

Cytokine production in thromboangiitis obliterans patients: New evidence for an immune-mediated inflammatory disorder

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Abstract

Objective

To study IL-6, IL-10, IL-12 levels and circulating immune complexes (CIC) containing IgG, IgM or IgA in sera of 14 TAO patients and 12 healthy blood donors. To evaluate the ability of TAO PBMC to produce IL-6, IL-10, IL-12, as well as to detect PBMC apoptosis after stimulation with different stimuli.

Methods

In vitro stimulation of PBMC with lipopolysaccharide (LPS), phytohemagglutinin (PHA), C3 binding glycoprotein from Cuscuta europea (C3bgp), pokeweed mitogen (PWM), and dexamethasone (DM) were performed. The quantities of the secreted cytokines in sera and in culture supernatants, as well as CIC were detected by ELISA. The apoptosis was assessed according to nuclear morphology, after acridine orange staining, by fluorescence microscopy.

Results

Significantly higher IL-6 levels in the patients' sera were found. An increased production of IL-6 and IL-12 in TAO PBMC supernatants was detected, regardless of the stimuli used. A hyporeactivity of TAO PBMC toward IL-10 production was found after C3bgp, LPS, PHA and PWM stimulation, compared to the controls' PBMC. The spontaneous and induced apoptosis was significantly higher in TAO compared to the control group. Increased CIC quantities were detected in 75% of the patients tested. According to the CIC isotype, the IgG CIC positives (75%) prevailed over IgA CIC positives (50 %).

Conclusion

The altered production of IL-6, IL-12 and IL-10, the increased apoptosis as well as the elevated levels of CIC could be a reason for the persisting immune inflammation in TAO.

Key words

TAO, PBMC, IL-6, IL-12, IL-10, apoptosis, immune complex.

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Abbreviations:

TAO: thromboangiitis obliterans;
LPS: lypopolysaccharide;
PHA: phytohemagglutinin;
C3bgp: C3 binding glycoprotein from
Cuscuta europea;
PWM: pokeweed mitogen;
DM: dexamethasone;
IgG CIC: circulating immune complexes;
containing IgG;
IgA CIC: circulating immune complexes;
containing IgA;
IgM CIC: circulating immune complexes;
containing IgM;
PBMC: peripheral blood mononuclear
cells.

Introduction

The thromboangiitis obliterans (TAO) is an inflammatory arteriopathy which predominantly affects the distal small and medium sized arteries of young smokers (1-3). The disease pathogenesis is still unknown and the role of the immune system in the disease progression is not completely elucidated.

At present, limited data exist for disturbances in the immune regulation in TAO patients, affecting the humoral as well as the cellular immunity (4, 5). Adar *et al.* found an increased cellular sensitivity to human types I and III collagen in patients with thromboangiitis obliterans (6). Kroger *et al.* reported for elevated serum levels of the circulating immune complexes, as well as alteration of the leukocyte count and their subpopulations in patients suffering from TAO (4).

Recent studies show the presence of anti-elastin, anti-cardiolipin and anti-endothelial autoantibodies in TAO patients (5, 7, 8). The role of the HLA genotype (especially HLA-A9, HLA-B5 and HLA-DRB1) as a linked factor with TAO susceptibility was also suggested (5, 9). The newest data reveal increased expression of cell adhesion molecules ICAM-1, VCAM-1 and E-selectin on the endothelial and mononuclear cells from the affected arteries, and suggest the role of the angiogenesis for the persistence of the inflammatory process in TAO (10). However, these facts are insufficient to precisely determine the place of Buerger's disease in the classification of the autoimmune vasculitis and to bring about a consensus in order for the diagnostic criteria to be achieved (11).

Meanwhile, the pathogenesis of many autoimmune diseases, including the autoimmune vasculitis, is closely related with disturbances in the cytokine regulation, elevated levels of the lymphocyte apoptosis and persistence of circulating immune complexes in the body fluids (12-15). All these data directed our attention to investigate cytokine production, apoptosis and levels of circulating immune complexes in TAO diseases patients.

