Human Interleukin 2 Analogues That Preferentially Bind the Intermediate-Affinity Interleukin 2 Receptor Lead to Reduced Secondary Cytokine Secretion: Implications for the Use of These Interleukin 2 Analogues in Cancer Immunotherapy

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ABSTRACT

Cancer patients undergoing interleukin (IL)-2-based immunotherapy frequently experience dose-limiting side effects believed to be caused by the actions of such cytokines as IL-1β, tumor necrosis factor (TNF)-α and -β, and interferon-γ (IFN-γ). Human peripheral blood mononuclear cells (PBMC) or monocyte-depleted peripheral blood lymphocytes were stimulated for up to 7 days by either of 2 IL-2 analogues (R38A or F42K) that bind to the intermediate-affinity IL-2Rβγ receptor but have reduced abilities to bind the high-affinity IL-2 receptor. We previously reported that these IL-2 analogues retain the ability to generate lymphokine-activated killing by PBMC. In this study, we analyzed the cytokine content of supernatants from stimulated PBMC and peripheral blood lymphocyte cultures by enzyme-linked immunosorbent assay. The secretions of IL-1β, TNF-α, -β, and IFN-γ induced by either R38A or F42K were markedly reduced compared with secretions produced in response to recombinant wild-type IL-2. In 4 experiments, secretion was reduced an average of 39% for IL-1β, 57% for TNF-α, 83% for TNF-β, and 86% for IFN-γ. Polymerase chain reaction analysis of recombinant wild-type IL-2 or analogue-stimulated PBMC did not reveal the presence of IL-2 mRNA; thus, differential production of endogenous IL-2 could not account for these findings. These data suggest the interaction of IL-2 and the high-affinity IL-2 receptor on human PBMC or peripheral blood lymphocyte is required for maximal secretion of IL-1β, TNF-α, TNF-β, and IFN-γ. Because such cytokines are believed to mediate the toxicity seen with IL-2-based immunotherapies, IL-2 analogues with reduced binding to the high-affinity IL-2 receptor may prove to be an effective and less toxic means of cancer treatment.

INTRODUCTION

The potent oncolytic properties of IL-2 as a single agent and in combination with IL-2-stimulated LAK cells have been used in many cancer immunotherapy protocols. In a number of cancer centers, the overall response rates for these IL-2-based regimens have been between 15 and 30% with complete responses in approximately 5–10% of patients (1–4). The problem with these protocols, however, has been the high incidence of multisystem toxicity that occurs in an IL-2 dose-dependent manner. Patients treated with IL-2-based therapies may experience such difficulties as hypotension, cardiac arrhythmias, pulmonary insufficiency, and renal failure with treatment-related mortality rates of about 2% (1, 5–8).

Many of the signs and symptoms of IL-2-induced toxicity are thought to be caused by the actions of cytokines such as IL-1β, TNF-α, TNF-β, and IFN-γ released by IL-2-activated PBMC (9–14). If secretion of these secondary cytokines could be reduced, patients receiving IL-2 immunotherapy might be able to tolerate higher and more effective doses of IL-2.

R38A and F42K, 2 human IL-2 analogues that have altered IL-2Rα binding domains, differ from human rIL-2 by a single amino acid substitution. In this report, we examined the capacity of R38A and F42K to induce the secretion of IL-1β, TNF-α, TNF-β, and IFN-γ. Compared with cultures stimulated by rIL-2, cultures exposed to either of the IL-2 analogues showed significant reductions in the amounts of these cytokines present. Previously, we showed that analogue-stimulated PBMC retain substantial LAK activity (15) (Table 1). Thus, the use of IL-2 analogues specific for the intermediate-affinity IL-2R may prove to be an effective and less toxic means of cancer immunotherapy.

