Vol. 169, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1990 Pages 184-191

AUTOCRINE REGULATION OF TERMINAL DIFFERENTIATION BY INTERLEUKIN-6 IN THE PLURIPOTENT KU812 CELL LINE

Sébastien Navarro[1], Fawzia Louache[2], Najet Debili[2], William Vainchenker[2], and Janine Doly[1]*

[1]Unité CNRS UPR37, IRSC, 7 rue Guy Moquet, 94802 Villejuif Cedex, France

[2]Unité INSERM U91, Hôpital Henri Mondor, 94010 Créteil, France

Received April 12, 1990

SUMMARY: The human KU812 leukemic cell line is a model for studying cell commitment towards different hematopoietic lineages. Indeed, this cell line is characterized by both a capacity for self-renewal and the ability to differentiate spontaneously along erythroid and basophilic cell lineages. In this study we show that interleukin-6 (IL-6) and its specific receptor (IL-6-R) are spontaneously expressed in the human KU812 cell line. Addition of antibody against IL-6 weakly inhibited its cell proliferation (20 to 30%) suggesting that the endogenous production of IL-6 was partially responsible for the growth of the cell line. In contrast, the spontaneous terminal differentiation of this cell line towards the erythroid and basophilic lineages was inhibited by an antibody against IL-6 and this effect was reversed by addition of recombinant human IL-6 (rIL-6). These results suggest that IL-6 is involved more in differentiation than in the proliferation of KU812 cells. After several passages, KU812 cells lose their capacity to differentiate spontaneously. In these cells, the IL-6-R was no more detectable. We therefore suggest that this loss of spontaneous differentiation is associated with an interruption of the IL-6 autocrine loop.

The human leukemic cell line KU812 is able to differentiate spontaneously along two cell lineages, the erythroid and basophilic cell lineages. Basophilic differentiation in KU812 cells can be revealed by the presence of histamine and of metachromatic granules after toluidine blue staining. Terminal erythroid differentiation is associated with the appearance of a significant percentage of mature fully hemoglobinized erythroblasts. This differentiation is associated with the appearance of erythroid proteins, including adult and fetal hemoglobins and glycophorins. The mechanisms which sustain this terminal differentiation remain unknown (1).

IL-6 is a cytokine involved in immunoregulation, inflammatory responses, proliferation and differentiation processes which appears to be identical to interferon-ß2, hybridoma plasmacytoma growth factor, B cell stimulatory factor 2, hepatocyte-stimulating factor, T cell activating factor and cytotoxic T cell differentiation factor (2). Recent data indicate that IL-6 can behave as a colony-stimulating factor for hematopoietic cells (3-5). A few years ago, some authors suggested that an interferon β-like might be involved in the autocrine regulation of terminal differentiation of several lineages including the Friend murine erythroleukemic cell line

* To whom correspondence should be addressed.

The abbreviations used are: IL-6, interleukin-6; IL-6-R, IL-6-receptor; rIL-6, recombinant human IL-6; NADHd, NADH dehydrogenase; GP, glycoprotein, GPA, glycophorin A.

0006-291X/90 $1.50

(6). Here we show that autocrine production of IL-6 by the KU812 cell line favors its terminal differentiation towards the basophilic and erythroid lineages.

MATERIALS AND METHODS

Cell lines and culture conditions. The KU812 cell line and one of its clones, KU812 F, generous gifts from Dr Kishi, were grown at 37°C in RPMI 1640 (Boehringer, Mannheim, FRG) containing 10% (v/v) fetal calf serum (Boehringer) in a fully humidified atmosphere with 5% CO2.

Stimulating factors and antibodies. Recombinant E. coli human IL-6 was obtained from Drs. T. Kishimoto and T Hirano (Osaka, Japan) and Dr. S. Clark (Genetics Institute, Cambridge, MA). Rabbit and sheep polyclonal antibodies against IL-6 were obtained from Genzyme (Boston, MA) and Drs. T. Kishimoto and T. Hirano (Osaka, Japan) respectively.

[^{3}H] thymidine assay. Cells were grown at low density in order to reduce endogenous IL-6 production. The cell concentration was adjusted to 1000 cells per 150 µl culture medium and the KU812 F cells were grown for 36 h in 96-well flat-bottomed microplates in the presence of their own supernatant or control culture medium, with or without anti-IL-6 antibody and rIL-6. After 36 h, 0.4 µCi of [^{3}H] thymidine was added in 50 µl of culture medium to each well for 6 h. The growth rate was the ratio of the mean [^{3}H] thymidine incorporation determined on four replicate wells compared with the value obtained with control culture medium.

