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# SOCS *in situ* expression in tuberculous lymphadenitis in an endemic area

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ABSTRACT

Background: The immune response to *Mycobacterium tuberculosis* is complex and multifactorial, the cytokine system being a major factor in *M. tuberculosis* immunity.
Aim: To analyze the immunohistochemical aspects of tuberculous lymph nodes in immunocompetent patients and search for associations between SOCS and cytokine expression in human tuberculous lymphadenitis.
Methods: Thirteen lymph nodes were assayed by immunohistochemistry for SOCS-1 and 3, STAT-3, RANTES, MIP-1-α, ICAM-1, IFN-γ as well as CD45RO, CD20, CD34, CD68, trypsin and lysozyme. Additionally, the RT *in situ* PCR was performed for SOCS-1 and 3 mRNA detection.
Results: Decreased MIP-1α expression together with reduced SOCS-3 ($p=0.042$), lysozyme ($p=0.024$) and CD45RO ($p=0.05$) was observed in the TB lymph nodes compared to the control lymph nodes. In conclusion, the lymphadenitis due to *M. tuberculosis* was associated with a downregulation of memory T cells (CD45RO), activated lysozymes and SOCS-3 compared to controls, which may play a role in the long-term bacterial replication and altered immune modulation characteristic of the disease.


## Introduction

*Mycobacterium tuberculosis* is the etiologic agent of tuberculosis (TB), one of the world's most widespread infectious diseases, responsible for more than 3 million deaths per year (WHO, 2006). Lymphadenitis is the most common form of extrapulmonary TB in children and adults. Tuberculous lymphadenitis, mainly affecting cervical lymph nodes, and is the principal cause of lymphadenopathy worldwide (Flynn and Chang, 2001). Furthermore, the incidence is increased among persons with compromised immuno function, particularly AIDS.

Lymph nodes are the major sites of immune response, therefore a primary target of infection or malignant transformation and lymphocyte trafficking (Frizzera and Seo, 1993). The coordination between macrophages and T lymphocytes is critical in limiting *M. tuberculosis* infection. The initiation and maintenance of adaptive immunity to TB require a high frequency of specific cellular interactions. Granuloma formation is the hallmark of *M. tuberculosis* infection, being associated with a highly activated cell-mediated immune response. This creates a local environment for the cell interaction producing an effective immune response where cytokine production, macrophage activation and CD8 T lymphocyte effector functions combine to eradicate *M. tuberculosis* (Algood et al., 2003). Chemokines and adhesion molecules are also involved in this process and play an important role in cellular trafficking (Tedla et al., 1999).

Cytokines are an integral component of the innate and adaptive immune responses. Suppressors of cytokine signalling (SOCS) proteins, as their name indicates, they are negative modulators of cytokine activity. These molecules, a family of cytoplasmic proteins, complete a negative feedback loop to attenuate signal transduction from cytokines that act through the janus kinase/signal transducer and activator (JAK/STAT) pathways. Cytokines, including interferons, activate the JAK–STAT pathway (Darnell, 1997; Kubo et al., 2003; Yoshimura et al., 2007). Interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) are crucial for the immune response to *M. tuberculosis* infection (Flynn et al., 1993, 1995; Ellner, 1997). Although IFNγ has been proven to be an important mediator of activation in intracellular pathogens, many studies have demonstrated that the expression of IFNγ-inducible gene is decreased in macrophages infected with mycobacteria (Hmamma et al., 1998; Ting and Kim, 1999; Imai et al., 2003) and that IFNγ is unable to activate human macrophages to restrict or kill virulent *M. tuberculosis* (Douvas et al., 1985; Rook et al., 1986). One conceivable hypothesis to explain this phenomenon could be the SOCS-induced inhibition of IFNγ expression (Imai et al., 2003), as especially, SOCS-1 and SOCS-3 appear to negatively regulate IFNγ signal transduction pathways (Alexander et al., 1999; Song and Shuai, 1998; Sakamoto et al., 1998). In fact, many studies have suggested

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that SOCSs are inducible genes in mycobacterial infection, in part responsible for the inhibition of IFNγ responses in mycobacterial infection. Furthermore, the trehalose 6,6′-dimycolate (TDM; cord factor), a glycolipid associated with a *M. tuberculosis* cell wall (Guidry et al., 2007), induces expression of SOCS-1 and SOCS-3 genes as well as inhibiting phosphorylation of STAT-1 stimulated by IFN-γ in the cells (Imai et al., 2003).

