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

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 acid-fast bacillus (AFB) microscopy and/or AFB culture [19]. Patients with ETB were diagnosed on the basis of histo-pathological findings, supportive radiological evidence on X-rays, contrast-enhanced Computerized Topography (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 (5µg/ml) (BEI Resources, American Tissue Culture Collection, USA) for 18 hours. Cell supernatants were harvested and stored at -80 ºC for subsequent IFN- γ measurement.

Table1. Characteristics of the study group

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

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

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 (1μg) was reverse transcribed using MulV 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 C_t values for all cDNA samples were obtained. HuPO was used as a control gene to calculate the ΔC_t values for individual samples. The relative amount of gene/ HuPO transcripts was calculated using the $2^{-(\Delta\Delta Ct)}$ 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

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.

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 .

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.

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].

Figure 1. ESAT6 induced IFN-γ expression is greater in EC ESAT6 IFN-γ (+) than EC ESAT6 IFN-γ (-).
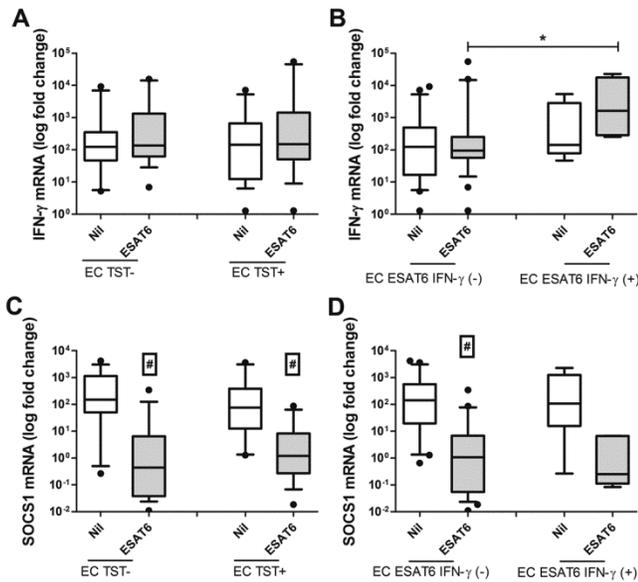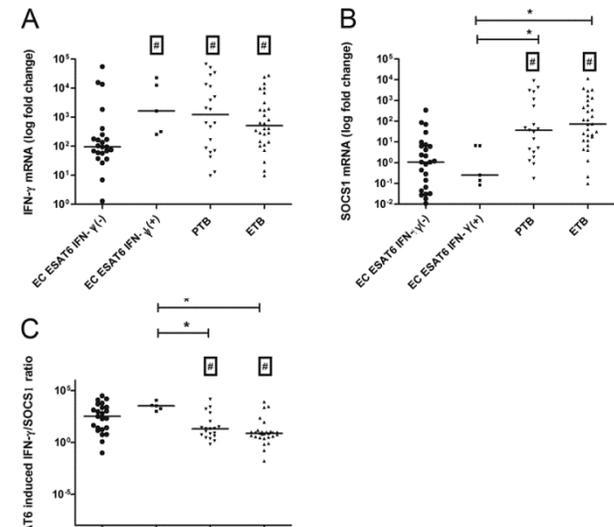

PBMCs (10^6) from healthy asymptomatic volunteers comprising of EC TST- (n=16), EC TST+ (n=13), EC ESAT6 IFN-γ (-), n=24 and EC ESAT6 IFN-γ (+), n=5 were stimulated with ESAT6 (5μg/ml) for 18 h. Total RNA was harvested and mRNA transcripts were determined by RT-PCR. IFN-γ and SOCS1 gene expression was 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

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

PBMCs (10^6) from ESAT6-IFN-γ negative responders (EC ESAT6 IFN-γ (-), n=24; ESAT6-IFN-γ positive responders (EC ESAT6 IFN-γ (+), n=5; and TB patients with pulmonary (PTB, n=21) and extra-pulmonary (ETB, n=30) involvement were stimulated with ESAT6 (5μg/ml) for 18 h. Total RNA was harvested and mRNA transcripts for IFN-γ and SOCS1 gene expression were determined as in Fig 1.. Ratio was calculated by dividing IFN-γ mRNA with SOCS1 mRNA expression value for each donor. Data is represented by scatters with median values of each group indicated by a horizontal line. '#' denotes significant differences (p ≤ 0.05) as compared with EC ESAT6 IFN-γ (-), * denotes significant differences (p ≤ 0.05) as compared with EC ESAT6 IFN-γ (+) using Mann Whitney U test. [A] IFN-γ [B] SOCS1 mRNA expression and [C] IFN-γ/SOCS1 ratio.

