

Targeting novel antigens in the arterial wall in thromboangiitis obliterans

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Abstract: Thromboangiitis obliterans is an inflammatory disease possibly resulting from cigarette smoking as a primary etiologic factor, perhaps as a delayed type of hypersensitivity or toxic angiitis. As little is known about the pathogenesis of the disease, we aimed to determine novel antigens that might be responsible from the local inflammatory reactions and structural changes observed in this disease. An indirect immunoperoxidase technique is used to examine the tissue samples obtained from the dorsalis pedis artery of affected individuals with twenty monoclonal antibodies. Among these several antigens which are not previously reported in TAO like CD34, CD44 and CD90 were determined in the tissue samples examined. On the other hand, many other antigens like cytokine/chemokine receptors, several enzymes and leukocyte/lymphocyte antigens were lacking giving some clues about the local pathological reactions. We briefly discussed our findings for several critical antigens those first described in the present work, possibly having roles in the development of the disease. Expression of the CD90/CD11c receptor/ligand pair seems to play an important role in mononuclear cell recruitment to the damage site. Vascular invasion of not only tunica intima but also the tunica media in affected vessels is clearly demonstrated using endothelial cell specific antigens.

Key words: Thromboangiitis obliterans, TAO, Buerger's disease, vasculitis, immunohistochemistry, CD90, CD11c

Introduction

Thromboangiitis obliterans (TAO), or Buerger's disease, is a non-atherosclerotic segmental, thrombotic, inflammatory disease most commonly affecting the small and medium sized arteries and veins of the upper and lower distal extremities [1-4]. The disease was proposed in 1908 by Buerger [1]. Although the exact underlying cause of TAO is still unknown, the disease is strongly associated with tobacco abuse, implicating cigarette smoking as a primary etiologic factor [3,5,6,7]. Affected patients are mostly young male smokers, who develop ulcers and gangrene of the toes

and fingers as a result of the vascular ischaemia [5,8]. However, cases of women with TAO are becoming more common, coinciding with an increased incidence of smoking in this sex [9,10]. Although distal vessels of lower and upper extremities are the most commonly involved, other vessels such as cerebral, mesenteric, and coronary arteries can be rarely affected [11-14].

The diagnosis is based on characteristic clinical criteria as well as pathological findings in arteriography and histopathology; and there is no specific marker of the disease yet [5,15]. Acute Buerger's disease is characterized histopathologically by intensely cellular vessel wall inflammation, total luminal obliteration by the thrombus, together with a varying degree of recanalization; however, the internal elastic lamina and the overall vascular wall architecture remain preserved [5,16]. Cell infiltration was observed mainly in the thrombus and the intima. Macrophages expressing

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HLA-DR and dendritic cells are preferentially located in the intima and these cells may present antigens brought by the blood stream to CD4⁺ T lymphocytes predominating in the infiltrate [17,18].

The pathogenesis of this disease is poorly understood and most hypotheses are controversial [4,7,16,18-20]. Accumulating clinical evidence point to an inflammatory and immunological pathogenesis. TAO is found to be associated with HLA-A9 and HLA-B5 antigens as well as polymorphism of the MICA gene [21,22]. A prothrombotic genetic factor was claimed to be associated with the risk of TAO [23]. Although most investigators speculate about an autoimmune mechanism, no direct causative antigen has yet been discovered [24,25]. However, an association between anticardiolipin antibodies and this disease was reported [26]. Antiendothelial cell antibodies were proposed to play a role in the pathogenesis of TAO [27]. Various studies were undertaken to examine the mechanism of the immune injury. An increased cellular and humoral response to collagen [28,29], circulating immune complexes [30,31], elevated anti-elastin titers [32] and abnormal elastin degradation [33] were reported. The altered production of IL-6, IL-12 and IL-10, the increased apoptosis as well as the elevated levels of circulating immune complexes were proposed to be the reason for the persisting immune inflammation in TAO [34]. The specificity of all these findings remains to be established.

As little is known about the pathogenesis of the disease we aimed to determine novel antigens that might be susceptible for the local reactions observed in this disease with the reference of previous reports. For this purpose we studied frozen sections of tissue samples from dorsalis pedis artery of affected individuals performing a screening using twenty monoclonal antibodies. Among these were several antigens which are not previously reported in TAO like CD34, CD44 and CD90. Some cytokine/chemokine receptors, several enzymes and leukocyte/lymphocyte antigens were also targeted. We discussed the findings indicating possible roles of these antigens or their absence in the development of the disease aiming to direct future studies including several novel antigens of yet unknown specificity.

