Transforming growth factor-β promotes 'death by neglect' in post-activated human T cells

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SUMMARY

Transforming growth factor-β (TGF-β) is central to the wound repair processes that follow local trauma and inflammation. In order to mimic the early events of wound-healing, we studied the effects of TGF-β on mitogen-stimulated peripheral blood cells. TGF-β added at the initiation of mitogenesis did not significantly alter T-cell activation, proliferation, CD45 isoform switching, or activation-induced cell death. By contrast, TGF-β added 72 hr post-activation (or later) enhanced the cumulative increase in apoptotic T cells. TGF-β had no effect on mitogen-induced up-regulation of Fas (CD95) or Fas ligand and did not enhance killing of the Fas-sensitive Jurkat cell line by activated T cells. Furthermore, TGF-β had no direct effect on levels of mRNA for members of the bcl family (bcl-X, bfl-1, bik, bak, bax, bcl-2 and mcl-1). These findings suggest that TGF-β does not directly induce apoptosis via the Fas system or by direct effects on bcl proteins. However, interleukin-2, which can 'rescue' lymphocytes from spontaneous apoptosis due to cytokine deprivation, abolished the pro-apoptotic effects of TGF-β on post-activated T cells, thus demonstrating that TGF-β increases the cytokine-dependence of T cells for survival. We propose a novel role for TGF-β in the suppression of inflammation by promoting the elimination of post-activated T cells once the initiating stimulus has been resolved.

INTRODUCTION

Local infection or injury provokes inflammation and a subsequent wound repair response, during which transforming growth factor-β (TGF-β) secretion is increased and the TGF-β zymogen is activated by the locally secreted proteases. TGF-β is a morphogenetic cytokine which is thought to be central to the tissue remodelling processes that occur during the wound-healing response, as well as being a major factor in scarring and fibrosis (recently reviewed in refs 1–6). In the immune system, TGF-β is thought to be antiproliferative.[1–3,7–9] However, there are numerous reports that it can promote, rather than inhibit, the primary response of antigen-naive T helper cells and the subsequent expansion of effector cell populations.[10–17] Thus, it can be hypothesized that up-regulation of TGF-β activity during wound healing may suppress the preceding inflammatory response, without necessarily preventing the generation of effector or memory cells from their unprimed precursors.

The expansion of activated lymphocytes during inflammation is followed by elimination of most of these cells by apoptosis. The role of TGF-β in this process is not well understood and there are conflicting reports that TGF-β can either inhibit[15–20] or promote[21–24] T-cell apoptosis. Although it has been shown that TGF-β does not alter T-cell expression of bcl2 or Fas/CD95,[18] there is disagreement in the reports concerning the effects of TGF-β on the expression of Fas ligand (FasL).[20,25] In order to help resolve the literature discrepancies, our experiments were designed to determine the effects of TGF-β on T-cell activation and survival, such as to mimic the early events of a wound-healing response. Here, we report that TGF-β promotes apoptosis in T cells if added at, or after 72 hr post-activation, a situation which would mimic the local increase in active TGF-β that occurs following extravasation of peripheral T cells to the site of injury or infection and subsequent activation.

MATERIALS AND METHODS

Lymphocyte culture
Blood samples were taken by venepuncture from normal volunteer laboratory staff and mixed with preservative-free heparin at 10 U/ml of blood. Peripheral blood mononuclear cells (PBMC) were isolated by density barrier centrifugation over 'Lymphoprep' (Nycomed UK Ltd, Sheldon, UK), washed

three times and cultured at 5×10^5 cells/ml in RPMI-1640 medium (Sigma, Poole, Dorset, UK) supplemented with 10% fetal bovine serum (FBS; Sera-Lab Ltd, Crawley Down, UK). Lymphocytes were activated with phytohaemagglutinin (PHA; Murex Diagnostics Ltd, Kent, UK) at a pretitred optimal concentration of 2·5 μg/ml added at the start of culture.[26–28] Control cultures with no mitogen were always included. For flow cytometry determinations, 1-ml cultures were set up in duplicate in 48-well 'tissue culture' grade plates; for mitogenesis assays, six replicate cultures were set up in 0·2-ml volumes in 96-well plates (Corning Costar, High Wycombe, Bucks, UK). In each experiment, blood from at least two donors was used.

