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<https://escholarship.org/uc/item/12060932>

Journal

Cytokine, 4(5)

ISSN

1043-4666

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Publication Date

1992-09-01

DOI

10.1016/1043-4666(92)90083-4

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THE AUTOCRINE ROLE OF TUMOR NECROSIS FACTOR IN THE PROLIFERATION AND FUNCTIONAL DIFFERENTIATION OF HUMAN LYMPHOKINE-ACTIVATED T KILLER CELLS (T-LAK) IN VITRO

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The autocrine role of tumor necrosis factor α (TNF) in the proliferation and functional differentiation of human lymphokine-activated T-killer cells (T-LAK) in vitro was investigated. Human peripheral blood lymphocytes initially stimulated with IL-2 and phytohemagglutinin-P (PHA) for 48 h will proliferate for long periods in vitro in the presence of IL-2. These T-LAK cells have been shown to be 95% CD3 positive. Employing ELISA techniques, greater than 500 pg/ml of TNF was found to be released in the supernatants of these cells during the first 5 days of culture. However, the levels dropped to 100–200 pg/ml by days 7–10. T-LAK cells grown from days 7 to 10 in the presence of IL-2 and rabbit anti-TNF were significantly growth inhibited (up to 23%). The cytolytic activity of T-LAK cells grown from days 0 to 7 in the presence of anti-TNF was also decreased (up to 75%). Phenotypic analysis of these anti-TNF treated T-LAK cells revealed a decrease in CD8 expression (up to 12%) and increase in CD4 expression (up to 27%) when compared with control cells. The data suggest that TNF has a regulatory role in the growth and functional differentiation of these human T-LAK cells.

Lymphocytes collected from peripheral blood or tumor sites can be co-cultured with IL-2 to become lymphokine-activated killer cells (LAK),¹ or tumor-infiltrating lymphocytes (TIL),² respectively. These cells have the ability to lyse a wide range of tumor targets, and are currently being used in a variety of clinical trials. Because LAK and TIL cells have been found to secrete cytokines,^{3,4} study has focused on the role(s) that cytokines may play in the proliferation, differentiation and cytotoxic activity of these cells.

There are two general classes of LAK cells: those which originate predominantly from NK precursors

and those which have T-cell precursors. Lymphocytes from peripheral blood which are initially stimulated with phytohemagglutinin-P (PHA) and IL-2 become long term, non-genetically restricted lymphokine-activated T killer cells (T-LAK).^{1,5} These T-LAK cells are 95% CD3 positive by day 7 of culture, and their numbers can be greatly expanded by co-culture with IL-2 in vitro over long periods. They are currently being investigated for their effectiveness in the intralesional immunotherapy of patients with recurrent brain tumors.⁶ Dett et al. showed that upregulation of the expression of tumor necrosis factor α (TNF) and its receptors occurred when the cells were co-cultured with IL-2 plus:IL-1 β , IL-4 or IL-6.⁷

TNF is a pleiotropic cytokine produced predominantly by activated macrophages and to a much lesser degree by lymphocytes.^{8–10} TNF is known to first bind to specific cell surface receptors, two of which different have recently been cloned and sequenced.^{11,12} TNF has many biologic activities, including such diverse actions as cytotoxicity on transformed cells in vitro and in vivo, growth stimulation of fibroblasts, control of cell surface antigen expression, and immunoregulation of macrophages, neutrophils,

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Received 21 January 1992; accepted for publication 22 April 1992

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1043-4666-92/050391+06 \$08.00/0

KEY WORDS: TNF/LAK/growth/phenotype/cytolysis

B cells, T cells, and NK cells *in vitro*.¹³⁻¹⁹

This study examines the autocrine role of human TNF in the proliferation and differentiation of IL-2 stimulated human T-LAK cells *in vitro*. We found reducing the levels of endogenous TNF with polyclonal antisera to TNF affects the proliferation, differentiation and cytolytic activity of T-LAK cells. These data suggest that TNF may have important roles in both the growth and functional differentiation of IL-2 stimulated human T-LAK cells.

