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IL-2 Responsiveness of CD4 and CD8 lymphocytes: further investigations with human IL-2Rβ transgenic mice

Franck Gesbert[1,2]*, Jean-Louis Moreau[1]* and **Jacques Thèze[1]**

[1]Unité d'Immunogénétique Cellulaire, Département de Médecine Moléculaire, Institut Pasteur, 25-28, rue du Dr. Roux, 75724 Paris Cedex 15, France

[2]Present address: Unité de Biologie des Interactions Cellulaires, Institut Pasteur, Paris, France

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Abstract

Responsiveness to IL-2 varies from one lympho-mononuclear subset to another. NK lymphocytes and monocytes spontaneously respond to IL-2 whereas it is generally accepted that T and B lymphocytes need to be activated to fully acquire this competence. To further investigate this phenomenon, we studied human IL-2Rβ (hIL-2Rβ) transgenic mice constitutively expressing heterospecific, intermediate-affinity IL-2R (hIL-2Rβ/mouse IL-2Rγ_c). We noted that the B lymphocytes and monocytes from spleens of these hIL-2Rβ transgenic animals failed to grow when cultured in IL-2-containing medium. Under the same experimental conditions, CD4 lymphocytes survived, again without growth, whereas CD8 lymphocytes and NK cells were able to proliferate and develop potent LAK cytotoxicity. The properties of these CD4 and CD8 lymphocytes were then compared after purification. Both subsets expressed functional IL-2R able to induce global protein phosphorylation and, more precisely, signal transducer and activation of transcription 5 and Erk phosphorylation. Therefore, the differential growth potential of these CD4 and CD8 lymphocytes cannot be explained by the lack of IL-2R-dependent early signaling events. When the entrance of purified CD4 and CD8 lymphocytes into the cell cycle was analyzed, we found that the CD4 lymphocytes were unable to enter the G1 phase in the absence of anti-CD3 stimulation. This correlates with the effect of IL-2 on cyclin-dependent kinase inhibitor $p27^{kip1}$. In CD4 lymphocytes, IL-2 does not affect $p27^{kip1}$ expression. But in CD8 lymphocytes, IL-2 down-modulates $p27^{kip1}$. These results indicate that, aside from IL-2R expression and function, IL-2 responsiveness is also controlled by lineage-specific mechanisms.

Introduction

IL-2 is a major cytokine that possesses inflammatory and immune properties (1–3). It acts on many cell types including T and B lymphocytes, NK cells and monocytes. Its effects on T lymphocyte activation and proliferation have been extensively studied. IL-2 is also involved in the control of activation-induced cell death and/or apoptosis (4–6). More recently, its role in the development of $CD4^+CD25^+$ regulatory T cells has also been discussed (7).

Responses to IL-2 are mediated by a multimeric receptor (IL-2R) and a complex cascade of signaling events. The high-affinity (10^{-11} M) receptor is composed of three chains: IL-2Rα, β and γ_c (8–13). In humans, IL-2 can also bind to a functional, intermediate-affinity receptor (10^{-9} M) formed after the dimerization of human IL-2Rβ (hIL-2Rβ) with hIL-2Rγ_c chains. This intermediate-affinity receptor is absent in the murine model and consequently, in mouse lymphocytes, the IL-2Rα chain is required to bind IL-2 (14, 15). However, in mouse cell lines, exogenous expression of the hIL-2Rβ chain in the presence of the endogenously expressed mouse IL-2R (mIL-2R)γ_c chain is sufficient to obtain a functional, intermediate-affinity IL-2R (15). Binding of IL-2 to IL-2R triggers a cascade of signaling events that include the Jak/signal transducer and activation of transcription (STAT) and Erk pathways, both of which play essential roles (16–20). It is

*These authors contributed equally to this work

Correspondence to: J. Thèze; E-mail: jtheze@pasteur.fr

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generally believed that responses to IL-2 are controlled by the expression of IL-2R, but this concept is far from being firmly established. We have shown that anti-μ-stimulated B lymphocytes expressing high-affinity IL-2R do not respond to this cytokine. These B lymphocytes require activation by anti-μ in the presence of IL-2 to acquire the competence to respond to IL-2 (21). This suggests that responsiveness to IL-2 is also controlled by additional, unknown mechanisms induced after appropriate lymphocyte activation. As far as T lymphocytes are concerned, if they are to respond to IL-2, they need to be activated to express IL-2R. But, IL-2R expression may not be sufficient to control IL-2 responsiveness (22). More specifically, the correlation in CD4 and CD8 lymphocytes between IL-2R expression, IL-2R function and responsiveness to IL-2 remains an open issue.

The hIL-2β transgenic mice described by Asano *et al.* (23) constitute an ideal model to address these issues. In these animals, IL-2R is constitutively and ubiquitously expressed and the response to IL-2 can be studied independently of the regulation of IL-2R expression. In parallel, we also further analyzed the effect of constitutive IL-2R expression on the development of lympho-mononuclear cells and on their IL-2 responsiveness. More importantly, we characterized some of the mechanisms that may explain the markedly different IL-2 responsiveness of CD4 and CD8 lymphocytes in this experimental model. Our studies further demonstrate that, aside from IL-2R expression, other cell lineage-specific mechanisms also control IL-2 responsiveness.

Methods

Mice and reagents

The hIL-2Rβ transgenic mice used have already been characterized (23). They constitutively express the hIL-2Rβ gene under the control of the mouse H-2K^d promotor. C57BL/6 and hIL-2Rβ transgenic mice were bred under routine conditions in the animal facilities at the Institut Pasteur (Paris, France). Male hIL-2Rβ transgenic animals were crossed with C57BL/6 females. Animals expressing the hIL-2Rβ transgene were identified by FACS analysis on peripheral blood cells using anti-CD122 mAb (see below). All animals were 2–3 months old at the time of the experiments.

Human IL-2 obtained from Chiron-Europe (Amsterdam, The Netherlands) was used in all cases at 10 ng ml^{-1} of culture medium. Anti-p27^{kip1}, anti-STAT5a, anti-STAT5b, anti-phospho-STAT5, anti-Erk and anti-phospho-Erk antibodies were purchased from BD-PharMingen (BD-France, Le Pont de Chaix, France). Anti-actin antibodies were obtained from Sigma (Paris, France). 4G10 anti-phosphotyrosine mAb was obtained from Upstate Biotechnologies (Lake Placid, NY, USA).

