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Regulation of tumor necrosis factor and interferon-gamma release from lymphokine-activated killer cells

Ybarrondo, Ana-Belen, Ph.D.

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U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

REGULATION OF TUMOR NECROSIS FACTOR AND INTERFERON-GAMMA RELEASE FROM LYMPHOKINE ACTIVATED KILLER CELLS

by

Ana-Belen Ybarrondo

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and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

David E. Yocum — 11/16/90 Date

Kenneth J. Ryan — 11/16/90 Date

Charles R. Sterling — 11/16/90 Date

Evan M. Hersh — 11/16/90 Date

Date

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SIGNED: Ana-Belen Ybarrondo

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ABSTRACT

Peripheral blood lymphocytes cultured in interleukin 2 (IL-2) acquire the ability to lyse tumor cell targets in a non-major histocompatability complex (MHC) restricted manner. These lymphokine activated killer (LAK) cells release tumor necrosis factor-alpha (TNF) and interferon-gamma (IFN-γ) in culture and when stimulated by interaction with tumor cells *in vitro*. The capacity of several suppressive factors to affect the release of TNF and IFN-γ from LAK cells which have been stimulated with K562 erythroleukemia cells was investigated. A number of agents known to inhibit mononuclear phagocyte secretion of TNF were tested, including prostaglandin E_2 (PGE_2), alpha-globulins and the synthetic protease inhibitor tosyl-arginine methyl ester (TAME). The alpha globulins and TAME are thought to suppress TNF release from monocytes by inhibiting proteolytic cleavage of the cytokine from the cell surface. The addition of PGE_2, whole plasma alpha globulins, purified alpha-1-acid glycoprotein, and TAME inhibited TNF and IFN-γ release in a concentration dependent manner. These inhibitory factors appear to act directly on the lymphocytes to suppress cytokine secretion, as the presence of monocytes or metabolically active tumor cells was not required. The effects of alpha-1-acid glycoprotein on LAK cells were also investigated. The addition of this alpha globulin fraction inhibits the incorporation of ^{3}[H]thymidine by LAK cells stimulated with

tumor cells, and results in a detectable decrease in TNF mRNA. The capacity of alpha-1-acid glycoprotein to suppress the release of TNF production also resulted in an inhibition of LAK cell generation which was reversed by the addition of exogenous TNF. In contrast to their suppressive effect on peripheral blood monocytes, the protease inhibitors alpha-1-antitrypsin and alpha-2-macroglobulin enhanced TNF secretion from LAK cells exposed to leukemia cells. Analysis of cell surface TNF expression by fluorescence activated cell sorting (FACS) suggest that the observed differences in regulation reflect the capacity of particular phenotypes to express TNF as a transmembrane molecule. The data presented here indicate that the regulation of TNF release by LAK cells stimulated with tumor cells differs significantly from that previously observed in monocytes and suggest a regulatory role for alpha-1-acid glycoprotein in TNF secretion by LAK cells.

CHAPTER 1

INTRODUCTION

Background

Grimm et al. originally described the lymphokine activated killer (LAK) cell phenomenon in 1982 (40). Partially purified lectin-free supernatant from phytohemagglutinin (PHA) stimulated lymphocytes, containing interleukin 2 (IL-2), was used to activate peripheral blood leukocytes (PBL). The result of activation was the generation of effector cells with an enhanced capacity to lyse tumor cells *in vitro* (40, 70). Since the crude IL-2 preparations could contain a number of cytokines, a requirement for other factors in addition to IL-2 could not be ruled out. However, subsequent studies provided evidence that IL-2 alone was sufficient for direct activation of PBL. LAK cell stimulatory activity in crude IL-2 preparations could be adsorbed by an IL-2 dependent cell line and blocked with an antibody to the IL-2 receptor (42). Experiments with recombinant IL-2 (rIL-2) confirmed that IL-2 was the only stimulus necessary to generate LAK cell activity (104).

IL-2 may be responsible for the anomolous killers generated in various other culture systems as well, including effectors in

the allogenic mixed lymphocyte reaction (MLR) (122, 115, 75), lectin-activated peripheral mononuclear cells (PBMC) (74), and anti-CD3 activated PBMC (57). The spectrum of susceptible cell types of these anomolous killers is similar to that of LAK cells, and IL-2 is known to be produced under the culture conditions described. The lytic activity observed may be due to activation of LAK cells by endogenously produced IL-2.

IL-2 activated lymphocytes exhibit non-major histocompatability complex (MHC) restricted killing of autologous and allogenic tumor cells as well as modified autologous nontransformed cells, but not normal cells (43). LAK cell activity can be generated from peripheral blood leukocytes as well as from cells of the thymus, bone marrow, spleen, and thoracic duct fluid (41, 100). Development of this activity is dependent on proliferation (40). The LAK phenomenon is distinguished from cytotoxic T lymphocyte killing (CTL) on the basis of kinetics of development and activation signals. The LAK cell phenomenon has a more rapid kinetics of induction (2-3 versus 6-7 days), no known antigenic stimulus is required, and cytolysis is not MHC-restricted (41). NK killing of target cells is also non MHC-restricted, but in contrast to the LAK system, NK activity is present in fresh PBL, is augmented by IFN-γ, does not lyse solid tumors, and does not require an activation step (47).

Immunotherapy

The ability of rIL-2 to expand non-specific cytotoxic lymphoid populations *in vitro* has lead to new possibilities in the development of adoptive immunotherapy of cancer. The use of rIL-2-activated autologous peripheral blood lymphoid cells in addition to IL-2 or high doses of IL-2 alone have met with limited success in the treatment of metastatic and localized cancers (24, 105, 132). Anti-tumor responses have been seen in patients with metastatic renal cell cancer, melanoma, colorectal cancer and non-Hodgkin's lymphoma (106). Cohen et al. reported a greater than 50% reduction in measurable tumor volume in approximately 40% of the patients treated (24).

Unfortunately, the administration of IL-2 and activated LAK cells is associated with significant toxicity, primarily due to increased capillary permeability causing fluid extravasation and organ dysfunction (107). A better understanding of human LAK cell activation and target cell lysis as well as IL-2 induced toxicity may provide a rationale for the development of optimal adoptive immunotherapy protocols using LAK cells or rIL-2 in the treatment of cancer.

LAK Cell Generation

Although IL-2 is the only stimulus required for LAK cell activation, other cytokines may augment LAK cell induction and cytotoxicity. Tumor necrosis factor-alpha (TNF) is produced in

LAK cell culture (88, 35). Addition of high concentrations of neutralizing antibody to TNF has an inhibitory effect on development of cytotoxicity (88, 23). Evidence supports a functional interaction between TNF and IL-2 in the generation of LAK activity, which results in a reduction of the IL-2 concentration required for activation (35, 23, 88). IL-2 can induce TNF receptor expression on both large granular lymphocytes (LGL) (23) and T cells (110), and low concentrations of IL-2 are sufficient for development of LAK activity from LGL if exogenous TNF is added (23). The synergy of IL-2 and TNF in the induction of LAK cells is not blocked by neutralizing antibodies to IL-1-α, IL-1-β, or IFN-γ, suggesting a direct effect of TNF and IL-2 on LGL (23). Itoh et al. provide evidence that endogenously produced IFN-γ plays a role in LAK generation (52). They have shown that IFN-γ is produced in LAK culture, and anti-IFN-γ antibody has an inhibitory effect on development of cytotoxicity in response to IL-2 (52). However, there are conflicting reports that the addition of interferon does not affect LAK cell development (28, 50). Addition of IL-1, a macrophage product, may have an enhancing effect on LAK generation (51). There is evidence that murine IL-4 alone can mediate induction of LAK activity of murine splenocytes against fresh tumor cells and can augment generation of LAK activity induced by IL-2 (80). However, Widmer and colleagues have reported that IL-4 has no detectable LAK cell-inducing activity for human PBMC (133).

They showed that when present together with IL-2 at the initiation of culture, IL-4 inhibited the development of effector cells (133).

LAK cell activation by IL-2 can be down regulated by several factors. Normal human monocytes/macrophages have an inhibitory effect on LAK cell induction in the early phase (51, 119), but potentiate it when added 1 day after the start of culture (119). Inhibitory activity of monocytes is most likely due to the production of prostaglandin E_2 (PGE_2) . The down regulatory effects of adherent cells are reversed by addition of indomethacin, which blocks PGE_2 synthesis (51). Espevik et al. have shown that addition of transforming growth factor-β-1 (TGFβ-1) significantly suppresses LAK generation, inhibits production of IFN-γ, TNF and lymphotoxin (LT) in a dose dependent manner, and can reverse the augmentation of LAK cell generation by TNF (35). The addition of TGFβ-1 with rIL-2 at the initiation of culture significantly inhibits the generation of LAK cell activity, while only minimal inhibition is seen when TGFβ-1 is added 24 hours or more after IL-2 addition, suggesting that TGFβ-1 affects an early stage of LAK cell development (35).

LAK Cell Phenotype

Grimm et al. originally described the LAK cell as a "null" cell lacking B cell and T cell markers which acquires the pan T cell antigens CD3 and CD5 upon stimulation with IL-2 (40). They

examined the phenotype of the LAK precursor and effector primarily by negative selection of PBL subpopulations (40, 41). The precursor phenotype was investigated by complement mediated lysis of cells expressing specific cell surface antigens prior to IL-2 stimulation of the cells. See Table 1 for a description of relevant monoclonal antibodies and the phenotypic markers for which they are specific.

Table 1: Phenotypic Markers

Designation	MoAb/Clone	Specificity
CD3	Anti-Leu4	T cells
CD5	Anti-Leu1	T cells
CD4	Anti-Leu3a	T helper/inducer cells
CD8	Anti-Leu2b	T cytotoxic/suppressor cells
CD16	Anti-Leu11	NK cells, neutrophils
CD56	Anti-Leu19/NKH-1	NK cells, cytotoxic T cell subset
CD57	Anti-Leu7/HNK-1	T cells, NK cell subset
CD11b	Anti-Leu15/OKM1	T suppressor cells, NK cells, monocytes, granulocytes

Depletion of cells expressing CD3, CD5, OKM1, CD57 or CD16 did not significantly decrease LAK cell activity, leading Grimm et al. to the conclusion that the precursor was a "null" cell. Grimm and colleagues also reported that the precursor was not removed by T cell rosetting and appeared morphologically similar to NK cells, sedimenting with the LGL fraction between 35 and 44% on Percoll density gradients (40, 41, 43). The phenotype of the LAK effector was investigated by complement-mediated lysis of subpopulations after culture with IL-2. Treatment of effector cells with anti-CD3 or anti-CD5 plus complement moderately decreased LAK activity, a combination of anti-CD3 and anti-CD8 significantly decreased it, as did anti-Tac antibody (specific for

the IL-2 receptor) (40, 41). They concluded that the LAK effector was a CD3+ lymphocyte.

Fluorescence activated cell sorting (FACS) techniques have allowed a more thorough investigation of the LAK cell phenotype. FACS purification of cells after activation with IL-2 demonstrates that LAK cell activity is enriched in the CD5- CD16+ (NK) subpopulation (125, 96). Lanier and Phillips report that CD56+ CD3+ cells show low levels of activity, while CD56- CD3+ cells show none (96). The LAK cell precursor and effector do not appear to differ phenotypically. Analysis of precursors reveal that the majority of LAK effectors arise from a precursor population that is CD5-, CD56+, and CD16+, i.e. NK, with a subset of CD16+ cells expressing low density CD8 (96, 66, 53, 125). This NK population lacks CD3 and does not show rearrangement of T cell antigen receptor beta genes (53, 65). A small CD56+ subset lacking CD3 and CD16 can develop LAK cell activity, possibly representing a stage of NK differentiation before acquisition of CD16 (96, 66). Phillips and Lanier report that CD56+ CD3+ cells may develop lytic activity after culture in IL-2, while CD56- CD3+ do not (96). In contrast, Tilden et al. report that only CD16+ cells can function as LAK cell precursors (125). They suggest that in the presence of accessory cells some T cells may develop LAK cell activity, but peripheral blood T cells cultured alone do not. Data from experiments using FACS isolation of cells confirm the report by Grimm et al. that the LAK cell precursor is

predominantly a CD3- lymphocyte. However, the LAK cell precursor does not appear to acquire T cell antigens after culture in IL-2, nor does the CD3+ T cell population contribute significantly to LAK cell activity derived from peripheral blood. In contrast to rIL-2 stimulation of FACS purified cells, Grimm and colleagues generated LAK cells with supernatant from PHA-activated PBL and depleted cell subpopulations by complement mediated lysis. The discrepancies between their work and more recent reports may reflect the different culture conditions as well as the different techniques used to evaluate antigen expression on LAK cells.

Evidence does not support the the existence of a unique LAK cell phenotype, rather a lytic ability that can be acquired by different cell populations stimulated with IL-2. Although NK cells have been established as the major contributors to LAK activity derived from peripheral blood, the role of T cells is controversial. Some reports demonstrate that T cells are unable to take part in the LAK phenomenon (53), while others show that several CD3+ T cell subpopulations, CD56+ (96), CD4+, CD8+ (27) and thymocytes (100) can be activated with IL-2 to display LAK activity. In contrast to NK cells, generation of LAK activity from T cells may require signals from monocytic accessory cells in addition to IL-2 (103, 125, 117). Silvennoinen et al. report that NK cells were activated by IL-2 alone while T cells were relatively unresponsive to even high concentrations of IL-2 in the

absence of accessory cells (117). The ability of different cell types to develop LAK activity appears to depend on the activation and culture conditions employed.

Several groups of investigators have attempted to correlate function and phenotype of LAK effectors in cancer patients receiving IL-2 (76). LAK activity, as detected in a standard 4 hour radioactive chromium (^{51}Cr) release assay with Daudi cell target, appears in PBL of cancer patients receiving rIL-2 4 to 6 weeks after initiation of therapy (76). Using FACS purification, the effectors were reported to be CD56+, CD57+, CD5- and heterogenous with respect to CD8 and CD16 expression (54). McMannis and colleagues conclude that circulating LAK effectors are a heterogenous population expressing NK associated antigens (76). Cohen et al. have studied the immunopathology of responsive tumors in patients treated with IL-2 and report that the tumors are infiltrated with T cells (primarily CD8+), while CD16+ or leu7+ cells are virtually absent (24). However, another group has provided evidence that both NK-like and T cell-like cells coexist at the tumor site, and that both cell types can be expanded in IL-2 (99). More information is needed to clarify the relationship of LAK cell phenotype and function *in vivo*.

LAK Cell Clones

A LAK cell clone is a population of cells that expresses lytic activity and that is derived from a single precursor cell

activated with IL-2 and expanded *in vitro*. Although there are a variety of methods, the cloning of LAK cells involves activating the cells with IL-2 plus PHA, plating them under limiting dilution conditions, and expanding the cells in culture with irradiated feeder cells from a variety of sources. Generally, clones are supplemented with irradiated feeder cells and media containing IL-2. The rationale for development of LAK cell clones is that they provide an isolated, phenotypically defined cell population for the study of the LAK cell phenomenon. Several groups of investigators have reported the generation of LAK cell clones and it is clear that the phenotype and specificity of these clones is determined by the precursor cell population, the method of stimulation, and the culture conditions in which the cloned cells are maintained. LAK clones can be derived from both T cell (with either alpha/beta or gamma/delta T cell receptor) and NK cell populations (36, 48, 78, 79). At the clonal level, LAK activity is always associated with killing of NK resistant targets regardless of the CD16 expression of the precursor (36). Individual clones are capable of killing multiple targets, suggesting a shared determinant on tumor cells (101).

LAK cells clones are capable of releasing cytokines in response to the appropriate stimuli. CD3+ clones release IL-2 and IFN-γ upon stimulation with PHA, and CD3- clones are reported to release IFN-γ upon stimulation, but not IL-2 (78). Allavena and colleagues demonstrate that LGL clones can produce

IFN-γ, IL-1, and B cell growth factor (BCGF) (3). It appears that lymphokines which may be important in the induction and/or amplification of anti-tumor activity can be produced by LAK effector cells themselves.

LAK cell clones are useful in correlating LAK cell phenotype with cytotoxicity and specificity. Working with clones may eliminate the problem of heterogeneity inherent in experiments done with LAK cells derived from PBL. However, there are limitations to this methodology. Clones cannot be maintained in culture for long periods of time. Depending on culture conditions, the cells lose lytic ability after 1 to 4 months (71). Also, the phenotype and functional characteristics of LAK clones may undergo significant changes due to the altered activation and culture conditions of the cells. This must be considered when comparing information on clones to the activity of conventionally derived LAK cells or *in vivo* LAK activity.

Target Cell Lysis

The mechanisms of LAK mediated target cell recognition and lysis are not well understood. LAK cytotoxic activity can be mediated by both NK and T cells activated with IL-2, and it has been proposed that the LAK cell phenomenon is distinct from CTL and NK cell activity at the level of target recognition rather than tumor cell lysis (15). Target cell lysis by NK and T cells involves

contact mediated cytotoxicity as well as the release of soluble cytotoxic molecules by the effector cell.

Cell Contact Mediated Lysis

Henkart et al. have examined the properties of isolated murine LAK cytoplasmic granules (46). The LAK cytolysin has properties similar to that of CTL and LGL with respect to non-specific killing, rapid kinetics and dependence on calcium. The activity is undetectable until after 2 days of culture with IL-2 and increases until day 7 (46). Contact mediated killing by LAK, like that of CTL and NK, is thought to involve exocytosis of cytotoxic granules and formation of transmembrane structures on the target cell surface (97). Isolated granules from cloned T cells and LGL are cytotoxic and can induce membrane lesions in the presence of Ca^{2+} (140). Pore forming proteins (perforins) purified from the granules have been shown to depolarize the membrane potential of cells and form functional channels in the bilayer (141, 77). The formation of channels is thought to damage cells by making them "leaky". Also, it is possible that the channels can act as a transmembrane conduit for other toxic molecules such as nucleases or esterases (140). This may be a factor in the DNA fragmentation observed in lymphocyte-mediated killing (141). The cytolytic pore forming molecules purified from granules of LGL and CTL produce similar pore-like

structures inserted in the target membrane, and are thought to be similar if not identical (140, 141).

Soluble Mediators

The recognition of target cells by CTL and NK may stimulate the release of soluble mediators such as TNF, lymphotoxin (LT), natural killer cell cytotoxic factor (NKCF), or IFN-γ from effectors. These cytokines can have direct cytostatic or cytotoxic effects on target cells. In addition to effects on the stimulating target cell, the cytokines may also affect tumor cell growth some distance from the site and possibly influence the activity of immunocompetent cells of the patient.

