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R. Dellalibera-Joviliano,*† E. E. Joviliano,† J. S. Silva‡ and P. R. B. Evora†
*University Center UNIFAFIBE, Bebedouro, †Department of Surgery and Anatomy, Ribeirão Preto Faculty of Medicine, University of São Paulo, Ribeirão Preto, and ‡Department of Biochemistry and Immunology, Ribeirão Preto Faculty of Medicine, University of São Paulo, Ribeirão Preto, São Paulo, Brazil

Summary
Thromboangiitis obliterans (TAO) is a segmental inflammatory occlusive disorder that affects the arm and leg arteries of young smokers. The immune system seems to play a critical role in the aetiology of TAO; however, knowledge of the aspects involved in the progression of vascular tissue inflammation and, consequently, the evolution of this disease is still limited. This study was carried out to investigate the cytokine levels of tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-4, IL-17 and IL-23 in the plasma of TAO patients presenting with acute clinical manifestations. The study included 20 TAO patients (n = 10 women; n = 10 men) aged 38–59 years under clinical follow-up, classified into two groups: (i) TAO former smokers (n = 11) and (ii) TAO active smokers (n = 9); the control groups included normal volunteer non-smokers (n = 10, active smokers (n = 10) and former smokers (n = 10). Patients’ plasma samples were measured using the sandwich enzyme-linked immunosorbent assay. Statistical analyses were performed using the non-parametric Mann–Whitney U-test, with parameters significant at \( P < 0.05 \). The activities of all cytokines were different in groups of TAO patients when compared with normal controls, and decreased for control smokers. Increased levels of TNF-α, IL-1β, IL-4, IL-17 and IL-23 were significant in patients with TAO when compared to the controls (\( P < 0.005 \), all parameters). The results presented here indicate an increased production of cytokines in TAO, possibly contributing to the inflammatory response observed in the patients’ vascular levels. In addition, the increased levels of IL-17 and IL-23 suggest that the disturbance of TAO is involved with mechanisms of autoimmunity. Thus, the discovery of IL-17 and its association with inflammation and autoimmune pathology has reshaped our viewpoint regarding the pathogenesis of TAO, which was based previously on the T helper type 1 (Th1)–Th2 paradigm.

Keywords: autoimmune disease, Buerger’s disease, cytokines, inflammation, thromboangiitis obliterans
increased cellular immunity to types I and III collagen when compared with patients who have atherosclerosis [4,9]. In addition, high titres of anti-endothelial cell antibodies have been detected in patients with this disorder [10]. Otherwise, cytokines studies involving TAO patients are relatively scarce.

Cytokines are small soluble mediators released by various immune cell subsets and tissues, and have a particularly critical role in modulating both the innate and adaptive immune responses. Both a deficiency and an excess of cytokine production, as well as unusual responsiveness of immune cells to cytokines, can favour the development of immune-mediated disease, suggesting the constant requirement of a fine balance among cytokines to support immune homeostasis.

Adaptive immunity has two responses: (i) a humoral immune response by stimulating B lymphocytes to produce antibodies and (ii) a cellular immune response, where CD8+ T cells with cytotoxic and macrophages are activated. CD4+ lymphocytes participate in both responses by antigen recognition and their subsequent differentiation into effector T helper type 1 (Th1) or Th2 subsets. Th1 cells facilitate the cytotoxic cellular immune response or cytokine production by interleukin (IL)-2, IL-12, interferon (IFN)-γ and tumour necrosis factor (TNF)-α, while Th2 cells secrete primarily IL-4, IL-5 and IL-10, which help B lymphocytes to produce antibody, facilitating the humoral immune response. Regulatory T cells (Treg) are responsible for enforcing limits on the cell-mediated immune response and exert this function through immunosuppressive cytokines such as IL-10 and transforming growth factor (TGF)-β. The T lymphocytes CD4+ and CD8+ cells are capable of producing cytokines in line with Th1 or Th2. Stimulation by IL-12, released by activated dendritic cells, induces differentiation in the direction of cytokine production, Th1 and Th2 and suppression of Th17. IL-4 induces Th2 differentiation. CD4+ and CD8+ cells, which release Th2 cytokines, have a regulatory role, because high concentrations of Th2 cytokines can suppress the actions of Th1 and Th17. Th17 cells are a subset of T helper cells producing IL-17; they are considered developmentally distinct from Th1 and Th2 cells, and excessive amounts of the cell are thought to play a key role in autoimmune disease. On initial characterization, Th17 cells have been broadly implicated in autoimmune disease, and autospecific Th17 cells have been shown to be highly pathological. A more natural role for Th17 cells is suggested by studies that have demonstrated preferential induction of IL-17 in cases of host infection with various bacterial and fungal species. Th17 cells primarily produce two main members of the IL-17 family, IL-17A and IL-17F, which are involved in the recruitment, activation and migration of neutrophils; these cells also secrete IL-21 and IL-22 [11].

