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IMMUNOLOGICAL ASPECTS

Gender biased immune-biomarkers in active tuberculosis and correlation of their profiles to efficacy of therapy

Karina Chavez [1], Resmi Ravindran [1], Ali Dehnad, Imran H. Khan*

Department of Pathology and Laboratory Medicine, University of California, Davis, USA

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SUMMARY

Active pulmonary TB is an inflammatory disease and is increasingly viewed as an imbalance of immune responses to *Mycobacterium tuberculosis* (*M. tb.*) infection. In addition, this immune imbalance may be gender biased (males have a higher prevalence of TB) but reasons for such bias are uncertain. We hypothesized that studies on profiles of immune-biomarkers will not only provide insight into molecular basis of gender bias but may also help identify biomarkers to monitor efficacy of TB therapy. We examined 10 plasma cytokine/chemokine/growth-factor and 8 antibody (against 8 *M. tb.* antigens) biomarkers (elevated in TB patients) by multiplex microbead immunoassays. In addition, we examined these biomarkers in patients under anti-tuberculosis therapy (ATT). The results showed that female patients contained significantly higher levels of CXCL9 (MIG) and CXCL10 (IP-10), while males contained higher levels of PDGF-BB. In contrast, more males than females contained antibodies against several antigens. Our results also show that there are progressive and substantial decreases in plasma levels of CXCL9, CXCL10, PDGF-BB, IFNγ, and IL-18, correlating with treatment success. Our results suggest that studies on gender bias in immunebiomarkers will enhance understanding of host responses in TB and would be valuable as biomarkers for monitoring efficacy of ATT.

1. Introduction

It is estimated that one third of the world's population (approximately 2 billion) is infected with *Mycobacterium tuberculosis* (*M. tb.*), the etiologic agent of tuberculosis (TB) (WHO Global TB Report, 2014). Approximately 10% of those infected develop active TB at some stage during their lifetime, while the remainder may carry a lifelong latent infection that is asymptomatic and not transmissible [1,2]. The active disease is generally manifested by symptoms such as cough, fever, night sweats, weight loss, and hemoptysis. Countries, where TB is endemic (e.g., India, China, Nigeria, Pakistan, Brazil, and regions of sub-Saharan Africa), carry the highest active disease burden with annual incidence of several hundred cases per 100,000 population (WHO Global TB Report, 2014). Active TB is a debilitating disease that severely affects the quality of life of patients and often leads to death if not correctly diagnosed and treated in a timely manner. The largest proportion of TB patients are young, in the economically productive age group (35–54 years) and thus, the actual societal impact of TB in endemic countries is crushingly high (WHO Global TB Report, 2014). The underlying mechanisms that trigger the shift from latent infection to active disease are presently not clear. But immune imbalance involving alterations in the dynamics of factors such as cytokines, chemokines and growth factors appears to play a key role [3–8].

Like many other infectious diseases, active TB has long been known to have disproportionate occurrence in men [9,10]. Well documented historical records of TB in New York and rural Wales clearly show male to female patient ratio of 2:1 [10,11]. Recent data from the endemic countries are consistent with such historical records showing that there may be twice as many males than females with active disease among the TB patient population [12,13]. In 2013, men were estimated to account for 63% of the 9 million new cases worldwide (WHO Global TB Report, 2014). More specifically, in Nigeria, the third highest TB prevalence country, for bacteriologically confirmed TB in 2013, there were twice as many men (751) than women (359) per 100,000 population (WHO Global TB Report, 2014). A variety of factors may influence this gender bias including differences in exposure to other pathogens, sex

* Corresponding author. Department of Pathology and Laboratory Medicine, University of California at Davis, 4400 V Street, Sacramento, CA 95817, USA. Tel.: +1 916 734 6538.
E-mail address: ihkhan@ucdavis.edu (I.H. Khan).
[1] These authors contributed equally.

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hormones and their role in modulating the response of the immune system, genetics etc. [12]. It has been reported that even after factoring in a higher exposure of males, due to social conditions in the TB endemic countries that increase the likelihood of *M. tb.* transmission, the worldwide gender bias in active TB is primarily influenced by host biology [12].

Consistent with the observation of gender bias in TB patients, studies on other infectious diseases where more prevalent and severe disease occurred in the males in comparison to females have been reported [14,15]. The bias appears to be related to differential immune responses. Females mount more robust and protective immune responses to microbial stimuli [12,15]. In amoebiasis, caused by *Entamoeba histolytica*, the protozoan may cause disease after years of latency (amoebic liver abscess; ALA) but with a characteristic gender bias of 7:1 towards males [14]. The reported gender bias in ALA and other parasitic diseases (e.g., malaria, leishmaniasis and paracoccidiodomycosis) appears to be related to the influence of sex hormones on the immune system exerted through cytokines/chemokines [12,14]. Similarly, in TB, influence of sex hormones on the immune system appears to reflect the gender bias toward males in the pathogenesis of active disease [12].

