

A NOVEL ADDITION TO THE T CELL REPERTORY
Cell Surface Expression of Tumor Necrosis Factor/Cachectin
by Activated Normal Human T Cells

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Tumor necrosis factor/cachectin (TNF), initially perceived as a secretory product of activated macrophages, is a potent proinflammatory polypeptide (1, 2). A wide range of biological outcomes have been ascribed to this hormone that is now recognized to be secreted by additional cells of the immune system, including T cells (3). TNF, on the one hand, is thought to contribute to the host armamentarium in the arena of defence against infectious agents and in the control of tumor growth. On the other hand, TNF has also been implicated as a major mediator of septic shock and of cachexia associated with chronic disease states. A possible reason for the differential outcomes might reside in the quantitative rather than qualitative aspects of TNF physiology: "appropriate" amounts of TNF being protective to the host and an excess being deleterious. A qualitative explanation might be that the cell-bound rather than secreted version of TNF is advantageous to the host. In this line of reasoning, we have examined whether activated normal human T cells display TNF on their cell surface. Our line of enquiry was prompted also by an earlier report of induced human monocytes expressing TNF on their cell surface (4).

Our first demonstration that normal human T cells, signaled with a synergistic combination of 12-*o*-tetradecanoyl phorbol-13-acetate (TPA) and ionomycin, display TNF on their cell surface forms the basis of this report. Furthermore, we show that the cell surface TNF is ~26 kD, and that cyclosporine (CsA) and methylprednisolone (MP), block the expression of TNF in T cells at a pretranslational site (by Northern analysis for TNF-specific transcripts). Our observations confirm and extend the observation that induced human monocytes express a membrane version of TNF (4), and are in broad agreement with an elegant study (published while this manuscript was in preparation) that murine cytotoxic cell lines express a membrane-bound cytotoxin that is immunologically related to TNF (5). More importantly, our data

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provide a mechanism by which the effects of TNF can be realized locally in an antigen-specific fashion.

Materials and Methods

Isolation and Activation of T Cells. Highly purified T cells were isolated from normal human PBMC with a sequential four-step procedure: depletion of monocytes by sephadex-G 10 gel filtration and by L-leucine methyl ester (5 mM; Sigma Chemical Co., St. Louis, MO) treatment, depletion of residual DR⁺ cells and NK cells by pretreatment with anti-DR and anti-NKH-1 mAbs, by "panning" the antibody-labeled cells in petri dishes coated with goat anti-mouse IgG, and utilization of SRBC-rosetting method as the final preparative step. FACS analysis of cells isolated revealed >99% of cells to be positive for the CD2 antigen and <1% of cells to be positive for the DR, NKH-1, or monocyte (M3) antigens.

T cells were activated with TPA (1 ng/ml) and the calcium ionophore, ionomycin (0.5 μ M). The cells (10⁶ cells/ml) were suspended in RPMI 1640 medium supplemented with 5% heat-inactivated autologous or pooled human AB serum, and antibiotics, and were incubated at 37°C in 5% CO₂, 95% air humidified atmosphere.

FACS Analysis for Cell Surface TNF. T cells were incubated with murine anti-human TNF mAb (SDW-18.1.1, ATCC cell line, HB9228) for 45 min at 4°C, washed twice in PBS, and stained with FITC-conjugated goat anti-mouse IgG1 antibody. A purified mouse monoclonal IgG1 was used as a relevant isotype control. The percentage of cells positive for cell surface TNF and the logarithmic mean channel fluorescence intensity of TNF-positive cells were determined by analysis of immunofluorescence histograms, with Epics C multi-data acquisition and display system, software version 3.1, as described (6).

Cell Surface Labeling of Intact Cells. Stimulated or unstimulated T cells were incubated on ice for 20 min in glass vials coated with lodogen, prepared as described by supplier (Pierce Chemical Co., Rockford, IL), to which 1 mCi ¹²⁵I had been added, and the reaction was terminated by removing the cells from the vials. Cells were washed with 150 mM NaI and then three times in cold, phosphate-buffered NaCl (150 mM). The cells were lysed by the addition of 1 ml of lysis buffer (50 mM Tris, pH 7.4, 1% NP-40, 0.25% Na deoxycholate [DOC], 150 mM NaCl, 5 mM EDTA, and 1 mM PMSF). Nuclei and other cellular debris were removed by centrifugation. 10 μ l murine anti-TNF mAb preabsorbed to protein A-sepharose was added to the cleared lysates, and immunoprecipitation was performed for 2 h at 4°C. The immunoprecipitates were washed three times in cold RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 1% DOC, 0.1% SDS), solubilized in SDS-PAGE loading buffer, and subjected to electrophoresis in a 12% polyacrylamide gel. The gel was fixed, dried, and exposed to film for 4 d with an intensifying screen.