Materials and methods

Patients and controls

14 patients (mean age 44.3 ± 10.2 yrs) with critical limb ischemia and ulcers, and/or necrotic lesion, were included in the investigation. The diagnosis of Buerger's disease was considered reliable when all 5 clinical criteria of Shionoya (16) were accessible: smoking history, onset of the symptoms before the age of 50, infrapopliteal arterial occlusion, upper extremity involvement and/or phlebitis migrans and absence of the risk factors for atherosclerosis (Table I). The diagnosis was confirmed by angiography and histological examination of arterial wall, obtained during arterial reconstructions or after amputation. The mean duration of the disease was 4.7 ± 2.6 years.

Twelve healthy individuals (mean age 46.2 ± 12.1 years), consisting of staff from the University Hospital were used as a control group. All of them were found to be with normal biochemical data and without anamnesis for any internal diseases.

All patients and controls had been informed about the study, and were included in the study after their informed consent was obtained.

Collection and storage of samples

Blood samples were obtained after venepuncture from all TAO patients and healthy controls. The serum samples were separated at room temperature within 4 h of collection and stored at -70°C until use. The blood for PBMC isolation was routinely processed within 2 h of collection.

Isolation and stimulation of human PBMC

The PBMC were isolated from the healthy donors' and patients' peripheral blood by gradient separation with Histopaque 1077. Immediately after isolation, the cells were cultured *in vitro* for 24 hours with various stimuli. All cultures were carried out in RPMI 1640 media, supplemented with penicillin G (200 U/ml), gentamycin (10 µg/ml) and L-glutamine (0.3 mg/ml). The following triggering/activating agents were employed for the *in vitro* stimulation: lypopolysaccharide from *E.*

Table I. Clinical characteristics of 14 patients with TAO disease.

Patients	Sex	Age	Age at onset (years)	Disease duration (years)	Smoker	Limbs affected	Ulcers and/or gangrene	Phlebitis	Angiography	Hystology
1	M	56	49	7	yes	lower	yes	yes	yes	yes
2	M	56	48	8	yes	lower	yes	no	yes	yes
3	M	46	40	6	yes	lower	yes	no	yes	yes
4	M	46	42	4	yes	upper & lower	yes	yes	yes	yes
5	M	43	40	3	yes	upper & lower	yes	no	yes	yes
6	M	49	47	2	yes	lower	yes	no	yes	yes
7	M	54	47	7	yes	upper & lower	yes	no	yes	yes
8	F	39	35	4	yes	upper & lower	yes	yes	yes	yes
9	M	24	22	2	yes	lower	yes	no	yes	yes
10	F	26	24	2	yes	upper & lower	yes	no	yes	yes
11	F	41	36	5	yes	lower	yes	no	yes	yes
12	M	43	38	5	yes	lower	yes	no	yes	yes
13	M	41	38	3	yes	lower	yes	yes	yes	yes
14	M	57	49	8	yes	upper & lower	yes	yes	yes	yes

coli serotype 026:B6 (LPS) - 30µg/ml; phytohemagglutinin (PHA) - 20µg/ml; C3 binding glycoprotein from *Cuscuta europea* (C3bgp) - 30µg/ml; pokeweed mitogen (PWM) - 30 µg/ml, and dexamethasone (DM) - 500µg/ml.

After stimulation, the culture supernatants were collected and stored at -70°C until use. The PBMC fraction of each culture was used for the assessment of apoptosis.

Cytokine determination

The quantitative determinations of IL-6, IL-12p40 and IL-10 were performed in culture supernatant by ELISA with commercially available kits purchased from BioSource, Austria, following the instructions of the manufacturer. The developed color reaction was measured as OD units at 450 nm on an ELISA reader (Rosys Anthos 2010, Austria), and the concentration of cytokines was expressed in pg/ml by kit's standard curve.

