Transforming growth factor-β negatively modulates T-cell responses in sepsis

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Abstract Sepsis is associated with depressed T-cell functions and increased circulating levels of immunosuppressive agents. TGF-β is a potential anti-inflammatory cytokine that can modify T-cell growth and differentiation. The up-regulation of TGF-β and the mechanism of its action on the T-cells during septic injury have not been resolved. We hypothesized that in sepsis TGF-β produced by macrophages acts on T-cells in a paracrine manner to suppress interleukin (IL)-2 production and proliferation. In this study, we examined the circulating TGF-β levels in a rat model of Gram-negative bacterial sepsis, and compared the abilities of adherent and non-adherent splenocytes to produce TGF-β. Additionally, we investigated the causal relationships of hrTGF-β to concanavalin A (ConA)-induced T-cell responses and the intracellular mechanism of the generation of these responses in normal splenic rat T-cells. Sepsis was induced in rats by intra-abdominally implanting fecal pellets containing *Escherichia coli* (150 CFU) and *Bacteroides fragilis* (10 000 CFU). Adherent and non-adherent splenocytes were isolated by differential adherence using Ficoll gradient centrifugation. T-cells were purified by use of Nylon wool columns. We observed a 3–6-fold increase in the circulating levels of TGF-β in sepsis. Western blots and ELISA determinations revealed a 2.5–3-fold increase in cell-associated TGF-β protein levels in adherent splenic cells. Northern analyses also showed a marked increase in TGF-β mRNA expression in adherent cells during sepsis. On the other hand, a significant change was not observed in the TGF-β protein and mRNA expression in non-adherent splenocytes. Pretreatment of control rat T-cells with hrTGF-β decreased both ConA-induced proliferation (by 35–40%) and IL-2 mRNA expression (by >50%). Further, whereas incubation of control rat T-cells with either ConA or TGF-β for 24 h resulted in a 10–15-fold increase in cAMP generation, the addition of hrTGF-β along with ConA resulted in a 50–60-fold increase in cAMP. These results suggest that in sepsis, TGF-β produced by splenic macrophages can act in a paracrine manner on T-cells to depress their IL-2 mRNA expression, IL-2 production and proliferation after up-regulation of cAMP which can interfere with T-cell signaling for proliferation.

Key words: Rat; Gram-negative infection; T-lymphocyte; Macrophage; Adherent cell; Cell proliferation; IL-2 mRNA; IL-2 production; Cell signaling; Cyclic AMP

1. Introduction

Severe trauma and sepsis are associated with depressed T-cell functions and increased levels of putative immunosuppressive agents such as PGE_2, IL-4 and IL-10 [1–4]. The immunosuppressive agents are released by macrophages/monocytes [5] and certain T-cells [6], and could adversely affect cell-mediated immunity via interference with the T-cell receptor-mediated early signal transduction steps [7,8]. Previous studies from our laboratory indicated that the sepsis-related decrease in the T-cell proliferative response could result from an alteration in $Ca^{2+}{}_i$ mobilization in these cells [9]. Furthermore, our recent studies have ascertained a potential role of decreased Ca^{2+} influx in the PGE_2-induced T-cell proliferative suppression in sepsis [10].

Like PGE_2, TGF-β might also effect a decrease in cell-mediated immunity during sepsis [7]. An elevation in circulating TGF-β has been correlated with a depression in splenocyte IL-2 release in trauma patients [11,12] and in a murine cecal ligation and puncture model of sepsis [13,14]. Further evidence of a role of TGF-β in sepsis-related suppression in immune function comes from experiments which show that addition of human TGF-β to human trauma patients' monocytes results in a significant elevation of PGE_2 levels [11]. The factors contributing to elevated levels of TGF-β in sepsis and the mechanism of TGF-β-related T-cell suppression are not clearly understood. We hypothesized that in sepsis TGF-β produced by activated macrophages contributes to its elevated circulating levels and that in the spleen it may act in a paracrine manner on T-cells to negatively modulate both their IL-2 production and proliferation. Therefore, along with ascertaining the circulating TGF-β levels in sepsis, in this study, we compared the ability of adherent splenocytes isolated from septic and non-septic rats to produce TGF-β. Additionally, we tested the ability of rTGF-β to modulate T-cell signaling events via cyclic AMP (cAMP) generation after their stimulation with concanavalin A (ConA). The generation of cAMP was correlated with changes in T-cell IL-2 mRNA expression and proliferative responses.