Northern Blot Analysis. Total RNA was extracted from T cells by the guanidine isothiocyanate/cesium chloride method and quantitated by absorbance at 260 nm. RNA samples (10 μ g) were subjected to electrophoresis through a 1.2% agarose/2.2 M formaldehyde gel, transferred onto a nylon membrane by capillary action, and baked at 80°C for 2 h. After prehybridization, the blot was allowed to hybridize with a ³²P nick-translated pUC-9 plasmid containing the mouse cachectin complementary DNA insert (7). Hybridization was carried out at 42°C for 12 h in the presence of 50% formamide, 5 \times SSC, 0.1% SDS, and 2.5 \times Denhardt's. The membrane was washed in 0.1 \times SSC, 0.1% SDS at 42°C, and exposed to Kodak film at -70°C for 48 h using an intensifying screen.

Results and Discussion

Activated Normal Human T Cells Express TNF on their Cell Surface. T cells execute a variety of immune effector functions via a direct cell to cell contact. This unique functional attribute is critical to the T cell's antigen-specific immunosurveillance capabilities. Given T cells' ability to secrete TNF, and TNF's role in tumor lysis, we considered the possibility that activated T cells might be equipped with TNF on their cell surface.

We tested the hypothesis that stimulated normal human T cells express surface TNF by FACS analysis of highly purified T cells signaled with a synergistic combination of TPA, an activator of protein kinase C (PKC), and a highly specific calcium ionophore, ionomycin. Results from a representative experiment are shown in Fig. 1 to illustrate several consistent findings: (a) activated normal human T cells express TNF on their cell surface; (b) TPA and ionomycin are synergistic in promoting cell surface expression of TNF; and (c) neither activation of PKC alone with TPA nor mobilization of calcium alone with ionomycin is sufficient for the induction of cell surface expression of TNF in T cells.

Several features of our experimental approach are worthy of emphasis: (a) use of normal human T cells rather than T cell clones or lines for the detection of a cell surface version of TNF; (b) use of highly purified T cells preparations, obviating the possibility of monocyte contamination of the T cell samples. This is an important consideration in light of an earlier demonstration of a cell surface version of TNF in human monocytes (4); (c) use of a mAb rather than polyclonal antibody directed at human TNF for the demonstration of cell surface TNF; and (d) use of an isotypic control mAb during FACS analysis to distinguish nonspecific (Fc receptor-related) fluorescence signal from the specific fluorescence signal resulting from the binding of anti-TNF mAb to the cell surface TNF.

T Cell Surface TNF Is ~26 kD. Kriegler et al. (4) have shown that a TNF-producing cell line has a membrane version of TNF of ~26 kD (size predicted by TNF cDNA). Herein, we have determined the relative molecular mass of cell surface TNF displayed by stimulated normal human T cells.