Flow cytometric analysis

Spleen cells from C57BL/6 and hIL-2Rβ transgenic mice were used to characterize lymphocyte subsets. Single-cell suspensions (5×10^6 cells in 0.1 ml PBS, 0.5% FCS, 0.2% sodium azide) were stained with PE-conjugated mAb (30 min on ice) and washed before analysis. The following mAbs were PE conjugated at the Institut Pasteur: anti-CD4 mAb (clone GK1.5), anti-CD8 mAb (clone H35b), anti-B220 mAb (clone 14.8) and anti-Mac-1 mAb (clone M1/70). PE-conjugated anti-NK 1.1 mAb (clone PK136) was obtained from PharMingen–Clinisciences (Montrouge, France). The expression of murine IL-2R chains was measured by labeling with anti-IL-2Rα mAb (clone 5A2), anti-IL-2Rβ mAb (clone TMβ1) or anti-IL-2Rγ_c mAb (clone TUGm2) followed by staining with FITC-labeled MARK-1 mAb (P.A.R.I.S Compiègne, France). The expression of hIL-2Rβ was measured by staining with FITC-conjugated anti-CD122 mAb (Immunotech, Marseille, France). Flow cytometry was performed using a FACScan flow cytometer and CELLQuest software for data analysis (Becton Dickinson, Mountain View, CA, USA).

Analysis of the growth and cytotoxicity of IL-2 cultured lymphocytes

Spleen cells from C57BL/6 or hIL-2Rβ transgenic mice were cultured in complete medium supplemented or not with IL-2. Cells were counted for up to 10 days of culture. After 3 and 7 days of culture, the growing cells were phenotyped and the proportion of CD4, CD8, B, NK lymphocytes and monocytes was determined.

The cytotoxicity of the cultured cells was tested on YAK target cells (5×10^3 in 0.1 ml) labeled with $Na_2{}^{51}CrO_4$ (Amersham–Pharmacia, Buckingamshire, UK). These were mixed in round-bottomed microwells with an equal volume of effector cells at various effector to target cell (E/T) ratios. After 4 h of incubation at 37°C, the plates were centrifuged at $2000 \times g$ for 2 min and cell-free supernatants were collected using a Luma plate 96-cell harvester (Lumac-LSC, Groningen, The Netherlands). Supernatant radioactivity was assayed using an automated microbeta 1450 Trilux γ_c-counter (Wallac, Turku, Finland). Spontaneous release was determined by incubating target cells in medium alone. Maximum release was determined by adding 0.1 ml of 1 M HCl to the target cell suspension. For each E/T ratio, the percentage of specific lysis was calculated as follows: 100 × (experimental ^{51}Cr release − spontaneous ^{51}Cr release)/(maximum ^{51}Cr release − spontaneous ^{51}Cr release).

CD4 and CD8 lymphocyte purification

Splenocytes from IL-2Rβ transgenic mice were labeled for 15 min at 6–12°C in PBS (1% FCS, EDTA 2 mM) with anti-CD4 (L3T4) microbeads or with anti-CD8α (Ly-2) microbeads from Miltenyi Biotec (Germany) and purified by separation through a 25LS MACS separation column.

Purity of the CD4 and CD8 cells was verified by double staining with FITC-conjugated anti-CD4 mAb and PE-conjugated anti-CD8 mAb and analysis on a FACScan flow cytometer. The ability of the spleen cells, purified CD4 lymphocytes and purified CD8 lymphocytes to proliferate *in vitro* was verified. Cells were cultured (10^5 cells per well) in 96-well flat-bottomed microtiter plates in a final volume of 200 μl. Anti-CD3 mAb (clone 145-2C11; BD-PharMingen, Paris, France) was used at a sub-optimal concentration (0.2 μg ml^{-1}). IL-2 (Chiron) was used at 10 ng ml^{-1}. After 3 days of incubation, the cultures were pulsed with [^{3}H]thymidine ([^{3}H]TdR) and harvested 16 h later.

Cell cycle progression analysis

Splenocytes and purified CD4 and CD8 lymphocytes from IL-2Rβ transgenic mice were cultured in the presence of IL-2 or anti-CD3 mAb plus IL-2. After 3 and 6 days the cells were harvested, fixed with PFA (1%), permeabilized with 0.05% saponin (in 5 mM HEPES pH 7.5, 150 mM NaCl and 4% FCS) and stained for 30 min at room temperature with propidium iodide (50 μg ml^{-1}) in PBS with RNAse (25 μg ml^{-1}). The cells were then analyzed using a FACScan flow cytometer. The percentage of cells in each stage of the cell cycle (G0/G1, S, G2/M) was determined by ModFit LT software (Becton Dickinson Immunocytometric Systems).

Protein analysis

The cells were harvested, washed in PBS and lysed for 30 min at 5×10^7 cells ml^{-1} in cold lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.5% Triton-X100, 10 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulphonylfluoride, 1 mM $NaVO_3$ and 1 μg ml^{-1} leupeptin and aprotinin). Lysates were clarified by centrifugation at 15 000 × *g* for 20 min at 4°C. Protein concentrations were determined by Bradford assay (Biorad protein assay), and equivalent amounts of protein were separated by gel electrophoresis and transferred to a PVDF membrane (Millipore, Bedford, MA, USA). The filters were blocked for 2 h at room temperature with either 5% non-fat dried milk or 3% BSA in Tris-buffered saline, 0.5% Tween (TBS-T). The filters were washed three times in TBS-T and incubated for 1 h with optimal concentrations of primary antibodies diluted in TBS-0.1% Tween. After four additional washes in TBS-T, the filters were incubated for 45 min with HRP-conjugated secondary antibodies (Amersham Pharmacia Biotech. Inc., Piscataway, NJ, USA). Proteins were visualized using ECL reagents and ECL hyperfilms (Amersham–Pharmacia).

Results

Lympho-mononuclear subsets and IL-2R chain expression in splenocytes from hIL-2Rβ transgenic mice

The effect of constitutive hIL-2Rβ chain expression on the *in vivo* development of the different splenocyte sub-populations is shown in Fig. 1(A). Proportions of CD4 and CD8 lymphocytes were comparable in the spleens of the transgenic and wild-type animals. Contrary to the results obtained by Suwa *et al.* (24), the proportion of NK cells was comparable in the spleens of IL-2Rβ transgenic and wild-type animals. But surprisingly, the hIL-2Rβ transgenic animals developed less B lymphocytes and more monocytes than their wild-type counterparts. About 30% of the cells in the spleens of the IL-2Rβ transgenic animals expressed Mac-1 cell-surface antigen whereas, as expected, only 10% of the cells in the spleens of the C57BL/6 animals expressed this marker.