Cytokines
TGF-β (Genzyme, West Malling, Kent, UK) was added to the cultures either at the start of the experiments, or at time-points up to 8 days after initiation of culture. Following dose–response experiments (see Fig. 1), TGF-β was routinely used at a final concentration of 100 U/ml, consistent with previous observations.[28,29] Both TGF-β$_1$ and TGF-β$_2$ isoforms were assessed. For some experiments, interleukin-2 (IL-2; R & D Systems, Abingdon, Oxfordshire, UK) was used at up to 1000 U/ml, added simultaneously with TGF-β at either the onset of mitogenesis or after 72 hr culture.

Phenotypic analysis of lymphocyte activation
PBMC were analysed by 'two-colour' flow cytometry to assess expression of cell-surface markers using an (FACScan)
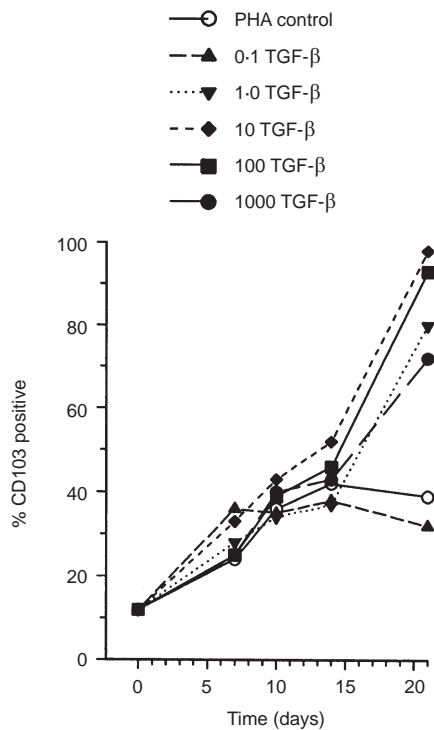

Figure 1. Up-regulation of CD103 by TGF-β on activated T cells. Two-colour flow cytometry analysis showed that CD103 (integrin α_E/β_7, HML-1 antigen) was up-regulated by TGF-β on T cells in a dose-dependent manner following PHA stimulation of peripheral blood lymphocytes. Representative experiment illustrated; data are means of replicates ± SD.

analytical flow cytometer with CELLQUEST software for acquisition and analysis (Becton Dickinson, Cowley, Oxord, UK). A direct-conjugated CD2 antibody was used to identify the T-cell fraction, together with a second direct-conjugated antibody of interest.[26–28] Although expressed weakly by natural killer (NK) cells, CD2 was used in preference to CD3 to 'gate' the T-cell population as CD3 is down-regulated during T-cell activation. Both fluorescein isothiocyanate (FITC) and phycoerythrin (PE) conjugates were used, as appropriate. A panel of antibodies was used to study T-cell phenotype. FITC-conjugated CD4, CD5, CD8, CD25, CD28, CD45RA, CD45RB, CD69, CD95, CD45RO-PE, CD95-FITC and biotin-conjugated CD95L (FasL) were purchased from Dako (High Wycombe, Buckinghamshire, UK). Biotin-conjugated HML-1 antibody (CD103, integrin α_E/β_7) was obtained from Immunotech (Coulter Electronics, Luton, Bedfordshire, UK). A streptavidin-Quantum red second layer (Sigma) was used to detect biotin-conjugated antibodies. PE- and FITC-conjugated antibodies against *Aspergillus niger* glucose oxidase (Dako) were used as negative controls. Three thousand $CD2^+$ cells were acquired per tube in duplicate and the amplifiers were set so that $\geq 95\%$ of negative-control cells fell within the first decade. For analysis of $CD4^+$ and $CD8^+$ populations, results were expressed as '% positive'; expression of other surface markers was quantified as the median fluorescence channel.

