Characterization of a Novel Subset of CD8$^+$ T Cells That Expands in Patients Receiving Interleukin-12

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Abstract

IL-12 has significant antitumor activity in mice that may be mediated by CD8$^+$ T cells. We show in this report that repeated subcutaneous injections of IL-12 in patients with cancer resulted in the selective expansion of a subset of peripheral blood CD8$^+$ T cells. This T cell subset expressed high levels of CD18 and upregulated IL-12 receptor expression after IL-12 treatment in vivo. In normal subjects, these CD3$^+$CD8$^+$CD18^{bright} T cells expressed IL-12 and IL-2 receptors and adhesion/costimulatory molecules to a greater degree than other CD8$^+$ and CD4$^+$ T cells. They appeared morphologically as large granular lymphocytes, although they did not express NK cell markers such as CD56. In addition, CD8$^+$CD18^{bright} T cells were almost exclusively T cell receptor (TCR) $\alpha\beta^+$, and exhibited a TCR Vβ repertoire that was strikingly oligoclonal, whereas the Vβ repertoire of CD18^{dim} T cells was polyclonal. Although CD8+CD18^{bright} T cells demonstrated little functional responsiveness to IL-12 or IL-2 alone in vitro, they responded to the combination of IL-12+IL-2 with strong IFN-γ production and proliferation and enhanced non–MHC-restricted cytolytic activity. In contrast, CD18^{dim} T cells were not activated by IL-12 or IL-2, alone or in combination. These findings demonstrate that CD8+CD18^{bright} T cells are a unique population of peripheral blood lymphocytes with features of both memory and effector cells that are capable of TCR-independent activation through combined stimulation with IL-12+IL-2. As this activation results in IFN-γ production and enhanced cytolytic activity, these T cells may play a role in innate as well as acquired immunity to tumors and infectious pathogens. Additional studies will be necessary to determine whether CD8+CD18^{bright} T cells mediate the antitumor effect of IL-12 or IL-2 administered to cancer patients, and if so, whether maximal activation of these T cells with the combination of IL-12+IL-2 in vivo can augment the clinical effectiveness of these cytokines. (*J. Clin. Invest.* 1998. 102: 561–575.) Key words: lymphocytes • activation • cytokines • immunotherapy • cancer

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Introduction

IL-12 is a cytokine derived from antigen-presenting cells that stimulates NK cells and T cells to proliferate, produce IFN-γ, and lyse tumor cells (1–5). IL-12 promotes the development of cellular immune responses by inducing CD4$^+$ T cells to differentiate into T helper type 1 (Th1) cells (6, 7) and by stimulating cytolytic activity and cytokine production among CD8$^+$ T cells and NK cells. This effect on cellular immunity appears to underlie the ability of IL-12 to effectively eradicate viral and parasitic infections (8–11) as well as established tumors in mice (12). The antitumor activity of IL-12 in mice has formed the basis for phase I and II clinical trials involving the administration of IL-12 to patients with cancer (13, 14). In mice, some studies have demonstrated that the depletion of CD8$^+$ T cells diminishes the antitumor effect of IL-12 (12), whereas another recent report suggested that Vα14 NKT cells are responsible for the antitumor effect (15). However, in murine models of ovarian carcinoma and melanoma, tumor regression in response to IL-12 was associated with the migration of CD8$^+$ T cells into tumor sites (16). Furthermore, there is evidence in mice that CD8$^+$ T cells mediate the graft-versus-leukemia effect of IL-12 (17). In humans, as in mice, there are data which suggest that CD8$^+$ T cells may have an essential role in antitumor immunity. In melanoma patients receiving hapten-modified autologous tumor cell vaccines, lymphocytic infiltrates within regressing metastases are comprised largely of CD8$^+$ T cells (18). In patients with AIDS, autologous IL-2–activated CD8$^+$ T cells can cause significant regression of Kaposi's sarcoma lesions (19). Taken together, these studies in animal models and the results of clinical trials suggest that CD8$^+$ T cells play an important role in antitumor immunity, and that the antitumor activity of IL-12 may be mediated by these cells.

Although it has been demonstrated that IL-12 has potent immunomodulatory effects on T cells, freshly isolated peripheral blood T cells exhibit little functional responsiveness to IL-12. Instead, T cell IL-12 responsiveness in vitro is dependent on activation via the T cell receptor (TCR)[1]/CD3 complex or with a mitogen such as phytohemagglutinin (2, 3). This activation is required to upregulate the expression of IL-12 receptors (20–22) and render cells responsive to IL-12. In addition, T cell activation is required to induce the expression of signal transducer and activator of transcription (STAT) 4 (23), an essential component of IL-12 signaling.

In light of the observations derived from mouse tumor models and from attempts to stimulate cellular immune responses directed against malignancies in humans, the clinical potential of IL-12 in the treatment of human cancer may rest

1. *Abbreviations used in this paper:* STAT, signal transducer and activator of transduction; TCR, T cell receptor.

in part on its ability to activate in vivo those T cell subsets capable of mediating TCR-dependent and/or TCR-independent killing of tumor cells. However, because human peripheral blood T cells demonstrate little IL-12 responsiveness in the absence of in vitro activation, the mechanism by which $CD8^+$ or $CD4^+$ T cells would be capable in vivo of mediating the antitumor effect of parenterally administered IL-12 is unclear. One possibility is that only a small subset of peripheral blood T cells is capable of responding to IL-12, making it difficult to detect IL-12–induced activation in vitro when intermixed with T cells that are unresponsive to IL-12. Nonetheless, this small T cell subset may be capable of potent antitumor activity. This would be analogous to the IL-12–induced antitumor activity observed in C57BL/6 mice in the small Vα14 NKT lymphocyte subset (15).

The concept that subpopulations of T cells can differ from the majority of T cells in their ability to be activated in vivo has gained credence from observations involving $CD8^+$ and $CD4^+$ T cells. In humans, T cells with rapid recall responses to antigens such as tetanus toxoid have been termed memory cells. These memory cells, which have been identified among the $CD4^+$ and $CD8^+$ subsets, are characterized immunophenotypically by the strong expression of CD2, LFA-1, LFA-3, CD45RO, and CD29 relative to other T cells (24). In addition to demonstrating strong recall responses to tetanus, these T cells also produce large amounts of IFN-γ in response to mitogens. Bystander T cell proliferation induced by viruses and type I interferon (25) and LPS-induced T cell stimulation (26) appears to primarily involve a small subpopulation of $CD8^+CD44^{hi}$ memory T cells.

To determine whether distinct subsets of peripheral blood lymphocytes in humans are capable in vivo of responding to IL-12, we performed an immunophenotypic analysis of T and NK cells in patients with cancer receiving subcutaneous injections of IL-12. Through this approach, we identified a subpopulation of $CD8^+$ T cells ($CD8^+CD18^{bright}$) that expanded in vivo and strongly upregulated IL-12 receptor expression in response to IL-12. Although in normal subjects, these $CD8^+$ T cells constitutively expressed IL-2 and IL-12 receptors, they responded poorly in vitro to either cytokine alone, but, like NK cells, were strongly activated by the combination of IL-12+IL-2. This identifies $CD8^+CD18^{bright}$ T cells as a unique subset of peripheral blood T cells that can be activated by IL-12+IL-2 in a TCR-independent manner and may play a role in mediating the antitumor effect of these cytokines when administered to patients with cancer.

Methods

Isolation of PBMC and T cell subsets. Heparinized blood samples were obtained from patients and normal volunteers. All patients had solid tumors and were receiving subcutaneous injections of IL-12 on a multicenter phase I dose escalation trial. This protocol was approved by the Human Subjects Protection Committee of the Dana-Farber Cancer Institute (DFCI), and informed consent was obtained from all patients. Blood samples enriched for white blood cells were also obtained from volunteers undergoing platelet pheresis in the DFCI Blood Bank. PBMC were isolated from blood samples through density gradient centrifugation using Ficoll–Hypaque (Pharmacia, Uppsala, Sweden).

For the isolation of $CD18^{bright}$ and $CD18^{dim}$ T cells subsets, PBMC were first depleted of monocytes through passage over a nylon wool column. For the preparation of CD8-enriched T cells, monocyte-depleted PBMC were depleted of $CD4^+$ T cells, B cells, and NK cells using antibodies to lineage-specific markers (anti-CD4, anti-CD20, and anti-CD56) and magnetic beads coated with anti–mouse Ig antibodies (Advanced Magnetics, Cambridge, MA). CD8-enriched T cell preparations were then stained with CD18-FITC and CD8-PE, and PE positive cells were separated on a fluorescence activated cell sorter into $CD18^{bright}$ and $CD18^{dim}$ populations based on CD18-FITC fluorescence intensity ($CD18^{bright}$ cells routinely had a CD18-FITC mean fluorescence intensity five to six times greater than $CD18^{dim}$ cells). $CD18^{bright}$ and $CD18^{dim}$ T cells were 95–98% pure after sorting.

Antibodies and cytokines. The following unconjugated and PE- or FITC-conjugated antibodies were obtained from Coulter (Hialeah, FL): negative control, anti-CD4, anti-CD8, anti-CD56 (NKH1), anti-CD28, anti-CD45RA, anti-CD20 (B1), and anti-CD29. Anti-CD49d was purchased from T Cell Diagnostics (Woburn, MA). The anti–IL-2Rβ antibody (341, IgG1) was a gift from Stefan Voss (Beth Israel Deaconess Medical Center, Boston, MA). Anti-CD2 (20B6, IgG1), anti-CD2R (T11-3, IgG3), anti-CD18 (8C12, IgM), anti-CD56 (3B8, IgM and N901, IgG1), anti-CD6 (T12, IgM), anti–IL-2Rγ (3B5, IgG1) and anti–IL-2Rα (IgG2a) have been previously described (27, 28) and were used as dilutions of mouse ascites. MOPC-21 (Sigma Immunochemicals, St. Louis, MO), used as a dilution of ascites, served as a mouse IgG1 negative control antibody. Anti–IL-12Rβ1 (12Rβ.3F12, IgG1) is a non-neutralizing antibody specific for the β1 subunit of the IL-12 receptor (29). PE- and FITC-conjugated isotype-specific goat anti–mouse antibodies, biotin-conjugated goat anti–mouse IgG1, and streptavidin-PE were purchased from Southern Biotechnology (Birmingham, AL).

Recombinant human IL-12 (specific activity 1.7×10^7 U/mg) was generously provided by Steven Herrmann at Genetics Institute (Cambridge, MA). IL-2 (specific activity 3.9×10^6 U/ml) was generously provided by Amgen (Thousand Oaks, CA).