Immune factors influencing progression to active TB remain poorly defined, and few studies have analyzed the immunopathological aspects of tuberculous lymphadenitis in HIV-seronegative patients. Therefore, in the present study we analyze the histological and immunohistochemical aspects of tuberculous lymph nodes in immunocompetent patients in order to elucidate possible associations between SOCS and some immune mediators in human tuberculous lymphadenitis.

## Materials and methods

### *Collection of lymph nodes*

Thirteen formalin-fixed, paraffin embedded archival lymph nodes were analyzed in this study, nine of which were TB positive based on clinical, bacteriological and histopathological findings as well as antituberculosis treatment response. All were collected from the Laboratory of Pathology of Evandro Chagas Clinical Research Institute, FIOCRUZ, Rio de Janeiro, Brazil. Four cases of benign reactive hyperplasia were obtained from the files of OSU Medical Center, USA and were adopted as controls. These lymph nodes were from the same anatomic region from patients in the same general age group. Additional data concerning clinical and laboratory results of these patients were obtained by reviewing clinic charts. HIV antibody testing was required for all patients after counseling. The study was approved by the Ethical Committee from Evandro Chagas Institute of Clinical Research, Fiocruz, in Rio de Janeiro, Brazil, and the institutional review board regulations of OSU Medical Center in USA.

Serial 5 μm sections were taken for Haematoxylin & Eosin (H&E), Grocott Methenamine Silver (GMS) and Ziehl-Neelsen staining for light microscopic assessment according to routine procedures, histopathologic diagnosis and immunoperoxidase analysis. All tissues were treated with the same exact method in regards to formalin fixation, paraffin embedding and sectioning.

### *Immunohistochemistry*

Immunohistochemistry reactions were performed on silane-coated slides (Sigma, St. Louis, MO, USA), dried overnight at 37 °C and then dewaxed, rehydrated and subsequently treated in hydrogen peroxide 3% in methanol for 10 min to eliminate endogenous peroxidase activity. The LSAB system HRP (Dakocytomation, Carpinteria, CA, USA) method was adopted for immunolabelling. Tissue sections were sequentially incubated for 1 h at room temperature with specific antibodies and dilution (Table 1), followed by the biotinylated link universal antibody and then the streptavidin–HRP conjugate for 30 min. Slides were washed three times in Tris pH 7.6 between each incubation step. Antibody binding was visualized with 3.3 diaminobenzidine (Sigma Chemical Co., St. Lois, MO, USA) at 5 mg/ml and 85 μl of hydrogen peroxide 0.3%. Finally slides were counterstained with hematoxylin, dehydrated and mounted in a resinous mountant (Merck, Darmstadt, Germany) for light microscopy. Negative controls included all but the primary antibody incubation.

**Table 1**
Antisera used for immunohistochemistry

| Antibody | Target cell type/cytokine | Source/Code | Dilution |
|---|---|---|---|
| CD 68 | Macrophage | Dakocytomation/M0718 | 1/100 |
| CD20 | B cells | Dakocytomation/M0755 | 1/100 |
| CD34 | Endothelial cells | Dakocytomation/M7165 | 1/100 |
| CD45RO | Leucocyte common antigen RO | Dakocytomation/M0742 | 1/100 |
| Antitrypsin | Serine protease inhibitor and trypsin inhibitor | Dakocytomation | Ready to use |
| Lysozyme | Cytoplasmic granules of the PMN | Dakocytomation | Ready to use |
| ICAM-1 | Intercellular adhesion molecule-1 | Santa Cruz-Sc-8439 | 1/1000 |
| MIP-1α | Macrophage inflammatory protein-1 α | Santa Cruz-Sc-1381 | 1/900 |
| RANTES | RANTES | Santa Cruz-SC-20731 | 1/70 |
| SOCS-1 | Suppressor of cytokine signaling -1 | Santa Cruz-SC-20731 | 1/70 |
| SOCS-3 | Suppressor of cytokine signaling -3 | Santa Cruz-SC-20731 | 1/70 |
| STAT-3 | Signal transducer and activator of transcription-3 | Santa Cruz-Sc-7179 | 1/70 |

The immunohistochemical staining for CD34 was evaluated by a semi-quantitative score ranging <+> to <++++>, considering both positive cell number and immunohistochemical reaction intensity.

