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Advances in Brief

Interleukin-18 (IL-18) Synergizes with IL-2 to Enhance Cytotoxicity, Interferon-γ Production, and Expansion of Natural Killer Cells[1]

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Abstract

We investigated the *in vitro* effects of combining interleukin-18 (IL-18) and IL-2 on human lymphocytes. The combined use of these two cytokines synergistically enhanced the proliferation, cytolytic activity, and interferon-γ production of peripheral blood mononuclear cells. Phenotypic analysis revealed a preferential expansion of $CD56^+CD3^-$ cells and an up-regulation of IL-2 receptor-α expression on natural killer cells. Isolated natural killer cells showed a substantial increase in proliferation and cytotoxicity compared with $CD4^+$ and $CD8^+$ T cells. The combined use of IL-18 and IL-2 should be considered a viable strategy to induce an antitumor response *in vivo*.

Introduction

IL-2[3] was first identified in 1975 as a T-cell growth factor (1). Extensive studies of IL-2 administration in murine tumor models have led to the common clinical utilization of this molecule in patients with advanced cancer (2). IL-2, a 15.5-kDa glycoprotein produced by activated T cells, enhances the proliferation, cytokine production, and cytolytic activities of T and NK cells (3). IL-2 used in high, intermittent bolus has a durable antitumor effect in ~15–20% of the patients with metastatic melanoma and advanced renal cell carcinoma (4, 5). However, it is also associated with significant systemic side effects, such as capillary leakage, fever, tachycardia, renal dysfunction, and hypotension (6, 7). The substantial toxicities associated with IL-2 therapy have defined the limits of its clinical application. Because attempts to reduce toxicity by lowering the IL-2 dosage have resulted in decreased antitumor potency as well, current research is being directed at developing combination regimens having additive or synergistic antitumor effects with less associated toxicities (8).

IL-18 is a relatively nontoxic cytokine (9) that was originally identified in 1989 as an IFN-γ-inducing factor (10). IL-18 is an 18.3-kDa proinflammatory cytokine produced by activated macrophages and dendritic cells, and it plays an important role in the Th1 response, primarily based on its ability to induce IFN-γ production in T cells and NK cells (11). IL-18 induces proliferation of activated T cells, activation of NK cells, secretion of several chemokines and cytokines, and it participates in both innate and acquired immunity (12). Recently, the role of IL-18 in tumor immune surveillance has been suggested in human colon adenocarcinoma (13). Systemic administration of IL-18 in murine tumor models has induced significant NK-dependent antitumor effects with no apparent toxicities (14, 15). Attempts have been made to increase the antitumor effect of IL-18 through simultaneous use with IL-12. The combination of these two cytokines has resulted in a more potent antitumor response. However, more importantly, coadministration of IL-12 and IL-18 has also been associated with lethal organ damages, attributable in part to extremely high levels of IFN-γ (16).

In this study, we explored the *in vitro* effects of combining IL-2 and IL-18 on human lymphocytes in the hope of developing regimens to decrease the toxicity of IL-2 while maintaining its antitumor effectiveness. Human bulk PBMCs, and isolated NK cells and $CD4^+$ and $CD8^+$ T cells were cultured with either cytokine or in combination and then analyzed for proliferation, cytolytic activity, and cytokine production.

Materials and Methods

Preparation of PBMCs. PBMCs were obtained from normal healthy donor leukapheresis products or whole blood buffy coat preparations supplied by the Central Blood Bank of Pittsburgh (Pittsburgh, PA). Mononuclear cells were isolated by Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) gradient separation and cultured in 6-well plates (5×10^6 cells/5 ml/well; Costar, Corning, NY) in a humidified incubator with 5% CO_2 at 37°C. Culture was maintained in AIM-V medium (Life Technologies, Rockville, MD) containing various concentration combinations of IL-18 (0–1000 ng/ml) and IL-2 (0–6000 IU/ml). The human rIL-2 and rIL-18 were generously provided by Chiron (Emeryville, CA) and SmithKline Beecham (King of Prussia, PA), respectively.

