FUNCTIONAL CONSEQUENCES OF INTERLEUKIN 2 RECEPTOR EXPRESSION ON RESTING HUMAN LYMPHOCYTES

Identification of a Novel Natural Killer Cell Subset with High Affinity Receptors

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In the initial characterization of the role played by IL-2 during immune responses many investigators questioned how an antigen-nonspecific lymphocytotrophic hormone could promote an antigen-dependent clonal expansion of T cells. However, when IL-2 receptors were found to be expressed primarily following antigen activation, it was immediately apparent that the T cell antigen receptor complex determines which clones become responsive to IL-2 (reviewed in reference 1). In contrast, NK cells respond to IL-2 without any prior antigenic stimuli, as evidenced by an increased capacity to lyse a variety of target cells (2–4). NK cells are thought to be responsible for much of the therapeutic efficacy of lymphokine-activated killer cells (LAK),[1] currently being used for cancer treatment (5, 6). In addition, some cells in freshly isolated PBL populations may also be capable of proliferating in response to IL-2, even in the absence of exogenous antigen activation (7). However, precisely what proportion of circulating cells are IL-2 responsive, and which cell types among the mixture of T cells, B cells, and NK cells actually account for this IL-2 reactivity have remained obscure.

It is now accepted that the IL-2 receptor is comprised of at least two chains, one 75-kD (p75) and another 55 kD (p55) (8–12). Each chain can be expressed in its isolated form or noncovalently combined as a heterodimer (1). The cDNA encoding these proteins have been cloned and sequenced, revealing that both chains have extracellular domains of ~215 residues. In addition, the p75 chain has a large in-

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tracytoplasmic domain (286 residues), while the p55 chain has only a small (13 residues) cytoplasmic anchor (13–16). This structural information is consistent with functional data, which indicate that the p75 chain participates in signal transduction in both its isolated and heterodimeric form, whereas the p55 chain only serves to facilitate IL-2 binding. When expressed together on the cell surface, the p75 chain and the p55 chain associate noncovalently to form the high affinity IL-2 receptor (K_d = 10^{-11} M). By comparison, when constitutively expressed in its isolated form, the p75 chain binds IL-2 with intermediate affinity (K_d = 10^{-9} M), while the nonfunctional isolated p55 chain binds IL-2 with low affinity (K_d = 10^{-8} M) (17).

Elucidation of the structure of the IL-2 receptor has been facilitated by the development of various mAbs directed against the receptor itself. Anti-p55 mAb (CD25) block the binding of IL-2 to the high affinity IL-2 receptor by disrupting the noncovalently bound heterodimer, thereby creating isolated p75 chains to which IL-2 binds with intermediate affinity (17). Recently, Takeshita et al. isolated a mAb (TU27) which competitively inhibits radiolabeled IL-2 binding, both to the high affinity IL-2 receptor and to the isolated intermediate affinity p75 chain (18). Tsudo et al. have also developed mAbs reactive with the p75 chain both in its isolated intermediate affinity and heterodimeric high affinity form (19), and in collaboration with Hatakeyama et al. these antibodies were used to isolate and clone cDNA encoding the p75 chain (16).

With the availability of mAbs reactive with both components of the IL-2 receptor, and cDNA encoding both receptor chains, we examined the expression of the different IL-2 receptor chains on purified lymphocyte populations in unstimulated peripheral blood. Our results, detailed in this report, indicate that most T cells (>98%) express neither the high affinity IL-2 receptor nor the functional intermediate affinity p75 chain of the IL-2 receptor without prior activation. In contrast, most resting NK cells, which make up ~10% of PBMC, express the isolated intermediate affinity p75 IL-2 receptor. In addition, a subpopulation of NK cells, distinguished by a high density expression of the NKH1 molecule, constitutively express both the p75 and p55 chain, thus forming the high affinity IL-2 receptor. These cells exhibit a brisk proliferative response to IL-2, similar to that seen with antigen-activated T cells, yet do so in the absence of any known antigenic stimuli.

Materials and Methods

^{125}I-IL-2 Binding Studies. Binding of radioiodinated IL-2 was performed as described previously (17), except that 10^7 cells/tube were used for unactivated PBMC, whereas 10^6 cells/tube were used for cells activated with anti-CD3 for 72 h. Radioiodinated IL-2 was prepared with two different specific activities (10^6 cpm/pmol and 10^5 cpm/pmol) so that binding assays could be carried out at IL-2 concentrations ranging from 1 pM to 100 nM. The number of intermediate affinity p75 IL-2 receptor binding sites was determined by performing the ^{125}I-IL-2 binding assay in the presence of a saturating concentration of anti-p55 as described previously (17).

Isolation and Activation of Cell Populations for Northern Blot Analysis. PBMC were obtained from the blood of normal individuals by Ficoll/Hypaque centrifugation and cultured at 10^6 cells/ml in RPMI 1640 medium containing 10% FCS supplemented with glutamine and antibiotics. PBMC were then activated with soluble anti-CD3 (OKT3; Ortho Diagnostic Systems, Raritan, NJ) at a 1:10,000 dilution. An enriched T cell population was obtained from PBMC using a modification of the method of Theile and Lipsky (20) to remove macrophages and NK cells, as well as positive selection with anti-CD3 to retain T cells. PBMC were resuspended

at 5 × 10^6 cells/ml in serum free medium containing 5 mM L-leucine methyl ester hydrochloride (Sigma Chemical Co., St. Louis, MO) and incubated at 22°C for 60 min. This procedure selectively lyses monocytes and NK cells (20). Cells were washed twice with serum-containing medium, then incubated with 5 μg/ml anti-CD3 mAb (64.1; a generous gift of Dr. E. Vitetta, University of Texas Health Science Center at Dallas) for 60 min at 4°C. Unbound antibody was removed by spinning cells over a layer of FCS. Cells were incubated on goat anti-mouse Fc-coated petri dishes for 60 min at 4°C. Unbound cells were removed by gentle aspiration and washed 3× with cold media. Adherent T cells were immediately harvested for t = 0 RNA isolation, and adhered aliquots were subsequently incubated at 37°C for 24 h to initiate T cell activation.