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/SOCS1 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].

References
1. WHO. World Health Organisation. Global Tuberculosis Control. Geneva: World Health Organisation, (2011). ISBN: 978 924 156380 2.
2. Machado A, Jr., Emodi K, Takenami I, Finkmoore BC, Barbosa T, Carvalho J, Cavalcanti L, Santos G, Tavares M, Mota M, Barreto F, Reis MG, Arruda S, Riley LW (2009) Analysis of discordance between the Tuberculin skin test and the Interferon-gamma release assay. Int J Tuberc Lung Dis 13: 446-453.
3. Cooper AM, Dalton DK, Stewart TA, Griffin JP, Russell DG and Orme IM (1993) Disseminated tuberculosis in Interferon gamma gene-disrupted mice. J Exp Med 178: 2243-7
4. Dalton DK, Pitts-Meek S, Keshav S, Figari IS, Bradley A and Stewart TA (1993) Multiple defects of immune cell function in mice with disrupted Interferon-gamma genes. Science 259: 1739-1742.
5. Arend SM, Geluk A, van Meijgaarden KE, van Dissel JT, Theisen M, Andersen P, Ottenhoff THM (2000) Antigenic equivalence of human T-cell responses to *Mycobacterium tuberculosis*-specific RD1-encoded protein antigens ESAT-6 and Culture filtrate protein 10 and to mixtures of synthetic peptides. Infect Immun 68: 3314-3321.
6. Pollock JM, Andersen P (1997) Predominant recognition of the ESAT-6 protein in the first phase of Interferon with *Mycobacterium bovis* in cattle. Infect Immun 65: 2587-2592.
7. van Ingen J, de Zwaan R, Dekhuijzen R, Boeree M and van Soolingen D (2009) Region of difference 1 in nontuberculous *Mycobacterium species* adds a phylogenetic and taxonomical character. J Bacteriol 191: 5865-5867.
8. Harboe M, Oettinger T, Wiker HG, Rosenkrands I and Andersen P (1996) Evidence for occurrence of the ESAT-6 protein in *Mycobacterium tuberculosis* and virulent *Mycobacterium bovis* and for its absence in *Mycobacterium bovis* BCG. Infect Immun 64: 16-22.
9. Hughes AJ, Hutchinson P, Gooding T, Freezer NJ, Holdsworth SR and Johnson PD (2005) Diagnosis of *Mycobacterium tuberculosis* infection using ESAT-6 and intracellular cytokine cytometry. Clin Exp Immunol 142: 132-139.
10. Andersen P, Andersen AB, Sorensen AL and Nagai S (1995) Recall of long-lived immunity to *Mycobacterium tuberculosis* infection in mice. J Immunol 154: 3359-3372.
11. Pathan AA, Wilkinson KA, Klenerman P, McShane H, Davidson RN, Pasvol G, Hill AV, Lalvani A (2001) Direct ex vivo analysis of antigen-specific IFN-gamma-secreting CD4 T cells in *Mycobacterium tuberculosis*-infected individuals : associations with clinical disease state and effect of treatment. J Immunol 167: 5217-5225.
12. Lange C, Pai M, Drobniewski F and Migliori GB (2009) Interferon-gamma release assays for the diagnosis of active tuberculosis: sensible or silly? Eur Respir J 33: 1250-1253.

13. Tamiya T, Kashiwagi I, Takahashi R, Yasukawa H and Yoshimura A (2011) Suppressors of Cytokine Signaling (SOCS) proteins and JAK/STAT pathways: regulation of T-cell inflammation by SOCS1 and SOCS3. Arterioscler Thromb Vasc Biol 31: 980-985.

