Original Article

Differential Early Secreted Antigen Target (ESAT) 6 kDa–induced IFN-γ and SOCS1 expression distinguishes latent and active tuberculosis

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Abstract

Introduction: Expression of Suppressor of cytokine signaling (SOCS)-1 molecules is increased in patients with tuberculosis (TB). Early Secreted Antigen Target (ESAT)-6 kDa – induced IFN-γ responses indicate Mycobacterium tuberculosis infection. The effect of ESAT6-stimulation on SOCS1 in the host is not known.

Methodology: Healthy asymptomatic controls had a negative (n = 16) or a positive (n = 13) tuberculin skin test (TST). ESAT6-induced IFN-γ responses classified these controls as positive (EC ESAT6 IFN-γ (+), n = 5) or negative (EC ESAT6 IFN-γ (-), n = 24) responders. Patients had pulmonary (n = 21) or extra-pulmonary (n = 30) tuberculosis. Peripheral blood cells were stimulated with ESAT6 and mRNA expression of IFN-γ and SOCS1 was determined.

Results: ESAT6-induced IFN-γ expression was raised in EC ESAT6 IFN-γ (+) as compared with EC ESAT6 IFN-γ (-), p = 0.019. ESAT6-induced SOCS1 mRNA expression was increased in both pulmonary TB and extra-pulmonary TB patients as compared with both EC groups. ESAT6-induced IFN-γ/SOCS1 mRNA expression ratio was decreased in TB patients as compared with both EC groups.

Conclusion: M. tuberculosis infection induces increased ESAT6-induced IFN-γ responses in both latent and active TB. Our data shows down-regulation of IFN-γ /SOCS1 expression to be induced only in active TB cases, distinguishing them from healthy individuals likely to have latent TB. A decreasing IFN-γ /SOCS1 ratio may leads to reduced Th1 immunity which contributes to inability of the host to control clinical disease.

Key words: ESAT6; SOCS1; tuberculosis; IFN-γ; pulmonary TB; extra-pulmonary TB


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Introduction

In 2010 the WHO reported 8.8 million incident cases of tuberculosis (TB), 1.1 million reported deaths from TB among HIV-negative individuals and an additional 0.35 million deaths in the case of HIV-associated TB [1]. A major challenge in the treatment and management of TB remains the early identification of Mycobacterium tuberculosis infected individuals and especially those who have progressed to develop tuberculosis. The Tuberculin skin test (TST) reaction is still commonly used to identify individuals who have been exposed to M. tuberculosis but this has been shown to be a non-specific test due to cross reactivity with other mycobacterial antigens and also because it is affected by BCG vaccination of the host [2].

IFN-γ plays a key role in regulating mycobacterial clearance by its ability to coordinate appropriate defense mechanisms required for M. tuberculosis (MTB) control [3,4]. The early secretory antigen target protein (ESAT6) is released by M. tuberculosis and can activate M. tuberculosis specific IFN-γ producing CD4+ T cells. ESAT6 is present in the RD1 region of pathogenic mycobacteria including, M. tuberculosis and M. bovis but is absent in avirulent M. bovis BCG and non-tuberculous mycobacteria (NTMs) with the exception of M. flavescens, M. szulgai, M. kansaii and M. marinum [5-8] and is therefore considered relatively MTB specific. A positive ESAT6 –induced IFN-γ response has been shown to be indicative of M. tuberculosis infection [9]. In murine and human models of tuberculosis ESAT-6–specific, IFN-γ–secreting CD4 T cells have been shown to play a role
in protection against *M. tuberculosis* in vivo [10,11]. ESAT6 in combination with CFP10 and TB7.7 antigens are included in the Interferon gamma release assays (IGRAs) used for detection of latent TB [12]. Discordant results have been reported between IGRAs and TST in areas highly endemic for TB due to prior BCG vaccination and the exposure to non-pathogenic mycobacteria [2].