Chong et al. have found that LAK effectors (IL-2 activated PBMC) release both TNF and IFN-γ when stimulated with tumor cells *in vitro* (21). Increased levels of TNF and IFN-γ were detected in culture supernatants as early as 4 hours after combining LAK cells and tumor cells and reached a maximum after 12-20 hours. The cytokines were detected by specific ELISA. Biological activity of secreted TNF and IFN-γ was assessed using a tumor cell line, MCF-7, sensitive to the synergistic effects of TNF and IFN-γ in a 72 hour cytotoxic assay. Based on neutralizing antibody experiments and ELISA measurement of TNF and IFN-γ concentration, Chong and colleagues conclude that the majority of cytotoxic activity released by tumor cell-stimulated LAK cells is due to IFN-γ and TNF acting synergistically (21). Release of these

slow acting factors by LAK cells is dependent on the length of coincubation with tumor cells and the stimulator to effector cell ratio. The optimum effector to target cell ratio was 2:1. A variety of tumor cell lines (Daudi, U937, A375M, CEM-C3, MOLT-4, K562) were found to stimulate release of TNF and IFN-γ. Unstimulated, fresh peripheral blood mononuclear cells could not be activated by the same tumor cell lines to secrete TNF and IFN-γ.

Chong et al. also investigated the ability of LAK cell clones to release TNF and IFN-γ upon stimulation with tumor cells. The clones were derived from peripheral blood T cells (CD3+) activated with IL-2. They found that 5 of 6 clones produced TNF and IFN-γ when cultured with K562 tumor cells (22). These findings suggest that the release of IFN-γ and TNF may play an important role in the interaction of LAK effectors and tumor cells and warrant a more detailed discussion of these cytokines.

TNF

TNF is a pleiotrophic cytokine secreted by macrophages (26), T cells (121), and LGL (93). It was originally described when it was discovered that the necrosis of tumors in Bacille-Calmette-Guerin (BCG) primed mice injected with endotoxin was due to a factor (TNF) produced by the host (17). TNF is an etiologic factor in septic shock (86), and is identical to

cachectin, a serum factor associated with cachexia, a wasting condition observed in chronic illness (7, 9).

Structure

TNF is a 17kD non-glycoslyated protein in its secreted form and forms trimers (91). It contains an internal disulfide bridge which does not appear to be essential for biological activity (2, 38). Human TNF is initially synthesized as a prohormone with 76 extra amino acids at the amino terminus (91). This sequence contains an unusually long hydrophobic region of 26 amino acids which is thought to constitute a transmembrane domain (91).

Kreigler et al. have described a novel 26kD cell surface form of TNF that can function as a cytotoxic transmembrane protein on human macrophages (62). The 26kD molecule was found to be the precursor of the 17kD secreted form of TNF. Luettig et al. have also demonstrated the existence of TNF as an integral membrane protein in mouse peritoneal macrophages (72). It appears that TNF can be expressed as a cell surface integral transmembrane protein which must be proteolytically cleaved to be released into the serum. It may be that membrane TNF is responsible for either cell contact mediated lysis of target cells by activated macrophages or the local release of TNF at an inflammatory site. This mechanism of secretion would avoid the detrimental effects associated with the presence of high levels of TNF in the circulation.

Biological Activities

TNF has been shown to cause necrosis of meth A sarcomas *in vivo*, as well as inhibition of growth of sensitive tumor cells *in vitro* (9, 17, 124). The mechanism by which TNF lyses tumor cells is not known. Suffys et al. report that inhibitors of arachadonic acid metabolism reduce TNF mediated cytotoxicity and suggest that phospholipase A2 and arachadonic acid are involved in lysis. However, the exact role of these molecules is unknown (123). Lysosomal enzymes and hydroxy radicals may also play a role in the lysis of tumor cells by TNF, as lysosomotropic agents and hydroxy radical scavengers reduce the cytotoxicity of TNF *in vitro* (131). Expression of specific TNF receptors is necessary but not sufficient for sensitivity (109, 127). The TNF receptor has been identified as a single class of high affinity receptors with a molecular size ranging from 50 to 140kD (109). More recently, evidence for the existence of two major types of TNF binding proteins has been reported (14). The action of TNF involves binding to specific receptors, rapid internalization and intracellular degradation (127). However, Hofsli and Niisen-Meyer report that internalization of TNF is not necessary for TNF to exert its lytic effects, suggesting that the cytokine acts on cells through receptor mediated events and the generation of second messengers (49). Pretreatment of sensitive target cells with transcription or protein synthesis inhibitors

greatly increases the effect of TNF (84). This finding suggests that in the absence of the ability to undergo cellular repair the tumor cell is more susceptible to lysis by TNF.

In addition to its anti-tumor activity, TNF has multiple effects on normal cells. Recombinant TNF stimulates the growth of normal fibroblasts *in vitro* (124, 130). It induces release of IL-1 from endothelial cells (83). Lung fibroblasts, vascular endothelial cells and smooth muscle cells can produce granulocyte-monocyte colony stimulating factor (GM-CSF) when stimulated with TNF (81). Navroth and Stern suggest that TNF promotes clot formation on endothelial cells *in vivo*, which may contribute to the hemorrhagic necrosis in the tumors of mice injected with TNF (83). TNF induces the production of collagenase and PGE_2 by synovial cells and dermal fibroblasts, suggesting it may have a role in the tissue destruction seen in chronic inflammatory diseases (30). TNF suppresses lipoprotein lipase activity in adipocytes (8), an effect associated with cachexia. The expression of HLA-A and B on fibroblasts and endothelial cells is increased by TNF (25). TNF has been shown to enhance phagocytosis, cytotoxicity, and adherence of granulocytes (37, 59). Scheurich et al. report that TNF has multiple stimulatory effects on T cells: it enhances IL-2 receptor expression, as well as HLA-DR antigens and acts synergistically with IL-2 in stimulating T cell proliferation and IFN-γ production (1987). TNF increases the lytic capacity of NK

cells (87), and it potentiates lymphokine induced macrophage activation (45).

Recent studies suggest that TNF plays a role in the induction of the acute phase response. The acute phase response is characterized by a rapid increase in levels of certain plasma proteins in response to tissue damage or stress, such as surgery or cancer (61). TNF can stimulate synthesis of acute phase proteins by hepatocytes directly (29), or through induction of IL-6 release form hepatocytes, which would also lead to stimulation of the acute phase response (60, 67).

Regulation of TNF Release

TNF release by macrophages is induced by endotoxin, IFN-γ and IL-1 (84, 95). IFN-γ may stimulate TNF production by itself or may enhance its production by cells activated with other agents (82). Macrophages are stimulated to release TNF by coincubation with some cytocidal targets such as microbial pathogens and antibody-opsonized targets (69, 31)

Neoplastic cells can also induce TNF release from monocytes. Several groups of investigators have shown that tumor cells or tumor cell membranes can directly stimulate TNF release by human monocytes (J56, 44, 137). Macrophages appear to be capable of recognizing tumor cells and responding with release of TNF *in vitro*, although the mechanism by which macrophages distinguish normal cells from tumor cells is not yet

known. Evidence suggests that the release of TNF in response to tumor cells may occur *in vivo* as well. Some cancer patients have increased levels of circulating TNF and elevated levels of TNF mRNA in peripheral blood monocytes (6). Further, Biessert et al. have shown that macrophages present within human colorectal adenocarcinoma biopsies had increased levels of TNF protein and mRNA (12).

Kunkel et al. demonstrate that PGE_2 suppresses lipopolysaccharide induced TNF production by macrophages at the level of TNF gene expression (63, 64). This suggests that PGE_2 acts as an autoregulatory factor in TNF production by macrophages. TGFβ-1 has been shown to inhibit both TNF and IFN-γ production in LAK cell cultures as discussed previously (35).

Scuderi et al. report that whole human plasma alpha-globulins, alpha-1-antitrypsin, alpha-1-acid glycoprotein and alpha-2-macroglobulin are all potent suppressors of TNF release by monocytes stimulated with LPS or IFN-γ (114). The alpha globulins constitute a major fraction of circulating plasma proteins and are part of the acute phase response. Serum alpha-1-antitrypsin, alpha-1-acid glycoprotein and alpha-2-macroglobulin are normally present at concentrations of 0.93-2.24 mg/ml, 0.25-1.4 mg/ml and 1.5-3.5 mg/ml, respectively (61). During an acute phase response, plasma levels of acute phase reactants rise 2-5 fold, with the primary site of synthesis being the liver. Both Alpha-1-antitrypsin and alpha-2-

macroglobulin are anti-proteases, and it is thought that they act to control the inflammatory response and restore homeostasis *in vivo*. Scuderi et al. propose that they inhibit TNF release by inhibiting proteolytic cleaving of the transmembrane precursor molecule from the monocyte cell membrane. Alpha-1-acid glycoprotein, while it has no anti-protease activity, is reported to be an immunosuppressive protein. In addition to its effect on TNF production, it has been shown to inhibit the T cell response to mitogens (19), antibody production by B cells (18), NK cell lysis of tumor cells (85), as well as monocyte phagocytosis (129). The mechanism of action is not yet known.

The synthetic protease inhibitor, *p*-toluenesulfonly-L-arginine methyl ester (TAME) has been reported to completely suppress the release of TNF from LPS stimulated monocytes (113). Again, it is thought to act at the cell surface to inhibit clipping of TNF from the cell membrane.

IFN-γ

IFN-γ is produced by CTL, NK, and LAK cells (32, 52, 108). It is a member of the interferon family which contains at least 20 distinct proteins divided into three classes (alpha, beta, and gamma) according to their biological and physiochemical properties. The interferons were first described and purified on the basis of their ability to interfere with viral replication in fibroblasts. Originally known as immune interferon, IFN-γ was

separable from alpha and beta interferons due to its instability at low pH (92).

IFN-γ is a 20-25kD glycoprotein, depending on the extent of glycosylation (138, 102). It is thought to be processed as a typical secretory protein; there is no evidence that IFN-γ exists as a transmembrane molecule on leukocytes.

IFN-γ has cytostatic/cytotoxic activity for sensitive cell lines (128, 98). The mechanism of action is unknown. However, target cell protein synthesis is involved, as cyclohexamide, an inhibitor of protein synthesis, abrogates lytic effects. A synergistic cytotoxic effect has been observed when certain tumors cells are incubated with LT and IFN-γ (135). IFN-γ also synergizes with TNF, possibly by enhancing TNF receptor expression (1). It is possible that IFN-γ plays an important role in target cell lysis by synergizing with TNF also produced by the effector cells.

In addition to its anti-tumor activity, IFN-γ has immunomodulatory effects. It enhances NK function *in vitro* and *in vivo* (47, 126). IFN-γ induces expression of class I and class II histocompatability antigens, and can synergize with TNF in this activity (39). It activates macrophages (4, 118), regulates T cell activity (92), and can stimulate production of TNF and IL-1 from monocytes (4, 112). There is some evidence that IFN-γ may serve as a modulator of the acute phase response. In contrast to the

effect of TNF, IFN-γ has been shown to inhibit synthesis of acute phase proteins by a human hepatoma cell line (Hep G2) (73).

IFN-γ is synthesis is induced by IL-2 (139) and TNF (110). Production of IFN-γ is inhibited by PGE_2 (33), TGFβ-1 (35), and IL-4 (90).

OBJECTIVES

I. To determine the role of TNF in LAK cell generation and cytotoxicity
   A. To determine the effect of exogenous TNF on the generation of LAK cell cytotoxicity
      1. Effect on cell recovery
      2. Effect on proliferation
   B. To quantitate the production of TNF by LAK cells in culture
   C. To determine the role of endogenously produced TNF by adding neutralizing antibody
      1. Inhibition of LAK cell generation
      2. Inhibition of LAK cell cytotoxicity

II. To investigate the regulation of TNF and IFN-γ release by LAK cells stimulated with tumor cells *in vitro*
   A. To test potential suppressors of TNF and IFN-γ release for their effect on cytokine release from LAK cells stimulated with tumor cells
      1. Effect of PGE_2
      2. Effect of alpha globulins
         a. Whole alpha globulin
         b. Alpha-1-acid glycoprotein
         c. Alpha-1-antitrypsin

d. Alpha-2-macroglobulin

3. Effect of TAME

B. To determine if suppression of TNF and IFN-γ release is the result of direct action of regulatory factors on lymphocytes in the LAK cell-tumor cell interaction

1. Requirement for monocytes

A. FACS purified CD14- cells

B. LAK cell clone

2. Requirement for metabolically active tumor cells

C. To analyze the ability of lymphocytes to express cell surface TNF

III. To investigate the effect of alpha-1-acid glycoprotein on LAK cell function

A. To determine the effect on tumor cell stimulation of LAK cells

1. Incorporation of ^{3}[H]thymidine

2. Expression of TNF mRNA

C. To determine the effect of alpha-1-acid glycoprotein on the generation of LAK activity

1. Generation of cytotoxic activity

2. Production of TNF in culture

CHAPTER 2. EXPERIMENTAL METHODS

Cells and Culture Conditions

Media

Leukocytes and tumor cells were cultured in complete media consisting of RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal calf serum (Gibco Laboratories, Grand Island, NY), 1% penicillin-streptomycin (Irvine Scientific), and 1% glutamine (Irvine Scientific). Hanks balanced salt solution without calcium or magnesium (HBSS) was purchased from Irvine Scientific. Phosphate buffered saline (PBS) was made by adding 1.44g/L KH_2PO_4, 90g/L NaCl, and 7.95g/L Na_2PO_4 to double distilled deionized H_2O (ddH_2O).

Tumor Cells

Tumor cell lines were purchased from the American Type Culture Collection (Rockville, MD). Daudi (a cell line derived from a Burkit's lymphoma), K562 (from an erythroleukemia), and U937 (from a monocytic leukemia) were maintained as suspension cultures and passaged every 3-4 days. The adherent cell lines MCF-7 (from a breast carcinoma) and A375M (from a melanoma) were subcultured every 3-4 days. Adherent cells were removed from the plastic culture flasks (Corning, Corning, NY) by a 2 minute treatment with

0.05% trypsin (Sigma, St. Louis, MO) in HBSS, followed immediately by the addition of complete media.

Preparation of LAK Cells

Peripheral blood mononuclear cells were obtained by venous puncture from healthy donors after informed consent was given, using vacutainer tubes containing sodium heparin (Becton Dickinson, Rutherford, NJ). The mononuclear cells were separated from whole blood by Ficoll Paque (Pharmacia, Pistcataway, NJ) density centrifugation. The mononuclear leukocytes were collected from the interface, washed twice in HBSS and resuspended at $1x10^6$ cells/ml in complete media in tissue culture flasks (Corning, Corning, NY). Recombinant human IL-2 (Cetus Corp., Emeryville, CA) was added to the culture at 10^3 U/ml, and the cells incubated at 37°C with 5% CO_2.

Antibody blocking studies were done using polyclonal rabbit anti-human TNF and IFN-γ antibodies provided by Dr. Philip Scuderi. The polyclonal antibodies were produced by immunizing New Zealand rabbits with recombinant human cytokines emulsified in Freund's complete adjuvant (Difco, Detroit, MI) using previously described methods (111) Heat inactivated normal rabbit serum (Sigma, St. Louis, MO) was used as a control.

Lymphocyte Purification

Plastic Adherence

LAK cells were washed three times in HBSS and resuspended in complete media at $5x10^7$ cells/ml. The cell suspension was added to a tissue culture flask placed on its side to maximize surface area for monocyte adherence. The flask was incubated at 37°C for one hour and the nonadherent cells (lymphocytes) harvested by gently rinsing the flask with complete media.

Nylon Wool Adherence

Nylon wool (NEN, Claremont, CA) was boiled in 0.2N HCl for 30 minutes and then rinsed continuously with ddH_2O for 30 minutes. The nylon wool was washed for 2 days before being allowed to air dry. A column was prepared by placing 0.6g "teased" nylon wool in a 6ml syringe (Becton Dickinson) fitted with a 3 way stop cock and an 18 gauge needle. The column and stopcock were autoclaved prior to use.

Before the addition of leukocytes, the column was rinsed with 100ml of 37°C complete media. The stopcock was closed and media added to cover the nylon wool. The column was covered and incubated for 1 hour at 37°C. The nylon wool was rinsed with an additional 20 ml of 37°C complete media, allowed to run dry, and the stopcock closed. Leukocytes were added to the column at a concentration of $5x10^7$ in complete media in a volume of 1-3ml. The column was incubated at 37°C for 30 minutes to allow for monocyte

adherence. Nonadherent cells (T cells and NK cells) were eluted from the column with 20ml of complete RPMI added dropwise.

Fluorescence Activated Cell Sorting

For the purification of LAK cell subpopulations, leukocytes were stained with antibodies to phenotypic markers and sterilely sorted by flow cytometry. Phycoerythrin conjugated monoclonal antibodies specific for CD3, CD16, CD14 were purchased from Becton Dickinson. Table 2 summarizes these surface markers and the cell populations expressing them.

Table 2: Leukocyte Cell Surface Antigens

Designation	Monoclonal Ab	Specificity
CD3	Leu4	T cells
CD16	Leu11	NK cells and neutrophils
CD14	LeuM3	monocytes/macrophages
CD20	Leu16	B cells

Cells to be sorted were harvested and washed three times in 4°C HBSS. To $1x10^6$ cells resuspended in 0.5ml 4° C PBS, 0.2ml of antibody was added. The cells were incubated at 4°C for 30 minutes, washed twice in 4°C PBS, and resuspended at $2x10^6$ cells/ml in complete media for sorting.

To eliminate residual monocytes, the LAK preparations were stained with LeuM3 and the nonstaining cells collected.

To obtain purified T cell and NK cell populations, the leukocytes were stained with both Leu4 and Leu16 and sorted into CD3+ (T cells) and CD16+ (NK cells) populations. Alternatively, the T cells could be purified by negative selection. To do this, the LAK cells were stained with antibodies specific for CD16, CD14 and CD20, and the nonstaining cells were collected.

FACS analysis of stained cells was performed on a Becton Dickinson FACSTAR analyser equipped with a water cooled 5 watt argon-ion laser, used at 200 milliwatts of power. Extinction wavelength was 488nm with green fluorescence detected using a 530nm, filter with a band width of 44nm. The fluorescent signal was read on a 256 channel linear scale. Forward and ninety degree light scatter was used to determine the cell populations present. Data analysis was performed on Hewlett packard 9000 series computer (model 310). The instrument was standardized daily using CaliBRITE beads (Beckton Dickinson, Mountain View, CA). Ten thousand events per sample were collected and the percent positive cells (percent gated events) as well as the mean fluorescent intensity was determined. The sterilely sorted cells were resuspended in complete media.

Cell Surface TNF Expression.