The automated immunohistochemical system from Ventana Medical Systems (Benchmark) was employed for RANTES (dilution 1:50, protease pretreatment), macrophage inflammatory protein-alpha (MIP-1α) and IFN-γ detection (each 1:500, protease pretreatment) according to manufacturer recommendations.

### *Cell counts*

Microscopic analysis of the slides was independently performed by two investigators, without previous knowledge of the H&E evaluation. Digitalized photographs were taken from positive stains with a Nikon COOLPIX Camera DP12, storing images in a computer-based software (ImagePro 4.5) for histological analyses. Positive stained cells were counted in ten fields. Counts were achieved with a grid inserted into the lens (1 square cm divided into 10×10 of 1 mm squares) of 400× magnification, as previously published (Nicol et al., 2006).

### *In situ RT PCR for SOCS-1 and SOCS-3*

Detection of PCR-amplified for SOCS-1 and 3 mRNA was carried out as previously described by Nicol and Nuovo (2005). In brief, optimal protease digestion was first determined with a signal from the incorporation of the labeled nucleotide (digoxigenin dUTP) and its elimination by overnight DNase digestion. Negative controls for the TB infected tissues included lymph nodes from benign reactive hyperplasia cases. The application of irrelevant primers (HPV positive primers, as this virus cannot infect lymphoid cells) was also adopted as an additional negative control. In each case, no signal was evident. The primer pairs for the human mRNAs *in situ* RT PCR reactions were: SOCS-1, forward (5′-CACCTTCTTGGTGCGCGGACA-3′); and reverse (5′-GCAGCTCGAAAAGGCAGTCG-3′) as described by Kawazoe et al. (2001); and SOCS-3, forward (5′-TGCGCCTCAAGACCTTCAGC-3′), and reverse (5′-ACCAGCTTGAGCACGCAGTC-3′) as described by Dogusan et al. (2000). The RT step proceeded as follows: 65 °C for 30 min, followed by 94 °C for 3 min, and 20 cycles of 94 °C for 3 min, 60 °C for 1 min, 30 s and 72 °C for 30 s and an extension step at 72 °C for 7 min.

### *Statistical analysis*

Data analysis was carried out using the Statistical Package for the Social Sciences (SPSS version 13.0). Student *t* test was adopted to compare means of positive cells per field in the tissue. Levene's test for equality of variances were applied to investigate correlation to all immune markers in this study. Differences were considered significant at $p \leq 0.05$.

## Results

### *Clinical findings*

We analyzed lymph nodes of nine patients with tuberculous lymphadenitis. The average age of this group was 44.8 years (range of 34–73 years ± 13.7 SD). Seven of the 9 patients (78%) had positive tuberculin skin tests. The presence of acid fast bacilli consistent with M. tuberculosis was documented by Ziehl-Neelsen stain; as expected, few organisms were evident in a given tissue, and only three patients (3/9) were positive by Ziehl-Neelsen stain. Six of 9 TB patients had positive culture for *M. tuberculosis*; two patients the data were not available (the one patient with a negative culture had a positive Ziehl-Neelsen stain). Cervical lymph nodes were the most frequent sites (89%). All nine patients were HIV negative, normal with respect to chest radiography and responded to standard antituberculous treatment. Four control cases of benign reactive hyperplasia in the head and neck region, ranging in age from 15–45 years old (36.5 ± 14.3 SD), had enlarged lymph nodes for benign, noninfectious reasons. Absence of infection was determined after testing by either serology and/or *in situ* hybridization/immunohistochemistry for Human Immunodeficiency Virus (HIV-1), Cytomegalovirus (CMV), Epstein Barr-virus (EBV), Hepatitis C, bacterial and fungal infections. Follow up information demonstrated that each of these control patients had benign clinical courses with resolution of their adenopathy.

### *Histological findings*

Sections from all tuberculous lymphadenitis cases exhibited well-formed epithelioid granulomas with numerous Langhans type giant