We first verified that all splenocytes from hIL-2Rβ transgenic mice expressed the hIL-2Rβ chain. Figure 1(B) shows the corresponding profile and the mean fluorescence intensity (MFI) for each subset. It clearly demonstrates that all the subsets expressed the hIL-2Rβ chain with comparable intensity. As an additional control, it was shown that FITC-labeled anti-CD122 mAb specific for hIL-2Rβ chain did not stain splenocytes from C57BL/6 mice (data not shown). We also studied the effect of constitutive hIL-2Rβ chain expression on the expression of the three mIL-2R chains (Table 1). All mIL-2R chains were expressed in CD4 and B lymphocytes, as

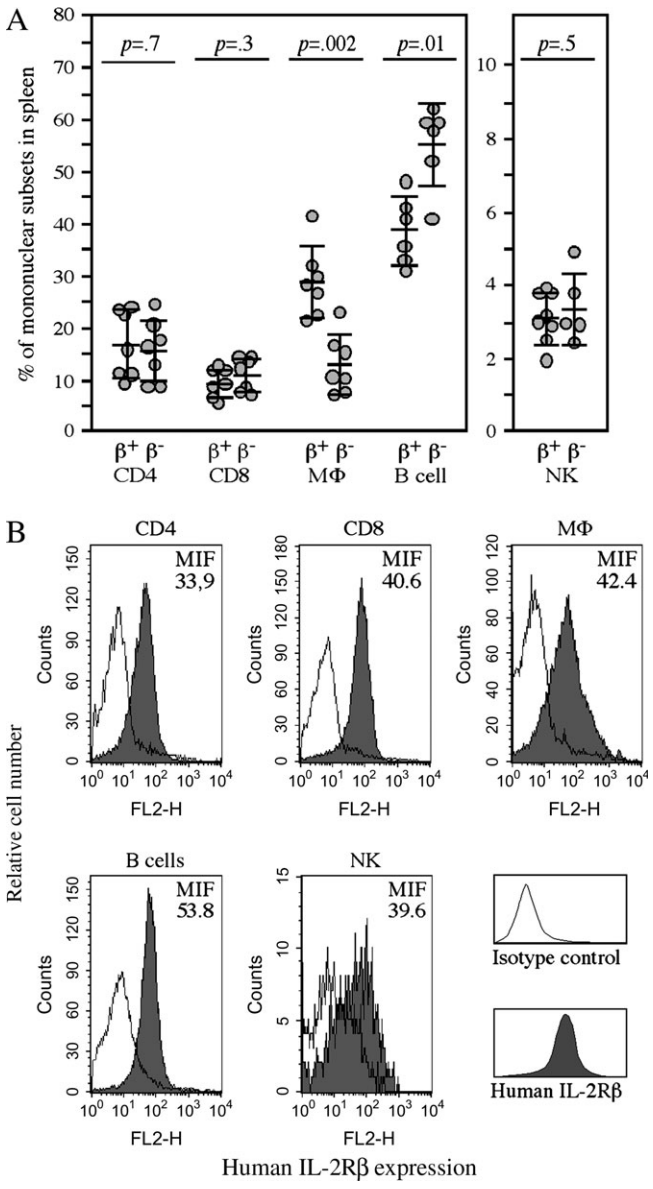


Fig. 1. Lympho-mononuclear subsets and IL-2R chain expression in spleen cells from C57BL/6 and hIL-2Rβ transgenic mice. (A) Spleen cells from C57BL/6 and hIL-2Rβ transgenic mice were analyzed by flow cytometry after labeling with PE-conjugated mAb specific for CD4, CD8, B, NK lymphocytes and monocytes. The experiment reported was performed with seven animals of each strain. Mean percent ± SD is reported for each subset. For B lymphocytes and monocytes the percentages were found to be statistically different ($P < 0.005$). (B) Expression of hIL-2Rβ chains was analyzed in the different lympho-mononuclear subsets from spleens of hIL-2Rβ transgenic mice. Each cell subset was first labeled with specific PE-conjugated mAb (see above). Cells were then stained with FITC-labeled anti-hIL-2Rβ chain mAb (anti-CD122 mAb). Control mAb of the same IgG subclass as anti-CD122 was used as a control.

Table 1. Expression of murine IL-2R chains in lympho-mononuclear subsets from spleen cells of C57BL/6 and hIL-2Rβ transgenic mice

Murine IL-2R chains		Control	α	β	γ
CD4	Normal	10.3	12.9	15.9	34.7
	TGh β	12.0	11.7	23.2	34.0
CD8	Normal	5.0	13.0	32.3	38.5 II
IL-2R low	TGh β	5.1	14.1	35.8	38.7
CD8	Normal	5.0	13.0	345.3	469.5
IL-2R high	TGh β	5.1	14.1	375.3	598.1
B cell	Normal	6.2	19.9	272.5	469.5
	TGh β	9.8	21.5	291.1	220.1
Monocytes	Normal	12.9	29.9	229.1	220.1
	TGh β	13.1	23.0	285.7	327.5
NK	Normal	6.3	5.4	47.7	9.0
	TGh β	3.5	7.7	46.1	9.9

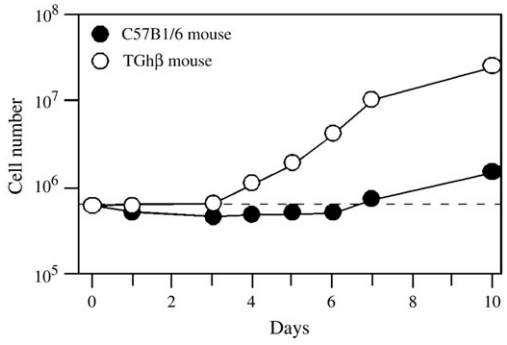
Each cell subset was first labeled with PE-conjugated mAb specific for CD4, CD8, B, NK lymphocytes and monocytes. mAbs specific for mIL-2Rα, mIL-2Rβ and mIL-2Rγ were used to label the three murine IL-2R chains. The cells were then stained by FITC-conjugated mAb specific for the κ chain of the anti-mIL-2R chains and for the κ chain of the control mAb. A single fluorescence peak was observed for CD4 and B lymphocytes, as well as for NK cells and monocytes. CD8 lymphocytes were divided into IL-2R low (~50%) and IL-2R high (~50%). The results for each subset are reported as MFI for IL-2Rα, IL-2Rβ and IL-2Rγ chains. Four experiments were performed with hIL-2Rβ transgenic mice and with C57BL/6 mice, and all gave similar results.