Proliferation assays and measurement of IFN-γ production. Sorted T cells were incubated in 96-well U-bottom plates at 3×10^4 cells/well with medium alone or the indicated concentration of cytokines at 37°C. Proliferation assays were performed as previously described (28) and consisted of a 96-h incubation, with 1 μCi [^{3}H]thymidine (DuPont NEN, Boston, MA) added 8 h before harvesting. For IFN-γ assays, supernatants were harvested after a 72-h incubation and the IFN-γ concentration assayed using an IFN-γ ELISA (Endogen, Cambridge, MA).

Cytotoxicity assays. Sorted $CD8^+CD18^{bright}$ or $CD8^+CD18^{dim}$ T cells were first incubated overnight in U-bottom well plates with either medium alone or the indicated cytokines at 37°C. COLO or OKT3 cells were then labeled with ^{51}Cr and added to the T cells at a 5:1 effector:target ratio. T cells were incubated with the labeled target cells for 4 h at 37°C, and the supernatants were then harvested. ^{51}Cr release was assayed using a gamma counter, and percent of specific cytotoxicity calculated as previously described (5).

Immunofluorescence analysis. For direct staining, 2.5×10^5 cells were incubated with the indicated PE- or FITC conjugated antibody for 20 min on ice. For indirect staining, cells were incubated with the primary, unconjugated antibody for 20 min on ice, washed twice, and then incubated with a PE- or FITC-conjugated isotype-specific secondary antibody for 20 min. Cytokine receptor staining was performed with a three-step method using the primary unconjugated antibody, a biotin-conjugated isotype-specific secondary antibody, and streptavidin-PE. Isotype-specific negative controls were used with direct and indirect staining as well as with the three-step staining involving biotin/streptavidin-PE. After staining, cells were fixed with 1% formaldehyde and analyzed by flow cytometry.

Cytospin preparations. Immediately after sorting, $CD8^+CD18^{bright}$ T cells, $CD8^+CD18^{dim}$ T cells, or NK cells were spun onto glass slides and stained with Wright-Giemsa. Stained cells were examined using an Olympus BX60 microscope.

RNA extraction, reverse transcription and PCR. RNA was extracted from sorted $CD8^+CD18^{bright}$ and $CD8^+CD18^{dim}$ T cells using the RNAStat-60 kit (Tel-test Inc., Friendswood, TX) according to the

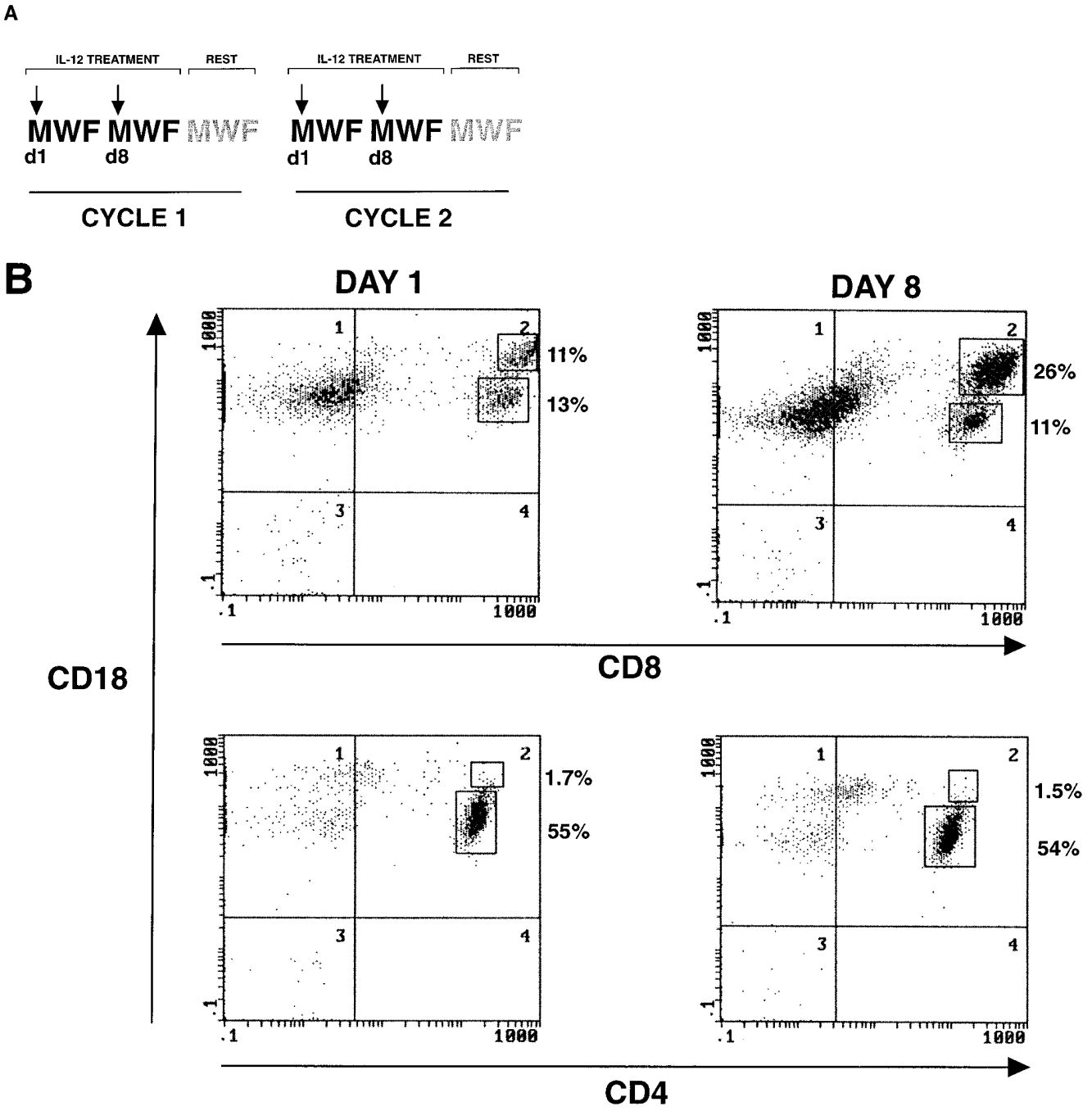

Figure 1. A subset of human CD8$^+$ T cells expands in vivo in response to subcutaneously administered IL-12. (*A*) Subcutaneous injections of IL-12 were administered to cancer patients as part of a phase I dose escalation study. Treatment cycles were begun 14 d after receiving a single test dose of IL-12. A cycle of treatment consisted of single injections of IL-12 on Monday, Wednesday, and Friday for two consecutive weeks, followed by a week of rest. Arrows point to the days (days 1 and 8) within each cycle when blood samples were obtained for immunophenotypic analysis. Blood samples were drawn just before receiving the IL-12 injection. (*B*) PBMC were isolated from patient blood samples drawn before the administration of IL-12 on days 1 and 8 of each treatment cycle. PBMC were two-color stained with CD18-FITC (*y-axis*) and either CD4-PE or CD8-PE (*x-axis*) and subjected to FACS analysis, gating on the lymphocyte population. Results shown are from one patient at the 500 ng/kg dose level during cycle 1, and are representative of the results obtained with two other patients (one at the 500 ng/kg dose level and one at 700 ng/kg). (*C*) PBMC were isolated from three patients at the 500 ng/kg and 700 ng/kg dose levels on days 1 and 8 of cycle 1 and stained with either CD56-PE/CD18-FITC, CD4-PE/CD18-FITC, or CD8-PE/CD18-FITC. Cell number was obtained by multiplying the lymphocyte subset percentage (calculated by FACS™ analysis) by the absolute number of lymphocytes (calculated by performing a complete blood count on the blood sample).

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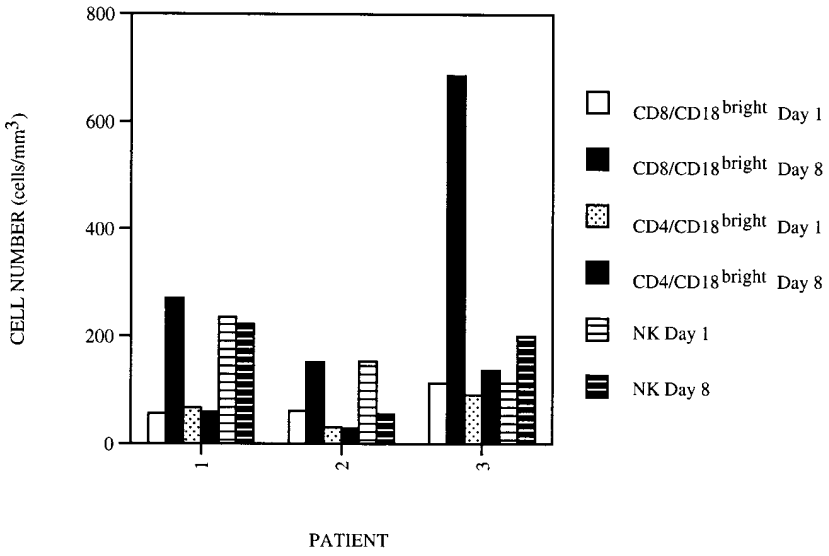


Figure 1 (Continued)

manufacturer's protocol. First strand cDNA was generated from 2 μg of total RNA using random hexanucleotides (Pharmacia Biotech Inc., Piscataway, NJ) and reverse transcriptase (Superscript, Gaithersburg, MD). Each Vβ segment was amplified with one of the 26 Vβ subfamily specific primers and a Cβ primer recognizing both Cβ1 and Cβ2 regions. PCR amplification of Vβ5 and Vβ13 each required the use of two primers to identify the entire Vβ subfamily. The sequences of primers used in these experiments were described by Choi et al. (30) except for the Cβ and Vβ3, Vβ19, Vβ21, Vβ22, Vβ23, and Vβ24 primers that were described by Puisieux et al. (31). The Cβ primer was conjugated to fluorescent dye 6-FAM (Applied Bioethicists, Foster City, CA) for CDR3 size analysis. The amplification was performed over 30 cycles in a final volume of 100 μl on a DNA thermal cycler (Perkin-Elmer Corp., Norwalk, CT). The PCR cycle profile was as follows: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, and a final polymerization step of 10 min at 72°C. Experiments in which template DNA was titrated and in which the number of cycles was varied were carried out to ensure that the PCR reactions were linear under the conditions used, as demonstrated by a dose-response relationship between initial amounts of cDNA and quantity of the final products.

Quantitation of TCR Vβ subfamily representation in peripheral blood T cells. 25 μl (1/4) of each PCR reaction was electrophoresed on a 2% agarose gel and blotted to nylon membranes (Boehringer Mannheim, Mannheim, Germany). The membrane was hybridized at 42°C overnight with a [^{32}P]γ-ATP–labeled Cβ internal probe (5′-GAGGACCTGAACAAGGTGTTCCCACCCGAG-3′) and washed twice at 4°C for 30 min in 6×SSC and twice at 65°C for 20 min in TMAC. The membrane was exposed to a Phosphor screen and radioactive intensity of each specific band was quantified on a PhosphorImager using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Representation of each Vβ subfamily was calculated as a percentage of the sum of all Vβ signals detected on the autoradiogram.

