A purified protein from *Salmonella typhimurium* inhibits high-affinity interleukin-2 receptor expression on CTLL-2 cells

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**Abstract**

Previously, we have demonstrated that the immunosuppression induced by the purified substance *Salmonella typhimurium*-derived inhibitor of T-cell proliferation (STI) involves T-cell non-responsiveness to interleukin-2 (IL-2). In the present study, it was found that STI inhibited the growth of CTLL-2 cells, which are an IL-2-dependent cytotoxic T-cell line. Analysis of IL-2 receptor (IL-2R) function showed that STI inhibited high-affinity receptor expression and internalization by CTLL-2 cells. Furthermore, FACS analysis demonstrated that STI inhibited both β chain and γ chain expression of IL-2R on the cells. These results suggest that the suppression of T-cell proliferation induced by STI results from a defect in IL-2R function.

**Keywords:** *Salmonella typhimurium*; Immunosuppression; Interleukin-2 receptor; CTLL-2 cell

1. **Introduction**

*Salmonella typhimurium* is a facultative intracellular pathogen that causes systemic infection in mice [1]. The host defence in murine salmonellosis, a model system of human typhoid fever, involves both humoral and cellular immune responses [2,3]. Immunization with a sublethal dose of viable bacterial cells induces high levels of protection against virulent strains, although recent studies have demonstrated that such *Salmonella*-immunized mice show signs of profound immunosuppression, which is reflected by a greatly reduced ability of their spleen cells to respond to a T-cell-specific mitogen such as phytohaemagglutinin (PHA) [4]. Previously, we obtained the purified substance *S. typhimurium*-derived inhibitor of T-cell proliferation (STI) as a product which was considered to explain the immunosuppression of T-cells in *Salmonella*-infected mice [5]. STI was purified from the bacterial cell ultrasonicate, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis revealed that the purified active substance migrated as a single band corresponding to a molecular mass of 87 kDa. Furthermore, we found that the suppression of T-cell proliferation induced by STI involved a defect in the T-cell responsiveness to interleukin-2 (IL-2) [6]. In this study, we further analyzed the mechanisms of T-cell non-responsiveness to IL-2 using the murine T-cell line, CTLL-2. The results showed that STI inhibited IL-2-induced proliferation of T-cells by down-regulation of IL-2 receptor (IL-2R) function.
2. Materials and methods

2.1. Preparation of STI

STI was prepared from Salmonella typhimurium LT2. The LT2 cells were grown on L-agar for 18 h at 37°C, harvested, and then suspended in phosphate-buffered saline (PBS, pH 7.2). The whole-cell protein was collected as a filtrate (filter size, 0.22 µm) of bacterial cell ultrasonicates (Kubota INS 200 M; 9 kHz, 200 W, 20 min, 4°C) and separated into soluble (cytosolic) and insoluble (membrane) fractions by ultracentrifugation (150,000 × g for 30 min at 4°C). STI was purified from the soluble fraction as described previously [5].

2.2. Tissue culture medium and reagents

The tissue culture medium comprised RPMI 1640 (Nissui, Tokyo) supplemented with 10% (v/v) fetal calf serum (FCS; HyClone Laboratories, Logan, UT), 2 mM glutamine (Nissui), 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (Sigma Chemical Co., St. Louis, MO), penicillin (100 U/ml; Gibco, Grand Island, NY) and streptomycin (100 µg/ml; Gibco). Human recombinant IL-2 (rIL-2) was purchased from Genzyme, Cambridge, MA.

2.3. Proliferation assay

The murine CTLL-2 cells were cultured with 0.1–1.0 µg/ml STI in the tissue culture medium supplemented with human rIL-2 (20 U/ml). The IL-2-dependent growth of the cells was assayed in 96-well plates at 5 × 10³/well for 24, 48 or 72 h at 37°C in 5% (v/v) CO₂ and 95% (v/v) air. During the last 6 h of culture, 0.5 µCi [³H]thymidine were added to each well. The cells were harvested on glass fiber filters (Skatron, Inc., Sterling, VA) and the amount of incorporated radioactivity was determined by liquid scintillation counting.

2.4. IL-2 binding assay

CTLL-2 cells (1 × 10⁶/ml) were incubated for 24 h with 0.2 µg/ml STI in the tissue culture medium supplemented with human rIL-2 (20 U/ml), then washed twice with chilled culture medium and resuspended in chilled acidic culture medium (pH 3.0) for 30 s to remove cell-bound IL-2. The cells were washed once, then diluted to 1 × 10⁶ per 100 µl in microtubes and mixed with 100 µl of human recombinant 125I-IL-2 (DuPont, Wilmington, DE) serially diluted in culture medium. After shaking for 30 min and incubating for 1 h at 4°C, the cell suspensions were layered over 0.2 ml of binding oil consisting of 84% silicone oil and 16% paraffin oil in a microtube and centrifuged at 10,000 × g for 1 min. These preparations were frozen to −80°C and the tips of the tubes containing the cell pellets were cut off. The radioactivity present in the pellet and in the supernatant was counted in a gamma counter. Specific binding was calculated by subtracting nonspecific binding, assessed in the presence of a 200-fold molar excess of unlabelled IL-2, from the total binding. The calculated values for the number of binding sites per cell (capacity) and dissociation constants (K_d) were derived by Scatchard analysis.