2. Experimental procedures

2.1. Rat model of sepsis

Male Sprague-Dawley rats (250–275 g) obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN) were used. Sepsis was produced in rats by implanting into their abdominal cavities fecal pellets (1 ml) impregnated with Gram-negative bacteria, *Escherichia coli* (150 colony-forming unit (CFU)) and *Bacteroides fragilis* (10^4 CFU). The rats implanted with pellets without the bacteria are referred to as 'sterile-implanted'. Details of this rat model of Gram-negative bacterial sepsis have been described previously [15–17].

2.2. Preparation of rat splenic cells

Rats were killed and their spleens were aseptically removed and processed to obtain T-cells as described previously [9]. Briefly, spleens were gently ground to prepare single-cell suspensions, which were then subjected to density gradient centrifugation with Ficoll-Paque (Pharmacia, Sweden) to remove red blood cells and dead cells. Splenocytes appearing at the interphase of Ficoll and HBSS medium (Hank's

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balanced salt solution) were collected. Splenocytes were then cultured for 2–3 h in RPMI-1640 medium (complete with L-glutamine (2 mM), 2-mercaptoethanol (50 μM), HEPES (10 mM), gentamicin (50 μg/ml) and fetal calf serum (10%) in plastic Petrie dishes to separate adherent (macrophage-rich) cells from non-adherent cells. For the isolation of T-cells, splenocytes were passed through a Nylon wool column pre-equilibrated with HBSS supplemented with 10 mM HEPES (*N*-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid), 5% fetal calf serum, and 50 μg gentamicin/ml. After 1 h incubation of the columns containing cells at 37°C, T-cells were obtained by eluting the columns with 30–35 ml of warm HBSS at a flow rate 1 drop/s. T-cells were washed and resuspended in RPMI-1640 (with complete medium) for further use.

2.3. TGF-β determinations in rat plasma and spleen cells

Blood was drawn into EDTA-coated tubes via cardiac puncture from the non-operated control, sterile- and septic-implanted rats at 24 and 48 h, and platelet-free plasma separated and stored at −70°C until use. Acid-activated plasma TGF-β levels were measured by ELISA (R&D Systems, Minneapolis, MN) which is specific for the determination of the TGF-β_1 isoform. The assay was sensitive enough to detect a minimum of 5 pg/ml of TGF-β standard in the ELISA protocol.

Approximately 10×10^6 adherent or non-adherent cells were lysed with lysis buffer followed by cell lysate acid activation for the quantification of TGF-β with ELISA.

2.4. Determination of T-cell proliferation

The method used for the determination of T-cell proliferation was the same as described recently [9]. Briefly, T-cells suspended in complete RPMI-1640 medium and were cultured at a density of 5×10^5 cells per well in a 96-well plate at 37°C under 5% CO_2 in air in the presence or absence of ConA (5 μg/ml; Sigma Chem. Co., St. Louis, MO). After 66 h culture, a trace quantity of [^{3}H]thymidine was added to each well, and cultures were maintained for an additional 6 h. Cells were harvested on glass fiber filter strips using a 24-well PHD Cell Harvester (Cambridge Technology, Cambridge, MA), and thymidine radioactivity was determined in a liquid scintillation counter. The proliferative response was expressed as radioactivity (disintegrations per minute, DPM) incorporated in the cells.

2.5. Cyclic AMP assay

cAMP levels of rat splenic T-cells incubated in the absence or presence of TGF-β (hrTGF-β_1, Collaborative Biomedical Products, Bedford, MA) and/or ConA were measured using the cAMP[^{125}I] radioimmunoassay (RIA) kit (Advanced Magnetics, Inc., Cambridge, MA). To extract cAMP, T-cells (10×10^6/ml, pre-washed in ice-cold PBS) were treated with 1 ml of 95% ethanol/5% 0.1 N HCl (pH 3.0) for 2 h at 4°C. The resulting cAMP-containing supernatant was lypophilized to powder by speed-vac and re-dissolved in a cAMP assay buffer (100 mM Tris-HCl, containing 2.6 mM EDTA and 10 mM theophylline). A cAMP standard curve was constructed using cAMP in a concentration range of 0.25–50.0 pmol/ml.

