TAK. Nonetheless, macrophages markers were equally found in all layers, and this observation may indicate that the disease duration of TAK patients evaluated in this study may have been much longer than what we expected as macrophages had already migrated from the adventitia to other layers. Conversely, a recent study showed that inflammatory mononuclear cells aggregate in the adventitia of the aorta from TAK patients, especially surrounding the *vasa vasorum* [34].

The thoracic aorta is a vascular site frequently affected by atherosclerosis in TAK [35], and we found concomitant atherosclerotic lesions in the aorta in one-third of our TAK patients. However, the presence of atherosclerotic lesions in the aorta from TAK patients did not yield significant differences in the expression of cell markers, only a tendency for a higher expression of CD206 was found in those with atherosclerotic lesions. The meaning of these findings is uncertain. Previous evidence in atherosclerotic disease has shown that M2 macrophages are associated with plaque stability, while M1 macrophages are associated with the progression of atherosclerotic lesions and with the development of ischemic cardiovascular events [36].

In this study, there was no association between disease activity based on clinical assessment of TAK patients and the presence of active inflammatory infiltration in the aorta, and no cell markers were increased in TAK patients presenting with clinically active disease. This dissociation between systemic and arterial inflammation is well-known in

TAK patients, who keep presenting arterial inflammation and may develop new angiographic lesions even after the remission state has been achieved [18,37]. Alternatively, for TAK patients presenting signs and symptoms of active disease, the absence of inflammation in the aorta may be due to an active inflammatory process in arteries other than the aorta. Beyond the analysis between the expression of cell markers in arteries from TAK patients and overt signs of disease activity, the analysis of correlations with systemic arterial remodeling would also disclose interesting findings. The angiographic score is a validated tool developed to assess the degree of arterial involvement, including stenosis and dilatation in large-vessel vasculitis, and it may be used as a surrogate marker of systemic remodeling for future studies assessing arterial inflammation in TAK [38].

The higher expression of CD3 in the aorta from TAK patients presenting active histologic lesions indicates that T cells are important players in the pathogenesis of TAK as these cells activate macrophages, induce the formation of giant-cells, and sustain granulomatous inflammation in arterial walls [6,7]. Indeed, a previous study has shown that $CD4^+$ and $CD8^+$ T cells comprise approximately 30% of cells found in the inflammatory infiltrate in arteries from TAK patients [39]. On the other hand, prednisone use had a significant impact on the expression of $CD3^+$ cells in the aorta from TAK patients. The induction of T cells apoptosis is one of the glucocorticoid immunoregulatory properties that may be involved in decreasing T cells in the aorta from TAK patients [40]. In GCA, prednisone use led to the suppression of Th17 $CD4^+$ T cells in temporal arteries with the persistence of Th1 $CD4^+$ T cells [41]. This issue is still controversial in TAK since the *in vitro* production of Th1 cytokines is inhibited by prednisone use, whereas serum levels of Th17 cytokines are decreased in TAK patients on prednisone [8,42]. We could not find significant differences in the expression of other cell markers, including macrophage markers regarding histologic disease activity or prednisone use in the aorta from TAK patients.

The expression of the B cell marker CD20 was relatively low in the aorta from TAK patients. This finding is the opposite of what was reported for large-vessel GCA, that massive B cell infiltration in the aorta, especially in the adventitia outnumbered T cells and led to tertiary lymphoid organ formation in the artery [43]. However, this issue is still

controversial, since another study observed higher expression of CD20 and CD138 in the aorta from TAK patients compared to temporal arteries from GCA patients [34]. B cell disturbances are not well-characterized in TAK yet as they are in GCA patients [21]. The findings of circulating autoantibodies and the increased number of newly formed plasmablasts in peripheral blood from TAK patients presenting active disease indicate a role of B cells in the pathophysiology of TAK, but this issue needs to be further explored [44,45].

The limitations of this study include the relatively low number of aorta specimens evaluated as well as the lower frequency of females and the higher median age in the control groups compared to TAK patients. These differences may be due to the epidemiologic profile of TAK, a disease that affects mostly young females, while older males are affected more frequently by atherosclerotic disease. Moreover, the higher median age in the heart transplant donors' group may be associated with an increased number of co-morbidities and even subclinical atherosclerotic disease, and these features would increase the chance of finding more inflammatory cells in the aorta.

M1 macrophages were more frequent in the aorta from TAK and atherosclerotic disease patients compared to heart transplant donors. Conversely, the expression of CD206 was higher than CD86 in the aorta from TAK patients and this finding may indicate the presence of either M2 macrophages or the atypical M2-like macrophages induced by the Th17 response. Macrophages are found in all layers of the aorta in TAK. Histologic disease activity and prednisone use are both of influence on the expression of CD3 but not on macrophage markers. Clinical disease activity and concomitant atherosclerotic disease had no impact on the expression of macrophage markers in the aorta from TAK patients.