Assessment of circulating immune complexes

The quantity of CIC was performed by CIF-ELISA according to the protocol, previously described (17, 18). Flat bottom polystyrene microtiter plates were coated with 20 µg/ml CIF in 0.2 M carbonate-bicarbonate buffer, pH 9.6 and incubated overnight at 4°C. After washing with 50 mM Tris-HCl, pH 7.2, con-

taining 0.05% Tween 20 (2 x 5 min), the plates were blocked for 20 min with the same buffer. 100 µl of each tested serum, diluted 1:50 in sample buffer (0.1M PBS, containing 1% BSA, 0.2% Tween 20, 1mM CaCl₂ and MgCl₂) was added in duplicate and incubated for 60 min at room temperature (RT). After three washes, 100 µl of the appropriate anti-human immunoglobulin antibodies peroxidase conjugate were added to each well for detection of IgG CIC (goat anti-human IgG), IgM CIC (goat anti-human IgM), IgA CIC (goat anti-human IgA) or total CIC level (goat anti-human polyvalent IgG, IgM and IgA). The developed color reaction was stopped with 10% H₂SO₄, and OD units at 492 nm were measured on an ELISA plate reader (Rosys Anthos 2010, Austria). All sera were tested in parallel for quantitative assessment of IgG CIC, IgM CIC, IgA CIC and total CIC.

Assessment of apoptosis

For nuclear morphology staining, the harvested cells were washed in PBS. To 20 µl of cell culture PBMC 20 µl of acridine orange (100 µg/ml) was added. The cell suspension was incubated in the dark at room temperature for 5 min and was mounted on a slide. The nuclear morphology was examined by a fluorescence microscope. At least 500 cells per slide were counted. According to Evans *et al.* (19), cells stained with

acridine orange and exhibiting brightly stained, condensed, and/or fragmented nuclei viewed by fluorescence microscopy were interpreted as apoptotic. The apoptotic cells were distinguished from viable cells since the latter displayed diffuse nuclear staining patterns. The apoptotic cells were expressed as a percentage of the total cell number.

Special reagents and purified proteins

All stimuli (except C3bgp), conjugates and reagents were purchased by Sigma Chemical Co. The C3bgp was isolated from the *Cuscuta europea* seeds as described previously (20).

Statistical analysis

Values obtained from patients and controls were compared by the unpaired Student's t-test or its nonparametric equivalent, the Mann-Whitney U test and Spearman correlation analysis, and statistical significance differences were determined. A *p* < 0.05 was considered statistically significant. Results obtained from 12 healthy individuals were used to define the mean plus 2SD (cut-off value) for each of the tested cytokines and CIC. All patients' sera with IL-6, IL-12 or IL-10, as well as IgG CIC, IgM CIC or IgA CIC serum levels, exceeding the cut-off point, were accepted as a positive. When the *in vitro* cytokine production

from TAO PBMC exceeded the cut-off point the data were accepted as a positive.

Results

Cytokine level detection

The IL-6, IL-10 and IL-12 serum levels, detected in the TAO patients as well as in the group of healthy blood donors are shown in Figure 1. The IL-6 quantity was found to be significantly higher in the patients' than in the control group. The levels of IL-12 and IL-10 were similar in both groups studied ($p > 0.05$).

The distribution of IL-6, IL-12 and IL-10 positive sera are presented in Table II. 50% of the patients were found to be IL-6 positive. IL-12 serum level was elevated only in two patients (14.3%). The IL-12 positive sera showed also elevated IL-6 levels. IL-10 was presented with undetectable levels in 85.1 % (12/14) and with elevated levels in 14.3% (2/14) of the patients studied. The two patients with elevated IL-10 were negative for IL-6 and IL-12.

IL-6, IL-10 and IL-12 production were also detected in the culture supernatant of PBMC, stimulated with C3b, LPS, DM, PHA and PWM. It was found that the levels of all cytokines studied depended on the stimuli used. Results for IL-6 production are shown in Figure 2. In both groups the level of IL-6, detected after stimulation with C3b, LPS, PHA or PWM was significantly higher, compared to the unstimulated PBMC ($p < 0.01$). In parallel, the LPS, PHA and PWM stimulated PBMC from TAO patients showed elevated IL-6 production, compared to the healthy controls.

