BRIEF COMMUNICATION

Suppressor of cytokine signaling-1 and chemokine (C-X-C Motif) receptor 3 expressions are associated with caseous necrosis in granulomas from patients with tuberculous lymphadenitis

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Abstract We investigated the role of suppressor of cytokine signaling-1 (SOCS1) and SOCS3 molecules in lymph nodes from tuberculous lymphadenitis patients (LNTB). Fewer T cells were noted in LNTB cases, which also had raised chemokine (C-X-C motif) receptor 3 (CXCR3) levels. In addition, we observed a positive correlation between CXCR3 and SOCS1 expression. Our data suggest that upregulation of SOCS1 molecules may contribute to the dissemination of Mycobacterium tuberculosis from granulomas.

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Tuberculosis (TB) causes significant morbidity and mortality worldwide and is primarily a disease affecting the lungs, but extrapulmonary TB has also been commonly reported. Tuberculous lymphadenitis is the most common form of extrapulmonary TB. TB is caused by Mycobacterium tuberculosis (Mtbd), and alveolar macrophages provide the first line of defense against its infection. Activated T cells and macrophages are required for containment of Mtbd within granulomas, and cytokines [e.g., interferon-γ (IFN-γ), tumor necrosis factor-α, interleukin-12] and chemokines [C-X-C motif chemokine 9 (CXCL9) and CXCL10] play an important role in granuloma maintenance. These chemokines bind to chemokine (C-X-C motif) receptor 3
(CXCR3), which is responsible for migration, activation, and homing of T lymphocytes and macrophages to the site of infection, constituting a positive regulatory loop. Although granulomas can restrict the growth of Mtb within them, these bacilli may continue to remain in either an active or a latent state.

Granulomas consist of CD68+ macrophages, epithelioid cells that have a reduced phagocytic capacity, and multinucleated (or Langhan) giant cells, surrounded by a layer of lymphocytes. Granulomas can be cellular (sarcoid), associated with containment, or necrotic, which may favor both containment and bacterial growth. Solid cellular granulomas are encapsulated by fibrosis and upon encapsulation, such fibrotic granulomas will develop a caseous necrotic center. Fibrosis can be followed by calcification and cavitation, both of which allow for Mtb dissemination.

Suppressor of cytokine signaling (SOCS) molecules are a family of eight intracellular proteins that regulate cytokine responses through the Janus-activated kinases/signal transducers and activators of transcription pathway. Of these, SOCS1 and SOCS3 are the most widely studied. SOCS1 and SOCS3 levels have been shown to be upregulated in the peripheral blood cells of patients with TB. SOCS1 and SOCS3 are transducers and activators of transcription pathway, both of which allow for protective IFN-γ responses in both murine and human macrophages. During chronic infection, the regulation of immunopathology seems to be a preponderant role of SOCS1 and SOCS3.

We hypothesized that SOCS1 and SOCS3 participate in the regulation and maintenance of the TB granuloma. Thus, we investigated the expression of SOCS1 and SOCS3 in lymph node specimens from patients and healthy controls in relation to T cell and macrophage markers.

This study was approved by the Ethical Review Committee of The Aga Khan University, Karachi, Pakistan.

Prior to the initiation of therapy, lymph node biopsy specimens were collected from patients with tuberculous lymphadenitis (LNTB) admitted at The Aga Khan University Hospital. Diagnosis was made by acid-fast staining, radiology, and histological analysis showing granulomatous inflammation predictive of TB, or chemotherapeutic response to antituberculous therapy.

The patients were included only if their TB diagnosis was confirmed between the age of 15 years and 65 years. Patients with comorbid conditions (human immunodeficiency virus infection, diabetes mellitus, chronic renal failure, and chronic liver disease or corticosteroid therapy) were excluded.

Moderate caseous necrosis (m-LNTB, n = 13) was defined as a case in which the granulomatous lesion occupied < 75% of the total area. Extensive caseous necrosis (e-LNTB, n = 12) was defined as a case in which ≥ 75% of the granulomatous lesion area showed caseous necrosis. Lymph nodes in the m-LNTB group were from the cervical, omentum, and axillary region, whereas those in the e-LNTB group were from the left inguinal, mesenteric, cervical, and mediastinal lymph nodes. In the control group, lymph node from patients with reactive hyperplasia only (r-LN, n = 10), derived from cervical and axillary regions, was selected.

