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# The IL-2 Mediated Amplification of Cellular Cytotoxicity

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**Abstract** High dose [>1 nM or 30 IU] interleukin-2 (IL-2) can induce MHC-unrestricted killing from various lymphoid populations. Although it is well established that CD16+ NK cells are the major source of blood-derived LAK precursors, other lymphoid cells, including several CD3+ T subsets can be a source of precursor activity. We hypothesize that most, if not all, lymphocytes with cytolytic potential may eventually express MHC-unrestricted killing, when provided with adequate IL-2 to initiate required secondary cytokine production. This perspective article presents our cumulative data supporting the role of secondary cytokines in the IL-2 initiated activation of MHC-unrestricted killing, first by our observations of synergy with the exogenously added TNFs or IL-1s in combination with low dose IL-2, and then by the evidence of endogenous cytokine production and response in lymphocytes stimulated with high dose IL-2.

Understanding the amplification mechanism(s) of the various effector arms of the immune system is critical to the eventual regulation of graft rejection, autoimmune phenomena, and potentially to the treatment of cancer. Our studies have focused on the cytotoxic lymphocyte effector system, and have addressed the molecular pathways by which IL-2 induced cytokines influence the quantity and quality of the cytotoxic lymphocyte response.

This article will review the pivotal role that IL-2 plays in the development of CTL (MHC-restricted antigen-specific cytotoxic lymphocytes), followed by the description of how studies in the CTL system led to the observation that IL-2 alone can activate a heterogeneous collection of MHC-*unrestricted* killer lymphocytes, originally known as "Lymphokine Activated Killers" or LAK. We will then describe experiments performed in our laboratory over the past several years demonstrating the positive regulation of LAK activity by exogenous addition of TNF-α, TNF-β, IL-1α or IL-1β. Finally, we will summarize our data and propose, in the context of the current literature, the *endogenous autocrine/paracrine* amplification network of secondary cytokines operative in the generation of LAK.

**Key words:** cytotoxic lymphocyte regulation, IL-2 activated lymphocytes, cytokine networks, tumor necrosis factor, interleukin-1

## INTERLEUKIN-2 AS A DIFFERENTIATION FACTOR FOR CYTOTOXIC LYMPHOCYTES

It is well known that the bioactivities of IL-2 extend beyond that originally described for T cell growth and include the ability to act as a "second signal," or "antigen-independent differentiating factor," in the development of antigen-specific CTL. During an immunogenic response, the T-helper cell secretes IL-2 after accessory cell antigen presentation and thereby drives the antigen-primed preCTL through the G1 stage of the cell cycle. This IL-2 promoted G1 progression has been recently reviewed by Smith and coworkers [1]. It was during our initial (and unproductive) attempts to utilize IL-2 in the production of in vitro sensitized CTL to fresh human tumors that LAK activity was first identified. These initial studies have been described in detail previously [2–4]. The essence of this observation is described in Table I.

The ability of IL-2 alone to generate lymphocyte cytotoxicity against tumor led some investigators to propose that LAK was merely the secondary activation of primed CTL. The consistent activation of LAK from normal lymphocytes, and the absence of MHC-restricted recognition, however, dispelled this theory. Numerous subsequent reports confirmed our observation that IL-2 alone, in the absence of other exogenously added factor(s), directly activates a heterogeneous collection of lymphocytes to become tumoricidal. This activation can be detected within 24 hours of IL-2 culture in the absence of proliferative differentiation [5]; however, maximal cytotoxicity is demonstrated by the aggregate lymphocyte population between 3 and 5

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**TABLE I. IL-2 Alone Culture Activates Tumoricidal Lymphocytes**

| | Activation conditions | | |
|---|---|---|---|
| Responder | Stimulator | IL-2 | Resultant cytotoxicity to tumor |
| PBMC | AUTO TUMOR | – | 0 |
| PBMC | AUTO TUMOR | + | + |
| PBMC | None | + | + |
| PBMC | None | – | 0 |

days after a wave of cellular proliferation. It is apparent that LAK can also occur during in vitro antigen-specific responses, as was suggested by the earlier reports of anomalous killers by Seeley and Golub [6,7].