The cell surface expression of TNF on monocytes, T cells and NK cells was investigated. To determine surface TNF expression, $1x10^6$ cells in 50µl PBS were incubated at 4°C for 30 minutes with 8µl of polyclonal rabbit anti-human TNF antibody, then washed and incubated with 2µl of goat anti-rabbit fluorescein (FITC) conjugated secondary antibody (American Qualex, La Mirada, CA). At the time of secondary antibody addition, they were also stained with one of several phycoerythrin conjugated monoclonal antibodies specific for a phenotypic marker. Phenotypic markers analyzed included CD3, CD16, and CD14. The cells were washed and analyzed by FACSTAR as described above.

LAK Cell Cloning

FACS purified T cells (CD3+) were seeded in limiting numbers (0.5-8 cells/ml)in round bottom 96 well plates containing 10^4 irradiated (3000R) allogenic peripheral blood mononuclear cells/well, 0.1µg/ml PHA and 1000U/ml IL-2. The cells were fed twice weekly with complete media plus IL-2 and PHA at the concentrations noted above. Irradiated feeder cells were added once a week. After two to three weeks of culture, each well was microscopically assessed for growth. , The phenotype of the cells was determined by FACS analysis.

LAK Cell Cytotoxicity Testing

LAK cells were harvested, washed three times in HBSS (Irvine Scientific) and resuspended in complete RPMI. Cytotoxicity of the cells was assayed in a standard four hour ^{51}Cr release assay (68). Daudi, an NK resistant cell line, was the target. Target cells were washed and resuspended at $1x10^6$ cells/ml in complete RPMI + 100μCi/ml of ^{51}Cr (NEN Products, Boston, MA). The cells were incubated overnight at 37°C + 5% CO_2. After labelling, target cells were washed three times with HBSS and resuspended in complete media for 1 hour. The cells were then centrifuged and resuspended at $1x10^5$ cells/ml in complete media. Target cells were plated at $1x10^4$ cells/well in 100μl volumes in 96 well round bottom plates. Concentrations of LAK cells in a volume of 100μl were added to target cells to give effector:target ratios ranging from 1.25:1 to 25:1. To determine spontaneous release, 100μl complete media was added, and for maximum release 100μl of 0.1N HCl was added to the labelled targets. Plates were incubated at 37°C + 5% CO_2 for 4 hours. After 4 hours, 100μl of the supernatant was harvested from each well. The samples (in triplicate) were counted in a Packard gamma counter. Percent cytotoxicity was calculated as follows:

$$\frac{\text{experimental release - spontaneous release}}{\text{maximum release - spontaneous release}} \times 100$$

LAK-Tumor Cell Interaction Supernatants

Tumor Cell Stimulation of LAK Cells

To investigate the secretion of TNF and IFN-γ by activated lymphocytes, ficoll separated PBL were cultured with IL-2 for 72 hours, washed three times in HBSS and resuspended in complete medium at $1x10^6$ cells/ml. Nonadherent tumor cells (K562, Daudi or U937) cultured in complete media were washed 2 times in HBSS and resuspended in complete media at $1x10^6$ cells/ml. Equal volumes of the two cell suspensions were combined for a LAK to tumor cell ratio of 2:1. The cells were incubated at 37°C with 5% CO_2 for 12-20 hours. Cell free supernatants were then collected for the quantitation of TNF and IFN-γ and stored at -20°C. LAK and tumor cells alone were set up as controls. The viability of the cells was determined by trypan blue (Gibco) exclusion.

Experiments using adherent tumor cell lines to stimulate LAK cells were performed as described above, with the following exceptions. The adherent tumor cells were trypsinized, washed and resuspended at $5x10^5$ cells/ml in complete RPMI. The tumor cells were then allowed to adhere for 8 hours prior to the addition of LAK cells. Equal volumes of the LAK and tumor cell suspensions were combined in 96 well plates, with a total volume per well of 200μl. Control wells contained either LAK or tumor cells alone. LAK viability was determined by trypan blue exclusion. To determine the viability of the tumor cells alone, adherent cells were quantitated with the Biorad protein assay. The plates were washed

3 times with PBS to remove dead, non-adherent cells. The remaining adherent cells were then lysed by the addition of 50µl H_2O, followed by two cycles of freeze-thaw. Protein remaining in the wells was quantitated by the addition of 200µl of a 1:5 dilution of Biorad Protein Assay reagent (Biorad Corp., Richmond, CA). The plates were read at 590nm with a Dynatech Microplate Reader. Percent cytotoxicity is calculated as follows:

$$1 - \frac{\text{experimental O.D.}}{\text{control O.D.}} \times 100$$

Control O.D. is the mean value for wells with complete media added.

In some experiments, tumor cell lysate was used in place of viable tumor cells to stimulate cytokine release by LAK cells. Tumor cell lysate was prepared from a cell suspension of K562 cells at a concentration of $1x10^6$ cells/ml in complete RPMI. The cells were frozen and thawed twice.

Regulation of Cytokine Release

A number of factors were tested for potential regulation of cytokine release: PGE_2 (Sigma, St. Louis, MO), whole human alpha globulin (Sigma), alpha-1-acid glycoprotein (Sigma), alpha-1-antitrypsin (Sigma), alpha-2-macroglobulin (Sigma), TAME (Sigma) and LPS (Sigma). For each factor, a range of concentrations was added to the coculture of LAK and tumor cells. At each concentration, the viability of LAK cells alone and tumor cells alone was determined as described above.

The effect of the pretreatment of LAK with suppressive factors on cytokine release in response to tumor cell stimulation was investigated. The LAK cells were washed as described previously and resuspended at $2x10^6$ in complete media or media containing PGE_2, alpha-1-acid-glycoprotein or TAME. After a 4 hour incubation, the cells were harvested, washed 3 times and resuspended in complete media at $2x10^6$ cells/ml. The LAK cells were then stimulated with K562 and the supernatant collected for quantitation of cytokine release.

Also, the effect of addition of alpha-1-acid glycoprotein at several time points following the mixing of LAK and tumor cells was investigated. LAK and K562 were prepared as described previously. Alpha-1-acid glycoprotein was added to the cells at 4, 8, or 12 hours after the initiation of LAK-tumor cell culture. Cell free supernatants were collected for TNF and IFN-γ quantitation.

Enzyme Linked Immunoabsorbent Assays (ELISAs)

TNF ELISA

Murine anti-human TNF monoclonal antibody (Olympus, Lake Success, NY) was used to coat 96 well Immunlon II plates (Fisher, Pittsburg, PA). These coated plates were washed with PBL + 0.05% Tween 20 (Biorad, Richmond, CA). The washing was repeated between each step in the assay. Assay plates were sequentially exposed to serum, rabbit anti-TNF, horseradish peroxidase-conjugated goat anti-rabbit Ig (American Qualex, La Mirada, CA), and

then 2,2'-azinobis-3-ethylbenz-pthiazoline sulphonic acid substrate (Sigma, St. Louis, MO). To generate a standard curve, rTNF (Genentech Inc., S. San Francisco) was diluted in complete media. Within 10 minutes of substrate addition, the optical density of the plates was read with a Dynatech Microplate Reader at 405nm. The sensitivity of the assay is 40pg/ml (111).

IFN-γ ELISA

Assay plates were coated with murine anti-human IFN-γ monoclonal antibody and then sequentially exposed to serum, rabbit anti-IFN-γ, horseradish peroxidase-conjugated goat anti-rabbit Iγ, and 2,2'-azinobis-3-ethylbenz-pthiazoline sulphonic acid substrate. A standard curve was generated using recombinant IFN-γ obtained from Genentech Inc. (S. San Francisco, CA). The sensitivity of the assay is 100pg/ml (111).

LPS Assay

The LPS content of several alpha globulin preparations was measured using a Limulus Amoebocyte Assay kit purchased from Associates of Cape Cod (Woodshole, MA.) The assay is performed as follows. The control standard of LPS (*E. Coli* 0113) was diluted in endotoxin free water. (Sigma) A series of dilutions, closely bracketing the reported concentration of LPS in the standard were prepared. A 0.2ml volume of each dilution was added to a Pyrotell tube. The tubes were mixed thoroughly and then incubated at 37°C

for 60 minutes. At that time, the tubes were slowly inverted. If the contents had coagulated, the tube was graded as positive. (This endpoint must be determined because different lots of Pyrotell can vary slightly in sensitivity.)

Each sample to be tested was also diluted in LPS free water and each dilution added to a Pyrotell tube. Following a 1 hour incubation at 37°C, the tubes were graded as positive or negative for coagulation. The approximate concentration of LPS in the samples was determined by comparison to the set of dilutions of the standard and accounting for the dilution factor of the sample.

^{3}[H]Thymidine Incorporation

LAK Cells Cultured in IL-2/TNF

The proliferative response of LAK cells cultured in IL-2 was compared to that of LAK cells cultured in IL-2/TNF. The LAK cells were cultured for five days in either 1000U/ml IL-2 or 1000U/ml IL-2 and 1000U/ml TNF. The cells were then harvested, washed three times in HBSS, resuspended at $1x10^6$ in complete media, and plated at $1x10^5$/well in a 96 well plate. The cells were then cultured for 12 hours in the presence of PHA (1, 10, or 20mg/ml) or IL-2 (10, 50, 100, or 1000U/ml). Control wells had no PHA or IL-2 added. The LAK cells were then pulsed with ^{3}[H]thymidine (1μCi/well) for 4 hours. The cells were harvested onto glass fiber filter strips (Cambridge Technology, Cambridge, MA) with a Cambridge Technology Cell Harvester. The filters were allowed to

air dry overnight in liquid scintillation vials (Research Products International, Mount Prospect, IL), and then Betamax liquid scintillation cocktail (ICN, Irvine, CA) was added to the filters. Counts per minute were determined with a Packard Liquid Scintillation Counter.

Stimulation with Tumor Cell Lysate

The uptake of ^{3}[H]thymidine (NEN, Boston, MA) by LAK cells stimulated with K562 tumor lysate for 4, 8, and 12 hours was determined. LAK and tumor cell lysate, prepared as described previously, were combined in equal volumes to give a final volume of 200µl/well in a 96 well plate. LAK cells alone and K562 lysate alone were set up as controls for each time point. LAK cells which had been exposed to lysate for 4, 8, or 12 hours were pulsed with ^{3}[H]thymidine (1µCi/well) for 4 hours. The cells were then harvested and cpm determined as described above.

After the exposure time required for maximum stimulation of LAK by tumor cells was determined, the effect of alpha-1-acid glycoprotein on ^{3}[H]thymidine incorporation was investigated. LAK cells were incubated with K562 lysate in the presence of 5mg/ml alpha-1-acid glycoprotein. Controls consisted of LAK cells alone in media or in media with 5mg/ml alpha-1-acid glycoprotein. After 12 hours, the cells were pulsed with 3[H]thymidine and incorporation determined.

Detection of TNF mRNA

Preparation of Cells

The effect of alpha-1-acid glycoprotein on levels of TNF mRNA in LAK cells alone and LAK cells stimulated with tumor lysate was investigated. Several cell preparations were examined for the expression of TNF mRNA. The cells were prepared as follows:

1. LAK cells were harvested after 3 days of culture.
2. Three day LAK were washed and resuspended at $2x10^6$ cells/ml in complete media containing 5mg/ml alpha-1-acid glycoprotein. After an 8 hour incubation at 37°C + 5% CO_2, the cells were harvested.
3. Three day LAK were washed, and resuspended at $2x10^6$ cells/ml in complete media. Tumor cell lysate was prepared as described previously and equal volumes of LAK cell suspension and K562 cell lysate were combined. The cells were harvested after an 8 hour incubation at 37°C + 5% CO_2.
4. Alpha-1-acid glycoprotein was added to the mixture of LAK and K562 lysate at a concentration of 5mg/ml. The cells were incubated for 8 hours.

RNA Extraction and Dot Blotting

RNA was isolated by acid guanidinium thiocyanate-phenol-choroform extraction (20). The cells were harvested by centrifugation, and for each $5x10^6$ cells 1.0ml of guanidinium

isothiocyanate solution (Sigma) was added to the pellet. The cells were vortexed and sheared with an 18 gauge needle and syringe. A volume of 0.5ml of a solution of 0.1M Na acetate (Sigma), 10mM Tris base (Sigma), 1mM EDTA (Sigma) was added and the solution vortexed. To each tube, 1.0ml of equilibrated phenol (BRL, Gaithersburg, MD) was added, the solution vortexed, and 1.0ml of chloroform:isoamyl-OH (24:1) (Sigma) added. The contents of each tube were covered and vigorously shaken for 10 minutes at RT. The cell extracts were then transferred to Corex ultracentrifuge tubes (Corning, NY) and centrifuged at 5000xg at 4°C for 10 minutes in a Sorvall centrifuge. The aqueous phase was collected and the phenol-chloroform extraction repeated until the protein interface between the aqueous and organic layers was gone. Two extractions with a 1.0ml volume of chloroform-isoamyl-OH were performed. To the collected aqueous phases, 2.5ml of -20°C absolute ethanol (Midwest Grain Products, Weston, MO) was added. The tubes were covered and the RNA allowed to precipitate at -20°C for 2 hours. To collect RNA, the tubes were centrifuged at 12,000xg for 30 minutes at 4°C. The pellets were washed once with 70% ethanol and then allowed to dry before resuspension in 0.5ml diethyl pyrocarbonate treated water (DEPC-H_2O) (ICN, Costa Mesa, CA). A 1:100 dilution of each RNA sample in DEPC-H_2O was used to determine O.D. at 260nm. The concentration of RNA was calculated as follows:

$$\frac{\text{O.D. at 260nm} \times \text{dilution} \times 40 \times \text{ml}}{\text{volume}} = \text{RNA mg/ml}$$

A 1:2 dilution series of each sample in DEPC-H_20 was prepared in eppendorf tubes with a final volume of 225µl per tube. To each tube, 75µl of a solution of 37% formaldehyde (Aldrich, Milwaukee, WI) in SSC buffer was added. The SSC buffer consisted of 3M NaCl and 0.3M Na_3 citrate in H_2O The samples were incubated in a 65°C water bath for 15 minutes and then put on ice. Each sample was blotted onto nitrocellulose (Schleicher and Schuell, Keene, NH) using a Minifod dot blot apparatus (Schleicher and Schuell). The blot was baked at 80°C for 2 hours in a vacuum oven.

Hybridization

The hybridization buffer contained 50% formamide (Aldrich, Milwaukee, WI), 50mM KPO_4, 5x SSC, 2x Denhardt's solution, 0.25mg/ml yeast tRNA (Sigma), and 0.2% SDS (Biorad, Richmond, CA). Denhardt's consisted of 20g ficoll 400g (Pharmacia), 20g polyvinyl pyrrolidone (Sigma) and 10g fraction IV BSA (Sigma) in 1L SSC buffer. The blot was prehybridized with buffer for 4 hours at 42°C, and the buffer removed. The nitrocellulose was then hybridized with 1-2ng/ml of random labelled probe with approximately $5x10^6$ cpm/ml for 18 hours at 42°C. The cDNA probe for TNF was provided by Philip Scuderi. The probe was labelled with ^{32}P (NEN) using a Multiprime labelling kit (Amersham,) After hybridization, the blot was washed with 1x SSC twice followed by one wash with 0.1x SSC. The blot was placed at -80°C for 30 hours with X ray film (KODAK, Rochester, NY).

To strip the nitrocellulose of bound TNF probe, the blot was then rinsed with a solution of 25mM Tris, 0.1% SDS, 0.5x Denhardt's at 95°C for 1 hour, followed by a rinse with 65°C H_2O. The blot was reprobed with random labelled beta actin cDNA (provided by Dr. Roger Meisfeld).

Exposed film was analyzed by laser densitometry (Biorad Video Densitometer) and the TNF data normalized to the beta-actin controls.

Statistical Analysis

Data was analysed by a Students T-test using the Statview 512+ program (BrainPower Inc., Calabasas, CA) on a Macintosh SE/30 computer.

CHAPTER 3. RESULTS

THE ROLE OF TNF IN LAK CELL GENERATION AND CYTOTOXICITY

The Effect of Addition of Exogenous TNF on Development of LAK Effectors

Although IL-2 is the only stimulus required for the development of LAK cell activity, the addition of TNF to LAK can have a enhancing effect. Figure 1 illustrates the effect of the addition of exogenous TNF on the development of LAK cell cytotoxicity. PBMC were stimulated with IL-2 and cultured for 5 days in complete media or media containing either 100U/ml or 1000U/ml TNF. The cytotoxicity of the LAK cells was assessed in a ^{51}Cr assay with effector to target (E:T) ratios of 10:1 and 2:1. The data are expressed as percent cytotoxicity ± standard deviation. When LAK cell activity was assessed at an E:T ratio of 10:1, the addition of either 100U/ml TNF or 1000U/ml TNF significantly enhanced the generation of cytotoxicity at optimal IL-2 concentration ($p=0.026$ and $p=0.018$, respectively).

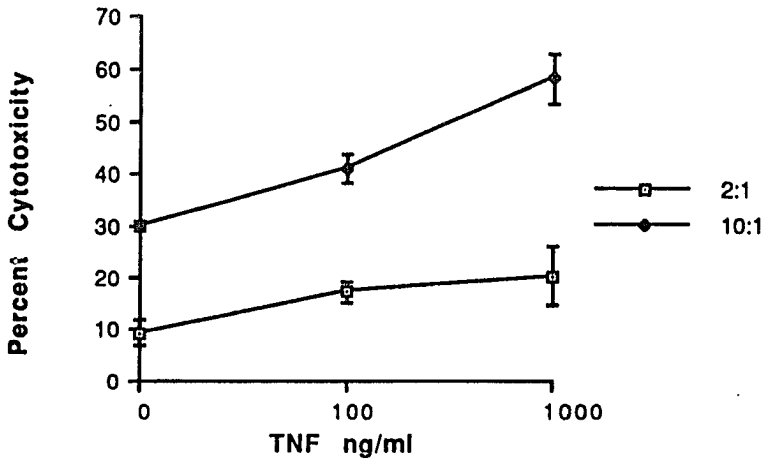

Figure 1: Stimulation of LAK Cell Generation by Exogenous TNF

To investigate the possibility that the enhancement of LAK generation was due to a proliferation-driven enrichment of effectors, the cell recovery and proliferative response of IL-2/TNF stimulated PBMC was examined. As shown in Figure 2, the cell recovery from PBMC cultured with IL-2/TNF did not differ from that of PBMC cultured in IL-2 alone (Control). Cell number was determined by trypan blue exclusion and is expressed as viable cells/ml.