The pathogenesis of TAO is poorly understood; most hypotheses are controversial and the above-mentioned modern immunology concepts have not yet been applied to TAO patients. Therefore, this investigation was carried out to evaluate some components of the levels of selected cytokines in the plasma of patients with TAO (smokers or former smokers).

Materials and methods

Study participants

Informed consent was obtained from all the patients, and the study protocol was approved by the Ethics Committee of the University Hospital, Ribeirão Preto Faculty of Medicine, University of São Paulo, Brazil (no. 12810/2008).

The study included 20 TAO patients (n = 10 female, n = 10 male) aged 38–59 years under clinical follow-up. The TAO diagnosis was based on the Shionoya and Olin criteria that are used routinely in our vascular division [9]. The five classic Shionoya criteria include a history of tobacco abuse, the onset of symptoms before the age of 50 years, infra-popliteal arterial occlusive disease, either upper limb involvement or phlebitis migrans and a lack of atherosclerotic risk factors other than smoking [9]. The Olin criteria consider the onset of disease before the age of 45 years; current tobacco use; distal (i) clinical data: extremity ischaemia (infra-popliteal and/or infrabrachial), such as claudication, rest pain, ischaemic ulcers; (ii) gangrene documented with non-invasive testing; (iii); laboratory tests for exclusion of autoimmune or connective tissue diseases and diabetes mellitus; (iv) exclusion of a proximal source of emboli by means of echocardiography and arteriography; and (v) demonstration of consistent arteriographic findings in the involved and clinically non-involved limbs [4]. All selected patients reported the use of cigarettes for more than 20 years, and TAO was diagnosed at a mean age of 40 years. Ninety per cent of the patients exhibited evidence of critical limb ischaemia and 60% presented leg amputations (below- or above-knee amputation) in the contralateral leg. Thus, the patients were classified into two groups: (i) TAO former smokers with clinical remission (n = 11) and (ii) TAO active smokers with clinical exacerbation (n = 9); the control groups included normal volunteer non-smokers (n = 10), former smokers (n = 10) and active smokers (n = 10). All smokers analysed in this study (control and TAO) had used cigarettes for at least 3 years and smoked a minimum of 10 cigarettes per day. All the subjects classified as TAO former smokers were ex-smokers who had quit 10 years before or even earlier. Patients presenting with anti-phospholipid syndrome were excluded. Standard treatment was applied to all TAO patients, including anti-platelet treatment with aspirin (100 mg/day), pain management (orally 5–7 days) with anti-inflammatory (ibuprofen 400 mg thrice-daily) and opioid drugs (tramadol 100 mg thrice-daily), and advice to cease smoking immediately.

Blood collection

A trained biomedical technician collected a 10-ml venous blood sample from each participant. Blood samples were
collected in trace metal-free tubes (BD Vacutainer; BD Vacutainer, Franklin Lakes, NJ, USA) that contained ethylenediamine tetraacetic acid (EDTA) anti-coagulants. Two milliliters of blood were then pipetted into an Eppendorf tube previously cleaned in a class 100 clean room and frozen immediately at −70°C before analysis.

Cytokines
Quantitative determinations of TNF-α, IFN-γ, IL-1β, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17 and IL-23 were performed on plasma samples using the sandwich enzyme-linked immunosorbent assay (ELISA) [DuoSet® ELISA Development Systems; R&D Systems, Minneapolis, MN, USA]. The cytokine concentrations in plasma were determined by a double-ligand using an ELISA plate scanner (Molecular Devices SpectraMax 250, El Cajon, CA, USA). The cytokine concentration was expressed in pg/ml by the kit’s standard curve.