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Disclosures

The authors declare no conflicts of interest related to this study.

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Figure legends

Figure 1 – Immunohistochemistry in the aorta from a TAK patient.

Legend – Representative images from a TAK patient with staining at 20x magnification for the pan-macrophage marker CD68 (1A), CD86 for M1 macrophages (1B), CD206 for M2 macrophages (1C), CD3 for T cells (1D), CD20 for B cells (1E) and CD56 for NK cells (1F). The images shown in Figure 1A, 1B, 1D, and 1E were taken in the media layer of the aorta, and the images shown in Figures 1C and 1F were taken at the boundaries between the intima and the media layer. Cell nuclei were stained in blue by hematoxylin, and each slide was stained with a brown specific marker (HRP-conjugated secondary antibodies).

Figure 2 – Active inflammatory infiltration in the aorta from a TAK patient.

Legend – Representative images in the aorta from a TAK patient with staining at 20x magnification for the pan-macrophage marker CD68 showing multinucleated giant cells (white arrows) in the media layer (2A) and the adventitial inflammatory infiltrate surrounding the *vasa vasorum* (2B).

Figure 3 – Expression of cell markers in the aorta from TAK patients.

Legend – Macrophages are the most frequent cells in the inflammatory infiltration in the aorta from TAK patients, followed by T cells. The median and IQR expressions of CD68, CD3, CD20 and CD56 were 2.121 (1.938-2.663) vs. 0.943 (0.562-1.749) vs. 0.176 (0.077-0.687) vs. 0.305 (0.139-0.637) $p < 0.0001$, respectively. The crossbar represents the median expression of cell markers. Data were presented as median and interquartile range and the analysis was performed by the Kruskal-Wallis test and by the Mann-Whitney's U test.

Figure 4 – M1 and M2 macrophage markers in the aorta from TAK patients.

Legend – CD206 vs. CD86: 1.672 (0.667-2.230) vs. 0.581 (0.366-0.949); $p = 0.0007$. The cross bar represents the mean expression of macrophage markers. Data were presented as median and interquartile range and the analysis was performed by the Mann-Whitney's U test.

Figure 5 – Expression of macrophage markers in the three layers of the aorta from TAK patients.

Legend – No significant differences were observed for the expression of macrophages markers in three layers of the aorta, as follows: CD68 [Intima: 1.174 (0.963-1.316) vs. Media: 1.253 (0.697-1.522) vs. Adventitia: 1.720 (1.129-2.776); p = 0.328], CD86 [Intima: 0.676 (0.223-0.980) vs. Media: 0.746 (0.316-1.513) vs. Adventitia: 0.947 (0.358-1.353); p = 0.700] and CD206 [Intima: 1.222 (0.658-1.918) vs. Media: 1.095 (0.793-1.510) vs. Adventitia: 2.204 (1.107-2.454); p = 0.326]. The cross bar represents the median expression of macrophage markers. Data were presented as median and interquartile range and the analysis was performed by the Kruskal-Wallis test.

Table

Table 1 – The expression of cell markers in the aorta from patients in all groups.

Variables	TAK (n = 22)	AD (n = 9)	HTD (n = 8)	*p*	*TAK vs. AD* *p*	*TAK vs. HTD* *p*	*AD vs. HTD* *p*
CD68	2.120 (1.937-2.662)	1.771 (1.465-2.229)	0.807 (0.351-1.538)	0.001*	0.164	<0.0001*	0.007*
CD86	0.581 (0.366-0.949)	0.631 (0.476-1.086)	0.257 (0.178-0.380)	0.003*	0.354	0.005*	0.001*
CD206	1.671 (0.667-2.229)	1.364 (1.279-1.729)	1.278 (0.324-1.468)	0.273	0.543	0.121	0.338
CD3	0.943 (0.562-1.749)	0.930 (0.634-1.177)	0.462 (0.153-0.784)	0.033*	0.728	0.017*	0.021*
CD20	0.176 (0.077-0.687)	0.242 (0.202-0.299)	0.047 (0.025-0.100)	0.004*	0.896	0.002*	0.004*
CD56	0.305 (0.139-0.637)	0.283 (0.134-0.454)	0.072 (0.002-0.268)	0.016*	0.500	0.007*	0.021*

AD – atherosclerotic disease; HTD – heart transplant donors; n – number of patients; TAK – Takayasu arteritis. Data are presented as median and interquartile range. Comparisons among groups were performed by the Kruskal-Wallis test, and by the Mann-Whitney's U test as a *post hoc* test. * flags significant results.

