

Angiogenic Activity of Sera from Pulmonary Tuberculosis Patients in Relation to IL-12p40 and TNF α Serum Levels

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Abstract The role of angiogenesis in the pathogenesis of tuberculosis (TB) is not clear. The aim of this study was to examine the effect of sera from TB patients on angiogenesis induced by different subsets of normal human mononuclear cells (MNC) in relation to IL-12p40 and TNF α serum levels. Serum samples from 36 pulmonary TB patients and from 22 healthy volunteers were evaluated. To assess angiogenic reaction the leukocytes-induced angiogenesis test according to Sidky and Auerbach was performed. IL-12p40 and TNF α serum levels were evaluated by ELISA. Sera from TB patients significantly stimulated angiogenic activity of MNC compared to sera from healthy donors and PBS ($p < 0.001$). The number of microvessels formed after injection of lymphocytes preincubated with sera from TB patients was significantly lower compared to the number of microvessels created after injection of MNC preincubated with the same sera ($p < 0.016$). However, the number of microvessels created after the injection of lymphocytes preincubated with sera from healthy donors or with PBS alone was significantly higher ($p < 0.017$). The

mean levels of IL-12p40 and TNF α were significantly elevated in sera from TB patients compared to healthy donors. We observed a correlation between angiogenic activity of sera from TB patients and IL-12p40 and TNF α serum levels ($p < 0.01$). Sera from TB patients constitute a source of mediators that participate in angiogenesis and prime monocytes for production of proangiogenic factors. The main proangiogenic effect of TB patients' sera is mediated by macrophages/monocytes. TNF α and IL-12p40 may indirectly stimulate angiogenesis in TB.

Keywords Angiogenesis · Tuberculosis · Pathogenesis · Interleukin-12p40 · Tumor necrosis factor alpha

Introduction

Angiogenesis from preexisting vasculature occurs in many pathological conditions such as tumors [1], cardiovascular diseases [2], chronic inflammation [2, 3], rheumatoid arthritis [4], diabetic retinopathy [5], endometriosis [6], and obesity [7]. In pulmonary nonmalignant diseases angiogenesis has been implicated in the pathogenesis of idiopathic interstitial pneumonia [8], lung fibrosis induced by bleomycin [9], and sarcoidosis [10]. Vascular endothelial growth factor (VEGF) is a fundamental regulator of normal and pathological angiogenesis [11]. Ischemia induces both VEGF production and angiogenesis [12]. Although there have been reports concerning angiogenesis in infectious diseases, its role in the pathogenesis of TB has not been determined [13, 14]. Serum VEGF might be a useful marker as a prognostic indicator in sarcoidosis [15] and other granulomatous diseases, including TB. Increased VEGF levels in serum [16–18], pleural fluid [19, 20] and cerebrospinal fluid [21] have been found in

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patients with active TB. VEGF therefore may be associated with the pathogenesis of pulmonary TB in the development of the chronic inflammatory reaction [18]. *M. tuberculosis* antigens are considered an important factor participating in the pathogenesis of Eales' disease—an idiopathic retinal periphlebitis characterized by capillary nonperfusion and angiogenesis [22]. In one study, an intense angiogenesis was shown ultrastructurally in active pulmonary tuberculosis lesions [23], but the meaning of this phenomenon is not completely understood yet. An important role in the pathogenesis of TB and also in the modulation of angiogenesis is played by some cytokines, especially IL-12 and TNF α [24–26]. The aim of this study was to examine the effect of sera from TB patients on angiogenesis induced by different subsets of normal human mononuclear cells (MNC) in relation to IL-12p40 and TNF α serum levels.

Materials and Methods

Study Population

The study was approved by the local ethics committee. Serum samples were obtained from 36 patients with active pulmonary TB and from 22 healthy volunteers. The TB group consisted of 26 male and 10 female patients aged 49.8 ± 14.9 years (range = 23–75 years). All were HIV-negative and 23 were smokers. The diagnosis of pulmonary TB was confirmed by a conventional sputum culture. No systemic or extrapulmonary TB was observed. Blood samples were taken from patients before antituberculosis treatment was started. As a control, sera from 22 healthy nonsmoking volunteers were used (12 women and 10 men, mean age = 38.7 ± 11.4 , range = 20–58 years). None of the control volunteers had any past medical history of tuberculosis, other pulmonary diseases, or cancer.