Materials and methods

Specimens and sections. After obtaining an informed consent and receiving approval from the institutional Ethics Committee on human research, we have taken the tissue samples as partial biopsy specimens from Department of Pathology, Dicle University Faculty of Medicine from two patients who were both men and fulfilled all of Shionoya's clinical criteria for the diagnosis of Buerger's disease: (1) smoking history; (2) onset before the age of 50 years; (3) infrapopliteal arterial occlusions; (4) either upper limb involvement or phlebitis migrans; and (5) absence of atherosclerotic risk factors other than smoking [15]. Histopatho-

logical diagnosis of both cases were confirmed by the same department.

The 43 year old patient had a smoking history of 13 years (20-30/day). There was gangrene in the big toe and necrosis on the tips of the 2nd and 3rd toes of his right foot. Big toe amputation and right lumbar sympathectomy operations were performed, because there were no convenient distal arterial vessel detected with Doppler USG and conventional angiography for the arterial by-pass surgery. The part of the medial tarsal branch of the dorsalis pedis artery which was within the amputated tissue was used for the study. The other patient, 37 years old, had been a smoker for 9 years (20/day) prior to admission in our clinic. He was suffering from pain and redness in the left foot and critical ischemia (in the extreme) was observed on the big toe of his left foot when he was admitted to the clinic. Doppler USG and conventional angiography showed that the blood supply from the left popliteal artery was only by weak, thin collaterals in some parts. Because there was no convenient distal vessel for the arterial by-pass surgery, only left lumbar sympathectomy and left big toe amputation were performed. The distal part of the dorsalis pedis artery within the amputated tissue was taken for the study also in this case.

Tissue samples from the dorsalis pedis artery of affected individuals were immediately frozen in liquid nitrogen, and stored in a liquid nitrogen tank for 2-3 days until sectioned. Cryo-sections (6-8µm thick) were taken with cryostat (Leica Frigocut 2800E, Wetzlar, Germany) on gelatin-coated slides and dried at room temperature. Then the sections were kept in humidity-free containers at room temperature until the staining was performed the day after.

Antibodies and staining procedure. In order to perform the screening, immunohistochemistry was performed using about twenty monoclonal primary antibodies which are all generated in mice including HI302, anti-vimentin, anti-CD10, anti-CD11c, anti-CD19, anti-CD20, anti-CD22, anti-CD34, anti-CD44, anti-CD62E, anti-CD80, anti-CD90, anti-CD104, anti-CD106, anti-CD116, anti-CD117, anti-CD123, anti-CD132, anti-CD195 and anti-TARC as listed in the Table 1. The immunostaining procedure previously described is applied as follows [35]. Sections were fixed in acetone for 10 minutes and air-dried for at least 30 minutes. Sections were then incubated for 60 minutes at room temperature with monoclonal antibodies (mAbs) in a humidity chamber. The dilutions of the mAbs used in the study are also given in Table 1. For negative controls, primary antibodies were replaced by their non-immune isotypes. After washing in phosphate buffered saline (PBS) pH 7.4, the slides were incubated with a 1:200 dilution of anti-mouse IgG peroxidase (Sigma Cat no: A9044) in PBS containing 0.2% bovine serum albumin (Sigma Cat no: A-7034) and 1% normal human serum. After washing the slides with PBS, they were stained for peroxidase activity with 3,3'-diaminobenzidine-tetrahydrochloride (DAB) (Sigma Cat no: D3939). Counterstaining with hematoxylin was done. Sections were then examined by using a research microscope (Leica DMR, Wetzlar, Germany) and images were obtained by a digital camera (Leica DC500, Wetzlar, Germany).

Results

Histopathological findings

In the samples examined the lumina of the affected vessels were completely obliterated and microvessels in intima serving as recanalizing vessels were evident. Varying degrees of infiltrating mononuclear cells in tunica intima were also present in both of the samples. The tunica media was rather thick which is usually considered as a characteristic feature of TAO when compared to other obliterating pathologies like athero-

Table 1. The specificity, clone name, source and dilutions of primary antibodies used in this study are listed. HLDA WS VIII, Human Leukocyte Differentiation Antigens Workshop VIII; CD, cluster of differentiation.