The PBMC production of IL-12 and IL-10 after stimulation was found to be significantly different in the healthy individuals and TAO patients. PBMC from healthy donors produced significantly elevated levels of IL-12 after stimulation with C3b and LPS compared to unstimulated PBMC ($p < 0.05$). PHA and PWM failed to induce significant production of IL-12 from PBMC of healthy individuals (Fig. 3). However, when PBMC from TAO patients were cultivated, a significantly higher IL-12 production was observed

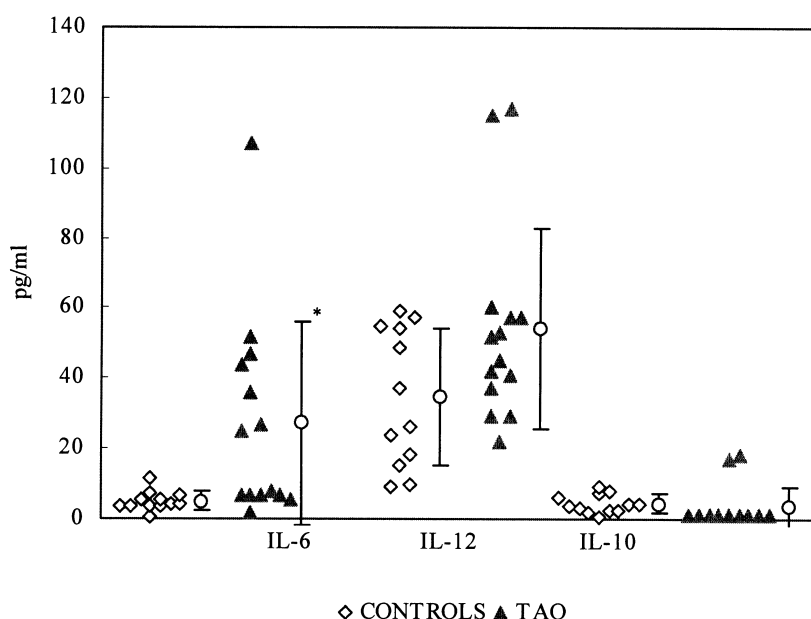


Fig. 1. Serum levels of IL-6, IL-12 and IL-10 in TAO patients and healthy controls. * $p < 0.05$ TAO versus control group.

Table II. Distribution of CIC (IgG, IgM, IgA), IL-6, IL-12 and IL-10 positive sera among TAO patients. The individual production of each patient is presented as a positive, only when the determined quantity exceeded the cut-off value.

Pts.	IgG CIC	IgM CIC	IgA CIC	IgG, IgM, IgA CIC	IL-6	IL-12	IL-10
1	+	+	-	+	-	-	-
2	-	-	-	-	-	-	+
3	+	-	+	+	+	-	-
4	+	+	-	+	+	-	-
5	+	-	+	+	-	-	-
6	+	+	+	+	+	+	-
7	+	-	+	+	+	-	-
8	-	-	-	-	-	-	-
9	+	+	+	+	+	-	-
10	-	-	-	-	-	-	-
11	+	-	+	+	+	-	-
12	+	-	+	+	-	-	-
13	+	+	-	+	+	+	-
14	-	-	-	-	-	-	+

after stimulation with C3b, LPS, PHA and PWM ($p < 0.01$). In parallel, all stimuli used induced significantly higher IL-12 production from PBMC of TAO patients compared to the healthy controls.

Results for detected quantities of the anti-inflammatory IL-10 are presented in Figure 4. A significantly enhanced IL-10 production in the controls' PBMC after stimulation with LPS and PWM ($p < 0.01$), was shown. The TAO PBMC failed to produce IL-10 upon all

stimuli used.

Our results show that the production of IL-6, IL-12 and IL-10 from both healthy and TAO PBMC was significantly suppressed after treatment with dexamethasone (Figs. 2, 3 and 4).

In Table III are presented the data of IL-6 and IL-12 production of each single patient, before and after stimulation. The data show that all patients were IL-6 positive after stimulation with PWM. 12/14 patients (85.7%) were IL-6 positive after stimulation