Preparation of Peripheral Blood Mononuclear Cells

Blood from healthy donors attending the Warsaw Central Blood Bank was collected in a heparinized syringe, then diluted 1:1 in phosphate-buffered saline (PBS) and layered over Lymphoprep separation medium (Sigma). After spinning the tubes for 20 min at 500 *g*, the mononuclear fraction was aspirated from the interface. MNC viability estimated by trypan blue stain was $\geq 98\%$. This method yielded MNC preparation containing 10–15% monocytes and 85–90% lymphocytes based on morphologic criteria and myeloperoxidase staining. MNC were depleted of monocytes by glass adherence and phagocytosis of iron particles using 15 μg for 10×10^6 cells.

Leukocyte-induced Angiogenesis Assay (LIA)

To assess the effect of sera from TB patients on angiogenesis induced by different fractions of MNC, a leukocyte-induced angiogenesis test described by Sidky and Auerbach in an animal model [27] with modification [10, 28] was used. The local ethical commission for experimenting on animals approved all procedures involved in this study. Female inbred 8-week-old BALB/c mice served as recipients of MNC or lymphocytes preincubated in PBS supplemented with 25% of serum from TB patients or from healthy volunteers for 1 h at 37°C. As a control, other mice were given MNC or lymphocytes that were preincubated only in PBS. Mice were anesthetized with 3.6% chloral hydrate (POCH, Poland) and injected intradermally with 5×10^5 cells in Parker liquid (6 injections per mice and 3 mice for each tested patient). The mice were killed after 3 days. The newly formed blood vessels were identified and counted on the inner surface of the skin of each mouse using a microscope (Nikon, Japan) at $6 \times$ magnification. In all cases identification was performed by one expert (UD) based on the previously described criteria [27].

Cytokines

IL-12p40 and TNF α in sera were evaluated by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (BIOSOURCE Europe S.A.) according to the manufacturer's instructions. Recombinant TNF α (NIBSC, Hertfordshire, UK, EN6 3 QG) was used as a standard. Concentrations of IL-12p40 in sera were determined by sandwich ELISA using specific IL-12p40 MoAb. Optical density was measured at 450 nm using spectrophotometric reader Elx800 (Biotek Instruments, Inc., USA).

Statistical Analysis

The value of the angiogenesis test was expressed as an angiogenesis index (AI):

$$AI = A_{\text{ex}}/A_{\text{contr}}$$

where A_{ex} is the mean number of micro blood vessels created after the injection of MNC or lymphocytes preincubated in PBS with the serum of a patient or healthy donor, and A_{contr} is the mean number of micro blood vessels created after the injection of MNC or lymphocytes preincubated with PBS alone

All results are presented as mean \pm SD. Statistical evaluation of the results was performed using Pearson's test, Student's *t*-test, and Mann-Whitney test (Statistica 6 for Windows, Statsoft, Tulsa, OK, USA). A *p* value < 0.05 was used to indicate statistical significance.

Results

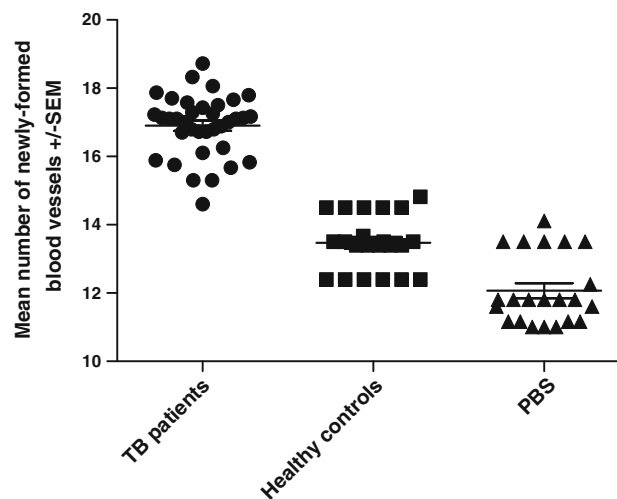
Angiogenic activity of MNC was measured by the mean number of newly formed vessels after injection of MNC preincubated with sera and PBS or with PBS alone. Sera from TB patients significantly indirectly stimulated angiogenesis to a greater extent than sera from healthy donors or PBS alone ($p < 0.001$) (Fig. 1). Proangiogenic activity of sera from TB patients (mean number of microvessels = 16.9 ± 0.9) was significantly higher than that of sera from healthy donors (13.99 ± 0.73 vessels; $p < 0.001$). However, sera from healthy donors also significantly ($p < 0.001$) stimulated angiogenic activity of normal MNC compared to MNC preincubated only with PBS (12.7 ± 1.16 vessels).