well as in monocytes and NK cells, and a single fluorescence peak was observed. The results were therefore expressed as MFI. In Table 1 it can be seen that CD4 lymphocytes showed low-level but significant expression of mIL-2Rβ and γ_c, with comparable expression in transgenic and wild-type animals. B lymphocytes and monocytes expressed high levels of mIL-2Rβ and γ_c chains both in hIL-2Rβ transgenic and C57BL/6 animals. Similarly in NK cells, the hIL-2Rβ chain did not affect the pattern of mIL-2Rβ and mIL-2Rγ_c expression. But the pattern of mIL-2R expression was different in CD8 lymphocytes where two cell groups could be distinguished, as previously suggested (25). Half the CD8 cells expressed large amounts of IL-2Rβ and γ_c (IL-2R high) whereas the other half expressed only small amounts of IL-2Rβ and γ_c (IL-2R low). The MFI for IL-2α, IL-2Rβ and IL-2Rγ is given for these two subtypes in Table 1. Here again, we observed that constitutive expression of the hIL-2Rβ chain did not modify the mIL-2R phenotype of the CD8 lymphocytes from hIL-2Rβ transgenic mice. The data reported here confirm and extend some of our previous observations concerning the pattern of IL-2R chain expression in the mouse (21, 26) and demonstrate that over-expression of hIL-2Rβ has no major effect on mIL-2R chain expression.

Overgrowth of CD8 lymphocytes and NK cells in IL-2 cultured spleen cells from hIL-2Rβ transgenic mice

A comparison was made of the growth of spleen cells from C57BL/6 and hIL-2Rβ transgenic mice cultured in the presence of IL-2 (Fig. 2A). Despite the expression of high levels of mIL-2Rβ and γ_c chain in some CD8 T lymphocytes, B lymphocytes and monocytes, spleen cells from C57BL/6

A



B

		Day 0	Day 3	Day 7
Total cell number		500,000	631,000	10,280,000
Total cell / subset	CD4	82,500	121,200	82,200
	CD8	74,000	256,200	5,109,200
	NK	30,000	94,700	5,345,600
% of population	CD4	16.5	19.2	0.8
	CD8	14.8	40.6	49.7
	NK	6.0	15.0	52.0
Growth coefficient	CD4		1.5	1.0
	CD8		3.5	69.0
	NK		3.2	178.2

C

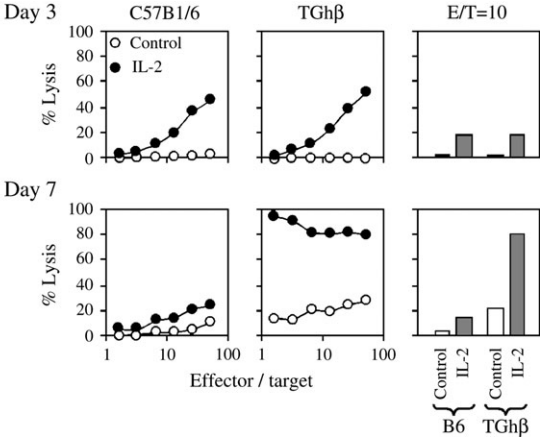


Fig. 2. IL-2 responsiveness of lympho-mononuclear cell subsets from spleens of C57BL/6 and hIL-2Rβ transgenic mice. (A) Comparison of the IL-2-induced growth of splenocytes from C57BL/6 and hIL-2Rβ transgenic mice. Splenocytes were seeded in IL-2-containing medium (10 ng ml^{-1}). The cell count was recorded over a period of 10 days. (B) IL-2-induced growth of CD4, CD8 and NK cells from hIL-2Rβ transgenic mice. Cells were seeded in IL-2-containing medium. The number of cells in each cell subset was evaluated with specific mAb as in Fig. 1(A). No B lymphocytes or monocytes were recovered in the cultures on day 7. Results are expressed in terms of total number of cells or percentage of each cell subset recovered. The growth coefficient indicates the increase in cell number: 1 indicates that the cell number did not change. (C) Induction of LAK cells in splenocytes from C57BL/6 and hIL-2R transgenic mice stimulated with IL-2 (10 ng ml^{-1}). Cultures were tested for their ability to lyse YAK target cells. Percentage of lysis at different E/T ratios is shown. A schematic representation of the data is given on the right side of the figure: after 7 days of culture, data for percentage lysis at an E/T ratio of 10 are shown as histograms.

mice were found to grow very poorly even after 10 days of culture. This was consistent with previous results demonstrating that the IL-2Rβγ_c is not functional in mice. In a representative experiment, the cell population consisted of 0.1% CD4 lymphocytes, 25.2% CD8 T lymphocytes and 53.1% NK cells after 10 days of culture. In agreement with previous observations, the growth of these cells was attributed to induction of the IL-2Rα chain (15).

By contrast, lymphocytes from hIL-2Rβ transgenic mice grew strongly in IL-2-containing medium since the total number of cells increased 10-fold after only 1 week of culture (Fig. 2A). The cells were phenotyped on days 3 and 7 of culturing (Fig. 2B). The total number of CD4 lymphocytes did not vary whereas, as previously described, the CD8 lymphocytes grew very strongly (23). Under these experimental conditions, the NK lymphocytes expressing the NK 1.1 marker also grew very strongly and amounted to 15% of the population by day 3 and >50% by day 10. After 7 days of culture, the cells were either CD8 or NK lymphocytes (Fig. 2B). No B cells or monocytes were recovered in IL-2-cultured splenocytes from hIL-2β transgenic mice.

To further characterize the extensive growth of NK cells, we checked the LAK activity of our cultures using YAK targets. On day 3, activity was comparable in C57BL/6 and hIL-2Rβ transgenic spleen cells cultured in the presence of IL-2, but after 10 days the LAK activity of spleen cells from hIL-2Rβ transgenic mice was very pronounced and probably reflects both the functional activation of the cells and their growth (Fig. 2C).