MATERIALS AND METHODS

PBMC Preparation. PBMC were obtained from normal, healthy volunteers by leukopheresis. The contents of the pheresis bags were diluted with Hank's balanced salt solution (Hazleton Biologicals, Inc., Lenexa, KS) and fractionated on Histopaque (Sigma Chemical Co., St. Louis, MO) by centrifugation at 450 × g for 30 min. PBMC were isolated at the interface, washed 5 times in Hank's balanced salt solution before being resuspended in serum-free AIM-V medium (Gibco Laboratories, Grand Island, NY) containing 2 μM L-glutamine. For experiments in which PBMC were used, PBMC were either subjected to plastic adherence for 2 h or passed through a nylon wool column. PBMC were analyzed for contaminating monocyte content by fluorescence-activated cell sorter using anti-LeuM3 antibodies. The percentage of residual macrophages in each sample was less than 0.4%.

IL-2 and IL-2 Analogues. Clinical-grade human wild-type rIL-2 was obtained from Dr. F. Khan of the Bioprocess Department at Hoffmann-LaRoche, Inc. (Nutley, NJ). IL-2 analogue proteins were prepared by site-directed mutagenesis, expressed in Escherichia coli, and purified as described by gel filtration (16). This gel filtration technique also extracts any endotoxin that is present in the purified protein preparations. The IL-2 analogue protein R38A contains an alanine substitution for Arg38 and the analogue F42K has a substitution of lysine for Phe42 (17). Binding of the analogues to the human IL-2R was evaluated as described by Sauve et al. (17). As previously determined in murine cytotoxic T-lymphocyte line proliferation assays, the specific activities of the analogue proteins were 2 × 10⁷ units/mg R38A, 7.6 × 10⁶ units/mg R38A, and 3.2 × 10⁵ units/mg F42K (17). Because of these differences in specific activity, equimolar concentrations of each protein were used in all experiments. Dose titration curves for the analogues’ induction of LAK activity were presented previously (15). In those studies, maximal activity was found at the concentration of 1 μg/mL; therefore, 1 μg/mL concentrations were used in all further experiments. Conversion factors for rIL-2 and the analogues are: 1 μg = 308 units/mL rIL-2 = 117 units/mL R38A = 5 units/mL F42K.

Activation of LAK Cells. PBMC or PBL were cultured at 10⁶ cells/ml in AIM-V medium and incubated at 37°C in a humidified 5% CO₂ atmosphere with 1 μg rIL-2 or IL-2 analogue. The AIM-V medium was selected because it contains an alanine substitution for Arg38. and the analogue F42K has a substitution of lysine for Phe42 (17). Binding of the analogues to the human IL-2R was evaluated as described by Sauve et al. (17). As previously determined in murine cytotoxic T-lymphocyte line proliferation assays, the specific activities of the analogue proteins were 2 × 10⁷ units/mg R38A, 7.6 × 10⁶ units/mg R38A, and 3.2 × 10⁵ units/mg F42K (17). Because of these differences in specific activity, equimolar concentrations of each protein were used in all experiments. Dose titration curves for the analogues’ induction of LAK activity were presented previously (15). In those studies, maximal activity was found at the concentration of 1 μg/mL; therefore, 1 μg/mL concentrations were used in all further experiments. Conversion factors for rIL-2 and the analogues are: 1 μg = 308 units/mL rIL-2 = 117 units/mL R38A = 5 units/mL F42K.

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Received 1/18/93; accepted 3/2/93.

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1 Supported by NIH Grant CA45445 (E. A. G.), National Cancer Institute General Surgery Training Grant 5 T32 CA09950-04 (K. M. H.), and Core Grant CA16672.

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3 The abbreviations used are: IL, interleukin; LAK, lymphokine-activated killer; PBMC, peripheral blood mononuclear cells; TNF, tumor necrosis factor; IFN, interferon; rIL-2, recombinant wild-type human interleukin 2; IL-2R, interleukin 2 receptor; PBL, peripheral blood lymphocytes; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction.

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it is chemically defined and serum-free, enabling us to avoid serum-induced secondary cytokine production from the stimulated cells.

