SERUM INFLAMMATION MARKERS IN TUBERCULOSIS

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Abstract

Tuberculosis remains one of the leading infectious cause of death in the world. The goals of screening are to detect active tuberculosis early enough and to identify individuals eligible for preventive therapy to reduce a potential co-infection by tuberculosis. Plasma/serum screening for selected potential biomarkers could represent a suitable method of tuberculosis diagnosis and treatment outcome. Furthermore, monitoring of tuberculosis treatment is crucial to clinical decision-making and besides the plasmatic concentration of administered antituberculosis drugs, the biomarkers appear to play a significant role in the estimation of the real therapeutical impact.

The current standard remains focused on culture conversion, especially two-month culture status, which has a relatively low sensitivity. Identification of non-sputum-based biomarkers of the treatment response would be beneficial for individual monitoring of tuberculosis patients.

This mini-review describes several serological/plasmatic markers that can be analyzed by simple immunoassays as ELISA method, e.g. C-reactive protein, soluble intercellular adhesion molecule-1, soluble urokinase plasminogen activator receptor, soluble lymphocyte activation gene-3, granzyme B and soluble tumor necrosis factor receptor one and two as reliable enough as an indicator of successful treatment of tuberculosis.

Key words: tuberculosis, inflammatory markers, CRP, sICAM-1, suPAR, sLAG-3, granzyme B, sTNFR I, II

INTRODUCTION

Tuberculosis (TB) is currently considered as one of the most common infectious diseases causing death (1). Even though the prevalence of tuberculosis in Slovakia is at a low level, the incidence in Eastern European countries has been increasing during the last decades. More of that, antituberculosis drug resistance has been also relentlessly increasing as in their the most severe forms – multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) (2).

Patients diagnosed with TB are generally put under a fixed four-drug regimen (isoniazid, rifampicin, pyrazinamide, and ethambutol) for 2 months, known as an intensive phase. The next phase, the maintenance phase, consists of 4 months with isoniazid and rifampicin. Despite of a long-term therapy, some patients experience a recurrence of infection and a type of resistance to first-line antituberculosis drugs (ATs), e.g. rifampicin. The origin of the resistance is based on gene mutations in mycobacteria, which leads to a change of the target molecule of the drug. The most frequent type of these mutations is a single nucleotide polymorphism (SNP) in core genes or promoters (3, 4). One of the main reasons for the resistance is the occurrence of suboptimal drug concentrations. As the patients with a resistant form of TB suffer from longer duration of treatment, the biomarkers that indicate

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the treatment efficacy at the early phase of the treatment as well as at the end of the treatment could greatly improve the clinical prognosis (5).

Accurate and early diagnosis is important for controlling the infection and an effective treatment of TB. There are many diagnostic tools to combat this infection (6,7). These tools include confirming the presence of the bacteria (mycobacterium or its components) or testing the response of the host (including the measurement of inflammatory biomarkers). Tools for the diagnosis of active disease include clinical suspicion, staining for acid-fast bacilli (AFB), culture for mycobacteria, and, more recently, nucleic acid amplification assays (7).

However, there are some limitations to the current standards. For instance, chest X-ray scans rely on the subjective judgment of the physicians. The sensitivity of sputum smear examination is limited even in well-equipped laboratories (8, 9). Furthermore, an absence of *M. tuberculosis* in sputum does not mean it is completely eradicated (8). Failure of sputum smear testing due to its low sensitivity may have serious consequences such as a continuation of the intensive phase of the therapy (10).

Therefore, there is a high demand for establishing a rapid and efficient method. One of the reasons is to confirm successfully cured TB. The other reasons are to confirm new cases of TB and differentiate it from a latent infection (7). In this context, there have been several attempts to determine the role of soluble disease markers that could play diagnostic and prognostic markers in TB (11).

This mini-review describes a brief insight into inflammatory markers in serum during TB infection which that could be used for diagnostics and especially for monitoring of the treatment advantage.

PATHOPHYSIOLOGY OF INFLAMMATION IN TUBERCULOSIS

Tuberculosis may involve any or all organs, but the lungs are the most commonly involved. Approximately 85% cases are in the form of pulmonary tuberculosis (63). TB outside the lung, known as extrapulmonary TB, usually results from a hematogenous dissemination (64). Active tuberculosis is caused by a primary infection or as a reactivation of latent tuberculosis known as post-primary pulmonary tuberculosis (65).