In contrast to the NK activity constitutively expressed by freshly isolated peripheral blood lymphocytes, LAK activity is not normally observed in resting lymphoid cells isolated from humans or other animals. LAK activity is inducible in vivo and has been consistently observed following the intravenous injection of IL-2 into cancer patients. Recently, Vanhaesebroeck et al. [8] reported that LAK activity was detectable in the lymph nodes of experimentally immunized mice. These results provide compelling evidence that LAK may be part of the normal immune amplification pathway.

## IL-2 SYNERGY WITH EXOGENOUS CYTOKINES

An understanding of the IL-2 receptor system is crucial to resolving the regulatory steps in LAK activation. Two major functional components of the IL-2 receptor system are the p55 alpha chain, originally named Tac, and the p75 beta chain. These two components bind IL-2 either separately, or in tandem, but with distinct affinities. The recently described 100Kd accessory component has not yet been associated with IL-2 responsiveness [9,10]. The p55 receptor component exhibits the lowest IL-2 binding affinity (Kd $10^{-8}$), the p75 receptor demonstrates an intermediate affinity (Kd $10^{-9}$), and together (p55 + p75) they form a bimolecular noncovalently linked complex with high affinity for the IL-2 ligand (Kd $10^{-11}$). Experimental evidence indicates that the p75 receptor alone is sufficient for LAK activation [11]. Therefore, the standard quantity of IL-2 used by our laboratory for LAK activation is 2 nM, or $2 \times 10^{-9}$M, exactly double that expected to saturate the p75 receptor. The involvement of ancillary receptor components appears likely, however, based on our observation that the concentration of IL-2 can regulate the magnitude of the resultant cytotoxic lymphocyte response. These observations led in part to the search for regulatory steps subsequent to the IL-2/p75 interaction, and especially to that involving secondary cytokine production.

The first indication that a cytokine other than IL-2 may be involved in LAK activation was that of IFN-γ/IL-2 synergy by Itoh et al. [12]. Upon repeating these studies, we observed that synergy was apparent only when monocyte-containing PBMC populations were employed, and not with monocyte-depleted lymphocytes. Therefore, it was hypothesized that an IFN-γ induced monocyte product may actually be responsible for the synergy with IL-2, and not the IFN-γ itself. Our studies led to examination of the major monocyte cytokine products, TNF-α and IL-1-α and -β.

These experiments were initially carried out using suboptimal IL-2 concentrations (0.2 nM or 10 Cetus units/ml) and varying amounts of recombinant TNFs. When lymphocytes were cocultured with IL-2 and TNF and their resultant LAK activity measured, an augmentation of tumor-directed cytotoxicity was observed [13]. The ability to boost LAK was noted when either TNF-α or -β was used in combination with IL-2 for LAK activation. These results indicated the presence of TNF receptors on lymphocytes, as well as possibility that both forms of TNF could participate in the activation of cytotoxic lymphocytes. As both TNF-α and -β are known to compete for the same cell surface receptor [14], it was not surprising that both TNFs could stimulate the same function. Because TNF-β is produced solely by lymphocytes and not monocytes, its synergy with IL-2 suggested the existence of a paracrine or autocrine lymphocyte response. For example, one can envisage that after an initial response to IL-2, lymphocytes secrete endogenous TNF-β which then synergizes with the waning IL-2 levels to maintain expression of LAK activity. The temporal production of TNF-α and subsequently TNF-β during CTL generation has also been observed, supporting our proposal of such an amplification mechanism in the antigen-specific responses.

The optimal concentration of exogenously added TNF-α required for enhanced IL-2 driven lymphocyte cytotoxicity was determined to be approximately 500 U/ml or 0.6 nM. In the ab-