Cytochemical staining. Cells were examined after May-Grunwald Giemsa or toluidine blue staining. The percentage of hemoglobinized cells was determined by the benzidine reaction as previously described (7).

Immunological markers of differentiation. A panel of monoclonal and polyclonal antibodies recognizing both cell-surface and cytoplasmic differentiation markers was used. The monoclonal antibodies recognizing cell surface antigens were FA6-152 (anti-GPIV) (8) and CLB-Ery 1 (anti-GPA) (9). The expression of hemoglobin at the cellular level was investigated using a monoclonal antibody against the ß globin chain (10) and two polyclonal antibodies directed respectively against the ß and γ globin chains (11). Antibodies directed against platelet GPIIb (Tab) (12), and GPIIIa (C17) (13) were also tested. Indirect immunofluorescence for cell surface antigens was performed on unfixed cells or after fixation in methanol for cytoplasmic antigens. Cells were examined by fluorescence microscopy or analyzed by flow cytometry.

IL-6 assay. IL-6 activity in the culture supernatants was measured using an IL-6 dependent hybridoma clone (7TD1), as described by Van Snick (14). 7TD1 cells were cultured in triplicate for 72 h in various concentrations of culture supernatants with or without antibody against IL-6. Growth rates were estimated by colorimetric determination (15). rIL-6 was used as an internal standard.

Hybridization analysis. RNA extraction and Northern blot were performed as previously described (16). The IL-6 probe used in the present studies comprised a 700 bp XmnI-BanI (17) insert of human IL-6 cDNA (a generous gift from Drs. C. Vaquero and J. Sanceau, Paris, France) cloned into the pGEM4 plasmid (Promega Biotec). The IL-6-R probe was a 900 pb SphI-NcoI (18) insert of human IL-6-R cDNA (kindly provided by Drs. T. Kishimoto and T. Hirano) cloned into the pGEM5Z plasmid (Promega Biotec). For RNA quantification, the human NADH dehydrogenase (NADHd) cDNA probe cloned into the pUC13 plasmid was used (a gift from Dr. N. Modjtahedi, Villejuif, France). These probes were labeled and the hybridizations were performed as previously described (16).

IL-6-R characterization. Binding was initiated by the addition of 5x10^6 cells in 100 µl of Hank's medium containing 1% bovine serum albumin (Sigma, Cohn fractionV) and various concentrations of highly purified recombinant human [^{125}I] IL-6 (1200 Ci/mmol, Amersham) in the absence or the presence of a 200-fold excess of unlabeled rIL-6. The cells were shaken for 2 h at 4°C, then centrifuged through a layer of a mixture of 40% dinonyl phtalate and 60% dibutyryl phtalate (Merck). Specifically bound radioactivity was calculated by substracting the radioactivity associated with cells incubated with an excess of unlabeled rIL-6 (non specific radioactivity) from that of cells incubated with labeled rIL-6 only (total radioactivity). Equilibrium experiments were analyzed according to the Scatchard plot technique.

RESULTS

Spontaneous expression of IL-6 and IL-6-R in KU812 cells. We first studied IL-6 and IL-6-R mRNA expression in the KU812 F cell line during the first passages, when the cells were still able to differentiate spontaneously (Fig. 1). A specific hybridization to the KU812 F cell line was found with both probes (i.e., 1.3 kb for IL-6 mRNA and approximately 5 kb for IL-6-R mRNA). We then examined IL-6 protein secretion in the supernatant of KU812 F cells and found it to be present with an activity within 20 and 50 U/ml IL-6 varying with the cell density. The presence of IL-6-R on KU812 F cells was investigated using [^{125}I] labelled IL-6. Scatchard analysis showed evidence of receptors for IL-6 with a dissociation constant of 2.5 x 10^{-10} M and 104 free receptors per cell were expressed at 4°C (Fig. 2). This number was increased when the binding assay was performed at a higher temperature (data not shown).

Autocrine regulation of KU812 cell growth by IL-6. The effect of IL-6 on the growth of KU812 F cells was studied using different techniques. First, by the thymidine incorporation assay, we showed that the supernatant of the KU812 F cell line contained an autostimulatory growth activity that increased its own proliferation 2- to 3-fold in cells cultured at low density. This effect was partially reproduced by the addition of rIL-6 (1.5- to 2-fold).