14. Croker BA, Kiu H and Nicholson SE (2008) SOCS regulation of the JAK/STAT signalling pathway. Semin Cell Dev Biol 19: 414-422.

15. Piganis RA, De Weerd NA, Gould JA, Schindler CW, Mansell A, Nicholson SE, Hertzog PJ (2011) Suppressor of cytokine signaling (SOCS) 1 inhibits type I Interferon (IFN) signaling via the Interferon alpha receptor (IFNAR1)-associated tyrosine kinase Tyk2. J Biol Chem 286: 33811-33818.

16. Almeida AS, Lago PM, Boechat N, Huard RC, Lazzarini LC, Santos AR, Nociari M, Zhu H, Perez-Sweeney BM, Bang H, Ni Q, Huang J, Gibson AL, Flores VC, Pecanha LR, Kritski AL, Lapa e Silva JR, Ho JL (2009) Tuberculosis is associated with a down-modulatory lung immune response that impairs Th1-type immunity. J Immunol 183: 718-731.

17. Carow B, Qun Ye X, Gavier-Widen D, Bhuju S, Oehlmann W, Singh M, Sköld M, Ignatowicz L, Yoshimura A, Wigzell H, Rottenberg ME (2011) Silencing Suppressor of Cytokine Signaling-1 (SOCS1) in Macrophages Improves *Mycobacterium tuberculosis* Control in an Interferon-{gamma} -dependent Manner. The Journal of biological chemistry 286: 26873-26887.

18. WHO. Treatment of Tuberculosis: Guidelines for National Programmes, 3rd edn. Geneva: World Health Organisation, (2003) WHO/CDS/TB/2003, 313.

19. Crofton J (1991) Clinical features of tuberculosis. Crofton and Douglas's respiratory diseases, 4th edn. London: Wiley-Blackwell scientific, ISBN: 978 06320 19731.

20. Hasan Z, Zaidi I, Jamil B, Khan MA, Kanji A and Hussain R (2005) Elevated ex vivo monocyte Chemotactic protein-1 (CCL2) in pulmonary as compared with extra-pulmonary tuberculosis. BMC Immunol 6: 14.

21. Hasan Z, Jamil B, Ashraf M, Islam M, Yusuf MS, Khan JA, Hussain R (2009) ESAT6-induced IFNgamma and CXCL9 can differentiate severity of tuberculosis. PLoS One 4: e5158.

22. Hasan Z, Cliff JM, Dockrell HM, Jamil B, Irfan M, Ashraf M, Hussain R (2009) CCL2 responses to *Mycobacterium tuberculosis* are associated with disease severity in tuberculosis. PLoS One 4: e8459.

23. Hasan Z, Jamil B, Khan J, Ali R, Khan MA, Nasir N, Yusuf MS, Jamil S, Irfan M, Hussain R (2009) Relationship between circulating levels of IFN-gamma, IL-10, CXCL9 and CCL2 in pulmonary and extrapulmonary tuberculosis is dependent on disease severity. Scand J Immunol 69: 259-267.

24. Yang T, Stark P, Janik K, Wigzell H and Rottenberg ME (2008) SOCS-1 protects against *Chlamydia pneumoniae*-induced lethal inflammation but hampers effective bacterial clearance. J Immunol 180: 4040-4049.

25. Masood KI, Rottenberg ME, Carow B, Rao N, Ashraf M, Hussain R, Hasan Z (2012) SOCS1 Gene Expression is Increased in Severe Pulmonary Tuberculosis. Scand J Immunol 76 : 398-404.

26. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408.

27. Brandt L, Oettinger T, Holm A, Andersen AB and Andersen P (1996) Key epitopes on the ESAT-6 antigen recognized in

28. Doherty TM, Demissie A, Olobo J, Wolday D, Britton S, Eguale T, Ravn P, Andersen P (2002) Immune responses to the *Mycobacterium tuberculosis*-specific antigen ESAT-6 signal subclinical infection among contacts of tuberculosis patients. J Clin Microbiol 40 : 704-6.

