Transforming Growth Factor \( \beta 1 \) Inhibits Fas Ligand Expression and Subsequent Activation-induced Cell Death in T Cells via Downregulation of c-Myc

By Laurent Genestier, Shailaja Kasibhatla, Thomas Brunner, and Douglas R. Green

From the Division of Cellular Immunology, La Jolla Institute for Allergy and Immunology, San Diego, California 92121

Summary

Activation-induced cell death (AICD) is a process that regulates the size and the duration of the primary immune T cell response. In this report, we investigated the mechanisms involved in the regulation of AICD by transforming growth factor \( \beta 1 \) (TGF-\( \beta 1 \)). We found that TGF-\( \beta 1 \) decreased apoptosis of human T cells or T cell hybridomas after activation by anti-CD3. This decrease was associated with inhibition of Fas (Apo-1/CD95) ligand (FasL) expression, whereas Fas signaling was not affected by TGF-\( \beta 1 \). In parallel, TGF-\( \beta 1 \) inhibited c-Myc expression in T cell hybridomas, and ectopic expression of a chimeric molecule composed of c-Myc and the steroid binding domain of the estrogen receptor (Myc-ER) blocked both the inhibition of FasL and the decrease of AICD induced by TGF-\( \beta 1 \), providing that 4-hydroxytamoxifen was present. These results identify one mechanism by which TGF-\( \beta 1 \) blocks AICD to allow the clonal expansion of effector T cells and the generation of memory T cells during immune responses.

Key words: apoptosis • T cells • transforming growth factor \( \beta 1 \) • Fas ligand • c-myc
presses constitutive and inducible c-Myc expression in two constitutively activated murine T cell clones (29).

In this report, we investigated the mechanisms involved in the regulation of AICD by TGF-β1. We determined that TGF-β1 inhibits FasL expression at the level of mRNA expression. TGF-β1 also inhibits the constitutive c-Myc expression in A1.1 T cell hybridomas, and since c-Myc has been demonstrated to regulate AICD, we prepared stable transfectants constitutively expressing a chimeric molecule composed of c-Myc and the steroid binding domain of the estrogen receptor (Myc-ER). In these cells, TGF-β1 did not inhibit FasL expression and subsequent AICD after anti-CD3 antibody treatment, providing that 4-hydroxytamoxifen (4-OHT) was present. These results demonstrate that TGF-β1 inhibits FasL expression and subsequent AICD via downregulation of c-Myc expression.

Materials and Methods

Cell Cultures and Reagents. The T cell hybridomas A1.1 and 2B4.11 have been described previously (17, 30). PBMCs were isolated from healthy donors by density gradient centrifugation on Histopaque (Sigma). All cells were grown in RPMI 1640 medium containing 10% FCS, 5 × 10⁻³ M β-mercaptoethanol, 2 mM l-glutamine, and 100 U/ml each of penicillin and streptomycin (complete medium). PMA, ionomycin, cyclosporine A (CsA), 4-OHT, and M2 anti-Flag antibody were purchased from Sigma. The mouse anti-human CD3 e antibody was obtained from Becton Dickinson. Dead cells were identified as those taking up the dye. Apoptosis was measured by flow cytometry using a FACScan® (Becton Dickinson). Dead cells were identified as those taking up the dye. Apoptosis was confirmed by morphological inspection by fluorescent microscopy. The expression of c-Myc was determined by reverse transcription (RT) of total RNA followed by PCR amplification (RT-PCR). Approximately 3 × 10⁶ cells were homogenized with 1 ml Trizol reagent (GIBCO BR), and total RNA was isolated according to the manufacturer's protocol. cDNAs were synthesized by extension of (dT)₃ primers with 200 U of SuperScript II reverse transcriptase (GIBCO BR) in a mixture containing 1 μg of total RNA digested by R Nase-free DNase (2 U/μg of RNA) for 15 min at 37°C. PCR of the cDNA was performed in a final volume of 50 μl containing all four dNTPs, 2 mM MgCl₂, 2.5 U of AmpliTaq (GIBCO BR), and each primer at 0.2 μM using the geneAmp 2400 PCR system (Perkin Elmer Corp.). The amplification cycles were 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The PCR products were separated by electrophoresis on a 15% agarose gel after 27–33 cycles for mouse fas, fasL, and cMyc or 22–28 cycles for mouse β-actin and visualized by ethidium bromide staining.