Following the injection of lymphocytes preincubated with sera from 20 TB patients, the mean number of microvessels significantly decreased (16 ± 1.33 ; $p < 0.05$) compared to the number of microvessels after the injection of a full suspension of MNC preincubated with the same sera (17 ± 0.79) (Fig. 2a). However, the mean number of microvessels after injection of lymphocytes preincubated with sera from healthy donors significantly increased (from 13.99 ± 0.73 to 14.8 ± 1.0 ; $p < 0.01$) compared to the

number of microvessels after the injection of a full suspension of MNC preincubated with the same sera (Fig. 2b). A similar effect was observed in the control group when MNC or lymphocytes were preincubated with PBS alone (increase from 12.7 ± 1.16 to 13.8 ± 1.63 ; $p < 0.05$) (Fig. 2c). The results were expressed as an angiogenic index and the following relationships were observed: After injection of MNC preincubated with sera from TB patients, AI was 1.29 ± 0.03 but it significantly decreased (1.12 ± 0.09 ; $p < 0.001$) following preincubation of lymphocytes with sera from TB patients. However, following the injection of lymphocytes preincubated with sera from healthy volunteers, AI remained the same as after the injection of MNC preincubated with sera from healthy volunteers (1.06 ± 0.04 vs. 1.07 ± 0.06).

The IL-12p40 serum level was significantly elevated in TB patients (355 ± 279 pg/ml; $p < 0.01$) compared to that of healthy controls (64 ± 70 pg/ml) (Fig. 3a). A significant correlation was found between the IL-12p40 serum level and angiogenic properties of sera of the examined patients and healthy donors ($r = 0.57$, $p < 0.01$) (Fig. 4a). TNF α serum level from TB patients (31 ± 8 pg/ml; $p < 0.002$) was significantly elevated compared to that of healthy controls (21 ± 3.4 pg/ml) (Fig. 3b). A significant

Fig. 1 Mean number of new vessels after injection of MNC preincubated with sera from TB patients ($n = 36$), sera from healthy controls ($n = 22$), and PBS ($n = 22$). The means are indicated by horizontal bars



One-way analysis of variance			
P value	< 0,0001		
Are means signif. different? (P < 0.05)	Yes		
F	215,8		
R square	0,8486		
Bartlett's test for equal variances			
Bartlett's statistic (corrected)	1,077		
P value	0,5835		
P value summary	ns		
Do the variances differ signif. (P < 0.05)	No		
ANOVA Table			
Treatment (between columns)	SS	df	MS
	359,8	2	179,9
Residual (within columns)	64,18	77	0,8336
Total	424,0	79	
Tukey's Multiple Comparison Test			
	Mean Diff,	q	Significant? P < 0,05? Summary
TB patients vs Healthy controls	3,431	19,64	Yes ***
TB patients vs PBS	4,835	27,67	Yes ***
Healthy controls vs PBS	1,404	7,213	Yes ***