Comparison of the early IL-2 responses of CD4 and CD8 lymphocytes from hIL-2Rβ transgenic mice

Early signaling events were studied in CD4 and CD8 lymphocytes purified from the spleens of hIL-2β transgenic mice. The first step in the study was to investigate the IL-2 reactivity of these purified populations. Figure 3(A) shows that purified CD4 lymphocytes proliferated only weakly in response to IL-2 whereas purified CD8 lymphocytes divided after IL-2 stimulation alone. After stimulation by sub-optimal doses of anti-CD3 plus IL-2, both subsets proliferated intensely. Furthermore, it was verified that the proliferation of the CD8 T cells from hIL-2β transgenic mice was independent of IL-2Rα expression (Fig. 3B).

Since the CD4 lymphocytes were unable to proliferate in the presence of IL-2, the functionality of their IL-2R was checked by comparing the tyrosine phosphorylation profiles of purified CD4 and CD8 cells after IL-2 stimulation. Purified CD4 and CD8 lymphocytes, initially maintained in culture in the absence of IL-2 for 8 h, were then exposed for 10 min to IL-2 (10 ng ml^{-1}), sub-optimal doses of anti-CD3 antibody or sub-optimal doses of anti-CD3 plus IL-2. The tyrosine phosphorylation profiles of the corresponding cell lysates were then analyzed. Figure 4(A) shows that the profiles were similar in the CD4 and CD8 cells. Despite relatively high background noise, two tyrosine-phosphorylated proteins were observed to be specifically induced by IL-2. These proteins, indicated by arrows in Fig. 4(A), were observed in the presence of IL-2 in cells pre-treated or not with anti-CD3. These results indicate that both cell subsets express a functional IL-2R able to induce the early stages of the IL-2-induced signaling cascade.

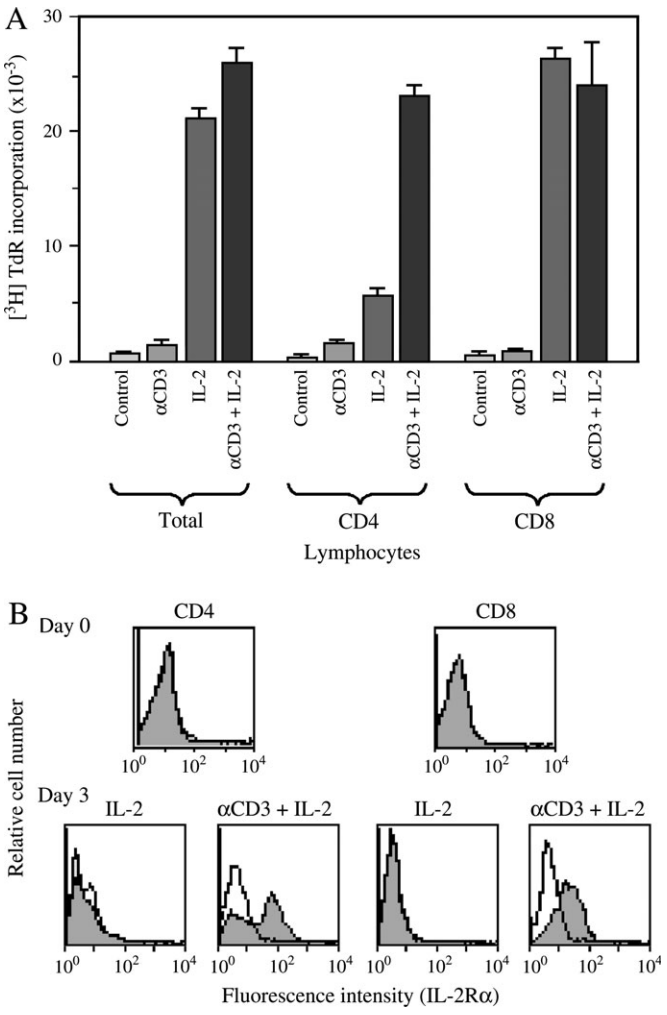


Fig. 3. Proliferation and IL-2Rα chain expressive after IL-2 stimulation of purified CD4 and CD8 lymphocytes from hIL-2Rβ transgenic mice. (A) Proliferation of purified CD4 and CD8 lymphocytes from IL-2Rβ transgenic mice after anti-CD3, IL-2 or anti-CD3 plus IL-2 stimulation. [^{3}H]TdR incorporation was measured on day 3 of the culture as described in Methods. (B) IL-2Rα expression by purified CD4 and CD8 lymphocytes from hIL-2R transgenic mice. The purified cells were challenged for 3 days with either IL-2 or sub-optimal doses of anti-CD3 plus IL-2. IL-2Rα expression was measured after labeling with 5A2 mAb and staining with FITC-conjugated MARK-1 mAb (see Methods).

As mentioned earlier, several extensively studied signaling pathways play a major role in the propagation of IL-2 signaling. The Jak/STAT cascade is one of the pathways that is strongly activated in response to IL-2. In Fig. 4(B), purified CD4 and CD8 lymphocytes were treated as described above. The use of an antibody specific for phosphorylated STAT5 molecules (upper panel) showed that both the CD4 and CD8 populations responded rapidly to IL-2 and evidenced marked phosphorylation of STAT5, both in the presence of IL-2 alone and in the presence of IL-2 plus a sub-optimal dose of anti-CD3. STAT5α and STAT5β expression profiles were also analyzed as a control (Fig. 4B, middle and lower panels). Under the same experimental conditions, we then studied the phosphorylation status of Erk kinase using a specific phospho-Erk antibody (Fig. 4C, upper panel). Erk phosphorylation was