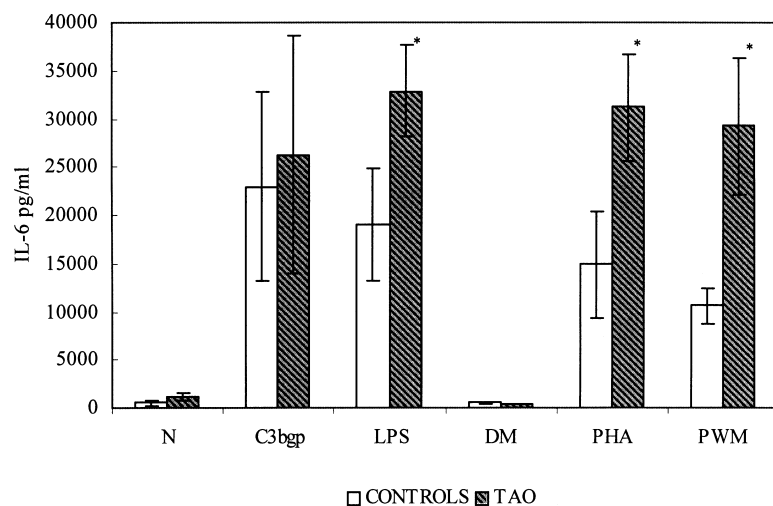


Fig. 2. IL-6 quantity in cell culture supernatants of PBMC from TAO patients and healthy controls. Cells were treated with C3bgb, LPS, PHA, PWM and DM. N: unstimulated cultures. * $p < 0.05$ TAO patients versus control group.

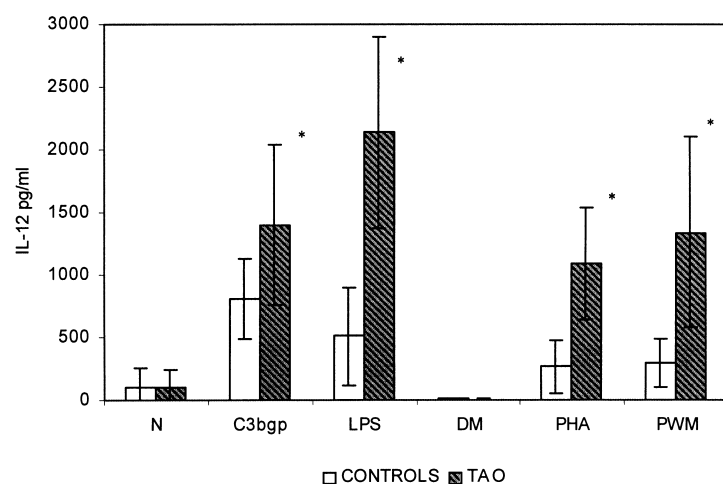


Fig. 3. IL-12 quantity in cell culture supernatants of PBMC from TAO patients and healthy controls. Cells were treated with C3bgb, LPS, PHA, PWM and DM. N: unstimulated cultures. * $p < 0.05$ TAO patients versus control group.

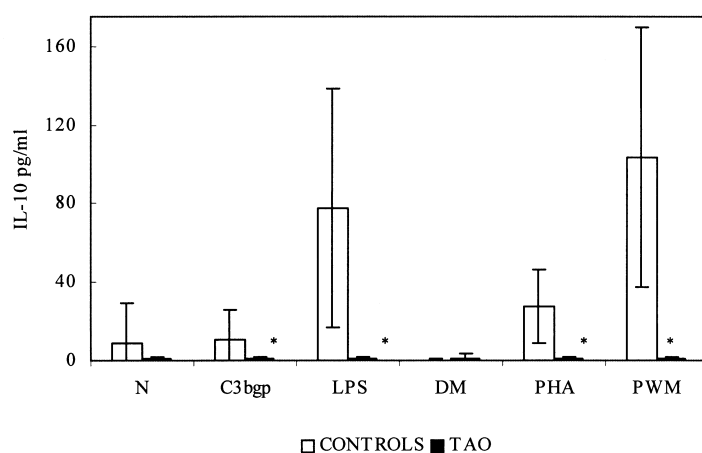


Fig. 4. IL-10 quantity in cell culture supernatants of PBMC from TAO patients and healthy controls. Cells were treated with C3bgb, LPS, PHA, PWM and DM. N: unstimulated cultures. * $p < 0.05$ TAO patients versus control group.

with PHA and LPS. In relation to IL-12 production it was found that 9/14 (64.3%) patients were positive after stimulation with PWM and PHA. 7/14 (50%) patients were positive after stimulation with C3bgb. Four out of 14 (28.6%) patients were IL-12 positive after treatment with all stimuli. Only one patient was found as an IL-12 negative after all stimuli used.