Preparation of $CD4^+$, $CD8^+$ T Cells and NK Cells. $CD4^+$, $CD8^+$ T cells and NK cells were enriched from PBMCs by negative immunomagnetic selection. PBMCs were labeled with the enrichment antibody cocktails for human T cells ($CD4^+$, $CD8^+$) and NK cells, and then with magnetic colloid according to the product insert supplied by StemCell Technologies (Vancouver, BC, Canada). The cell suspension was then passed through a high-gradient magnetic column (0.5-inch diameter) of stainless steel mesh to remove unwanted magnetically labeled cells. Cell purity was determined to be 85 ± 5% by flow cytometry. Enriched cells were cultured in 24-well plates (1×10^6 cells/ml/well) in AIM-V medium with or without the cytokines.

Antibodies and Flow Cytometry. Surface phenotypes were determined by flow cytometry using a FACScan (Becton Dickinson, San Jose, CA). Data were analyzed using WinMDI Version 2.8 software (Joseph Trotter, Scripps Research Institute, La Jolla, CA). Fluorescently (FITC and phycoerythrin) labeled antibodies (mouse antihuman CD3, CD25, and CD56) were purchased from PharMingen (San Diego, CA). A total of 5×10^5 cells were suspended in 100 μl of cold staining buffer consisting of PBS containing 0.1% BSA and 0.05% sodium azide. Antibodies were added for 30 min at 4°C, followed by two washes with staining buffer. Cells were fixed in 2% paraformaldehyde, and data were acquired immediately.

Proliferation Assay. To measure viability and proliferation of PBMCs, cultured cells were counted manually in a hemocytometer every 3 days for 2 weeks. Trypan blue exclusion staining was used to determine the numbers of viable cells. The proliferation of PBMCs, $CD4^+$, $CD8^+$ T cells, and NK cells was determined by [^{3}H]thymidine incorporation (NEN Life Technologies, Boston, MA). At day 3 of culture, the cells (5×10^4 cells/well) were plated in a 96-well round-bottomed plate with [^{3}H]thymidine (1 μCi/well) after several

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[3] The abbreviations used are: IL, interleukin; NK, natural killer; IFN, interferon; PBMC, peripheral blood mononuclear cell; rIL, recombinant IL; LU, lytic unit; IL-2R, IL-2 receptor.

washes. Twenty-four h later, the cells were harvested on fiberglass filter mats. The nuclear bound radioactive [^{3}H]thymidine was measured by a liquid scintillation counter and recorded as cpm. A synergism curve was graphed at [^{3}H]thymidine uptake = 10,000 cpm.

Cytotoxicity Assay. Cytolytic activity was determined by standard 4-h ^{51}Cr-release assays. Cells from the EBV-transformed human B-cell lymphoma line Daudi, which was kindly provided by Dr. Theresa Whiteside (University of Pittsburgh Cancer Institute, Pittsburgh, PA), were used as target tumor cells. PBMCs, CD4$^+$, CD8$^+$ T cells, and NK cells were cultured for 3–5 days as described above and used as effector cells. Target tumor cell pellets (2×10^6 cells) were suspended in 100 μCi of ^{51}Cr (NEN Life Technologies) and placed in a humidified incubator with 5% CO_2 at 37°C for 1 h. The targets were then washed free of excess ^{51}Cr and plated in 96-well round-bottomed plate at 5×10^3 cells/well. Effectors were added to the wells at various effector-to-target ratios (30:1 to 1:1). Wells containing target cells alone were used to determine spontaneous release, and 2% Triton X-100 was added to determine maximum target ^{51}Cr release. Assay plates were incubated at 37°C with 5% CO_2 for 4 h. The Skatron Harvesting System (Skatron Instruments INC., Sterling, VA) was used to harvest the well supernatants. A gamma counter was used to measure the radioactivity released from the lysed tumor cells. The percentage of cytolysis was converted to lytic units ($LU_{20}/10^7$cells), and a synergism curve was graphed at $LU_{20}/10^7$cells = 500.

ELISA for IFN-γ. All ELISAs were performed with reagents and procedures described in the product literature inserts supplied by Endogen (Woburn, MA). For ELISAs, high binding 96-well flat-bottomed plates (Costar) were used. In brief, the ELISA plates were coated with anti-IFN monoclonal antibody. After blocking of nonspecific binding sites, supernatants from 3 day cultures were added to the wells and incubated for 1 h. After several wash steps, biotin-labeled anti-IFN monoclonal antibody was added. Finally, color was developed using horseradish peroxidase-conjugated streptavidin and tetramethylbenzidine substrate solution. The absorbance was determined at 450–550 nm with an ELISA plate reader. A synergism curve was graphed at IFN-γ = 500 pg/ml.