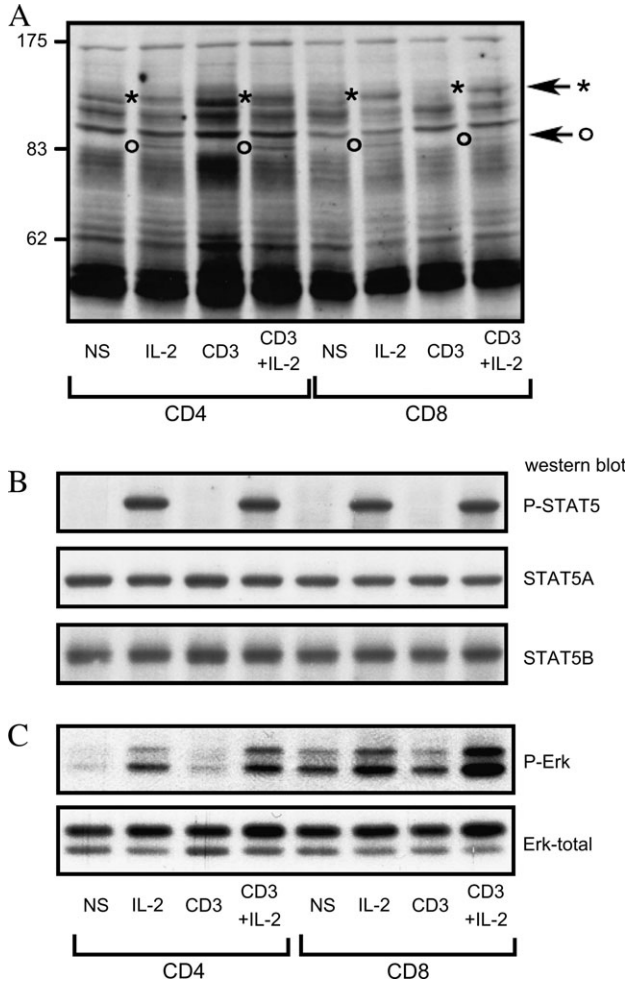


Fig. 4. IL-2-induced early signaling events in CD4 and CD8 lymphocytes from hIL-2Rβ transgenic mice. (A) Cells were incubated for 8 h in IL-2-free medium. The cells were then exposed for 10 min to IL-2, to sub-optimal doses of anti-CD3 or to sub-optimal doses of anti-CD3 plus IL-2. Cell lysates were separated by gel electrophoresis, transferred to PVDF membranes and blotted with anti-phosphotyrosine mAb (4G10). Two proteins phosphorylated in the presence of IL-2 are indicated by arrows. (B) Lysates prepared as above were separated by gel electophoresis, transferred to PVDF membranes and blotted with an anti-phosphorylated STAT5 (P-STAT5, upper panel). The same membrane was dehybridized and subsequently treated with anti-STAT5α or anti-STAT5β antibodies, as indicated (lower panels). (C) Lysates prepared as above were visualized using an anti-phosphorylated Erk antibody (P-Erk, upper panel). After dehybridization, the membrane was treated with anti-Erk antibodies.

induced by IL-2 in both the purified CD4 and CD8 populations. A western blot performed against total Erk protein is shown as a control (Fig. 4C, lower panel). Despite the fact that purified CD4 and CD8 lymphocytes expressed the same amount of Erk, purified CD8 lymphocytes showed a higher basal level of Erk phosphorylation than CD4 lymphocytes in the absence of stimulation. But, the enhanced phosphorylation of Erk appeared to be equivalent in both cell subsets. In conclusion, it was shown that although the CD4 lymphocytes failed to proliferate, they expressed a functional IL-2R alone able to induce early signaling events comparable to those noted after anti-CD3 plus IL-2 stimulation. The same signals were found in IL-2-activated purified CD8 lymphocytes.

CD4 lymphocytes from hIL-2Rβ transgenic mice do not enter the cell cycle after IL-2 stimulation

An analysis was performed of the ability of CD4 and CD8 T lymphocytes from hIL-2Rβ transgenic mice to progress through the cell cycle. As shown in Fig. 5(A), when stimulation was provided by IL-2 alone, only the CD8 cells progressed through the cell cycle: 38% of $CD8^+$ cells were in the S phase after 6 days of culture in the presence of IL-2. Under the same conditions, the number of CD4 lymphocytes in the S phase was very similar to the response in the absence of IL-2. It was observed that CD4 lymphocytes entered the S phase only in response to sub-optimal doses of anti-CD3 plus IL-2. These results are consistent with those obtained by $[^3H]$TdR incorporation. However, for unknown reasons, the response measured with propidium iodide was weaker than when proliferation was measured by $[^3H]$TdR incorporation (Fig. 3A). By contrast, when stimulated by sub-optimal doses of anti-CD3 plus IL-2, the CD8 cells showed intense entry into the S phase of the cell cycle. No sub-diploid cells were observed in this study, indicating that CD4 and CD8 lymphocytes from hIL-2Rβ transgenic mice are equally susceptible to apoptosis.

We then studied some of the regulatory proteins that control progression through the cell cycle, e.g. the well-known CDK inhibitors of the Kip family (27, 28). No difference was detected in the p21 expression of CD4 and CD8 lymphocytes from hIL-2Rβ transgenic mice (data not shown). By contrast, clear differences were observed between CD4 and CD8 for $p27^{kip1}$ expression. CD4 lymphocytes showed down-regulation of $p27^{kip1}$ after anti-CD3 plus IL-2 stimulation whereas $p27^{kip1}$ continued to be strongly expressed after stimulation by IL-2 alone. On the other hand, $p27^{kip1}$ was decreased under both conditions in CD8 lymphocytes (Fig. 5B, upper panels). If purified CD4 and CD8 lymphocytes are not stimulated, they do not survive well *in vitro*. Therefore, the levels of $p27^{kip1}$ expression were examined as a control at early time points (cells starved for 8 h). Under these conditions, we failed to detect any significant difference in $p27^{kip1}$ levels of expression between CD4 and CD8 lymphocytes (Fig. 5B, lower panels). These results establish a correlation between the ability of CD4 and CD8 lymphocytes to progress through the cell cycle after IL-2 stimulation and the regulation of $p27^{kip1}$ levels.

Discussion

This study confirms and extends the notion that CD4 and CD8 lymphocytes show different response patterns to IL-2. It also provides new insights into the mechanisms involved. Our investigations were based on the use of hIL-2Rβ transgenic mice that we further characterized in the course of the work undertaken.

Unexpectedly, constitutive expression of the IL-2Rβ chain affected the *in vivo* development of lymphocytes and monocytes in hIL-2Rβ transgenic mice. It was shown that hIL-2Rβ transgenic mice possess more monocytes and less B lymphocytes than their wild-type counterparts. The mechanisms possibly explaining this observation remain hypothetical.