2.6. Determination of TGF-β protein levels by Western analyses

Adherent and non-adherent splenocytes (10×10^6 cells) from the control, sterile and septic rats on days 1 and 2, post-implantation, were lysed with the lysis buffer, and the total protein content of the lysates were determined by the dye-binding method [18]. Aliquots of equal amount of lysate proteins (usually 10 μg) from the various experimental groups were electrophoresed and transferred to nitrocellulose membranes and blocked. Using Western blot analysis (Bio-Rad, Richmond, CA), the membranes were probed with anti-TGF-β_1 polyclonal antibody (Promega, Madison, WI) to determine the cell-associated TGF-β protein levels.

2.7. RNA preparation and Northern blot analyses

Total RNA from $100–150\times10^6$ adherent and non-adherent splenocytes as well as from the Nylon wool-purified T-cells of various group of animals (treated with ConA for 6 h, and/or TGF-β for 2 h) was isolated using isopycnic centrifugation over a cesium chloride gradient [19]. All the RNA sample preparations had A_{260} and A_{280} ratio greater than 2.0. Aliquots containing equal amounts of RNA (usually 20 μg) were electrophoretically separated on a formaldehyde–agarose gel (1%). RNAs were blot transferred to Nytran membrane (Northern transfer), and UV-cross linked. To detect the IL-2 and TGF-β mRNA expressions, the membranes were prehybridized overnight in an appropriate pre-hybridization solution and then hybridized with ^{32}P-labeled cDNA probes of rat IL-2 (400 bp fragment in PCR TMII vector; kindly supplied by Dr. Jersey Kupiec-Wiglinski, Harvard Medical School, Boston, MA) and TGF-β (985 bp fragment in pBluescript 2KS+ vector; obtained from Dr. G.L. Engelmann of our institution) at 42°C, overnight. The hybridized filter-membranes were washed, and then autoradiographed by exposing the X-ray films at −70°C, overnight or as appropriate.

Uniform RNA loadings were ascertained by stripping and re-probing the same membrane blots with ^{32}P-labeled β-actin cDNA probe in the same manner. The relative intensity of each band of the mRNA expression was quantitated by means of densitometry. Data were statistically analyzed using ANOVA.

3. Results

Fig. 1 shows plasma TGF-β levels in septic and non-septic rats. The circulating TGF-β levels in septic-implanted rats were significantly higher on days 1 and 2 compared to the controls (day 0) and corresponding sterile groups on days 1 and 2. Compared to controls, the septic animals' TGF-β levels were 3-fold higher on day 1 and more than 6-fold on day 2. Although, compared to controls, there was no significant change in the sterile rat group on day 1, there was a 3-fold increase in the TGF-β level in sterile rats on day 2.

The TGF-β protein contents determined by Western analyses of the adherent splenocytes from control, sterile and septic rats are shown in Fig. 2. A representative immunoblot is shown in Fig. 2A, and the average values obtained after densitometry of the immunoblots are shown in Fig. 2B. During sepsis on day 2, there was an approximately 2.7-fold increase in cell-associated TGF-β protein level compared to the controls. No significant changes were observed between the septic and sterile groups on day 1, or between the controls (day 0) and the two experimental groups on day 1. Also, no significant change was observed in TGF-β protein levels in the day 2 sterile groups compared to the control or the day 1 experimental groups. TGF-β protein levels in the non-adherent sple-

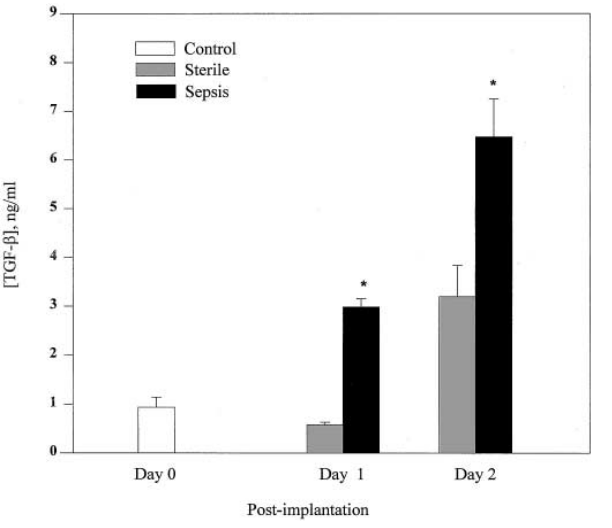

Fig. 1. Plasma levels of TGF-β in control, sterile and septic rats on days 1 and 2 after implantations. Values are means (±SE) for at least seven animals in each group. *$P<0.01$ sterile versus septic groups on each day.