Statistics
The non-parametric Mann–Whitney U-test, Kruskal–Wallis and Wilcoxon’s tests were used for cytokine data analysis. The null hypothesis was rejected when the possibility of chance occurrence of observed differences did not exceed 5% (P < 0.05).

Results
Proinflammatory cytokines
Figure 1 shows the values of proinflammatory cytokine activities (IL-1β, TNF-α and IL-6) in the plasma of control individuals (non-smoker, ex-smoker and active smokers) (n = 10 for each group) and patients with TAO (active smokers and former smokers) (n = 10 for each group) expressed in pg/ml. The data results revealed an increase of these cytokines in the plasma of TAO patients compared with control subjects (P < 0.05 for each comparison). A significant difference was also observed between the TAO groups (P < 0.05).

Th1 cytokines
Figure 2 shows the values of the determinations of Th1 cytokine profiles (IFN-γ and IL-12) in the plasma of normal individuals (smoker, ex-smoker and non-smokers) and patients with TAO (smokers and former smokers). The data results show an increase of these cytokines in the plasma of TAO patients compared with control subjects (P < 0.05 for each comparison). A significant difference was also observed between the TAO groups (P < 0.05).

Th2 cytokines
Figure 3 shows values of the determinations of Th2 cytokine profiles (IL-4, IL-10, IL-13 and IL-5) in the plasma of normal individuals (smoker, ex-smoker and non-smokers) and patients with TAO (smokers and former smokers). The data results show increased levels of IL-4, IL-5 and IL-13 in the
plasma of TAO patients compared with control subjects ($P < 0.05$ for each comparison). Decreased levels of IL-10 were found in patients with TAO active smokers compared to control individuals and TAO former smokers ($P < 0.05$ for each comparison).

**Th17 cytokines**

Figure 4 shows the values of the determinations of Th17 cytokine profiles (IL-17 and IL-23) in the plasma of normal individuals (smoker, ex-smoker and non-smokers) and patients with TAO (smokers and former smokers). The data results show an increase of these cytokines in the plasma of TAO patients compared with control subjects ($P < 0.05$ for each comparison).

**Discussion**

Because the development and aetiology of TAO have not yet been elucidated, and as the direct action of inflammatory mediators has been observed in the vascular endothelium of TAO patients, in this study we have evaluated some components of the cytokines in the plasma of TAO patients who presented with acute symptoms. To the best of our knowledge, this is the first complete investigation including cytokines with proinflammatory, Th1, Th2 and Th17 profiles.

The precise cause of TAO is still unknown, and different hypotheses have been suggested. A reaction to the constituents of cigarettes is recognized as a factor in the initiation, progression and prognosis of this disease. It is possible that genetic modifications or autoimmune disorders are implicated [5,12,13]. Thus, the strong relationship with smoking seems to involve direct toxicity to the endothelium by certain tobacco products (nicotine) or an idiosyncratic immune response to some agents. Most patients with TAO have hypersensitivity to extracts of tobacco. Peripheral endothelium-dependent vasodilation is impaired in the non-diseased limbs of TAO patients, and this vascular dysfunction may contribute to such characteristics as segmental proliferative lesions or thrombus formation in the peripheral vessels [14].

The immune system seems to play a critical role in the aetiology of TAO. However, knowledge about immunological aspects involved in the progression of vascular tissue inflammation, and consequently the evolution of this disease, is still limited. TAO may be an autoimmune disorder, probably initiated by an unknown antigen in the vascular endothelium, possibly a component of nicotine. The presence of different antibodies such as anti-nuclear, anti-elastin, anti-collagens I and III and anti-nicotine antibodies, as well as identification of deposits of immunoglobulin (Ig)G, IgC3 and IgC4 in the blood vessels of patients, provide evidence for the theory of the immune character of TAO. Accordingly, the formation of immune complexes, activation of cell-mediated phagocytosis and the release of toxins stimulated by nicotine are the main agents responsible for vascular damage [14].