TCR β chain CDR3 fragment size analysis. The size distribution of each fluorescent PCR product was determined by electrophoresis on an automated 373 DNA sequencer (Applied Biosystems) using a 5.5% polyacrylamide gel and data analyzed by GeneScan software (Perkin Elmer, Foster City, CA). Because the positions of the 5′ and 3′ primers are fixed, fragment size differences within each Vβ subfamily are due entirely to different CDR3 region lengths, reflecting junctional diversity and *N*-random nucleotide insertions in the V-D-J region. Peaks corresponding to in-frame transcripts are detected at three nucleotide intervals. As described previously (32), a normal transcript size distribution, reflecting polyclonal cDNA, contains 8 to 10 peaks for each Vβ subfamily with a Gaussian size distribution. The appearance of dominant peaks indicates the presence of excess cDNA of identical size, suggesting the presence of an oligoclonal or clonal T cell population.

Results

IL-12 administration induces in vivo the selective expansion of a subpopulation of CD8$^+$ T cells and the upregulation of lymphocyte IL-12 receptor expression. Patients with solid tumors received subcutaneous injections of IL-12 given as part of a multi-center phase I dose escalation study. A single injection of IL-12 was given 2 wk before the first cycle of therapy, as this has been shown to diminish the toxicity of subsequent repetitive IL-12 dosing. A cycle of therapy consisted of a single injection of IL-12 administered three times a week for two consecutive weeks followed by a week of rest (Fig. 1 *A*). To determine whether subcutaneous injections of IL-12 had any effect on populations of circulating lymphocytes, we obtained blood samples from three patients enrolled at the two highest dose levels (two patients at the 500 ng/kg dose level and one patient at the 700 ng/kg dose level) at the Dana-Farber Cancer Institute. Blood was drawn from patients before IL-12 injections on days 1 and 8 of each cycle. PBMC were isolated from blood samples and subjected to immunophenotypic analysis using antibodies against lineage-specific markers on T, NK, and B cells, an antibody against the CD18 adhesion molecule (part of the CD11a/CD18 complex, referred to also as LFA-1), and an

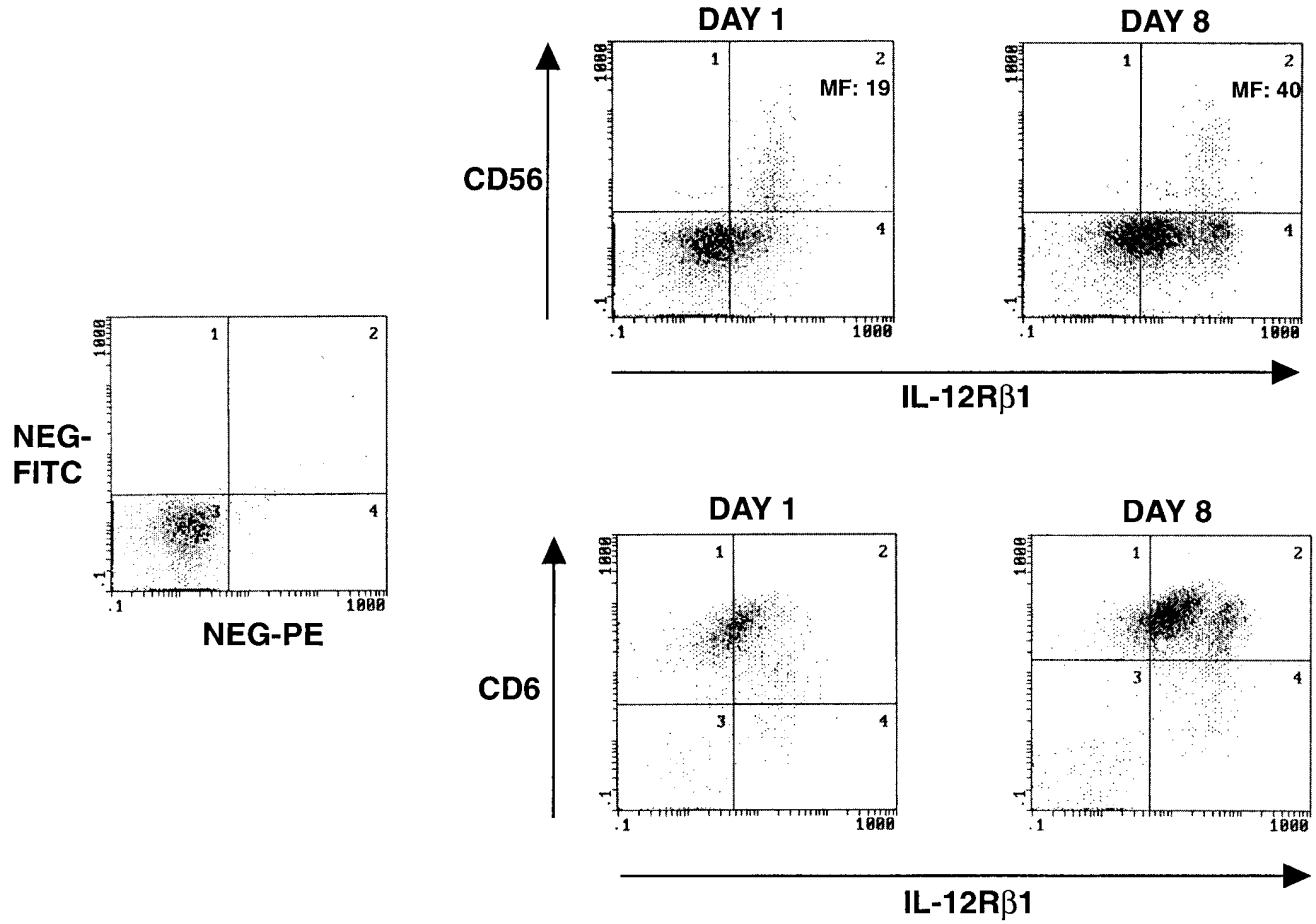
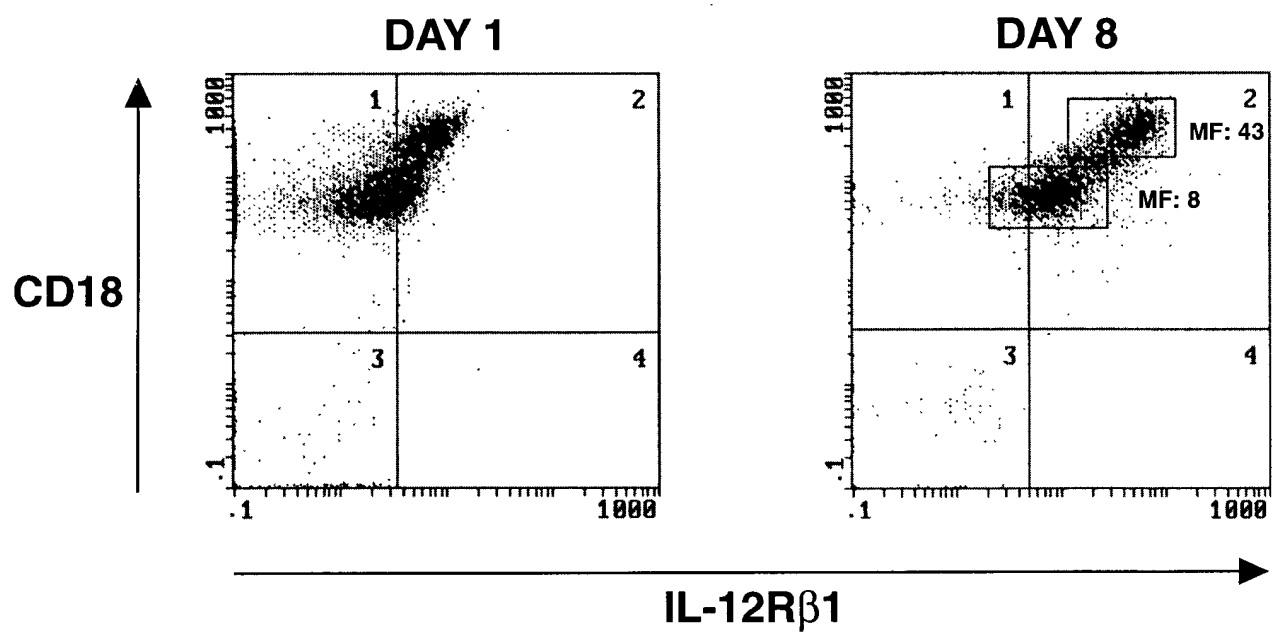


Figure 2. IL-12Rβ1 expression is upregulated on NK cells and T cells in response to subcutaneously administered IL-12. (*A*) PBMC were isolated from patients on days 1 and 8 of a cycle of IL-12 therapy and stained with IL-12Rβ1-PE (*x-axis*) and either CD56-FITC (NK cells) or CD6-FITC (T cells) (*y-axis*). Quadrants were set using isotype-matched negative control antibodies. MF = mean fluorescence intensity. (*B*) PBMC were isolated from patients on days 1 and 8 and stained with IL-12Rβ1-PE (*x-axis*) and CD18-FITC (*y-axis*). Results shown for *A* and *B* are for one patient at the 500 ng/kg dose level during cycle 1 and are similar to the results obtained with two other patients at the 500 ng/kg and 700 ng/kg dose levels.

antibody against the β1 subunit of the IL-12 receptor (IL-12Rβ1). Among peripheral blood T cells, two populations of $CD8^+$ cells could be discerned based on the intensity of staining with a CD18 antibody (Fig. 1 *B*). $CD18^{bright}$ and $CD18^{dim}$ $CD8^+$ T cells differed four- to fivefold in their CD18 mean fluorescence intensity, and the ratio of dim/bright $CD8^+$ cells was usually 1–2:1. $CD4^+$ T cells were more homogeneous in their CD18 staining, with the majority (> 95%) of $CD4^+$ T cells staining dimly for CD18.

In patients receiving IL-12 at the highest dose levels (500–700 ng/kg/d), $CD8^+CD18^{bright}$ T cells were the only lymphocyte subset consistently observed to expand (by percentage and absolute number) by the second week of each cycle (Fig. 1, *B* and *C*). During the week of rest within each cycle, the number of circulating $CD8^+CD18^{bright}$ T cells partially diminished, only to expand again during the second week of the subsequent cycle (data not shown). As seen during cycle 1 (Fig. 1 *C*), the N-fold change in the $CD8^+CD18^{bright}$ T cell number between days 1 and 8 (ranging from 2.6- to 6-fold for the three patients studied) was significantly increased over the N-fold change in the $CD4^+CD18^{bright}$ T cell (range: 0.9- to 1.4-fold) and NK cell (range: 0.34- to 1.3-fold) numbers. This was assessed by examining the ratio of the N-fold change in $CD8^+CD18^{bright}$ number divided by the N-fold change in either the $CD4^+CD18^{bright}$ or NK cell number for each patient. Testing was performed using a one-sided Student's *t* test, against a null hypothesis of equal increases (ratio = 1). The *P* value for assessing the $CD8^+$ $CD18^{bright}$ T cell change relative to the $CD4^+CD18^{bright}$ T cell change was 0.05; the *P* value assessing the $CD8^+CD18^{bright}$ T cell change relative to the NK cell change was 0.02.

