

# Propagation of lewis rat encephalitogenic T cell lines: T-cell-growth-factor is superior to recombinant IL-2

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## Abstract

This study was designed to test the process of selecting encephalitogenic T cell lines in the Lewis rat using recombinant human IL-2 (rhIL-2) in comparison to TCGF. The lines were tested for growth, antigen induced proliferation, cytokine production, V-beta 8.2 expression and pathogenicity. We now report that rhIL-2 and TCGF were equally effective in supporting short-term pathogenic T-cell lines with similar proportion of V-beta 8.2 usage. For the maintenance of long term lines, however, TCGF was superior to IL-2. The concentration of rhIL-2 influenced the cultures: 10 units/ml led to more T-cell proliferation than either 2 or 50 units/ml. However, 50 units/ml of IL-2 led to enhanced Th1 polarization. Thus, the type and concentration of growth factors can influence both the propagation of T cells and their phenotype. © 2002 Elsevier Science B.V. All rights reserved.

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## 1. Introduction

The ability to grow long term functional T cell lines and clones has considerably enhanced the study of experimental autoimmune diseases (Ben-Nun et al., 1981; Gold, 1994; Olive, 1995). Characterization of the TCR restriction of pathogenic cells (Gold, 1994) as well as the T helper type (Lafaille et al., 1997), migration patterns (Naparstek et al., 1983), anti-clonotypic T-cells (Lider et al., 1988) and T cell–tissue interactions (Wekerle et al., 1991) were achieved using T cell lines and clones. The basic reagent that has enabled this progress was the discovery of T cell growth factors (TCGF) (Gillis et al., 1978a,b) later characterized as interleukin-2 (Smith, 1988). The basic methodology of using the supernatant of Concanavalin-A stimulated spleen cultures as a source of IL-2 was introduced over 20 years ago (Ben-Nun et al., 1981; Gillis et al., 1978a,b). The art of derivation of T cell lines and clones did not significantly change over this period of time: many laboratories still use the same methodology (Flugel et al., 2001; Linington et al., 1984; Ratcliffe et al., 2000; Stienekemeier et al., 1999; Sun et al., 1999). It is well known that the TCGF preparation contains many molecules in addition to IL-2, such as INF- $\gamma$ , IL-4, IL-5, IL-9 (Bancroft

et al., 1993), but their identity and actual concentration in the preparation are not known and could vary between preparations. Moreover, the recent search for alternatives to materials obtained from laboratory animals (Hagelin et al., 1999) points at rIL-2 as a potential alternative to the traditional TCGF.

We initiated this study to compare the culture of encephalitogenic T cells in the traditional manner to culture in varying concentrations of recombinant human IL-2 (rhIL-2). We found that T cell lines propagated on rhIL-2 were similar in proliferative capacity, cytokine secretion pattern, V-beta 8.2 expression and pathogenicity. The long-term viability and cell numbers were better using TCGF followed by a 10 units/ml of rhIL-2. Thus, functional pathogenic T cell lines can be cultured in rhIL-2, the optimal IL-2 concentration is 10 units/ml, but for extended culture TCGF appears to be superior to rhIL-2.

## 2. Materials and methods

### 2.1. Animals

Inbred female Lewis rats were supplied monthly by the animal breeding center at the Weizmann Institute of Science (under the supervision of Harlan Laboratories) and were used at 2–3 months of age.

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## 2.2. Antigens and antibodies

*Mycobacterium tuberculosis* H37Ra was purchased from Difco, Detroit, MI. The myelin basic protein peptide p71–87 was synthesized using the F-MOC technique with an automatic multiple peptide synthesizer (AMS 422, ABIMED, Langenfeld, Germany). Guinea-pig myelin basic protein p71–87 is the major encephalitogenic determinant in the Lewis rat (Mor and Cohen, 1993) and its sequence is SLPQKSQRSQDENPVVH. The purity of the peptide was analyzed by HPLC and amino acid composition. Monoclonal antibodies to V $\beta$  8.2 and V $\beta$  16 were purchased from Pharmingen, San Diego, CA. FITC conjugated rabbit-anti-mouse antibody was obtained from Jackson Immuno Research, West Grove, PA.