Table 2 – Correlation analysis between the expression of cell markers in the aorta from TAK patients.

	Macrophage markers		Cell markers of lymphocytes and NK cells		
	CD86	**CD206**	**CD3**	**CD20**	**CD56**
CD68	Rho = 0.429 *p* = 0.053	Rho = 0.798 *p* < 0.0001*	Rho = 0.617 *p* = 0.002*	Rho = 0.493 *p* = 0.020*	Rho = -0.126 *p* = 0.577
CD86	--	Rho = 0.044 *p* = 0.855	Rho = 0.621 *p* = 0.003*	Rho = 0.586 *p* = 0.005*	Rho = 0.047 *p* = 0.841
CD206	Rho = 0.044 *p* = 0.855	--	Rho = 0.397 *p* = 0.083	Rho = 0.177 *p* = 0.454	Rho = -0.111 *p* = 0.640

Correlation analysis were performed by the Spearman's correlation coefficient. * flags significant results.

Supplementary files

Figure S1. Expression of cell markers in the aorta from a patient with atherosclerotic disease.

Figure S2. Expression of cell markers in the aorta from a heart transplant donor.

Figure S3. Significant correlations between cell markers in the aorta from TAK patients.

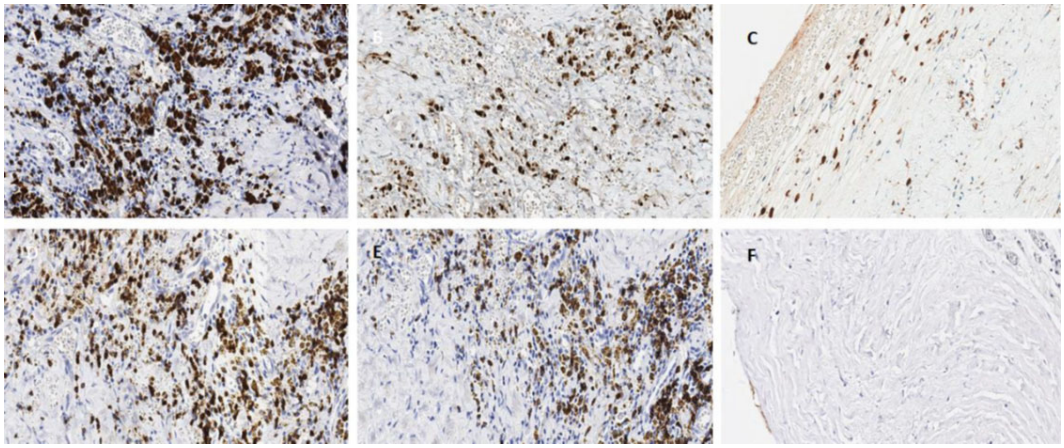
Table S1. Disease features of patients with Takayasu arteritis.

Table S2. The expression of cell markers in the aorta of TAK patients and disease activity.

Table S3. The expression of cell markers in the aorta of TAK patients and histologic disease activity.

Table S4. Atherosclerotic lesions in the aorta from TAK patients and the expression of cell markers.

Table S5. The expression of cell markers in the aorta of TAK patients and prednisone use.