Changes in IL-12 receptor expression were also analyzed during each cycle of IL-12 therapy. Whereas all NK cells were uniformly positive for IL-12Rβ1 before starting IL-12 therapy, most T cells were only weakly positive, with a small subset expressing IL-12Rβ1 at a level more comparable to NK cell expression (Fig. 2 *A*, Day 1). By the second week of IL-12 injec-



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tions, there was upregulation of IL-12Rβ1 expression on NK cells, with a twofold increase in the mean fluorescence intensity (Fig. 2 *A*, Day 8). IL-12Rβ1 expression was also upregulated on all T cells, but this upregulation was strongest on a small subpopulation of T cells (IL-12Rβ1^{bright}) that now expressed IL-12Rβ1 with a mean fluorescence intensity that was five to six times greater than that which was observed on the majority of T cells (Fig. 2 *A*, Day 8). The mean fluorescence intensity of these IL-12Rβ1^{bright} T cells during the second week of IL-12 injections paralleled NK cell IL-12Rβ1 staining.

Because IL-12 responsiveness is determined in part by IL-12 receptor expression, we hypothesized that the CD18^{bright} T cells observed to expand in vivo in response to IL-12 might be the same T cells that stained the brightest for IL-12Rβ1 during the second week of IL-12 therapy. As shown in Fig. 2 *B*, CD18^{bright} lymphocytes stained brightly for IL-12Rβ1 relative to CD18^{dim} lymphocytes before IL-12 therapy and upregulated IL-12Rβ1 expression to the greatest extent during IL-12 therapy. As CD18^{bright} lymphocytes consisted of NK cells and predominantly CD8+ T cells (Fig. 3 *A*), it was clear that the differential expansion of CD8$^+$CD18^{bright} T cells relative to other T cells was associated with the differential expression of IL-12Rβ1.

CD18^{bright} T cells and NK cells express higher levels of adhesion molecules and receptors for IL-12 and IL-2 than CD18^{dim} T cells. Having observed the selective expansion of CD8$^+$CD18^{bright} T cells in patients receiving subcutaneous injections of IL-12, we further characterized this subpopulation of T cells in normal subjects. As shown in Fig. 3 *A*, CD18 expression on NK cells and T cell subsets in normal individuals was similar to the pattern observed in patients with solid tumors. Specifically, CD18^{bright} lymphocytes were comprised primarily of NK cells and CD8$^+$ T cells, along with a smaller number of CD4$^+$ T cells (Fig. 3 *A*). CD18^{bright} lymphocytes stained brightly relative to CD18^{dim} lymphocytes for adhesion molecules other than CD18, such as CD2R and CD49d (Fig. 3 *B*) as well as CD29 (data not shown). However, CD18^{bright} lymphocytes exhibited more variable expression of CD2 and CD28. Three-color staining demonstrated that the CD18^{bright} lymphocytes strongly expressing CD2 were T cells, whereas those with weaker expression of CD2 were NK cells. In addition, whereas CD18^{bright} and CD18^{dim} T cells expressed CD28 to a similar degree (Fig. 3 *B*), three-color staining showed that the CD18^{bright} lymphocytes with weak CD28 expression were NK cells (data not shown).

As was observed for patients receiving IL-12, IL-12Rβ1 expression was stronger on CD18^{bright} T and NK cells compared to CD18^{dim} T cells in normal subjects (Fig. 3 *C*). Because IL-2 can augment the functional response of T and NK cells to IL-12, we also examined IL-2 receptor expression on peripheral blood lymphocytes. As shown in Fig. 3 *C*, both the β and γ chains of the IL-2 receptor were expressed to a greater extent on CD18^{bright} T and NK cells compared to CD18^{dim} T cells, whereas IL-2Rα was only weakly expressed to a similar extent on CD18^{bright} and CD18^{dim} lymphocytes.

CD8$^+$CD18^{bright} T cells morphologically resemble NK cells. To obtain pure populations of CD8$^+$CD18^{bright} and CD8$^+$ CD18^{dim} T cells, preparations of CD8-enriched lymphocytes from normal subjects were stained with CD8 and CD18 antibodies and sorted into two populations using fluorescence acti-

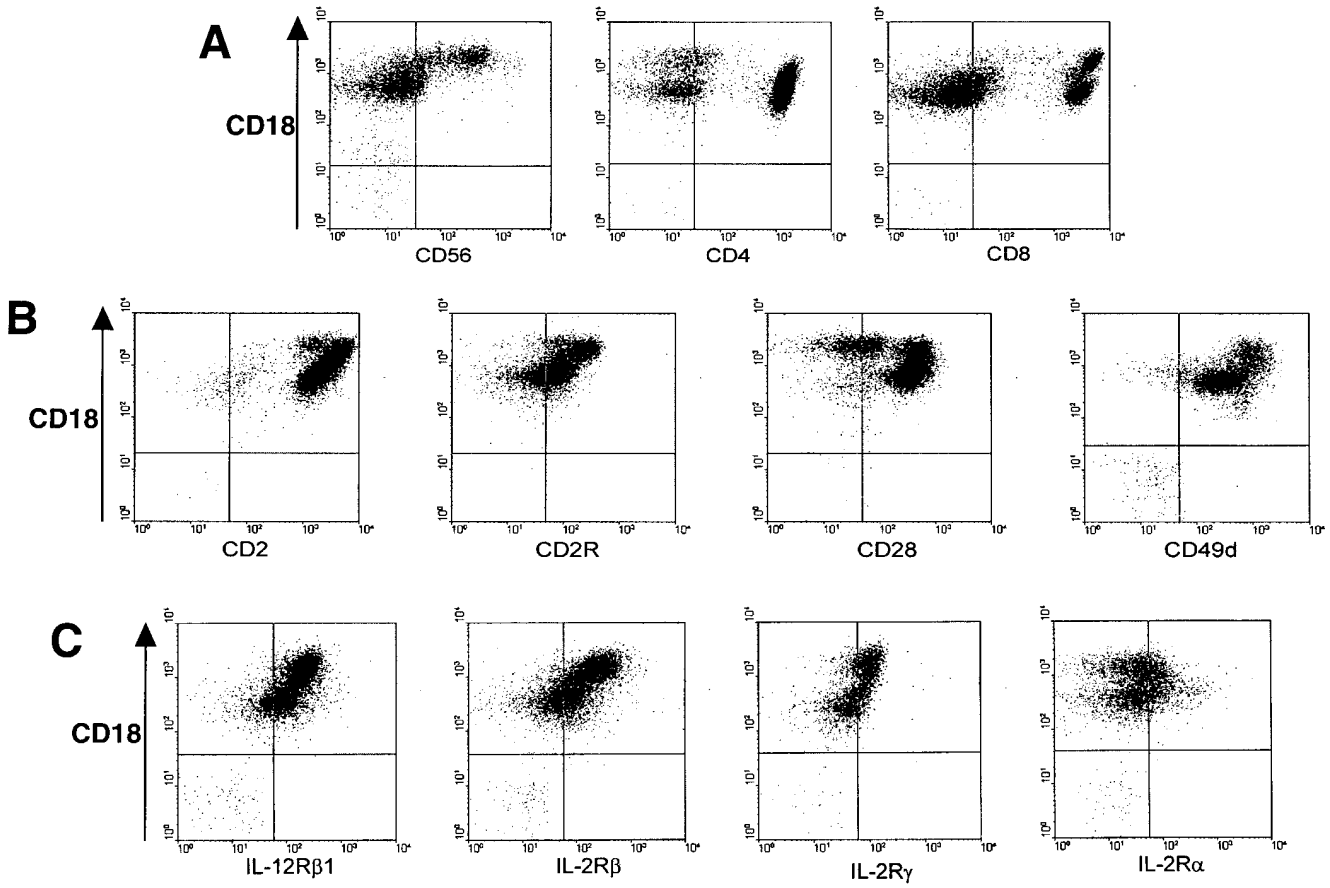

Figure 3. Adhesion molecules and receptor subunits for IL-12 and IL-2 are differentially expressed on $CD18^{bright}$ and $CD18^{dim}$ lymphocytes. PBMC were isolated from blood samples obtained from normal subjects and stained with CD18-FITC (*y-axis*) and lineage-specific NK or T cell markers (*A*), antibodies to adhesion molecules (*B*), or antibodies specific for the β1 subunit of the IL-12 receptor or the α, β, or γ subunits of the IL-2 receptor (*C*) conjugated with PE (*x-axis*). Results are representative of six separate experiments using blood from different volunteer donors.

vated cell sorting (Fig. 4 *A*). The CD18 antibody used for cell sorting does not activate resting lymphocytes and neither augments nor inhibits the functional response of mitogen-activated T cells to IL-12 or IL-2 (28). Sorted $CD18^{bright}$ and $CD18^{dim}$ T cells were > 95% pure and differed five- to sixfold in their CD18 mean fluorescence intensity.

To examine these cell populations morphologically, cytospin preparations of sorted $CD18^{bright}$ and $CD18^{dim}$ $CD8^+$ T cells were made immediately after sorting and stained with Wright-Giemsa. $CD8^+CD18^{dim}$ cells appeared morphologically as resting T cells, exhibiting scant cytoplasm without granules and round, homogeneous nuclei with dense chromatin (Fig. 4 *B*). In contrast, $CD8^+CD18^{bright}$ T cells were larger cells with abundant cytoplasm, prominent azurophilic cytoplasmic granules, and large pleomorphic nuclei with open chromatin (Fig. 4 *C*). Morphologically, $CD8^+CD18^{bright}$ lymphocytes closely resembled $CD56^+CD16^+$ NK cells. However, sorted $CD8^+CD18^{bright}$ lymphocytes were $CD3^+TCR\alpha\beta^+$ $CD56^-$ (data not shown), demonstrating that these lymphocytes were T cells rather than NK or NKT cells.