## 2.3. Immunizations

The p71–87 peptide was dissolved in PBS (1 mg/ml), and an oil emulsion was prepared (1:1 ratio) with IFA containing 4 mg/ml *Mycobacterium tuberculosis* H37Ra. Naive female Lewis rats were immunized in both hind footpads with 50  $\mu$ l of the emulsion; each rat was injected with 50  $\mu$ g of peptide. Draining popliteal lymph node cells were removed on day 12 after injection and a single cell suspension was prepared by pressing the organs through a fine wire mesh, and the cells were studied *in vitro*.

## 2.4. T cell proliferation assay

T-cell proliferation assay was performed by seeding  $5 \times 10^4$  Line cells (at the 4–5 day in propagation phase) with  $5 \times 10^5$  irradiated thymocytes as antigen presenting cells, in stimulation medium for 3 days, in 96 micro-titter round bottom wells (Nunc, Roskilde, Denmark). Stimulation medium was composed of RPMI supplemented with 2 mercaptoethanol ( $5 \times 10^{-5}$  M), L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (100 units/ml), streptomycin (100  $\mu$ g/ml), non-essential amino acids (1 ml/100 ml, Bio Lab Jerusalem, Israel), and autologous serum 1% (v/v) (Mor et al., 1990). The cultures were incubated in triplicate for 72 h at 37 °C in humidified air containing 7% CO<sub>2</sub>. Each well was pulsed with 1  $\mu$ Ci of [<sup>3</sup>H] thymidine (10 ci/mmol sp.act. Amersham, Buckinghamshire, England) for the final 4 h. The cultures were then harvested using a MicroMate 196 Cell Harvester and cpm were determined using a Matrix 96 Direct Beta Counter using avalanche gas (98.7% helium; 1.3% C<sub>4</sub>H<sub>10</sub>) ionization detectors (Packard Instrument Company, Meriden, CT, USA). The results of proliferation are expressed as cpm.

## 2.5. T cell lines

Antigen-specific T cell lines were established from lymph node cells that had been stimulated with Gp71–87 (10  $\mu$ g/ml) for 3 days in stimulation medium as described above.

Following stimulation, the T-cell blasts were isolated on Lympho-prep (Nycomed Pharma, Oslo, Norway) and seeded in propagation medium. Propagation medium was identical to stimulation medium without autologous serum, but supplemented with fetal calf serum 10% and T cell growth factors from the supernatant of Con A stimulated spleen cells 10% (Mor et al., 1990), or 2, 10 or 50 units/ml recombinant human IL-2 (Tecin™ from Hoffmann-LaRoche; generously provided by Dr. C.W. Reynolds, Biological Resources Branch, National Cancer Institute Preclinical Repository). Four days after seeding, the cells ( $5 \times 10^5$ /ml) were re-stimulated with peptide, and irradiated thymocytes as antigen presenting cells ( $10^7$ /ml) for 3 days in stimulation medium. T-cell lines were expanded by repeated stimulation with antigen and irradiated thymocytes as antigen presenting cells every 10–12 days (Mor and Cohen, 1993). After each stimulation cycle, the lines were tested in a proliferation assay at the end of the propagation phase. Recombinant mouse IL-2 was purchased from Pharmingen, with the activity of  $2.5 \times 10^6$  units per mg protein.

## 2.6. Induction of EAE

Adoptive EAE was transferred by intraperitoneal injection of  $1.2 \times 10^7$  peptide-activated cells of the lines as described (Mor and Cohen, 1995). Clinical EAE was observed 4–5 days following administration of T cell lines. Clinical scoring was: +1, paralysis of tail; +1.5, paresis of posterior paws and ataxia; +2, paraplegia; +3, paralysis extending to thoracic spine; +4, a moribund state (Mor and Cohen, 1995).