The Suppressor of Cytokine Signaling (SOCS) family comprises eight members involved in the regulation of cytokine responses [13-14]. Among these, one of the best characterized is the SOCS1 molecule [15]. SOCS1 acts as a negative regulator of cytokine signaling by inhibiting Janus activated kinase (JAK)/STAT1 activation, a key molecule for IFN-γ signaling. SOCS1 expression has been shown to be increased in patients with TB as compared with healthy controls [16]. In a murine model of *M. tuberculosis* infection we found that SOCS1 expression by non-macrophage cells protected the host from infection-induced detrimental inflammation [17].

As IGRAs rely on a competent IFN-γ response which is affected by SOCS1 molecules we investigated ESAT6-induced IFN-γ and SOCS1 mRNA expression levels in patients with TB and healthy endemic controls. The healthy control group was stratified both according to their TST response and also according to their ESAT6-induced IFN-γ response. As such this data shed light on differential SOCS1 regulation in individuals who have a positive ESAT6-induced IFN-γ response regardless of their TST status.

**Methodology**

**Ethical approval**

This study was conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the Institutional Review Board of Aga Khan University, Indus Hospital, Karachi and Dow University of Health Sciences, Karachi. All patients provided written informed consent for the collection of samples and subsequent analysis.

**Subject selection**

Fifty one patients with TB were recruited from Aga Khan University Hospital (AKUH), Karachi; OJHA Institute for Chest Diseases, DOW University of Health Sciences (DUHS), Karachi and Indus Hospital, Karachi Pakistan using a cross-sectional study design. Inclusion criteria were: patients with confirmed TB diagnosis that had not received anti-tuberculous therapy (ATT); male or female; aged between 15-65 years; un-related study subjects. Exclusion criteria were: pregnancy; co-morbid conditions compromising the immune system (such as HIV infection, diabetes mellitus, chronic renal failure, chronic liver disease or corticosteroid therapy) and patients with relapsed TB.

Patients were classified as Pulmonary (PTB, \( n = 21 \)) and Extra-pulmonary (ETB, \( n = 30 \)) TB as per WHO guidelines for treatment of TB [18]. PTB patients were diagnosed by clinical examination, chest X-ray, sputum fast acid bacillus (AFB) microscopy and/or AFB culture [19]. Patients with ETB were diagnosed on the basis of histopathological findings, supportive radiological evidence on X-rays, contrast-enhanced Computed Tomography (CT) scan and/or Magnetic Resonance Imaging (MRI). Diagnostic criteria of patients with PTB or ETB are depicted in Table 1. ETB cases comprised those with tuberculous lymphadenopathy (\( n = 14 \)), unilateral (\( n = 9 \)) or bilateral (\( n = 1 \)) pleural effusion, spinal (\( n = 3 \)), abdominal (\( n = 1 \)), ovarian (\( n = 1 \)) and bilateral pleural effusion (\( n = 1 \)) TB.

Healthy asymptomatic control subjects (\( n = 29 \)) were BCG vaccinated volunteers recruited from AKUH.

**Tuberculin skin testing**

TST reactivity in each healthy endemic control (EC) subject was tested by intra-cutaneous administration of five tuberculin units of purified protein derivatives (PPD) solution and read after 48 h. An induration of < 10 mm was used as a cut-off for negative responses. Thirteen healthy volunteers were TST positive (EC TST+) and 16 TST negative (EC TST-).

**Stimulation of peripheral blood mononuclear cells (PBMCs) with recombinant ESAT6**

Ten ml of venous blood was used to obtain a buffy coat layer containing peripheral blood mononuclear cells (PBMCs) using a Ficoll-Histopaque (Sigma Aldrich, St. Louis, USA) density gradient. PBMCs were plated as \( 10^6 \) cells per well into 24 well tissue culture plate (Corning, New York, USA) and stimulated with ESAT6 antigen (5\( \mu \)g/ml) (BEI Resources, American Tissue Culture Collection, USA) for 18 hours. Cell supernatants were harvested and stored at -80°C for subsequent IFN-γ measurement.
**Measurement of IFN-γ**

IFN-γ was measured in culture supernatants by ELISA (Endogen, Rockford, USA) as previously reported [20]. Recombinant human IFN-γ was used to obtain a dose response curve with a range of detection from 3.9–1000 pg/ml. All experimental samples were tested in duplicate [21].