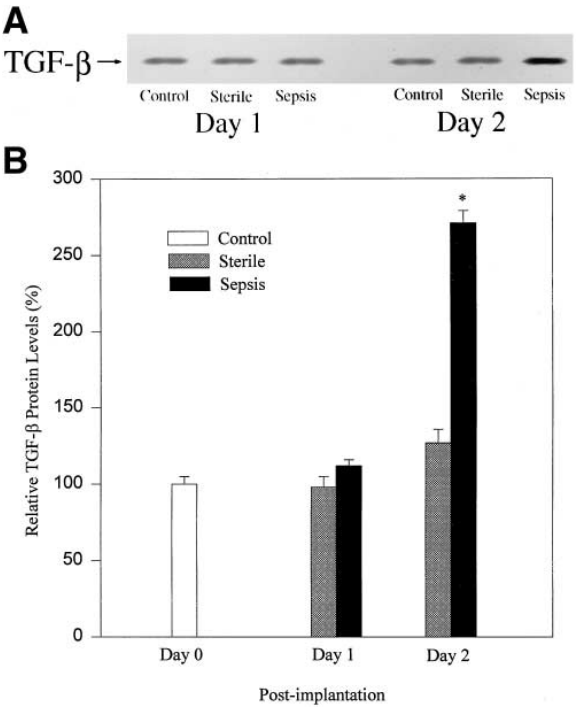

Fig. 2. A: Western blot detection of TGF-β from adherent splenic cells of control, sterile and septic rats on days 1 and 2, post-implantation. The blot is representative of experimental observations in four different animals. B: Densitometric quantitation of Western blot analyses of adherent cells from the experimental group of rats on days 1 and 2. The values obtained in the control rats were taken as 100%. Values are mean (±SE) for four animals in each group. $^{*}P < 0.01$ control or sterile versus sepsis on day 2, post-implantation.

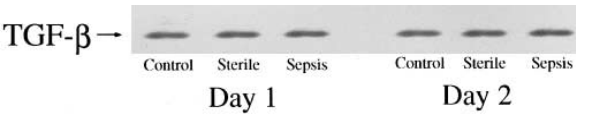

Fig. 3. Western blot detection of TGF-β from non-adherent splenic cells of control, sterile and septic rats on days 1 and 2, post-implantation. The blot is a representative of four similar experiments. No significant differences were noted among the experimental groups on either time points.

nocytes were not significantly different in either the septic or sterile groups on days 1 and 2 from those in controls (Fig. 3).

Cell-associated TGF-β determinations using ELISA (Table 1) showed quantitative changes in the adherent and non-adherent splenocytes from sterile and septic rats compared to controls. On day 1 after the implantations, there was no significant change in TGF-β in the sterile group non-adherent or adherent cells compared to controls. However, on day 2, TGF-β levels in both the adherent and non-adherent cells from sterile-implanted rats were 35–40% higher compared to the respective controls. A comparable increase in TGF-β was found in both the non-adherent and adherent cells of the septic rats on day 1 compared to the controls. In contrast, whereas the septic rat non-adherent cells showed only a 60% increase on day 2, the septic rat adherent cells of the septic rat, the septic rat adherent cell TGF-β contents were about 3-fold higher compared to controls. The TGF-β ELISA evidently provided for a more sensitive and quantitative determination than that using Western analyses. Whereas Western analyses failed to show effects of sterile implantation on days 1 and 2, or of the septic implantation on day 1 on the TGF-β contents of non-adherent or adherent splenocytes compared to controls, ELISA determinations showed 35–60% elevations in cell-associated TGF-β levels in these groups. However, it should be noted that the substantially greater effect of sepsis on day 2 in the adherent cell TGF-β levels than in the non-adherent cells was clearly demonstrateable with both Western analysis and ELISA. These results support that a distinct day 2-sepsis-related increase in TGF-β protein expression occurs in the macrophage-rich adherent splenocyte population and that a relatively modest increase occurs in the sterile and the day 1-sepsis group adherent as well as non-adherent cells. The modest increases in TGF-β levels may represent effect of non-septic inflammatory responses on the macrophages and a contamination of non-adherent cells with macrophage products.