The following primers were designed to discriminate between the amplification of cDNA (low size PCR products) and contaminating genomic cDNA (large size PCR products): mouse fas sense, 5'-CAG-CAG-TTG-CAC-ATT-GG-3'; mouse fas antisense, 5'-TTC-ATT-CAT-GAG-ATC-AGC-3'; mouse fas sense, 5'-GAG-GAT-TAC-GAA-ATA-TTG-G-3'; mouse fas antisense, 5'-GAC-ATT-GC-GGT-3'; mouse fas antisense, 5'-GAA-ACC-ATA-GAC-GTT-TGG-3'; mouse c-myc sense, 5'-ACA-ACC-ATA-GAC-GTG-AGC-GGA-3'; mouse c-myc antisense, 5'-TCT-ACC-TGT-GAG-GGC-3'; mouse β-actin sense, 5'-GGA-ACT-TGT-GCA-GTA-3'; mouse β-actin antisense, 5'-TAA-ACC-GCA-GCT-CAG-TAA-3'.

Plasmids and Transfections. Moloney retroviral viros were produced as described previously (33). In brief, amphotropic packaging cell line was plated at 2.5 × 10⁶ cells/10-cm² culture dish for 18–24 h before transfection as described (34). Cells were transfected with 7.5 μg of pBABE puroMyc-yr-E G525R construct (35) using a standard calcium phosphate protocol except for chloroquine (25 μM final), which was added to the cells 5 min before addition of calcium phosphate DNA precipitate. After 24 h the cells were gently washed, and fresh medium was added. Viral containing supernatant was harvested at 24 and 48 h after transfection, filtered, and stored at 4°C. For virus infection, A1.1 cells (0.5 × 10⁶/ml) were resuspended in 3 ml of viral supernatant containing 5 μg/ml polybrene for 12 h. Cells were then washed and resuspended in RPMI medium without phenol red.
(GIBCO BRL) containing 10% steroid-free FCS, 5 × 10⁻⁵ M β-mercaptoethanol, 2 mM l-glutamine, and 100 U/ml each of penicillin and streptomycin. 48 h after transfection, cells were selected with puromycin (1.5 μg/ml) for 7 d. 

Assessment of cMyc and Myc-ER Proteins. 2 × 10⁶ A1.1 or A1.1 Myc-ER cells were incubated with or without 10 ng/ml TGF-β1 for 8 h. Cells were then harvested, washed once in PBS, and lysed in 100 μl × 1X SDS-PAGE sample buffer (50 mM Tris, pH 6.8, 100 mM dithiothreitol, 2% SDS, 10% glycerol). Equal volumes of samples (30 μl) were separated on an 8% gel and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech). Membranes were blocked with 5% nonfat dry milk in TBS, 0.02% sodium azide for 2 h and then incubated with mouse anti-human c-Myc 9E10 (1:1,000; Santa Cruz Biotechnology, Inc.) for 4 h at room temperature. Membranes were then washed three times in TBS, 0.1% Tween 20 for 15 min and incubated with a rabbit anti-mouse horseradish peroxidase conjugate (1:2,000; Amersham Pharmacia Biotech) for 1 h. After three washes, membranes were incubated with ECL chemiluminescence detection solutions (Amersham Pharmacia Biotech) and exposed to x-ray films for 1 min.

Cell Cycle Analysis. Cell cycle analysis was performed as described previously (36), with slight modifications. 10⁶ A1.1 cells were washed twice in PBS and resuspended in 0.1% sodium citrate). After 4 h at 4°C in the dark, the fluorescence of gated live cells was analyzed using a FACScan® flow cytometer (Becton Dickinson).

Results

TGF-β1 Decreases AICD in Human T Cells as well as M Ham T Cell Hybridomas. TGF-β1 has been reported to increase long-term cell expansion of Th2 cells by decreasing activation-induced apoptosis in human T cells (14). To confirm these data, we activated PBMCs for 6 d with anti-CD3, before restimulation with PMA plus ionomycin for 16 h in the presence or absence of increasing concentrations of TGF-β1. CsA, which has been shown to effectively inhibit AICD (37), was used as a positive control. As illustrated in Fig. 1 A, activated PBMCs restimulated by addition of PMA plus ionomycin showed ~50% death. In contrast, if the cells were stimulated in the presence of TGF-β1, the percentage of apoptotic cells was dramatically decreased.

To study the mechanism by which TGF-β1 inhibits AICD, we used the T cell hybridomas A1.1 and 2B4.11, two cell lines used extensively to examine AICD (17, 30). We first investigated the effect of TGF-β1 on AICD in these T cell hybridomas. As expected, TGF-β1 decreased anti-CD3-induced apoptosis in both cell types, as measured by propidium iodide uptake (Fig. 1 B) or DNA laddering (Fig. 1 C).