sence of IL-2, TNF alone did not result in the development of LAK activity. The ability of exogenously added TNF to boost IL-2 driven cytotoxic lymphocyte function was critically dependent upon the IL-2 concentration used for activation (Table II). TNF augmentation was rarely observed when IL-2 concentrations above 1000 u/ml were employed, suggesting the endogenous production of TNF. The effect of exogenously added TNF was shown to be on lymphocyte differentiation and not at the effector stage, as TNF was unable to potentiate lymphocyte killing of tumor targets when added directly into the chromium release assay. The mechanism whereby TNF potentiates IL-2 activated lymphocyte killing of tumor targets has not been fully resolved. It is well established that TNF can upregulate IL-2 receptor expression, presumably via the induction of NFκB transcriptional factors. Therefore, TNF may function to increase cellular proliferation in the IL-2 activated lymphocyte population. Indeed, we have demonstrated that both the IL-2 α receptor (Tac) and proliferation are increased in those lymphocytes cultured in IL-2 with TNF when compared to those cultured in IL-2 alone. In addition to the direct effects of proliferation, however, we have observed a selective enrichment of cells involved in tumor recognition and killing in those lymphocytes cultured in IL-2 and TNF; a representative experiment is presented in Table III. For the studies such as the one reported in Table III, lymphocytes were cultured for five days in either IL-2 alone or IL-2 and TNF and the frequency of effectors measured by single cell assay. The data indicate that those lymphocytes cultured in IL-2 and exogenously added TNF have a greater number of effectors able to recognize and kill the tumor target than those lymphocytes cultured in IL-2 alone.

**TABLE II. Effect of Exogenous TNF on IL-2 Activated Lymphocyte Cytotoxicity***

| Culture conditions: IL-2 (U/ml) | TNF (U/ml) | Cytotoxicity LU against Raji target |
|---|---|---|
| None | None | <0.1 |
| 5 | None | 7.8 |
| 5 | 100 | 9.7 |
| 5 | 1000 | 14.0 |
| 5 | 10,000 | 11.2 |
| 100 | None | 15.2 |
| 100 | 100 | 22.2 |
| 100 | 1000 | 30.7 |
| 100 | 10,000 | 18.2 |

*Lymphocytes were cultured with the indicated cytokines for 5 days in serum-free medium and their cytotoxicity measured against the Raji target using a chromium release assay. Cytokines were generously provided by Cetus Corporation, Emeryville, CA. The indicated cytokine concentrations are in Cetus units. LU (lytic units) were calculated using 30% lysis of the Raji target/$10^6$ effectors. Lymphocytes cultured in TNF alone, in the absence of IL-2, did not demonstrate cytotoxicity toward the Raji target (LU <0.1).

**TABLE III. Conjugate Formation and Target Killing in Lymphocytes Activated With IL-2 Alone Versus IL-2 and Exogenously Added TNF***

| Culture condition | Raji Target: Conjugate formation (%) | Raji Target: Target death (%) |
|---|---|---|
| 10 U/ml IL-2 alone | 6.4 | 20 |
| 10 U/ml IL-2 + TNF | 26.0 | 65 |
| 100 U/ml IL-2 alone | 28.4 | 40 |

*Lymphocytes were cultured in serum-free medium for 5 days with the indicated cytokines. Cytokines were the generous gift of the Cetus Corporation, Emeryville, CA. TNF was used at a concentration of 500 U/ml for these studies. The IL-2 concentrations indicated are Cetus units. At the end of the culture period, lymphocyte conjugate formation with the Raji target was measured using the single cell assay. The percentage target cell death was determined after a four hour incubation with lymphocytes (at 37°C) by trypan blue exclusion. For each assay, a minimum of 200 conjugates were counted.

Upon further investigation a similar, but not identical, functional enhancement was observed when lymphocytes were cultured in IL-2 and IL-1α or IL-1β [15]. Reminiscent of our findings with exogenously added TNF, the ability of the IL-1s to boost IL-2 driven cytotoxic lymphocytes was dependent upon the IL-1 dose employed (Figure 1A). Unlike that of the TNF system, however, the IL-1s alone were able to generate lymphocyte-mediated tumor killing in some cases (Figure 1B). The mechanisms underlying this phenomena have not been fully elucidated, but may involve the ability of IL-1 to stimulate endogenous IL-2 production.