Because both the circulating as well as the cell-associated TGF-β protein levels were found to be elevated in the septic animals on day 2 (post-implantation) relative to on day 1, the assessments of TGF-β mRNA expressions in adherent and non-adherent splenic cells from sterile and septic animals were carried out on day 2 only. Fig. 4A is a representative ^{32}P-labeled Northern blot showing a marked increase in TGF-β mRNA expression in adherent splenocytes from septic rats compared to the control and sterile rat groups. The densitometric analyses of these blots showed an approximately 1.8-fold increase in the sterile group, and a 3.5-fold increase in mRNA abundance in the septic group of animals (Fig. 4B). Unlike the changes in TGF-β mRNA expressions in the adherent splenic cells, no significant differences were noted in the mRNA abundance in the non-adherent splenic cells from the septic and non-septic animals (Fig. 5A,B).

To investigate causal relationships between TGF-β and

Table 1
Effect of sepsis on non-adherent and adherent splenocyte TGF-β contents (pg/10^6 cells) as determined by ELISA

Cell types/days-post-implant	Animal groups		
	Control	Sterile	Septic
Non-adherent cells			
Day 0	161.6 ± 7.1	–	–
Day 1	–	177.1 ± 16.4	236.3 ± 12.1
Day 2	–	223.1 ± 11.3	259.6 ± 6.6
Adherent cells			
Day 0	164.5 ± 7.3	–	–
Day 1	–	190.8 ± 14.9	229.3 ± 16.7
Day 2	–	232.2 ± 11.1	474.8 ± 19.6

Values are means (±SE) of 3–4 unoperated control (Control), sterile-implanted (Sterile), or septic implanted (Septic) rats.

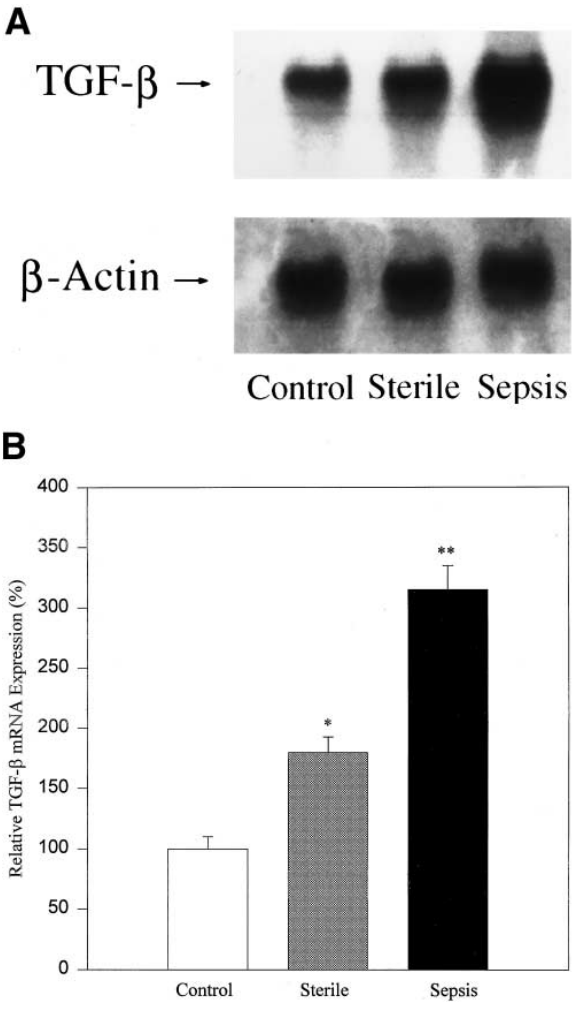

Fig. 4. A: ^{32}P-labeled Northern blot detection of TGF-β mRNA expression in adherent cells obtained from control, sterile and septic rats on day 2, post-implantation. The blot is representative of four experimental observations. B: Densitometric analyses of the four such Northern blots of TGF-β mRNA expressions in adherent cells. The values of the mRNA abundance computed in the controls were taken as 100%. Values are mean (±SE) for four experiments in each group. $^{*}P<0.05$, control versus sterile or sterile versus sepsis; $^{**}P<0.01$, control versus sepsis.

T-cell proliferation, lymphokine production and signaling, we studied the effect of exogenously added hrTGF-β on control rat T-cell proliferative response to ConA, and ConA-mediated IL-2 mRNA expression, in vitro. The proliferative responses of T-cells to ConA were assessed in the presence of different doses of rTGF-β and its absence as shown in Fig. 6. At 72 h of incubation, the ConA-mediated T-cell proliferative responses were significantly depressed (35–40% decrease, $P<0.05$) in the presence of TGF-β at concentrations of 2.5 and 25.0 ng/ml. Since 2.5 ng/ml TGF-β concentration was sufficient to inhibit the T-cell proliferative response, we chose to use this concentration for the experiments on the effect of TGF-β on T-cell IL-2 mRNA expression and the generation of cyclic AMP.

