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ENHANCEMENT OF LYMPHOKINE-ACTIVATED T KILLER CELL TUMOR NECROSIS FACTOR RECEPTOR mRNA TRANSCRIPTION, TUMOR NECROSIS FACTOR RECEPTOR MEMBRANE EXPRESSION, AND TUMOR NECROSIS FACTOR/LYMPHOTOXIN RELEASE BY IL-1β, IL-4, AND IL-6 IN VITRO

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Co-culture with IL-2 can induce human T lymphocytes to proliferate and become nongenetically restricted, lymphokine-activated killer (LAK) cells in vitro. Our studies were conducted with long term cultured, human T-LAK cells from peripheral blood, which are 95 to 99% CD3+. We found that proliferating 7- to 10-day human T-LAK cells express TNF, by using a 125I-TNF binding assay. Additional treatment of these cells with the cytokines IL-1β, IL-4, or IL-6 rapidly up-regulated 55-kDa TNFR mRNA transcription and doubled TNFR membrane expression. Further studies revealed that these cytokines also increased the release of TNF and lymphotoxin (LT). Antibody neutralization studies indicated that IL-1 induces release of both TNF and LT; however, IL-4 and IL-6 induce primarily LT release. These results further support the concept that these cytokines are involved in the regulation of TNF/LT release, TNF synthesis, and TNFR membrane expression. It is apparent that cytokines and their membrane receptors are involved in the autocrine/paracrine control of T cell proliferation, differentiation, and expression of functional activity after IL-2 stimulation in vitro.

The stimulation of PBMC from normal human donors with PHA for 2 days and co-culture with IL-2 (1) leads to the generation of long term, proliferating, nongenetically restricted T effector cells (2), which we have termed T-LAK3 cells. Kruse et al. (1) demonstrated that, by day 7, 90-99% of the cells in these cultures are proliferating CD3+ T cells. T-LAK cells are under current investigation for use in experimental intralesional immunotherapy of patients with recurrent brain tumors (1, 3). TNF and LT are cytokines produced by activated lymphoid cells (4, 5) that cause a wide range of biologic activities in vivo and in vitro. In vivo, TNF is known to be involved in inflammation (6), wasting, and shock (7). In vitro, TNF has direct cytotoxic effects on certain transformed cell lines (8) and is also known to stimulate fibroblast growth (9, 10) and regulation of cell surface Ag expression (11, 12). This cytokine also possesses multifunctional immunoregulatory activities (13), which include activation of NK cells (14) and macrophages (15) and induction of growth and function of B and T lymphocytes in vitro (13, 16).

The biologic effects of TNF/LT, both in vivo and in vitro, are initiated by their first binding to specific cell membrane receptors (17). It has recently been shown that mammalian cells can express one or both of two different TNF/LT receptors, one of 55 kDa (18) and one of 68 to 75 kDa (19). These membrane proteins show some homology in their extracellular domains, but their intracellular domains are totally different in amino acid sequence. The fact that they are two different receptors was not detected by almost all previous investigators. It has been demonstrated that human T-LAK cells express the TNFR but unstimulated T cells do not (13, 20). However, it is not yet known whether these cells express one or both receptors. It has also been shown that TNF and other cytokines are released by human T cells after activation in co-culture with IL-2 in vitro (21). Furthermore, it is known that TNF and IL-2 synergize to augment proliferation of T cells (13) and cytotoxic activity of human T-LAK cells (22, 23). It is becoming apparent that released cytokines and their membrane receptors are involved in T cell proliferation, differentiation, and expression of cytolysis in vitro. We were interested in further examining the role of cytokines and their membrane receptors in the differentiation of T-LAK cells in vitro.

We established the baseline expression of the TNFR on T-LAK cells and then determined the effects of selected cytokines on mRNA transcription of the 55-kDa TNFR, membrane TNFR expression, and TNF/LT release. We found that cytokines IL-1β, IL-4, and IL-6 up-regulate 55-kDa TNFR transcription. TNFR expression, and TNF/LT release in human T-LAK cells in vitro. TNF and LT may be important immunoregulatory molecules in the function and growth of T-LAK cells.
IL-1β, IL-4, AND IL-6 UP-REGULATE TNFR ON T-LAK CELLS

MATERIALS AND METHODS

Cells, cytokines, and reagents. L929 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS (GIBCO Laboratories Life Technologies, Inc., Grand Island, NY). Human PBL were cultured in AIM-V (GIBCO) supplemented with 2% FCS. rTNF/ LT and rIL-1β were supplied by Genentech Corp. (South San Francisco, CA). rIL-2 was obtained from the Cetus Corp. (Emeryville, CA). rIL-4 and rIL-6 were obtained from Genzyme (Boston, MA). BoltonHunter reagent was purchased from DuPont (Wilmington, DE). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Collection of human PBMC. Human PBMC were collected from peripheral venous blood of normal donors, which was defibrinated, diluted with an equal volume of PBS, and separated by density gradient centrifugation on 1.077 Histopaque (Sigma). The PBMC were collected from the interface of the plasma and Histopaque and for medium supplemented with rTNF at 37°C. A cell sample in trypan blue was counted on a Spotlite hemacytometer and resuspended in complete medium, which contained AIM-V with 2% FCS plus 400 U/ml rIL-2. These T-LAK cell cultures were recultured every 48 h in fresh complete medium, at a density of 0.5 x 10⁶ cells/ml.