RNA Isolation and Quantitation. Total cellular RNA was isolated from cells by a modification of the method of Ausubel et al. (21). Cells were washed with normal saline and suspended in 7.5 ml cold lysis buffer: 4 M guanidine thiocyanate, 20 mM sodium acetate (pH 5.2), 150 mM 2-β-mercaptoethanol, and 0.4% Sarkosyl. The lysate was sheared through a syringe containing an 18 g needle and layered over 2.5 ml of a 5.7 M cesium chloride and 25 mM sodium acetate (pH 5.2) solution. RNA was pelleted at 160,000 *g* at 20°C for 20 h. The RNA pellet was resuspended in water and ethanol, precipitated overnight at −20°C, pelleted by centrifugation, and resuspended in water. Total RNA was quantified by measuring A260/280.

Hybridization Analysis. 15 μg total RNA was denatured with formaldehyde (10 m 70°C), fractionated by electrophoresis through 1% agarose gels containing 2.2 M formaldehyde and visualized by ethidium bromide staining to assess the integrity of the RNA and to verify that equal amounts of RNA were loaded in all wells. The RNA was transferred to nitrocellulose filters by capillary transfer in 10× SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0). The filters were vacuum baked at 80°C for 90 min. Prehybridizations and hybridizations were carried out for 22 and 72 h, respectively, at 42°C in a solution containing 50% formamide, 5× SSC, 5× Denhardt's solution, 0.2% SDS, and 100 μg/ml of heat denatured sheared salmon sperm DNA. DNA fragments were ^{32}P labeled by the oligonucleotide primed elongation method (22) and 1.5 × 10^6 cpm of heat denatured ^{32}P-labeled probes (specific activity 10^9 cpm/μg DNA) were added per milliliter of hybridization solution. Filters were washed 10 min at room temperature with 1× SSC and 0.1% SDS, 30 min at 42°C with 0.1 SSC and 0.1% SDS and 30 min at 60°C with 0.2× SSC and 0.1% SDS, air dried, and exposed to Kodak XAR-5 film using Dupont Cronex intensifying screens. The autoradiographs were scanned with an EC densitometer (EC Apparatus Corp., St. Petersburg, FL) using a Hewlett Packard 3390 integrator (Palo Alto, CA). The p75 cDNA probe was removed from the nitrocellulose filter by treatment with 0.1× SSC and 0.1% SDS at 100°C for 20 min before subsequent p55 hybridization. Plasmid pBSβ2 containing a full-length human p75 chain cDNA was obtained from Dr. Warren Leonard (National Institutes of Health, Bethesda, MD). pBSβ2 was cleaved with Eco RI to generate a 1.8-kb fragment that was purified from agarose gels. Plasmid pKCR Tac-2a, containing a full-length human p55 chain cDNA was obtained from Dr. Tasaku Honjo (Kyoto University, Kyoto, Japan), and was cleaved with Hind III to generate a 2.9-kb fragment.

Monoclonal Antibodies (mAb). NKH1 (anti-CD56), B1 (anti-CD20), IL-2R (anti-CD25), T1 (anti-CD5), T4 (anti-CD4), and T8 (anti-CD8) are commercially available from Coulter Immunology (Hialeah, FL). TU 27-FITC (anti-p75) was the generous gift of Dr. Kazuo Sugamura (18, 23). T12 is a pan-T cell antibody (anti-CD6) from our laboratory previously described (24).

Immunofluorescence Studies. PBL were obtained from fresh blood after Ficoll-Hypaque separation and adherence to plastic for 1 h at 37°C. PBL were 82% ± 1.9 $CD3^+$ for six individuals. For T cell phenotypic analysis, cells were then washed twice and stained with an anti-CD3 mAb directly conjugated to phycoerythrin (CD3-RD1) and TU-27-FITC (anti-p75) or CD3-RD1 and IL-2R-FITC (anti-p55). Similar staining was performed with CD5-RD1 instead of CD3-RD1 with identical results (not shown). To obtain similar numbers of NK cells, nonadherent PBL were first depleted of mature T cells using anti-CD6 mAb coupled to immunomagnetic beads (Coulter Immunology), and then stained with NKH1-RD1 and sorted for $NKH1^+$ cells on an EPICS 752 flow cytometer (Coulter Electronics, Hialeah, FL). Sorted cells, which were 92% ± 9.2 $NKH1^+$ for five individuals, were then stained with either TU-27-FITC (anti-p75) or IL-2R-FITC (anti-p55). For phenotypic studies performed

on $CD4^+$ T cells, freshly isolated PBMC were depleted of monocytes by adherence for 1 h at 37°C and then 10^6 cells were labeled with T4-RD1 and IL-2R-FITC or with T4-RD1 and TU27-FITC. 10^4 $CD4^+$ cells were then analyzed for their reactivity with IL-2R-FITC and TU27-FITC. The same procedure was repeated for $CD8^+$ T cells using T8-RD1, except that these cells were first depleted of $NKH1^+$ cells (which can also express CD8) using NKH1 coupled to magnetic beads (Dynabeads, Fort Lee, NJ). Background immunofluorescence using nonreactive directly conjugated mAbs Mslg-FITC and Mslg-RD1 was <1% for all analyses. Populations of $NKH1^{bright+}$ and $NKH1^{dim+}$ cells were separated on the basis of visually defined boundaries of staining intensity as seen in Fig. 5 and as previously described (25). Variation between five different donors was minimal. Since $CD6^+$ T cells were depleted by immunomagnetic beads before immunofluorescent cell sorting of $NKH1^+$ cells, purified NK populations did not contain cells that coexpressed CD3.

Proliferation Assays. Sorted populations of lymphocytes were plated shortly after their isolation from resting human peripheral blood in U-bottomed wells at a concentration of 15–25 × 10^3 cells/well in RPMI 1640 with 18% human AB serum in the presence of varying concentrations of rIL-2 (sp act 1.5 × 10^7 U/mg protein; Hoffmann-La Roche, Inc., Nutley, NJ). Cells were incubated at 37°C for either 4 or 7 d as specified below and proliferation was measured in triplicate by methyl-[^{3}H]thymidine incorporation. Anti-p55 (26) was used as sterile mouse ascites in blocking studies at a 1:400 dilution. Ascites from a nonreactive murine hybridoma was used as a control in blocking studies at a 1:400 dilution. Each mAb was added 30 min before the addition of rIL-2.