The effect of hrTGF-β (2.5 ng/ml) on T-cell cAMP generation is shown in Fig. 7. It is clear from the figure that a significant increase (10–15-fold, $P<0.001$) in cAMP levels was observed in T-cells exposed to ConA or rTGF-β (compared to the control) for 24 h, but not in the cells exposed for only 2 or 8 h. The addition of hrTGF-β along with ConA resulted in a much greater increase in cAMP generation (50–60-fold, $P<0.0001$) after 24 h incubation of T-cells (Fig. 7).

Because of the marked increases in ConA-induced cAMP generation by TGF-β after the incubation of T-cells for 24 h, the effect of TGF-β on the lymphokine (IL-2) production was also examined at this time point. Fig. 8A showing a Northern blot of the ^{32}P-labeled IL-2 mRNA expression level in normal rat splenic T-cells. The relative percentage of the IL-2 mRNA expression as quantitated by densitometry is shown in Fig. 8B. Data suggest that pre-treatment of control rat T-cells with rTGF-β (2.5 ng/ml) leads to a decrease in ConA-induced IL-2 mRNA expression by more than 50% ($P<0.001$).

4. Discussion

The present study demonstrated significantly elevated levels of circulating TGF-β in septic rats, and correlatable changes

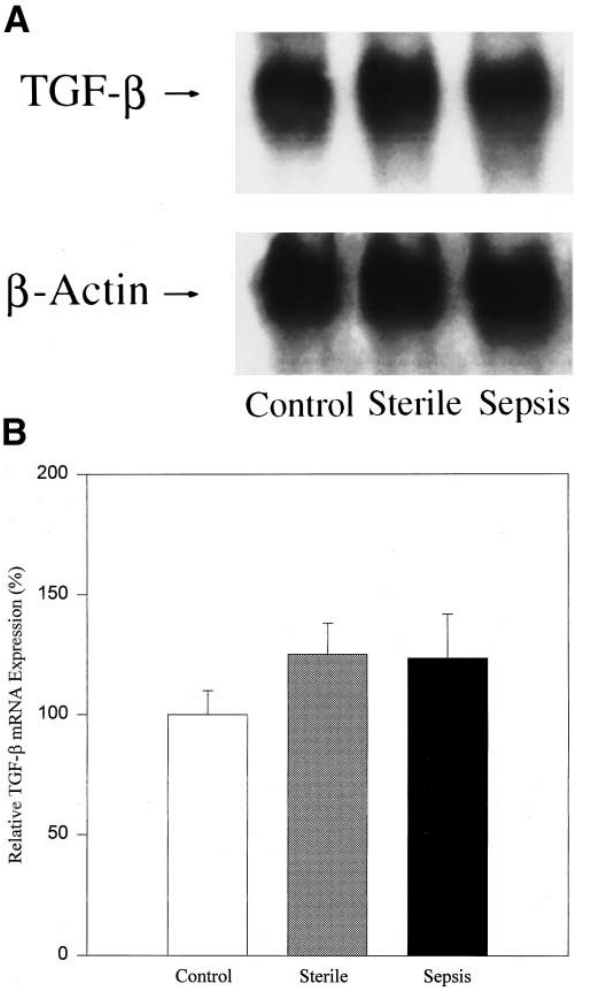

Fig. 5. A: Northern blot of TGF-β mRNA expression in non-adherent splenic cells from the control, sterile and septic rats on day 2, post-implantation. The blot is representative of observations in four different animals. B: Densitometric quantitation of four such blots of TGF-β mRNA expressions in non-adherent cells. The values taken as 100% were from the mRNA expression detected in the control animals. Values are mean (±SE) for four animals in each group. No significant differences ($P>0.05$) were observed among the experimental groups.