Statistical Analysis. The Mann-Whitney rank-sum test was used to analyze the significance of difference between the combined use of cytokines (IL-2 and IL-18) and each cytokine alone (IL-2 or IL-18). SigmaStat for Windows Version 2.03 software (SPSS Science, Chicago, IL) was used for the calculation.

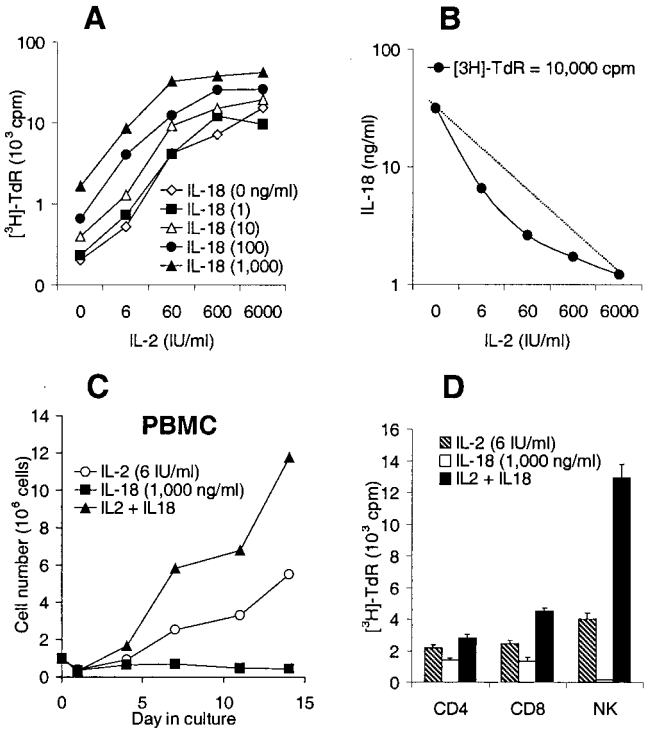

Fig. 1. Synergistic proliferation of PBMCs and NK cells. *A*, [^{3}H]thymidine incorporation was measured in PBMCs that were cultured for 3 days with various concentration combinations of IL-18 and IL-2. *B*, IL-2 and IL-18 concentrations were plotted at [^{3}H]thymidine ([^{3}H]-*TdR*) = 10,000 cpm, using the results shown in *A*. This demonstrates that the effect is synergistic, not merely additive. *C*, PBMCs were cultured for 14 days with IL-2 (6 IU/ml) and IL-18 (1000 ng/ml) or either cytokine alone, and viable cells were counted. *D*, CD4$^+$, CD8$^+$ T cells and NK cells were enriched from PBMCs using negative immunomagnetic selection. These cells were cultured for 3 days with IL-2 (6 IU/ml) and IL-18 (1000 ng/ml) or either cytokine alone, and [^{3}H]thymidine uptake was compared. *Bars*, SD.

Results

Combined Use of IL-2 and IL-18 Promotes Synergistic Proliferation of PBMCs. [^{3}H]Thymidine incorporation was compared in PBMCs that were cultured for 3 days with various concentration combinations of IL-2 and IL-18, and combined use of these two cytokines clearly promoted the proliferation of PBMCs. When 100 ng/ml IL-18 was combined with 6, 60, and 600 IU/ml IL-2, [^{3}H]thymidine uptake increased 6-, 19-, and 38-fold, respectively, compared with using IL-18 alone (Fig. 1*A*). This increase in proliferation was not simply additive but synergistic, as seen in a synergism plot (Fig. 1*B*). PBMCs were cultured for 14 days with IL-2 (6 IU/ml) and IL-18 (1000 ng/ml), or either cytokine alone. These cytokines were added again on days 6 and 12 of culture, and viable cell numbers were counted every 3 days. When compared with IL-2 alone, cultures with both IL-2 and IL-18 showed an increase in cell number by 40% at day 14. IL-18 was able to maintain the survival of PBMCs only when combined with IL-2 (Fig. 1*C*).