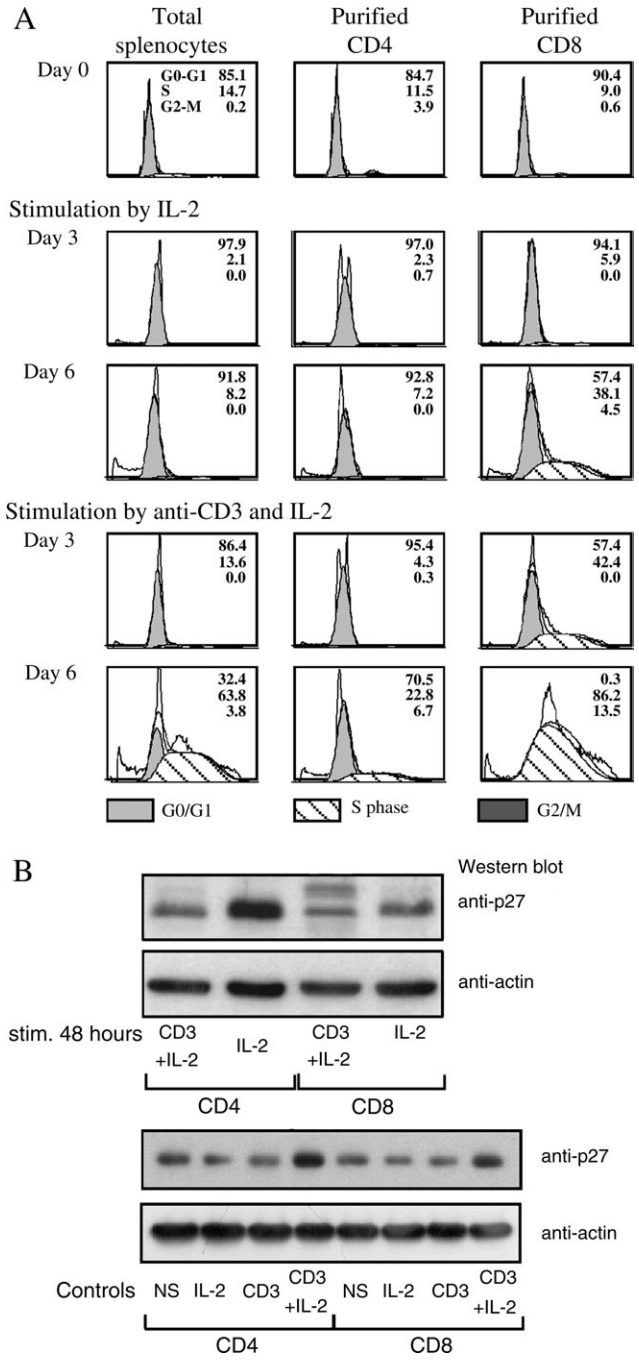


Fig. 5. Cell cycle progression of purified CD4 and CD8 lymphocytes from hIL-2Rβ transgenic mice and $p27^{kip1}$ expression after IL-2 stimulation. (A) Purified CD4 and CD8 lymphocytes were cultured for 6 days in the presence of IL-2 or sub-optimal doses of anti-CD3 antibodies plus IL-2. The proportion of cells in each phase of the cell cycle was measured on days 3 and 6 as described in Methods. The proportion of cells in the G0/G1, S and G2/M phases of the cell cycle is indicated in each panel. (B) Purified CD4 and CD8 were cultured in the presence of the indicated stimulus (IL-2 or sub-optimal doses of anti-CD3 plus IL-2). After 48 h, the cells were collected, lysed, separated by SDSP and transfered onto PVDF filters. The filters were blotted with anti-$p27^{kip1}$ antibodies. To measure the initial level of $p27^{kip1}$ expression, similar blots were performed with lysates from cells treated as described in Fig. 4 (A and B). Anti-actin blots were performed as a loading control.

Concerning NK cell development, our results differed from those reported by Suwa *et al.* (24) who showed that no NK 1.1 cells were detectable in mIL-Rβ transgenic animals. This discrepancy is unexplained. However, it is possible that the level of IL-2Rβ expression may affect NK cell development. The promotor used by Suwa *et al.* (chicken β-actin with CMV-IE enhancer) may induce more IL-2Rβ expression than the $H\text{-}2^k$ promotor used by Asano *et al.* (23). Furthermore, Suwa *et al.* used the mIL-2Rβ gene for their construct whereas Asano *et al.* used the hIL-2Rβ gene. Since mIL-2Rβ does not bind IL-2, this may also affect the formation of IL-15R and as yet unknown receptors that impact NK cell development.

Each IL-2R gene is independently regulated. IL-2Rα is induced by IL-2 (26, 29–32) whereas IL-$2R\gamma_c$ is constitutively expressed (33–35). The regulation of IL-2Rβ has been studied less extensively. Here, we studied the effect of constitutive IL-2Rβ expression on the three mIL-2R chains and noted that the cell-surface expression of mIL-2Rα, β and γ_c chains was unaffected by the hIL-2Rβ transgene. We previously discussed the hypothesis that IL-$2R\gamma_c$ precursor stored in the cytoplasm of CD4 lymphocytes may use activation-induced IL-2Rβ expression for maturation and translocation to the cell surface (35). The results presented here do not support this hypothesis since IL-$2R\gamma_c$ expression was comparable in CD4 lymphocytes from both C57BL/6 and hIL-2Rβ transgenic mice.

When we compared the IL-2-induced growth of spleen cells from C57BL and hIL-2Rβ transgenic mice, we noted that spleen cells from C57BL/6 animals started to grow only by day 10 whereas those from hIL-2Rβ transgenic animals started to grow by day 3. The growth of spleen cells from C57BL/6 is delayed by the absence of mIL-2Rα expression since mouse lymphocytes need this chain for IL-2 recognition (15). Therefore, cells from C57BL/6 mice growing *in vitro* must be selected from the rare lymphocytes expressing the mIL-2Rα chain. By contrast, the hIL-2Rβ–mIL-$2R\gamma_c$ complex forms a functional receptor enabling spleen cells from hIL-2Rβ transgenic mice to start their growth early. The regulation of IL-2Rα expression has already been the subject of laboratory studies. *In vivo*, IL-2Rα is only expressed by activated cells of secondary lymphoid organs (36). *In vitro*, IL-2 stimulation of mouse T cell lines transfected by hIL-2Rβ does not lead to IL-2Rα induction (15, 37). These data and the results presented in Fig. 3(B) indicate that other signals in addition to IL-2 are required for IL-2Rα induction. The results obtained with hIL-2Rβ transgenic mice highlighted the intrinsic capacity of CD8 and NK cells to multiply in the presence of IL-2. Multiple experiments confirmed that CD8 lymphocytes grew very strongly (23). Furthermore, we found that the growth of NK lymphocytes was even more intense. By contrast, despite high levels of IL-2R expression, CD4 lymphocytes, B cells and monocytes failed to grow. CD4 lymphocytes survived without entering into apoptosis whereas B cells and monocytes progressively disappeared from the cultures. Our results do not support the notion that IL-2 in hIL-2Rβ transgenic mice induces a sub-population of cells able to suppress the growth of CD4 lymphocytes.