RESULTS

TNF Levels and Phenotypic Expression of Developing Human T-LAK Cultures

In-vitro PBMC were stimulated with PHA and co-cultured with IL-1 as described in the Materials and Methods. As shown previously,⁷ cell division began in 4-5 days and cells doubled every 20-24 h during the period of these studies. Phenotypic expression of CD3, CD4 and CD8 was measured by FACS analysis on days 2, 5 and 7. The results of a representative experiment are shown in Table 1. CD3 and CD8 expression increased (from 84% to 96%, and from 34% to 51%, respectively) during this culture period, while CD4 expression decreased from 55% to 40%. This pattern was seen in all cultures tested, although the absolute % of antigen expression differed from donor to donor. Supernatants from these cultures were assayed by ELISA for TNF on days 2, 5, 7 and 9. The results of a representative experiment are shown in Fig. 1. TNF was present in high concentrations (> 500 pg/ml) for the first 5 days of culture; however, levels dropped thereafter. This pattern was seen in each of at least 3 T-LAK cultures tested, although absolute cytokine concentrations varied from donor to donor.

The Effect of Reduced TNF Levels on T-LAK Cell Proliferation

Polyclonal rabbit anti-TNF or NRS was added to 7-day growing T-LAK cultures at final concentrations of 1:200, 1:2000 and 1:20 000. After 4 days at 37°C,

the resulting viable cell numbers were calculated and compared with NRS-treated control cultures. Figure 2 shows the results of a representative experiment and is expressed as % growth inhibition. Anti-TNF dilutions of 1:200, 1:2000 and 1:20 000 inhibited T-LAK growth 23%, 9% and 3%, respectively, when compared to NRS cultures. Dye exclusion tests showed no significant viability differences between cultures incubated with anti-TNF and those incubated with NRS (data not shown).

The Effect of Reduced TNF Levels on T-LAK Cytolytic Activity

T-LAK cells grown continuously from the start of culture (days 0-7) in the presence of 1:200 anti-TNF or NRS were tested for their capacity to induce cytolysis of K562 cells *in vitro* on day 7 of cul-

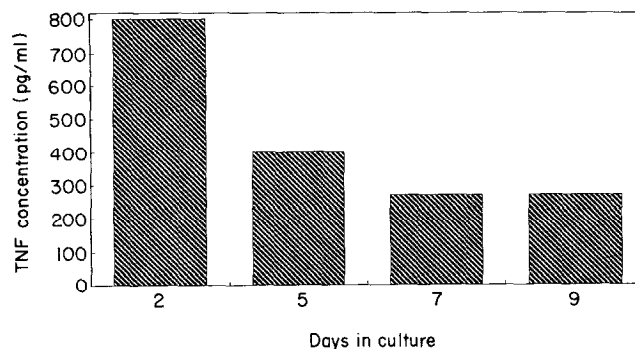


Figure 1. TNF production by IL-2 stimulated human T-LAK cells *in vitro*.

T-LAK culture supernatants were assayed by ELISA for TNF concentrations on days 2, 5, 7 and 9 of culture.

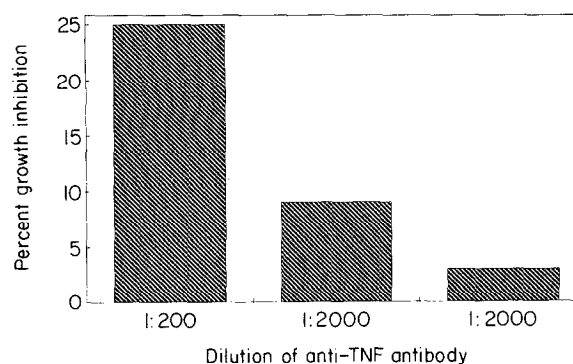


Figure 2. The effect of polyclonal anti-TNF on the proliferation of T-LAK cells.

Seven day T-LAK cultures were supplemented with polyclonal anti-TNF at final dilutions of (A) 1:200, (B) 1:2000 or (C) 1:20 000. Following a 3-day incubation with these antibodies, viable cell numbers were calculated and compared to cultures supplemented with NRS. Data are expressed as % growth inhibition.

TABLE 1. Phenotype expression developing T-LAK cells *in vitro*.

Antigen	Day 2	Day 5	Day 7
CD3	84	95	96
CD4	55	47	40
CD8	34	44	51

T-LAK cells were analyzed by FACScan flow cytometry for expression of surface antigens on days 2, 5 and 7 of culture. Results are expressed as % positive cells.

ture. The data presented in Fig. 3 are representative of the results obtained from three separate experiments, conducted with T-LAK cells from three different donors. The cytolytic activity of T-LAK cells which were treated with anti-TNF were inhibited from 17–45% at effector/target (E/T) ratio of 25:1 and 45–75% at E/T ratio of 10:1. The percentage of inhibition of cytolysis is even higher at lower E/T ratios (data not shown). In additional studies, we found almost the same degree of suppression of cytolytic activity when these cells were tested on another target cell line, RAJI.