Preferential Expansion of NK Cells. The synergistic proliferation resulting from the combined use of IL-2 and IL-18 was most prominent in NK cells. Isolated NK cells were cultured for 3 days with IL-2 (6 IU/ml) and IL-18 (1000 ng/ml) or either cytokine alone. [^{3}H]Thymidine incorporation by NK cells cultured with both cytokines increased 3- and 92-fold compared with those cultured with IL-2 or IL-18 alone, respectively (Fig. 1*D*).

PBMCs were cultured for 3 days with IL-2 (6 IU/ml) and IL-18 (1000 ng/ml) or either cytokine alone. The side and forward scatter of the lymphocyte population increased (Fig. 2*A*, *gate R1*) with the combined use of the two cytokines. This increase in membrane complexity and cellular size signifies an activated state. The lymphocyte gate (Fig. 2*B*) contained a higher percentage of CD56$^+$CD3$^-$ (NK) cells compared with IL-2 or IL-18 alone (34% *versus* 16 and 2%, respectively).

Up-Regulation of IL-2Rα in NK Cells. Isolated NK cells were cultured for 3 days in the presence of IL-2 (6 IU/ml), IL-18 (1000 ng/ml), or a combination of both cytokines. When cultured with both cytokines, IL-2Rα (CD25), which normally is not expressed on resting NK cells, was substantially up-regulated. The relative percentage of CD56$^+$CD25$^+$ cells was 80% compared with 2 and 22% for the IL-2 and IL-18 groups, respectively (Fig. 2*C*). The mean fluorescence intensity for the CD25-FITC staining was 59.9 for the CD56$^+$CD25$^+$ cells compared with 4.7 and 10.8 for the IL-2 and IL-18 groups, respectively.

Synergistic Enhancement of the Cytolytic Activity in PBMCs, Specifically NK Cells. PBMCs were cultured for 5 days at various concentration combinations of IL-2 and IL-18, and their cytolytic activities were tested against the Daudi cell line. The combined use of these two cytokines substantially enhanced the cytolytic activity of PBMCs even when a very low dose of IL-2 was used. The combination of 6 IU/ml IL-2 and 1000 ng/ml IL-18 significantly increased the cytolytic activity ($LU_{20}/10^7$cells) of PBMCs compared with the same concentrations of IL-2 and IL-18 alone (1271 *versus* 41 and 115, respectively; $P < 0.001$; Fig. 3*A*). This increase in cytolytic activity was synergistic as seen in a synergism plot (Fig. 3*B*). Data from one representative 4-h ^{51}Cr-release assay are shown in Fig. 3*C*. When IL-2 and IL-18 were used concurrently, the cytolytic activity (percentage of