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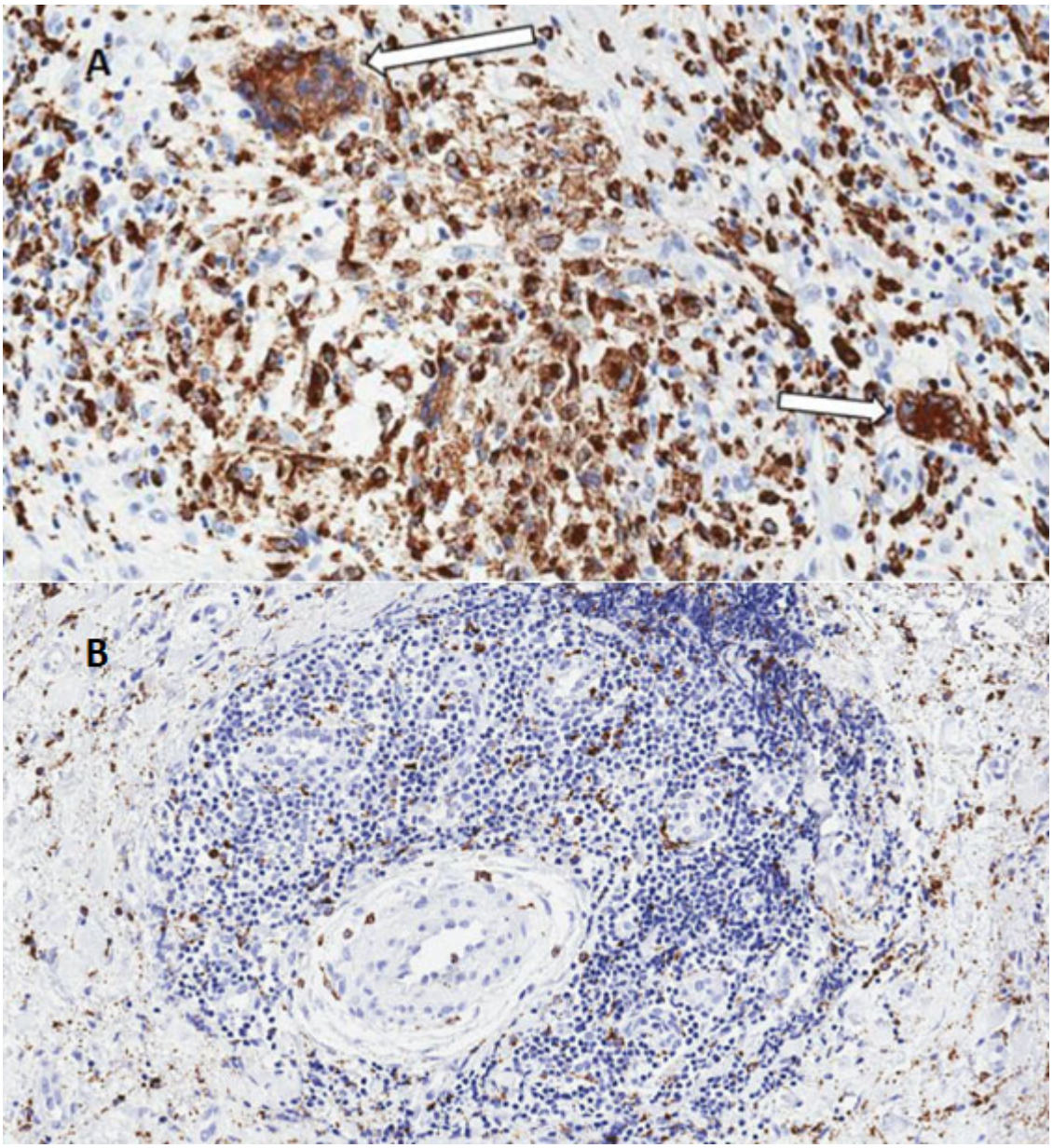

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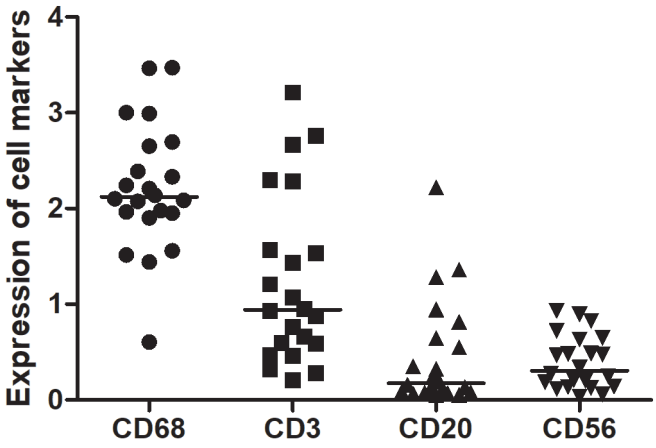

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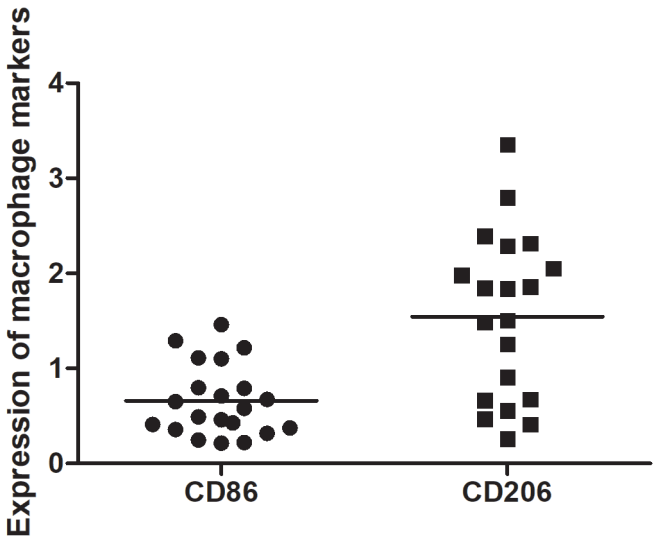

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