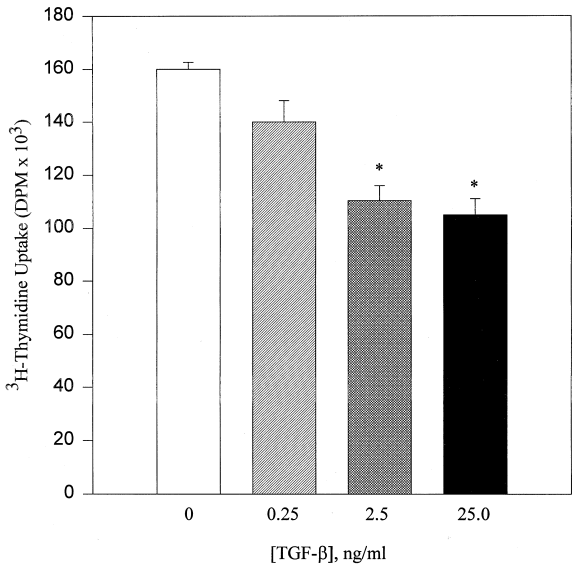

Fig. 6. Effect of varying concentration of hrTGF-β on ConA-induced T-cell proliferative response in control rats. Values are means (±SE) for six animals. $^*P<0.01$, T-cells incubated with rTGF-β versus T-cells without rTGF-β.

in the levels of TGF-β protein and mRNA expressions in adherent splenocytes. Some of these observations are in keeping with the previous studies on highly in vivo activated macrophages from trauma patients [11], and in a murine cecal ligation and puncture model of polymicrobial sepsis [14]. Miller-Graziano et al. [11] showed that following the injury, patients who were immunosuppressed and would typically die due to septic complications possessed blood monocytes that exhibited an enhanced capacity to produce TGF-β. Other studies [20–23] have indicated that the enhanced TGF-β production under injury conditions is related with the increased release of another potent immunosuppressant such as PGE_2. TGF-β can act as a stimulus for prostanoid release which in turn could contribute to immunosuppression [11,24]. Thus, it is clear that the release of TGF-β in sepsis could play a role in the network of events that lead to host immunosuppression.

Although not of the same magnitude as found in the septic rats, the observed increase in circulating TGF-β levels in the sterile-implanted animals on day 2, but not on day 1, could be due to the surgical stress to animals because simple laparotomy in itself can affect immune functions [25]. While no substantial alterations could be observed during sepsis in non-adherent splenic cells in terms of TGF-β protein contents on days 1 or 2, we observed an approximately 2.5–3.0-fold increase in TGF-β protein levels in adherent cells of septic rats compared to the controls or sterile groups on day 2 post-implantation. Similarly, we observed an overexpression of TGF-β mRNA abundance in adherent splenic cells in sepsis on day 2 without any significant changes in non-adherent cells of the experimental groups. The observed overexpression of TGF-β mRNA abundance in adherent splenic (macrophage-enriched) cells in sepsis on day 2, but not in non-adherent splenic cells could be due to a transcriptional up-regulation of TGF-β in sepsis. The ability of adherent cells to produce greater quantities of TGF-β protein as well as its mRNA after the onset of sepsis implies that TGF-β released from tissues such as spleen could be the source of circulating TGF-β in septic animals. We can not, however, rule out the possibility of release of TGF-β into plasma from other sources such as macrophages and monocytes.

Several studies have suggested the inhibitory effect of TGF-β on T-cell proliferation in clinical and experimental injury conditions such as trauma, burn and hemorrhagic shock [11,14,26,27]. However, to date, there is little information available on the potential mechanisms of action of TGF-β on T-cells. In our studies, we investigated through in vitro experiments the causal relationships of TGF-β to T-cell responses and the intracellular mechanism of the generation of these responses. Our hypothesis has been that in sepsis, TGF-β produced by splenic macrophages acts on adjacent T-cells to modulate their IL-2 mRNA expression, IL-2 production and proliferation after affecting T-cell signaling events that are responsible for the elicitation of these responses. Our results on the capacity of rTGF-β to modulate ConA-induced T-cell proliferation and IL-2 mRNA expression and to augment cAMP generation ascertained that this anti-inflammatory cytokine does play a role during the development of T-cell suppression in the course of sepsis. Our results support such a role of TGF-β via its paracrine effect on the T-cells within the spleen.

An elevation in cAMP production in T-cells subsequent to the action of PGE_2 on these cells is known to effect a decrease in T-cell activation, IL-2 production and proliferation [28–31]. Previous studies from out laboratory have shown an involvement of PGE_2 in the alteration of T-cell $Ca^{2+}{}_i$ signaling which leads to suppressed T-cell response in sepsis [10]. The present study showing the direct effect of rTGF-β in enhancing T-cell cAMP levels suggests that like PGE_2, TGF-β also effects T-cell cAMP accumulation to cause the suppression of the proliferative response in these cells. PGE_2 has been shown to down-regulate the *IL-2* gene transcription by interfering with $Ca^{2+}{}_i$-mediated activation of calcineurin [32]. We spec-