Cytotoxicity Assays. Sorted $NKH1^{bright+}$ and $NKH1^{dim+}$ cells were plated in V-bottomed wells in RPMI 1640 with 5% FCS at a concentration of 4 × 10^3 cells/well. Assays were then placed in media alone or media supplemented with 10 pM or 10 nM rIL-2 and incubated at 37°C for 18 h, at which time 4 × 10^3 ^{51}Cr-labeled K562 target cells were added to each well. Plates were then centrifuged and incubated for an additional 4 h at 37°C. Chromium release assays were then performed as described previously (27).

Results

IL-2 Binding to Resting and Activated PBMC. To characterize the unstimulated peripheral blood cells capable of responding to IL-2, we first assayed resting freshly isolated PBMC for IL-2 binding sites after optimizing the sensitivity of the radiolabeled IL-2 binding assay to detect small numbers of binding sites. Results in Table I show that freshly isolated PBMC express very low levels of all three classes of IL-2 binding sites, including the high affinity IL-2 receptor, intermediate affinity p75 chains, and isolated low affinity p55 chains. The number of intermediate affinity sites was determined by radiolabeled IL-2 binding assays performed in the presence of anti-p55 mAb (17). It is therefore likely that at least some of the p75 chains detected are, if fact, expressed as part of the high affinity heterodimer. However, when compared with the number of high affinity IL-2 receptor sites, a small but significantly greater number of isolated intermediate affinity p75 sites was detected, suggesting

TABLE I

IL-2R Binding Sites on Unstimulated and Stimulated Human PBMC

Activation signal	High affinity (p75/p55)	Intermediate affinity (p75)	Low affinity (p55)
None	53 ± 5*	177 ± 40	1,080 ± 325
Anti-CD3	1,099 ± 135	1,452 ± 373	8,993 ± 1,802

* The values shown represent mean sites per cell ± SEM for 4 to 7 separate experiments.

that at least some p75 chains are expressed in their isolated form within the resting PBMC pool.

The interactions of various IL-2 receptors within the T cell population was further evaluated by performing similar binding assays after activation with anti-CD3. As shown in Table I, an eightfold increase in the number of both p55 sites and p75 sites occurs after CD3 activation. However, in contrast to resting PBMC, it is likely that almost all available p75 chains noncovalently bind with the excess p55 chains to form the high affinity IL-2 receptor after CD3 activation, as there is no statistical difference between the number of high affinity sites compared with the number of intermediate affinity p75 IL-2 receptor sites detected.

Expression of IL-2 Receptor mRNA in Resting and Activated PBMC. To assess the qualitative distribution of the different IL-2 receptors on the various cells within PBMC before and after activation, we next examined freshly isolated PBMC and purified resting T cells for their expression of p75 and p55 IL-2 receptor mRNA using Northern blot analysis. Previous work from other laboratories suggested that resting T cells constitutively express the p75 chain of the IL-2 receptor, while only the p55 chain is actively synthesized after antigen activation to form the high affinity IL-2 receptor (28). As can be seen in Fig. 1 *A*, unstimulated PBMC contained low but significant amounts of p75 mRNA. However, after depletion of both monocytes and NK cells, p75 mRNA transcripts were barely detectable, indicating that the majority of p75 mRNA is expressed within the non-T cell compartment of resting PBMC. After activation of either PBMC or purified T cells with anti-CD3, there is a marked increase in the level of expression of p75 mRNA within 24 h, corresponding to similar increases in the surface expression of the p75 chain detected by IL-2 binding. When the same Northern blots were hybridized with a p55-specific probe, p55 mRNA was not detected in either PBMC or purified T cells (Fig. 1 *B*). However, 24 h after CD3 activation, p55 mRNA was readily detected in both populations. In other experiments not shown, very low levels of p55 mRNA were detectable in resting PBMC after prolonged exposure of the Northern blots. In all experiments, p55 mRNA was consistently less prevalent than p75 mRNA in unstimulated PBMC.

Expression of p55 and p75 IL-2 Receptor in Unstimulated PBL. To determine whether p75 mRNA is translated into a stably expressed protein product and to identify the cells in resting peripheral blood that constitutively express p75, we examined resting

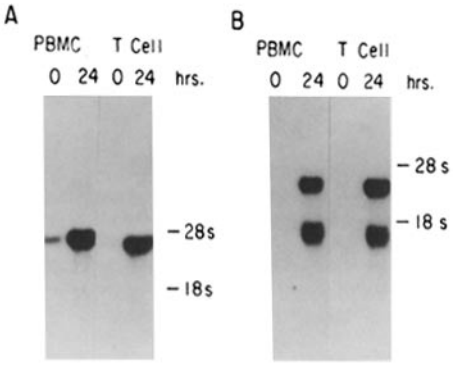

FIGURE 1. Northern blot analysis of IL-2 receptor p75 (*A*) and p55 (*B*) expression in resting and CD3-activated populations of human PBMC and purified T cells. Cells were isolated as described in Materials and Methods. Total RNA was harvested from resting cells (t = 0) or after an incubation in the presence of anti-CD3 antibodies at 37°C for 24 h. 15 μg of total RNA per lane was subjected to Northern blot analysis. The p75 (*A*) hybridization probe used detected a specific mRNA of 4.0 kb. The p55 (*B*) cDNA probe detected mRNAs of 3.5 and 1.5 kb in length. p75 and p55 blots were exposed using intensifying screens for 20 and 48 h, respectively.

T cell and purified NK cell populations using flow cytometry. An example from a single individual is shown in Fig. 2. Dual immunofluorescence of CD3 versus p75 or p55 revealed that resting $CD3^+$ cells have no detectable p75, and only a very small number of cells express p55. In contrast, ~50% of $NKH1^+$ cells in this individual expressed p75, while p55 expression was very low, similar to that seen on resting T cells. Similar studies carried out on six individuals confirmed these findings, showing that only 0.2% ± 0.5 of resting $CD3^+$ T cells express p75 and only 1.8% ± 0.6 of resting $CD3^+$ T cells express p55. In contrast, 28.9% ± 19.8 of $NKH1^+$ cells express p75, while only 1.4% ± 1.1 of NK cells express p55. An analysis of IL-2 receptor expression within the $CD8^+$ and $CD4^+$ T cell subsets was also performed (Fig. 3), showing that 6% of $CD4^+$ cells constitutively expressed the isolated p55 chain, while p55 was not expressed in the $CD8^+$ subset. Isolated p75 IL-2 receptor was not detectable on either subset. It should be noted that $CD8^+$ NK cells (which express CD8 in low density), were first removed using immunomagnetic bead depletion with NKH1 mAb before immunofluorescence analysis. Thus, the phenotypic analyses demonstrate that the vast majority of T cells found in resting human peripheral blood lack expression of both p55 and p75 chains of the IL-2 receptor.