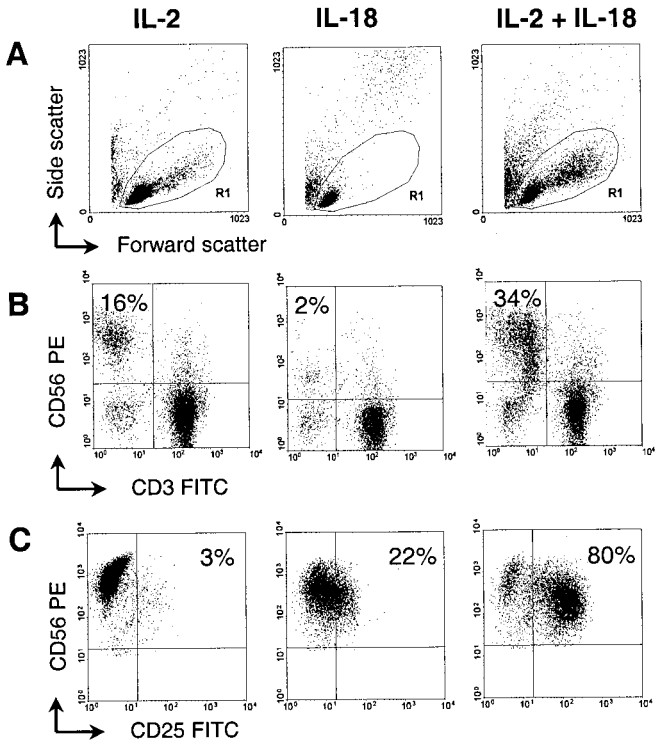

Fig. 2. Preferential expansion of NK cells and up-regulation of IL-2Rα in NK cells. *A*, PBMCs were cultured for 3 days with IL-2 (6 IU/ml) and IL-18 (1000 ng/ml) or either cytokine alone. With combined use of the two cytokines, activated lymphocytic population increased on the dot plot of forward and side scatter. *B*, when gated on lymphocytes (*R1* in *A*), the percentage of $CD56^+CD3^-$ cells was higher with combined use of IL-2 and IL-18 compared with either cytokine alone. *C*, $CD56^+CD3^-$ cells were enriched from PBMCs and cultured for 3 days. Surface expression of CD25 (IL-2Rα) was compared, which was clearly up-regulated with combined use of both cytokines. *PE*, phycoerythrin.

cytolysis) was significantly greater compared with IL-2 and IL-18 alone (61% *versus* 5 and 2%, respectively; $P < 0.001$) at a 30:1 effector-to-target ratio. When tested on isolated T cells ($CD4^+$, $CD8^+$) and NK cells, a synergistic increase in cytolytic activity was most notable in the NK cell population. When the two cytokines were used together, cytolytic activity of NK cells ($LU_{20}/10^7$cells) increased 5- and 20-fold compared with those cultured with IL-2 or IL-18 alone (Fig. 3*D*).

Synergistic Enhancement of IFN-γ Production. We compared the concentration of IFN-γ in culture supernatants of PBMCs (1×10^6 cells/ml) that were exposed to various concentration combinations of IL-2 and IL-18. The combination of these two cytokines clearly enhanced IFN-γ production in PBMCs. When IL-18 (1000 ng/ml) was combined with IL-2 (6, 60, 600, and 6000 IU/ml), IFN-γ production increased to 0.7, 2.9, 3.6, and 4.8 ng/ml, respectively (Fig. 4*A*). As shown in Fig. 4*B*, this increase in IFN-γ production was synergistic. One representative IFN-γ ELISA is shown in Fig. 4*C*. When IL-2 (6 IU/ml) and IL-18 (1000 ng/ml) were used together, the IFN-γ concentration in day 3 culture supernatants of PBMCs (1×10^6 cells) was significantly higher compared with IL-2 or IL-18 alone (478 *versus* 9 pg/ml and nondetectable; $P < 0.001$; Fig. 4*C*). When the combination of IL-2 and IL-18 was tested on isolated T cells ($CD4^+$, $CD8^+$) and NK cells, a synergistic increase in IFN-γ production was observed in all of these cells (Fig. 4*D*).

Discussion

Immunotherapy with high-dose rIL-2 has been reported to produce objective responses in ~15–20% of treated patients and is associated with durable complete responses and prolonged survival in responding patients (4, 5). However, the clinical utility of high-dose i.v. rIL-2 therapy is limited by severe toxicity, including vascular leak syndrome (6, 7). In an attempt to reduce the risks of IL-2 therapy, current research is being directed at developing combination regimens with additive or synergistic antitumor effects and reduced toxicity (8).

Similar to IL-2, IL-18 has potent antitumor effects (5, 16) and effects on the activation and proliferation of NK and T cells (3, 11), but it is also a relatively nontoxic cytokine (9). In this study, we explored the *in vitro* effect of combining IL-2 and IL-18 on human lymphocyte populations. Synergistic increases in proliferation, cytotoxicity, and IFN-γ production in PBMCs were all observed. Similar results were found *in vitro* in tumor-infiltrating lymphocytes (data not shown) that were freshly isolated from a patient with melanoma. These findings suggest that whether injected systemically or locally at the site of tumor, the synergistic activity of these two cytokines may promote the activation, expansion, and possibly the survival of effector cells that interact with tumor targets, thus enhancing the antitumor response.

We determined the optimal concentrations of IL-18 and IL-2 by attempting to maximize their cytolytic activities while minimizing the dosage of IL-2 and, therefore, its toxicity. When we used 6 IU/ml IL-2 with 1000 ng/ml IL-18, a comparable level of proliferation (89% of $[^3H]$thymidine uptake) as well as even a greater level of cytolytic activity (192% of $LU_{20}/10^7$ cells) could be evoked compared with using a 1000-fold higher concentration (6000 IU/ml) of IL-2 alone (Figs. 1*A* and 3*A*). These findings provided us with the rationale to choose 6 IU/ml IL-2 and 1000 ng/ml IL-18 for the subsequent experiments on isolated subsets of lymphocytes.