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. Particles with unusually low forward and side scatter were considered to be nonviable and were not counted. Surface phenotypes on lymphocytes were analyzed by staining the cells for surface Ag. L929 cell culture supernatants were assayed for TNF or LT activity against the L929 mouse fibroblast cell line by the method of Gatagan et al. (24). Briefly, L929 cells (8 x 10⁶ cells/well), culture supernatant, and actinomycin D (0.2 µg/well) were incubated at 37°C for 18 h in 96-well flat-bottom microtiter plates, in 200 µl/well RPMI 1640 medium supplemented with 10% FCS. After incubation, the supernatants were aspirated and the plates were stained with 1% crystal violet for 5 min at room temperature. Excess dye was rinsed from the plate with water, and the plate was allowed to dry and was solubilized with 100 µl of modified methanol (100 mM HCl in methanol). Absorbance was measured at 600 nm. The amount of TNF and LT bioactivity was determined by comparing activity with a standard curve made with rTNF-α and rLT (Genentech). Antibody neutralization studies were carried out in the same manner; however, antiserum was added in conjunction with the culture supernatants. Immune polyclonal antisera were generated against human rLT and rTNF in NZW rabbits, by the method of Vaitukaitis et al. (25).

Southern blot analysis. The T-LAK cell RNA was isolated from T-LAK cells by the guanidinium isothiocyanate/cesium chloride method and was quantitated spectrophotometrically. The integrity of the RNA was confirmed as previously described (25). The mRNAs were hybridized with 32P-labeled TNFR-specific synthetic probe. After hybridization, the blots were then exposed to X-Omat XAR-5 (Kodak Co., Rochester, NY) X-ray film at -70°C.

RESULTS

Expression of T lymphocyte surface phenotypic markers by 7–10-day T-LAK cells. T-LAK cells were generated as described in Materials and Methods and were subjected to phenotypic analysis on day 7 and day 10 of culture. Shown in Figure 1 are the data obtained from phenotyping of one individual donor; however, these results are representative of data from all normal individuals (10 subjects) examined. These data reveal that CD3+ T cells dominate (90–95%) the culture by days 7 to 10 and, although not shown, remain the predominant cell type throughout the culture period (days 15 to 21). The ratio of CD4+ to CD8+ cells in these cultures from a single donor remained relatively constant; however, they vary from individual donor to donor.

Capacity of IL-1β, IL-4, and IL-6 to induce the release of TNF/LT by T-LAK cells in vitro. T-LAK cells were incubated with the cytokines IL-1β, IL-4, or IL-6 (in addition to IL-2) for 24 h, and then cell-free supernatant cytotoxic activity was measured in the L929 assay, as described in Materials and Methods. Supernatants from cells incubated with cytokines for a period of 4 h did not

![Figure 1. Phenotypic analysis of day 7 and day 10 T-LAK cells. T-LAK cells were phenotypically analyzed by staining (the cells for surface Ag, with mAb to CD3 (pan T), CD4 (helper-inducer), and CD8 (cytotoxic-suppressor). Data are expressed as percentage of positive cells for the corresponding surface Ag.](image)
show any cytolytic activity. Figure 2 shows that supernatants from T-LAK cells incubated with these cytokines for 24 h produce cytotoxic activity against L929 cells. Supernatants from T-LAK cells incubated with IL-4 produced the highest amount of cytotoxic activity, followed by IL-6 and IL-1β. However, control T-LAK cells incubated with IL-2 alone produced much less. Additional studies revealed that the cytotoxic activity of all T-LAK supernatants was blocked 75 to 95% by the anti-LT antiserum, and 45% of the cytolytic activity produced by IL-2- and IL-1β-stimulated cells was neutralized by anti-TNF antiserum. Thus, the majority of the cytolytic activity of these supernatants was due to LT and not TNF.

Effects of IL-1β, IL-4, and IL-6 on the expression of the TNFR on T-LAK cells. The expression of TNFR on human T-LAK cells (days 7 to 17) was examined in a 125I-TNF-binding assay, as described in Materials and Methods. The expression of TNFR was examined at 4 and 24 h after incubation with the cytokines IL-1β, IL-4, and IL-6 (in addition to IL-2). Results from one experiment are shown in Figure 3, A and B. Control T-LAK cells (cultured with IL-2 alone) expressed 674 receptors on day 10 of culture (Fig. 3B); however, when these cells were incubated with IL-1β, IL-4, or IL-6 (in addition to IL-2) for 4 h, an increase of 1341, 1241, and 1172, respectively, in receptor number (74 to 99%) was detected. The apparent Kd values were $1.8 \times 10^{-10}$ M for IL-2, $1.3 \times 10^{-10}$ M for IL-1β, $1.4 \times 10^{-10}$ M for IL-4, and $1.1 \times 10^{-10}$ M for IL-6, as determined by Scatchard analyses. There were clearly no significant changes in receptor affinity. When T-LAK cells were treated with these cytokines for 24 h, no further up-regulation of TNFR was detected (data not shown). A slight variation in receptor number was noted in experiments conducted on cells from different donors; however, results from multiple experiments were essentially identical.