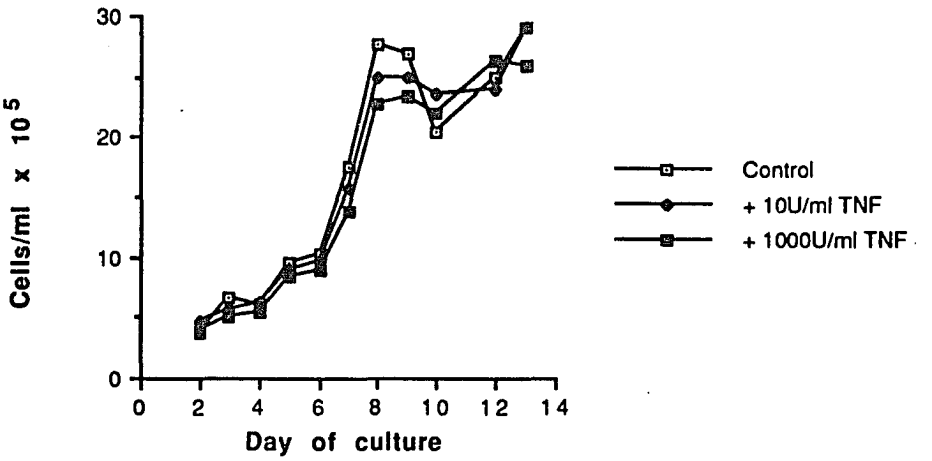

Figure 2: Effect of Exogenous TNF on LAK Cell Recovery

To determine the responsiveness of LAK cells cultured in IL-2/TNF, the effectors were washed and resuspended in media alone, media with IL-2 or media with PHA. The cells were then pulsed with ^{3}[H]thymidine and counts per minute (cpm) determined as described previously. No significant increase in ^{3}[H]thymidine incorporation in response to stimulation with IL-2 (Table 3) or PHA (Table 4) was observed in LAK cells generated in the presence of 50, 100, or 1000U/ml TNF as compared to LAK cells generated without TNF, indicating that the addition of TNF did not affect the proliferative response to subsequent stimulation with PHA or IL-2.

Table 3: Effect of Exogenous TNF on LAK Cell Responsiveness to IL-2

IL-2 U/ml	0 TNF	50 U/ml TNF	100U/ml TNF	1000U/ml TNF
0	45.6±4.4*	39.1±3.9	36.5±2.6	39.3±1.6
10	62.8±1.7	60.5±2.2	63.0±7.8	59.2±4.3
50	59.9±2.8	67.8±21.2	67.9±2.6	68.7±1.4
100	66.6±7.2	76.3±4.1	68.4±4.2	73.5±2.4
1000	79.9±3.9	77.8±4.7	72.4±7.1	71.8±1.2

*Counts per minute ± standard deviation

Table 4: Effect of Exogenous TNF on LAK Cell Responsiveness to PHA

PHA µg/ml	0 TNF	50U/ml TNF	100U/ml TNF	1000U/ml TNF
0	38.2±1.0*	43.4±0.75	37.6±3.7	39.3±2.2
1	38.2±2.8	43.5±3.3	34.6±2.1	40.2±3.3
10	50.2±4.5	48.2±1.7	45.4±1.6	49.2±2.0
20	64.7±3.4	64.5±5.2	60.7±2.9	64.4±3.6

*Counts per minute ± standard deviation

These results suggest that augmented LAK function in IL-2/TNF stimulated PBMC is not due to increased proliferation, but rather a functional activation. This conclusion is supported by the findings of others. It has been reported that the enhancement by TNF

of LAK cell activity derived from PBMC (88) as well as the activity derived from purified LGL (23) does not involve increased cell cycling, as determined by cell recovery and ^{3}[H]thymidine incorporation. Owen-Schaub et al. found that the addition of TNF to IL-2 stimulated PBMC resulted in enhanced IL-2 receptor expression (88). Chouaib et al. report that the stimulatory effect of TNF on the generation of LAK activity from LGL involves the induction of high affinity IL-2 receptors. The induction of high affinity IL-2 receptors was observed exclusively on LGL stimulated with the combination of TNF and IL-2 and reflected a shift from expression of the p75 component to the high affinity p75+p55 complex (23). They found that monoclonal antibodies to either the p55 or the p75 component would inhibit LAK generation by IL-2/TNF and result in a parallel decrease in expression of lymphocyte TNF receptors. In the model proposed by Chouaib and colleagues, IL-2 would bind to intermediate affinity IL-2 receptors (p75) present on circulating lymphocytes. This interaction would result in TNF receptor induction. The subsequent binding and activity of TNF would result in formation of the high affinity IL-2 receptor, lowering the IL-2 concentration required for differentiation of lymphocytes into LAK effectors.

Effect of Exogenous TNF on the Effector Phase

As it is known that membrane-associated TNF can lyse sensitive cell lines, the possibility that the enhanced LAK cell

function upon stimulation with IL-2/TNF was due to membrane-associated TNF expressed on the effectors was investigated. Figure 3 shows the result of the addition of exogenous TNF (1000U/ml) to LAK effectors during the 4 hour ^{51}Cr assay. TNF was added to the LAK cells at either 15 minutes, 30 minutes or 60 minutes prior to the cytotoxicity assay and was present throughout the 4 hours. In the control, no TNF was added. The data represents the mean of three replicates and is expressed as per cent cytotoxicity ± standard deviation. The addition of TNF to the interaction of LAK and tumor cells did not have any effect on cytotoxicity.

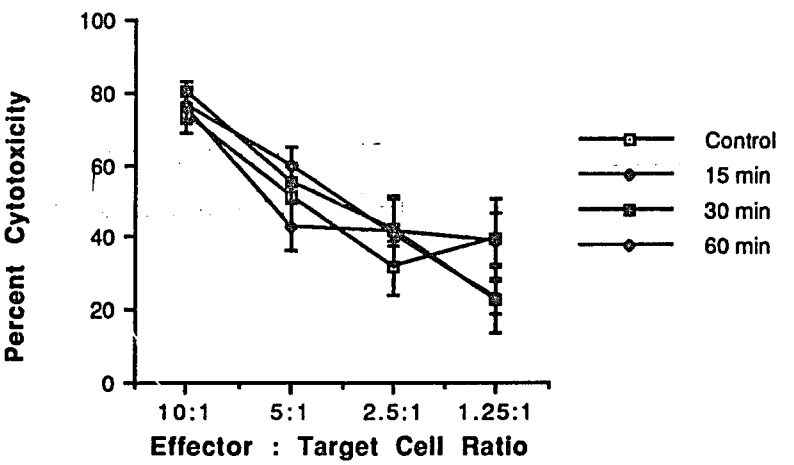

Figure 3: Effect of Exogenous TNF on the Lytic Phase of LAK Activity

The data presented here suggest that the enhancement of LAK cell cytotoxicity does not involve "arming" of the LAK effectors with membrane associated exogenous TNF. This conclusion is supported

by the findings of Blay et al. (13). They found no stimulatory or inhibitory effect on LAK function when either exogenous TNF or neutralizing antibody to TNF was added to the lytic phase. Also, Daudi were found to be insensitive to lysis by TNF in a 4 hour ^{51}Cr assay.

Production of TNF by LAK Cells in Culture

Because it is known that both T cells and NK cells are capable of producing TNF, the production of TNF in LAK cell culture was quantitated by specific ELISA. It was found that TNF is secreted by LAK cells in culture. To examine the kinetics of this production, PBMC activated by 10, 100, or 1000U/ml IL-2 were cultured in complete media and the TNF released into culture supernatant was measured daily by ELISA. Figure 4 shows that TNF released by LAK cells cultured in 10U/ml or 100U/ml IL-2 increased over time of culture and reached a maximum at 4-7 days. When PBMC were activated by 1000U/ml IL-2, TNF release was maximal by 24 hours.

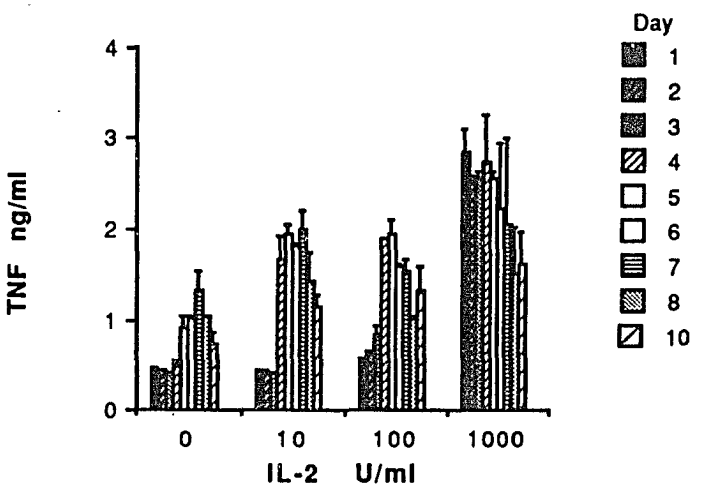

Figure 4: TNF Release by LAK Cells in Culture

The finding that IL-2 induced TNF release by LAK cells is in agreement with data reported by Owen-Schaub et al. (88). However, PBMC cultured without IL-2 also show significant TNF production, reaching a maximum on day 6. In this experiments, cells were cultured in complete media supplemented with fetal calf serum, and release of TNF by PBMC in the absence of IL-2 addition is thought to be due to activation of the cells by the foreign proteins present in the media.

Antibody Blocking Studies

The possible involvement of endogenously produced TNF in LAK cell generation and target cell lysis was investigated in antibody blocking studies. Polyclonal rabbit anti-human TNF antibody was added at 1000 neutralizing units (nU)/ml together with 1000U/ml

IL-2 at the initiation of culture. Normal rabbit serum (NRS) was added to controls. Cells were harvested and washed on day 5 and cytotoxicity was determined in a standard 4 hour ^{51}Cr release assay with Daudi target cells. Figure 5 shows that the addition of anti-TNF antibody had an inhibitory effect on the generation of LAK cell activity at all effector to target cell ratios (E:T), ranging from 15-34%. The inhibitory effect was statistically significant at each E:T ratio ($p \leq 0.01$).

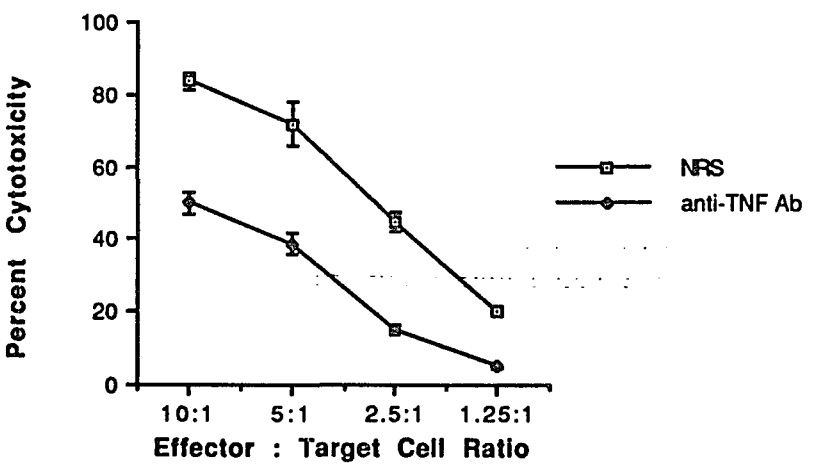

Figure 5: Inhibition of LAK Cell Generation: Addition of Anti-TNF Antibody at the Initiation of Culture

It was postulated that neutralizing activity of the antibody could decay in culture or be inactivated over time by culture conditions. To maintain a high concentration of neutralizing antibody throughout LAK cell development, 1000nU/ml of anti-TNF antibody was added each day, starting at the initiation of culture

with 1000 U/ml IL-2. Cells were harvested and cytotoxicity was assayed on day 6 in a ^{51}Cr release assay. As seen in Figure 6, anti-TNF had an inhibitory effect ranging from 26-39%, depending on the effector to target cell ratio. Again, the effect was significant at all E:T ratios ($p<0.01$).

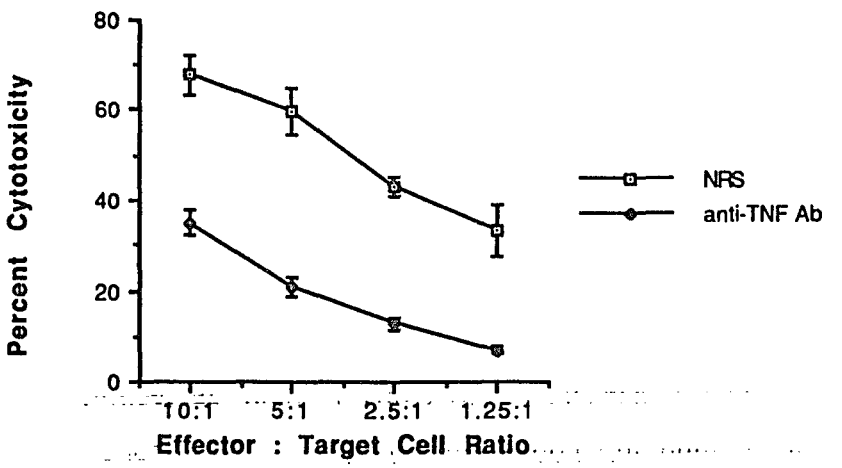

Figure 6: Inhibition of LAK Cell Generation: Daily Addition of Anti-TNF Antibody

To determine the stage of LAK cell development in which TNF is involved, neutralizing antibody was added to cells at initiation of LAK cell culture, or on subsequent days. Cells were harvested and cytotoxicity assayed on day 6. Figure 7 demonstrates that the addition of anti-TNF antibody on day 2 or 3 resulted in maximum inhibition of LAK cell generation.

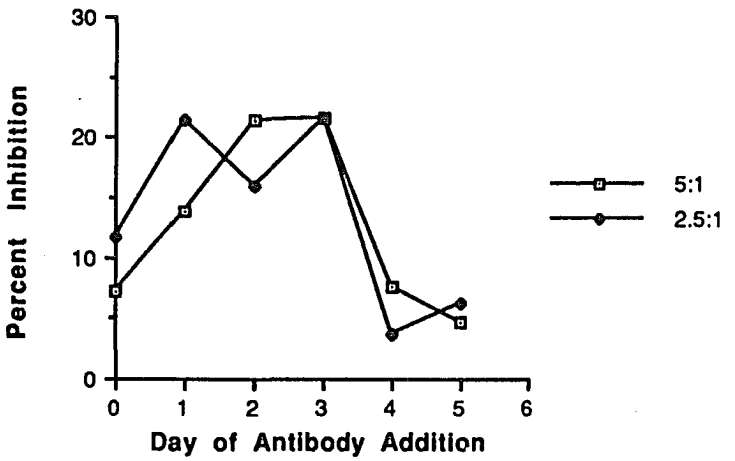

Figure 7: Inhibition of LAK Cell Generation: Addition of Anti-TNF Antibody on Different Days

The addition of anti-TNF antibody had no effect on the lytic phase, as seen in Table 5. In this experiment, the indicated concentrations of polyclonal rabbit anti-human TNF or NRS were added at the initiation of the four hour ^{51}Cr assay.

Table 5: Effect of Anti-TNF Antibody on the Cytotoxicity Assay

nU/ml	NRS	Anti-TNF Ab
0.01	89.97±6.75*	90.26±3.66
0.1	92.59±2.58	95.08±3.87
1.0	87.47±10.99	97.39±5.77
10.0	98.56±0.47	93.80±5.33

*Percent cytotoxicity ± standard deviation

The observation that anti-TNF antibody inhibits the generation of LAK effectors suggests that endogenously produced TNF plays a role the developments of LAK effectors. The inhibitory effect was only partial, even when high levels of antibody were maintained with the daily addition of 1000nU/ml. This suggests that TNF is involved in augmenting LAK generation, but is not essential for it. This conclusion is supported by the findings of others. It has been reported that the addition of neutralizing anti-TNF antibody to the initiation of culture partially inhibits the generation of LAK cytotoxicity from both LGL (13) and PBMC (88). Here, it was found that antibody added on day 2 or 3 had the strongest inhibitory effect, evidence that TNF acts in LAK cell development at this stage of culture.

The finding that addition of neutralizing antibody to the ^{51}Cr assay had no effect on cytotoxicity suggests that endogenously

produced TNF is not involved in the effector phase of LAK cell activity, as measured in a 4 hour cytotoxicity assay. However, the data presented here does not rule out a role for cytokine release in slow killing of tumor cells. As discussed previously, TNF and IFN-γ are released by LAK effectors coincubated with tumor cells, and supernatant containing these cytokines lyses tumor cells in a 72 hour cytotoxicity assay (21). For this reason, the secretion of TNF from tumor cell stimulated LAK cells and the regulation of this secretory response were investigated.

REGULATION OF CYTOKINE RELEASE BY LAK EFFECTORS STIMULATED WITH TUMOR CELLS

Suppression of TNF and IFN-γ Release

While very little is known about the regulation of cytokine release from LAK cells, a number of studies have examined the regulation of TNF release from monocytes. Several factors have been reported to inhibit TNF secretion, including the alpha globulins (114), TAME (113) and PGE_2 (63). The effect of these suppressive agents on the release of TNF and IFN-γ from IL-2 activated PBMC stimulated with K562 erythroleukemia cells was investigated. As described previously, each of the factors to be tested for potential regulation of cytokine release was added to the coincubation of LAK cells and K562 tumor cells. The supernatant was subsequently harvested and assayed for TNF and IFN-γ concentration. Data are expressed as ng/ml and represent the mean of two replicates.

PGE_2

To determine the effect of PGE_2 on cytokine secretion by LAK cells stimulated with tumor cells, various concentrations of the prostaglandin were added to LAK cells which were cultured together with K562 erythroleukemia cells. Figure 8 shows that the addition of PGE_2 to the mixture of LAK cells and tumor cells suppressed TNF and IFN-γ in a concentration dependent manner. The suppressive

effect on TNF release was significant at each concentration of PGE_2 ($p \leq 0.01$), while the effect on IFN-γ release was significant at 1.0 and 10.0mM only ($p \leq 0.03$). The viability of leukocytes, as determined by trypan blue exclusion was unaffected by exposure to PGE_2 at all concentrations tested.