TGF-β1 Inhibits Activation-induced FasL Expression. We demonstrated previously that AICD in both A1.1 and 2B4.11 T cell hybridomas proceeds via expression of FasL and subsequent Fas-FasL interaction (17). Therefore, we envisioned three nonexclusive possibilities for the mechanism of inhibition by TGF-β1: inhibition of FasL expression, inhibition of Fas expression, and/or inhibition of the Fas signaling pathway. To assess whether the inhibitory activity of TGF-β1 alters Fas or FasL gene expression, RNA was isolated and analyzed by semiquantitative RT-PCR. A1.1 T cell hybridomas constitutively expressed a low level of Fas mRNA, which increased approximately twofold after activation, and TGF-β1 only slightly decreased Fas expression at high doses (10–100 ng/ml; Fig. 2 A). In contrast, activation-induced expression of FasL in A1.1 cells was greatly reduced by TGF-β1 at 0.1 ng/ml and completely prevented at higher concentrations (Fig. 2 A). This decrease in FasL mRNA was reflected in the biological activity of this molecule, since A1.1 cells that were stimulated with anti-CD3 antibody in the presence of TGF-β1 did not induce apoptosis in FasL target cells (Fig. 2 B). Addition of TGF-β1 to the cocultures of L1210-Fas target cells and activated A1.1 cells (6 h after activation), rather than to the A1.1 cells at the onset of the activation, failed to inhibit cytotoxicity (Fig. 2 B). These data suggest
that TGF-β1 inhibits AICD by preventing FasL expression and not via a blockade in the Fas signaling pathway. To confirm the absence of effect of TGF-β1 on FasL expression, we treated A1.1 cells with recombinant soluble FasL cross-linked with anti-Flag antibody (31) in the absence or presence of TGF-β1. As shown in Fig. 3, addition of TGF-β1 did not prevent soluble FasL–induced apoptosis in A1.1 cells.

**Figure 2.** TGF-β1 inhibits activation-induced apoptosis by blocking FasL mRNA expression and functional activity. (A) TGF-β1 inhibits activation-induced FasL mRNA expression. RT-PCR analysis of total mRNA obtained from A1.1 cells incubated in medium alone or activated for 4 h with anti-CD3 antibodies in the presence or absence of TGF-β1 or CsA (100 ng/ml) was performed using different primers pairs indicated. The products were electrophoresed on 1.5% agarose gel and stained with ethidium bromide. (B) TGF-β1 inhibits AICD by modulating the expression of FasL functional activity but not by blocking the signaling through Fas receptor. A1.1 cells were activated at time 0 with coated anti-CD3 antibodies in the presence or absence of different concentrations of TGF-β1. L1210 or L1210-Fas target cells were not added at this time. The T cell hybridomas were then cultured for 6 h to allow FasL expression, harvested, washed twice, and incubated for an additional 8 h with [3H]TdR–labeled L1210 or L1210-Fas target cells. In a parallel experiment, A1.1 cells were activated with anti-CD3 alone and TGF-β1 was added when A1.1 and target cells were mixed (T = +6 h). Percentage of DNA fragmentation was calculated as described in Materials and Methods.

**Figure 3.** TGF-β1 does not block recombinant soluble FasL–induced apoptosis. A1.1 T cell hybridomas were preincubated for 1 h in the presence of TGF-β1 (10 ng/ml) and then treated with different doses of soluble recombinant human FasL (sFasL). 15 min after addition of soluble FasL, anti-Flag M2 antibody (1 μg/ml) was added to cross-link soluble FasL. After 12 h incubation, percentage of apoptosis was assessed by propidium iodide uptake and analyzed using a FACScan.

**Figure 4.** TGF-β1 inhibits endogenous c-myc mRNA and protein expression but not ectopic expression of the chimeric Myc-ER protein. (A) TGF-β1 inhibits c-myc mRNA expression. RT-PCR analysis of total mRNA obtained from A1.1 cells incubated in medium alone or activated for 4 h with anti-CD3 antibody with the indicated concentration of TGF-β1 or CsA (100 ng/ml) was performed using different primers pairs indicated. The products were resolved by agarose gel electrophoresis. (B) TGF-β1 inhibits constitutive endogenous c-Myc protein expression but not ectopic expression of the chimeric Myc-ER protein. Total cell extracts were prepared from A1.1 or A1.1 MycER cells treated for 8 h in the presence or absence of TGF-β1 (1 ng/ml). Samples were analyzed by immunoblot analysis using anti-human c-Myc antibody.
Abbreviations

CD3 antibodies (lanes 2, 3, and 4). 1 ng/ml TGF-β1 or CsA (100 ng/ml).