Circulating immune complex level assessment

The levels of CIC, containing IgG, IgM or IgA antibodies are shown in Figure 5. All three isotypes CIC were found elevated in TAO, compared to the control group. Elevated levels of IgG CIC were measured in 71.4 % of TAO patients (10/14), IgM CIC positive were 35.7% (5/14) and IgA CIC – 50.0% (7/7). Moreover, TAO patients with elevated IgA CIC or IgM CIC were also IgG CIC positive (table II). The screening test for CIC detection (simultaneous detection of IgG, IgM and IgA CIC), showed elevated CIC levels in 71.4% of the patients, compared to 0 % of the healthy controls.

A well expressed correlation between CIC and serum IL-6 ($r = 0.84$; $p = 0.018$) was established.

Assessment of PBMC apoptosis

Results for apoptotic cells percentage are shown in figure 6. The apoptosis, detected in TAO PBMC, was higher than that of the healthy controls. The unstimulated PBMC from TAO patients presented a significantly higher percentage of spontaneous apoptosis than that of the healthy controls ($p < 0.05$). This tendency was preserved after stimulation with C3bgb, LPS and PWM, as well as after DM treatment.

Discussion

Based on the unclear pathogenesis of TAO, particularly the involvement of immune mechanisms as a vascular inflammatory trigger, we consider that further studies could be performed to reveal the role of the immune disturbance in TAO progression.

At the same time, several immune disturbances were unravelled in other types of vasculitis (21-23). They affect

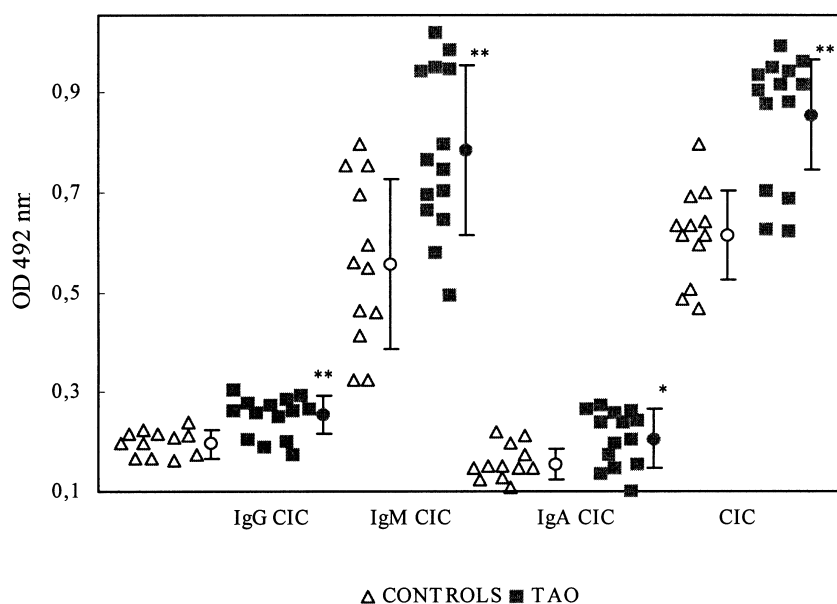
Table III. IL-6 and IL-12 production from PBMC of TAO patients after treatment with C3bgp, LPS, DM, PHA and PWM. N: unstimulated PBMC. The individual IL-6 and IL-12 production of each patient are presented in pg/ml, only when the determined quantity exceeded the cut-off value.

Stimuli	IL-6 pg/ml						IL-12 pg/ml					
	N	C3bgp	LPS	DM	PHA	PWM	N	C3bgp	LPS	DM	PHA	PWM
Cut- off Pts.	1177	42460	30591	770	25895	14299	398	1451	1287	30	680	676
1	< 1177	43987	36697	< 770	35733	32028	< 398	2375	2705	< 30	2057	2067
2	< 1177	44090	34680	< 770	34791	35621	< 398	< 1451	< 1287	< 30	1586	< 676
3	< 1177	< 42460	33197	< 770	28448	28236	< 398	1917	3554	< 30	1147	3294
4	< 1177	< 42460	30702	< 770	< 25895	29798	< 398	1829	2824	< 30	1097	1030
5	1311	< 42460	36031	1706	27700	30355	< 398	< 1451	< 1287	< 30	< 680	954
6	< 1177	< 42460	< 30591	< 770	34559	19415	< 398	< 1451	< 1287	< 30	< 680	< 676
7	1267	43090	33556	< 770	43231	25621	< 398	1685	3079	< 30	< 680	< 676
8	< 1177	< 42460	37748	< 770	< 25895	27655	< 398	< 1451	< 1287	< 30	1100	1230
9	< 1177	< 42460	35214	< 770	28448	31905	< 398	2100	2270	< 30	1787	1120
10	< 1177	< 42460	41239	< 770	33723	20783	< 398	< 1451	< 1287	< 30	1348	2472
11	< 1177	< 42460	33936	< 770	26834	32028	< 398	< 1451	< 1287	< 30	1634	< 676
12	< 1177	43781	< 30591	934	34998	33732	< 398	< 1451	2999	< 30	< 680	2506
13	< 1177	< 42460	40769	< 770	33120	22300	< 398	1743	2855	< 30	< 680	1784
14	< 1177	< 42460	36513	< 770	39954	34007	< 398	2256	< 1287	< 30	1420	< 676