Specificity	Clone Name	Antigen Synonyms	Source	Dilution
-	HI302	-	HLDA WS VIII	1/100
Vimentin	V9	-	Abcam	1/300
CD10 (CALLA)	56C6	Common acute lymphocytic leukemia antigen	Abcam	1/100
CD11c	Bu15	Integrin alpha X	Abcam	1/100
CD19	LT19	B-lymphocyte antigen	Abcam	1/100
CD20	L26	Fc epsilon receptor I beta chain	Abcam	prediluted
CD22 (BL-CAM)	RFB4	B lymphocyte cell adhesion molecule	Millipore	1/100
CD34	BI-3C5	Hematopoietic progenitor cell antigen	Abcam	1/100
CD44	F10-44-2	Hyaluronate receptor	Abcam	1/100
CD62E	P2H3	E-selectin	Millipore	1/200
CD80	MAB104	Activation B71 antigen	Abcam	1/50
CD90	AF-9	Thy 1 cell surface antigen	Abcam	1/100
CD104	ASC-3	Integrin β 4	Millipore	1/100
CD106 (VCAM1)	1.4C3.	Vascular cell adhesion molecule 1	Abcam	1/100
CD116 (CSF2RA)	4H1	Granulocyte-macrophage colony-stimulating factor receptor alpha	Bio Legend	1/100
CD117 (c-Kit)	57A5D8	Stem cell factor receptor	Abcam	1/300
CD123 (IL3RA)	6H6	Interleukin 3 receptor alpha chain	Abcam	1/100
CD132 (IL2RG)	5J92	Common cytokine receptor gamma chain	LifeSpan BioSciences	1/100
CD 195 (CCR5)	1M2D7	CC chemokine receptor type 5	BD Sciences	1/100
TARC	6SN	Thymus and activation-regulated chemokine	Leica Microsystems Inc.	1/30

sclerosis. Prominent internal elastic lamina also helps to delineate intima and media layers. In addition, the number of microvasculature was determined to be increased also in tunica media. No significant pathological change was observed in tunica adventitia.

Immunohistochemical findings

Control: In both control stainings (which the primary antibodies were replaced by their non-immune isotypes) no reaction was determined in all layers of affected vessels.

No significant reaction was detected for the following antigens: CD10 (CALLA- zinc metallo-peptidase), CD19, CD20, CD22, CD62E, CD80, CD116 (GM-CSF receptor alpha chain), CD132 (IL2RG; common cytokine receptor gamma chain; common gamma) and TARC (Thymus and Activation-Regulated Chemokine).

Reactive antigens and their reactivity pattern are summarized below.

A- Structural/stromal/endothelial antigens

Vimentin: A weak to moderate reaction was present in the vascular smooth muscle including the tunica media

of the artery itself (Fig. 1). Vimentin immunoreactivity helped to reveal all calibers of vasculature in the tissue samples examined. Intimal and adventitial fibroblasts were also reactive for the antigen.

CD104: Weak to moderate expression on the vascular smooth muscle and endothelial layer was observed, though not typically reflecting the linear tissue distribution of this integrin chain along the basal lamina.

CD34: The antigen is well recognized as a hemopoietic stem cell marker and it was strongly expressed by all of the microvascular endothelial cells present in the sections (Figs. 2a and 2b). These vessels were located in intima (recanalizing vessels), media and adventitia of the affected vessel. Reaction to this antigen was clearly restricted to the endothelial lining and revealed how extensive were the newly formed vessels in tunica intima. A significant finding observed by the expression of this antigen was the presence of extensive microvasculature in tunica media (which was somehow surprising being at this extent), in addition to intima and adventitia. This finding was more prominent at a higher magnification in Fig. 2b.

CD44: CD44 antigen was expressed by some vascular endothelial cells, fibroblasts in intima, media and

especially in adventitia and elongated cells (fibroblasts) along internal elastic lamina.

CD90: A moderate to strong reaction was present on fibroblasts but the antigen was also strongly expressed by all of the endothelial cells and vascular smooth muscle of newly formed vasculature in the lumen (Fig. 3).

CD106 (VCAM-1): This antigen was expressed by the microvascular endothelial cells and some mononuclear cells in the samples examined. Those vessels reactive for the antigen were the recanalization vessels in the intima.