Effects of IL-1β, IL-4, and IL-6 on 55-kDa TNFR mRNA expression by T-LAK cells. Human T-LAK cells were exposed to the different cytokines and then tested for 55-kDa TNFR mRNA expression by Northern blot analysis, as described in Materials and Methods. Results in Figure 4A demonstrate that T-LAK cells cultured with IL-2 expressed mRNA for the 55-kDa TNF/LTR. However, after a 4-h incubation with IL-1β, IL-4, or IL-6 (in addition to IL-2), an up-regulation in 55-kDa mRNA was evident. Duplicate samples were run on the same gels to permit quantitation of the amount of RNA loaded/lane. The gel was cut and stained with ethidium bromide, to ensure the quantity of samples loaded.
The generation of T-lymphokine-activated killer cells, expressing T cell phenotypes, from human peripheral blood has been described (31). These non-MHC-restricted T-LAK cells exhibit a broad range of cytotoxic activity against panels of target cells in vitro [2]. Whereas IL-2 stimulates proliferation of T-LAK cells, other cytokines, such as TNF, have been shown to be involved in the expression of cytolytic activity in vitro [22, 23]. Little is known, however, with regard to the effects of various cytokines on the control of TNF/LT release and membrane receptor regulation.

Much effort is now being aimed at defining the function and regulatory activity of cytokine receptors on T lymphocytes. Others have suggested that up-regulation of TNF membrane receptors is involved in the autocrine/paracrine pathway operative after IL-2 stimulation and that interaction of TNF/LT with these receptors is important in T cell proliferation, differentiation, and expression of functional ability [13, 20]. Fresh unstimulated PBL do not express TNFR of any type [13, 20]. Although it is not yet apparent which class of receptors are expressed, Owen-Shaub et al. [20] demonstrated that lymphocyte TNFR expression is regulated by IL-2 concentration in vitro. However, although the presence of surface TNFR on activated T-lymphocytes has been established, the mechanisms of receptor regulation are unknown.

It has been shown that various cytokines can influence both proliferation and expression of T cell function. IL-1β, IL-4, and IL-6 have been shown to induce human and murine thymocyte proliferation (32–34). Suda et al. [34] reported that IL-4 induced murine T cell proliferation and IL-1β together with IL-6 enhanced proliferation induced by IL-2. It is also known that activated T cells have the capacity to produce IL-2, IL-4, IL-6, and TNF/LT and that these cytokines are involved in an autocrine loop of T cell expression of cytotoxicity and proliferation in vitro (22, 23, 35).

We report here that TNFR number, 55-kDa mRNA expression, and TNF/LT release can be up-regulated, in response to IL-1β, IL-4, and IL-6 incubation, on human T-LAK cells co-cultured with IL-2. Although these studies do not distinguish whether up-regulation of receptors involves 55- or 75-kDa receptors, or both, we noted dramatic up-regulation within 4 h. In fact, by Scatchard analysis the number of sites/cell for IL-1β, IL-4, and IL-6-treated cells increased almost 2-fold. In addition, we found that 4-h incubation of day 10 T-LAK with IL-1β, IL-4, and IL-6 resulted in an up-regulation of 55-kDa TNFR mRNA expression. This finding agrees with the Scatchard analysis data, in that TNFR mRNA enhancement correlates with an increase in TNFR membrane expression. It is interesting that opposite results are observed with stimulated human monocyte-like cell lines; namely, LPS, PMA, and IL-1β rapidly down-regulate TNFR membrane expression. It is also interesting to note that T-LAK cell TNF/LT release was not apparent at 4 h but was up-regulated by each cytokine after 24 h. IL-4 was the most effective in up-regulating TNF/LT release. Both IL-2 and IL-1β induced about equal amounts of TNF and LT; however, IL-4 and IL-6 induced predominantly LT. It is evident and perhaps slightly significant that there is some selective induction of LT/TNF by these cytokines.

The evidence presented here further supports the concept that IL-2 is sufficient to induce T cell proliferation but is not the sole cytokine involved in the regulation of TNF/LT release or TNFR synthesis and membrane expression. IL-1β, IL-4, and IL-6 can also be involved in the up-regulation of the TNFR at the level of both mRNA transcription for the 55-kDa receptors and membrane expression. In the latter case, it is not yet clear whether this is selective expression of 55-kDa or 75-kDa receptors or up-regulation of both. It is not yet clear whether the T-LAK cell can express the 75-kDa receptor. The exact mechanism involved in the immunoregulation and differentiation of T-LAK effector cells and the specific role of the TNFR is unclear; however, it is apparent that cytokines are important in regulating the magnitude of the immune response.

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IL-1β, IL-4, AND IL-6 UP-REGULATE TNFR ON T-LAK CELLS


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