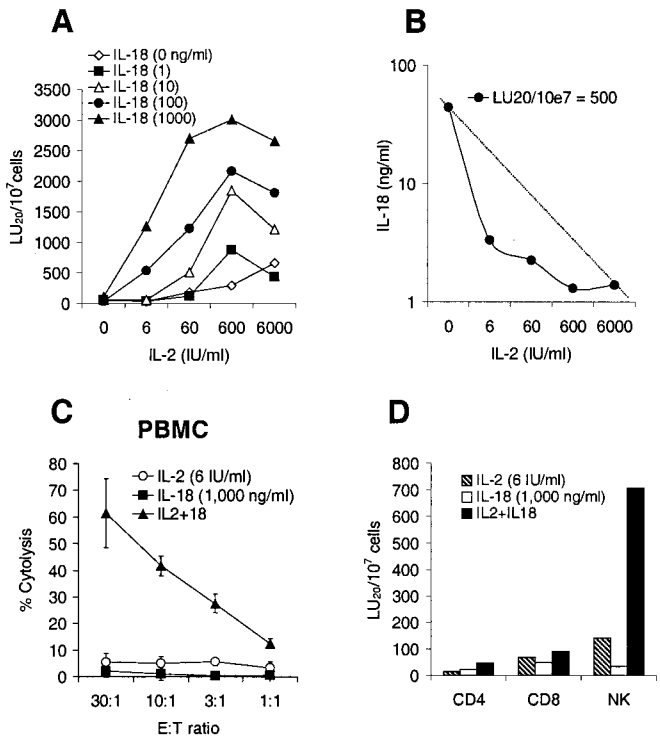

Fig. 3. Synergistic enhancement of the cytolytic activity of PBMCs, specifically NK cells. *A*, standard 4-h ^{51}Cr-release assays were performed against the Daudi cell line. PBMCs cultured for 5 days in various concentration combinations of IL-2 and IL-18 were used as effector cells. *B*, IL-2 and IL-18 concentrations were plotted at $LU_{20}/10^7$ cells = 500, using the results shown in *A*. Again, this demonstrates a synergistic rather than an additive effect. *C*, representative standard 4-h ^{51}Cr-release data are shown against the cell line Daudi. PBMCs were used as effectors after a 3-day culture period in the presence of IL-2 (6 IU/ml) and IL-18 (1000 ng/ml) or either cytokine alone. *E:T*, effector-to-target ratio. *Bars*, SD. *D*, enriched T cells ($CD4^+$, $CD8^+$) and NK cells were cultured for 3 days and tested for cytolytic activity. A synergistic increase in cytolytic activity was most notable in NK cells.