T-cell activation was assessed by up-regulation of CD69 at 24 hr and CD25 at 72 hr, as described previously.[26–28] The biological effects of TGF-β were monitored by assessing the TGF-β-induced up-regulation of the integrin α_E/β_7 on post-activated T cells.[13]

Assessment of apoptosis
Apoptosis was routinely assessed by flow cytometry by using Annexin V (FITC conjugate, BioWhittaker, Wokingham, Bucks, UK)[30] in conjunction with propidium iodide, and by criteria of forward and side scatter.[31] To confirm nuclear fragmentation, a characteristic of the late stages of apoptosis, preparations were stained with acridine orange and examined by fluorescence microscopy (Zeiss Axioplan) using a $\times 63$ oil-immersion objective.

T-cell mitogenesis assays
Cell cultures were established at 5×10^5 lymphocytes/ml in 96-well microtitre plates in 0·2 ml volumes as described previously.[26,27] PHA was used as the mitogen and TGF-β was added either at the onset of culture or after 72 hr, as above. Positive and negative controls consisted of PHA alone and no mitogen, respectively. Six replicates were used for each condition. [³H]Thymidine (0·5 μCi; Amersham, Bucks, UK) was added to each well after 72 hr culture and cells were harvested onto glass-fibre filter mats after a further 18-hr incubation. Precursor incorporation was measured by liquid scintillation spectrometry and results were expressed as a stimulation index (SI) where: SI = (test counts per minute)/(control counts per minute) after subtraction of background counts.

Apoptosis assay of Jurkat cells
In addition to flow cytometric analysis (above), the 'JAM' test of DNA fragmentation[32] was used to determine if TGF-β functionally promoted the ability of activated T cells to kill the
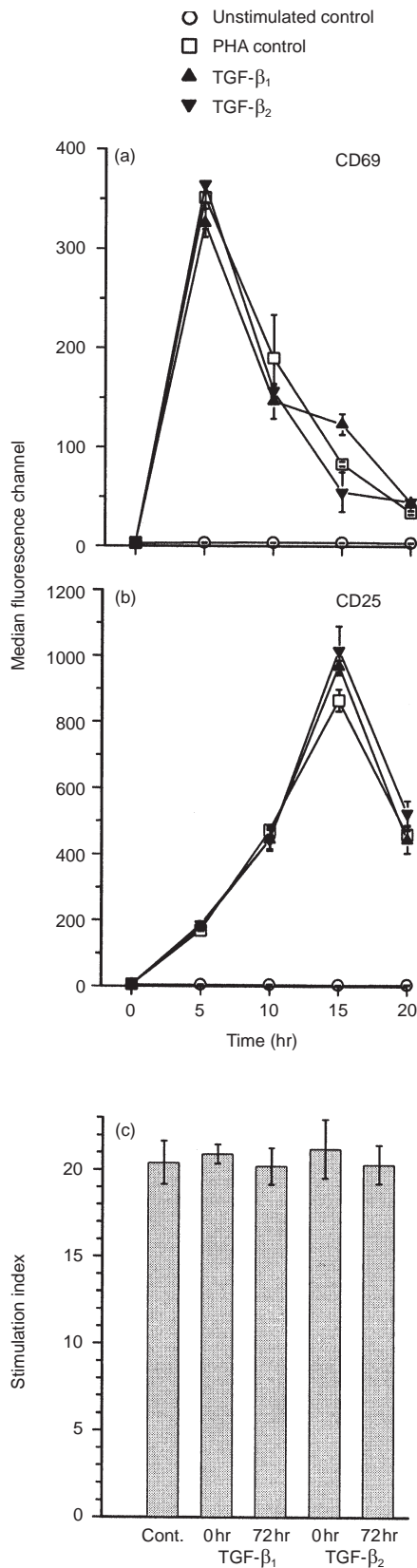
Fas-sensitive Jurkat T lymphoblastoid cell line. Jurkat cells [obtained from the European Collection of Cell Cultures (ECACC), Porton Down, Wiltshire, UK] were maintained in suspension culture in RPMI-1640 medium with 10% FBS. Cells in log-phase growth were prelabelled overnight with $[^3H]$thymidine, washed and incubated for 24 hr with lymphocytes taken at various stages after activation, using a ratio of $1:1$ effector:target cells. Cultures were harvested onto glass-fibre filter mats and the amount of intact DNA retained was estimated by liquid scintillation spectrometry.