## 2.7. Flow cytometry

Line cells were incubated at 4 °C for 45 min. with anti-V $\beta$  8.2 or anti-V $\beta$  16 antibodies that were obtained from Pharmingen. Secondary rabbit anti-mouse F.I.T.C. conjugated antibodies were incubated at 4 °C for 30 min. The cells were then washed and fluorescence was measured using the FACSCAN (Becton Dickinson). To exclude apoptotic cells from the analysis, cells were stained with propidium iodide (Sigma), and the gating was performed to unstained cells (Carter et al., 1992). Analysis of the results was done using Lysis II software.

## 2.8. Cytokine ELISA assays

Supernatants were collected after three days of stimulation of the T-cell lines with p71–87 peptide. IL-10 and IFN $\gamma$  in the culture supernatants was measured by ELISA using Pharmingen's OPTEIA kit (Pharmingen). Pharmingen recombinant rat cytokines were used as standards for calibration curves. Briefly, ELISA plates (Maxisorp; Nunc) were coated overnight at 4 °C with anti-rat cytokine monoclonal capture antibodies. Non-specific binding was blocked by incubation with BSA 1% for 1 h at room temperature, and

the culture supernatants or recombinant cytokines were added for 2 h at RT. After washing the plates, biotinylated detection antibodies were added for 1 h at room temperature, and then extensively washed and incubated with streptavidin conjugated alkaline phosphatase (Jackson Labs) for 30 min at room temperature. The plates were washed, and substrate for alkaline phosphatase (Sigma, Rehovot, Israel) was added, and the samples were read at 405 nm after 45 min of incubation at room temperature. The results are expressed as pg/ml based on calibration curves constructed using recombinant cytokines as standards. The lower limits of sensitivity for the experiments described were 30 pg/ml for both IL-10 and IFN $\gamma$ .

### 3. Results

#### 3.1. Proliferation of rat T cells to recombinant human IL-2

Upon activation, T cells express high affinity IL-2 receptors composed of  $\alpha$ ,  $\beta$  and  $\gamma$  chains (Takeshita et al., 1992). To verify that the human recombinant IL-2 is mitogenic to rat T cells, a rat antigen-specific T cell line in the propagation phase ( $5 \times 10^4$ /well) was incubated with graded concentrations of rhIL-2, and in comparison recombinant mouse IL-2. The results are shown in Fig. 1. It is evident that the human cytokine is mitogenic to the rat T cells, and the activity of the mouse IL-2 is very similar to the human cytokine. The IL-2 content in rat TCGF was measured and found to be 20 units/ml, thus the final concentration of IL-2 in the prepared TCGF medium is the equivalent of 2 units/ml of rhIL-2. Since the proliferative effects of mouse and human IL-2 were similar (Fig. 1), and in several experimental settings the human cytokine was found to be active in the rat system (Bellinger et al., 2001; Thornton et al., 2000; Xing et al., 2000; Zhang et al., 1993) in the long term culture experiments we tested rhIL-2 in comparison to rat TCGF.

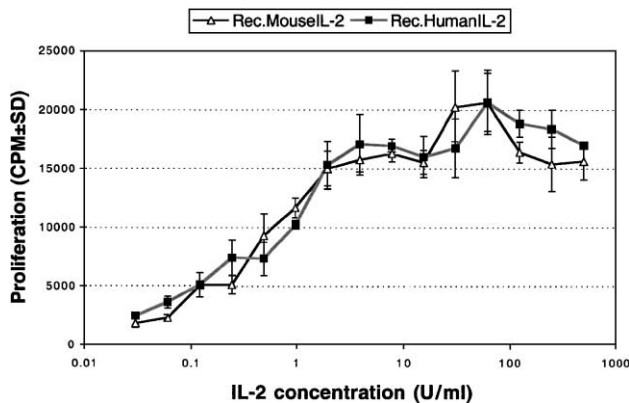


Fig. 1. Rat line T cells respond to rhIL-2. Proliferative response of anti MBP line cells in the propagation phase to recombinant human or mouse IL-2.  $5 \times 10^4$  line cells were plated in micro-titer round bottom 96 well plates, in doubling dilutions of IL-2, from 500 to 0.03 units/ml for 72 h. In the last 4 h, the wells were pulsed with  $3[H]$  thymidine and harvested.