All lymph node sections were paraffin-embedded and stained with hematoxylin and eosin. Subsequently, immunostaining was performed using rabbit polyclonal anti-SOCS1 (ab62584), rabbit polyclonal anti-SOCS3 (ab16030), mouse monoclonal anti-CXCR3 (ab64714), mouse monoclonal anti-CD3 (ab17143), or mouse monoclonal anti-CD68 (ab783; Abcam, Boston, Massachusetts, USA). Stains were developed using the EnVision Dual Link system-HRP (Dako-Cytomation, Glostrup, Denmark) according to manufacturer's instruction and counterstained with hematoxylin. Slides were analyzed in a blinded manner, and photographed using a Leica photomicroscope. Positive cells were counted in three microscope fields using a grid. Results were expressed as the average number of positive cells per square millimeter area.

The expression of each molecule in the LNTB and r-LN groups was statistically compared using the Student t test and the correlation between the numbers of cells stained for different molecules was estimated by Spearman rank correlation coefficient using the GraphPad Prism software version 5 (GraphPad Software, Inc., San Diego, CA, USA).

We studied 25 tuberculous lymphadenitis (LNTB) biopsy specimens with m-LNTB or e-LNTB and eight cases of benign reactive hyperplasia (r-LN). In r-LN, there were germinal centers with a high density of lymphocytes and macrophages along the periphery of granuloma. The LNTB sections lacked these discrete germinal centers but contained granulomas (data not shown). The m-LNTB cases displayed granulomas with moderate necrosis, well-developed epithelioid cells, and a variable number of multinucleated or Langhan-type giant cells interspersed with lymphocytes and plasma cells. By contrast, the LNTB cases had granulomas with extensive caseating necrosis, fewer discrete granulomas containing epithelioid cells, and a central core with caseating necrosis as described previously.

A comparison of r-LN and LNTB cases indicated that CD3 staining was reduced in LNTB as compared with r-LN (Table 1). In r-LN cases, T cells were located in both the germinal centers and the peripheral regions of the granuloma (Figure 1A). In m-LNTB cases, T cells were present only in the periphery of granulomas (Figure 1B). In e-LNTB cases, the frequency of CD3+ cells was reduced in e-LNTB and there was increased caseous necrosis (Figure 1C), suggesting a negative association between T cells and caseous necrosis.

The CD68+ macrophages were interspersed within the lymph nodes in both r-LN and LNTB cases, with a comparable density (data not shown). SOCS1 expression was detected in the cytoplasm of cells in both r-LN and LNTB cases. In r-LN, SOCS1-positive cells were apparent in the germinal centers (Figure 1D). However, in m-LNTB (Figure 1E) and e-LNTB (Figure 1F) cases, SOCS1-positive cells were found in the peripheral tissue as well as in granulomatous lesions. This increase in the intensity of SOCS1 expression was found to be associated with caseous necrosis. Overall, the frequency of SOCS1-positive cells was slightly increased in m-LNTB and e-LNTB, compared with r-LN, but this difference did not reach statistical significance (Table 1).
SOCS3 expression was confined to the follicles in r-LN cases (Figure 1G). By contrast, in m-LNTB (Figure 1H) and e-LNTB (Figure 1I) cases, SOCS3-positive cells were located within the granulomas and based on CD3 and CD68 staining, they appeared to be colocalized with lymphocytes and macrophages. CXCR3-positive cells were interspersed within the tissue in both r-LN and LNTB cases. When healthy reactive and TB lymph nodes were compared, it appeared that the intensity of CXCR3-positive cells was lower in r-LN, but moderate to strong in LNTB. However, the density of CXCR3-positive cells was similar in both r-LN and LNTB cases (Table 1).

Table 1  Cells positive for SOCS1, SOCS3, CXCR3, CD3, and CD68 in lymph nodes from TB patients compared with normal reactive lymph nodes.

<table>
<thead>
<tr>
<th></th>
<th>r-LN (n = 10)</th>
<th>LNTB (n = 25)</th>
<th>m-LNTB (n = 13)</th>
<th>e-LNTB (n = 12)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Mean</td>
<td>Mean</td>
<td>Mean</td>
</tr>
<tr>
<td>CD3</td>
<td>34.0 ± 3.40</td>
<td>24.4 ± 2.04 *</td>
<td>21.2 ± 1.29 **</td>
<td>28.1 ± 1.29 ***</td>
</tr>
<tr>
<td>CD68</td>
<td>20.5 ± 1.01</td>
<td>21.4 ± 1.94</td>
<td>19.5 ± 2.31</td>
<td>24.7 ± 2.31</td>
</tr>
<tr>
<td>SOCS1</td>
<td>46.5 ± 22.07</td>
<td>54.5 ± 9.35</td>
<td>58.4 ± 16.60</td>
<td>50.7 ± 16.50</td>
</tr>
<tr>
<td>SOCS3</td>
<td>16.1 ± 2.90</td>
<td>21.9 ± 3.14</td>
<td>20.8 ± 4.68</td>
<td>24.1 ± 4.68</td>
</tr>
<tr>
<td>CXCR3</td>
<td>48.9 ± 17.71</td>
<td>33.5 ± 2.77</td>
<td>34.9 ± 4.15</td>
<td>31.8 ± 4.15</td>
</tr>
</tbody>
</table>