2.5. Measurement of internalization

CTLL-2 cells (1 × 10⁶/ml) were treated for 24 h with 0.2 µg/ml STI in the tissue culture medium supplemented with human rIL-2 (20 U/ml) and the cell-bound IL-2 was removed as described above. Cells were washed once, then diluted to 1 × 10⁶ per 100 µl in microtubes and incubated in the presence of a near-saturating concentration of human recombinant 125I-IL-2 (100 pM for high-affinity IL-2 receptors on non-treated CTLL-2 cells or 300 pM for high-affinity IL-2 receptors on STI-treated CTLL-2 cells) for 1.5 h at 4°C as described above. The cells were washed twice with chilled culture medium, then incubated to initiate the internalization of 125I-IL-2 in culture medium at 37°C for 0–60 min. The cells were resuspended in chilled acidic culture medium (pH 3.0) for 10 s to remove cell-bound IL-2, then the cell suspensions were layered over 0.2 ml of binding oil in a microtube and centrifuged at 10,000 × g for 1 min. These preparations were frozen to −80°C and the tips of the tubes containing the cell pellets were cut off. The radioactivity present in the pellet and in the supernatant was counted in a gamma counter. The pellet fraction was considered to represent internalized IL-2.
2.6. Flow cytometry

CTLL-2 cells (1×10^6/ml) were incubated for 24 h with 0.2 μg/ml STI in the tissue culture medium supplemented with human rIL-2 (20 U/ml), then washed twice with phosphate-buffered saline (PBS). The cells were incubated on ice for 30 min with R-phycocerythrin-conjugated monoclonal rat anti-mouse IL-2Rα, IL-2Rβ or IL-2Rγ antibody (Pharmlingen, San Diego, CA), then washed twice with PBS. The stained cells were analyzed using a FACSort (Becton Dickinson).

2.7. Statistics

The data were expressed as means ± S.E.M. and differences between them were analyzed using Student’s t-test with a two-tailed test of significance [7]. Differences at P<0.05 were considered to be significant.

3. Results

3.1. Effect of STI on CTLL-2 cell growth

Our previous data showed that STI suppressed the IL-2-induced proliferation of murine splenic T-lym-

![Fig. 1. Effect of STI on CTLL-2 cell growth. CTLL-2 cells were cultured with human rIL-2 (20 U/ml), and subsequently treated with 0.1–1.0 μg/ml STI in a 96-well plate at 37°C for 24 h. During the last 6 h of culture, 0.5 μCi [³H]thymidine were added to each well. Results are expressed as the mean counts/min of [³H]thymidine incorporation ± S.E.M. (n = 5).](https://academic.oup.com/femspd/article-abstract/17/3/155/486850)

![Fig. 2. Time course of inhibition of CTLL-2 growth by STI. CTLL-2 cells were cultured with human rIL-2 (20 U/ml) and 0.2 μg/ml STI in a 96-well plate at 37°C for 24, 48 or 72 h. During the last 6 h of culture, 0.5 μCi [³H]thymidine were added to each well. Results are expressed as percentage of inhibition relative to the control (n = 5). The counts/min for the non-STI-treated cultures were 28,956 ± 1,756 (24 h), 42,933 ± 2,855 (48 h) and 57,437 ± 3,758 (72 h), respectively.](https://academic.oup.com/femspd/article-abstract/17/3/155/486850)
3.2. STI down-regulates binding affinity of the high-affinity IL-2 receptor and internalization of IL-2 in CTLL-2 cells

To investigate the effect of STI on the function of cell surface IL-2R, CTLL-2 cells were incubated with 0.2 μg/ml STI and 20 U/ml human rIL-2 for 24 h and then tested for the number of high- and low-affinity IL-2 binding sites using an IL-2R competitive binding assay. Results of binding studies with $^{125}$I-IL-2 in CTLL-2 cells are shown in Fig. 3. Scatchard analysis gave a curvilinear profile, suggesting two types of receptors: high affinity (5,935 site/cell; $K_d = 58.2$ pM) and low affinity (49,400 site/cell; $K_d = 3.1$ nM). However, when CTLL-2 cells were treated with 0.2 μg/ml STI at 37°C for 24 h, binding affinity and the number of high-affinity receptors, but not that of low-affinity receptors, were decreased (4,328 site/cell; $K_d = 149.2$ pM). Measurement of internalization showed that cell-bound IL-2 which was bound through high-affinity IL-2 receptors was internalized as early as 5 min and reached a maximum after 40 min (Fig. 4A). However, the internalized IL-2 was also lower in CTLL-2 cells treated with 0.2 μg/ml STI at 37°C for 24 h (Fig. 4B). These results