**Reverse Transcription-PCR**

Cells were lysed with Trizol reagent (Invitrogen, USA) and total RNA isolated as described previously [22,23]. RNA (1 µg) was reverse transcribed using MuIV reverse transcriptase (Invitrogen, Grand Island, New York USA) as described [24]. Real time PCR was performed in duplicate 20 µl reactions containing Platinum® SYBR® Green qPCR Supermix-UDG (Invitrogen, Carlsbad, USA), 150 nM forward and reverse primers, and 2 µl of cDNA on an ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, USA). Sequence specific primers were used for HuPO (human acidic ribosomal protein), IFN-γ and SOCS1 as described previously [25].

Two-fold dilutions of cDNA samples were amplified to control amplification efficiency and the optimal concentration for each primer pair. Thereafter, the Ct values for all cDNA samples were obtained. HuPO was used as a control gene to calculate the ΔCt values for individual samples. The relative amount of gene/ HuPO transcripts was calculated using the 2^(-ΔΔCt) method [26] and these values were then used to calculate the relative expression of mRNA in un-stimulated and ESAT6-stimulated cells.

**Statistical analysis**

Data is depicted as median values for each group whereby IQR (inter quartile range 25th to 75th percentile) are indicated in each case. Paired data between un-stimulated and ESAT6 stimulated samples was analyzed by the Wilcoxon Rank test. Comparison of non-parametric data between EC ESAT6 IFN-γ (-), EC ESAT6 IFN-γ (+) and TB groups was performed using the Mann-Whitney U test. Analysis was performed and data plotted using GraphPad PRISM Version 5 (GraphPad Software, San Diego, USA).

**Results**

**Characteristics of study groups**

TB patients showed pulmonary (PTB) and extra-pulmonary (ETB) disease. Healthy volunteers were sub-divided according to their TST reactivity and ESAT6-stimulated IFN-γ secretion.

Overall, the healthy control group comprised of sixteen individuals with a negative TST reaction and thirteen with a positive TST reaction. On the basis of ESAT6 induced IFN-γ responses five individuals showed ESAT6-IFN-γ positive responses (EC ESAT6 IFN-γ (+)) while twenty-four individuals had a negligible IFN-γ response (EC ESAT6 IFN-γ (-)). We further considered the EC ESAT6 IFN-γ (+) cases as an *M. tuberculosis* -exposed group. Characteristics of all groups are illustrated in Table 1; the age and gender distribution amongst the study groups was similar.

**Identification of healthy individuals with ESAT6 induced IFN-γ (+) responses**

ESAT6-induced IFN-γ secretion in PBMCs from all healthy volunteers was determined. Five

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**Table1. Characteristics of the study group**

<table>
<thead>
<tr>
<th>Site</th>
<th>N</th>
<th>Age (Mean ± SD)</th>
<th>Gender (M/F)</th>
<th>Microscopy</th>
<th>AFBC</th>
<th>Histopathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC TST-</td>
<td>16</td>
<td>30.33±8.89</td>
<td>9/7</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>EC TST+</td>
<td>13</td>
<td>36.8±11.35</td>
<td>6/7</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>EC ESAT6 IFN-γ (-)</td>
<td>24</td>
<td>33.79±11.29</td>
<td>11/13</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>EC ESAT6 IFN-γ (+)</td>
<td>5</td>
<td>30.8±5.8</td>
<td>5/0</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>TB</td>
<td>51</td>
<td>29.6±13.4</td>
<td>25/26</td>
<td>8/24</td>
<td>16/18</td>
<td>15/23</td>
</tr>
<tr>
<td>PTB</td>
<td>21</td>
<td>26.38±13.6</td>
<td>11/10</td>
<td>8/14</td>
<td>8/9</td>
<td>1/2</td>
</tr>
<tr>
<td>ETB</td>
<td>30</td>
<td>30.51±13.9</td>
<td>14/16</td>
<td>0/10</td>
<td>8/9</td>
<td>14/21</td>
</tr>
</tbody>
</table>