Figure 5. Ectopic expression of chimeric Myc-ER protein prevents TGF-β1-mediated inhibition of FasL mRNA and subsequent AICD. (A) Functional Myc-ER interferes with the inhibitory effect of TGF-β1 on activation-induced FasL expression in A1.1 cells. A1.1 or A1.1 Myc-ER cells exposed to 4-OHT (50 nM) were first preincubated for 4 h with the

Thus, when c-Myc activity is maintained, no inhibitory effect of TGF-β1 on either FasL expression or AICD in T cell hybridomas is observed. These results formally demonstrate that the ability of TGF-β1 to inhibit FasL expression and subsequent AICD is dependent on its ability to downregulate c-Myc.

TGF-β1-mediated Inhibition of c-Myc Does Not Block Activation-induced FasL Expression via Perturbation of the Cell Cycle. In some models, TGF-β1 has been shown to cause cell cycle arrest in G1 by upregulation of CDK inhibitors (25) or by repression of Cdc25A, a CDK tyrosine phosphatase which activates CDK (26). Since Cdc25A is transcriptionally induced by c-Myc (41), we addressed whether TGF-β1-inhibited induction of c-Myc decreased FasL expression via cell cycle arrest. As shown in Fig. 7, addition of TGF-β1, AS-c-myc, or NS-c-myc oligonucleotides to A1.1 cells cultured in medium alone did not modify the proportion of cells in G0/G1, S, or G2/M phase of the cell cycle. When A1.1 cells were activated with anti-CD3 alone, live cells accumulated in G0/G1 phase with a decreased proportion of cells in S and G2/M phase at 24 h as described (30). Addition of TGF-β1, AS-c-myc, or NS-c-myc oligonucleotides did not significantly alter this profile (Fig. 7). Although TGF-β1 and AS-c-myc oligonucleotides did not modify the proportion of cycling cells, they decreased AICD in A1.1 cells from 60 to 20% and 60 to 29%, respectively, after 12 h of culture (21; data not shown). DNA synthesis studies using [3H]TdR incorporation also demonstrated that TGF-β1 did not inhibit proliferation of unactivated A1.1 cells (26,678 ± 2,343 cpm in control cells vs. 24,310 ± 4,121
cpm in TGF-β1-treated cells), whereas [3H]Thymidine incorporation of activated A1.1 cells was almost completely inhibited with or without addition of TGF-β1 (1,625 ± 243 cpm in anti-CD3-treated cells vs. 2,111 ± 328 cpm in anti-CD3 plus TGF-β1-treated cells).

Discussion

AICD in vivo has been proposed first to limit the expansion of an immune response by eliminating effector cells that are no longer needed, and second to eliminate potentially autoreactive T cells that have escaped thymic selection (15). Evidence from lpr or gld mice, deficient in functional Fas and FasL, respectively, suggested that Fas/FasL interactions are crucial for the regulation of these physiological processes. Experiments using antagonists of Fas/FasL interaction such as Fas-Fc fusion protein or a neutralizing anti-Fas mAb further demonstrated the direct involvement of these molecules in AICD of mature T cells (16–20). In this study, we have shown that TGF-β1, which is known to block the process of AICD in T cells (13, 14), does so by the selective inhibition of activation-induced FasL expression. This was demonstrated by analysis of FasL mRNA expression by RT-PCR and by functional assays. Indeed, many tumors, such as malignant astrocytoma, produce a high level of TGF-β1 (42) which might inhibit FasL expression on CD8+ T cells and therefore counteract their cytotoxic function.

Interestingly, there is also an inhibitory effect of TGF-β1 on perforin and granzyme B mRNA expression as well as serine esterase activity in CD8+ T cells, and this serves to block cytotoxic function of perforin-dependent killers (43, 44). Whether this represents a similar requirement for c-Myc in the expression of these important components of cytotoxic granules is not known.