Ribonuclease protection assays

Multitemplate ribonuclease protection assays were used to examine mRNA from lymphocytes cultured with and without TGF-β, using the template set hAOP-2 containing probes for bcl-X, bfl-1, bik, bak, bax, bcl-2 and mcl-1 (PharMingen, Becton Dickinson, Cowley, Oxford, UK). Control probes for L32 and GAPDH were included. Cells were harvested 24 hr after addition of TGF-β and the assay was performed using reagents and the protocol provided by Ambion (AMS Biotechnology, Abingdon, Oxford, UK). Protected fragments were separated and visualized by electrophoresis through 5% (w/v) denaturing polyacrylamide gels. Dried gels were analysed on a phosphorimager.

Statistics

Means and standard deviations were used as descriptive statistics. For determination of statistical significance, analysis of variance (ANOVA) was performed using the INSTAT software package (GraphPAD, San Diego) with Bonferroni correction for multiple comparisons.

RESULTS

Effects of TGF-β on T-cell activation and mitogenesis

The bioactivity of TGF-β was confirmed by the expected up-regulation of the CD103 integrin α_E/β_7 (Fig. 1) (cf. ref. 13). The effects of TGF-β$_1$ and TGF-β$_2$ isoforms on both cell activation and cell-surface phenotype were closely comparable (Fig. 2).

Irrespective of whether TGF-β was added at the initiation of culture or at 72 hr, there was no significant effect on T-cell activation or mitogenic responses (Fig. 2). TGF-β also had no significant effect on the subsequent development of a CD45R0^{hi} CD45Ra^{lo} CD45RB^{lo} 'memory' T-cell phenotype (Fig. 3). Furthermore, TGF-β did not preferentially affect the percentages of CD4$^+$ ($49 \pm 9 \cdot 6$ versus $52 \pm 2 \cdot 7$ and $51 \pm 7 \cdot 8\%$, in PHA only controls versus PHA with TGF-β at 0 hr and