M. tuberculosis bacteria are transmitted mostly via respiratory routes. The initial interaction of the bacteria with the host is through alveolar macrophages by their accumulation in a tuberculous lesion – granuloma, which is a base for the clinical manifestations. The bacteria are transmitted to the parenchyma of the lung, replicating within resident macrophages, which become infected (Figure 1A, I) (12). This process initiates an inflammatory response, including secretion of inflammation markers as tumor necrosis factor-alpha (TNF- α), several interleukins such as IL-12, IL-1, IL-6, and various chemokines producing by host immune cells. The most important chemokines include chemokine ligand 5 (CCL5, also known as RANTES), chemokine ligand 3 (CCL3, also known as macrophage inflammatory protein 1- α), macrophage inflammatory protein 2 (MIP2), monocyte chemoattractant protein-1 (MCP-1), Cytokine-induced neutrophil chemoattractant-1 (CINC-1) (13, 14). These cytokines and chemokines play active roles in the regulation of the immune response (15). The expression of these substances (IL-1, IL-6, TNF- α) modulates lipoarabinomannan (LAM), an integral component of the mycobacterial cell wall (16). They stimulate other cells to enter the lungs, especially monocytes and eventually lymphocytes.

After 2–3 weeks of the infection, a specific cell-mediated immunity develops and it is managed by T-cells. The major pathway is realized by T-helper cells (Th1) for tuberculosis. Th1 cells are responsible for the activation of macrophages by the production of cytokines, especially interferon- γ (IFN- γ) and TNF- α (17).

Cellular events shown in Figure 1B lead to the development of granuloma. The formation of granulomas is the key pathological feature of TB. If granulomas can control the growth of mycobacteria and their spread, it develops a clinically latent infection (12, 18). Patients

with latent infections are the largest reservoir for a potential transmission. The main complication of this state is the manifestation of the disease from the latent state to an active state, i.e reactivation (19). A high risk of reactivation is among patients on a long-term treatment by immunosuppressive treatment with TNF- α inhibitors (20).

However, if granulomas are impaired, an infection may progress and results in an active form of tuberculosis (12).

Fig. 1 Multiscale diagram of mechanisms of an immune response to tuberculosis infection. Adapted from (12)

(M_r – resting macrophage, M_i – infected macrophage, M_{ci} – chronically infected macrophage, M_a – activated macrophage, T_y – proinflammatory producing IFN- γ T-cell, T_c cytotoxic cell.)

Figure 1A: Schematically depicted interactions between immune cells and *Mycobacteria* in a nutshell on a tissue-level scale. These examples of processes are: (I) infection of a resting macrophage after phagocytosis of extracellular *M. tuberculosis* (*Mtb*), (II) intracellular growth of *Mtb* within an infected macrophage, (III) cytotoxic T cell-mediated killing of an infected macrophage, (IV) activation of macrophage as a result of interaction with IFN- γ producing T cells and TNF, (V) secretion of TNF. Figure 1B: Single-cell model represents binding TNF- α its signal transduction and various events such as synthesis, internalization, recycling, and degradation of ligands and receptors. Figure 1C: Molecular scale NF- κ B dynamics model and its regulation in the form of negative feedback is shown by red lines. There are also shown NF- κ B-mediated responses. (sTNF/TNFR – soluble TNF with receptor for TNF, IKKKn – a neutral form of IKKK, IKKKa – Active form of IKKK, IKKK – IKK kinase, IKKn – Neutral form of IKK kinase, I κ B – Cytoplasmic I κ B)

VARIOUS BIOMARKERS AS DETERMINANTS OF TUBERCULOSIS OUTCOME

Generally, biomarkers are described as objective characteristics indicating a physiological or, more often, a pathological process. Biomarkers can be used also to demonstrate or observe the pharmacological response to a therapeutic intervention (21). Furthermore, they provide information about the disease status and the progression of the infection and very often represent an objective basis for endpoints in clinical trial (7).

There have been studied several biomarkers derived from *M. tuberculosis* or human inflammatory immune response that can be potentially used for the diagnosis of TB. These biomarkers can describe various diagnostic events. For instance, the reactivation of a latent tuberculosis infection and its durable eradication in patients with an active disease (7).