HI302 monoclonal antibody (Endothelial cell marker): This endothelial cell antigen revealed the extensive vascular network along the whole thickness of the affected vessel wall (Fig. 4). As most endothelial cell antigens were also weakly reactive with vascular smooth muscle, this antigen reflected a unique sample which was absent on vascular smooth muscle cells.

B- Leukocyte/lymphocyte antigens

CD11c: CD11c antigen was detected on some cells with dendritic processes and also on blood mononuclear cells in the vessel lumen (Fig. 5). The cells observed in the vessel wall were macrophages; however, some of them may also represent dendritic cells which cannot be excluded.

CD195 (CCR5): This macrophage specific antigen revealed macrophages with their dendritic processes. Such reactive cells were present in all three layers of the vessel wall, but the presence of many reactive cells in tunica media was a bit surprising (Fig. 6).

CD117: Expression of c-KIT (stem cell factor receptor, SCFR) was found only in a few endothelial cells.

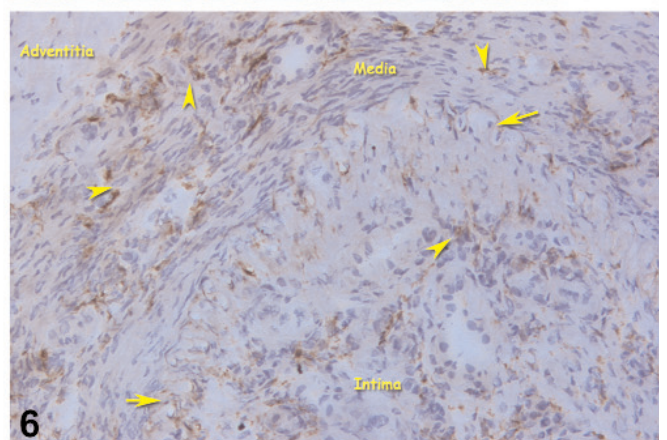