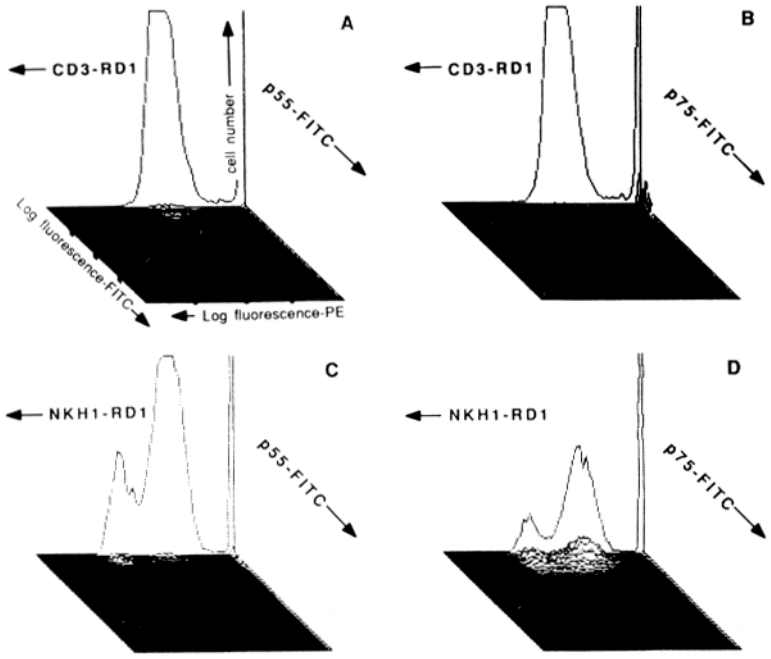

FIGURE 2. Two-color immunofluorescence analysis of resting peripheral blood lymphocyte subpopulations. For T cell analysis, nonadherent PBL were directly stained with phycoerythrin-conjugated anti-CD3 (CD3-RD1) and anti-p55-FITC (*A*), or anti-p75-FITC (*B*). NK cell populations were first purified from peripheral blood by sorting for $NKH1^+$ cells (using NKH1-RD1), and then stained with either anti-p55-FITC (*C*); or anti-p75-FITC (*D*) before two-color analysis. Note the biphasic intensity of NKH1 staining. Actual values are as follows: (*A*) $CD3^+p55^-$ 72%; $CD3^+p55^+$ 3%; CD3-$p55^+$ 0%; (*B*) $CD3^+p75^+$ 0%; $CD3^-p75^+$ 4%; (*C*) $NKH1^+p55^-$ 89%; $NKH1^+p55^+$ 3% $NKH1^-p55^+$ 0%; (*D*) $NKH1^+p75^-$ 46%; $NKH1^+p75^+$ 46%; $NKH1^-p75^+$ 0%.

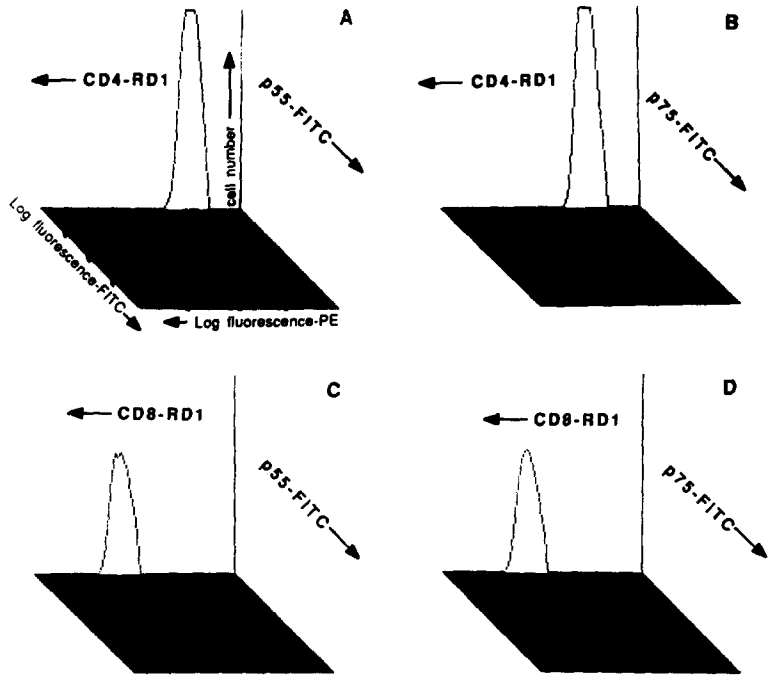

FIGURE 3. Two-color immunofluorescence analysis T cell subpopulations. $CD4^+$ or $CD8^+$ T cells were stained and analyzed. (*A*) CD4 vs. p55; (B) CD4 vs. p75; (*C*) CD8 vs. p55; (*D*) CD8 vs. p75. Note minimal (6%) staining of $CD4^+$ cells with anti-p55 (*A*). The remaining T cell subsets shown were negative for IL-2 receptor expression.

In striking contrast, a significant fraction of NK cells isolated from the resting PBMC population constitutively express the isolated intermediate affinity p75 IL-2 receptor, while expression of the p55 chain is limited to a very small fraction within this population.

Proliferation of Unstimulated PBL to Exogenous IL-2. To assess the functional significance of IL-2 receptor expression by T cells and NK cells, we examined the proliferative response of purified populations at increasing doses of IL-2 without additional activation stimuli. As shown in Fig. 4, T cells consistently display a very low degree of proliferation, as determined by [^{3}H]thymidine incorporation when compared with equal numbers of NK cells. This result was evident at high IL-2 concentrations, including those that saturate both high and intermediate affinity binding sites. In addition, it is evident that NK proliferation was noted at an IL-2 concentration of only 10 pM. Since this concentration of IL-2 saturates 50% of high affinity IL-2 receptor and <1% of isolated intermediate affinity receptors, it effectively stimulates cells solely via the high affinity IL-2 receptor (17). Given this discrimination, it is noteworthy that there is a significant NK cell response at this concentration, and that this response was fivefold greater than that of purified T cells that remained essentially unchanged compared with proliferation in media alone. Moreover, when a 10-fold higher IL-2 concentration (100 pM) was used to saturate all high affinity IL-2 receptors and ~10% of any isolated intermediate affinity p75 IL-2 receptors

(17), there was a marked increase in NK cell proliferation, again without any significant increase in T cell proliferation. 50% maximal proliferation was noted at 50 pM, a concentration that saturates nearly all high affinity sites and <5% of intermediate affinity sites. Further increases in the concentration of IL-2 to 5 nM, which results in ~85% saturation of the isolated intermediate affinity p75 IL-2 receptor (17), did not result in further increases in NK cell proliferation and actually resulted in a slight decrease. Collectively, these data suggested that a fraction of NK cells express the heterodimeric high affinity IL-2 receptor, which when saturated, accounts for much of the proliferation seen within the entire NK cell population.