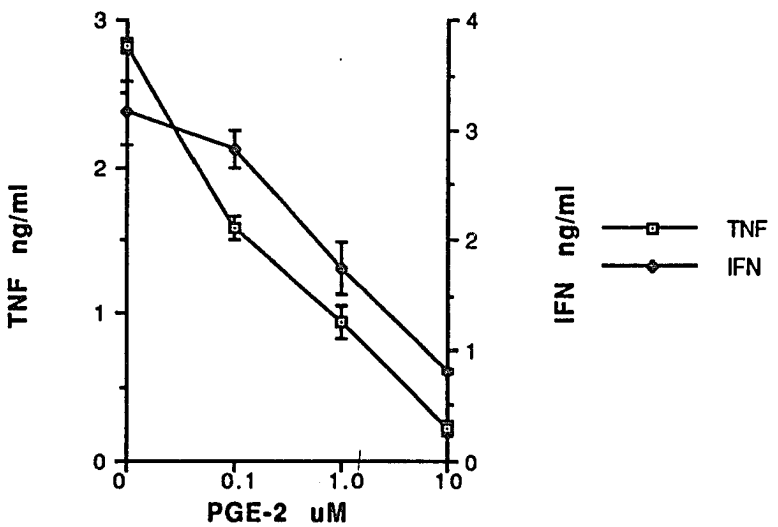

Figure 8: Suppression of TNF and IFN-γ Release by PGE_2

The observation that PGE_2 suppressed cytokine secretion by LAK cells was expected in view of the fact it has been demonstrated to suppress the secretion of TNF by macrophages at the level of gene expression (64), as well as the release of IFN-γ from activated T cells (33). The data presented here provide additional evidence that PGE_2 is a an important regulatory factor influencing production of inflammatory mediators such as TNF and IFN-γ. As TNF can induce

PGE_2 release (30), this prostaglandin may act as a feedback inhibitor in an autoregulatory loop.

Alpha Globulins

Whole alpha globulin, as well as the purified alpha-1-antitrypsin, alpha-2-macroglobulin, and alpha-1-acid glycoprotein fractions, suppress TNF release from endotoxin stimulated monocytes *in vitro* (114). Figure 9 shows that the addition of whole human alpha globulin to LAK cells stimulated with tumor cells suppressed both TNF and IFN-γ secretion in a concentration dependent manner. The inhibition was significant at 1.25 and 2.5 mg/ml for both TNF and IFN-γ ($p \leq 0.04$).

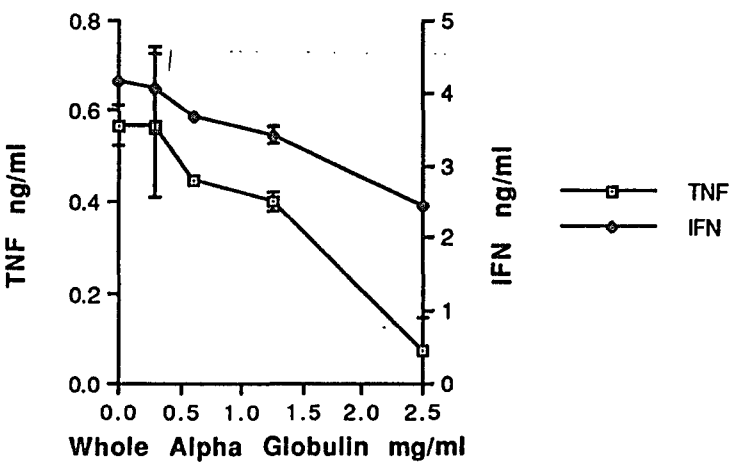

Figure 9: Suppression of TNF and IFN-γ Release by Whole Alpha Globulin

Purified alpha-1-acid glycoprotein also had an inhibitory effect on the release of TNF and IFN-γ (Figure 10). The addition of 4 or 8mg/ml significantly reduced the secretion of both TNF and IFN-γ ($p<0.05$). Alpha-1-acid glycoprotein did not affect viability of LAK or K562 cells at any of the concentrations tested.

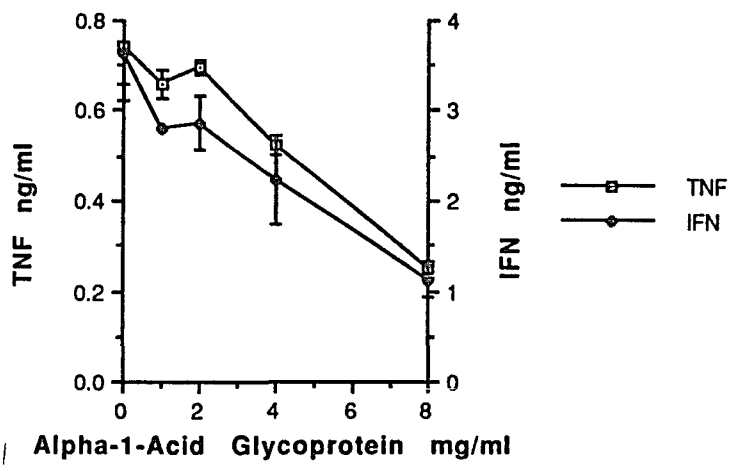

Figure 10: Suppression of TNF and IFN-γ Release by Alpha-1-Acid Glycoprotein

In contrast to the effects of whole alpha globulin and alpha-1-acid glycoprotein, the addition of alpha-1-antitrypsin or alpha-2-macroglobulin enhanced the release of cytokines from tumor cell-stimulated LAK cells. As shown in Figure 11, the addition of 1.5mg/ml or 3.0mg/ml alpha-1-antitrypsin stimulated TNF release ($p<0.03$). The presence of 3.0mg/ml had a stimulatory effect on IFN-γ release ($p=0.03$). The enhancement was seen despite a reduction in viable LAK cells at 3.0mg/ml alpha-1-antitrypsin (Figure 12)

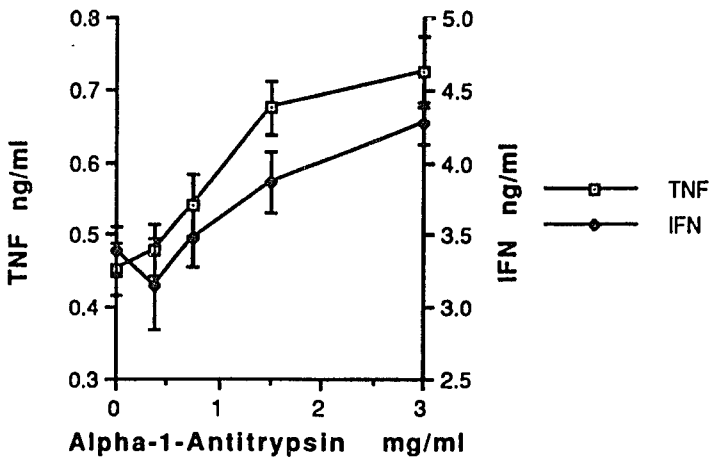

Figure 11: Stimulation of TNF and IFN-γ Release by Alpha-1-Antitrypsin

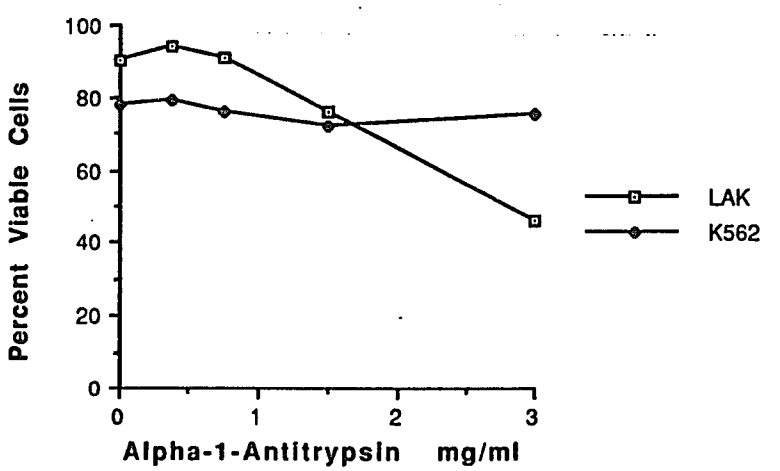

Figure 12: Effect of Alpha-1-Antitrypsin on Cell Viability

Purified alpha-2-macroglobulin enhanced IFN-γ secretion at a concentration of 0.3mg/ml ($p=0.04$) and TNF release at each concentration tested ($p \leq 0.01$) (Figure 13). Alpha-2-macroglobulin was toxic to LAK cells and tumor cells at concentrations greater than 1.5mg/ml (Figure 14).

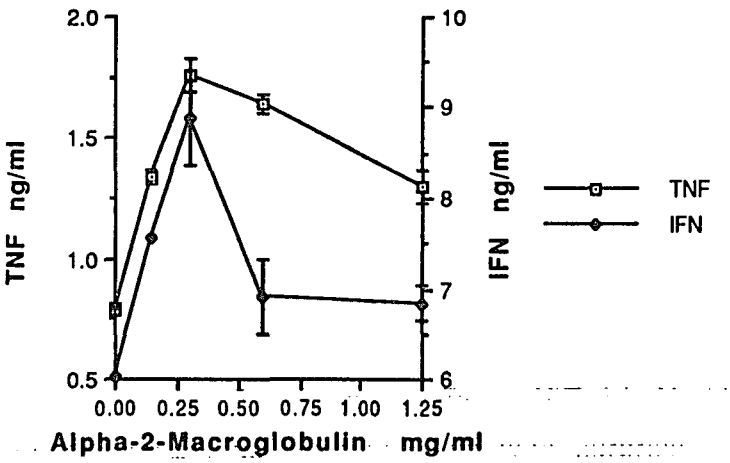

Figure 13: Stimulation of TNF and IFN-γ Release by Alpha-2-Macroglobulin

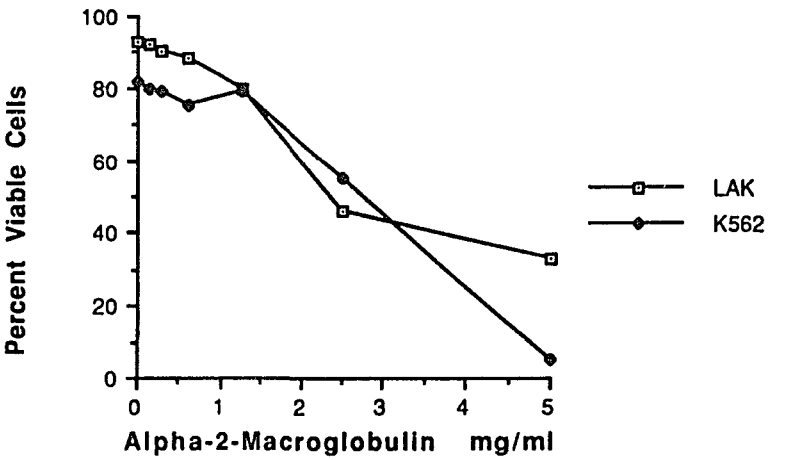

Figure 14: Effect of Alpha-2-Macroglobulin on Cell Viability

The finding that alpha-1-antitrypsin and alpha-2-macroglobulin had a stimulatory effect on cytokine release from tumor cell-stimulated LAK cells was surprising in view of the fact that these two alpha globulin fractions are reported to be potent suppressors of TNF release by monocytes. Several experiments were done to investigate the possibility that the enhancement was due to contamination of the purified fractions with LPS.

First, LPS was quantitated in the Limulus Ameobocyte Assay. In this assay, a $1x10^3$ µg/ml solution of each alpha globulin in complete media, as well as a series of dilutions of each ($1x10^2$, 10.0, 1.0, and 0.1µg/ml), were graded as positive (coagulated) or negative. This was compared to a set of dilutions of a standard to determine approximate concentration of LPS present. Levels of LPS in complete RPMI (RPMI), whole alpha globulin (Glob). alpha-1-acid

glycoprotein (AG), alpha-1-antitrypsin (AT), and alpha-2-macroglobulin (Mac) are shown in Table 6.

Table 6: LPS Levels of Alpha Globulins

	LPS	Samples					
ng/ml	Standard	µg/ml	RPMI	Glob	AG	AT	Mac
1.0	+	$1x10^3$	+	+	+	+	+
0.1	+	$1x10^2$	+	+	+	+	+
0.050	+	10	-	-	+	+	+
0.025	-	1.0	-	-	-	+	+
0.0125	-	0.1	-	-	-	-	-

Both alpha-1-antitrypsin and alpha-2-macroglobulin were found to have a 10 fold higher concentration of LPS (0.025-0.05µg LPS/mg alpha globulin fraction) than that present in alpha-1-acid glycoprotein.

Next, purified LPS was added to the interaction of LAK cells and tumor cells at concentrations comparable to those present in the alpha globulin fractions to determine if LPS would enhance TNF and IFN-γ secretion. Figure 15 demonstrates that the presence of 0.01 or 0.1µg/ml LPS did not have a significant effect on the release of either TNF or IFN-γ. Data represents the mean of two replicates ± standard deviation. Enhancement of cytokine secretion was observed only at a concentration of 10µg/ml LPS ($p \leq 0.03$), a level 200 fold higher than that detected in alpha-1-antitrypsin and alpha-2-macroglobulin.

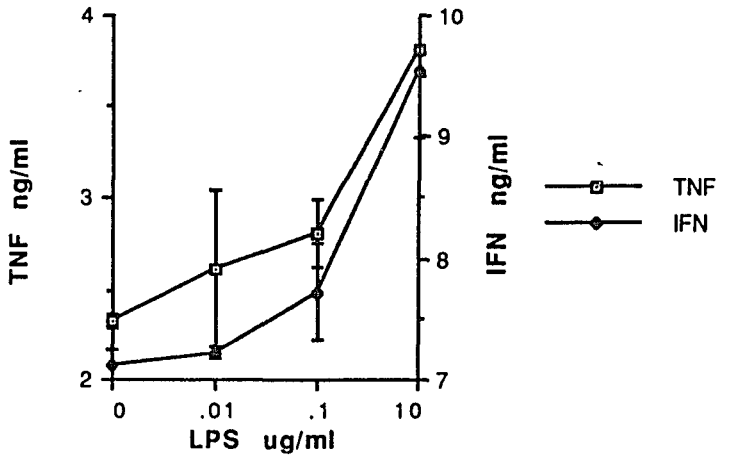

Figure 15: Addition of LPS

Also, polymixin B, an antibiotic known to bind to LPS and block its biological effects (120), was added to the interaction of LAK cells, tumor cells and alpha-1-antitrypsin. Polymixin B was added at 50μg/ml, a concentration sufficient to neutralize 0.05μg/ml LPS (136). LAK cells and K562 were cultured together with the indicated concentrations of alpha-1-antitrypsin in complete media (Control) or in media containing Polymixin B. The presence of 2mg/ml alpha-1-antitrypsin enhanced the release of TNF ($p<.03$) as observed previously, but had no significant effect on the release of IFN-γ. The addition of Polymixin B had no effect on the secretion of either TNF or IFN-γ in the presence of alpha-1-antitrypsin (16).

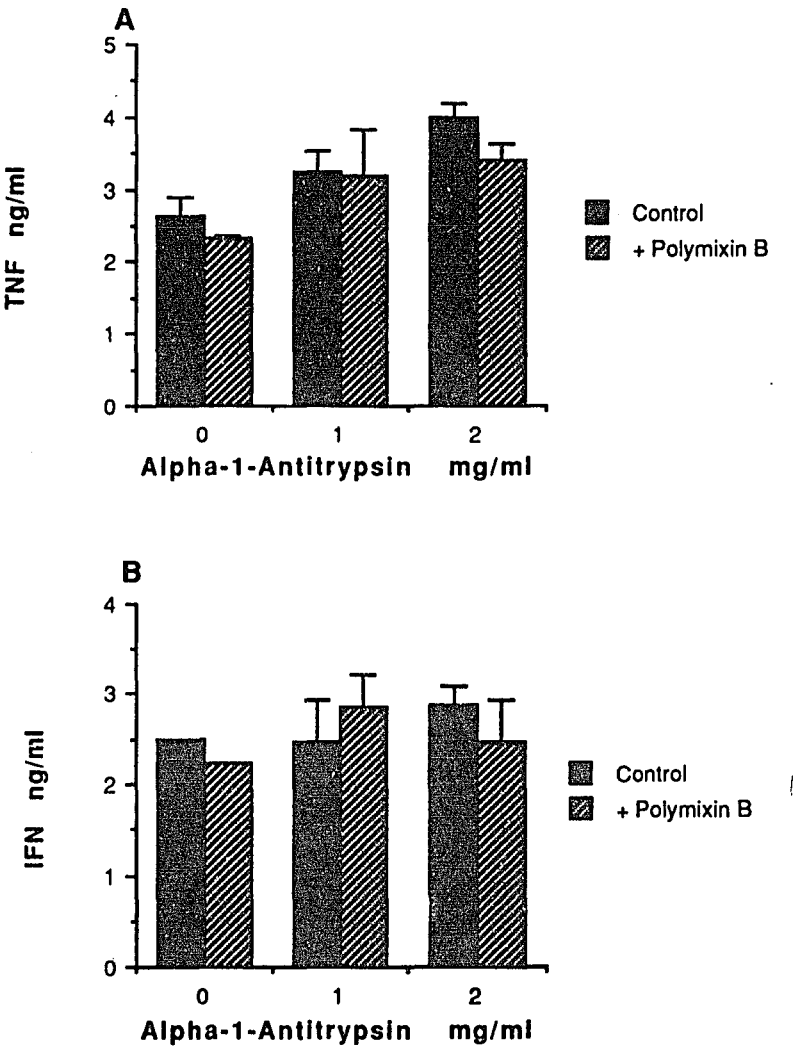

Figure 16: The Effect of Polymixin B on TNF (A) and IFN-γ (B) Release

Together, these experiments suggest that the stimulatory effect of alpha-1-antitrypsin and alpha-2-macroglobulin is not due to LPS contamination of the purified preparations.

From the data presented here, it is clear that the regulation of TNF release from LAK cells by alpha globulins is different than that reported for monocytes. While whole alpha globulin and alpha-1-acid glycoprotein were suppressive, alpha-1-antitrypsin and alpha-2-macroglobulin had an enhancing effect. The possibility that the observed differences were due to variation in the activity of the preparations between different lots was addressed in an experiment comparing regulation of release of TNF in LPS-stimulated PBMC (Table 7) to that of K562-stimulated LAK cells (Table 8). Fresh PBMC were cultured overnight in complete media or in media containing 0.1μg or 10.0μg/ml LPS. The indicated alpha globulins were added to the cultures at 5mg/ml. The alpha globulins were also added to LAK cells stimulated with K562. Whole alpha globulin (Glob) was found to inhibit TNF release by both PBMC and LAK cells ($p<0.01$). The addition of 5mg/ml alpha-1-acid glycoprotein (AG) suppressed secretion of TNF by LAK cells ($p<0.01$), but not by PBMC Alpha-1-antitrypsin (AT) inhibited TNF release by PBMC ($p<0.01$), but enhanced TNF secretion by LAK cells ($p<0.05$).