Figure 2. Effects of TGF-β on T-cell activation and proliferation. T-cell activation was determined by flow cytometry by gating on the CD2$^+$ population following PHA stimulation. Neither TGF-β$_1$ nor TGF-β$_2$ isoforms had any significant effect on either the kinetics or magnitude of up-regulation of CD69 (a) or CD25 (b). Irrespective of whether added at 0 hr or 72 hr, TGF-β did not affect proliferation, relative to mitogen-only controls, as determined by $[^3H]$thymidine incorporation (c). Results are expressed as a Stimulation Index (ratios of counts of PHA-stimulated to unstimulated cultures after background subtraction). Representative experiment illustrated; data are means of replicates $\pm$ SD.
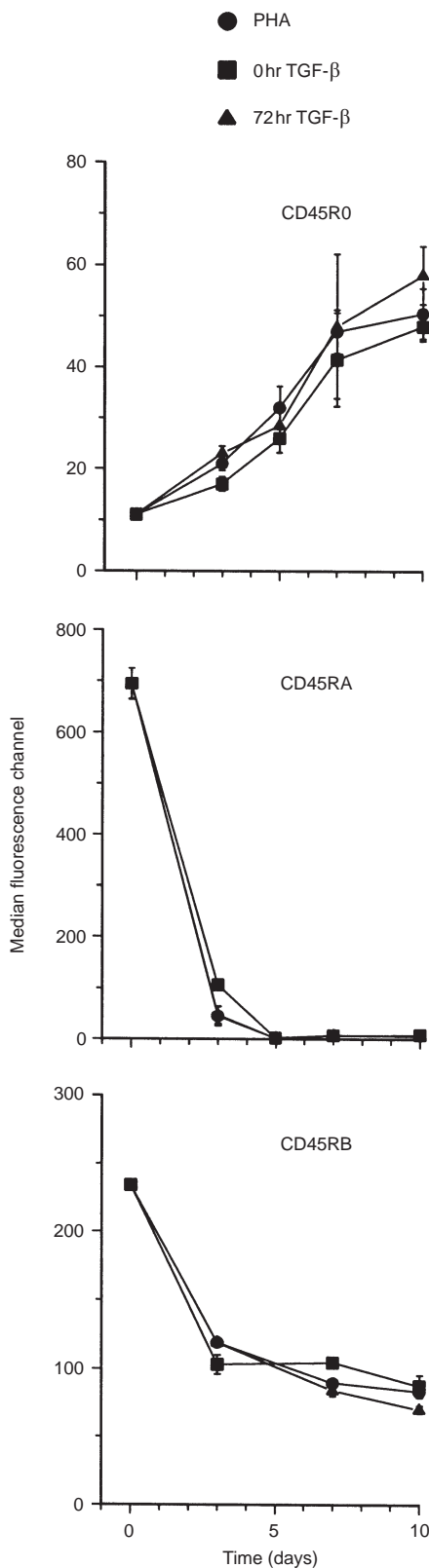
Figure 3. Effects of TGF-β on development of an antigen-committed phenotype. The development of the CD45R0^{hi} CD45Ra^{lo} CD45RB^{lo} phenotype of antigen-committed T cells following PHA stimulation was unaffected by the presence of TGF-β. Note the superimposition of some data points. Representative experiment illustrated; data are means of replicates ± SD.

72 hr, respectively, $n = 10$ donors) and CD8$^+$ cells ($37 \pm 12 \cdot 9$ versus $36 \pm 11 \cdot 8$ and $38 \pm 13 \cdot 6\%$, respectively, $n = 10$ donors) at 7 days. There was no significant effect of TGF-β on the expression of CD28 (median fluorescence values of $67 \pm 26 \cdot 6$ versus $73 \pm 31 \cdot 2$ and $75 \pm 35 \cdot 3$, respectively, $n = 6$ donors) and TGF-β had no significant effect on the activation-induced up-regulation of CD5 (median fluorescence values of 238 ± 133 versus 296 ± 143 and 263 ± 159, respectively, compared with $105 \pm 18 \cdot 7$ in unstimulated controls, $n = 10$ donors) at 7 days.

Effects of TGF-β on T-cell survival and apoptosis

When added at the same time as mitogen, TGF-β induced a slight (typically 4–5%) but consistent increase in the percentage of apoptotic cells during extended culture (up to 21 days), compared to PHA-activated T cells cultured in the absence of TGF-β. However, the difference was not statistically significant. (Fig. 4)

By contrast, when TGF-β was added at 72 hr or later post-mitogenesis, there was a profound slow, cumulative increase in the percentages of apoptotic T cells (Fig. 4). The effect became statistically significant within 2 days of culture after addition of TGF-β ($P < 0 \cdot 05$) and increased steadily with time. By the end of 14 days from the onset of mitogenesis, the percentage of T cells surviving in cultures where TGF-β had been added 72 hr post-mitogenesis was less than 50% of mitogen-only controls or cells where TGF-β had been added at the onset of mitogenesis (Fig. 4).