The earliest documented surrogate endpoint for efficacy at present is sputum culture status after 2 months of treatment. A meta-regression analysis conducted by Wallis and colleagues showed a highly significant relationship between 2-month status and a relapse risk, the treatment duration, and month 2 culture status. The report qualifying month 2 sputum culture status as a biomarker for relapse has helped with an early testing of new regimens containing rifampicin and pyrazinamide. (7, 22, 58)

Automated liquid culture systems such as mycobacterial growth indicator tube are used for the diagnosis of tuberculosis. They routinely report the time needed for the detection of the growth of *M. tuberculosis*. This variable is inversely correlated with the size of inoculum when tested with laboratory stock cultures (23). In patients, who responded on therapy, the time needed for the detection of growth (TTD) has been increased compared to non-responders, whose time did not increase in time. Thus, this parameter may show the possibility of treatment failure (24). Male sex, extensive parenchymal disease, presence and number of cavities, and baseline TTD less than 3 days were significantly associated with a delayed smear conversion at month 2 (25).

There are typically impaired lung functions associated with chest radiograph abnormalities. They can be easily measured by spirometry to evaluate the range of the impairment. The ratio of forced expiratory volume in 1 second (FEV1) to forced vital capacity (FVC) can show the severity of tuberculosis as the presence of obstruction, where the ratio FEV1/FVC is less than 70%, and the restriction is suggested by a low FVC (less than 80% of the predicted value) (59).

In terms of immunodiagnostic tools that could be useful for the diagnosis of active TB, there were attempts to identify novel antigens. Current IFN- γ release assays are not able to distinguish between active and latent tuberculosis (27, 57).

T cell-based analysis is another approach to diagnose TB (57).

Malnutrition is common among TB patients, associated with insufficient intake of micronutrients. Two decades ago it was found that zinc and vitamin A deficiency were more prevalent among sputum-positive patients than in healthy controls. Thus, it may contribute to a susceptibility for TB. These micronutrients may have an influence on the outcome of TB, but the mechanisms of that effects are still unclear. Plasma concentrations of retinol were associated with severity of TB (26, 29).

CHARACTERISTICS OF PLASMA/SERUM INFLAMMATORY MARKERS

At the World Health Organization (WHO) consensus meeting, non-sputum-based biomarker tests for all forms of TB were prioritized as a new generation technology for TB detection. A highly sensitive test based on a biological sample would help shorten the delay before the diagnosis and enable an early treatment. This test also would enable the diagnosis of extrapulmonary tuberculosis and tuberculosis in children (30).

There are several serological markers in tuberculosis disease, which reflect the activity of the disease, e.g. C-reactive protein (CRP), soluble intercellular adhesion molecule-1 (sICAM-1),

soluble urokinase plasminogen activator receptor (suPAR), soluble lymphocyte activation gene-3 (sLAG-3), granzyme B, and soluble tumor necrosis factor receptor one and two (sTNFR I and sTNFR II) (10).

C-reactive protein (CRP)

CRP, as an acute-phase protein produced in the liver, promotes phagocytosis in the case of a bacterial infection. CRP concentration is considered as a rapid non-specific indicator of an inflammation and a tissue necrosis (31). Thus, serum CRP levels rise in response to an inflammation induced by a disease such as tuberculosis. Significantly higher levels of CRP were found in a post-primary tuberculosis form compared to a primary tuberculosis (32). High levels of CRP have been observed in connection with the presence of multiple cavities and in patients with severe pulmonary destruction or suffering from millary tuberculosis (10, 32). Elevated CRP levels are usually significantly decreased after one month of treatment and by 6 months of treatment fall to normal values (33).

CRP levels are also used as a diagnostic tool in case of differential diagnosis between non-tuberculosis pneumonia and pneumonia caused by *M. tuberculosis*. Median CRP concentration was 3.2 mg/dL compared to the patients suffered from community-acquired pneumonia caused by other bacteria in which the median concentration was 8.3 mg/dL (60).

There is a term reported by Yoon et al. – so-called point-of-care CRP concentration. It is a concentration of 10 mg/l or more when it can be considered as screen positive patient for tuberculosis (36). This finger-prick test is provided by a point-of-care diagnostic test using POC fluorescent scanner (34). It is defined as a medical diagnostic testing at the time and place of the patient care (28). Another approach to analyze the levels of CRP is using a multiplex cytokine platform.

Nuclear factor-kappa B (NF- κ B)

Transcription factor NF- κ B is essential for the induction of a variety of genes in response to pathogens and mainly inflammatory cytokine tumor necrosis factor α (TNF- α). NF- κ B is bound to I κ B proteins (inhibitory proteins) in resting cells. The I κ B proteins maintain NF- κ B in a latent state. When TNF- α binds to its TNF receptor 1 (TNFR1), I κ B kinase (IKK) is being activated and phosphorylates I κ B proteins that leads to the degradation of I κ B proteins (Figure 1C) (12). Free NF- κ B starts to accumulate in the nucleus and mediates the transcription of the target genes (37). These genes are further transcribed and translated to extracellular signaling molecules (e.g. TNF- α , macrophage-activating proteins, etc.) as well as negative regulatory proteins of NF- κ B such as I κ B α and A20. NF- κ B activation is later linked to NF- κ B-mediated cell responses that include TNF expression, chemokine expression, macrophage activation, and inhibition of apoptosis (Figure 1C) (12).