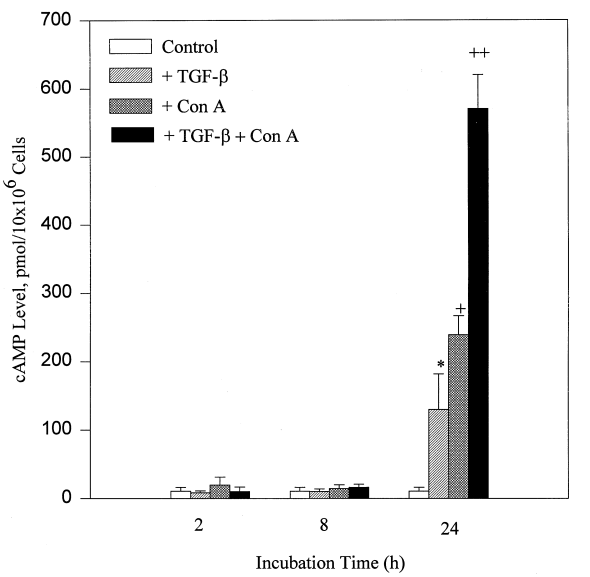

Fig. 7. Effect of hrTGF-β (2.5 ng/ml) and/or ConA (5 μg/ml) on control rat T-cell cAMP generation at various time points. Values are means (±SE) for six animals. $^*P<0.05$, control versus T-cells incubated with rTGF-β; $^+P<0.01$, control versus T-cells with ConA; $^{++}P<0.001$, control versus T-cells treated with both rTGF-β and ConA.

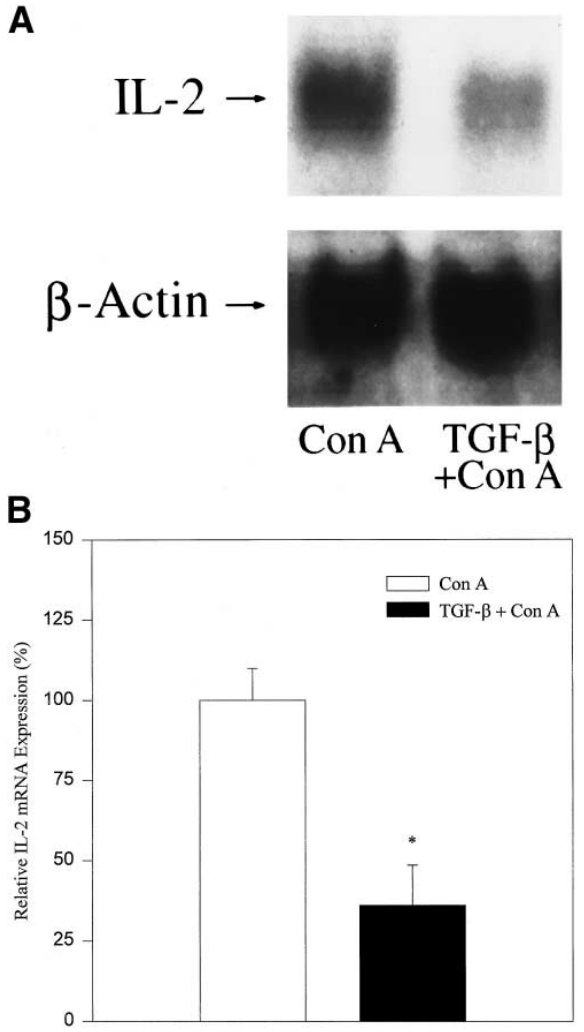

Fig. 8. A: ConA- and hrTGF-β+ConA-induced IL-2 mRNA expression in control rat T-cells. The blot is a representative of four independent Northern analyses. B: Densitometric quantitation of four such Northern blots of IL-2 mRNA expressions in control rat T-cells. The values of the mRNA abundance obtained in the T-cells incubated with ConA were taken as 100%. Values are mean (±SE) for four experiments. $^{*}P < 0.01$, T-cells incubated with ConA versus incubation with rTGF-β+ConA for 24 h.

ulate that TGF-β interferes with TCR-associated signal transduction pathways via generation of cAMP. The TGF-β-mediated activation of its own cell surface receptor and the pronounced increase in cAMP could be correlated with its negative modulation of $Ca^{2+}{}_i$-dependent signaling to lead to suppression of T-cell proliferation and IL-2 mRNA expression.

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