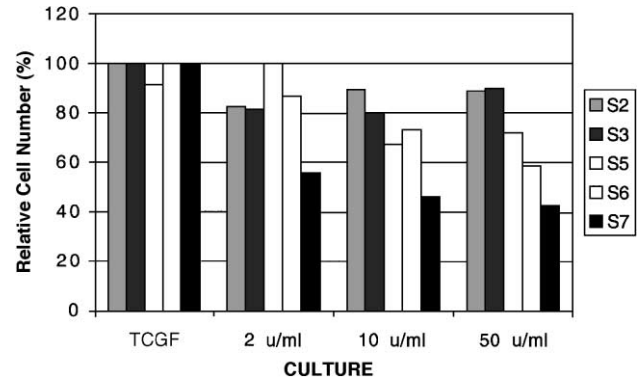


Fig. 2. Relative cell numbers of lines after successive cycles of stimulation. At the stimulations indicated, (S2 indicates the second stimulation, S3 the third, etc.) cells in the four propagation protocols (TCGF; 2, 10 and 50 units/ml indicate the IL-2 concentration in propagation medium in units/ml) were counted and expressed as percentage of the line with the maximal number of cells.

#### 3.2. Line growth in TCGF versus rhIL-2

Under optimal conditions, the growth of an antigen-specific T cell line behaves in an exponential manner with cells multiplying in number in both the stimulation phase (multiplication of 4–10 fold) and in the propagation phase (multiplication of 2–4 fold). The relative cell numbers after several in vitro stimulations in the various propagation protocols is shown in Fig. 2. The protocol that yielded the maximal number of cells was TCGF. At early stimulations, rhIL2 was similar to TCGF but after six to seven stimulations the cell numbers decreased especially in 50 units/ml IL-2. At the propagation phase, early stimulations in 50 units/ml IL-2 gave the maximal number of cells, while in later stimulations, the growth was maximal in TCGF (data not shown).

#### 3.3. T cell line proliferation to the 71–87 peptide

To further examine the effects of TCGF versus graded concentrations of rhIL-2, we performed T cell proliferation assays after consecutive cycles of peptide specific stimulation, at the end of the propagation phase.

In the early proliferations, the response to TCGF medium was equivalent to the 50 units/ml concentration of rhIL-2. At later cycles however, the proliferation of cells incubated in 50 units/ml was significantly reduced (Fig. 3). The cell numbers from these cultures were also reduced. Thus, the effect of IL-2 on T-cell proliferation showed a bell-shaped curve, with the optimal concentration of IL-2 at 10 units/ml. The lower proliferation of the cells at 50 units/ml could be explained by the induction of apoptotic genes, known to be induced by high concentration of IL-2 (Zheng et al., 1998). Examination of the background proliferation (line and APC without added antigen) shows, contrary to expectations, that cells grown in the highest concentration of IL-2 had the lowest background proliferation (Fig. 3).