Regardless of the time of disease onset, recent studies have shown a significant increase in the levels of components of the kinin system observed in patients when TAO active smokers were compared with TAO ex-smokers ($P < 0.01$ for all analysed parameters). Kinin can stimulate proinflammatory cytokines (for example, TNF-α and IL-1β), and activation of the kinin system in TAO patients may indicate the involvement of vasodilatation in an attempt to control vascular changes, thereby favouring the deposition of immune
complexes in the vascular level due to nicotine stimulation. Moreover, our results corroborate the idea that TAO can be an autoimmune disorder with specific mechanisms [15]. Additionally, to reinforce the autoimmune theory, increased matrix metalloperoxidase 9 (MMP-9) and reduced tissue inhibitor of metalloproteinases 1 (TIMP-1) activity has been found in TAO patients, especially in active smokers compared with non-TAO patients. These data suggest that compounds in the smoke could activate MMP-9 production or inhibit TIMP-1 activity [16].

The cytokines are mediators necessary to drive the local inflammatory response to infection and damage by promoting proper wound-healing. However, the over-production of proinflammatory cytokines from the lesion may manifest systemically with haemodynamic instability or metabolic disorders. After injury or serious infections, an exacerbated response and persistent Th1 cytokines may contribute to target organ damage, leading to multiple organ failure and death. Th2 cytokines can alleviate some of these adverse effects [11]. In inflammatory diseases, immunological injury is implicated strongly in the disruption of the vascular barrier, primarily through the secretion of cytokines which stimulate the proliferation or metabolic activity of several components. In this study, we observed that various plasma levels were increased significantly in TAO patients when compared to controls.

The present investigation has studied 12 cytokines; thus, the main data results of the present investigation, compared with control patients, revealed that TAO patients presented: (i) an increase of proinflammatory cytokines (IL-1β, TNF-α and IL-6); (ii) an increase of Th1 cytokines (IFN-γ and IL-12); (iii) with regard to Th2 cytokines, the TAO patients presented an increase of IL-4, IL-5 and IL-13 and a decrease of IL-10; and (iv) an increase of Th17 cytokine profiles (IL-17 and IL-23). These results indicate that patients with Buerger’s disease have an altered production of several cytokines in response to different stimuli. The disturbances in immune cell reactivity could be a reason for the persistent immune inflammation in TAO, and may confirm the role of immune dysregulation in TAO disease. It is essential to emphasize that the inflammatory response is closely related to tabagism, as the plasma cytokines of TAO former smoker patients were similar to the controls.

We did not find any studies concerning plasma cytokines in TAO patients. So far, we have found only one report that examines cytokines in patients with TAO [17]. In this ex-vivo study, the authors observed abnormal production of IL-6, IL-12 and IL-10, increased apoptosis and increased levels of circulating immune complexes, which may explain the persistence of TAO immune inflammation. Vascular endothelial growth factor (VEGF) strongly promotes angiogenesis, and monococyte colony-stimulating factor (M-CSF) regulates the differentiation, proliferation and survival of monocytes in TAO [18].

The data indicate that endothelial cells in TAO can be activated in TAO and that vascular lesions are associated with TNF-α secretion by tissue-infiltrating inflammatory cells, intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and E-selectin expression on endothelial cells and leucocyte adhesion via their ligands. The preferential expression of inducible adhesion molecules in microvessels and mononuclear inflammatory cells suggests that this is due probably to inflammation contributing to the persistence of the inflammatory process in TAO [19].

Although the cause of TAO disease remains unknown, a strong association with tobacco use has been established [3,20]. Use of or exposure to tobacco plays a central role in the initiation and progression of the disease. By using an antigen-sensitive thymidine-incorporation assay, Adar et al. [21] showed that patients with TAO have an increased cellular sensitivity to types I and III collagen compared to patients with arteriosclerosis obliterans or healthy males. De Moerloose et al. [22] found a marked decrease in the
frequency of human leucocyte antigen (HLA)-B12 in patients with Buerger’s disease (2.2% versus 28% in controls). Similarly to other autoimmune diseases, TAO may have a genetic predisposition without a direct ‘causative’ gene mutation. Most investigators believe that TAO is an immune-mediated endarteritis. Immunocytochemical studies have demonstrated a linear deposition of immunoglobulins and complement factors along the elastic lamina [20,23].