Table 7: Suppression of TNF Release from PBMC

PBMC	Control	0.036±.014*
	Glob 5mg/ml	0
	AG 5mg/ml	0.349±.037
	AT 5mg/ml	0.209±.086
PBMC + 0.1mg/ml LPS	Control	1.821±.044
	Glob 5mg/ml	0.479±.023
	AG 5mg/ml	1.750±.105**
	AT 5mg/ml	0.230±.001
PBMC + 1.0mg/ml LPS	Control	1.999±.133
	Glob 5mg/ml	0.348±.016
	AG 5mg/ml	1.806±.073**
	AT 5mg/ml	0.402±.022

*Data is expressed as ng/ml ± standard deviation
**Not statistically significant (p>0.05)

Table 8: Suppression of TNF Release from LAK Cells Stimulated with K562 Tumor Cells

Control	0.668±.057*
Glob 5mg/ml	0
AG 5mg/ml	0.002±.003
AT 5mg/ml	0.776±.037

*Data is expressed as ng/ml ± standard deviation

These findings indicate that the observed differences in regulation between that observed in LAK cells and that reported for PBMC are not the result of variation in the activity of the alpha globulin preparations tested.

TAME

The synthetic protease inhibitor TAME can suppress TNF release from monocytes stimulated with LPS, presumably by

inhibiting cleavage of the cytokine from the cell membrane (Scuderi 1989). Figure 17 shows that TAME also suppressed the release of both TNF and IFN-γ from the LAK cells stimulated with K562 cells. The presence of 5mM TAME completely suppressed TNF secretion and decreased release of IFN-γ by 82%. The viability of the LAK cells was unaffected by all concentrations of TAME used in this experiment.

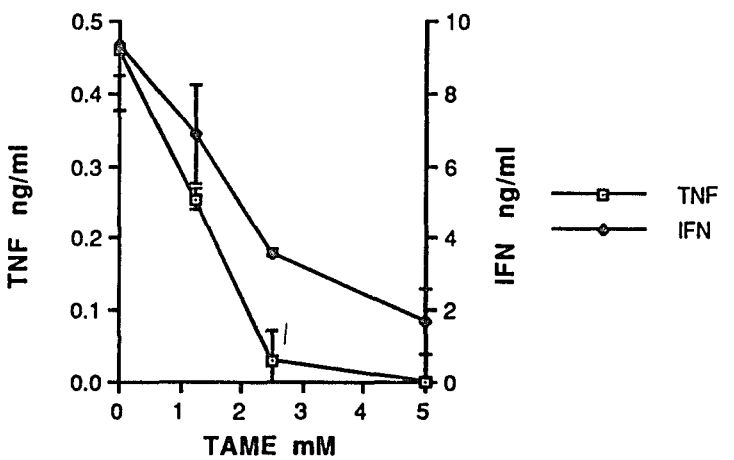

Figure 17: Inhibition of TNF and IFN-γ Release by TAME

The finding that TAME completely suppresses TNF secretion by LAK cells would tend to support the involvement of a serine protease in secretion. However, the finding of suppression of IFN-γ release was unexpected. Unlike TNF, IFN-γ is synthesized as a typical secretory protein and there is no evidence that it exists in a transmembrane form or that its release is dependent on protease activity.

As TNF can act synergistically with IL-2 in stimulating IFN-γ production by T cells (110), the possibility that the suppression of IFN-γ release under these condition was the result of a decreased levels of TNF was investigated. Polyclonal rabbit anti-human antibody specific for TNF and IFN-γ was added to the coincubation of LAK cells and tumor cells. Normal rabbit serum was used as a control. As shown in Figure 18, the addition of anti-TNF antibody decreased the concentration of TNF present in the supernatant, but had no effect on the IFN-γ concentration. Conversely, addition of anti-IFN-γ to the cultures resulted in decreased levels of IFN-γ, but had no effect on the concentration of TNF detected in the supernatants.

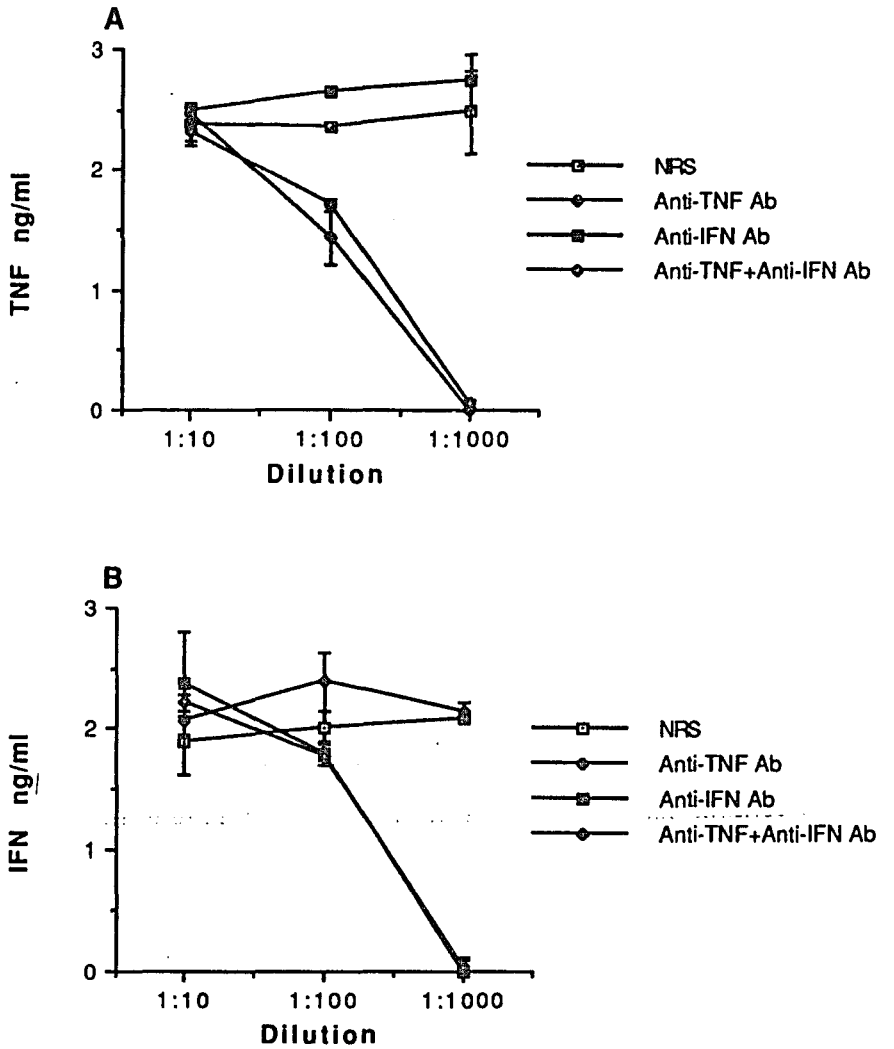

Figure 18: Effect of Neutralizing Antibody on TNF (A) and IFN-γ (B) Release

These findings suggest that TAME, at the concentrations tested, may have a wider range of biological activities than

previously recognized and that the suppression of cytokine release by TAME under these conditions is not due its anti-protease activity

LAK Cell-Tumor Cell Interaction

Stimulation of LAK Cells with K562 Lysate

The need for metabolically active K562 cells in the stimulation of cytokine secretion and the possibility that the suppressive agents were altering the way in which viable K562 interacted with activated lymphocytes was investigated. LAK cells were stimulated with viable K562, with K562 lysate (Ly) or with lysate diluted in complete media (Ly 1:2 and Ly 1:4). The presence of metabolically active K562 was not required for stimulation of cytokine secretion by the leukocytes. LAK cells cultured in the presence of tumor cell lysate secreted both TNF and IFN-γ, although at levels lower than that of LAK cells cultured with viable tumor cells (Figure 19).

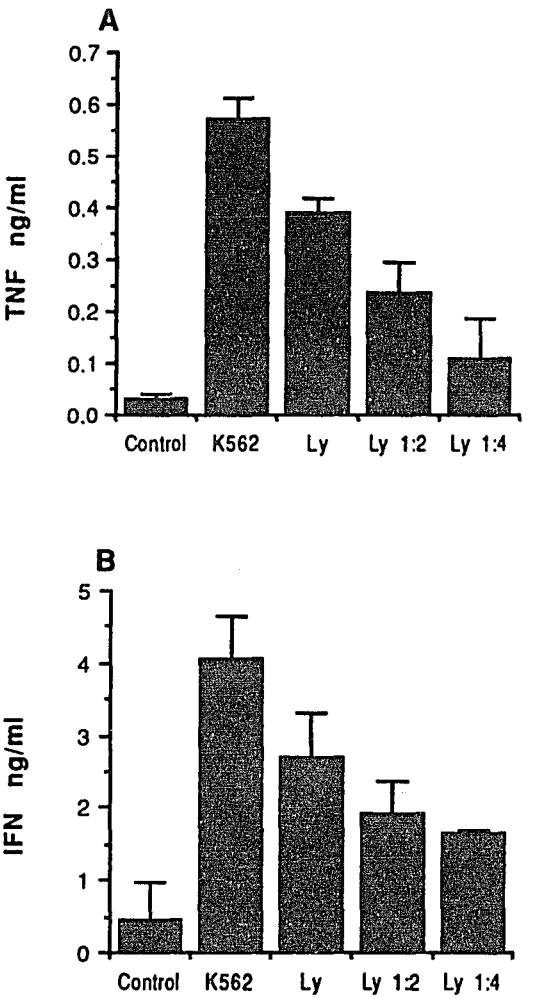

Figure 19: TNF (A) and IFN-γ (B) Release by LAK Cells Stimulated with K562 Lysate

The suppressive effects of 10mM PGE_2, 4mg/ml alpha-1-acid glycoprotein (AG), and 6mM TAME on tumor cell-induced as well as tumor cell lysate-induced secretion of TNF and IFN-γ from LAK cells were compared. In this experiment each factor tested inhibited both

TNF and IFN-γ release ($p<0.05$) with the exception of alpha-1-acid glycoprotein, which had no significant effect on the release of IFN-γ from either preparation of LAK cells (Figure 20). The susceptibility to suppression did not differ between LAK cells stimulated with viable tumor cells and LAK cells stimulated with tumor cell lysate.

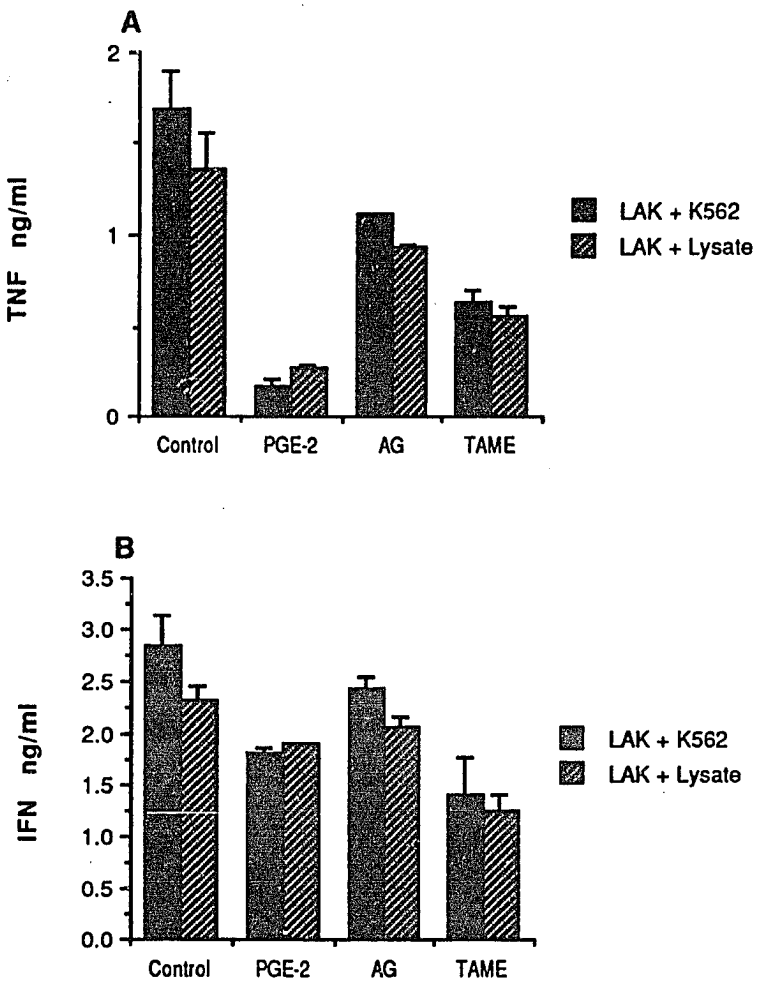

Figure 20: Suppression of TNF (A) and IFN-γ (B) Release by LAK Cells Stimulated with K562 Lysate

This data indicates that PGE_2, alpha-1-acid glycoprotein, and TAME act on the LAK cells to suppress the release of cytokines, not at the level of the stimulating tumor cells.

Pretreatment of LAK Cells with Suppressive Factors

To determine if the inhibition of cytokine release required constant exposure to PGE_2, AG, and TAME, LAK cells were pretreated with the suppressive factors for 1 hour then washed and coincubated with K562. As a control group, LAK cell-tumor cell cocultures were continuously exposed to PGE_2, AG or TAME. Pretreatment of LAK cells with 4mg/ml AG or 5mM TAME did not inhibit the secretion of TNF and IFN-γ (Figure 21). It appears that inhibition of cytokine release by AG or TAME require that they be present throughout the interaction of LAK cells and K562. However pretreatment with 10μM PGE_2 did result in a significant reduction in TNF ($p<0.01$)and IFN-γ secretion ($p<0.03$), although the effect was less dramatic than that observed when PGE_2 was present during the coincubation.

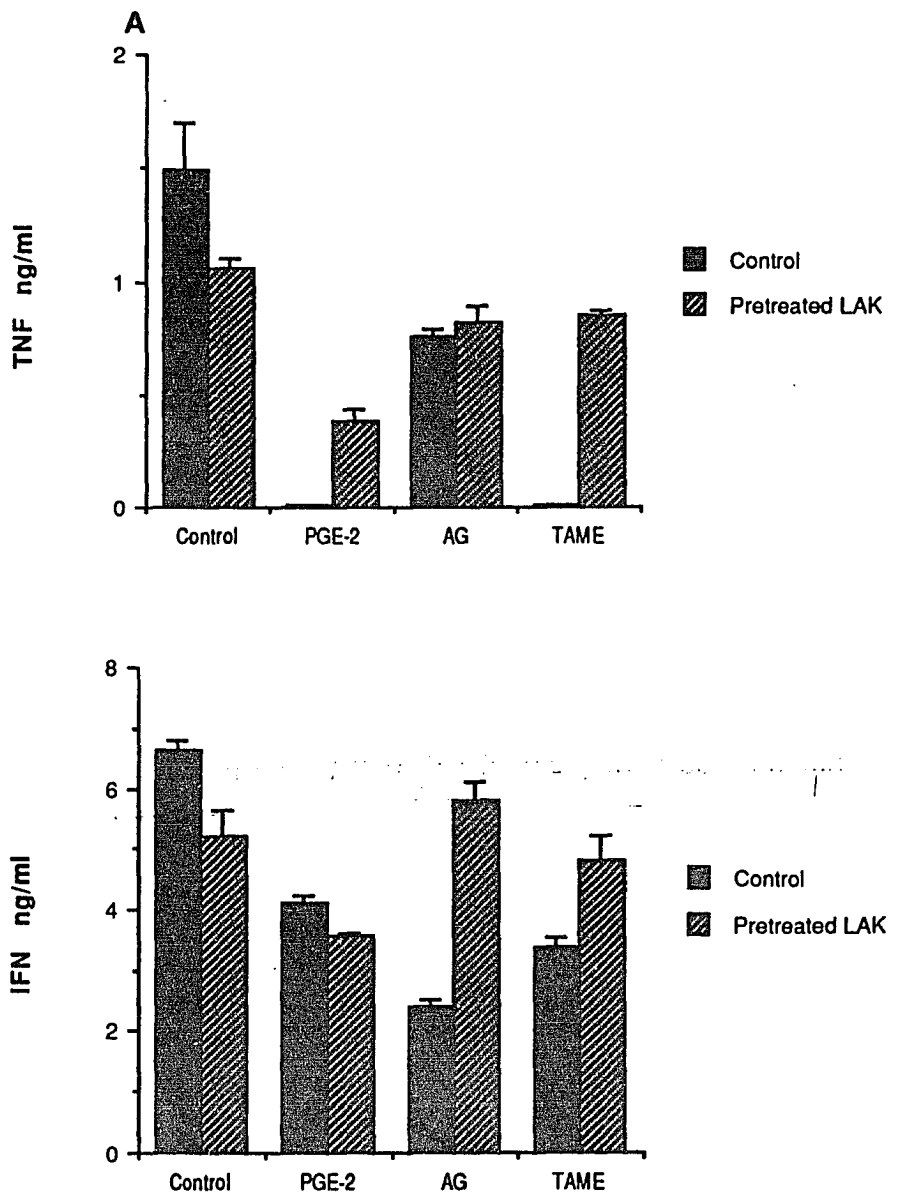

Figure 21: Pretreatment of LAK Effectors with Suppressive Factors

Cytokine Release from Monocyte Depleted LAK Cells

Since the regulation of cytokine release from tumor cell-stimulated LAK cells was being compared to that reported for TNF release by monocytes, the possibility that the cytokine production detected under these conditions was dependent on the presence of monocytes was investigated. To accomplish this, LAK cells were depleted of LeuM3+ cells (monocytes) by fluorescence activated cell sorting prior to stimulation with tumor cells. The LeuM3- LAK cells were then cultured with K562 alone (Control) or in the presence of 10μM PGE_2, 4mg/ml AG or 5mM TAME. Figure 22 shows that the elimination of monocytes did not eliminate production of TNF or IFN-γ in response to tumor cells, although levels were lower than that of unseparated LAK. The addition of PGE_2 or TAME inhibited the release of TNF ($p<0.01$) and IFN ($p<0.05$) from both LeuM3- and unseparated LAK cells. Alpha-1-acid glycoprotein suppressed TNF release ($p<0.01$), but not IFN-γ release from both LAK cell preparations.