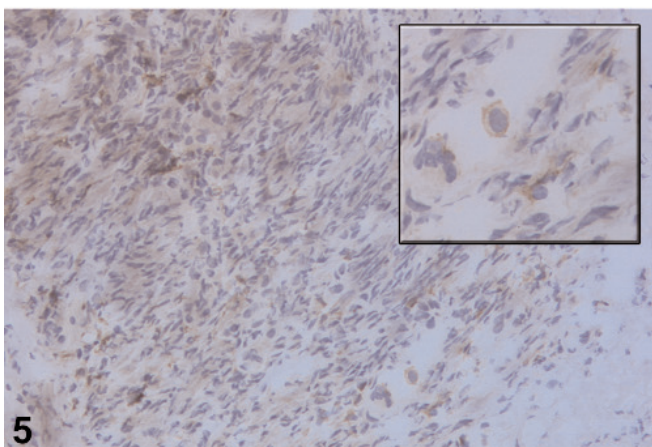
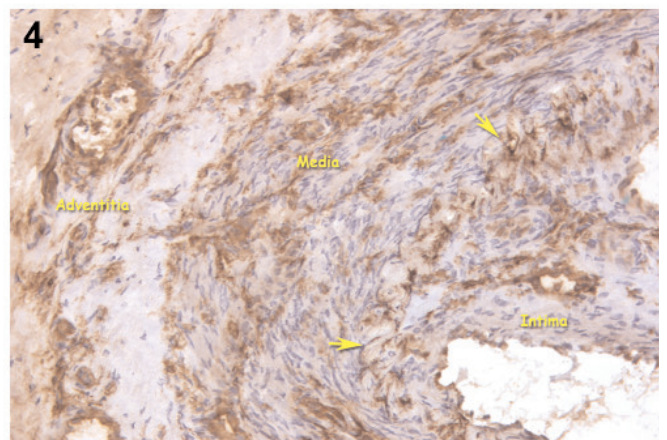
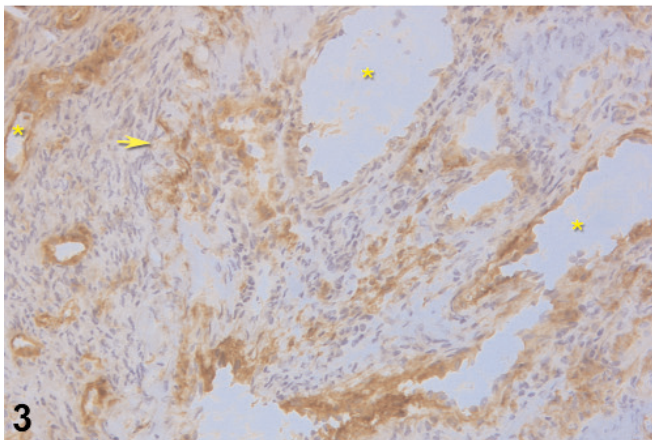
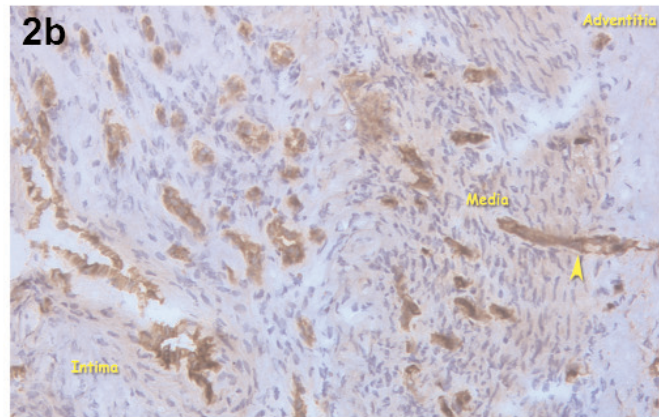
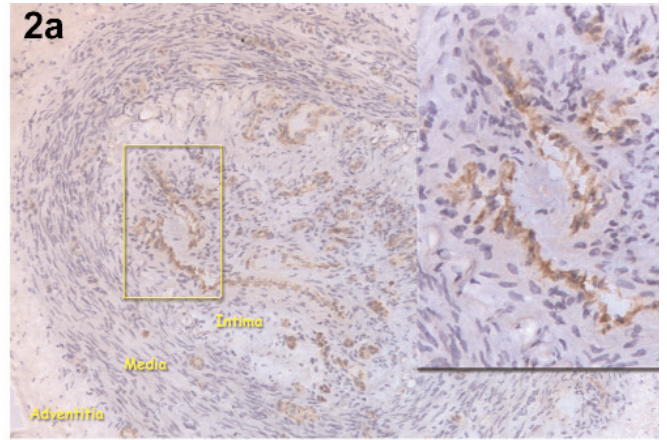
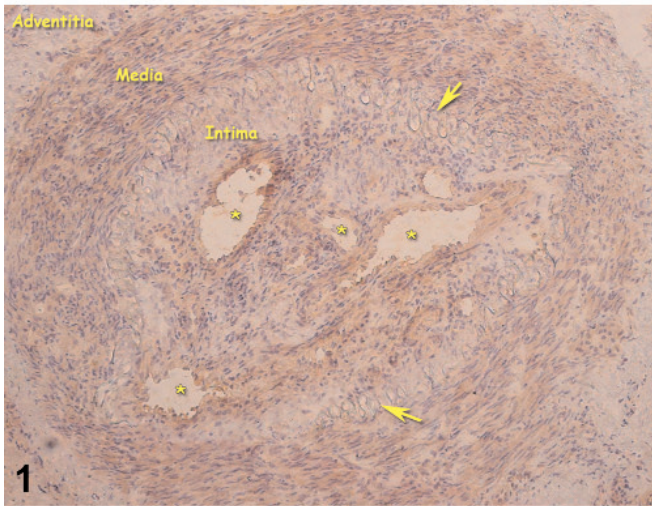
CD123: IL3-R alpha chain expression was determined only on the endothelial lining of some small vessels in the sections.

Discussion

Although the disease is well described about a century ago, we still know little about the mechanisms of the onset and progress of TAO, probably because only a restricted number of samples are available for examination. So far, only several immunohistochemical studies on this immune-induced, tobacco triggered disease were performed. In one of the studies, linear deposition of immunoglobulins and complement factors along the internal elastic lamina and presence of activated macrophages, B- and T-cells especially in the intima of the affected vessels have been demonstrated [17]. Kim *et al.* have reported that the immunologic injury to the internal elastic lamina is associated with T-lymphocytic infiltration and this might be the initial