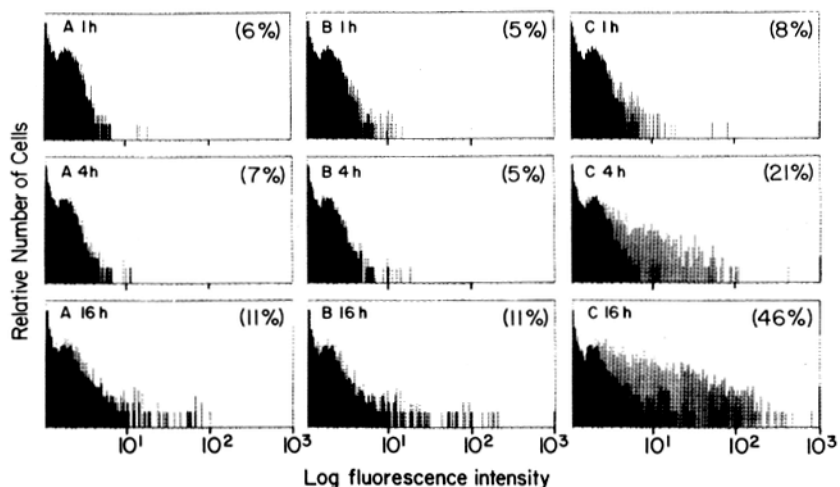


FIGURE 1. Activated T cells express TNF on their cell surface. Highly purified normal human T cells were incubated for 1, 4, or 16 h with 1 ng/ml TPA alone (A), 0.5 μ M ionomycin alone (B), or TPA + ionomycin together (C). FACS analyses is shown after labeling of T cells with IgG1 anti-TNF mAb and FITC-conjugated goat anti-mouse IgG1. The percentage of T cells positive for cell surface TNF (numbers in each panel) was determined by analysis of immunofluorescence histograms using Epics C multidata acquisition and display systems, software version 3.1. T cells incubated alone in the absence of TPA or ionomycin, and for similar duration, and then labeled with IgG1 anti-TNF mAb and FITC-goat anti-mouse IgG1 were used to determine the background fluorescence signal.

T cells triggered with TPA and ionomycin or unstimulated T cells were surface labeled with ^{125}I , lysed, and the cell lysates were immune precipitated with mAbs directed at TNF. Results from this experimental approach are illustrated in Fig. 2. It is evident that T cells signaled with TPA and ionomycin, and not unstimulated T cells, express the 26-kD polypeptide (Fig. 2, lane 2 vs. 1). Immune precipitation with anti-TNF mAb of cell lysates of stimulated T cells resulted also in the detection of two additional proteins of higher molecular mass, one polypeptide in the range of 50–60 kD, and the other in the range of 70–80 kD. In this context, it is worth noting that Liu et al. (5) have recently detected a 50–60-kD polypeptide in a murine cytotoxic T cell line by surface iodination and immune precipitation with polyclonal anti-TNF antibody. Also, the active form of TNF is thought to be a trimeric molecule formed by an unusual arrangement between individual subunits of TNF (8).

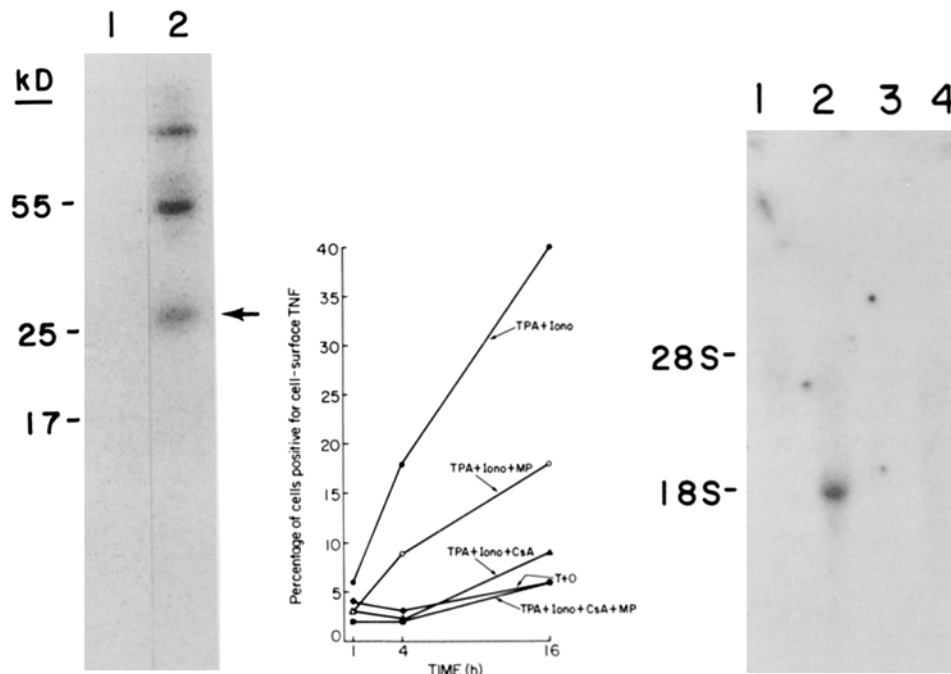
The secreted form of TNF is ~ 17 kD. The lack of a polypeptide with the relative molecular mass of 17 kD in our immune precipitates of stimulated T cells argues strongly against the possibility that the cell surface TNF identified in our studies is due to the secreted form of TNF that has been adsorbed to the T cell surface. Moreover, TPA and other activators of PKC prevent the binding of TNF to its receptor (9), and our experimental conditions (use of TPA to generate one of the signals required for T cell activation) negate the possibility that the secreted TNF bound to the T cell surface via the TNF receptor is responsible for the display of TNF by activated T cells.