Effects of TGF-β on the Fas/FasL system

In order to determine any involvement of the Fas/FasL system, expression of Fas (CD95) and FasL (CD95L) was determined on T cells activated in the presence or absence of TGF-β. However, no significant changes were observed in either Fas or FasL up-regulation attributable to TGF-β (Fig. 5).

To confirm these data, the functional ability of activated T cells to kill the Fas-sensitive Jurkat cell line was investigated. Activated T cells showed a potent ability to kill Jurkat cells at 72 hr post-activation, which was largely lost by 120 hr (Fig. 5). This closely mirrored the kinetics of FasL expression. Irrespective of whether TGF-β was added at the same time as mitogen or after 72 hr, there was no functional difference in the ability of activated cells to induce apoptosis in Jurkat cells (Fig. 5).

Bcl family transcipt expression

Ribonuclease protection assays showed that TGF-β did not directly affect levels of bcl family transcripts. Cultures of lymphocytes without TGF-β, with TGF-β added at 0 hr and with TGF-β added at 72 hr all showed comparable amounts of message after a further 24 hr for bcl-X, bfl-1, bik, bak, bax, bcl-2 and mcl-1 when normalized against mRNA for the 'housekeeping' genes L32 and GAPDH (not shown).

Effects of exogenous IL-2

Although several cytokines have been reported to 'rescue' post-activated T cells from spontaneous apoptosis due to cytokine deprivation, the archetypal cytokine in this respect is IL-2.
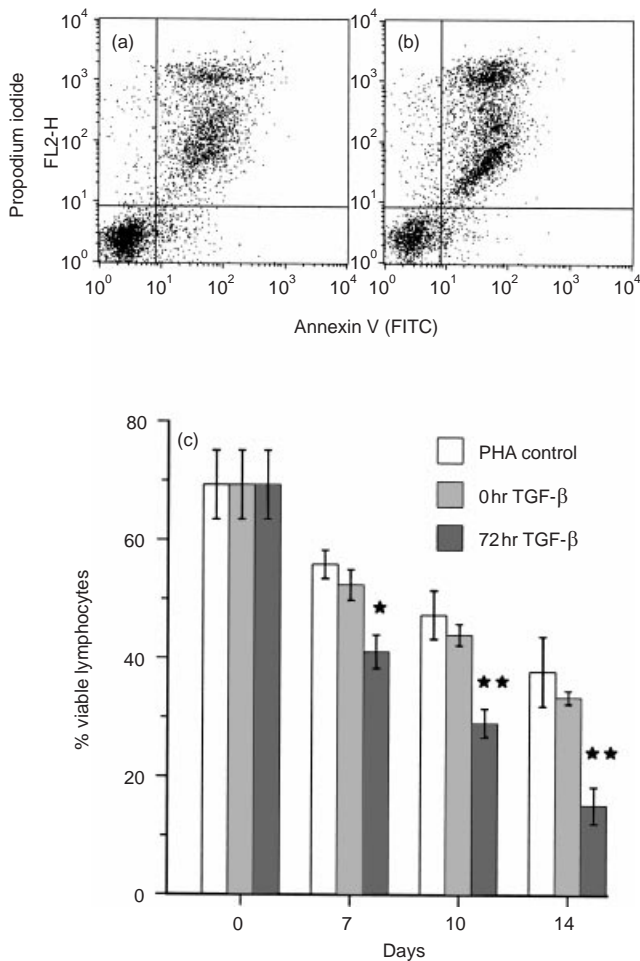
Figure 4. TGF-β promotes apoptosis in post-activated T cells. Apoptosis was assessed by flow cytometry following incubation with Annexin V and propidium iodide (a,b). The negative cells in the lower left quadrant were considered as viable (non-apoptotic), whereas apoptotic cells were Annexin V-positive, with the late apoptotic/dead cells showing the highest levels of propidium iodide staining. In the representative experiment illustrated, the percentage viable cells in the control culture (a) at 10 days was 46%, compared to 26% in the treated culture (b). (c) shows that the percentage of viable T cells decreased more rapidly following PHA stimulation if TGF-β was added at 72 hr post-activation, compared to TGF-β added at the onset of mitogenesis or omitted. Data represent mean values ($\pm$SEM) from 15 independent experiments.*$P < 0.05$; **$P < 0.01$.