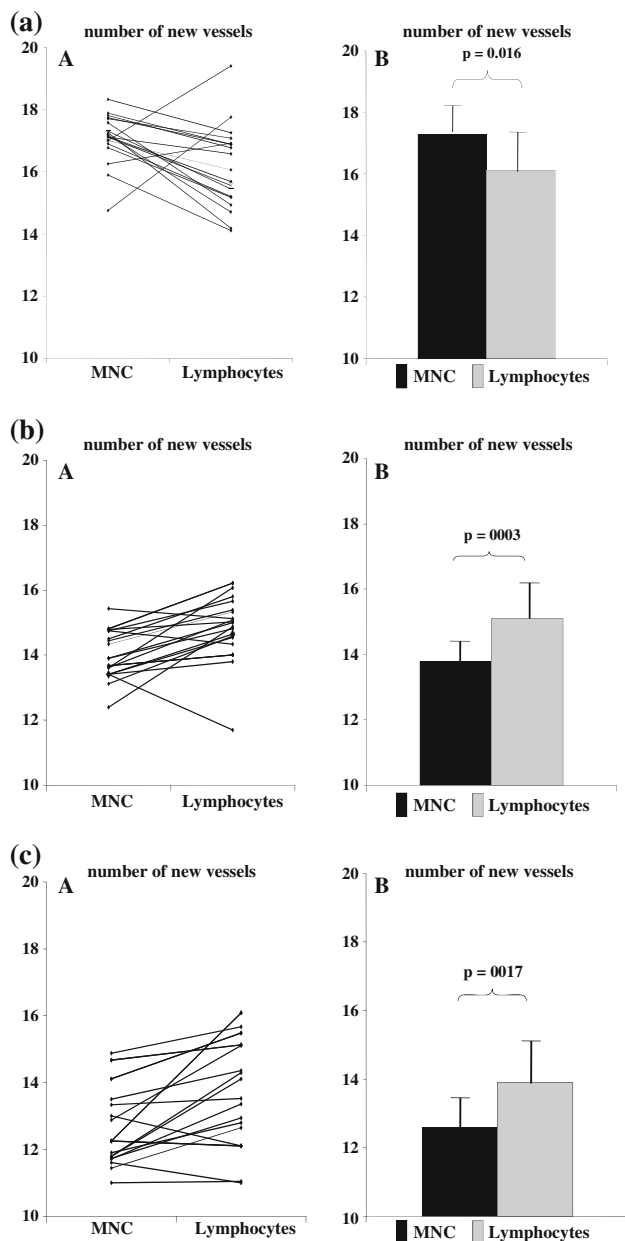


Fig. 2 Mean number of new vessels after injection of MNC and lymphocytes preincubated in the same time (A, individual results; B, mean result). **a** With sera from TB patients ($n = 20$). **b** With sera from healthy donors ($n = 22$). **c** Without sera (only in PBS as a control) ($n = 22$)

correlation ($r = 0.62$, $p < 0.01$) between $\text{TNF}\alpha$ serum level and angiogenic activity of sera was found (Fig. 4b).

Discussion

These results confirm suggestions [16, 17] of the involvement of angiogenesis in TB. The positive correlation between angiogenic activity measured by the number of

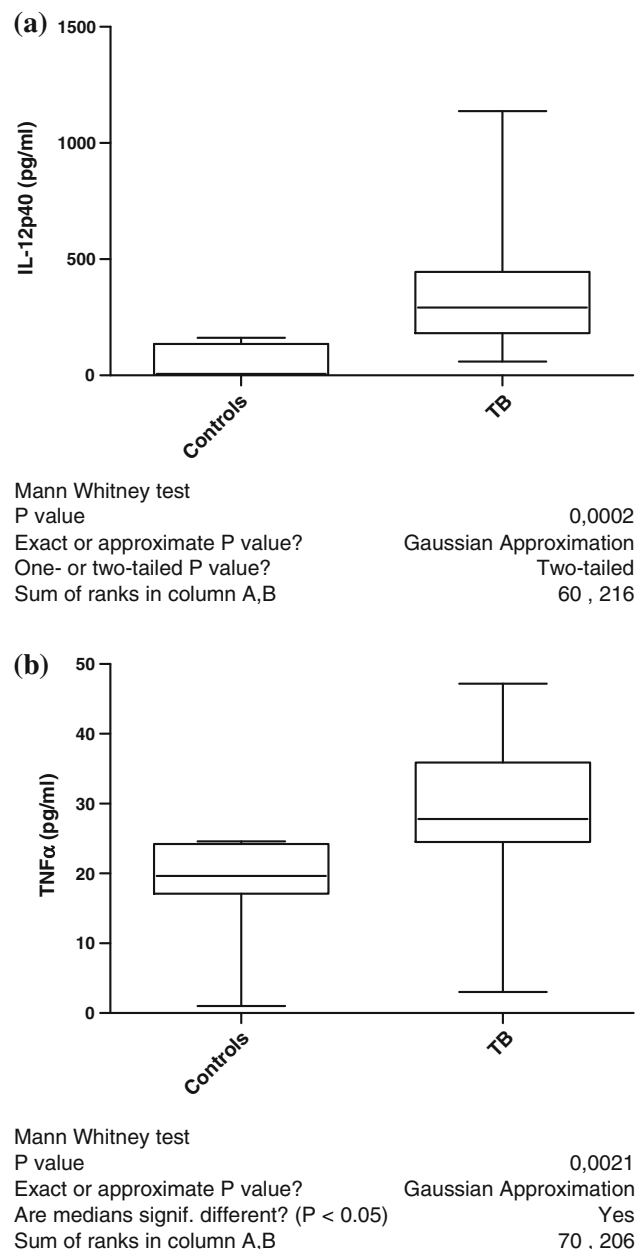


Fig. 3 **a** IL-12p40 serum level from patients with TB ($n = 13$) and from healthy controls ($n = 10$). **b** $\text{TNF}\alpha$ serum level from patients with TB ($n = 12$) and from healthy controls ($n = 9$)