mainly the regulation between Th1 and Th2 immune response and especially the role of IL-12 and IL-10. Most of the studies performed, however, elucidated the relationship between the serum levels of these cytokines, the disease onset and progression (13, 24, 25). In recent years, the new approaches related to studying the response of the immune cells *ex vivo*, after treatment with different stimuli, were explored (26, 27). In the literature available we did not find any work studying the *in vitro* production of cytokine from TAO peripheral blood mononuclear cells. In this regard, we directed our study towards the assessment of TAO PBMC cytokine production and apoptosis after stimulation with C3bgp, LPS, PHA, PWM, as well as treatment with dexamethasone. The serum levels of CIC, IL-6, IL-12 and IL-10 were detected in our attempt to search for a correlation between these parameters and the disease progression. IL-12 was selected as a pivotal cytokine which drives the differentiation of naive T cells into Th1 cells (28-30). IL-12 also mediates several biological activities on T and NK cells, including the induction of IFN-gamma production and the enhancement of cell-mediated cytotoxicity (31, 32). IL-6 was selected as a potent

inducer of the acute-phase protein response and as a marker for systemic activation of proinflammatory response (33). IL-10 was studied as the most important anti-inflammatory cytokine within the human immune response. Moreover this Th2 cytokine is a potent inhibitor of Th1 cytokines including IFN-gamma. (34).

We found that the IL-6 serum levels were elevated in 50% of the TAO patients. Meanwhile, patients' PBMC revealed an increased sensitivity towards IL-6 production after stimulation with LPS, PHA and PWM. The enhanced production of IL-6 of TAO PBMC and the elevated serum level of this cytokine indicate the involvement of im-

**Fig. 5.** Serum levels of CIC, containing IgG, IgM, IgA and total CIC levels, detected by CIF-ELISA in TAO patients and healthy controls. *p < 0.05 TAO versus control group; **p < 0.01 TAO versus control group.

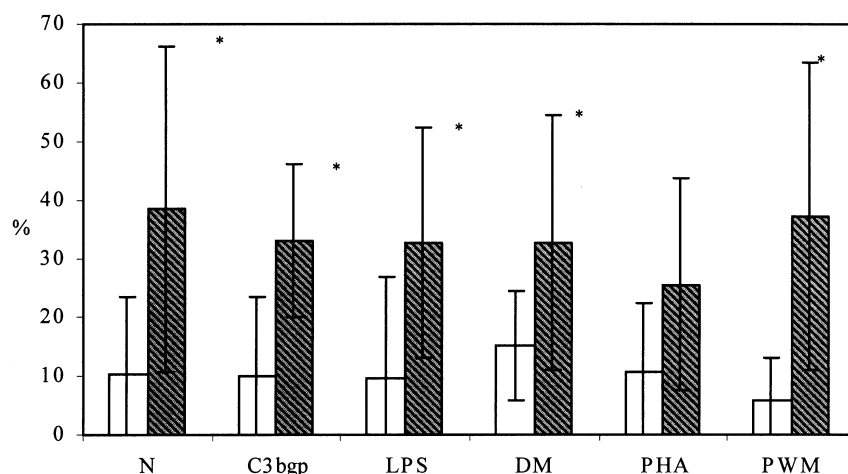


Fig. 6. Percent of apoptosis in TAO patients and healthy controls PBMC, after stimulation with C3bip, LPS, PHA, PWM and DM. * $p < 0.05$ TAO versus control group.