The TCR Vβ repertoire is oligoclonal in $CD8^+CD18^{bright}$ T cells but polyclonal in $CD8^+CD18^{dim}$ T cells. To determine whether $CD8^+CD18^{bright}$ T cells were comprised of a clonal or oligoclonal T cell population distinct from $CD18^{dim}$ T cells, we analyzed the Vβ repertoire of sorted $CD8^+CD18^{bright}$ and $CD8^+CD18^{dim}$ T cells. First, we examined the size spectrum of the CDR3 region for each Vβ subfamily to determine whether there was an indication of clonal expansion within a given Vβ subfamily. In T cells from normal subjects, the analysis of CDR3 size for each Vβ gene should show a Gaussian distribution of six to eight peaks, indicating the predominance of polyclonal T cells (32). As shown in Fig. 5, all 24 Vβ gene subfamilies were represented in both the $CD18^{bright}$ and $CD18^{dim}$ populations. Whereas the $CD18^{dim}$ Vβ subfamilies were all polyclonal (Fig. 5 *A*), the $CD18^{bright}$ T cells were notable for having a large number of Vβ subfamilies with either an oligoclonal or clonal pattern (Fig. 5 *B*). This finding was consistently observed in all of the individuals whose cells were analyzed. However, the Vβ subfamilies that were oligoclonal or clonal varied among the different normal subjects tested, with no discernible pattern.

In addition to finding abnormal Vβ patterns among $CD18^{bright}$ cells that suggested clonal predominance within Vβ subfamilies, we also found that there was a much more uneven distribution of Vβ subfamily usage among $CD18^{bright}$ cells compared with $CD18^{dim}$ cells (Fig. 6). In some cases this was strik-

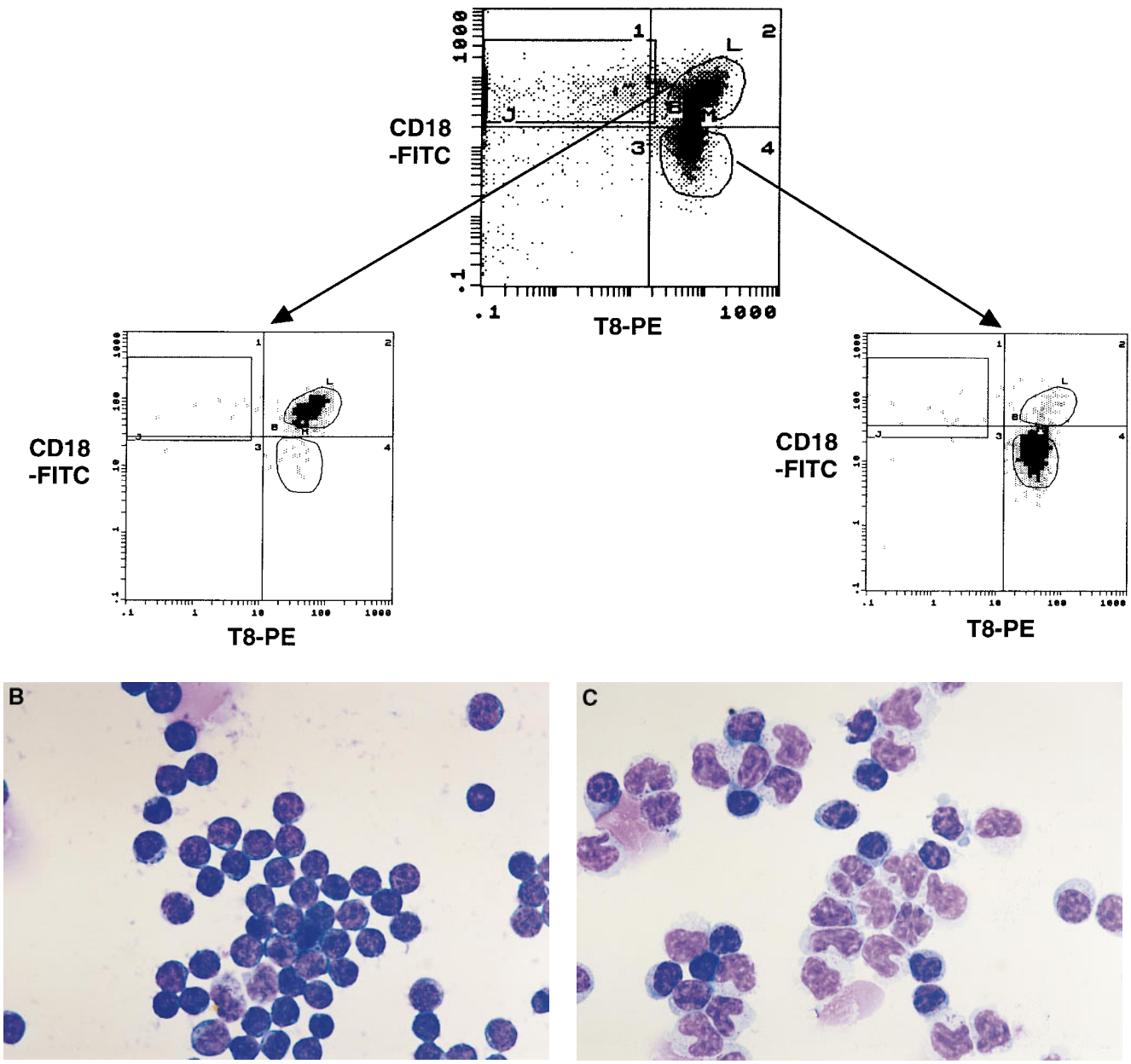

Figure 4. $CD8^+CD18^{bright}$ T cells morphologically resemble NK cells. (*A*) Among $CD8^+$ T cells, the $CD18^{bright}$ and $CD18^{dim}$ subsets were isolated separately through fluorescence-activated cell sorting. $CD8^+$ T cell–enriched peripheral blood lymphocytes were obtained by depleting PBMC of monocytes, NK cells, $CD4^+$ T cells, and B cells through passage of the cells over nylon wool columns followed by treatment with antibodies recognizing lineage-specific markers and magnetic beads coated with anti-immunoglobulin antibodies. CD8-enriched T cells were then stained with CD18-FITC and CD8-PE and sorted based on CD18-FITC fluorescence intensity. Sorted cell populations were 95–98% pure. (*B* and *C*) Cytospin preparations were made from sorted $CD18^{dim}$ (*B*) and $CD18^{bright}$ (*C*) T cells and stained with Wright-Giemsa. Stained cells were viewed under a light microscope at 100× magnification. Identical results were obtained in two separate experiments.

ing, with a single Vβ subfamily comprising 25–50% of the total $CD8^+CD18^{bright}$ T cells (Fig. 6). In contrast, each Vβ subfamily usually comprised no more than 3–8% of the total cells within the $CD18^{dim}$ subset.

$CD8^+CD18^{bright}$ T cells proliferate and produce IFN-γ in response to the combination of IL-12+IL-2, whereas $CD8^+$ $CD18^{dim}$ T cells do not. The in vivo expansion of $CD8^+$ $CD18^{bright}$ T cells in patients receiving subcutaneous injections

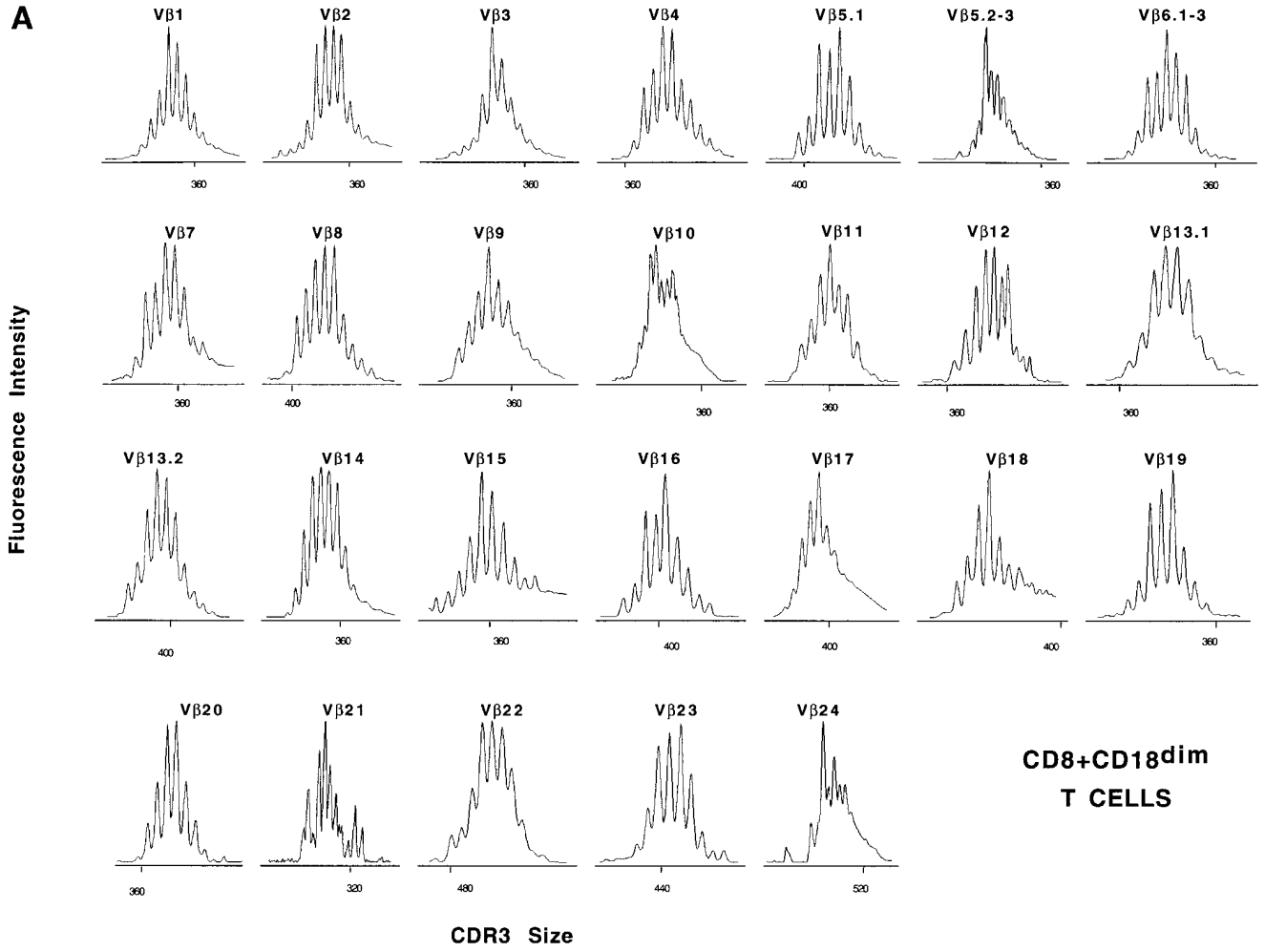

Figure 5. $CD8^+CD18^{bright}$ T cells exhibit oligoclonal and clonal Vβ subfamily patterns, whereas $CD18^{dim}$ T cells exhibit polyclonal Vβ patterns. CDR3 size patterns for 24 Vβ gene subfamilies are displayed for sorted $CD8^+CD18^{dim}$ (*A*) and $CD8^+CD18^{bright}$ (*B*) T cells. Results shown are from one normal donor, and are representative of results obtained from three different donors.

of IL-12, as well as the relatively strong expression of IL-12 and IL-2 receptors relative to $CD18^{dim}$ T cells, suggested that the in vitro responsiveness of $CD8^+CD18^{bright}$ T cells to IL-12 and IL-2 would be greater than $CD18^{dim}$ T cells. To test this hypothesis, sorted $CD18^{bright}$ and $CD18^{dim}$ $CD8^+$ T cells were stimulated with IL-12, IL-2, or the combination of IL-12+IL-2 in 96-h proliferation assays and 72-h IFN-γ assays. $CD8^+CD18^{bright}$ T cells did not proliferate or produce IFN-γ in response to any concentration of IL-12 alone (Fig. 7 *A*). Although these cells also did not produce IFN-γ in response to IL-2 alone, they exhibited a modest proliferative response to high concentrations of IL-2 (Fig. 7 *A*). The limited functional responsiveness to 1 nM IL-2 was consistent with the observed expression of intermediate affinity (Kd = 1 nM) βγ IL-2 receptors on $CD8^+CD18^{bright}$ T cells (Fig. 3 *C*). $CD8^+CD18^{dim}$ T cells could neither proliferate nor produce IFN-γ in response to IL-12 or IL-2 alone (Fig. 7 *B*).