EC, endemic controls; TST, tuberculin skin test negative (TST-) and positive controls (TST+); EC ESAT6 IFN-γ (-), ESAT6 induced IFN-γ negative responders; EC ESAT6 IFN-γ (+), ESAT6 induced IFN-γ positive responders; TB, patients with tuberculosis; PTB, pulmonary TB patients; ETB, patients with extra-pulmonary TB; N, number of subjects; M/F, Male/Female; Microscopy, acid fast bacillus smear using Ziehl Neelsen staining; AFBC, acid fast bacilli culture by MIGIT system (Becton Dickinson, USA); histo-pathological staining of biopsy material where relevant; N/A, not applicable.
individuals showed ESAT6-IFN-γ positive responses (IFN-γ median, 64.3 pg/ml, IQR 1.1-79.9 pg/ml) and were classified as EC ESAT6 IFN-γ (+) while twenty-four individuals had a negligible IFN-γ response (IFN-γ median, 0 pg/ml, IQR 0-0 pg/ml) and were classified as EC ESAT6 IFN-γ (-).

**ESAT6 induced IFN-γ expression is greater in EC IFN-γ (+) than EC IFN-γ (-) individuals**

We first determined ESAT6-induced IFN-γ and SOCS1 mRNA gene expression in PBMCs of healthy EC TST- and EC TST+, EC ESAT6 IFN-γ (-) and EC ESAT6 IFN-γ (+) groups. Incubation with ESAT6 did not result in increased IFN-γ mRNA expression in healthy EC TST- and EC TST+ group. IFN-γ mRNA levels were also comparable between EC TST- and EC TST+ groups (Fig. 1A). Similarly ESAT6 did not induce IFN-γ mRNA levels in EC ESAT6 IFN-γ (-) and EC ESAT6 IFN-γ (+) group as compared with un-stimulated levels. However, ESAT6 stimulated an increase in IFN-γ mRNA expression level in EC ESAT6 IFN-γ (+) cases as compared with the EC ESAT6 IFN-γ (-) group (p = 0.019) (Figure 1B).

ESAT6-induced SOCS1 mRNA levels were decreased in EC TST- (p = 0.0003) and EC TST+ (p = 0.0002) groups as compared with un-stimulated levels. While, SOCS1 mRNA levels did not differ between EC TST- as compared with EC TST+ group (Figure 1C). ESAT6-induced SOCS1 mRNA transcripts were decreased in EC ESAT6 IFN-γ (+) group as compared with un-stimulated levels (p = 0.006), while ESAT6 induced SOCS1 mRNA levels were similar in EC ESAT6 IFN-γ (-) as compared with EC ESAT6 IFN-γ (+) group (Figure 1D).

As we had observed that there was no difference between EC TST- and EC TST+ groups but that there was a difference in ESAT6-IFN-γ mRNA levels between EC ESAT6 IFN-γ (-) and EC ESAT6 IFN-γ (+) groups, we continued our further analysis with the latter two groups.

**Discussion**

While ESAT6-induced IFN-γ responses may be indicative of *M. tuberculosis* –infection in the host, our data indicates that ESAT6-induced SOCS1 regulation may distinguish between *M. tuberculosis* –infected individuals who are healthy and those with active TB. As IFN-γ in the host is essential for mycobacterial clearance, this highlights a balance which may differentiate protective responses in healthy asymptomatic individuals as compared with active TB.