29. Ribeiro S, Dooley K, Hackman J, Loredo C, Efron A, Chaisson RE, Conde MB, Boechat N, Dorman SE (2009) T-SPOT.TB responses during treatment of pulmonary tuberculosis. BMC Infect Dis 9: 23.

30. Demissie A, Wassie L, Abebe M, Aseffa A, Rook G, Zumla A, Andersen P, Doherty TM; (2006) The 6-kilodalton Early secreted antigenic target-responsive, asymptomatic contacts of tuberculosis patients express elevated levels of Interleukin-4 and reduced levels of Gamma Interferon. Infect Immun 74: 2817-2822.

31. Hasan Z, Rao N, Salahuddin N, Islam M, Ashraf M, Rottenberg ME, Hussain R (2012) *M. tuberculosis* sonicate induced IFN gamma, CXCL10 and IL10 can differentiate severity in tuberculosis. Scand J Immunol 75: 220-226.

32. Wu-Hsieh BA, Chen CK, Chang JH, Lai SY, Wu CH, Cheng WC, Andersen P, Doherty TM (2001) Long-lived immune response to Early secretory antigenic target 6 in individuals who had recovered from tuberculosis. Clin Infect Dis 33: 1336-1340.

33. Lalvani A, Pathan AA, McShane H, Wilkinson RJ, Latif M, Conlon CP, Pasvol G, Hill AV (2001) Rapid detection of *Mycobacterium tuberculosis* infection by enumeration of antigen-specific T cells. Am J Respir Crit Care Med 163: 824-828.

34. Cornish AL, Chong MM, Davey GM, Darwiche R, Nicola NA, Hilton DJ, Kay TW, Starr R, Alexander WS (2003) Suppressor of Cytokine Signaling-1 regulates signaling in response to Interleukin-2 and other gamma c-dependent cytokines in peripheral T cells. J Biol Chem 278: 22755-22761.

35. Dooms H, Wolslegel K, Lin P and Abbas AK (2007) Interleukin-2 enhances CD4+ T cell memory by promoting the generation of IL-7R alpha-expressing cells. J Exp Med 204 : 547-57.

36. Takahashi R, Nishimoto S, Muto G, Sekiya T, Tamiya T, Kimura A, Morita R, Asakawa M, Chinen T, Yoshimura A (2011) SOCS1 is essential for regulatory T cell functions by preventing loss of Foxp3 expression as well as IFN-{gamma} and IL-17A production. The Journal of experimental medicine 208: 2055-2067.

37. Hougardy JM, Place S, Hildebrand M, Drowart A, Debrie AS, Locht C, Mascart F (2007) Regulatory T cells depress immune responses to protective antigens in active tuberculosis. American journal of respiratory and critical care medicine 176: 409-416.

38. Scott-Browne JP, Shafiani S, Tucker-Heard G, Ishida-Tsubota K, Fontenot JD, Rudensky AY, Bevan MJ, Urdahl KB (2007) Expansion and function of Foxp3-expressing T regulatory cells during tuberculosis. The Journal of experimental medicine 204: 2159-2169.

39. Lienhardt C, Azzurri A, Amedei A, Fielding K, Sillah J, Sow OY, Bah B, Benagiano M, Diallo A, Manetti R, Manneh K, Gustafson P, Bennett S, D'Elios MM, McAdam K, Del Prete G (2002) Active tuberculosis in Africa is associated with reduced Th1 and increased Th2 activity in vivo. Eur J Immunol 32: 1605-1613.

40. Imai K, Kurita-Ochiai T and Ochiai K (2003) *Mycobacterium bovis* bacillus Calmette-Guerin infection promotes SOCS induction and inhibits IFN-gamma-stimulated JAK/STAT signaling in J774 macrophages. FEMS Immunol Med Microbiol 39: 173-180.
41. Vazquez N, Greenwell-Wild T, Rekka S, Orenstein JM and Wahl SM (2006) *Mycobacterium avium*-induced SOCS contributes to resistance to IFN-gamma-mediated mycobactericidal activity in human macrophages. J Leukoc Biol 80: 1136-1144.

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