Expression of the High Affinity IL-2 Receptor in Resting PBL. In previous studies (29), the NK cell population, purified from resting PBMC, was found to be heterogeneous with regard to the cell surface density expression of the NKH1 antigen. Two populations have been identified: one with low surface density expression of the NKH1 antigen ($NKH1^{dim+}$) and the other with higher surface density expression of NKH1 ($NKH1^{bright+}$). The $NKH1^{bright+}$ cells routinely make up <5% of the entire NK cell population and have been shown to lack expression of CD3 antigen. As shown in Fig. 2, constitutive expression of the p75 chain is phenotypically equivalent in both the $NKH1^{bright+}$ and $NKH1^{dim+}$ populations. Less than 2% of NK cells express sufficient p55 to be detected by immunofluorescence, but this appeared to be primarily within the $NKH1^{bright+}$ fraction. Given that isolated p55 and p75 chains, when simultaneously expressed, interact to form the heterodimeric high affinity IL-2 receptor, this suggested that the $NKH1^{bright+}$ population might express the high affinity receptor in addition to isolated intermediate affinity p75, and that this high affinity receptor might be responsible for the proliferation of NK cells seen at low concentrations of IL-2.

To investigate this further, we sorted $NKH1^+$ cells from fresh PBL into $NKH1^{bright+}$ and $NKH1^{dim+}$ fractions based on the surface density expression of the NKH1 antigen as shown in Fig. 5 *A*. We next examined proliferation of sorted cells at a dose of rIL-2 (10 pM) that would saturate only the high affinity IL-2 receptors expressed within each population (17). As can be seen in Fig. 5 *B*, the $NKH1^{bright+}$ cells isolated from unstimulated peripheral blood showed over a 40-fold increase in [^{3}H]thymidine incorporation when compared with the $NKH1^{dim+}$ cells that failed to respond. Further evidence that this proliferation was promoted solely via the high affinity IL-2 receptor is provided by the complete abrogation of proliferation seen

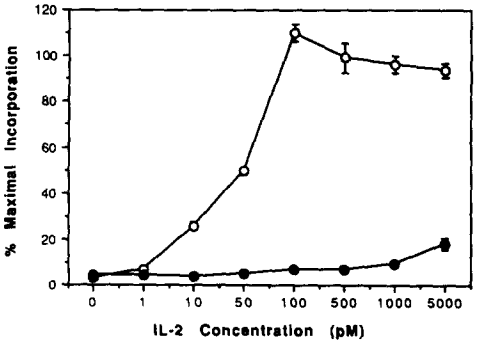

FIGURE 4. Proliferative response of resting populations T cells (●) vs. NK (○) cells cultured at varying concentrations of IL-2 for 96 h. $CD5^+$ T cells and $NKH1^+$ NK cells were purified by immunofluorescent cell sorting as described in Materials and Methods. Values displayed represent the percent of the maximal [^{3}H]thymidine incorporation ± SEM for triplicate samples. Actual value for the % maximal proliferation is the mean sum of CPM seen for NK cells at doses 100, 500, 1000, and 5,000 pM rIL-2.

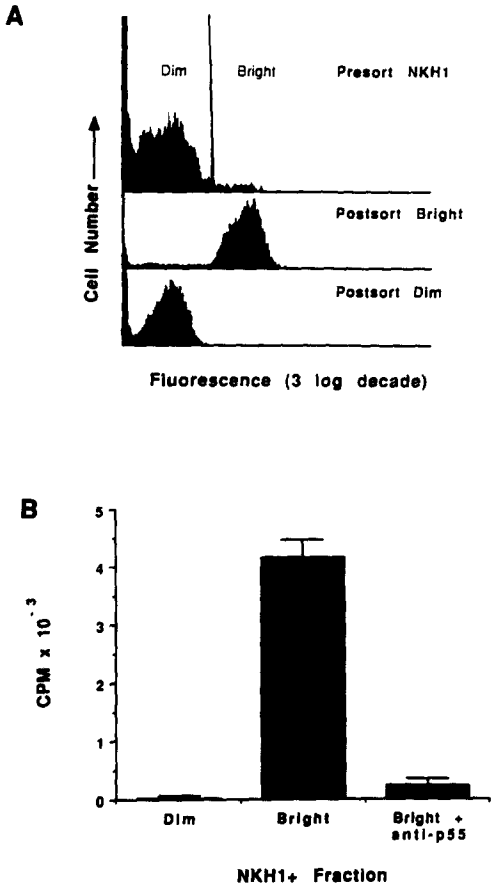

FIGURE 5. (*A*) Expression of NKH1 antigen on resting NK cells. Flow cytometric histograms showing NKH1 staining of high density (bright) and low density (dim) cells in peripheral blood before (*top*) and after (*middle and bottom*) the two $NKH1^+$ subpopulations were purified by cell sorting. (*B*) Proliferation of $NKH1^{dim+}$ and $NKH1^{bright+}$ cells in response to low concentrations of rIL-2. Subpopulations of $NKH1^+$ cells were isolated from fresh PBL and then plated in media alone or in media containing 10 pM rIL-2 for 96 h. Before the addition of IL-2, cells were incubated for 30 min with either a nonreactive mAb or anti-p55 mAb. cpm in media alone (246 ± 70 for $NKH1^{bright+}$ and 227 ± 67 for $NKH1^{dim+}$ cells) were subtracted as background. Results represent the mean ± SEM for triplicate samples.

after addition of anti-p55 mAb, which blocks binding of IL-2 to the high affinity IL-2 receptor but not to the intermediate affinity p75 IL-2 receptor (17).