immune inflammation in the Buerger's disease. Similar data for elevated production of IL-6 have been detected in sera, as well as in cell culture supernatants of stimulated PBMC from small vessel vasculitis (35). Since we first detected the *in vitro* IL-6 hyper-reactivity of TAO patients, we have further extended our study of the proinflammatory cytokine IL-12. Our observation demonstrated that the TAO PBMC response toward IL-12 production, after all stimuli used (C3bip, LPS, PHA and PWM), was significantly higher compared to the healthy controls. These results are quite similar to those obtained by Ludviksson *et al.* for another vasculitis – Wegener's granulomatosis, where increased *in vitro* monocyte production of IL-12 has been detected after treatment with the following stimuli: LPS + INF- γ , *Staphylococcus aureus* + INF- γ or CD40 + INF- γ (36). Moreover, Lamprecht *et al.* have demonstrated that immunosuppressive therapy with cyclophosphamide and corticosteroid normalized monocytic IL-12 production in patients with Wegener's granulomatosis (37). We suppose that the higher potential of PBMC from TAO patients to produce IL-12 may be involved in the pathogenesis of the disease.

In many autoimmune diseases with or without vascular pathology, elevated serum levels of IL-10 have been detected (24, 25, 38). Popa *et al.* consider that the strong IL-10 production may act as an inhibitory signal for T cell prolifera-

tion *in vitro* and may have an important immunoregulatory function *in vivo* (35). Myers *et al.* reported that the IL-10 levels increased in the first week after thrombosis (39). In parallel, Gunnert *et al.* found that in mouse experimental models the levels of superoxide are elevated in carotid arteries from IL-10 $^{-/-}$ mice, compared with IL-10 $^{+/+}$ mice after LPS injection (40). In this regard, they suppose that IL-10 protects the endothelial function after an acute inflammatory stimulus by limiting a local increase in superoxide. Our results showed a well expressed hyporeactivity of TAO PBMC toward IL-10 production, as well as a total lack of IL-10 in the serum. The observed IL-10 down-regulation, accompanied with the elevated levels of IL-6 and IL-12, indicated that in TAO patients a pro-inflammatory immune mechanism predominates. Recently some authors show that T-cell mediated immune inflammation is a significant event in the development of TAO (41). The results we obtained are consistent with the immunohistochemical evidence for T cell mediated immune inflammation as a trigger event in the development of TAO (42). Regardless of the accumulated data for prevailing of T cell immune response, some humoral immune mechanisms were also related with TAO pathogenesis (7, 42). We detected significantly higher serum levels of CIC in TAO group, compared to the healthy individuals. Analyzing the isotype of CIC antibodies, we established the highest per-

cent of IgG CIC, followed by IgA CIC and then IgM CIC. Comparing our results with the existing data for the presence of elevated IgA CIC in patients with Henoch-Sonlein purpura and other vasculitis, we suppose the involvement of different isotypes CIC in TAO pathogenesis (43, 44). We also found in TAO patients a well expressed correlation between CIC levels and IL-6 values, similar to some autoimmune diseases where IL-6 production was related with the immune complex mediated pathology (12, 45). Therefore, the simultaneous detection of elevated IL-6 and CIC serum levels in TAO patients could be used as an appropriate diagnostic feature.

We demonstrated that cultured TAO PBMC were more sensitive to both spontaneous and stimuli induced apoptosis, compared to the control group. The results we obtained are in line with the data for elevated level of apoptosis in many autoimmune diseases and particularly in autoimmune vasculitis (22, 46). The elevated apoptosis and the presence of an increased CIC quantity support our suggestion for autoimmune inflammation in TAO.

This study indicates that patients with Buerger's disease have altered production of IL-6, IL-12 and IL-10 in response to different stimuli. The disturbances in the immune cells reactivity could be a reason for the persisting immune inflammation in TAO and may confirm the role of the immune dysregulation in TAO disease.

Based on our observation, we suppose that stimulation of patients' PBMC with different agents and measurements of the produced IL-12 and/or IL-6 and IL-10 may provide a useful marker in defining the immune status of TAO patients.

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