CsA or MP Inhibit T Cell Surface Expression of TNF. Corticosteroids inhibit the secretion of several monocyte-derived inflammatory polypeptides, including TNF (7). CsA, a potent immunosuppressant, prevents the production of T cell-derived lymphokines but not the secretion of IL-1 or TNF by monocytes (10). It was therefore of interest to determine the effect of steroids and CsA on the expression of cell surface TNF by stimulated T cells.

Fig. 3 illustrates the inhibitory effects of CsA and MP on cell surface expression of TNF by T cells activated with TPA and ionomycin. At all time intervals tested, the immunosuppressants used clearly reduced the cell surface expression of TNF. The ability of CsA to prevent cell surface expression of TNF was also evident by radioiodination and immune precipitation of stimulated T cells (data not shown).

Transcriptional Activation of the TNF Gene in Stimulated T Cells and Reduction of TNF mRNA by CsA and MP. Transcriptional, as well as post-transcriptional, regulation of TNF expression have been reported. Beutler et al. (7) have demonstrated that dexamethasone prevents the increase in TNF mRNA content resulting from stimulation of murine macrophages with endotoxin. Granelli-Piperno et al. (10), on the other hand, found that CsA failed to prevent TNF mRNA accumulation in human monocytes triggered with TPA and Con A. Given our observation that CsA prevents the cell surface expression of TNF, it was of interest to locate the sites of action of CsA with respect to TNF gene expression and compare it with that of corticosteroids.

Fig. 4 summarizes the induction of TNF mRNA production in T cells activated with TPA and ionomycin, and the inhibitory activity of CsA and MP. Northern blot analysis revealed: (a) lack of TNF mRNA in unstimulated T cells (Fig. 4, lane 1); (b) accumulation of TNF mRNA in T cells triggered with TPA and ionomycin (lane 2); and (c) reduction of TPA and ionomycin induced TNF mRNA accumula-



FIGURES 2-4. (Fig. 2, left) Presence of 26-kD TNF on the surface of activated normal human T cells. Surface labeling and immune precipitation with anti-TNF mAb of cell lysates of T cells incubated alone for 16 h (lane 1) or T cells stimulated with TPA (1 ng/ml) and ionomycin (0.5 μ M). The 26-kD polypeptide, a size predicted by TNF cDNA and found only in stimulated T cells, is indicated by the arrow. (Fig. 3, middle) CsA and MP inhibit cell surface expression of TNF. The percentage of T cells expressing cell surface TNF is shown, as determined by FACS analyses of cells labeled with IgG1 anti-TNF mAb and FITC-goat anti-mouse IgG1. Nonspecific fluorescence was determined by labeling cells with IgG1 control mAb and FITC-goat anti-mouse IgG1 (TPA, 1 ng/ml; ionomycin, 0.5 μ M; CsA, 100 ng/ml; and MP, 100 μ M). (Fig. 4, right) Transcriptional activation of TNF gene and the inhibitory effect of CsA and MP on TNF mRNA accumulation. Total cellular RNA was isolated from T cells and assayed for TNF mRNA by Northern blot analysis. Test conditions: lane 1, T cells incubated alone; lane 2, T cells + TPA and ionomycin; Lane 3, T cells + CsA + TPA and ionomycin; Lane 4, T cells + MP + TPA and ionomycin. 18s and 28s indicate positions of ribosomal RNA (TPA, 1 ng/ml; ionomycin, 0.5 μ M; CsA, 100 ng/ml; MP, 100 μ M).

tion by CsA (lane 3) and by MP (lane 4). These results suggest that CsA or MP inhibit T cell surface expression of TNF at a pretranslational site.

Summary

Expression of the pluripotent molecule TNF in a focused and antigen-restricted fashion might provide an advantage to the host organism. Given the central role of T cells in antigen-specific immunity, we examined whether activated T cells express TNF on their cell surface. FACS analysis of highly purified normal human T cells labeled with an anti-TNF mAb revealed that T cells express cell surface TNF when signaled with the synergistic combination of a calcium ionophore, ionomycin, and a protein kinase C activator, 12-*o*-tetradecanoyl phorbol acetate. Cell surface

radioiodination studies of stimulated T cells demonstrated the presence of 26-kD transmembrane protein, a size predicted by TNF cDNA and different from that of the 17-kD secreted TNF molecule. The induced cell surface expression of TNF could be blocked with cyclosporine and/or methylprednisolone, and Northern analysis for TNF-specific transcripts revealed that this inhibitory effect occurs pretranslationally. Our demonstration for the first time that stimulated normal human T cells display cell surface TNF provides a mechanistic basis for the realization of effects of TNF in an antigen-specific fashion.

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