Data (mean) are presented for average number of cells positive for SOCS1, SOCS3, CXCR3, CD3, and CD68 staining counted in three high-power (40 × magnification) fields per millimeter area. Statistical analysis was performed using t test and p < 0.05 was considered significant.

Significant difference was observed between * LNTB and r-LN (p = 0.003), ** m-LNTB and r-LN (p = 0.003), and *** e-LNTB and r-LN (p = 0.004).

CXCR3 = chemokine (C-X-C motif) receptor 3; e-LNTB = lymph node of patients with tuberculous lymphadenitis with extensive caseation; LNTB = lymph nodes from patients with tuberculous lymphadenitis; m-LNTB = lymph node of patients with tuberculous lymphadenitis with moderate caseation; r-LN = reactive lymph nodes from control patients; SOCS = suppressor of cytokine signaling.

Figure 1. CD3, SOCS1, and SOCS3 protein expressions in lymph node biopsy specimens from patients with tuberculous lymphadenitis. Lymph node tissue specimens from control patients (r-LN) and tuberculous lymphadenitis patients (LNTB) with moderate (m-LNTB) and extensive caseation (e-LNTB) were stained with anti-CD3, anti-SOCS1, and anti-SOCS3 antibodies. Figures show representative immunohistochemical staining at 10× magnification for CD3: (A) r-LN, (B) m-LNTB, and (C) e-LNTB cases; SOCS1: (D) r-LN, (E) m-LNTB, and (F) e-LNTB; and SOCS3: (G) r-LN, (H) m-LNTB, and (I) e-LNTB cases. r-LN = reactive lymph nodes from control patients; SOCS = suppressor of cytokine signaling.
We further investigated the association between expression levels in different lymph node types. In r-LN controls, a strong positive correlation was observed between CXCR3 and CD68 expression ($\rho = 0.879$, $p = 0.002$). However, the correlation between the same markers was not significant in LNTB. Rather, in LNTB, a positive correlation was observed between CD3 and CD68 levels ($\rho = 0.720$, $p = 0.016$) and also between SOCS1 and CXCR3 ($\rho = 0.718$, $p = 0.016$) whereas the same was not apparent in r-LN cases.

Here, we describe the distribution and intensity of SOCS1 in relation to cells within the granuloma, and thus, further elucidate the mechanism by which SOCS1 molecules may regulate immune pathology at the site of mycobacterial infection.

The decreased T-cell frequencies in lymph nodes from patients with TB lymphadenitis compared with r-LN may be attributed to insufficient T-cell-mediated control during TB. A heterogeneous group of T cells including CD4$^+$ and CD8$^+$ effector as well as memory T cells showed increased expression of CXCR3 in contrast to naive precursors. CXCR3 is particularly expressed on Th1 cells. Hyper-responsiveness of chemokines and their receptors at the site of infection may cause exacerbated inflammatory responses, leading to tissue damage and necrosis, which may destabilize granulomas. Therefore, increased CXCR3 expression in LNTB with caseating lesions may lead to increased pathology at the site of infection.

IFN-\(\gamma\) produced by CD4 Th1 cells restricts mycobacterial infection. Our results suggest that an increase SOCS1 expression will hamper IFN-\(\gamma\) responses. Impaired cytokine responses have been associated with increased mycobacterial load.

Our data showing comparable expression of SOCS3 between LNTB and r-LN in lymph node tissues differ from reports that show increased SOCS3 messenger RNA expression in peripheral T cells from patients with TB. This difference may be attributed to differences in SOCS3 expression between circulating blood cells and local granuloma sites as well as to the fact that enlarged r-LN tissues occur as a consequence of inflammatory reaction, and therefore, are likely to have higher SOCS3 expression levels.

Overall, our data show that LNTB cases demonstrate a decrease in T-cell numbers in LNTB cases and a positive correlation between SOCS1 and CXCR3 expression levels. This suggests that SOCS1 may affect granuloma integrity and the extent of tissue necrosis, thereby influencing the outcome of Mycobacterium tuberculosis infection.

## Conflicts of interest

None declared.

## Acknowledgments

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## References