To further characterize the expression of functional high affinity IL-2 receptors within resting human PBL, we next examined the proliferation of various purified PBL populations as identified by lymphocyte specific antigens, again using 10 pM of IL-2. As can be seen in Fig. 6, the only population to show significant proliferation after 5 d of culture were the $NKH1^{bright+}$ cells. Only a minimal proliferative response was seen in the $NKH1^{dim+}$ population. This may be due to a small fraction of cells expressing the high affinity IL-2 receptor within this population since this activity is also completely abrogated in the presence of anti-p55. Most notably, there is virtually no proliferation seen within either $CD4^+$ or $CD8^+$ T cell subsets or the $CD20^+$ B cell population.

Activation of NK Cells through the Isolated Intermediate p75 IL-2 Receptor. Proliferation within the $NKH1^{bright+}$ and $NKH1^{dim+}$ populations was next examined following a 96-h incubation period and at concentrations of IL-2 that selectively saturated each of the two functional IL-2 receptors. A representative assay after isolation of these two populations from resting peripheral blood is shown in Fig. 7 *A*,

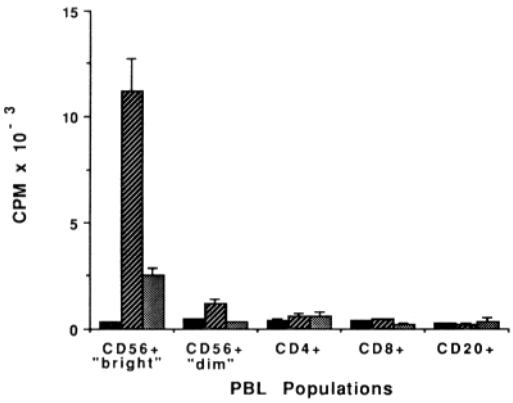

FIGURE 6. Proliferation of resting human PBL subpopulations as measured by [^{3}H]thymidine incorporation following a 96-h culture in either media + a nonreactive mAb (■), 10 pM rIL-2 + a nonreactive mAb (■), or 10 pM rIL-2 + anti-p55 mAb (■). Purified populations were isolated from resting peripheral blood using immunofluorescent cell sorting. Results represent the mean ± SEM for triplicate samples.

and serves to illustrate several points. First, there is maximal proliferation in the NKH1$^{bright+}$ population at 100 pM IL-2, a concentration that saturates 100% of high affinity IL-2 receptors and only ~20% of intermediate affinity IL-2 receptors (17). In contrast, the NKH1^{dim+} population, which expresses the isolated intermediate affinity IL-2 receptor, shows little or no proliferation at this concentration. This finding suggests that IL-2 binding to high affinity IL-2 receptors is entirely responsible for the proliferation seen within the NKH1$^{bright+}$ population. Second, further increases in IL-2 concentrations that saturate 50% (1 nM) and 100% (10 nM) of isolated intermediate affinity p75 IL-2 receptors (17) fail to increase the proliferative response in the NKH1$^{bright+}$ population. Surprisingly, the NKH1^{dim+} cells, which lack the high affinity IL-2 receptor but constitutively express the intermediate affinity p75 IL-2 receptor, showed only minimal increases in proliferation at these high concentrations of IL-2. In separate experiments, absolute cell counts were found to correlate closely with results of the [^{3}H]thymidine proliferation assay. After 7 d of in vitro culture with either 1 or 10 nM rIL-2, there was only a twofold increase in the number of NKH1^{dim+} cells, while the NKH1$^{bright+}$ cells showed more than a 15-fold increase in cell number (data not shown).

The constitutive expression of a functional isolated intermediate affinity p75 IL-2 receptor on NKH1^{dim+} cells was verified by examining non-MHC-restricted cytotoxicity following 18-h incubations in the presence or absence of increasing concentrations of rIL-2 (Fig. 7 *B*). While a minimal increase in cytotoxicity against K562 target cells was noted with 10 pM rIL-2, a more substantial increase in cytotoxicity was seen at IL-2 concentrations (10 nM) that saturate intermediate affinity p75 IL-2 receptors. It is noteworthy that NKH1$^{bright+}$ cells also show enhanced cytotoxicity at these higher doses of rIL-2, consistent with phenotypic studies that demonstrate a relative excess of isolated intermediate affinity p75 receptor compared with the expression of high affinity IL-2 receptors within this select population.

Discussion

The present study characterizes the consequences of IL-2 receptor expression by resting human peripheral blood cells by identifying which cells express receptors and by determining the functional responses of these cells to varying concentrations

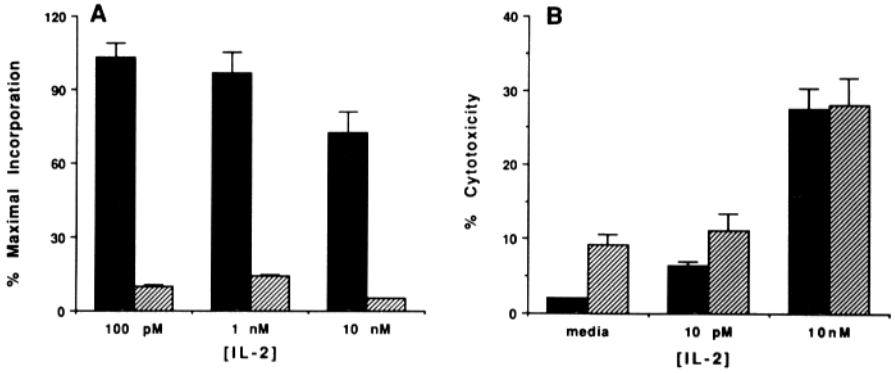

FIGURE 7. (*A*) Proliferative response of resting populations of NKH1$^{bright+}$ (■) and NKH1^{dim+} (□) cells cultured in varying concentrations of IL-2 for 96 h. Values displayed represent the percent of the maximal [^{3}H]thymidine incorporation ± SEM for triplicate samples. (*B*) Cytotoxicity of NKH1$^{bright+}$ (■) and NKH1^{dim+} (▨) cells. Effector populations were first cultured overnight in either media, 10 pM rIL-2 or 10 nM rIL-2. ^{51}Cr-labeled K562 targets were then added to each well at an E/T ratio of 1:1, and incubated for an additional 4 h. Results represent the mean percent lysis ± SEM for triplicate samples.