morphogenetic mechanism of the thrombotic occlusion and organization of medium-sized arteries in TAO [16]. In another study, among the infiltrating cells, T-helper cells outnumbered cytotoxic T cells and the expression of VCAM-1 as well as iNOS were observed in endothelial cells around the intima or occluded segment [18]. In an immunohistochemical light and electron microscopical study to analyze the expression of TNF α , ICAM-1, VCAM-1 and E-selectin in surgical biopsies obtained from femoral and iliac arteries of patients with TAO, it is indicated that endothelial cells are activated in TAO and that vascular lesions are associated with TNF α secretion by tissue-infiltrating inflammatory cells [36]. Kurata *et al.* presented histological and immunohistochemical characteristics of TAO that would be helpful in a differential diagnosis from arteriosclerosis obliterans (ASO) and thromboembolism [6,7]. In the subset of definite TAO cases, the macrophages, and B- and T-lymphocytes were mostly found in close vicinity to internal elastic lamina and adventitia, often following the recanalizing vessels and vasa vasorum, suggesting that minute vessels and elastic fibers are specific targets of the inflammatory reaction in TAO [7]. It has been suggested that immunovascular disorder and dysfunction of the microvascular endothelial cells are the unifying frame of this vaso-occlusive disease, which is superimposed and modified by immune responses to normally hidden antigenic determinants of the vascular intima [6,7]. Nishikimi *et al.* also postulated that there is a breakdown of the microvascular defense system from the beginning of the disease [37]. In the current study we aimed to determine novel antigens hoping to obtain evidence on the nature of the development of the disease with special regard to inflammatory process and structural changes.

From a structural point of view, both of the samples examined in this study fulfilled the previously reported histopathological characteristics of the disease. Expression patterns of vimentin, CD104 and CD44 reflected the expected features of stromal component organization. One of our most interesting findings was the presence of numerous vessels also in tunica media which is not reported as a distinct histopathological finding. Examining vasculature using endothelial cell reactive antibodies like anti-CD34, HI302 and anti-CD90 helped greatly to determine the extent of the vascular network as well as its structural organization. Similar findings were observed in the illustrations of a previous report by Kurata *et al.* [7], however authors did not stress this finding. In our opinion, it is not surprising to have a similar reaction in adjacent compartments of a vessel facing the same local stimuli for angiogenesis and/or vasculogenesis. Moreover, CD90 (Thy-1) was strongly expressed by vasculature and by some stromal cells (fibroblasts and mesenchymal stem



cells?) in the neighboring connective tissue compartments. It is suggested that this glycoposphatidylinositol (GPI)-anchored immunoglobulin family member has a signaling function in various physiological and pathological events including thymocyte maturation, axon regeneration, apoptosis, adhesion, cancer, fibrosis, leukocyte recruitment and angiogenesis. CD90 also helps to characterize mesenchymal stem cell populations [38-44]. In our study, a strong and broad expression of CD90 supports the view that this antigen is involved in the new vessel formation in tunica intima and media of the affected vessels in TAO. It was previously reported that human Thy-1 (CD90) on activated endothelial cells serves as a counterreceptor for the leukocyte integrin Mac-1 (CD11b/CD18) [42]. We examined CD11c, another leukocyte integrin, and a strong expression of this antigen was determined on mononuclear cells in some samples. This finding together with CD90 expression by vascular endothelial cells in the involved tissue suggests that CD11c probably serves as another ligand for leukocyte migration for this antigen in TAO. CD90 may have a role in leukocyte (and/or other cell types) recruitment to the effected site of the vessel. Taken together, both findings suggest that CD90 is at least one of the key molecules involved in the pathogenesis of the disease. Our attempt to find out other antigens like CD106 (VCAM) and CD62E with a similar documented role as CD90 was not fruitful, as CD62E was completely lacking, and CD106 was expressed by the endothelial lining of few vessels and few mononuclear cells. Therefore, these molecules may play roles in the earlier stages of the disease, but probably not after the disease is completely settled down.