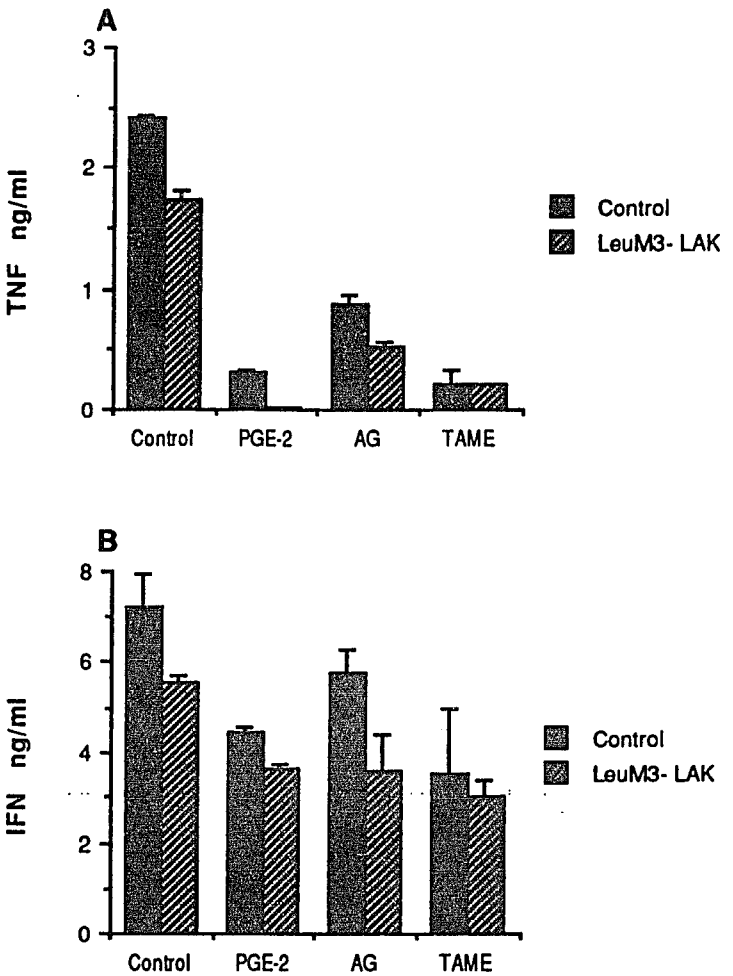

Figure 22: TNF and IFN-γ Release from LeuM3- LAK Cells

These data indicate that PGE_2, AG and TAME act directly on lymphocytes in the LAK cell preparation, not on residual monocytes and not indirectly via the release of monokines.

Cytokine Release from Cloned LAK Cells

The ability of tumor cell-stimulated lymphocytes to secrete TNF and IFN-γ in the absence of monocytes was also assessed using a LAK cell clone, IP12, which has a T cell phenotype. The IP12 cells were incubated with K562 alone or with the indicated concentrations of prostaglandin, alpha globulins or TAME. The pattern of suppression was identical to that observed for peripheral blood derived LAK cells, with the exception that alpha-1-antitrypsin did not significantly enhance IFN-γ release by IP12 (Table 9).

Table 9: Suppression of TNF and IFN-γ Release by LAK Cell Clone IP12

Additive	TNFng/ml±SD	IFN ng/ml±SD
Control	0.917 ± .10*	3.342 ± .22
PGE2 1mM	0.206 ± .01	2.831 ± .63
PGE2 10mM	0.195 ± .003	2.039 ± .17
Glob 2mg/ml	0.288 ± .01	3.068 ± .32**
Glob 4mg/ml	0.004 ± .006	2.393 ± .03
AG 2mg/ml	0.494 ± .03	3.095 ± .13**
AG 4mg/ml	0.151 ± .01	2.099 ± .07
AT 2mg/ml	1.331 ± .02	3.862 ± .49**
AT 4mg/ml	1.344 ± .09	3.573 ± .17**
TAME 5mM	0.397 ± .003	2.586 ± .04

*Data is expressed as ng/ml ± standard deviation
**Not statistically significant ($p \geq 0.05$)

Cell Surface TNF Expression

The capacity of the two plasma protease inhibitors, alpha-1-antitrypsin and alpha-2-macroglobulin, to block TNF secretion in fresh monocytes, yet have no suppressive effect on IL-2 activated

leukocytes stimulated with K562 cells, suggests that there may be alternative mechanisms for the release of TNF in the two cell populations. The responsiveness of leukocytes to the suppressive effects of the individual alpha globulin fractions may depend on the phenotype of the TNF secreting population and could reflect the capacity of particular phenotypes to express TNF as a transmembrane molecule. As discussed previously, the hydrophobic aminoterminus of the TNF molecule can function as a means by which the cytokine is inserted into the plasma membrane of peripheral blood monocytes (62). It is thought that the natural protease inhibitors, alpha-1-antitrypsin and alpha-2-macroglobulin, as well as the synthetic protease inhibitor TAME, block TNF secretion in monocytes by inhibiting the enzymatic hydrolysis of cell surface TNF necessary for secretion. In IL-2 activated T cells and NK cells, the mechanism of TNF release may not involve hydrolysis of transmembrane cytokine into the lower molecular weight secreted form. In these populations, the fusion of secretory vacuoles containing TNF that has been processed from the 26kD pro-TNF into the secreted 17kD form with the plasma membrane may be the dominant mechanism of cytokine release. To investigate this possibility, the cell surface expression of TNF on LPS stimulated PBMC and K562 stimulated LAK cells was examined

First, fresh PBMC and PBMC stimulated with 20μg/ml LPS for 12 hours were analyzed by flow cytometry. The analysis was gated on monocytes. The data is expressed as percent gated events,

representing the mean of four replicates ± standard deviation. Table 10 demonstrates that fresh PBMC increase surface TNF on CD14+ cells when stimulated with LPS, as expected. The percentage of monocytes positive for both CD14 and TNF increased from 2.3% to 48.9%, representing a 63.4 ± 6.6% increase in the number of CD14+ cells expressing surface TNF ($p<0.01$).

Table 10: PBMC Surface Expression of TNF

Phenotype	PBMC	PBMC + LPS
CD14+ TNF-	77.4 ± 1.9*	25.3 ± 9.5
CD14+ TNF+	2.3 ± 2.5	48.9 ± 7.3
CD14- TNF+	0	3.5 ± 3.8

*Percent gated events (monocytes) ± standard deviation

To determine if the LAK cells stimulated by interaction with tumor cells were expressing cell surface TNF, the cells were stained and analyzed by flow cytometry for expression of TNF and the phenotypic markers CD3 and CD16. Cell surface expression of TNF on LAK cells alone was compared to the expression on LAK cells cultured with tumor cell lysate overnight. Both preparations of LAK cells were depleted of adherent cells. The data is expressed as percent gated events and represents the mean of four replicates ± standard deviation. As shown in Table 11, a low percentage of the cells analyzed were found to be CD3+ and TNF+ in both the stimulated (9.21%) and unstimulated LAK (13.24%) There was no upregulation of TNF on CD3+ cells following incubation with tumor

cell lysate. In contrast, the percentage of CD16+ TNF+ cells increased from less than 1% of the cells analyzed to 2.43% (p=0.026). This data is represented in Figure 23 as the percent TNF+ cells in both the CD3+ and the CD16+ populations.

Table 11: LAK Cell Surface Expression of TNF

Phenotype	LAK	LAK + K562
CD3+ TNF-	73.26 ± 1.66*	77.43 ± 2.83
CD3+ TNF+	13.24 ± 4.87	9.21 ± 1.24
CD3- TNF+	3.66 ± 3.68	2.92 ± 1.25
CD16+ TNF-	6.0 ± 0.70	3.26 ± 0.58
CD16+TNF+	0.70 ± 0.32	2.43 ± 0.42
CD16- TNF+	0	0.68 ± 0.33

*Percent gated events ± standard deviation

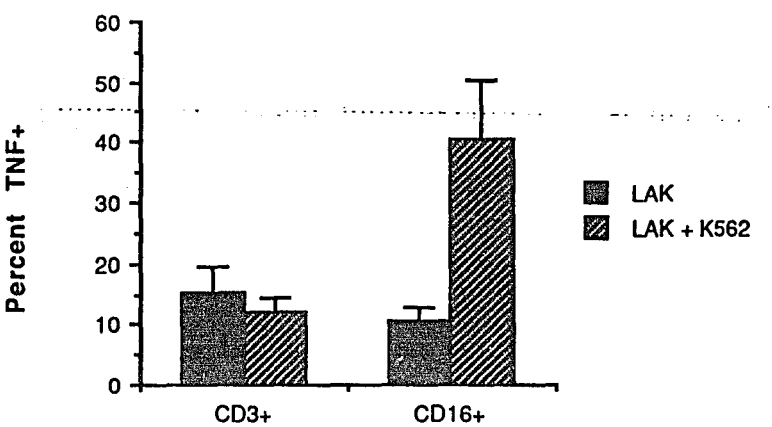

Figure 23: Percent of CD3+ and CD16+ Cells Expressing Cell Surface TNF

In addition to the number of positively staining cells, FACS analysis also provides information on the intensity of staining,

referred to as the mean fluorescence intensity. The CD3+ LAK cells which expressed TNF were very dimly stained for CD3+. This can be compared to the intensity of staining for CD16 on TNF- and TNF+ cells (Figure 24). The TNF+ T cells appear to represent a unique, very dimly CD3+ population.

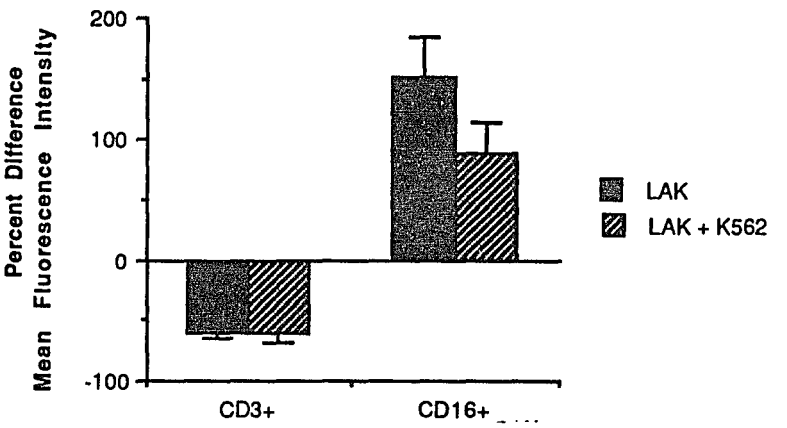

Figure 24: Mean Fluorescence Intensity of CD3 and CD16 on TNF+ Cells

FACS analysis of T cell TNF surface expression before and after exposure to K562 revealed that the number of CD3+ lymphocytes which expressed membrane bound cytokine did not increase after stimulation with leukemia cells, while the number of CD16+ NK cells expression surface TNF increased by 49% after exposure to K562 tumor cells. These data suggest that the release of TNF from T cells under these conditions, in contrast to monocytes and NK cells, does not involve hydrolysis of transmembrane cytokine.

ALPHA-1-ACID GLYCOPROTEIN

With respect to the factors that were found to suppress cytokine release by LAK cells, alpha-1-acid glycoprotein was of particular interest as the potential for suppression by this acute phase reactant may have important implications for the use of recombinant IL-2 and TNF in immunotherapy. The possible mechanism of action in inhibition of cytokine release, as well as the effect of alpha-1-acid glycoprotein on the generation of LAK cell activity were investigated.

Effect of Alpha-1-Acid Glycoprotein on TNF Secretion in Response to Stimulation with Different Tumor Cell Lines

Previous experiments to investigate the secretion of TNF from LAK cells involved the use of one tumor cell line, K562. To determine if the inhibition of cytokine release by alpha-1-acid glycoprotein was restricted to LAK cells stimulated with K562 or if the observed effect was a more general phenomenon, a panel of different tumor cell lines was used. LAK cells were cultured in the indicated concentrations of alpha-1-acid glycoprotein alone (Control) or with one of the tumor cell lines (K562, Daudi, U937, A375M, or MCF7) under conditions previously described. Viability of the LAK cells and the tumor cells was unaffected by the concentration of alpha-1-acid glycoprotein used here, as determined

by trypan blue exclusion (LAK, K562, Daudi, and U937) or the Biorad cytotoxicity assay (A375M and MCF7). Data is expressed as ng/ml ± standard deviation and represents the mean of two replicates. The stimulation of TNF release differed considerably between the tumor cell lines tested. As shown in Figure 25, LAK cells cultured with A375M secreted a greater than 10 fold higher concentration of TNF than LAK cells stimulated with Daudi. This variability may reflect the density of expression of HLA or tumor cell marker(s) recognized by the LAK effector. There was an inhibition of TNF release in response to K562 at each concentration of the alpha globulin tested ($p<0.03$). The inhibitory effect was statistically significant at 2.5mg/ml and 5mg/ml only for both Daudi ($p<0.01$) and U937 ($p<0.03$). When the LAK cells were stimulated with A375M or MCF7, TNF release was suppressed by 5mg/ml only ($p<0.01$ and $p<0.04$, respectively).

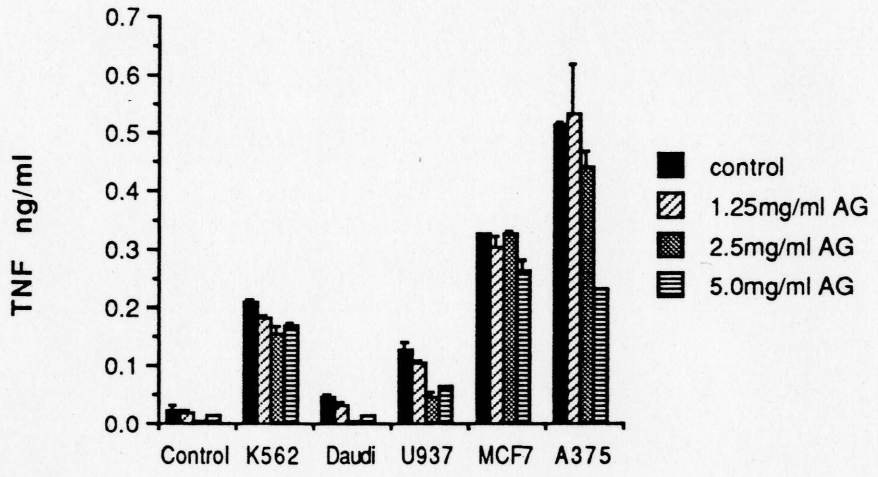

Figure 25: Inhibition of TNF Release by LAK Cells Stimulated with Different Tumor Cells Lines

The effect of alpha-1-acid glycoprotein is not restricted to cytokine release in response to K562 cells, but is observed when LAK cells are cultured with a variety of different tumor cell lines. This suggests that alpha-1-acid glycoprotein may be an important regulatory factor in the LAK cell mediated anti-tumor response.

Suppression of Lymphocyte Responsiveness

As alpha-1-acid glycoprotein is known to inhibit lymphocyte mitogenic responses (19), the effect of the alpha-globulin on the response of LAK cells to K562, as determined by ^{3}[H]thymidine incorporation was assessed.

First, a time course experiment was performed to determine the optimum time of coincubation with K562 lysate for uptake of ^{3}[H]thymidine by the LAK cells. The cells were pulsed with

^{3}[H]thymidine at 12 hours, 8 hours or 4 hours after the initiation of culture with tumor cell lysate. LAK cells alone were used as controls. All of the cells were pulsed for 4 hours and the counts per minute (cpm) determined. The data is expressed as cpm ± standard deviation and represents the mean of three replicates. A 12 hour exposure to tumor cell lysate was optimum, resulting in 1725±150 cpm (Figure 26).

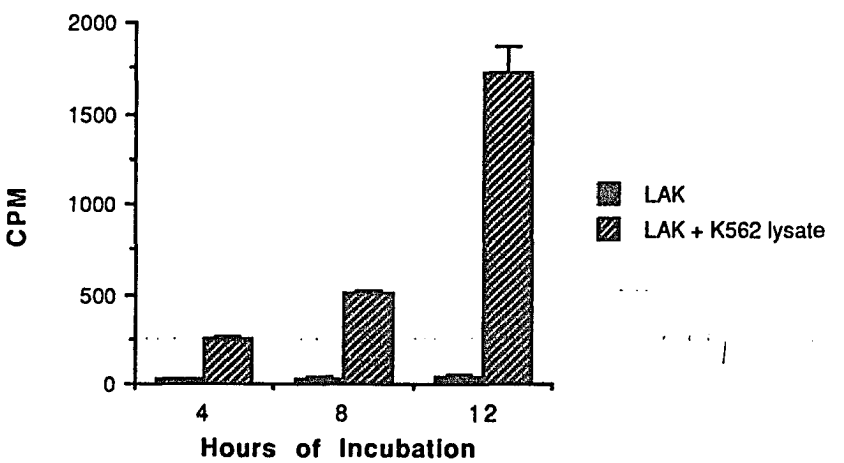

Figure 26: Incorporation of ^{3}[H]Thymidine by LAK Cells

After the optimum incubation time was determined, LAK cells were stimulated with K562 lysate in media or media containing 2.5mg/ml or 5mg/ml alpha-1-antitrypsin. The cells were pulsed with ^{3}[H]thymidine after 12 hours. Figure 27 demonstrates that the addition of the alpha globulin does result in an inhibition of ^{3}[H]thymidine incorporation. At a concentration of 5mg/ml $p<0.01$.

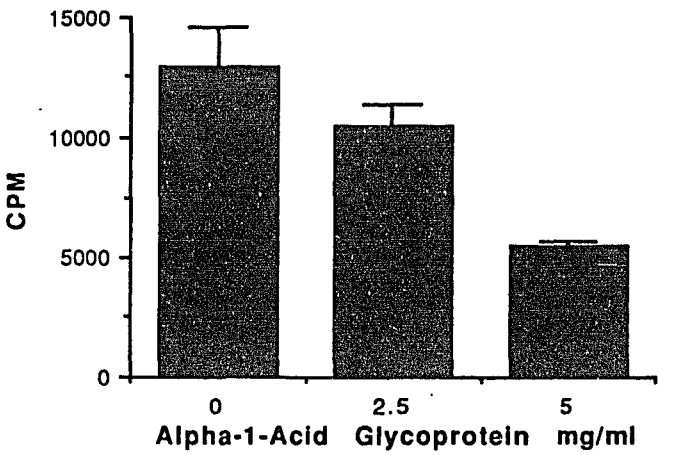

Figure 27: Inhibition of ^{3}[H]Thymidine Incorporation by Alpha-1-Acid Glycoprotein

The effect of alpha-1-acid glycoprotein on LAK cell responsiveness to tumor cells, as determined by ^{3}[H]thymidine incorporation, is not surprising in view of reports regarding its inhibition of other lymphocyte functions. This acute phase reactant appears to be a generally immunosuppressive molecule.