In contrast to its effects on FasL expression, we observed that TGF-β1 does not significantly affect activation-induced...
Fas expression in T cell hybridomas. T cell activation induces the appearance of Fas on naïve T cells and an increased expression on some T cell hybridomas (15, 17). In the latter, CsA does not inhibit the activation-induced increase in Fas mRNA expression, although it does partially inhibit cell surface Fas (39). Similar observations have been made for retinoic acid analogues, which inhibit FasL but not Fas expression (20, 45). Thus, Fas is regulated in T cells in a fundamentally different way from its ligand, and this includes the effect of TGF-β1. However, in contrast to T cells, progenitors of dendritic cells show reduced Fas expression when cultured with TGF-β1 (6). Whether or not this is related to a requirement for c-Myc or some other TGF-β1-regulated factors is not known.

In our studies, the inhibition of FasL by TGF-β1 in T cell hybridomas correlated with a decrease in c-myc mRNA levels. This is in agreement with previous studies showing that TGF-β1 is a potent inhibitor of c-Myc expression in various cell types and in particular in mature T cells (27–29, 46). Our experiments using ectopic expression of the chimeric Myc-ER protein have now formally shown that the inhibition of FasL induced by TGF-β1 is a direct consequence of its downregulation of c-Myc expression.

The c-myc oncogene has been implicated in the control of cell proliferation and differentiation, as well as neoplastic transformation (47). Overexpression or inappropriate expression of the c-myc gene has been found to promote apoptosis in fibroblasts (48, 49). Recently, Hueber et al. (23) reported that c-myc-induced apoptosis of serum-starved fibroblasts requires the expression and function of Fas and FasL. Although they showed that this was associated with an increased susceptibility to Fas-induced apoptosis, they did not rule out a role for c-Myc in driving expression of FasL. We demonstrated previously that c-Myc function is required for AICD in T cells, using c-myc antisense oligonucleotides (21) and dominant negative mutants of Myc or Max, which antagonize the functional Myc/Max heterodimer (22). Our finding that TGF-β1 inhibits FasL expression via downregulation of c-myc mRNA expression strongly suggests that the requirement for c-Myc during AICD may be at the level of FasL expression.

There is evidence that proliferating T lymphocytes are more susceptible to AICD during the S phase of their cell cycle (50). Although TGF-β1 causes cell cycle arrest in G1 in some models (24–26) and can also block constitutive c-Myc expression in T cell hybridomas, surprisingly we did not find a significant difference in cell cycle distribution when we compared cells stimulated for 3–24 h in the presence or absence of TGF-β1. This observation could be explained by the fact that residual c-Myc expression may be sufficient for cell cycle progression but not for FasL expression. Nevertheless, our findings argue against the possibility that TGF-β1 regulates FasL expression and subsequent AICD via perturbation of the cell cycle. This result is consistent with the finding that TGF-β1 promotes effector T cell expansion in association with IL-2 in murine Th2 clones (14).

In contrast to T cells, TGF-β1 promotes apoptosis in immature B cell lines such as WEHI 231 or CH31 cells (51–54). Apoptosis induced by TGF-β1 in these cells is preceded by a decline in c-Myc expression, and stabilization or ectopic expression of c-Myc prevented this cell death (52, 55). More recently, Arsuria et al. (51) demonstrated that inhibition of c-Myc expression and subsequent apoptosis in B cells after TGF-β1 treatment were a consequence of the transcriptional activation of IkBα, which prevented the direct regulation of c-Myc expression by NF-κB. Whether this plays a role in TGF-β1-induced downregulation of c-Myc in T cells is unknown.

The relevance of the antiapoptotic effect of TGF-β1 might be associated with the observation that addition of this cytokine together with IL-2 not only blocked AICD but also enhanced effector T cell expansion and long-term T cell survival (13, 14). These results strongly suggest that TGF-β1 may be one of the cytokines involved in T cell memory generation. The persistence of memory T cells has been suggested to be dependent on periodic restimulation by specific or cross-reacting antigens (56, 57). TGF-β1 might be necessary for inhibiting FasL expression, thus permitting the generation and survival of memory T cells (15).

The roles played in the development and function of the immune system by c-Myc and FasL, under the control of TGF-β1, are likely to be diverse. TGF-β1-deficient mice have smaller spleens with less distinct white pulp and fewer Peyer’s patches with less distinct germinal centers than those of normal littermates (58), and it is thus possible that this is a consequence of deregulated FasL expression, normally controlled by this cytokine.

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Address correspondence to Douglas R. Green, Division of Cellular Immunology, La Jolla Institute for Allergy and Immunology, 10355 Science Center Rd., San Diego, CA 92121. Phone: 619-558-3500; Fax: 619-558-3526; E-mail: dgreen5240@aol.com

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