newly created vessels after the injection of MNC preincubated with sera of TB patients and IL-12p40 and $\text{TNF}\alpha$ serum concentration levels seems to suggest that these cytokines play an important role in the pathogenesis of the neovascularization [29, 30]. Our previous studies carried out on the same model did not confirm any correlation between angiogenic activity of sarcoidosis patients' sera and IL-6 and IL-8 serum levels [10]. However, $\text{TNF}\alpha$, an important proinflammatory factor, may stimulate angiogenesis in interstitial lung diseases, and a weak correlation was observed between the serum level of IL-12 and the

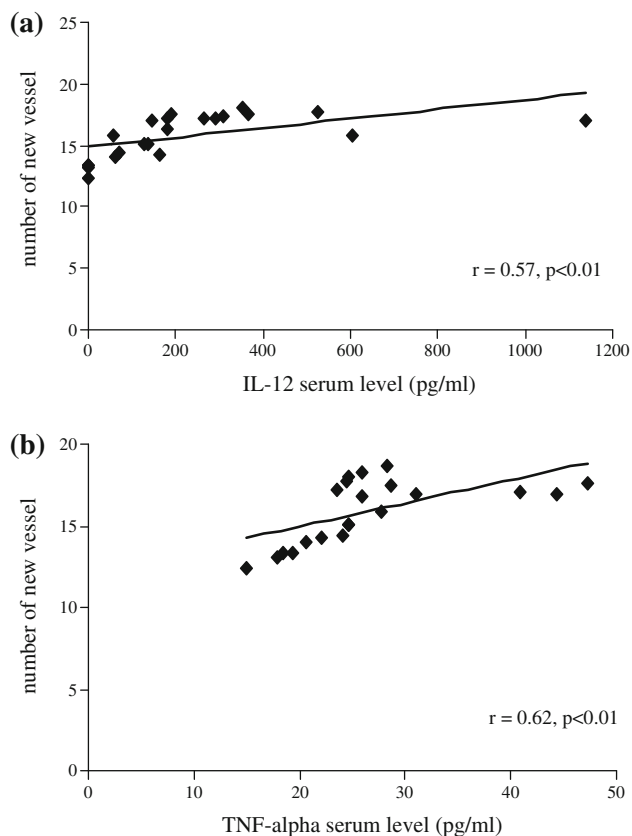


Fig. 4 **a** Correlation between angiogenic activity of sera from TB patients ($n = 13$) or from healthy controls ($n = 10$) and IL-12p40 serum level. **b** Correlation between angiogenic activity of sera from TB patients ($n = 12$) or from healthy controls ($n = 9$) and TNF α serum level ($r =$ Pearson's coefficient)

number of new vessels created after intradermal injection of MNC preincubated with sera from interstitial lung diseases patients [31, 32]. Our results support the hypothesis about the possible link between angiogenic activity of TB patients' sera and IL-12 and TNF α . These cytokines, which play an important role in the pathogenesis of TB, also modulate angiogenesis [25, 26, 29, 30]. Significant correlations between the concentration of these cytokines and radiological findings in TB patients were found [24]. Some reports point to neovascularization in the pathogenesis of tuberculosis. Thus, Abe et al. [16] suggest that the increased blood vessel formation and oxygen supply caused by the overexpression of VEGF inhibits cavity formation in TB. However, studies of Alatas et al. [17] did not confirm these findings. These authors observed a significant increase of VEGF serum level in active TB, and a decrease with effective antituberculosis treatment [17]. Matsuyama et al. [18] reported higher VEGF levels in the sera of patients with active pulmonary TB. *Mycobacterium tuberculosis*-infected monocytes develop a matrix-degrading phenotype [33]. Matrix degradation is the essential first step in the process of creating new vessels from the