of IL-2. Previous studies have shown that antigen activation of resting T cells results in the expression of high affinity IL-2 receptors which then signal the proliferation of antigen-selected cells (1). The expression of a functional IL-2 receptor on unactivated or resting populations of T cells has remained controversial, with several studies suggesting that some or all of these cells constitutively express at least one component of the IL-2 receptor (28, 30, 31). By comparison, it has been known for some time that freshly isolated NK cells respond to IL-2 with increases in non-MHC-restricted cytotoxicity (2–4) and proliferation (32–35), without apparent prior antigen activation. Despite the functional responsiveness to IL-2, the phenotypic expression of the p55 chain has never been detectable on resting NK cells (32–34, 36–38). In contrast, it has recently been shown that the majority of NK cells constitutively express the isolated intermediate affinity p75 IL-2 receptor, and that blocking of this receptor abrogates the IL-2-mediated enhancement of cytolytic activity (23). Even so, the proliferative response of NK cells that has been observed at very low concentrations of IL-2 has not been conclusively shown to occur through this intermediate affinity IL-2 receptor (23, 39). To assess the functional responsiveness of resting populations of T cells and NK cells and to identify which receptor form mediates these responses, we undertook a detailed analysis of these cells using radiolabeled IL-2 binding assays, Northern blot analyses, flow cytometric phenotypic analyses, as well as IL-2 dose-dependent functional assays of proliferation and cytotoxicity.

Taken together, our studies demonstrate that the vast majority of T cells lack constitutive expression of either p55 or p75 IL-2 binding sites before antigen activation. Consequently, incubation of resting T cells with exogenous rIL-2 does not result in proliferation. After activation with anti-CD3, T cells show a dramatic increase in the expression of both p75 mRNA and p55 mRNA, which accounts for an observed eightfold increase in the surface expression and subsequent formation of high affinity IL-2 receptors within the activated T cell population. This rapid induction of high affinity receptor expression, along with the concomitant production of IL-2

(40), accounts for the proliferative response observed following activation of T cells as shown in previous studies (40, 41). As activation of CD3 is thought to essentially mimic the signaling events that follow antigenic activation via the CD3-TCR complex, these studies serve to emphasize not only the role of IL-2 and the IL-2 receptor in determining the onset and duration of a clonal T cell response after antigen activation, but also the equally critical absence of IL-2 reactivity by T cells before antigen activation. Thus, the specificity of the T cell response appears to be tightly regulated by antigen-induced expression of both chains comprising the IL-2 receptor.

The expression of the IL-2 receptor within the NK cell population is distinctly different from that seen within the T cell population. Taken together, binding studies, Northern blot analyses, and phenotypic analyses strongly suggest that the majority of NK cells constitutively express isolated intermediate affinity p75 IL-2 receptor. The variable expression of p75 seen within the $NKH1^+$ population (i.e., 29 ± 19% positive cells) appears to be a limitation of the flow cytometric analysis (19, 23). These cells undoubtedly express numbers of isolated p75 IL-2 receptors that are barely detectable by standard flow cytometric methods. In addition to the constitutive expression of this intermediate affinity receptor, NK cells appear to be heterogeneous with regard to the expression of a functional high affinity receptor as defined by their marked proliferative response to low concentrations of IL-2 that only saturate high affinity IL-2 receptor sites. Again, due to low density expression of this receptor, this subpopulation of NK cells cannot be reproducibly detected using mAbs specific for the p55 chain of the heterodimeric high affinity IL-2 receptor. However, they can be isolated from the remainder of resting PBL by their unique high density surface expression of the NKH1 antigen. Careful purification of $CD4^+$ and $CD8^+$ T cell subsets, B cells, and NK cells (based on density expression of the NKH1 antigen) failed to demonstrate any other lymphoid population within resting human peripheral blood with constitutive expression of a functional high affinity IL-2 receptors, as measured by proliferation in response to exogenous IL-2 without additional activation.

Importantly, by examining the proliferative responsiveness of both $NKH1^{bright+}$ cells and $NKH1^{dim+}$ cells at increasing doses of IL-2, we were able to demonstrate that the constitutive expression of the high affinity IL-2 receptor found only within the $NKH1^{bright+}$ population is responsible for the great majority of NK cell proliferation seen when resting peripheral blood is incubated with IL-2. Moreover, the isolated intermediate affinity p75 IL-2 receptor, expressed in the absence of the high affinity IL-2 receptor on $NKH1^{dim+}$ cells, appears to transduce only a minimal growth signal, despite addition of saturating concentrations of IL-2 and the demonstration of the receptor's functional competency in cytotoxicity assays. Given that the $NKH1^{bright+}$ population represents only ~3–5% of NK cells and therefore only ~0.3–0.5% of PBMC, it is clear that only a very small fraction of resting peripheral blood cells have the capacity to generate a significant proliferative response to physiologic concentrations of IL-2 in the absence of antigen activation.

The present model of the IL-2 receptor suggests that signal transduction depends upon the p75 chain, whether expressed in its isolated intermediate affinity form or as part of the heterodimeric high affinity receptor. Accordingly, this model dictates that fully saturated isolated intermediate affinity p75 IL-2 receptors should transduce similar signals for growth as does the high affinity IL-2 receptor when fully

saturated. Data supporting this interpretation come from experiments measuring proliferation of activated T cells expressing high affinity IL-2 receptors: in the presence of excess anti-p55, the high affinity heterodimeric IL-2 receptor structure is disrupted, and the dose-response curve shifts, showing a similar maximal proliferative response but requiring ~100-fold greater concentrations of IL-2 to saturate the newly created isolated intermediate affinity IL-2 receptors. These data are also consistent with affinity binding studies performed on cell lines expressing only the intermediate affinity p75 IL-2 receptor (17). Nevertheless, recent attempts to construct a high affinity IL-2 receptor by transfection of both the p75 and p55 cDNA into COS cells failed to demonstrate IL-2 binding characteristic of the high affinity receptor, suggesting that this structure may have additional components (16). Using $NKH1^{dim+}$ cells freshly isolated from resting human peripheral blood, our current studies show that the IL-2 proliferative signal in cells constitutively expressing the intermediate affinity p75 IL-2 receptor without the high affinity IL-2 receptor is not equivalent to that seen in $NKH1^{bright+}$ cells that also express the high affinity IL-2 receptor, despite concentrations of IL-2 that resulted in 100% saturation of the isolated p75 IL-2 receptor. Additionally, $NKH1^{bright+}$ cells did not increase their proliferation at doses of IL-2 that saturate the isolated p75 chain, despite the phenotypically detectable excess of p75 IL-2 receptors expressed on their surface. While the explanation for this is not clear, these data strongly argue against receptor affinity as being the only functional difference between these two classes of receptors, and suggest that there may be additional, and as yet unknown, characteristics of the isolated p75 IL-2 receptor that affect its ability to transduce a proliferative signal. Data presented here, using populations of resting human peripheral blood NK cells, would suggest that additional undetected structures might be involved in the formation of the high affinity IL-2 receptor, or that the noncovalent bonding of the p55 chain to the p75 chain may somehow alter the post-ligand signal. Alternatively, it is also possible that the $NKH1^{dim+}$ and $NKH1^{bright+}$ cells represent distinct populations that might therefore transmit different post-ligand signals after IL-2 binding to the isolated intermediate affinity p75 IL-2 receptor. This latter explanation seems unlikely, as proliferation of the $NKH1^{bright+}$ population is not increased at doses of IL-2 that completely saturate the isolated 75 chain, known to be expressed in excess on the $NKH1^{bright+}$ cell surface.