ESAT6 is recognized by IFN-γ producing CD4+ T cells in individuals who have had prior exposure to *M. tuberculosis* [27]. Elevated IFN-γ responses to ESAT6 antigen in individuals in close contact with active TB cases are shown to be at increased risk of developing clinical disease [28]. Further clinical studies have shown an association between immune reactivity to ESAT6 with that of the increased bacterial load and disease pathology in tuberculosis [29]. ESAT6 has shown to induce altered cytokine profile with decreased IFN-γ and increased IL4 responses in individuals with advanced disease [30].

When ESAT6- induced SOCS1 mRNA expression levels were determined they were found to be raised in PTB (p = 0.001) and ETB (p < 0.001) as compared with EC ESAT6 IFN-γ (-) cases, Figure 2B. Similarly, ESAT6-induced SOCS1 mRNA titers were also higher in PTB (p = 0.023) and ETB (p = 0.004) as compared with EC ESAT6 IFN-γ (+). There was no difference in ESAT6-induced SOCS1 mRNA expression between EC ESAT6 IFN-γ (-) and EC ESAT6 IFN-γ (+) groups. To further investigate the relationship between IFN-γ and SOCS1 we studied the ratio between these two gene targets (Figure 2C). ESAT6 induced IFN-γ/SOCS1 mRNA expression ratio was decreased in PTB and ETB as compared with EC ESAT6 IFN-γ (-) (PTB, p = 0.034; ETB, p = 0.001) and EC ESAT6 IFN-γ (+) (PTB, p = 0.006; ETB, p = 0.002) groups.

**Differential ESAT6-induced SOCS1 expression between healthy asymptomatic individuals and active TB cases**

We further studied ESAT6-induced IFN-γ and SOCS1 mRNA titers in EC ESAT6 IFN-γ (-), EC ESAT6 IFN-γ (+) as compared with PTB and ETB cases. Concordant with the raised levels of ESAT6 induced IFN-γ secretion observed [23], ESAT6-induced IFN-γ mRNA expression levels were increased in the EC ESAT6 IFN-γ (+), as compared with EC ESAT6 IFN-γ (-) cases (p = 0.019), Figure 2A. Also, ESAT6-induced IFN-γ expression levels were raised in PTB (p = 0.039) and ETB (p = 0.010) as compared with EC ESAT6-induced IFN-γ(-) cases.

As IFN-γ is recognized by IFN-γ producing CD4+ T cells in individuals who have had prior exposure to *M. tuberculosis* [27]. Elevated IFN-γ responses to ESAT6 antigen in individuals in close contact with active TB cases are shown to be at increased risk of developing clinical disease [28]. Further clinical studies have shown an association between immune reactivity to ESAT6 with that of the increased bacterial load and disease pathology in tuberculosis [29]. ESAT6 has shown to induce altered cytokine profile with decreased IFN-γ and increased IL4 responses in individuals with advanced disease [30].
Figure 1. ESAT6 induced IFN-γ expression is greater in EC ESAT6 IFN-γ (+) than EC ESAT6 IFN-γ (-).

Figure 2. ESAT6-induced SOCS1 and IFN-γ mRNA expression in EC and TB patients.

PBMCs ($10^6$) from healthy asymptomatic volunteers comprising of EC TST- (n=16), EC TST+ (n=13), EC ESAT6 IFN-γ (-), n=24 and EC ESAT6 IFN-γ (+), n=5 were stimulated with ESAT6 (5 μg/ml) for 18 h. Total RNA was harvested and mRNA transcripts were determined by RT-PCR. IFN-γ and SOCS1 gene expression was normalized to the housekeeping gene HuPO. Data shown is representative of median target gene/HuPO for each sample as calculated by the relative quantification method. Box and whiskers plots indicate data between 10th-90th percentiles with median values of each group indicated by a horizontal line. * denotes significant difference (p≤ 0.05) between the groups using Mann-Whitney U non-parametric test. IFN-γ mRNA expression in [A] EC TST- and EC TST+ groups, [B] EC ESAT6 IFN-γ (-) and EC ESAT6 IFN-γ (+) groups; SOCS1 mRNA expression in [C] EC TST- and EC TST+, [D] EC ESAT6 IFN-γ (-) and EC ESAT6 IFN-γ (+) groups.