Tumor necrosis factor-alpha (TNF- α)

TNF- α is the main cytokine that propagates and enhances the functions of NF- κ B. The central role in the development of TB has been attributed to TNF- α . This cytokine contributes to several typical symptoms of TB, e.g. fever and weight loss (11). TNF- α , released from macrophages, is also responsible for host defense mechanisms in mycobacterial infections (38). It is crucial for granuloma formation and for its maintenance (13). Its functions as induction of macrophage activation, induction of chemokine, and cytokine production and apoptosis is mediated by NF- κ B (12). TNF- α acts as an auto-amplifier: it enhances its synthesis and release. Also, it is considered as an important signal for a macrophage activation with IFN- γ . These activities regulated by the NF- κ B pathway have made TNF- α a key factor for restricting bacterial growth in granulomas (12, 61).

TNF- α can be bind by soluble TNF receptors (sTNFRs), known as soluble TNF receptor type I and type II, respectively (sTNF-RI, sTNF-RII). sTNFRs serve as inhibitors of TNF, if its concentration is too high. According to that, it has been proposed, that the serum concentrations of sTNFRs as endogenous inhibitors may indirectly reflect the activity of TNF by a negative feedback. The concentrations of sTNFRs were significantly higher in patients with active TB in comparison with healthy controls. sTNFRs concentrations were elevated also in the number of infectious diseases and chronic diseases. However, there was no difference

between pulmonary and extrapulmonary TB (41). It was reported, that the elevation of serum sTNF-RI is associated with TB compared to uninfected patients with HIV. Thus, the elevated concentrations of sTNF-RI show TB disease specificity. In contrast, sTNF-RII was elevated both in HIV and TB infected patients (39).

IFN- γ and the latent state of TB

In most cases, the infection with *M. tuberculosis* is reflected by the host immune response, which results in the prevention of progression to an active disease. Thus, the latent form is defined as having an evidence of the infection by the immunologic test IFN- γ release assay (IGRA) without any clinical symptoms of the disease and normal chest radiograph. IGRA test detects sensitization to mycobacterial antigens (7, 19). This represents a "steady-state" when the host can control the infection but not completely get rid of the bacteria (19).

Other serological biomarkers

Intercellular adhesion molecule-1 (ICAM-1) is a glycoprotein, which helps the interaction of cells with each other or with the extracellular matrix in the processes of cell migration (40, 41). It acts as a ligand for leukocyte integrins (42), and is expressed on endothelial and epithelial cells, macrophages, and fibroblasts. Its expression (in the membrane and soluble form) is induced by TNF- α , IL-1 β , and IFN- γ , which are present in the inflammatory area (40).

The soluble form (sICAM-1) of intercellular adhesion molecule-1 is created from the form expressed in the cell membrane (mICAM-1) via a proteolytic breakdown (43). The concentrations of sICAM-1 were higher in patients with an extensive alveolar disease and in patients with TB at the time of the diagnosis in comparison to the controls (10, 42). The highest concentrations of sICAM-1 were obtained in patients with severe TB. Healthy patients had the lowest level of sICAM-1. At the end of the therapy, the patients had a lower level of sICAM-1 than at the time of the diagnosis. The soluble form can be used as a marker of the inflammation in TB and other diseases (e.g. diabetes mellitus type II, hepatitis B infection, and pneumonia). sICAM-1 is also increased in patients with latent TB compared to patients with pneumonia, lung cancer, and bronchiectasis characterized by less or small cytokine production (16).

Another marker, urokinase plasminogen activator receptor (uPAR, CD87), is mainly located on monocytes and macrophages. Its function is to participate in cell adhesion and migration. uPAR is bound to the cell surface. uPAR can be cleaved from the cell membrane, resulting in a soluble form suPAR, detectable in serum or plasma (44). It was found that uPAR is significantly higher on peripheral blood monocytes from 8 patients with tuberculosis compared to healthy controls (41). The suPAR levels were also significantly higher in the TB-positive patients (sputum positive) compared to TB-negative patients. Its levels seem to correlate with the number of Mycobacteria in sputum. Patients with high suPAR levels died more rapidly than patients with low suPAR concentrations. Furthermore, its concentrations correlate with the treatment effectiveness, as eight months of treatment was associated with a decrease of suPAR levels (45).