preexisting vasculature. In TB meningitis, monocytes secrete matrix metalloproteinase-9, which facilitates leukocyte migration across the blood-brain barrier [34]. TNF α plays an important role in this process as well [33]. Lipoteichoic acid, one of the main *M. tuberculosis* antigens, stimulates macrophages to release metalloproteinase-9, which, by breaking down proteins, not only causes formation of cavities but also induces angiogenesis [35]. A correlation between VEGF and TNF α was also observed in the pathogenesis of exudate in tuberculous pleuritis [20]. TNF α is produced by various cells in the inflammatory site and induces angiogenic cytokines, including IL-8, VEGF, and basic fibroblast growth factor (bFGF), all of which are involved in neovascularization [36]. Saita et al. [37] demonstrated that intracorneal challenge with trehalose 6,6'-dimycolate derived from *M. tuberculosis* induces an inflammatory response, including granuloma formation and neovascularization. The angiogenic reaction was inhibited by neutralizing antibodies targeting VEGF and IL-8, partially by anti-TNF α antibodies, but not by anti-IL-1 β [37]. Macrophages stimulated with cord factor produce proinflammatory type-1 helper-T-cell-inducing cytokines, including TNF α , IL-1, chemotactic factors, and IL-12 [38]. In time it leads to the increase of VEGF concentration [38].

Establishing the key role of the monocytes in inducing angiogenic activity when affected by sera of TB patients is an important result of this study. The angiogenic effect of TB patients' sera was different than that of sera from healthy donors. Healthy human sera stimulated stronger lymphocytes than monocytes. In the case of TB patients, depletion of monocytes from MNC decreased the proangiogenic effect of the sera; therefore, the proangiogenic factors present in the patients' sera stimulated mainly monocytes and had no such strong effect on lymphocytes. From this observation we may conclude that the main proangiogenic effect of TB patients' sera is mediated by macrophages/monocytes. Matsuyama et al. [18] showed by immunohistochemistry that the expression of VEGF occurred in the alveolar macrophages around active TB lesions. It has been demonstrated that VEGF plays a role in the pathogenesis of pulmonary *M. avium* complex infection [39]. Therefore, it can be assumed that activated macrophages are the main cells that secrete VEGF in TB lesions when affected by factors such as IL-12 and TNF α . Pulmonary TB and sarcoidosis are granulomatous diseases and their pathogenesis has been linked to monocytes and alveolar macrophages [40, 41]. Meyer et al. [42] pointed out the key role of monocytes from sarcoidosis patients in stimulating neovascularization. In an earlier study we also demonstrated that sera from sarcoidosis patients prime monocytes for production of proangiogenic factors [10]. Sakaguchi et al. [43] indicated that trehalose 6,6'-dimycolate augments VEGF production by neutrophils and

macrophages and induces neovascularization in granulomatous tissue. Chronic inflammation in TB is characterized by a localized accumulation of mononuclear cells, i.e., the formation of a granuloma that is surrounded by neovascularization [41]. Angiogenesis is required for the progression of chronic inflammation [3].

IL-12 is a multifunctional cytokine produced by macrophages and dendritic cells. As a factor promoting the commitment of naïve lymphocytes to a Th₁-type profile of cytokine expression, IL-12 may be pivotal in the cascade of proinflammatory events in the lungs [44]. The number of cells expressing Th₁-type cytokines such as INF γ and IL-12 is increased within the lungs of patients with granulomatous disease such as TB and sarcoidosis [29]. IL-12 not only stimulates the growth of T and NK cells but also inhibits angiogenesis in solid tumors [45]. IL-12 is a heterodimeric cytokine composed of p40 and p34 chains. The subunit of IL-12p40 is in a free form, which blocks specific receptors to stimulate angiogenesis; this determined the selection of this cytokine for this study. Heterodimeric IL-12 and free p40 were present in tuberculous pleural effusions but not in malignant pleural effusions, suggesting that IL-12 is produced in the local immune response to infection [46]. Cooper et al. [47] have demonstrated that IL-12p40 knockout mice are defective in their ability to induce INF γ production and to generate a protective Th₁-type of immunologic response in TB. IL-12 proved to be an effective and successful adjuvant to a standard treatment in patients suffering from progressive clinical TB [48].

It is difficult to determine whether neovascularization plays a beneficial or a harmful role in the course of TB. It can only be assumed that the growth of a localized inflammatory mass requires neovascularization. Insufficient angiogenesis resulting in hypoxia creates favorable conditions for tissue necrosis and cavity formation. Therefore, further research on neovascularization and its role in the pathogenesis of TB is necessary.

Conclusions

Sera from TB patients constitute a source of mediators participating in angiogenesis. Sera from TB patients prime monocytes for production of proangiogenic factors. TNF α and IL-12p40 as important proinflammatory factors may stimulate angiogenesis in TB.

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