Addition of IL-2 completely abolished the pro-apoptotic affect of TGF-β. In the presence of exogenous IL-2, there was no increase in the percentages of apoptotic cells with time in cultures treated with TGF-β either at the onset of mitogenesis or after 72 hr (Fig. 6).

DISCUSSION

Our observations suggest that TGF-β may play a hitherto unsuspected role in the elimination of activated T cells at the resolution of an immune response. The two major pathways involved are 'activation-induced cell death' (AICD), an active process mainly mediated by the Fas/FasL system, and 'death
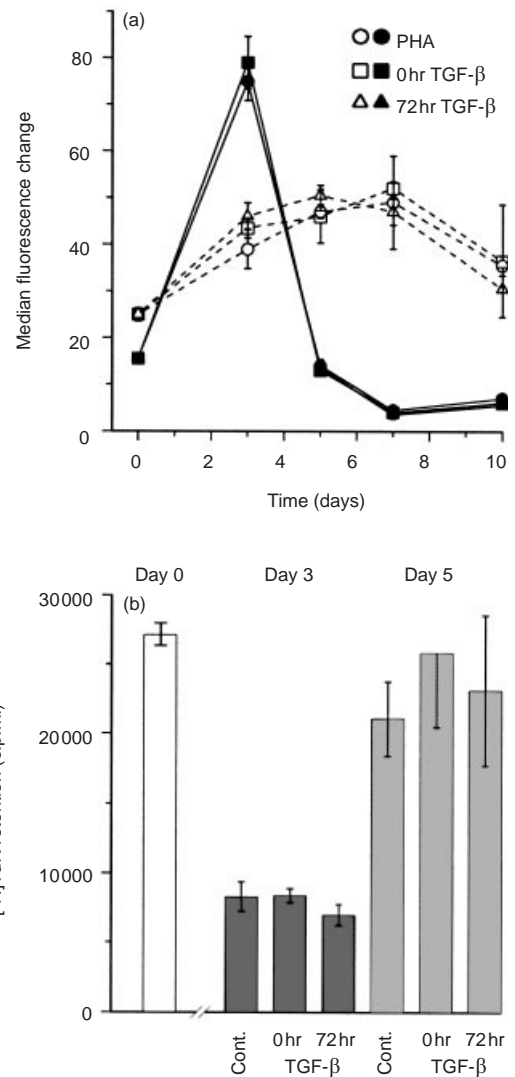

Figure 5. TGF-β does not promote Fas-mediated killing. Flow cytometry analysis (a) revealed that TGF-β had no significant effect on either the kinetics or the magnitude of PHA-induced up-regulation of CD95 (Fas) (open symbols) or FasL (solid symbols), whether added at the onset of mitogenesis or after 72 hr. The JAM test of DNA fragmentation (b) showed that activated T cells at day 3 (dark bars) induced apotosis in the Fas-sensitive Jurkat T cell line, as measured by the amount of [^{3}H]thymidine retained on filter mats. However, 5 days after activation, T cells had lost the ability to induce apoptosis in Jurkat cells (shaded bars), compared to the day 0 control (open bar). The effects were unaltered whether TGF-β was absent (Cont), added at the time of mitogenesis (0 hr) or added 72 hr after the onset of stimulation.