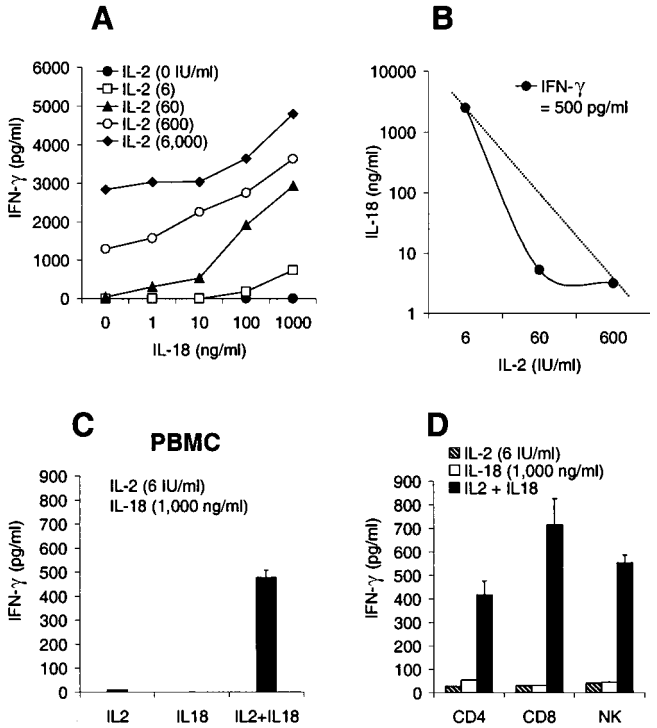

Fig. 4. Synergistic enhancement of IFN-γ production in PBMCs. *A*, ELISA for IFN-γ was performed using culture supernatants of PBMCs (1×10^6/ml) that were cultured for 3 days with various concentration combinations of IL-2 and IL-18. *B*, using the data shown in *A*, IL-2 and IL-18 concentrations were plotted at IFN-γ = 500 pg/ml, demonstrating a synergistic effect between the two cytokines. *C*, representative IFN-γ ELISA for PBMCs cultured for 3 days with IL-2 (6 IU/ml) and IL-18 (1000 ng/ml) or either cytokine alone. *D*, when tested on isolated T cells ($CD4^+$, $CD8^+$) and NK cells, synergistic increase in IFN-γ production was observed in all cells treated with the cytokine pair. *Bars*, SD.

IFN-γ, also known as type II IFN, regulates a variety of immunological responses in both innate and acquired immunity. It is the predominant cytokine secreted from activated T cells and NK cells during Th1-dominated immune responses (17). It has tumoricidal effects *in vivo*, which result either from a direct action on tumor cells or indirectly via the activation of several effector mechanisms. These include stimulation of MHC antigen expression, macrophage activation, and stimulation of T- and NK-cell activity (18). Several cytokines, including IL-2 and IL-18, are known as potent inducers and co-inducers of IFN-γ in NK and T cells (3, 10, 11). However, excessively elevated serum concentrations of IFN-γ are highly toxic and usually lethal, as shown in animals treated with IL-12 and IL-18 (9, 16). In contrast to these studies, a recent report demonstrated that IL-18 in combination with IL-2 enhances NK-cell activity without inducing large amounts of IFN-γ production and without visible side effects in a murine model (19). This strongly suggests the possible therapeutic application of combining the two cytokines in the clinic. In our study, combined use of IL-2 and IL-18 resulted in a synergistic increase in IFN-γ production in PBMCs. However, the day 3 IFN-γ concentration in the supernatant of cells cultured with 6 IU/ml IL-2 and 1000 ng/ml IL-18 was only 26% compared with the high concentration of IL-2 (6000 IU/ml) alone (Fig. 4*A*). This synergistic but relatively low level of IFN-γ production may be beneficial for the induction of an antitumor Th1 response while maintaining nontoxic levels of IFN-γ in the serum.

The functional IL-2 receptor is composed of three distinct membrane-associated subunits: a 55-kDa α chain (IL-2Rα, p55), a 70- to 75-kDa β chain (IL-2Rβ, p70), and a 64-kDa γ chain (IL-2Rγ, p64). Although these individual subunits alone bind IL-2 with very low affinity, heterodimerization and heterotrimerization of the subunits permit binding with intermediate and high affinity, respectively (3). NK cells are known to constitutively express IL-2Rβ (3, 20). It was observed that the surface expression of IL-2Rβ was comparable for both freshly isolated NK cells and the cytokine-activated NK cells. Because the IL-2Rα subunits of NK cells are substantially up-regulated by the combined use of IL-2 and IL-18 (Fig. 2*C*), the synergistic expansion and activation of NK cells can be partly explained by the αβ heterodimerization or αβγ heterotrimerization of IL-2R, with subsequent increased affinity and binding to IL-2. Therefore, relatively low concentrations of IL-2 would be necessary to evoke and maintain the strong antitumor activity of NK cells.

In summary, the combined use of IL-2 and IL-18 promoted synergistic *in vitro* expansion and activation of human PBMCs, specifically NK cells. Substantial up-regulation of IL-2Rα in NK cells may partly explain the mechanism of this synergism. The use of the two cytokines concurrently dramatically reduced the amount of IL-2 needed to obtain greater cytolytic activity and comparable proliferation of PBMCs compared with using a high concentration of IL-2 alone, while maintaining a relatively lower, and therefore possibly nontoxic, IFN-γ concentration. These findings suggest that we can obtain an enhanced antitumor effect of IL-2 while reducing the risks of related toxicity. Therefore, the combined use of IL-2 and IL-18 should be considered a viable strategy for the generation of antitumor responses *in vivo* and for future clinical applications.

Acknowledgments

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