**Cytokine Assays.** Supernatant cytokine concentrations were determined by ELISA detecting TNF-α and TNF-β (R & D Systems, Inc., Minneapolis, MN), IL-1β (AMAC, Inc., Westbrook, ME), and IFN-γ (Endogen, Inc., Boston, MA) following all manufacturer protocols. Briefly, supernatants were obtained from stimulated PBMC or PBL on the indicated day and centrifuged at 90 x g for 5 min to remove any cells. These supernatants were incubated in duplicate or in triplicate in antibody-coated wells of the supplied microtiter plates. A second distinct cytokine-specific antibody/enzyme conjugate was then added to each well followed by the appropriate chromogenic substrate. Absorbance was determined by spectrophotometry, and the concentration of cytokine was determined from a standard curve. Mean cytokine secretion was determined by standardizing the analogue-stimulated cytokine secretion as a percentage of the mean rIL-2-induced secretion for each day in each individual experiment. These percentages were then combined to give a mean 7-day secretion expressed as the percentage of rIL-2-induced cytokine secretion. Unless otherwise specified, 4 experiments were performed for each cytokine using PBMC from different donors. The sensitivity of each ELISA was 5-7 pg/ml.

**IL-2 PCR Analysis.** PBMC were stimulated for 24 or 72 h with 10 µg/ml phytohemagglutinin or 1 nM rIL-2, R38A, or F42K. Total RNA was isolated from 5 x 10⁶ stimulated PBMC by RNAzol B (Biotex Laboratories, Inc., Friendswood, TX) using the single-phenol extraction technique described by the manufacturer. The quantity of RNA used was 0.5 µg/reaction. Reverse transcription was performed at 22°C for 10 min and then at 45°C for 45 min. PCR was performed using a Perkin Elmer/Cetus PCR kit (Norwalk, CT) in 30-µl volumes with specific primers for IL-2 produced by The University of Texas M. D. Anderson Cancer Center macromolecular synthesis facility. The IL-2 primer sequences span an intron and were purified with an oligonucleotide purification cartridge (ABI, San Mateo, CA). The sequences are 5’ primer 5’ATGTCACAGGATCCAACCTCTCTCTTT 3’ and 3’ primer 5’GTATGTTGTTGATGATGATGCTTTGAC 3’.

The amplification procedure involved denaturation at 94°C for 1 min, annealing at 60°C for 1.5 s, and extension at 72°C for 80 s during 38 cycles in a Perkin-Elmer/Cetus GeneAmp PCR System 9600 thermal cycler. The PCR products were resolved on a 1% agarose gel and visualized by ethidium bromide fluorescence. HaeIII-digested X174 RF DNA fragments (Gibco Laboratories, Inc.) served as the molecular weight markers in defining the size of the PCR products.

**Statistical Analysis.** All data are presented as mean ± SE. An analysis of variance was performed with Dunnett’s t-test to compare the analogue-induced cytokine secretion to that produced in response to rIL-2. Statistical significance was reached with P < 0.05. Statistical analysis was performed using StatView SE + graphics (copyright 1988–1991; Abacus Concepts, Inc., Berkeley, CA) on an Apple Macintosh IIi personal computer.

**RESULTS**

To delineate the function of the IL-2R subunits in the generation of LAK activity, we used IL-2 analogue proteins that differentially bind the low-, intermediate-, and high-affinity IL-2 receptors on human lymphoid cells. The IL-2 analogue R38A was previously characterized as deficient in binding to the isolated low-affinity IL-2Rα subunit; however, this analogue is capable of fully binding to the IL-2Rβγ (intermediate-affinity) and of partially binding to IL-2Rαβγ (high-affinity) receptors on YT cells (17) (Table 1). The related IL-2 analogue F42K had no detectable binding to the low-affinity IL-2Rα, but in contrast to R38A, F42K has only 2–3% binding to the high-affinity IL-2Rαβγ complex. Thus, both analogues maintain full capacity to bind to the intermediate-affinity receptor but differ in ability to interact with the high-affinity receptor. Previously, we showed that these analogues generate substantial LAK activity as measured against the natural killer-resistant Daudi cell line (15) (Table 1).

**Analogue-stimulated PBMC or PBL Secrete Less IL-1β, TNF-α, TNF-β, and IFN-γ.** Human PBMC or PBL were stimulated for up to 7 days with 1 nM rIL-2 or IL-2 analogue. On the specified days, the cell-free culture supernatants were tested for cytokine concentration using ELISA methodology. In 2 of 4 experiments performed using rIL-2 or analogue-activated PBMC from different donors, no detectable IL-1β was found in the culture supernatants. In the 2 experiments in which IL-1β was present, however, the amount of IL-1β secretion induced by R38A averaged only 76%, and F42K-induced IL-1β was about 61% of that produced in response to rIL-2 (Fig. 1A). Fig. 1B shows the kinetics of IL-1β production for one representative experiment. IL-1β production peaked at 24 h and then subsequently decreased. Supernatants from the monocyte-depleted PBL cultures were also tested for IL-1β. No detectable IL-1β was present up to 7 days after rIL-2 or analogue stimulation in the 2 experiments performed.