Although numerous other cytokines have now been tested, only IL-6 and IL-7 appear to function in synergy with IL-2 for the development of cytotoxic lymphocytes. With the exception of a minority of IL-7 alone experiments (Yang S, unpublished observations), our preliminary experiments suggest that both IL-2/IL-6 and IL-2/IL-7 synergy requires the presence of monocytes. It is possible, therefore, that IL-6 and IL-7

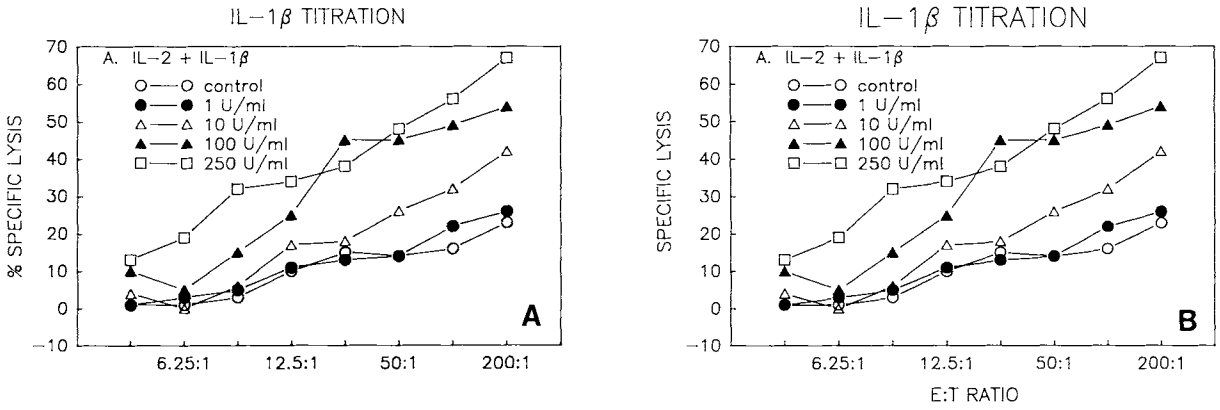


**Fig. 1.** Effect of IL-1β on activation of LAK. Normal PBL were co-cultured with IL-2 (10 U/ml) alone, IL-2 (10 U/ml) plus IL-1β, or IL-1β alone for 5 days. **A** is IL-2 with and without IL-1β, and **B** is the IL-1β alone results. The concentrations shown are for IL-1. On the fifth day the lymphocytes were harvested, counted, and then tested for killing activity directed to the Raji tumor target line in a 4 hour chromium release assay at the effector to target ratios (E:T) indicated.

may act indirectly through the TNFs and IL-1s. Experiments are currently in progress to test this possibility. It should be noted that several groups of investigators have observed non-IL-2 cytokines activating LAK in the absence of IL-2; however, these are awaiting confirmation by others. Our laboratory has not been able to confirm any of the findings using non-IL-2 cytokines with our conditions of serum-free medium and highly purified (monocyte-depleted) peripheral blood lymphocytes as responders. Thus, it is plausible that these other reports may indicate that lymphocytes are activated indirectly through the elaboration of various macrophage products.

## ENDOGENOUS CYTOKINES PROVIDE AN AUTOCRINE AND/OR PARACRINE IMMUNE AMPLIFICATION NETWORK

Because we observed that the ability to boost IL-2 driven lymphocyte cytotoxicity with exogenously added TNF was dependent upon the IL-2 concentration, we queried whether endogenous TNF production was regulated by IL-2 dose. As we have previously reported, IL-2 concentration can regulate transcription of the TNF-α gene and protein secretion in a dose-dependent manner [16]. Although IL-2 activated lymphocytes clearly produced endogenous TNF-α and -β, it was not known whether these cytokines were required for the functional development of LAK. To address this question, lymphocytes were activated with IL-2 in the presence and absence of neutralizing antibodies against the TNFs. The results of one such experiment is shown in Table IV. As shown, when neutralizing antibodies against TNF-α or TNF-β were included in the IL-2 activation culture, the resultant lymphocyte-mediated tumor cytolysis was greatly inhibited. In fact, using antibodies against both TNF species reduced lymphocyte cytotoxic function by almost 90%. These results demonstrate that the endogenously produced TNFs are obligatory for the development of LAK function. Antibody inhibition does not appear to be the sole result of reduced cellular proliferation as we have shown that antisense TNF oligonucleotides can also block "early" LAK function at 24 hours (a time at which no cell division is detectable). We have also demonstrated that IL-2 can regulate TNF receptor expression in lymphocytes [17], providing further strength to the hypothesis that TNF elaboration/response is a critical pathway for immune amplification in these cells.