Previous studies have demonstrated that resting NK cells, while producing little IFN-γ in response to IL-12 or IL-2 alone, can produce large amounts of IFN-γ in response to the combination of IL-12+IL-2 (1). It has also been shown that among resting T cells, a small fraction can substantially increase IFN-γ mRNA synthesis in response to IL-12+IL-2 (1). Although $CD8^+CD18^{bright}$ T cells did not proliferate or produce IFN-γ in response to IL-12 alone, concentrations of IL-12 as low as 1–10 pM synergized with 1 nM IL-2 to greatly increase proliferation and IFN-γ production (Fig. 7 *A*). In contrast, $CD8^+CD18^{dim}$ T cells remained functionally unresponsive to the combination of IL-12+IL-2 (Fig. 7 *B*). $CD4^+CD18^{dim}$ T cells also did not respond to IL-12 and IL-2, alone or in combination (data not shown). Although $CD4^+CD18^{bright}$ T cells were few in number and comprised only a small fraction (< 5%) of all $CD4^+$ cells, they too could be isolated through cell sorting and exhibited the same functional response to IL-12 and IL-2 as $CD8^+CD18^{bright}$ T cells (data not shown).

IL-12 and IL-2 augment the cytolytic activity of $CD8^+$ $CD18^{bright}$ T cells. The generation of LAK activity from peripheral blood lymphocytes has been ascribed to the cytokine-induced stimulation of non–MHC-restricted cytolytic activity among circulating NK cells (33). Overnight incubation of NK cells in vitro with either IL-2 or IL-12 can augment their lysis of certain tumor cells (5). The in vitro stimulation of resting

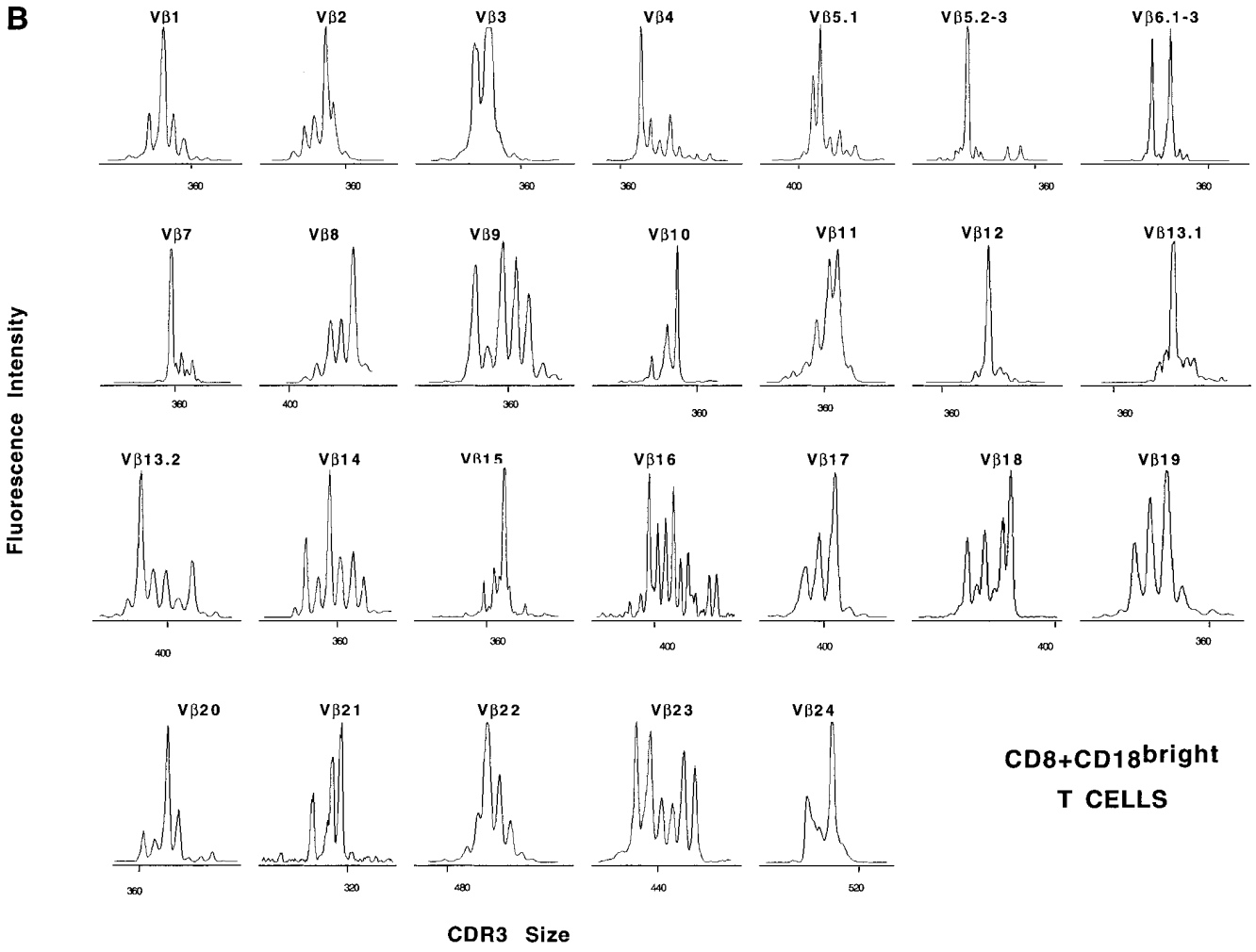

Figure 5 (Continued)

peripheral blood T cells with IL-12 or IL-2 has not been shown to generate non–MHC-restricted cytolytic activity. Because $CD8^+CD18^{bright}$ T cells appeared to differ from other circulating T cells in their ability to be activated by IL-12 and IL-2 and exhibited a striking morphologic, immunophenotypic, and functional resemblance to NK cells, we analyzed the ability of these cytokines to stimulate cytolytic activity by this T cell subset. To assess non–MHC-restricted killing, we measured the ability of $CD8^+CD18^{bright}$ T cells to kill the COLO cell line derived from a patient with colonic adenocarcinoma. Although freshly sorted $CD8^+CD18^{bright}$ T cells could not lyse COLO cells without in vitro activation, the killing of COLO cells was significantly augmented after an overnight incubation with either IL-12 or IL-2 alone (Fig. 8). Stimulation of $CD8^+CD18^{bright}$ T cells with the combination of IL-12+IL-2 further increased the killing of COLO cells in an additive manner. $CD8^+CD18^{dim}$ T cells, on the other hand, exhibited relatively little cytolytic activity against COLO, even when IL-12 and IL-2 were used together (Fig. 8). Neither $CD4^+CD18^{bright}$ nor $CD4^+CD18^{dim}$ T cells exhibited cytolytic activity against COLO in response to IL-12, IL-2, or the combination of IL-12+IL-2 (data not shown).

To assess CD3-mediated cytolytic activity, we measured the ability of $CD8^+CD18^{bright}$ T cells to kill the OKT3 hybridoma cell line (4). Fig. 8 demonstrates that CD3-triggered killing of OKT3 target cells by sorted $CD8^+CD18^{bright}$ effector cells was greater than that observed with $CD8^+CD18^{dim}$ cells. The further augmentation of CD3-triggered killing by IL-12 or IL-2 was also more substantial among $CD18^{bright}$ compared with $CD18^{dim}$ T cells, although stimulation of $CD18^{bright}$ T cells with IL-12+IL-2 did not have an additive effect on CD3-triggered killing. Although $CD4^+CD18^{dim}$ cells could not lyse OKT3 cells, even after overnight stimulation with IL-12 or IL-2, $CD4^+CD18^{bright}$ cells exhibited modest cytolytic activity against OKT3 target cells in response to IL-2 but not in response to IL-12 (data not shown).

Discussion

In this report, a subpopulation of $CD8^+$ T cells has been shown to expand in vivo in response to subcutaneously administered IL-12. Whereas previous studies have demonstrated that parenterally administered IL-2 can lead to NK cell expansion in cancer patients (34) or the modest expansion of $CD4^+$ T