**Table 1 Competitive binding and lytic activity of IL-2 analogues**

<table>
<thead>
<tr>
<th>% competitive binding</th>
<th>IL-2Rαβγ</th>
<th>IL-2Rα</th>
<th>IL-2Rαβγ</th>
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<tbody>
<tr>
<td>rIL-2</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>R38A</td>
<td>50–100</td>
<td>No IC₅₀</td>
<td>3–50</td>
</tr>
<tr>
<td>F42K</td>
<td>50–100</td>
<td>No IC₅₀</td>
<td>2</td>
</tr>
<tr>
<td>Lytic activity (lytic units)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>rIL-2</td>
<td>7.4 ± 0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R38A</td>
<td>5.4 ± 0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F42K</td>
<td>5.8 ± 1.0</td>
<td></td>
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*Percent competitive binding was calculated from values of analogue concentration required to inhibit rIL-2 binding 50% as described (16, 17). Data are as presented previously (15, 17). Competitive binding to IL-2Rα, IL-2Rβγ, and IL-2Rαβγ was assayed on immobilized purified human recombinant p55, human YT cells, and human YT-1 cells, respectively. Lytic activity was determined by 4-H ³¹Cr-release assays using PBMC stimulated for 3–5 days with 1 nM rIL-2, R38A, or F42K against Daudi target cells (mean ± SE calculated from 9 separate experiments).

*IC₅₀ concentration of analogue required to inhibit rIL-2 binding 50%.

Fig. 1. PBMC were stimulated for up to 7 days with 1 nM rIL-2, R38A, or F42K, and cell-free supernatants analyzed for IL-1β by ELISA (*P < 0.05). A, mean 7-day secretion of IL-1β by R38A- or F42K-stimulated PBMC. Data were analyzed as described in "Materials and Methods." B, cumulative IL-1β production by PBMC. Data shown are representative of the 2 experiments in which IL-1β was detectable. PBMC from 2 donors did not secrete IL-1β in response to rIL-2 or the analogues.
TNF-α secretion was also attenuated during activation of PBMC by the IL-2 analogues. R38A-induced secretion of TNF-α from PBMC cultures was only 61% of that produced in response to rIL-2, and only 43% of the TNF-α was secreted in response to F42K (Fig. 2A). Similarly, monocyte-depleted PBL cultures exposed to R38A secreted 57%, and those exposed to F42K secreted 35% of the TNF-α produced in response to rIL-2 (Fig. 3A). TNF-α secretion increased with time in both the PBMC and PBL cultures (Figs. 2B and 3B).

The TNF-β content was even more dramatically decreased when the IL-2 analogues were used. R38A-stimulated production of TNF-β from PBMC was only 52% of that secreted in response to rIL-2, while F42K cultures produced just 17% (Fig. 4, A and B).

IFN-γ was the cytokine with the largest reduction in secretion in response to stimulation by the IL-2 analogues. PBL cultures stimulated by R38A produced 49%, and those stimulated by F42K produced 33% as much IFN-γ as that produced in response to rIL-2 (Fig. 5, A and B). PBMC cultures exposed to R38A produced 46%, and those exposed to F42K produced only 14% of the IFN-γ secreted in rIL-2-stimulated cultures (Fig. 6, A and B).

Interestingly, while the secretion of IL-1β, TNF-α, TNF-β, and IFN-γ was reduced by as much as 86%, the lytic activity generated by PBMC stimulated by either R38A or F42K was only marginally reduced when the lytic activities from all experiments were averaged (Table 1). There was, however, one experiment (of the 4 performed) in which the lytic activities were equal (11 lytic units), and the same decreases in cytokine production were observed.