Patients with Buerger’s disease present a statistically significantly higher frequency of HLA-DR4 and a significantly lower frequency of the HLA-DRW6 antigen. This observation may serve as further evidence that an autoimmune mechanism is involved in TAO [22,24]. Our study complements these findings, further emphasizing the participation of autoimmune mechanisms in the aetiology of the development of TAO, as we show significant increases of IL-17 and IL-23, cytokines related closely to autoimmunity activation [25–27].

IL-17-producing T cells have been classified as a new effector T cell subset, termed Th17, which is distinct from Th1, Th2 and Treg subsets. There has been much progress in the past year, leading to identification of the molecular mechanisms that drive differentiation of Th17 T cells. This has helped to clarify many aspects of their role in host defence as well as in autoimmunity [28]. Additionally, exactly which cytokines contribute to Th17 formation remains unclear, but TGF-β, IL-6, IL-21 and IL-23 have been implicated in mice and humans [29,30]. It has recently been questioned, however, whether TGF-β is involved at all in humans, and it is assumed that IL-1β may also play a role. Other proteins involved in their differentiation are signal transducer and activator of transcription 3 (STAT3) and the retinoic acid receptor-related orphan receptors alpha (ROR-α) and gamma (ROR-γ) [31]. Effector cytokines associated with this cell type are IL-17, IL-21 and IL-22 [32,33]. Th17 cells are implicated in autoimmune disease, and autосpecific Th17 cells were shown to be highly disturbing. IL-23 is a member of the IL-12 family of cytokines with proinflammatory properties. Its ability to potently enhance the expansion of Th17 cells indicates responsibility for many of the inflammatory autoimmune responses. Emerging data demonstrate that IL-23 is a key participant in central regulation of the cellular mechanisms involved in inflammation. Both IL-23 and IL-17 form a new axis through Th17 cells, which has evolved in response to human diseases associated with immunoactivation and immunopathogeny, including bacterial or viral infections and chronic inflammation. Targeting of IL-23, the IL-23 receptor or the IL-23 axis is a potential therapeutic approach for autoimmune diseases including psoriasis, inflammatory bowel disease, rheumatoid arthritis and multiple sclerosis [27]. In addition to the Th17 profile, autoimmunity development could be defined clearly by monitoring autoantibodies and autoreactive T cells along the time course of TAO.

The cytokine environment in peripheral lymphoid tissues and the target organ (vascular) has a strong influence on the outcome of the initial events that trigger autoimmunity inflammation. In susceptible individuals, these events drive inflammation and tissue damage in the vascular system.

The increased proinflammatory and Th1 results indicate, as in other vasculitis, a contribution to the inflammatory response observed in the vascular levels of smoker patients. The observed increase of Th2 cytokines suggests that an imbalance in the Th1/Th2 cytokine immune response could be...
be related to TAO pathogenesis. In addition, the increased levels of IL-17 and IL-23 suggest that the disturbance of TAO is involved with mechanisms of autoimmunity. Finally, the IL-10 (Th2) reduction could be suggestive of its therapeutic use in TAO. Based on the unclear TAO pathogenesis, particularly the involvement of immune trigger mechanisms of vascular disease, further studies should be carried out to reveal the role of the immune disorder in TAO progression. Finally, the discovery of IL-17 and its association with inflammation and autoimmune pathology has reshaped our viewpoint regarding the pathogenesis of TAO, which was based previously on the Th1–Th2 paradigm.

Study limitations
The inflammatory profile status is not exclusive to TAO; in other diseases, where damage to the vascular wall is recorded, the scenario of increased proinflammatory and detrimental anti-inflammatory cytokines has also been described for other types of vasculitis. In order to analyse the behaviour/ onset of inflammatory status, it would be useful to have evidence of those cytokine profiles in healthy smokers along the time course of consumption and in non-diseased TAO patients. Unfortunately, we could not obtain this information from consulting the patients’ charts. In addition to the Th17 profile, the development of autoimmunity could be defined clearly by monitoring autoantibodies and autoreactive T cells along the time course of TAO, which was not performed in the present study.

Disclosure
None.

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