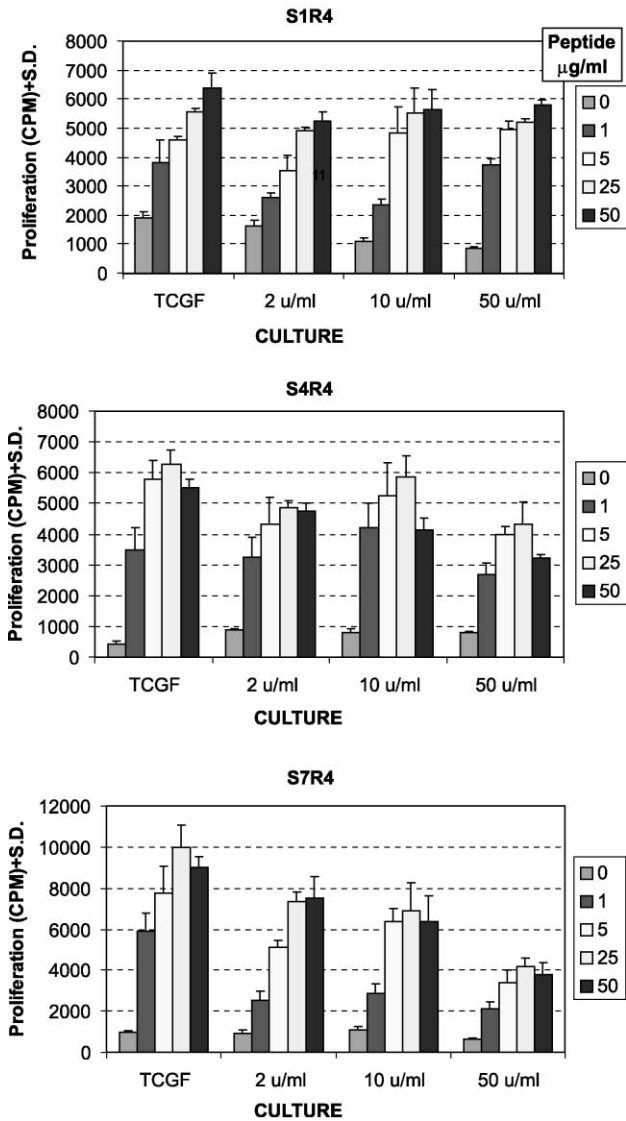


Fig. 3. Proliferation profiles of T cell lines to p71–87. The proliferation assays shown are after one (S1R4) three (S3R4) and seven (S7R4) cycles of stimulation. At the end of the propagation phase  $5 \times 10^4$  line cells were incubated in micro-titer wells with graded concentration of peptide 0, 1, 5, 25 and 50 mcg/ml) with irradiated thymocytes as antigen presenting cells. The cells were harvested after pulsing with  $^3\text{H}$  thymidine for the last 4 h in culture.

### 3.4. Secretion of cytokines by lines maintained on TCGF or IL-2

One of the main functional characteristics of T cells is their cytokine secretion pattern (Murphy et al., 2000). Encephalitogenic T cells in the rat are usually of the Th0 or Th1 phenotype (Weinberg et al., 1994). The results of measurements of  $\text{INF}\gamma$  or IL-10 after three and seven stimulations in vitro are shown in Fig. 4. The levels of  $\text{INF}\gamma$  were high in all the cultures at the two time points, while the levels of IL-10 were significantly lower in the

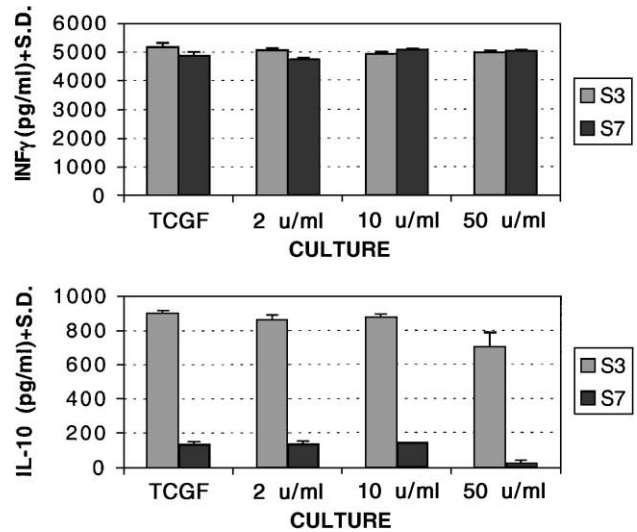


Fig. 4.  $\text{INF}\gamma$  and IL-10 secretion by anti-p71–87 T cell lines propagated in TCGF or IL-2 in the concentrations indicated. The lines were stimulated in vitro for 3 days and the supernatant was assayed for  $\text{INF}\gamma$  and IL-10 in ELISA. The supernatants were collected after the third stimulation (designated S3) and after the seventh stimulation (designated S7).

in 50 units/ml of IL-2. Thus,  $\text{INF}\gamma$  was secreted at high levels by all cultures, while IL-10 production was lower as the cells were repeatedly stimulated with antigen. Interestingly, both  $\text{INF}\gamma$  and IL-10 were detected in TCGF preparation. Thus, the in vitro conditions of raising T cell lines in the Lewis rat are more favorable for the selection of the Th0–Th1 phenotype.