**TABLE IV. Effect of Neutralization of Endogenous TNF Production on LAK Activation***

| Activation condition | LU/106 Raji | Inhibition (%) |
|---|---|---|
| MEDIA | <1 | |
| 100 U IL-2 | 66.9 | — |
| 100 U IL-2 + ANTI TNF-α | 21.0 | 68 |
| 100 U IL-2 + ANTI TNF-β | 13.8 | 79 |
| 100 U IL-2 + ANTI TNF-α + -β | 8.3 | 88 |

*Lymphocytes were cultured in serum-free medium for 5 days with IL-2 (100 U cetus = 2.2 nM) with or without the indicated neutralizing rabbit antibodies. Antibodies were present at 5000 neutralizing units/ml; control rabbit serum was included and had no effect.

## SUMMARY AND CONCLUSIONS

In contrast to merely supporting the clonal expansion of cytotoxic lymphocytes, IL-2 elicits the production of secondary cytokines which are regulatory for development of MHC-unrestricted cytotoxic lymphocytes. Not only does IL-2 induce the tumor recognition capacity and lytic competence necessary for MHC-unrestricted killing, but it also upregulates the expression of TNF receptors, and endogenous TNF production. Because the TNFs have been shown to participate in the amplification of lytic function and to be required for the development of LAK, we propose that these factors are required for autocrine/paracrine lymphocyte activation. As IL-2 also induces a number of secondary cytokines including the IL-1s, IL-4, IL-6, and IFN-$\gamma$, it is likely that complementary or additional amplification circuits exist. Clearly, much additional investigation will be required to elucidate the interrelationships of the secondary cytokines and their receptors. In conclusion, we propose that within the microenvironment of an immune response varying levels of IL-2 are produced dependent upon the strength of the immunogen and the status of the host immune response. The quantity of IL-2 produced will then determine the relative amount of the secondary cytokine production and eventually the magnitude and longevity of the cellular immune response.

## REFERENCES


1. Smith KA: Science. 240:1169-1176, 1988.
2. Grimm EA, Mazumber A, Rosenberg SA: Cell Immunol 70:248-259, 1982.
3. Strausser J, Mazumder A, Grimm EA, Lotze MT, Rosenberg SA: J Immunol 127:266-271, 1981.
4. Grimm EA, Rosenberg SA: In Pick E (ed): "The Lymphokines." New York: Academic Press, 1984, pp 279–311.
5. Owen-Schaub LB, Loudon WG, Yagita M, and Grimm EA: Cell Immunol 111:235-246, 1988.
6. Seeley JK, Golub SH: J Immunol 120:1415-1422, 1978.
7. Seeley JK, Mascucci G, Poros A, Klein E, Golub SH: J Immunol 123:1303-1311, 1979.
8. Vanhaesebroeck B, Grooten J, and Fiers W: J Immunol 143:1396-1402, 1989.
9. Colamonici OR, Neckers LM, Rosolen A: J Immunol 145:155-160, 1990.
10. Sharon M, Gnarra JR, Leonard WJ: Proc Natl Acad Sci USA 87:4869-4873, 1990.
11. Tsudo M, Goldmann CK, Bongiovanni F, Chan WC, Winton EF, Yagita M, Grimm EA, Waldmann TA: Proc Natl Acad Sci USA 84:5394-5398, 1987.
12. Itoh K, Shiiba K, Shimizu Y, Suzuki R, Kumagai K: J Immunol 134:3124-3129, 1985.
13. Owen-Schaub LB, Gutterman JU, Grimm EA: Cancer Res 48:788-792, 1988.
14. Aggarwal BB, Essalu TE, Hass PE: Nature 318:665-667, 1985.
15. Crump III WL, Owen-Schaub LB, Grimm EA: Cancer Res 149-153, 1988.
16. Owen-Schaub LB, de Mars M, Murphy Jr EC, Grimm EA: Cell Immunol (in press), 1991.
17. Owen-Schaub LB, Crump III WL, Morin GI, Grimm EA: J Immunol 143:2236-2241, 1989.