In TB, cytokine/chemokine dynamics play a key role in the disease outcome [16]. Cytokines and chemokines are secreted by specific cells of the immune system that mediate interactions between cells and are required for an integrated response to a variety of stimuli in immune and inflammatory processes [17]. They can be pro- or anti-inflammatory, are involved in both paracrine and autocrine pathways, and are grouped into different classes, such as interleukins, lymphokines, chemokines and cell signaling molecules [18]. Pro-inflammatory cytokines help in the control of *M. tb.* infection, but they also play a crucial role during the chronic (latent) infection stage, dictating the pathogenesis of the disease [19]. Tumor necrosis factor alpha (TNF-α), interleukin-12 (IL-12), and gamma interferon (IFN-γ) are central cytokines in the regulatory and effector phases of the immune response to *M. tuberculosis* [20]. Alveolar macrophages and dendritic cells release inflammatory cytokines such as TNF-α, IL-12, and IL-23 along with a variety of chemokines, including C-C motif ligand 2 (CCL2), CCL5, and C-X-C motif ligand 8 (CXCL8). The Th1 response, important for granuloma assembly, is triggered by the production of IL-12 and IL-23 by DCs [5]. Activated T cells regulate this flow of inflammatory events by secreting IFN-γ and IL-2, which activate alveolar macrophages to produce a variety of substances involved in growth inhibition and killing of mycobacteria [21]. Immune responses to *M. tb.* infection are downregulated by the production of anti-inflammatory cytokines such as IL-4, IL-10, and transforming growth factor β (TGF-β) [22]. In TB patients, patterns of cytokines and chemokines detected in the blood circulation can provide evidence of infection and/or disease without direct analysis of tissue from the affected organ(s) (e.g., lung biopsy) [23,24].

Immune responses to active TB can be assessed by studying profiles of immune biomarkers (cytokines/chemokines, and antibodies against the infectious agent) in patients. In a previous study, we found that samples from TB patients with limited antibody response against *M. tb.* antigens (e.g., Rv3881c, Rv2031c [HspX], Rv0934 [P38 or PstS1], Rv3804c [Ag85a], Rv1886c [Ag85b]) contained higher levels of plasma cytokines/chemokines consisting of IL-18, IFN-γ, CXCL10, CXCL9, G-CSF, IL-6, CXCL1, VEGF, and PDGF-BB [25]. Therefore, careful examination of gender differences in these immune biomarkers may be helpful in elucidating the gender bias underlying the immune responses. In this report we describe differences in profiles of cytokines/chemokines and anti-*M.t.b.* antibodies in males and female TB patients. In addition, because monitoring of immune biomarker profiles may be of value in evaluating a patient's response to therapy, we examined the effects of anti-tuberculosis treatment (ATT) with a cocktail of four first line drugs (isoniazid, rifampin, ethambutol, pyrazinamide, or streptomycin), according to WHO's Directly Observed Treatment Short-Course (DOTS) criteria, on alterations in plasma levels of immune biomarkers (antibodies and cytokines/chemokines) at times 0, 2, and 4 months after the start of therapy. The decreases in cytokine/chemokine levels (CXCL9, CXCL10, PDGF-BB, IFNγ, and IL-18) correlating with the success of treatment, and the related gender bias in such decreases are described. Because TB treatment is a lengthy process, typically lasting 6 months, these biomarker profiles as described above, in conjunction with the analysis of gender bias, could help understand the dynamics of immune responses in TB patients and potentially be utilized to enhance TB treatment strategies.

2. Materials and methods

2.1. Sample groups

Plasma samples from patients with active pulmonary TB (mixed gender, 21–35 years of age) were collected. For the measurements of plasma cytokines/chemokines (n = 104: 45 Female (F) and 59 Male (M)) and detection of anti-*M. tb.* antibodies (n = 225: 104 F and 121 M), blood samples were drawn at the time of diagnosis. All the patients were from Federal Government TB Hospital, Rawalpindi, Pakistan, that follows WHO guidelines, administered through the National TB Program (http://www.ntp.gov.pk). The patients were diagnosed for active pulmonary TB on the basis of positive result for sputum smear AFB-microscopy (Ziehl-Neelsen Technique), chest X-ray suggestive of TB, and physicians' assessment based on clinical presentation including persistent cough for more than two to three weeks, and other systemic symptoms when present e.g., fever (low grade and intermittent), weight loss, night sweats etc. For AFB-microscopy, sputum specimens were collected on three consecutive days, with at least one early morning sample. A patient was considered to be sputum smear positive if at least one sample gave a positive result, following the International Standards for Tuberculosis Care [26]. Appropriate treatment (WHO Guidelines for treatment of tuberculosis) according to DOTS was initiated at the time of diagnosis for all patients included in this study. To study the effects of treatment on plasma immune-biomarker profiles, samples from patients were collected at the time of diagnosis (n = 51: 16 F and 35 M), and at follow up of the same patients at 2 months (n = 51: 16 F and 35 M) and 4 months (n = 39: 12 F and 27 M) during treatment. At the 4 month time point, the number of patients decreased by 12 (4 F and 8 M) because of refusal to grant consent for blood draw. Treatment of all 51 patients, investigated for alterations in immune-biomarker profiles, was considered to be successful based on negative result of sputum AFB-microscopy and clinical evaluation (6 months of ATT).

Healthy group (n = 35) included mixed gender, between 21 and 30 years of age, with no history of active TB and no known medical conditions (infection, cancer, or metabolic disease). They had no apparent symptoms and signs of any respiratory tract disease. None of the healthy individuals had symptoms such as cough, sputum production, fever, weight loss etc. They were all from the same geographical area as the TB patients. To establish the cut-off values, samples from healthy individuals were tested for the same analytes (cytokines/chemokines and anti-*M. tb.* antibodies) as TB patients.

2.2. Sample collection

As previously described, blood samples (5 ml) were collected into a Vacutainer tube (EDTA, catalog # 367899; BD, Franklin Lakes, NJ) via venipuncture [27]. Plasma was separated (1000 × *g* for