### 3.5. Analysis of the V-beta 8.2 TCR

The availability of a monoclonal antibody to TCR V-beta 8.2 in the rat enables the direct quantitation of the proportion of T cells in culture expressing this TCR V beta family. In previous work, we found that after four to six in vitro stimulations the V-beta 8.2 cells are the dominant population (Mor et al., 2000). Fig. 5 shows the FACS analysis of four different lines at the fifth stimulation. The highest proportion

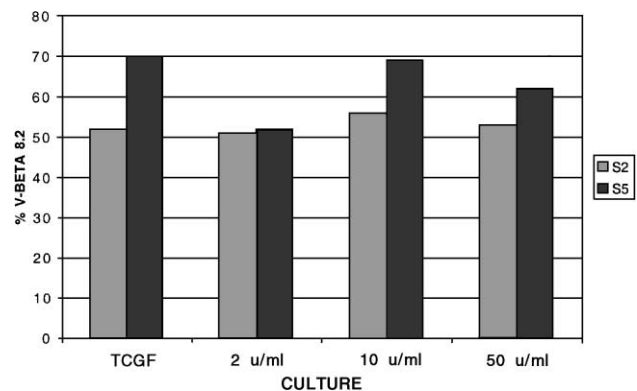


Fig. 5. Percentage of cells expressing V-beta 8.2 in T cell lines maintained in various protocols, at the second in vitro stimulation (indicated as S2) and the fifth stimulation as determined by FACS analysis.

of cells expressing V-beta 8.2 was found in the cells cultured in TCGF (70%), and the lowest was in the cells cultures maintained in 2 units/ml of IL-2 (52%).

### 3.6. Pathogenic potential of the lines

The lines were injected intra-peritoneally ( $10\text{--}12 \times 10^6$  per rat) to groups of naive rats after the third or after the sixth stimulation. All lines were pathogenic with scores of EAE at 1.5 (complete tail paralysis and posterior paws weakness leading to unsteady gait). Thus, culture in rhIL-2 is able to generate of pathogenic T cells similar to T cells raised in TCGF medium.

### 3.7. Transfer from TCGF to rhIL-2

To test the effect of changing the culture protocol from TCGF to rhIL-2, we took an MBP-reactive clone A6 (Mor et al., 1996), which had been raised using TCGF in the traditional manner. The clone cells were maintained in parallel in TCGF, or in 0, 10 or 100 units/ml of rhIL-2. After each cycle of stimulation and rest, the cells were counted, and in parallel, the cells were tested in a proliferation assay. As shown in Fig. 6, the proliferation profile of the cells in high IL-2 concentration deteriorated upon successive stimulations, similar to the phenomenon seen with the T cell lines. With regard to cell numbers, the TCGF and 10 units/ml IL-2 were superior to 0 or 100 units/ml IL-2.

## 4. Discussion

The methodology of raising T cell lines for the study of autoimmune diseases using TCGF was developed over 20 years ago (Ben-Nun et al., 1981), and has been followed ever since, despite the availability of pure recombinant IL-2 (Smith, 1988). The advantages of using rIL-2 include its purity, its controlled concentration and its availability from the NCI, without the need to sacrifice animals for its production.

The present study compared the traditional mode of T cell line culture using TCGF to culture in rhIL-2. For short term cultures (three to four cycles of antigen stimulation), the performance of rhIL-2 was similar to that of TCGF. In long-term cultures, the traditional TCGF medium was superior in supporting the cells. Moreover, at high concentrations of IL-2, there was a paradoxical finding that those cells had the lowest numbers and a larger proportion of the cells was found to be in apoptosis (Lenardo, 1991). A possible mechanism for the toxicity of long-term culture in high concentration of IL-2 could be the induction of apoptotic proteins by IL-2 (Zheng et al., 1998). Thus, IL-2 is essential for the maintenance of cells in culture (Mor and Cohen, 1996), but high doses of the cytokine are toxic to the cells, and lead to enhanced apoptosis and cell loss. The high IL-2 concentration culture also showed very low levels of IL-10 and the highest level of  $\text{INF-}\gamma$  at the seventh stimulation indicating a more polarized Th1 phenotype of the cells cultured using this protocol (Fig. 4). Thus, the specific culture conditions in cell propagation affect both cell survival and cytokine phenotype.