The reasons for the constitutive expression of the intermediate affinity IL-2 receptor on NK cells in resting peripheral blood remain unresolved. It is known that enhancement of cytotoxicity can occur exclusively through the isolated p75 chain (23, 39). Thus, for the $NKH1^{bright+}$ cells that constitutively express both the high affinity and intermediate affinity IL-2 receptors, low concentrations of IL-2 result in proliferation of this population, while higher concentrations of IL-2 are also able to activate the cytolytic mechanism of these cells. The fact that this process occurs in an incremental, dose-response fashion might well be advantageous to the host. Nevertheless, it is also evident that, in the absence of additional activation signals, the majority of NK cells express only the intermediate affinity IL-2 receptor, and it is unclear why IL-2 can readily promote the enhanced cytotoxicity of this subpopulation without comparable stimulation of their proliferation. It is certainly possible that additional cell–cell interactions and/or other lymphokines are involved in the generation of a proliferative response in vivo.

The constitutive expression of a functional high affinity IL-2 receptor on a subset of NK cells without prior activation suggests that these cells undergo a polyclonal proliferative response to physiologic concentrations of IL-2. Hence, although both activated T cells and NKH1$^{bright+}$ cells use the high affinity IL-2 receptor to proliferate in response to IL-2, the NK cell fraction constitutively expresses this receptor without prior antigen activation, while the T cell can only express this receptor and respond to IL-2 after antigen activation. This differential use of the receptor may lend some insights into the distinct functional roles for these two populations in vivo. There is extensive experimental (42–46) and limited clinical (47) information that suggest that NK cells play an important role in the early response to viral infection, acting as a first line of defense capable of responding nonspecifically to virus-infected cells before a specific T cell response can be generated. Our data demonstrating the constitutive expression of a functional high affinity IL-2 receptor within the NKH1$^{bright+}$ population of resting peripheral blood, while resting T cells lack any such receptor, would strongly support this hypothesis and provide a mechanism whereby this may occur in vivo. Also consistent with the idea that NK cells provide a first line of defense is the fact that NK cells do not express a clonotypic CD3-TCR (48). The recent finding that NK cells express the ζ chain of the TCR (49) suggests that NK cells may represent an evolutionary forerunner of clonotypic T cells, but NK cells have nevertheless retained a place in the human immune defense system (50). Our demonstration of a subset of NK cells that constitutively express a functional high affinity IL-2 receptor in resting human peripheral blood similar to that seen on T cells after antigen activation helps to explain how such cells may be able to respond quickly to the presence of virus-transformed cells or other target cells.

The expression of different IL-2 receptor forms with functionally distinct consequences as now seen on subpopulations of NK cells should help us to further characterize these lymphocytes with regard to their function in vivo. Thus, our data help to explain why freshly isolated NKH1$^{bright+}$ cells show a greater proliferative response to IL-2 when compared with the NKH1^{dim+} population as previously reported (25, 51, 52), and why clinical studies often show an increase in the NKH1$^{bright+}$ population after low dose infusion of rIL-2: presumably only cells expressing the high affinity IL-2 receptor can proliferate to what must be very low serum concentrations of IL-2. Furthermore, the information gained from the data presented here should now assist us in the future development of rational clinical trials whereby large numbers of NK cells may be generated in vivo with prolonged infusions of relatively low doses of IL-2 that saturate only the high affinity IL-2 receptor constitutively expressed on the NKH1$^{bright+}$ cells. Subsequent induction of non-MHC-restricted cytotoxicity via the isolated intermediate affinity p75 IL-2 receptor also expressed on these cells might then be undertaken at regular intervals to promote tumor cytolysis with less clinical toxicity.

Summary

In this study, we have used radiolabeled IL-2 binding assays, Northern blot analysis, immunofluorescent flow cytometry and cell sorting, as well as proliferation and cytotoxicity assays to perform an extensive phenotypic and functional characterization of the IL-2 receptor in normal resting human peripheral blood lymphocytes. Our results indicate that almost all T cells (>98%) express neither the high affinity

IL-2 receptor nor the functional intermediate affinity p75 chain of the IL-2 receptor without prior activation. In contrast, most NK cells constitutively express the isolated intermediate affinity p75 IL-2 receptor. In addition, a subpopulation of NK cells, distinguished by high density expression of the NKH1 antigen, constitutively express the high affinity IL-2 receptor, in addition to an excess of the isolated intermediate affinity p75 IL-2 receptor. These NKH1$^{bright+}$ cells exhibit a brisk proliferative response to IL-2, similar to that seen with antigen-activated T cells, yet do so in the absence of any known antigenic stimuli. No other resting peripheral blood lymphocyte population, including CD4$^+$, CD8$^+$, and CD20 cells, exhibits this property. The intermediate affinity p75 IL-2 receptor, as it exists in its isolated form on resting NK cells, does not transduce a growth signal equivalent to that seen in NK cells expressing the high affinity IL-2 receptor, despite doses of IL-2 that are known to fully saturate the isolated p75 chain. This strongly suggests that additional structural or functional components are involved in generating the proliferative response following the binding of IL-2 to the high affinity heterodimeric form of the IL-2 receptor. The constitutive expression of this functional high affinity IL-2 receptor on a small population of resting NK cells provides further evidence in support of a role for these cells in the host's early defense against viral infection or malignant transformation, before the more delayed but specific T cell response.

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