The Effect of Reduced TNF Levels on the Development of Cell Phenotype

The phenotype of T-LAK cells grown continuously from the start of culture (days 0–7) with anti-TNF, or NRS (final dilution 1:200) were analysed on day 7. Figure 4 shows the data from 3 representative experiments from three different donors. Cultures maintained in anti-TNF serum showed a 5–27% increase in CD4 expression when compared with cells cultured in NRS. In contrast, CD8 expression was decreased by 8–12% when compared with NRS supplemented cultures.

DISCUSSION

The role of endogenously produced cytokines in the autocrine control of the proliferation, differentiation and effector function of IL-2 stimulated lymphocytes is an important issue to analyse. It has been previously reported that IL-2-stimulated human PBMC

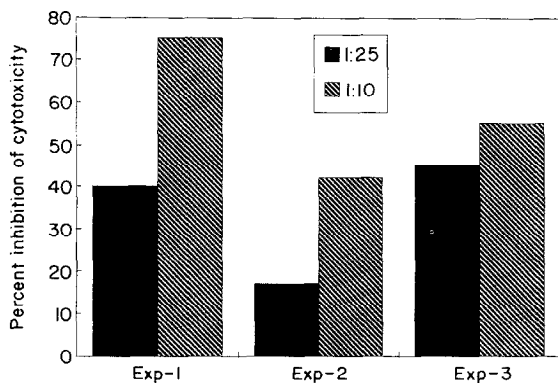


Figure 3. The effect of polyclonal anti-TNF on T-LAK cell cytolytic activity.

T-LAK cultures were supplemented with polyclonal anti-TNF at final dilution 1:200 from the start of culture (days 0–7), and were tested for their capacity to induce cytolysis of K562 cells in vitro. Data are expressed as % inhibition of killing as compared with cultures supplemented with NRS.

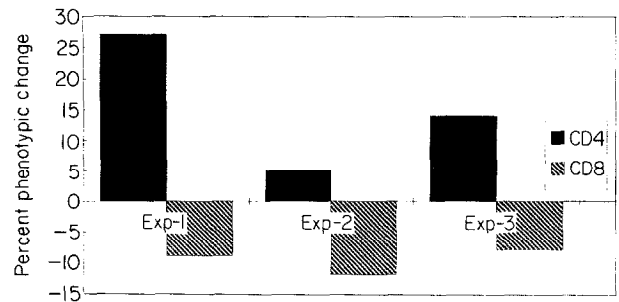


Figure 4. The effect of polyclonal anti-TNF on the expression of CD4 and CD8 phenotype in human T-LAK cell cultures.

T-LAK cultures were continuously supplemented with 1:200 polyclonal anti-TNF or NRS from days 0 to 7 of culture. Cells were then stained with FITC-labeled CD8 or CD4 and analyzed by FACScan.

cultures release various cytokines, including TNF, LT, IL-1 α , and IFN- γ .³ It is becoming apparent that TNF has a role in the maturation and expression of cytolytic activity of IL-2 stimulated human NK-derived LAK cells, and T-cell-derived tumor infiltrating lymphocytes (TIL) cells in vitro. Exogenous TNF has been found to synergize with low levels of IL-2 in the generation of LAK cell cytolytic activity in vitro.²⁰ This effect is thought to occur through the TNF-mediated upregulation of high affinity IL-2 membrane receptors, making these cells more responsive to IL-2. The increase in LAK cytotoxicity was not accompanied with changes in cell proliferation in these cultures, however. Recently, it has been reported that TNF receptors are involved in the autocrine control of both cytotoxicity and proliferation of IL-2 stimulated NK derived LAK cultures.²¹