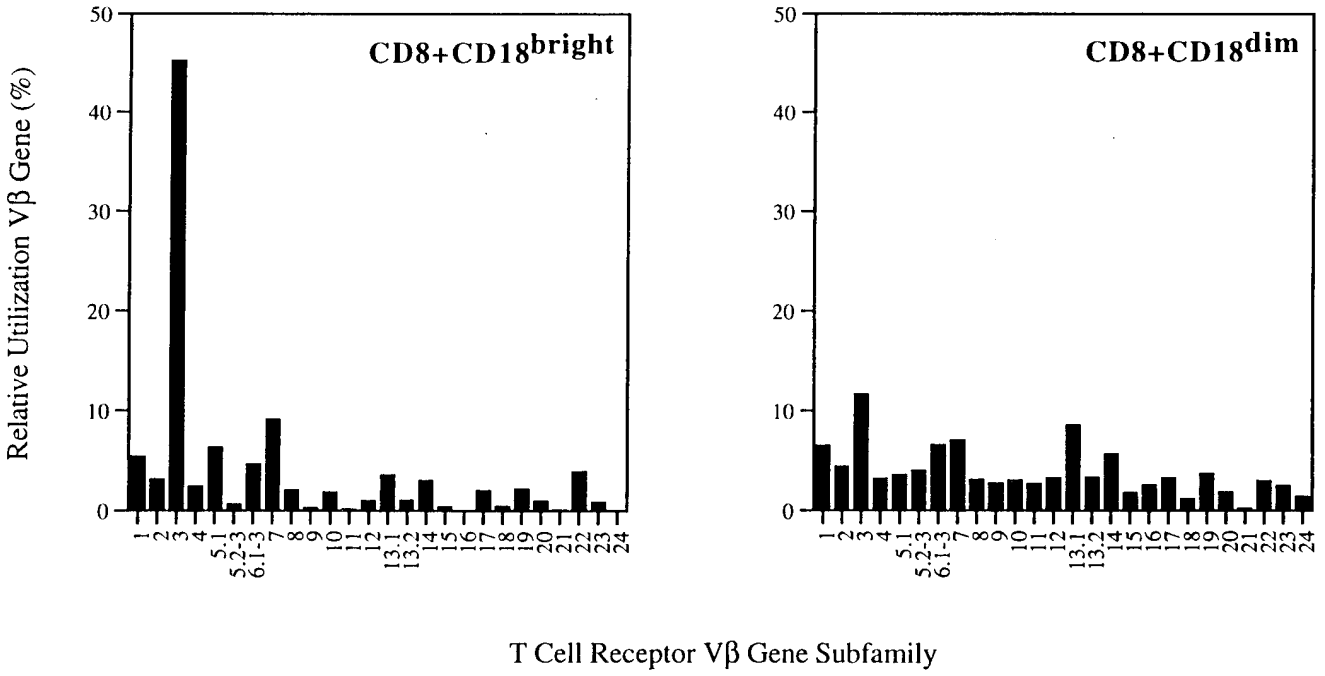

Figure 6. TCR Vβ gene representation in $CD8^+CD18^{bright}$ versus $CD8^+CD18^{dim}$ T cells. Use of 24 TCR Vβ gene subfamilies was evaluated in sorted $CD8^+CD18^{bright}$ and $CD8^+CD18^{dim}$ T cells. Results shown are from one donor, and are representative of results obtained from three different donors.

cells in patients infected with HIV (35, 36), this is the first study to show the in vivo expansion of $CD8^+$ T cells in humans in response to a cytokine.

In primates receiving doses of intravenous or subcutaneous IL-12 for five consecutive days, lymphopenia (including decreases in the number of $CD4^+$ and $CD8^+$ T cells) was observed by day 2 and was noted to resolve within a day of stopping the IL-12 (37). In humans receiving intravenous IL-12 on a similar schedule, transient lymphopenia was also observed 24 h after the first dose (13). In our study of patients with cancer receiving subcutaneous injections of IL-12, doses of IL-12 were given every other day (three times a week) for 2 wk instead of daily for 1 wk, and blood samples were drawn before administering the IL-12 dose at days 1 and 8 of a treatment cycle rather than 24–48 h after the start of dosing. Rather than observing lymphopenia, we noted the selective expansion of a $CD18^{bright}$ population of $CD8^+$ T cells that occurred by the second week of each cycle and usually diminished during the following week of rest before the start of the next IL-12 cycle. This cyclical expansion of $CD8^+CD18^{bright}$ T cells was accompanied by a parallel upregulation of IL-12Rβ1 expression (in the second week of a cycle, returning to baseline level during the week of rest). The strong, cyclical upregulation of IL-12Rβ1 was also seen on NK cells, but no NK cell expansion was detected. $CD18^{dim}$ T cells (including $CD8^+$ T cells and the majority of $CD4^+$ T cells) exhibited relatively weak IL-12Rβ1 upregulation and also did not increase in number. Whereas NK cells have been viewed as the primary lymphocyte population in the peripheral blood capable of responding to IL-2 or IL-12 without additional activation, our findings in patients receiving subcutaneous IL-12 suggest that $CD8^+CD18^{bright}$ T cells are a second lymphocyte population poised to respond to endogenous or exogenous Th1-type cytokines.

The immunophenotypic, morphologic, and functional analysis of $CD8^+CD18^{bright}$ T cells performed in this study demonstrates that this small population of circulating T cells bears a striking resemblance to NK cells. Like the majority of NK cells, $CD8^+CD18^{bright}$ T cells express intermediate affinity βγ IL-2 receptors as well as the β1 subunit of the IL-12 receptor, and stain brightly for LFA-1, CD2, and CD49d. This expression of cytokine receptors and adhesion molecules raises the possibility that $CD8^+CD18^{bright}$ T cells might, like NK cells, be capable of migrating to tumor sites and mediating non–MHC-restricted tumor cell lysis after activation by IL-2 or IL-12. $CD8^+CD18^{bright}$ T cells exhibit a modest proliferative response to IL-2 only at concentrations that are high enough to saturate their intermediate affinity IL-2 receptors, and demonstrate non–MHC-restricted cytolytic activity in response to IL-2 alone. However, they do not produce IFN-γ in response to IL-2 alone. Although $CD8^+CD18^{bright}$ T cells do not proliferate or produce IFN-γ in response to IL-12 alone, they do exhibit non–MHC-restricted cytolytic activity with IL-12 alone. Because it has been shown that expression of the IL-12Rβ1 subunit alone is not sufficient to mediate IL-12–induced signaling or functional responses (22, 38, 39), it is likely that $CD8^+CD18^{bright}$ T cells also express IL-12Rβ2 in order to form functional high affinity IL-12 receptors.

Although $CD8^+CD18^{bright}$ T cells demonstrated little functional responsiveness to IL-12 or IL-2 alone, the combination of IL-12+IL-2 synergistically augmented proliferation and IFN-γ production, while having an additive effect on non–MHC-restricted killing of tumor cells. NK cell activation with

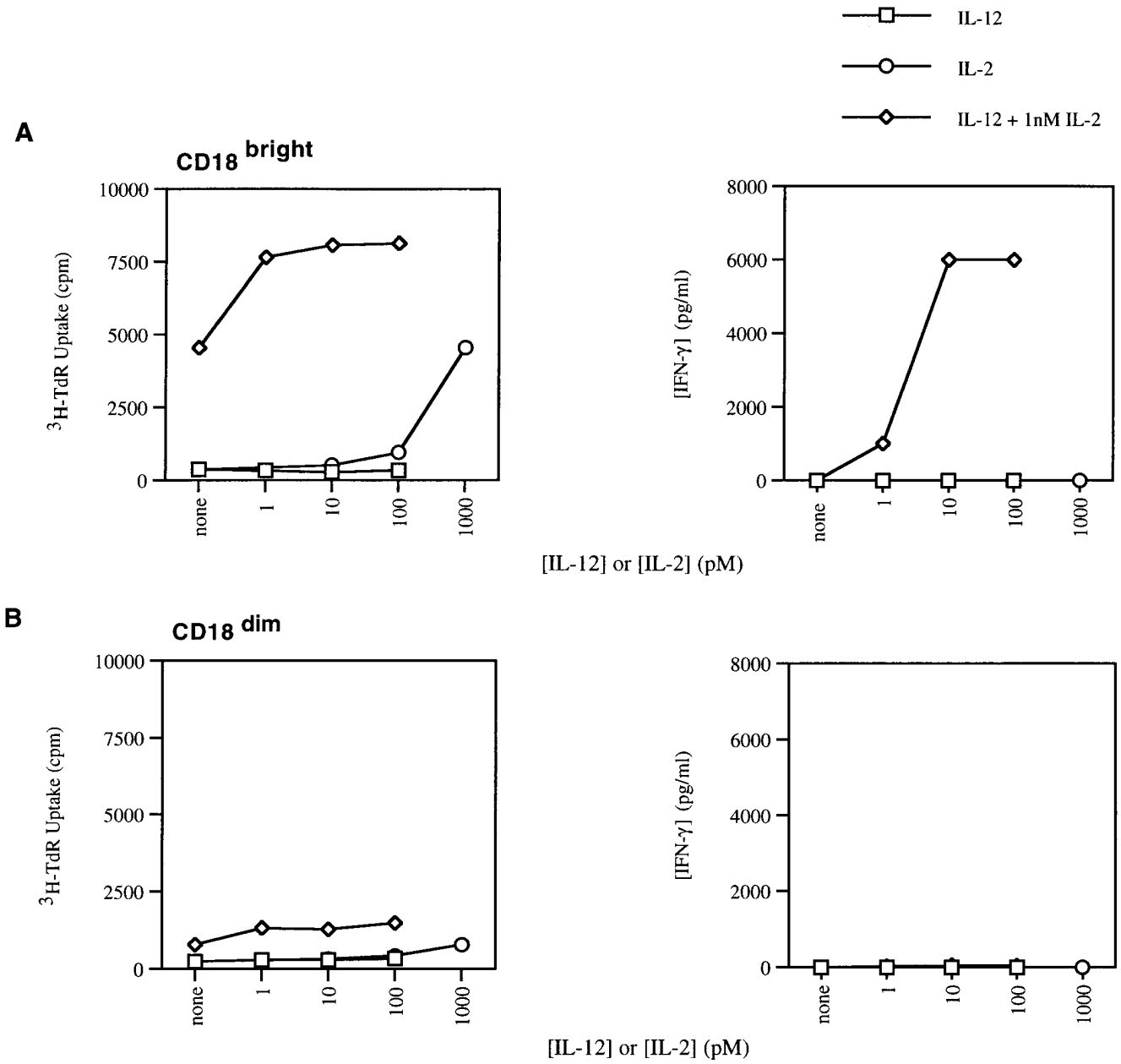

Figure 7. Differential functional response of $CD8^+CD18^{bright}$ and $CD8^+CD18^{dim}$ T cells to IL-12, IL-2, and IL-12+IL-2. Sorted $CD8^+CD18^{bright}$ (*A*) and $CD8^+CD18^{dim}$ (*B*) T cells were incubated with medium alone or the indicated concentrations of IL-12, IL-2, or IL-12+IL-2 in a 96-h proliferation assay or 72-h IFN-γ assay as described in Methods. Similar results were obtained in six separate experiments using cells from different volunteer donors.

IL-12+IL-2 has similar effects on IFN-γ production (1) and cytolytic activity (5). The observation that $CD8^+CD18^{bright}$ T cells only proliferate or produce IFN-γ in response to IL-12 in the presence of IL-2 suggests that signaling through the IL-12 receptor alone, though capable of stimulating modest cytolytic activity, is unable to mediate proliferation or IFN-γ production in these cells. Although IL-12 receptor expression is stronger on $CD18^{bright}$ compared with $CD18^{dim}$ T cells, it appears as though the differential expression of intermediate affinity IL-2 receptors on these T cell subsets also contributes significantly to the control of IL-12 responsiveness.