![Scatchard analysis of $^{125}$I-IL-2 binding to CTLL-2 cells cultured with STI. Cells were incubated with IL-2 and either medium or 0.2 μg/ml STI for 24 h. Following incubation, the cells were washed, divided into aliquots, and incubated for 1.5 h at 4°C in various concentrations of $^{125}$I-labelled and unlabelled IL-2. The radioactivity present in the cell pellet and in the supernatant was counted in a gamma counter, and subsequently specific binding of $^{125}$I-IL-2 was calculated.](https://academic.oup.com/femspd/article-abstract/17/3/155/486850)

![Influence of STI on internalization of IL-2 in CTLL-2 cells. Cells were incubated with IL-2 and either medium or 0.2 μg/ml STI for 24 h. Following incubation, the cells were washed, divided into aliquots, and incubated for 2 h at 4°C in the presence of 100 pM or 300 pM $^{125}$I-IL-2. The cells were then washed, incubated at 37°C for 0-60 min, and resuspended in chilled acidic culture medium (pH 3.0) for 10 s. The radioactivity present in the cell pellet and in the supernatant was counted in a gamma counter. The cell pellet fraction represents internalized IL-2.](https://academic.oup.com/femspd/article-abstract/17/3/155/486850)
suggest that STI can specifically down-regulate the binding affinity and number of high-affinity IL-2R on T-cells.

3.3. Influence of STI on IL-2R expression in CTLL-2 cells

The above results suggested that STI treatment inhibited the growth of CTLL-2 cells by decreasing the expression of high-affinity IL-2R. To gain information about each chain of the high-affinity IL-2R (α/β/γ heterotrimers), we examined the effect of STI on surface expression of IL-2Rα, -β and -γ chains by flow cytometry. CTLL-2 cells express homeostatically the α, β and γ chains of IL-2R on their surface, as shown in Fig. 5. However, when CTLL-2 cells were treated with 0.2 μg/ml STI at 37°C for 24 h, the expression of IL-2Rβ and IL-2Rγ was suppressed. In particular, the expression of IL-2Rγ showed significant disappearance from the surface of CTLL-2 cells. On the other hand, IL-2Rα expression was slightly augmented rather than decreased.

4. Discussion

Previously, we have demonstrated that the immunosuppression induced by the purified substance Salmonella typhimurium-derived inhibitor of T-cell proliferation (STI) involves T-cell non-responsiveness to IL-2 [6]. In this study, the mechanisms of T-cell non-responsiveness to IL-2 were analyzed using a murine T-cell line, CTLL-2 cells. The IL-2-dependent growth of CTLL-2 cells was significantly inhibited by treatment with STI in a dose-dependent manner. These results indicated that STI might down-regulate the function of IL-2R in CTLL-2 cells.

The interaction of IL-2 and IL-2 receptors regulates the magnitude and duration of the T-cell immune response [8]. Different combinations of three
distinct chains (α, β and γ) form three classes of IL-2 receptors [8]. Low-affinity IL-2R consist of IL-2Rα, intermediate-affinity receptors include IL-2Rβ and IL-2Rγ, and high-affinity receptors contain all three chains. Recent studies have demonstrated that only high-affinity and intermediate-affinity IL-2R can be internalized after ligand binding, and that only internalized receptors are capable of transducing the signals necessary for T-cell activation [9,10]. Thus, IL-2Rγ is a component of functional intermediate- and high-affinity receptors [11]. Analysis of the functions of IL-2 receptors showed that the number and binding affinity of high-affinity IL-2 receptors on CTLL-2 cells were decreased by STI treatment. This observation demonstrated that STI could down-regulate both the number and binding affinity of high-affinity IL-2 receptors on T-cells. Furthermore, our study showed that STI also inhibited the internalization of IL-2 on CTLL-2 cells. We therefore hypothesized that STI might inhibit the expression of IL-2Rγ on CTLL-2 cells.

The results of FACS analysis using monoclonal anti-IL-2Rα, -β and -γ antibodies demonstrated that STI inhibited the expression of both IL-2Rβ and IL-2Rγ on CTLL-2 cells. In particular, the expression of IL-2Rγ was significantly inhibited. This was a reasonable result, since down-regulation of high-affinity receptors was demonstrated by Scatchard analysis. Also, the results of FACS analysis showed that STI slightly augmented IL-2Rα expression rather than inhibiting it. A similar phenomenon has been observed in splenic T-lymphocytes from Salmonella-infected mice [12] and in murine splenic T-lymphocytes treated with STI [5]. Our further experiments showed that the above augmentation of IL-2Rα would not appear to be an important mechanism responsible for Salmonella infection-induced or STI treatment-induced inhibition of T-cell proliferation [6]. Although the role of augmentation of IL-2Rα expression is unknown, our data demonstrate that STI inhibits the growth of CTLL-2 by inhibiting IL-2Rβ and γ expression. Especially, since the expression of IL-2Rγ significantly disappeared from the surface of CTLL-2 cells, this would induce a decrease in the binding affinity of high-affinity receptors and inhibit the internalization of IL-2 through high-affinity receptors. Furthermore, this down-regulation of IL-2Rγ would result in a decrease of high-affinity receptors. Therefore, we feel that the inhibition of IL-2Rγ plays a key role in the T-cell suppression induced by STI. The effect of STI on the expression of IL-2Rγ mRNA in CTLL-2 cells is currently under investigation.

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References