Our studies are the first to examine the effects of endogenous TNF on the proliferation and differentiation of human (T-cell derived) T-LAK cells. In these cultures, we first upregulate IL-2 receptor expression on PBMCs with PHA and simultaneously co-culture with IL-2 for 48 h, then subsequent cultures are conducted in IL-2 alone. This results in a population of CD3 positive/CD16 negative T-LAK cells which will proliferate in the presence of IL-2 for long periods (up to 6–8 months) in vitro.^{6,22} We also detected that high levels of exogenous human recombinant TNF inhibit and low levels enhance the IL-2 driven cell proliferation in these cultures (data not shown). This result was further supported by the finding here presented that antisera which neutralize TNF bioactivity also inhibit the growth of T-LAK cells in these cultures. Preliminary data indicate that these anti-TNF treated cultures express increased 75 kDa TNF receptors and decreased IL-2 receptor levels compared with T-LAK cultures grown in NRS (data not shown). It should be noted that these cells produce

high levels of endogenous TNF which peak during the first 3-5 days of culture, and drop sharply thereafter. Accordingly, the inhibitory effects of anti-TNF sera were only noted 5 days after these cultures were induced, and after endogenous levels of TNF had declined to lower levels (data not shown). These results imply TNF had little or no growth regulatory effect during the first few days of culture, or that levels of TNF were so high that the antibodies were unable to completely neutralize TNF's proliferation effect.

We also found that endogenously produced TNF affects the expression of cytotoxic effector function of these T-LAK cells *in vitro*. Our data clearly indicate that lowering levels of endogenously produced TNF with polyclonal antibodies causes the downregulation of T-LAK cell killing activity. In addition, the presence or absence of TNF influenced the expression of CD8 and CD4 glycoproteins on the T-LAK cell surface. Cultures continuously supplemented with anti-TNF had decreased CD8 and increased CD4 expression by day 7 when compared with NRS treated control cells. Interestingly, preliminary work has shown that T-LAK cells grown in anti-LT (TNF-beta) have slightly lower cytotoxicity but the opposite effect on phenotype (increased CD8 and decreased CD4). Further studies are underway in our laboratory to investigate this apparently contrasting effect of these two largely homologous cytokines. The different levels of killing activity of these T-LAK cells may be due to selection of certain subsets present in the original PBMC culture. We find the addition of exogenous TNF had no effect on T-LAK phenotype when added after day 7 of culture (data not shown). These results suggest that TNF may also effect the differentiation and maturation of different populations of T-cells present in these cultures.

Collectively, these results suggest that while TNF is not a major lymphocyte product, it is important in the autocrine control of T-LAK cell growth, differentiation, maturation and expression of effector functional activity *in vitro*. Thus, IL-2 alone is not sufficient to induce functional activity but acts as a growth factor. It appears that endogenously produced TNF is needed for both the proliferation and differentiation of these cells *in vitro*. While the precise mechanisms operating *in vivo* are still unknown, these findings may be important in the understanding and eventual improvement of immunotherapies employing T-cell derived effector cells.

MATERIALS AND METHODS

Cytokines and Polyclonal Antibodies

Recombinant human tumor necrosis factor- α (TNF) (specific activity 1.0×10^7 U/mg) was supplied by

Genentech Corp. (South San Francisco, CA). Recombinant human IL-2 (specific activity 3.3×10^5 U/mg) was obtained from the Cetus Corp. (Emeryville, CA). Polyclonal antisera specifically reactive to human recombinant TNF was generated in New Zealand white rabbits after repeated injections according to the method of Vaitukaitis.²³ Each ml of these antisera were able to specifically neutralize 5×10^6 U of TNF cytolytic activity against the L-929 murine fibroblast line.

Collection of Human Peripheral Blood Mononuclear Cells (PBMC)

Human PBMC were collected from peripheral venous blood of normal donors which was defibrinated, diluted with an equal volume of phosphate-buffered saline (0.01 M phosphate 7.2 pH, 0.15 M NaCl) (PBS) and separated by density gradient centrifugation on 1.077 Histopaque (Sigma Chemical Co., St. Louis, MO) as described previously.²⁴ The PBMC were collected from the interface and further washed to remove the remaining Histopaque by alternate centrifugation ($500 \times g$ for 10 min) and resuspension in PBS. After the final wash, PBMCs were resuspended in AIM-V (GIBCO, Grand Island, NY) with 2% heat-inactivated fetal calf serum (FCS) (GIBCO) and the cells were counted using a Spotlite hemacytometer. These cells were 99 to 100% viable, as determined by the trypan blue exclusion test.

Generation of Human Lymphokine-activated T Killer Cells (T-LAK)

Human PBMC were initially cultured in AIM-V with 2% FCS supplemented with 400 U/ml of IL-2 and 0.4 μ g/ml phytohemagglutinin-P (PHA) (Sigma). The PBMC were cultured at a density of 2.0×10^6 cells/ml in 25 cm² tissue culture flasks in a manner similar to the method described by Ingram et al.⁶ After 48 to 72 h at 37°C, viable cells were counted and resuspended at 0.5×10^6 cells/ml in AIM-V with 2% FCS and 400 U/ml IL-2 (no PHA). These T-LAK cell cultures were then passed in these conditions every 48-72 h.