The molecular mechanisms behind the difference observed for IL-2 responsiveness in CD4 and CD8 lymphocytes were analyzed using highly purified cell sub-populations. The initial approach was to study the induction of early events following

the binding of IL-2 to its receptor. The Jak/STAT pathway is part of these early events. The phosphorylation of Jak-1 and Jak-3 kinases, constitutively associated with IL-2Rβ and IL-2Rγ_c subunits, induces the phosphorylation of a docking site in IL-2Rβ. This is followed by STAT5 recruitment to the phosphorylated receptor complex, STAT5 phosphorylation, dimerization, nuclear translocation and transactivation of target genes. The functional capacity of the heterospecific IL-2R (hIL-2Rβ/mIL-2Rγ_c) expressed by the purified CD4 and CD8 lymphocytes from hIL-2Rβ transgenic mouse was assessed by first measuring the phosphorylation pattern obtained after IL-2 stimulation. Two of these bands co-migrated with STAT5. We showed that STAT5 in IL-2-stimulated CD4 and CD8 lymphocytes was also phosphorylated. Furthermore, under the same experimental conditions, IL-2 in both populations also strongly and rapidly induced Erk phosphorylation. But, CD8 lymphocytes had a higher basal level of Erk phosphorylation in the absence of stimuli. At this time, we cannot rule out a possible relationship between this higher basal level of Erk phosphorylation and the ability of CD8 lymphocytes to show a rapid proliferative response in the presence of IL-2. In conclusion, it is very clear that the IL-2R expressed by the CD4 and CD8 lymphocytes from hIL-2Rβ transgenic animals is functional at least for inducing the early events in one of the well-known IL-2 signaling cascades.

To investigate the inability of CD4 lymphocytes from hIL-Rβ transgenic animals to enter the cell cycle despite STAT5b activation, we examined the expression of cyclin-dependent kinase inhibitors (CKIs) since these play a key role in regulating cell cycle progression from the G1 to S phase (27). These inhibitors fall into two categories: the INK4 family ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$) and the CIP/KIP family ($p21^{WAF1/CIP1}$, $p27^{Kip1}$ and $p57^{Kip2}$). INK4 family members specifically inhibit the activity of cdk4 and 6, whereas CIP/KIP family members have a broader action. The precise regulation of the level of expression of these CKIs has been demonstrated to be crucial for S-phase entry. However, the exact mechanism by which these proteins exert their action is still a matter of debate (28). CD8 lymphocytes from hIL-2Rβ transgenic mice showed down-modulation of $p27^{kip1}$ when exposed to IL-2. This was not the case for CD4 lymphocytes. This was confirmed by the fact that anti-CD3 plus IL-2 decreases $p27^{kip1}$ expression in both CD4 and CD8 lymphocytes. This correlation between IL-2 responsiveness and $p27^{kip1}$ down-modulation suggests that CD4 T lymphocytes, unlike CD8 lymphocytes, need to be appropriately activated to down-modulate $p27^{kip1}$ and enter the cell cycle under the effect of IL-2 alone.

Our results illustrate the pleiotropic effects of IL-2 at the molecular level. Many IL-2-regulated genes have been characterized, including those involved in cytoskeleton organization, oncogene regulation and transcriptional control (38–40). IL-2 has been shown to affect the regulation of cyclin D3 (41). In our study we observed that IL-2 also participates directly or indirectly in the regulation of the cell cycle through the regulation of CKI $p27^{kip1}$ expression. At the immunological level, our results further confirm that the growth of CD4 lymphocytes is strictly regulated, in compliance with the crucial function of these cells in the regulatory processes controlling both cellular and humoral responses. By contrast, our observations support the notion that regulation of the growth of the CD8 lymphocyte subset is more flexible. The requirement of CD8 lymphocytes for T_h is not stringent (42). Under some conditions, no help from T cells is necessary for CTL induction. More recently, in full agreement with our results, a model of *Listeria* infection was used to show *in vivo* that the proliferative capacities of CD4 and CD8 lymphocytes are very different (43, 44). Furthermore, a sub-population of CD8 lymphocytes (CD8CD44high) that can spontaneously respond to IL-2 (45, 46) has also been described. However, these responding cells are related to NK cells since they express NKRs, including 2B4 and NKG2D (46).

Finally, our study may be discussed both in fundamental and clinical terms. At the fundamental level, it further supports the concept that cytokine responsiveness in cells of the specific branch of the immune system is not strictly controlled by cytokine receptor expression; for though this is necessary, it is not sufficient to confer full competence to respond to a cytokine. Therefore, as yet unknown lineage-specific mechanisms are also involved. CD4 and B lymphocytes respond only weakly to IL-2 even though they express functional IL-2R. In order to fully respond to IL-2, they first need to be activated by TCR or BCR. The presence of IL-2 in B cells is necessary during the activation phase (21). In CD4 lymphocytes, this is impossible to study since these cells produce IL-2 after activation. Of the cells involved in acquired immunity, CD8 lymphocytes appear to be the only subset in which IL-2R expression is the sole parameter controlling IL-2 responsiveness. At the clinical level, despite several limitations, IL-2 is currently used to enhance T cell responses to viral or tumor antigens in patients, including those with HIV or metastatic cancer (47–53). Over and above an activation of NK cells and monocytes, our results suggest that IL-2-based immunotherapy could easily induce CD8 lymphocyte expansion when these cells express IL-2R. By contrast, expansion of the CD4 compartment would require appropriate antigenic stimulation in addition to IL-2R expression to fully recover an effective T_h function.

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Abbreviations

CKI	cyclin-dependent kinase inhibitor
E/T	effector to target cell
hIL-2Rβ	human IL-2Rβ
[^{3}H]TdR	[^{3}H]thymidine
MFI	mean fluorescence intensity
mIL-2R	mouse IL-2R
STAT	signal transducer and activation of transcription
TBS-T	Tris-buffered saline-Tween

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