IL-2-stimulated PBMC Did Not Generate IL-2 mRNA. The production of endogenous IL-2 by IL-2-stimulated cells was also evaluated. Because no monoclonal antibodies specific for either the IL-2-stimulated PBMC or F42K protein exist, ELISA analysis of the culture supernatants would not distinguish the exogenous rIL-2 or IL-2 analogue from endogenously produced IL-2. Therefore, PBMC were analyzed for the presence of IL-2 mRNA by PCR after being stimulated for 24 and 72 h. In 4 experiments using different individual cell donors, IL-2 mRNA was not present at either 24 or 72 h in rIL-2-, R38A-, or F42K-stimulated PBMC, although these same PBMC produced IL-2 mRNA in response to phytohemagglutinin stimulation (Fig. 7). Thus, it is probable that the differential secondary cytokine production measured here is a direct response to the exogenous IL-2 and not a secondary response to endogenously produced IL-2.

**DISCUSSION**

IL-2 has been used for cancer immunotherapy as a single agent and in combination with LAK cells, other immunomodulators, and the more classic chemotherapeutic agents such as cisplatin. In major cancer centers, clinical responses have been seen in 15–25% of patients with complete responses in 5–10% of patients receiving IL-2-based therapy (1–4). Despite these favorable outcomes and the recent approval of rIL-2 for the treatment of metastatic renal cell carcinoma, the use of IL-2-based immunotherapy has been severely limited because of its associated toxicity. Excessive weight gain, pulmonary failure, renal insufficiency, and hypotension are frequent consequences of an IL-2-induced vascular leak syndrome (1, 18), and this results in treatment-related deaths for approximately 2% of the patients treated with IL-2 (1).

Many of the toxic side effects of IL-2 are thought to be secondary to the actions of IL-1β, TNF-α, TNF-β, and IFN-γ, each of which is
well known to be secreted by IL-2-stimulated PBMC. IL-1, a proinflammatory cytokine, has been shown to cause fever, anorexia, and in some cases, hypotension (10, 18). Like IL-1, TNF-α and TNF-β have many systemic effects, including cachexia, malaise, and nausea. In addition, IL-1 and TNF synergize with IFN-γ in the production of nitric oxide (19), a primary mediator of the vasodilatation and resulting hypotension frequently observed in patients undergoing IL-2-based immunotherapy (20, 21). Furthermore, each of these cytokines (IL-1, TNF, and IFN-γ) is capable of modulating the adherence of IL-2-activated leukocytes to vascular endothelium by up-regulating the expression of cellular adhesion molecules (22, 23). Adherence of these leukocytes to the microvasculature and their subsequent release of secondary cytokines provide one possible mechanism for the pathogenesis of the vascular leak syndrome seen in IL-2-treated patients.

Because of the tremendous side effects that patients experience when given IL-2, there have been many efforts to reduce the toxicity of IL-2 treatments. In mice, anti-TNF antibodies administered concurrently with high-dose IL-2 partially abrogated IL-2 toxicity (12). In a murine model, pentoxifylline, an inhibitor of macrophage TNF transcription, was able to diminish the side effects of i.p. injections of IL-2 without reducing that agent’s antitumor efficacy (24). In addition, infusion of N-methylarginine, a nitric oxide synthase inhibitor, has been successful in eliminating hypotension in dogs given i.v. IL-2 (25).

Another way to decrease the toxicity of IL-2 would be to reduce the amount of IL-1, TNF, and IFN-γ produced by a patient’s IL-2-stimulated leukocytes. In this study, we evaluated the ability of 2 human IL-2 analogues to stimulate the secretion of IL-1β, TNF-α, TNF-β, and IFN-γ. The 2 analogues we selected (R38A and F42K) interact with the intermediate-affinity IL-2R but have reduced binding to the high-affinity receptor. We observed that the concentrations of IL-1β, TNF-α, TNF-β, and IFN-γ in the supernatants of R38A- or F42K-stimulated PBMC or PBL were dramatically decreased compared with those produced in response to rIL-2 (Figs. 1–6). In addition, we have previously shown that analogue-stimulated PBMC induce similar levels of lytic activity despite large differences in the specific activity of the analogues as determined in murine cytotoxic T-lymphocyte line proliferation assays (15) (Table 1). These findings are not surprising since the intermediate-affinity IL-2R to which rIL-2, R38A, and F42K bind with identical affinity has been shown to be responsible for the induction of lytic activity, while stimulation of the high-affinity receptor complex is necessary for cellular proliferation (15, 26). If such IL-2 analogues that retain LAK activity were used for cancer immunotherapy, the reductions in these cytokines could result in decreased toxicity to the patient.