TNF mRNA Expression

Currently, there is no information on the molecular mechanism of action of alpha-1-acid glycoprotein in any of its immunosuppressive activities. The effect of the alpha globulin on the level of TNF mRNA in LAK cells was investigated. To determine if the inhibitory effect of alpha-1-acid glycoprotein on TNF release was the result of decreased TNF mRNA expression, we examined TNF mRNA from both treated and untreated LAK cells and LAK cells

stimulated with K562 lysate with ^{32}P-labelled TNF probe. The blot shown in Figure 28 was analyzed by densitometry, normalized to beta-actin controls and is expressed as percent of the control (LAK alone). The LAK cells alone appeared to transcribe TNF mRNA, although they secrete little or no cytokine. The addition of alpha-1-acid glycoprotein did not cause a decrease in TNF message; the untreated (A) and treated (B) LAK alone had approximately equal levels of TNF mRNA. The addition of K562 lysate resulted in a detectable increase in TNF mRNA expression and leads to TNF secretion. However, when IL-2 activated cells were stimulated with K562 lysate in the presence of alpha-1-acid glycoprotein, the induced increase in TNF message was reduced by 32%.

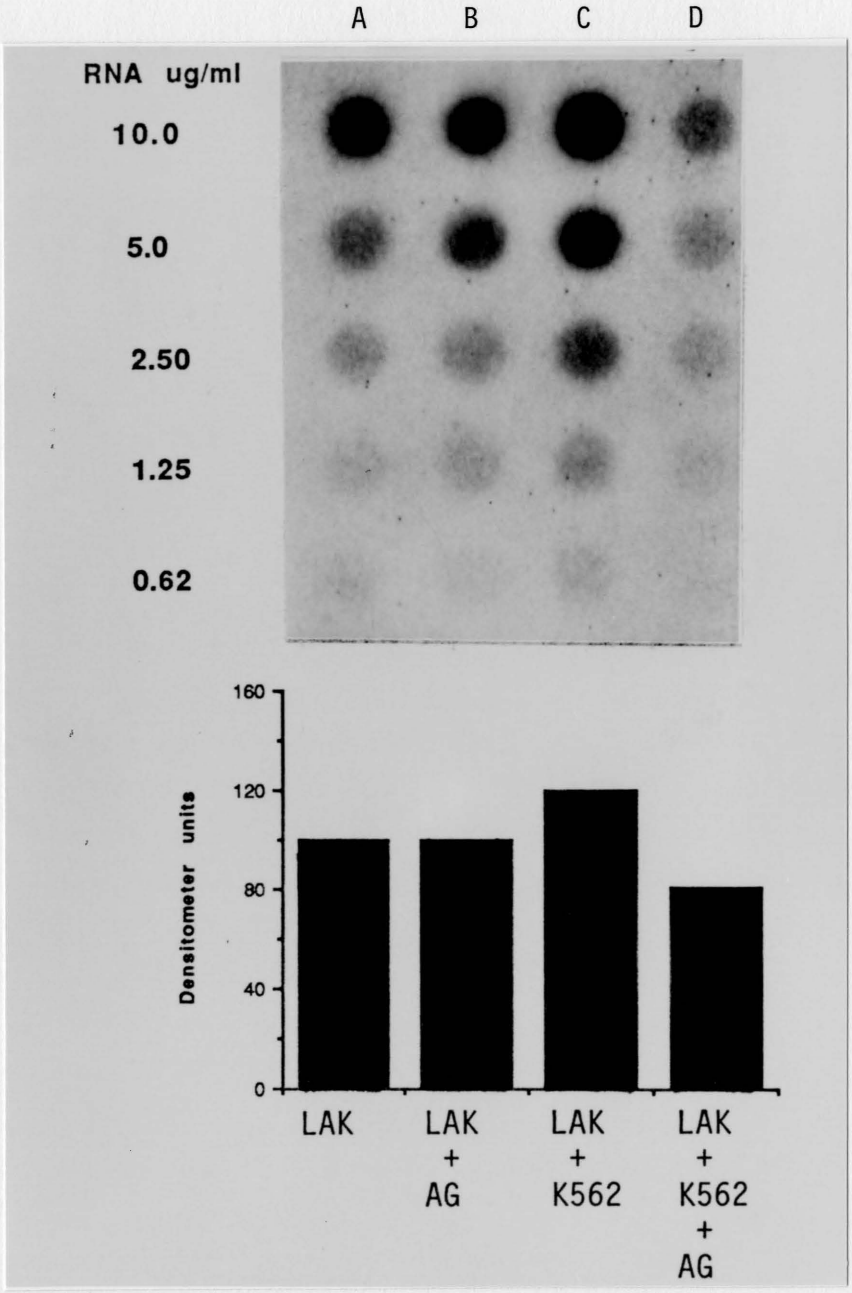

Figure 28: The Effect of Alpha-1-Acid Glycoprotein on TNF mRNA Expression

The alpha-1-acid glycoprotein induced decrease in TNF mRNA observed may reflect regulation of gene activation and transcription by the alpha globulin. It is also possible that the suppressive effect of alpha-1-acid glycoprotein is mediated by a reduction in the half life of the message. TNF mRNA, like that of a number of other cytokines, has a AU rich 3' untranslated sequence that is believed to confer instability upon mRNA's containing it (16, 116). It has been proposed that recognition of this sequence by ribonucleases with high selectivity leads to destruction of the mRNA's containing it (11). Alternatively, these 3' untranslated sequences may accelerate poly (A) shortening by base pairing with the poly (A) tail (134). Although the regulatory mechanisms controlling TNF secretion are not well understood, it is clear that regulation operates at multiple levels (reviewed in 10) and that the potential mechanisms of action discussed here are not mutually exclusive.

Generation of LAK Activity

The ability of alpha-1-acid glycoprotein to influence the generation of LAK cell cytotoxic activity was assessed. LAK cells were cultured in media or media containing 2.5 or 5mg/ml alpha-1-antitrypsin. After 5 days, cytotoxicity was determined in a 4 hour ^{51}Cr release assay with E:T ratio of 6:1 and 3:1 using Daudi target cells. The data are expressed as percent cytotoxicity ± standard deviation, and represent the mean of three replicates. As shown in Figure 29, the presence of the alpha globulin resulted in an

inhibition of the development of LAK cell cytotoxicity. The effect was significant at 5mg/ml at E:T ratios of both 6:1 and 3:1 ($p<0.05$)

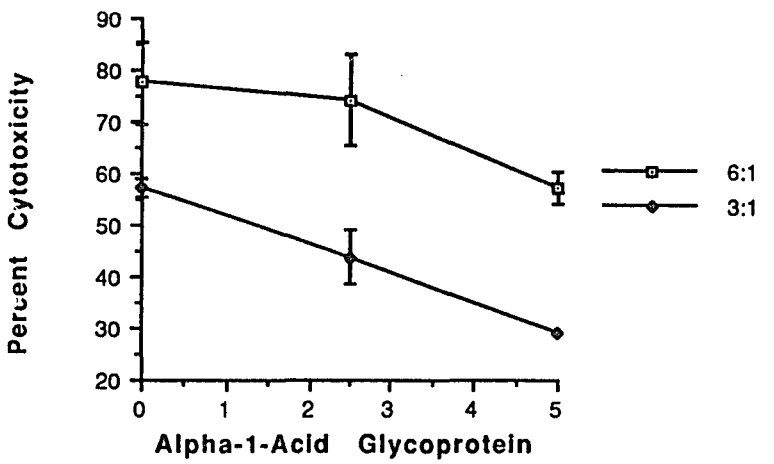

Figure 29 Inhibition of LAK Cell Generation by Alpha-1-Acid Glycoprotein

In the same experiment, the TNF produced by the LAK cells in culture was quantitated by ELISA on the fifth day of culture. The data is expressed as ng/ml ± standard deviation, representing the mean of two replicates. The addition of alpha-1-acid glycoprotein resulted in a decrease ($p<0.01$) in the concentration of TNF secreted by LAK cells in culture. (Figure 30)

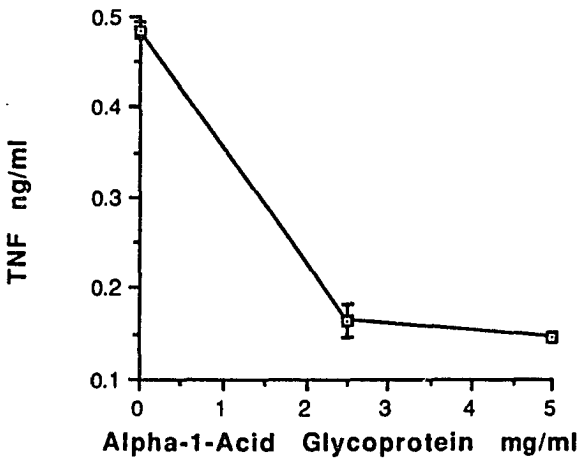

Figure 30: Inhibition of TNF Release from LAK Cells by Alpha-1-Acid Glycoprotein

Reversal of Inhibition

The possibility that the inhibition of LAK cell development mediated by alpha-1-acid glycoprotein was due to its specific down regulation of endogenously produced TNF was investigated. Exogenous TNF was added to the culture of LAK cells and the alpha globulin to determine if the suppression of LAK cell development could be reversed. In this experiment, LAK cells were cultured in media alone or media containing 5mg/ml of alpha-1-acid glycoprotein. The indicated concentrations of recombinant TNF were added at the initiation of culture (Figure 31) Also, LAK cells were cultured with 10U/ml or 100U/ml TNF alone as controls (B). The addition of 10U/ml TNF partially reversed the inhibition induced by alpha-1-acid glycoprotein, while the addition of 100U/ml almost

completely abrogated the effect. TNF alone had no effect on the generation of activity at 10U/ml or 100U/ml. (B)

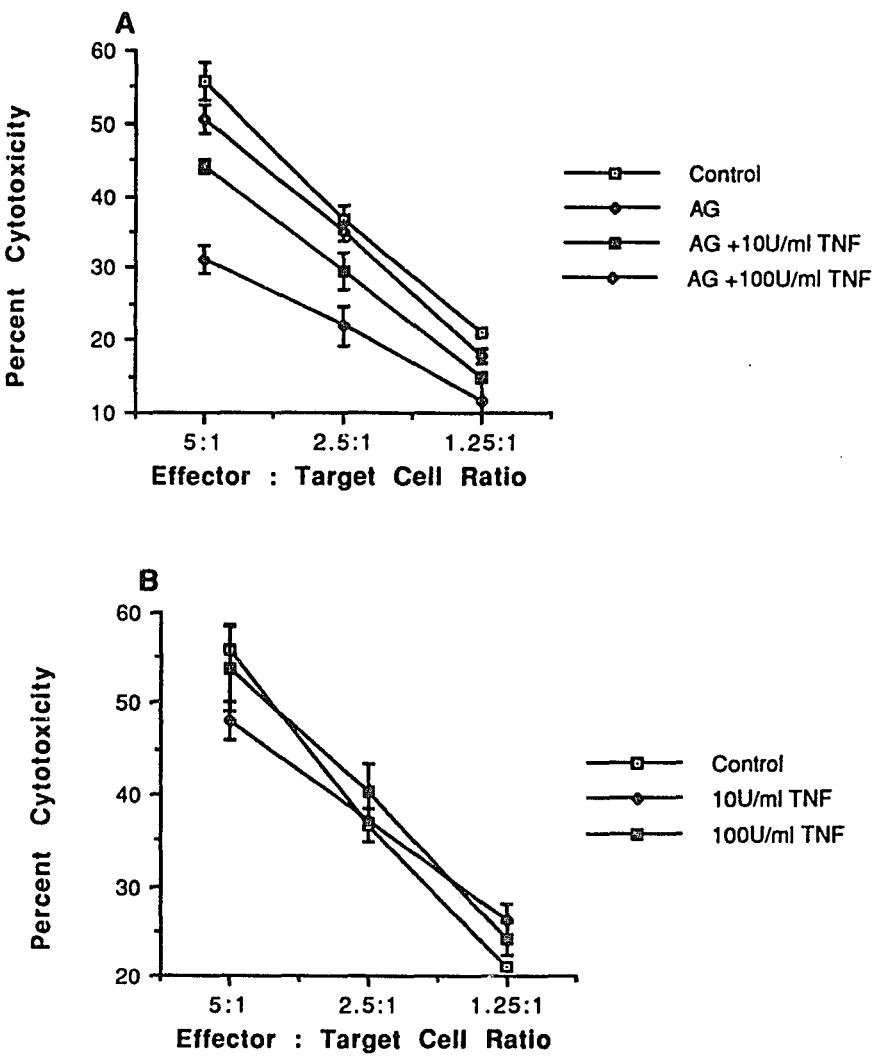

Figure 31: Reversal of Inhibition by Exogenous TNF

This finding supports the conclusion that the suppression of LAK cell generation by alpha-1-acid glycoprotein is mediated by a specific reduction of endogenous TNF production by the LAK cells in culture.

CHAPTER 4. CONCLUSIONS

The Role of TNF in LAK Cell Generation and Cytotoxicity

While IL-2 is the only stimulus required for the generation of LAK cells, the addition of exogenous TNF augments the development of cytotoxic activity. Work by others suggest that this augmentation is mediated by an increase in high affinity IL-2 receptors (23, 88).

TNF was found to be secreted by LAK cells in culture and Ab blocking studies indicate that endogenous TNF plays a role in an early stage of LAK cell development. The inhibitory effect of neutralizing antibody to TNF was only partial, even at high levels of Ab, indicating that TNF augments LAK generation, but is not essential for it. As the addition of neutralizing antibody to the ^{51}Cr assay had no effect on cytotoxicity, TNF production is not involved in rapid, cell-mediated LAK cell activity. This conclusion is supported by the findings of others (13).

Regulation of TNF and IFN-γ Release

While TNF is not a factor in short term LAK cell activity as measured in a ^{51}Cr release assay, it is a soluble, slow acting mediator of cytotoxicity by LAK cells. Both TNF and IFN-γ are released by LAK effectors coincubated with tumor cells, and supernatant containing these cytokines lyses tumor cells in a 72 hour cytotoxicity assay (21). The finding that many cancer patients

have elevated serum levels of TNF suggests that this secretory response may occur *in vivo* as well.

In this study, the secretion of TNF and IFN-γ from tumor cell stimulated LAK cells and the regulation of this secretory response *in vitro* were investigated. Factors known to regulate the release of TNF by monocytes were tested for their effect on the LAK cell cytokine secretion in response to stimulation with K562 tumor cells. The factors tested included PGE_2, alpha globulins, and TAME. This study demonstrates that PGE_2, alpha-1-acid glycoprotein and TAME suppress the release of TNF and IFN-γ by LAK cells. The inhibitory effect of each factor was seen in the absence of Leu M3+ cells, indicating that the TNF released under these conditions could be attributed to lymphoid cells rather than cells of the monocyte/macrophage lineage. This conclusion was supported by experiments using a T cell derived LAK cell clone, IP12. The stimulation of LAK cell TNF and IFN-γ release induced by K562 cell lysate instead of viable tumor cell was also inhibited; evidence that PGE_2, alpha-1-acid glycoprotein and TAME were acting directly on the lymphocytes to suppress TNF and IFN-γ secretion.

The regulation of TNF release from LAK cells by purified alpha globulin fractions differs from that reported for monocytes. While whole alpha globulin and alpha-1-acid glycoprotein were suppressive, alpha-1-antitrypsin and alpha-2-macroglobulin stimulated cytokine release. The enhancing effect of these two fractions was not the result of LPS contamination. Addition of

purified LPS, at levels comparable to those detected did not stimulate release of TNF and IFN-γ from LAK cells alone. Also, the addition of polymixin B to the interaction of alpha-1-antitrypsin, LAK cells and K562 cells had no effect on the stimulation. Nor was the difference in the activity of the fractions the result of variation in the activity of the lots tested, as the same lot of alpha-1-antitrypsin that stimulated TNF and IFN-γ release from LAK cells suppressed TNF release from LPS stimulated monocytes.

The finding that alpha-1-antitrypsin and alpha-2-macroglobulin, which inhibit TNF release by monocytes, did not suppress TNF release by LAK cells suggested that there may be alternative mechanisms for TNF secretion in the two cell populations. If LAK cells were not expressing transmembrane TNF when stimulated with tumor cells, then proteolytic clipping of the molecule from the cell membrane would not be involved in secretion, and antiproteases such as alpha-1-antitryspin and alpha-2-macroglobulin would not be inhibitory. FACS analysis of cell surface TNF expression in the different leukocyte populations suggests that this may be the case. While LPS-stimulated monocytes and tumor cell-stimulated CD16+ LAK cells upregulate cell surface TNF, CD3+ LAK cells do not.

In contrast to the effects of alpha-1-antitrypsin and alpha-2-macroglobulin, purified alpha-1-acid glycoprotein suppressed the release of both TNF and IFN-γ in a concentration dependent manner. This result was not unexpected as alpha-1-acid glycoprotein has

been shown to be capable of inhibiting various lymphocyte and monocyte functions, independent of any anti-protease activity (9, 18, 85, 129). Treatment of activated lymphocytes with alpha-1-acid glycoprotein resulted in a detectable decrease in TNF mRNA levels, indicating that this alpha globulin specifically regulates the production of TNF by influencing the level of mRNA expression. Alpha-1-acid glycoprotein was also found to inhibit the generation of LAK cell activity via its suppression of TNF release during LAK cell culture.

The finding that alpha-1-acid glycoprotein down regulates TNF release leads to speculation on the *in vivo* role of this acute phase reactant. TNF released from a localized site into the circulation can act directly, through IL-1, or through IL-6 to stimulate production of acute phase reactants. The resulting increase in alpha-1-acid glycoprotein concentration could lead to down regulation of TNF secretion. As alpha-1-acid glycoprotein has been shown to rise rapidly under acute phase conditions and can move out of the circulation (55), it would be well suited to a regulatory role as part of an intricate network of cytokines and plasma proteins which interact in the regulation of TNF secretion. The ability of alpha-1-acid glycoprotein to suppress TNF release may also contribute to a more localized regulation, as macrophages can produce this alpha globulin.

The potential for inhibition of TNF secretion among leukocytes subsets is particularly important with regard to the use of

transfused LAK cells plus high dose IL-2 in immunotherapy. Typically, IL-2 activated PBL are infused along with the recombinant cytokine. The introduction of recombinant IL-2 and the activated leukocytes triggers an acute phase response, characterized by a rapid increase in circulating alpha-globulins (5, 54). Such an acute phase response may block TNF and IFN-γ secretion *in vivo*, possibility influencing a number of biological processes occurring during tumor development. Both cytokines have been reported to be directly cytostatic or cytotoxic for sensitive tumor cell lines (39, 17) and can act synergistically to inhibit tumor cell growth (1). TNF (17, 89) and IFN-γ (34) can cause necrosis of tumors due to their effects on vascular endothelium In addition, TNF and IFN-γ can enhance expression of class I and II MHC on tumor cells which could lead to augmentation of the immune response to the tumor (94).

The data presented here serve to extend previous observations on the production of TNF by LAK cells and to bring to light an important and previously unrecognized aspect of LAK cell immunotherapy, the potential for suppression of LAK cell cytokine secretion.

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