Altered Th1/Th2 balance may be influenced by some regulatory molecules elevated in mycobacterial infection as a survival strategy and may determine the outcome of disease.

The absence of ESAT6 induced IFN-γ mRNA expression in peripheral blood cells of uninfected healthy controls is in agreement with previous data [31] as is ESAT6 stimulated IFN-γ mRNA upregulation in TB patients [32,33]. ESAT6-induced SOCS1 mRNA expression in healthy controls was reduced whether or not they had a positive ESAT6-induced IFN-γ response. Therefore, in ECs lowered SOCS1 mRNA expression with a positive ESAT6-induced IFN-γ response could be indicative of latent TB. Increase in SOCS1 mRNA expression in patients with TB corresponds with previous reports [25].

We speculate that decreased SOCS1 levels in EC ESAT6 IFN-γ (+) reflect more efficient IFN-γ-mediated responses by myeloid cells that probably control the infection in these individuals. The decreased IFN-γ/SOCS1 ratio found in PTB and ETB group as compared with EC ESAT6 IFN-γ (-) is most probably a consequence of active TB disease. However, in healthy individuals of the EC ESAT6 IFN-γ (+) group the raised IFN-γ/SOCS1 mRNA expression ratio may demonstrate a protective response against development of TB despite *M. tuberculosis* infection. SOCS1 negatively controls the signaling of many cytokines besides IFN-γ, such as IL-12 and IL-2 in turn regulating T cell responses [34,35]. Moreover, SOCS1 expression is necessary for the suppressor function of T regs cells in vivo by maintaining FoxP3 expression and hampering IFN-γ and IL-17 production in vivo [36]. T regs cells are in fact increased in active TB [37], and the experimental
manipulation of Foxp3+ cells indicate detrimental roles for Treg cells in host defence against *M. tuberculosis* [38].

Suppressed Th1 immune responses have been shown to be associated with TB *in vitro* and poor clinical outcome [39]. *M. tuberculosis* is known to promote down modulatory immune status and is shown to increase SOCS1 mRNA expression in patients with active TB as compared with patients with other infectious lung diseases or clinically healthy individuals [16]. Decrease in the levels of SOCS1 mRNA after chemotherapy in patients with TB suggests that *M. tuberculosis* alter the levels of these mediators to transit to active disease [16]. However differences in the level of expression of SOCS1 in healthy individuals with positive IFN-γ responses to ESAT6 antigen who might be latently infected and those who present with active TB are not yet known. Ours is the first observation that healthy subjects with ESAT6 positive IFN-γ responses, have lower SOCS1 mRNA with a concomitant increase in IFN-γ/SOC1 mRNA expression levels as compared with TB patients; indicating better protective Th1 responses against *M. tuberculosis*.

A limitation of this study was that our identification of EC ESAT6 IFN-γ (+) as an *M. tuberculosis* exposed group is based on the measurement of IFN-γ responses only to ESAT6 and we were not able to classify our healthy subjects as having latent TB according to the commonly used commercial Quantiferon-TB Gold assay which measures an IFN-γ response to ESAT6, CFP10 and TB7.7 as the Quantiferon assay has not been available in Pakistan.

Understanding the mechanisms which lead to the establishment of *M. tuberculosis* infection and development of active TB is of importance. Up-regulation of SOCS1 may be one of the several mechanisms used by *M. tuberculosis* to antagonize immune activation of Th1 protective responses and facilitate survival of pathogen [16,40,41].

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**References**


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