ELISA Assay for Human TNF

Supernatants were collected from three separate T-LAK cultures after 3, 5, 7 and 9 days in culture. ELISA assays for TNF (Endogen, Boston, MA) were performed according to manufacturer's instructions, and TNF levels were determined by comparison with TNF standards provided in these kits.

For determination of the effect of rabbit anti-TNF antibodies on the proliferation and differentiation of T-LAK cells *in vitro*, cell cultures were supplemented with dilutions of rabbit antisera (NRS or anti-TNF) of 1:200 (0.5% v/v), 1:2000, 1:20 000 for a 3-day interval (days 7-10) after the culture was established. At the end of this period, viable cell numbers were determined as described in Materials and Methods. Data were analyzed for % growth

inhibition according to the following formula:

$$\% \text{ growth inhibition} = [(\text{control cell number} - \text{test cell number}) / (\text{control cell number})] \times 100.$$

For determination of differentiation effects, cell cultures were supplemented with 1:200 NRS or anti-TNF every 48 h from days 0 to 7. At the end of this period, cell were analyzed for their phenotypic expression and cytolytic effectiveness as described below.

Cytolytic ^{51}Cr Release Assay

Cytolytic assays employing the non-adherent target cell K562 (American Type Culture Collection, Rockville, MD) were performed in 96-well round-bottomed microcytotoxicity plates as described previously.²⁵ Briefly, radiolabeling of the K562 target cells was accomplished by the addition of 100 μl of ^{51}Cr (NEN, Boston, MA) (specific activity 590 mCi/mg, 10 mCi/ml) to 10^6 cells in 1 ml of RPMI + 10% FCS for 18 h at 37°C. The cells were then washed three times by alternate centrifugations ($300 \times g$ for 5 min) and resuspensions in RPMI + 10% FCS. A constant number of labeled target cells (2×10^4 in 20 μl) was added to microtiter wells with various numbers of effector cells in 100 μl 10% RPMI to achieve different effector cell:target cell ratios (25:1, 10:1). Targets and effector cells were incubated at 37°C for 4 h in an atmosphere of 5% CO_2 . Cell-free supernatants were removed with Titerteks Supernatant Collection System (Skatron, Lier, Norway) and the amount of ^{51}Cr quantitated in an automated Clini-gamma counter (Pharmacia LKB Biotech Inc., Piscataway, NJ). The total ^{51}Cr releasable (90–95% of total counts) was determined by lysing the cells with 100 μl of 3% SDS. Spontaneous ^{51}Cr release was 1–2% per h. Percent specific cell lysis was determined by the following formula:

$$\% \text{ specific lysis} = [(\text{experimental release} - \text{spontaneous release}) / (\text{total release} - \text{spontaneous release})] \times 100.$$

Anti-TNF treated cultures were compared for their cytolytic effectiveness to NRS treated cultures; results are expressed as % inhibition of cytotoxicity as determined by the following formula:

$$\% \text{ inhibition} = [(\text{NRS control specific lysis} - \text{anti-TNF specific lysis}) / (\text{NRS control specific lysis})] \times 100.$$

Flow Cytometric Analysis

Cell suspensions were analyzed by FACScan (Becton Dickinson, Mountain View, CA). The FACScan was calibrated with the Autocomp program and optimized for each sample tested. Forward scatter thresholds were set in order to eliminate debris and dead cells. Lymphocytes were analyzed by staining the cells for surface antigens with the following fluorescein-labeled monoclonal antibodies obtained from Becton Dickinson: (a) CD3 (Pan T), (b) CD4 (helper-inducer), and (c) CD8 (cytotoxic-suppressor). Cells were incubated with monoclonal antibodies for 30 min on ice and washed twice with PBS with 0.2% sodium azide by alternate centrifugation ($500 \times g$ for 5 min) and resuspension. Cells were fixed in 1.0% paraformaldehyde in PBS and stored at 4°C, in the dark, until further analysis. For comparison of

NRS and anti-TNF treated cultures data are expressed as % phenotypic change according to the following formula:

$$\% \text{ phenotypic change} = (\% \text{ anti-TNF cells positive} - \% \text{ NRS cells positive}) / (\% \text{ NRS cells positive}).$$

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