Activated Th1 cells express on the membrane a special molecule called lymphocyte activation gene-3 (LAG-3) which can be detected as a soluble lymphocyte activation gene-3 (sLAG-3) (46). Therefore, soluble LAG-3 can be considered as a parameter of Th1 cells activity. Plasma levels of sLAG-3 were significantly lower in patients with untreated TB than levels detected in a healthy community and household controls. Furthermore, after 2–3 months of the treatment, the plasma levels of sLAG-3 were increased three to four times than before the treatment. At the end of the therapy, sLAG-3 concentrations were six times higher in comparison to the concentrations before treatment. Cured patients also showed higher levels of sLAG-3 compared to non-cured patients. Low concentrations of sLAG-3 suggest that TB is associated with a low activity of Th1 cells (47). The large size of cavities (more than 4 mm) was associated with higher concentrations of sLAG-3. However, there is no difference in its concentrations between fast and slow responders to the therapy.

However, the concentrations of sLAG-3 correlate with the radiological extent of pulmonary tuberculosis (10).

The primary cytolytic mechanisms of lymphocytes involve the release of granzyme-containing granules. Granzymes are proteases, which augment the damage of cells caused by a complement-like molecule perforin (48). In contrast to the other markers discussed above, granzyme B remains relatively constant during the therapy and declines at the end of the treatment. Just as in the case of sLAG-3, granzyme B levels were higher in patients with a large size of cavities (1). This suggests that there is a correlation between the levels of granzyme B and the radiological extent of disease.

Neopterin is another important marker. It is a non-specific marker of macrophage activation and its high levels are associated with a high risk of relapse or reactivation (53).

Laboratory analysis of serologic markers

Most of the inflammatory markers are usually measured by immunoassay methods. These methods are used to quantify biological molecules, mostly proteins based, on the specificity and selectivity of antibodies chosen for intended molecules that are produced intracellularly or secreted in any response. ELISA (enzyme-linked immunosorbent assay) is one of several methods used to detect specific molecules. The molecules of inflammation can be detected by a sandwich-type or a competitive type of ELISA. There are different modes of detections, for example, colorimetric, fluorescence, or chemiluminescence (54).

Modern measurement of CRP is carried out using light scattering from specific CRP-specific antibody aggregates (53).

The production of IFN- γ is carried out by IGRA test mentioned above. This test measures *in vitro* by whole blood ELISA or an enzyme-linked immunospot (ELISPOT) assay on peripheral blood mononuclear cells (PBMC), respectively (54,55).

CONCLUSIONS

Every method for the diagnosis of tuberculosis has its own limitations. Despite the sensitivity limitations of sputum smear microscopy, it remains the most commonly used diagnostic test for TB. *M. tuberculosis* culture, the reference standard test, has long turnaround time and other disadvantages such as the high price of test and a sensitivity to contamination. Tests based on the analysis of *M. tuberculosis* DNA (The GeneXpert MTB/RIF sputum test, whole-genome sequencing) have arguably the most important advance in the diagnosis of TB field as well as in the diagnosis of resistance of antituberculosis drugs to the mycobacteria. Whole-genome sequencing (WGS) provides information about complete genetic information of *Mycobacterium tuberculosis*. This method covers sequencing DNA (chromosomal and mitochondrial as well) (62). They are coupled also with fast detection of rifampicin and other resistance. However, due to relatively high operating costs this test is very popular only in developed countries (27).

Contrary to sputum-based examinations, serum/plasma-based biomarkers have several advantages. Blood is relatively easy to collect and remains as a stable option for biomarker measurements throughout the therapy. Blood-based biomarkers can be quantified and they could be predictive by combining multiple biomarkers as biosignatures. They can act as indicators of the disease severity and the effectiveness of the therapy for both clinical investigators and care providers (56). Another challenge of immunodiagnostic tests is to identify individuals at risk for possible development of tuberculosis (52).

ACKNOWLEDGMENTS: This work was supported by grants VEGA 1/0255/18, APVV-18-0084, Comenius University grant UK/81/2020, and by the project "Biomedical Center Martin" (ITMS code: 26220220187) – the project is co-financed from EU sources.

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Received: October, 1, 2020

Accepted: November, 9, 2020