by neglect', where the default apoptotic machinery is engaged in the absence of sufficient rescue factors, such as IL-2 (recently reviewed in refs 33–39). AICD alone cannot account for our observations, as TGF-β had no effect on the Fas/FasL system and did not directly alter the balance of the pro- and anti-apoptotic members of the bcl family. Conversely, the ability of exogenous IL-2 to abrogate the pro-apoptotic effects of TGF-β suggests that post-activated T cells exposed to TGF-β become more vulnerable to cytokine deprivation. This may be due to premature exit from the cell cycle, with consequent impairment
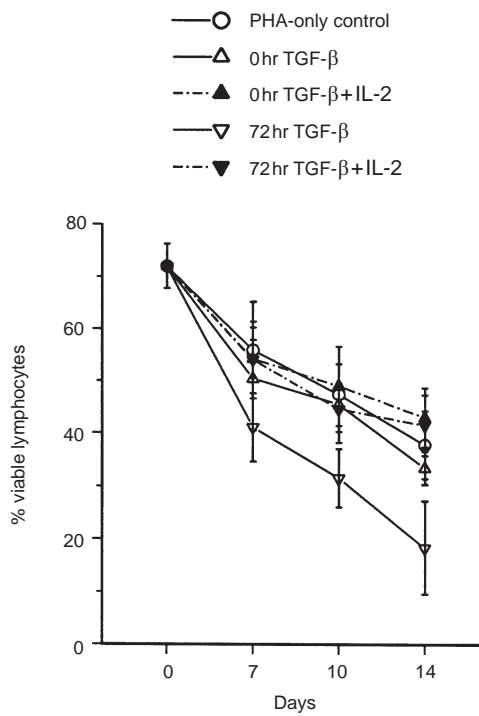
Figure 6. IL-2 overcomes TGF-β-mediated apoptosis in post-activated T cells. Addition of IL-2 together with TGF-β either at the onset of mitogenesis (0 hr) or at 72 hr abolished the ability of TGF-β to promote apoptosis in post-activated T cells. Representative experiment illustrated; data are means of replicates ± SD.

of IL-2 production. This view is supported by the relatively slow kinetics of the effect, as increased cell death did not occur immediately, but rather was a cumulative effect, increasing steadily with time. Another contributory factor may be inhibition of IL-2-induced phosphorylation and activation of the JAK/STAT pathway of T cells.[23] This, together with reduced expression of the α- and β-chains of the IL-2 receptor,[23] could also account for the increased requirement for IL-2 to permit T-cell survival.

Although we found that TGF-β added after mitogenic stimulation promoted apoptosis, it had minimal effects if present throughout the activation response. These observations provide direct confirmation of a previous report[13] and are consistent with the notion that TGF-β does not necessarily suppress T-cell activation or proliferation.[10–12,15,19,40–43]

Our observations imply that the timing of TGF-β signalling is an important determinant of cell fate. This hypothesis is in keeping with previous observations, which suggest that TGF-β has different effects on cells undergoing initial stimulation or a priming response, compared to cells undergoing re-stimulation. Thus, TGF-β can reduce apoptosis during re-stimulation, either if present in the initial phase[18] or if present during the restimulation phase.[20] Furthermore, we and others;[18,25] have shown that FasL expression is unaffected by TGF-β during first-round stimulation, whereas in cells undergoing restimulation, TGF-β can inhibit up-regulation of FasL.[20]

On the basis of the above, we propose that TGF-β plays a biphasic role in the modulation of local inflammatory

responses and the subsequent fate of post-activated lymphocytes during wound-healing. The published data suggest that during the initial phase of the inflammatory response, TGF-β enhances the recruitment of naive cells[10–12,16] and, during the ongoing reaction, promotes effector cell expansion and the generation of memory cells,[13–15,17,18] perhaps by down-regulation of FasL.[20] However, once the initiating stimulus has been eliminated and local production of IL-2 has ceased, our findings show that TGF-β promotes the removal of post-activated T cells by increasing the survival requirement for rescue factors. This is a novel role for TGF-β in the resolution of inflammatory responses and the promotion of wound-healing and failure of this mechanism may be a contributory factor in the perpetuation of chronic inflammatory conditions.

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