In contrast to the relative ease of raising T cell lines in rodents, many laboratories report difficulties in maintaining long-term human T cell lines. Since human lines are cultured using rhIL-2 at relatively high concentrations (Polak et al., 2001), it is possible that some of the difficulty may be due to the effects of high IL-2 concentration-early expansion followed by increased apoptosis. Our results would suggest lowering the IL-2 concentration in the propagation media or using mitogen-induced TCGF as a source of a mixture of cytokines that is more supportive for T-cell culture. However,

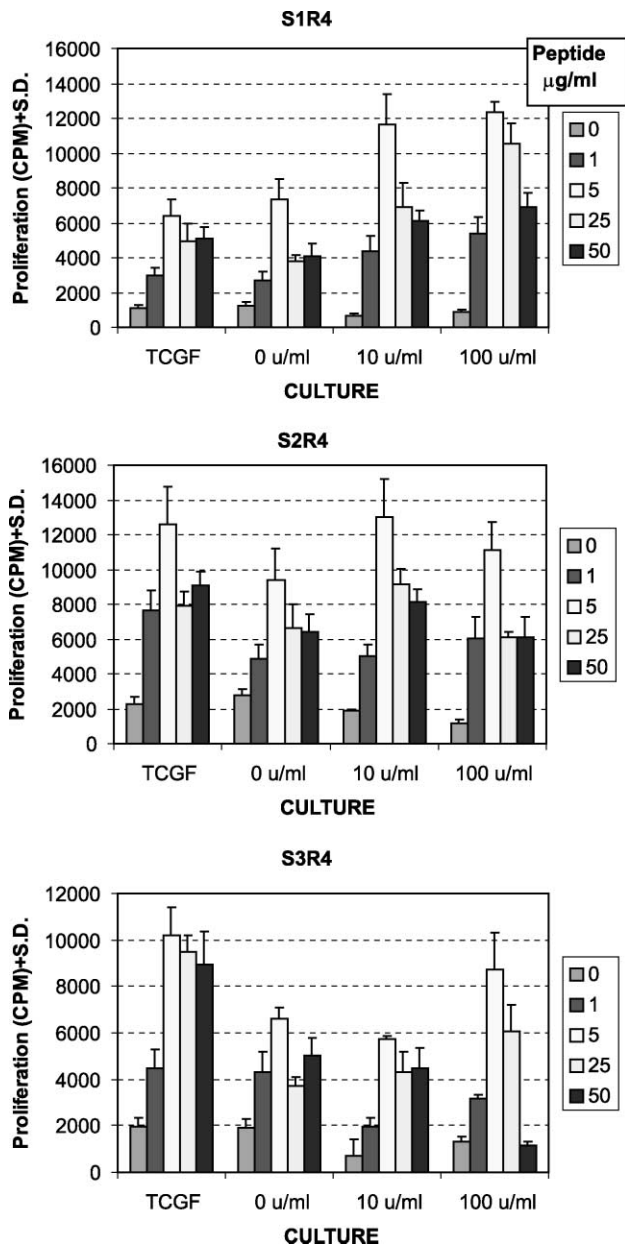


Fig. 6. Proliferation profiles of clone A6 after one (S1R4), two (S2R4) and three (S3R4) cycles of stimulation and propagation in medium containing TCGF or 0, 10 or 100 units/ml of IL-2.

since we tested the comparative effects of rhIL-2 versus TCGF in rat T cells, applicability to human T cell culture will necessitate direct testing of human cells in the same manner. The relative advantage of TCGF in extended cultures could be due to the presence of multiple cytokines acting in concert on many different receptors on the T cells. It is likely that the low level triggering of multiple receptors is superior to the strong stimulation of the IL-2 receptor, which is known to induce a mechanism of proapoptotic death in high concentrations (Lenardo et al., 1999). An enhanced induction of anti-tumor CTL has been described using a mixture of cytokines (including IL-1, IL-2, IL-4 and IL-6) compared to IL-2 or IL-2 and IL-4 (Tsujitani et al., 1995).

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