The functional synergy between IL-12 and IL-2 in $CD8^+CD18^{bright}$ T cells does not appear to be due to changes in the expression of IL-12 or IL-2 receptors. When $CD8^+CD18^{bright}$ T cells were cultured in IL-12 for up to 96 h, no increase in IL-2 receptor expression was observed (Gollob, J.A., and J. Ritz, unpublished observations). Likewise, when cells were cultured in IL-2, we did not observe any increase in IL-12Rβ1 expression. As IL-12Rβ2 antibodies are not currently available, we cannot exclude the possibility that IL-2 may upregulate IL-12Rβ2 expression on $CD8^+CD18^{bright}$ cells during a 72–96 h incubation with IL-12+IL-2, and that this may contribute to the

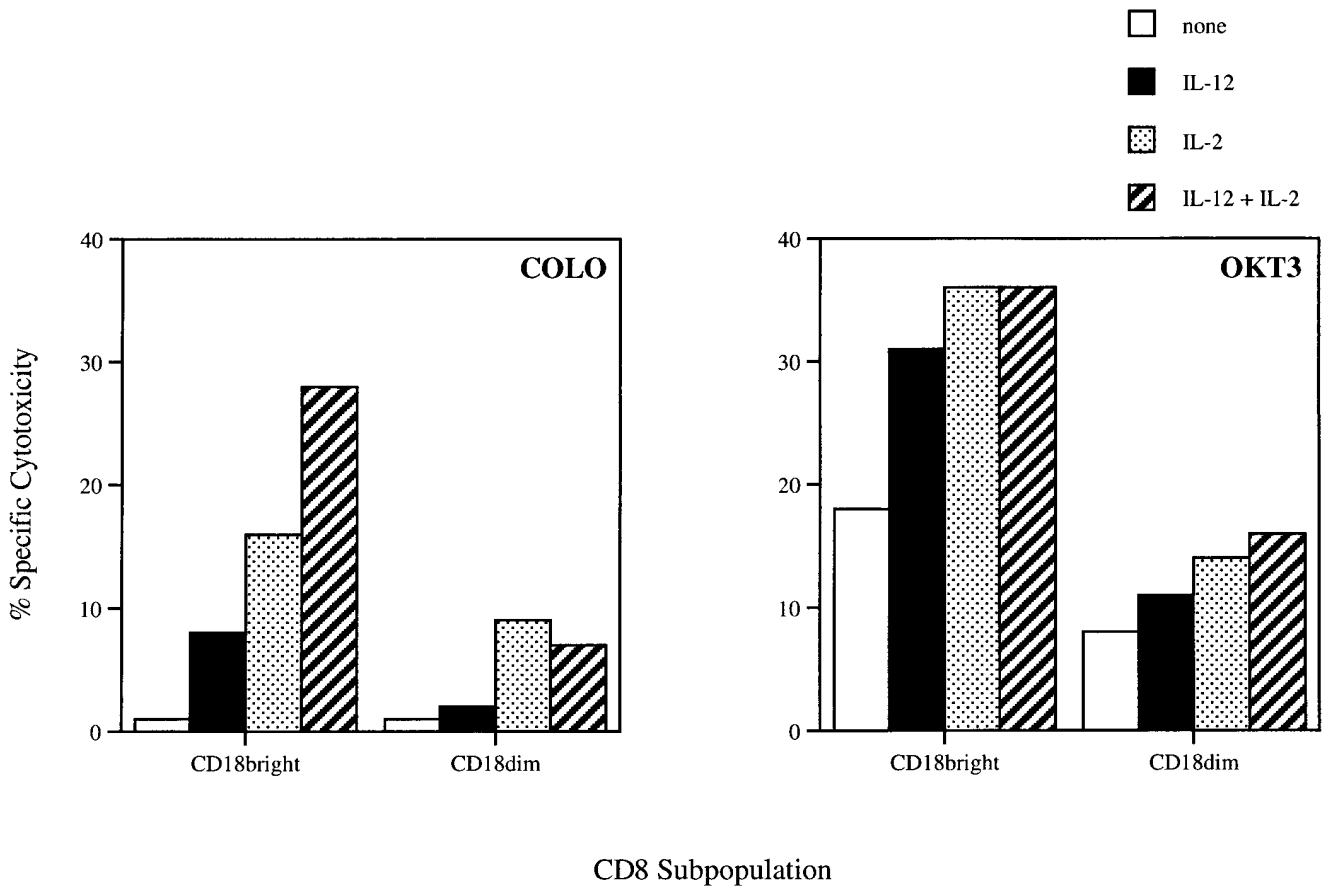

Figure 8. The cytolytic activity of $CD8^+CD18^{bright}$ T cells is augmented by IL-12 and IL-2. Sorted $CD8^+CD18^{bright}$ and $CD8^+CD18^{dim}$ T cells were incubated overnight with either medium alone, 1 nM IL-12, 1 nM IL-2, or IL-12+IL-2. ^{51}Cr-labeled COLO or OKT3 target cells were then added at a 5:1 effector:target ratio and a 4-h cytotoxicity assay was performed as described in Methods. Results are representative of four separate experiments.

functional synergy between IL-12 and IL-2. However, our analysis of signaling in sorted $CD18^{bright}$ T cells stimulated for only 15 min with IL-12, IL-2, or IL-12+IL-2 showed strong synergy between IL-12 and IL-2 at the level of p38 MAP kinase activation and STAT1 and STAT3 serine phosphorylation (Gollob, J.A., C.P. Schnipper, E.A. Murphy, J. Ritz, and D.A. Frank, manuscript submitted for publication). Furthermore, a specific inhibitor of p38 MAP kinase blocked the functional synergy between IL-12 and IL-2. These findings suggested that the functional synergy between IL-12 and IL-2 is occurring through the modulation of signaling at a point that is distal to IL-12 or IL-2 receptor expression.

The $CD18^{bright}$ T cells described in this report share phenotypic and functional similarities with T cells described in previous studies as memory T cells and effector T cells (24, 40–42). Like memory and effector T cells, $CD18^{bright}$ cells are capable of significant IFN-γ production and proliferation when provided with the appropriate stimuli. The strong expression of adhesion molecules such as CD2, CD11a, CD18, CD29, and CD49d is also characteristic of both memory and effector cells. The NK cell-like morphology and the ability to mediate CD3-directed cytotoxicity without pre-activation are more characteristic, however, of an effector rather than a memory T cell. Our analysis of the $CD8^+CD18^{bright}$ TCR Vβ repertoire provides evidence that this is an oligoclonal population of T cells, whereas the $CD4^+$ and $CD8^+$ cells that are $CD18^{dim}$ are polyclonal with Vβ subfamilies always evenly represented. Based on these findings, we believe that $CD8^+CD18^{bright}$ T cells are probably T cells comprised of effector and memory cells that have previously encountered antigen and have undergone selective clonal expansion in vivo. In contrast, $CD18^{dim}$ T cells appear to be naive, quiescent cells that have not undergone selective clonal expansion. We have shown that the effector function of $CD8^+CD18^{bright}$ T cells can be elicited in a TCR-independent manner through combined stimulation with IL-12+IL-2. It remains to be determined whether these T cells can also exhibit rapid recall responses to distinct antigens that would be consistent with a memory function as well.

Because $CD8^+CD18^{bright}$ T cells are capable of TCR-independent activation through stimulation with IL-12+IL-2, as well as activation through the TCR by antigen presented in the context of MHC class I molecules, what is their role in the immune response to neoplastic cells or infectious pathogens? NK cells are considered to be the primary effector cell in early, innate immunity to pathogens by virtue of their responsiveness to cytokines, production of IFN-γ and TNF, and ability to

mediate non–MHC-restricted cell lysis. However, although $CD8^+CD18^{bright}$ T cells have probably been previously activated through the TCR by specific antigens and are likely to be an important component of acquired immune responses when rechallenged with antigen, they may also play a key role in early, innate immunity that is not antigen specific. Both neutrophils and antigen-presenting cells are capable of producing IL-12 when pathogens are first encountered, but IL-12 by itself is not capable of mediating significant NK cell or T cell activation. However, antigen-presenting cells also produce IL-15, which like IL-2 activates T and NK cells through the IL-2Rβ and IL-2Rγ subunits (43, 44). Therefore, the early phase of a cellular immune response may consist of NK cell and $CD8^+CD18^{bright}$ T cell activation through combined stimulation with IL-12+IL-15, resulting in non–MHC-restricted killing and the production of Th1 cytokines such as IL-2 and IFN-γ. These cytokines would further enhance the innate immune response, and facilitate the formation of an acquired, TCR-dependent immune response.

Our observation that $CD8^+CD18^{bright}$ T cells upregulate IL-12 receptor expression and expand in patients receiving IL-12 suggests that they, along with NK cells, are being activated in vivo by IL-12. However, because our in vitro analysis demonstrated that IL-12 alone does not activate these $CD8^+$ T cells, it is likely that, in vivo, IL-12 is also inducing the production of IL-15 and/or IL-2, either of which could then activate $CD8^+CD18^{bright}$ T cells in conjunction with IL-12. Although the in vivo upregulation of IL-12 receptor expression on NK cells in response to IL-12 administration is an indication that they too are being activated along with $CD8^+CD18^{bright}$ T cells, only the $CD8^+$ T cells were observed to expand. If, as our in vitro data suggest, the proliferation of $CD8^+CD18^{bright}$ T cells is due to combined stimulation with exogenous IL-12 and endogenous IL-2 and/or IL-15, the lack of NK cell proliferation in vivo would be consistent with the in vitro response of NK cells to stimulation with IL-12+IL-2. Previous studies have shown that stimulation with IL-12+IL-2 augments both T and NK cell IFN-γ production. However, although stimulation with IL-12+IL-2 enhances T cell proliferation, the addition of IL-12 inhibits IL-2–induced NK cell proliferation (5).

Regarding the expansion of $CD8^+CD18^{bright}$ T cells in our patients receiving IL-12, we do not yet know whether this represents a polyclonal or oligoclonal/clonal expansion. Future studies with additional patients will be required to determine this. It is also important to consider that the expansion of $CD8^+CD18^{bright}$ T cells in patients treated with IL-12 may not be solely due to the proliferative effect of cytokines. For example, IL-12 alone or IL-12 in conjunction with IL-15 or IL-2 may also prevent activation-induced apoptosis in this $CD8^+$ T cell population. It will be important to determine whether these $CD8^+$ T cells are mediating the antitumor effect of IL-12, and if so, whether the antitumor effect is antigen-specific or nonspecific. Regardless of whether they mediate the killing of tumor cells through a TCR-dependent mechanism or through 'natural' killing that is non–MHC-restricted, the in vivo activation of $CD8^+CD18^{bright}$ T cells with combination cytokine therapy using IL-12+IL-2 or IL-12+IL-15 might be an effective way to expand these effector/memory cells in cancer patients with or without pre-existing antitumor responses or in conjunction with tumor vaccines. Through this approach, it might be possible to generate more clinically effective immune-based cancer therapies.

Acknowledgments

This paper is dedicated to the memory of Samuel J. Kowal, M.D.

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