The decreased production of IFN-γ by F42K-stimulated cells was particularly impressive and is important for several reasons. Lower IFN-γ levels in patients given i.v. IL-2 have correlated with the tumor’s response to treatment (14) and with decreased toxicity (9, 13). In addition, a theoretical benefit in reducing the production of IL-1, TNF, and IFN-γ exists because of their synergy in initiating the production of nitric oxide (19). If lower levels of these cytokines are present and nitric oxide synthesis is reduced (or not induced) by these IL-2 analogues, patients treated with the analogues may not experience the dose-limiting problems of hypotension and vascular leak syndrome.

The regulatory pathways and mechanisms behind the secretion of these cytokines are unknown. One apparent explanation for the dif-
The differential expression of cytokines is that stimulation of the high-affinity IL-2R is necessary for maximal secondary cytokine secretion. F42K, which interacts minimally with the high-affinity IL-2Rαβγ, produced only 14% of the IFN-γ made by rIL-2-stimulated cells (Fig. 6). R38A, on the other hand, with a more intermediate ability to bind to the high-affinity IL-2R, produced 46% as much IFN-γ (Fig. 6). Several other studies have yielded similar results that further demonstrate the importance of IL-2 interaction with the high-affinity receptor complex in regulating secondary cytokine secretion. Blocking the low-affinity IL-2Rα with anti-Tac antibodies and thereby prohibiting binding to the high-affinity receptor complex markedly reduced both the transcription of the IFN-γ gene (27) and secretion of the IFN-γ protein (27–29). The correlation between increased binding to the high affinity receptor complex and increased cytokine secretion was also apparent in our measurements of IL-1ß, TNF-α, and TNF-β. With the exception of the reports regarding IFN-γ production (27–29), our report is the first to link reduced high-affinity IL-2R binding to reductions in the secretion of these cytokines. Although our data clearly indicate that interaction of IL-2 with this receptor complex is important in cytokine regulation, some cytokine is clearly produced through stimulation of the intermediate-affinity βγ receptor. In addition, a complex interactive network of cytokine regulatory controls exists. For example, while it is known that IL-2 and TNF-α enhance the production of IL-1 from human PBMC (30–32), TNF secretion is stimulated by IL-2 alone (33–35) and can be synergistically increased using a combination of IFN-γ with IL-2 (36). Finally, the secretion of both TNF-α and IFN-γ (37, 38) requires the presence of both IL-1 and IL-2. Thus, if the production of one cytokine is reduced, the secretion of other cytokines will probably also be affected.

One other explanation for decreased cytokine production by analogue-stimulated leukocytes is the potential for differential production of endogenous IL-2 in response to rIL-2, R38A, or F42K. IL-2 has been shown to be produced upon stimulation of PBMC by phorbol esters (40), lectins (41), or IL-1 in conjunction with antigen exposure (42, 43). To the best of our knowledge, IL-2 has not been shown to stimulate its own synthesis in an autocrine fashion. Our PCR data are consistent with this finding. Endogenous IL-2 mRNA was not detected in response to stimulation of either the high-affinity IL-2R by rIL-2 or the intermediate-affinity receptor complex by R38A or F42K (Fig. 7). Thus, it appears that differential regulation of endogenous IL-2 is not responsible for the differences in secondary cytokine production that we observed.

In summary, our data show that maximal production of IL-1ß, TNF-α, TNF-β, and IFN-γ by human PBMC is regulated by the high-affinity IL-2R receptor and not by endogenous IL-2 production. Thus, we propose that analogues of human IL-2 that interact primarily with the intermediate affinity IL-2R and not the high affinity receptor complex may not only be useful as immunotherapeutic agents but may also evoke less systemic toxicity than that seen with native rIL-2.

REFERENCES


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