High endothelial venules are specific postcapillary venules located in diffuse lymphoid tissue compartments of secondary lymphoid organs and at chronic inflammatory sites, such as the synovial tissues in

rheumatoid arthritis [45,46]. In our samples, we did not observe a high endothelial venule, which indicates that continuous and pronounced lymphocyte migration to the lesion side is not present. B cell differentiation - maturation and/or activation markers, such as CD10, CD19, CD20 and CD22 were also lacking, suggesting that there is no B cell infiltration and antibody production locally. We also looked for TARC (Thymus and activation regulated chemokine) and CCR5 (CD195) expression to examine if this chemokine and chemokine receptor are involved in this process. TARC is reported to be a chemokine directing migration of CD4+Th2 lymphocytes which suppresses local inflammatory reaction [47]. CCR5 is a receptor for RANTES, MIP-1 α and MIP-1 β playing a dominant role in immune reactions like graft versus host disease, multiple sclerosis and rheumatoid arthritis [48-51]. TARC was not present in any of the TAO samples but CCR5 expression was determined on macrophages (some of which may represent dendritic cells). The absence of TARC may be considered as a sign of limited local suppression of inflammation. Similarly, the absence of several cytokine receptors, such as CD116 (GM-CSFR α chain), CD117 (Stem Cell Factor- cKIT receptor), CD123 (IL-3R α -chain), CD132 (IL-2R γ chain: common γ chain receptor for cytokine receptors) suggests that they are not involved in this process. On the other hand, the protein expression of CCR5 suggests a possible role for this receptor in TAO. CD80, a co-regulator of T cell activation with CD86, is involved in the alloactivation of T cells and known to play a critical role in autoimmune, humoral, and transplant responses. It is expressed on activated B cells, activated T cells and macrophages [52-55]. CD80 was also absent in TAO samples examined in this study.

In conclusion, in addition to the major involvement site intima, the involvement of tunica media is critical, regarding new vessel formation and macrophage / den-

Fig. 1. In a frozen section through dorsalis pedis artery the lumen of the vessel is seen to be completely obliterated due to TAO. Intact internal elastic lamina (arrows) indicates the border of tunica intima. Several sections of varying sizes of recanalizing vessels in the lumen of the vessel are observed (asterix). Vascular smooth muscle of both the artery itself and the newly developed recanalizing vessels is reactive for vimentin. Intimal and adventitial fibroblasts are also reactive. Indirect immunoperoxidase – hematoxylin counterstained, original magnification $\times 10$. **Fig. 2.** Immunohistochemical staining for CD34 antigen: (a) Strong CD34 reactivity on the endothelial lining of all vasculature is observed. Inset: Higher magnification of the area consisting of internal elastic lamina at lower left diagonal; several vessels in intima are evident. Indirect immunoperoxidase – hematoxylin counterstained, original magnification $\times 10$; inset $\times 20$. (b) Expression of the same antigen at a higher magnification. Intima consists of numerous recanalizing vessels. Internal elastic lamina delineates intima and media. More interesting finding is the presence of numerous vessels also in media. One of them is observed extending from adventitia into media (arrow head). Indirect immunoperoxidase – hematoxylin counterstained, original magnification $\times 20$. **Fig. 3.** A broader reactivity pattern for CD90 antigen is detected. In addition to endothelial cells, vascular smooth muscle and some fibroblasts are also reactive. Asterix: lumina of the vasculature. Arrow: internal elastic lamina. Indirect immunoperoxidase – hematoxylin counterstained, original magnification $\times 10$. **Fig. 4.** Immunoreactivity of HI302 mAb. Strong reactivity with almost all vascular endothelial cells is observed. Arrows: internal elastic lamina. Indirect immunoperoxidase – hematoxylin counterstained, original magnification $\times 10$. **Fig. 5.** Cells with dendritic processes are reactive (moderate to strong) for CD11c antigen. A large mononuclear cell in the lumen of a small vessel also exhibits a strong membranous reaction (inset). Indirect immunoperoxidase – hematoxylin counterstained, original magnification $\times 10$ (inset original magnification $\times 40$). **Fig. 6.** Cells with processes are reactive for CD195 antigen detected by 1M2D7 monoclonal antibody. As the antigen is specific to macrophages it is somehow surprising to observe many reactive cells in tunica media (arrow heads). Arrow: internal elastic lamina. Indirect immunoperoxidase – hematoxylin counterstained, original magnification $\times 10$.

dratic cell infiltration presumably participating in the complex pathogenesis of the disease. Among all the screened antigens, CD90 (Thy1) represents the most prominent one. Taken together with the expression of CD11c on macrophages/dendritic cells it can be suggested that this molecule pair is possibly involved in cellular recruitment to the affected vessel wall. CCR5 expression also suggests a role for this chemokine in this process. The present findings provide preliminary data indicating further examinations in greater detail will be valuable.

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