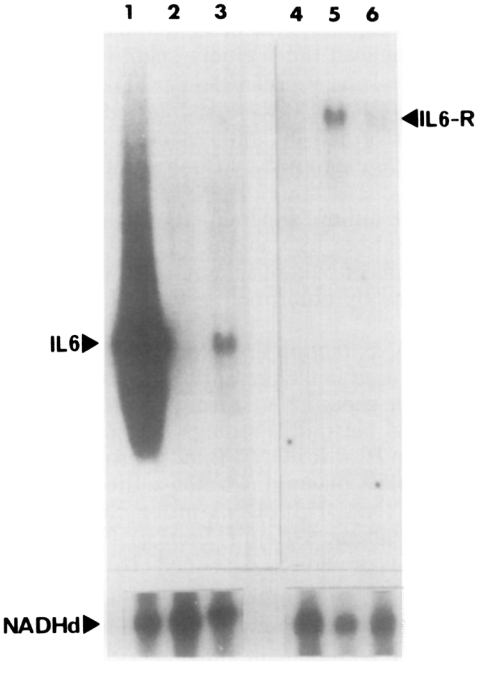

Fig. 1. **IL-6 and IL-6-R mRNA in KU812 F cell line.** Northern blot hybridization analysis of poly (A)$^+$ mRNA (5 µg per lane) from polyinosinate polycytidinate-treated human fibroblasts (FS-4 cells) (lane 1), U937 cell line (lanes 2 and 5), KU812 F cell line (lanes 3 and 6) and K562 cell line (lane 4). Hybridization was carried out first against IL-6 and IL-6-R RNA probes. The filters were then dehybridized and reprobed against NADHd probe. Exposure times were five days for IL-6 and IL-6-R hybridization and 5 h for NADHd probe.

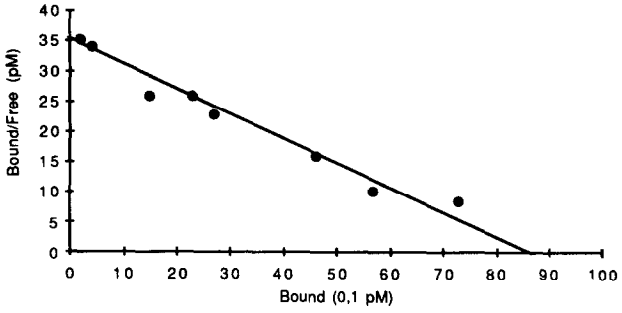

Fig. 2. Characterization of receptors for IL-6 on KU812 F cells. Scatchard plot analysis of the specific binding of [^{125}I] IL-6 to KU812 F cells as detailed in Materials and Methods. Concentrations of the labeled ligand ranged from 5.8 pM to O.86 nM.

Furthermore, part of the stimulatory effect of the supernatant was neutralized by antibody against IL-6 and this partial neutralization was reversed by adding rIL-6 (Table 1). This result suggests the presence of one or more autostimulatory factors other than IL-6 in the cultured supernatant of the KU812 F cell line. Secondly, the proliferation of KU812 F cells was partially inhibited by rabbit polyclonal antibodies against IL-6 (between 20% and 30% in four experiments). This inhibition was found to be specific by the use of rIL-6, which reversed the effect of the polyclonal antibody (data not shown). However, the cell cycle was not modified by the addition of antibody against IL-6 or by addition of rIL-6, suggesting that autocrine IL-6 production shortens all phases of the cell cycle (data not shown).

Autocrine regulation of spontaneous differentiation of the KU812 cell line. The effect of IL-6 on the basophilic differentiation of KU812 F cells was studied using antibodies against IL-6 and rIL-6 (Table 2). In the presence of the anti-IL-6, the percentage of basophilic cells decreased dramatically: from 14% in the untreated population to an undetectable level after 5 days. This effect was partially reversed when the cells were grown in the presence of both anti-IL-6 and rIL-6 (6% of basophilic cells). The effect of IL-6 on erythroid differentiation in the KU812 F cell line was investigated using the benzidine reaction to detect

Table 1. [^{3}H] Thymidine incorporation in KU812 F cells cultured for 36 h under various conditions

Addition to culture	Growth rate	
Supernatant (1/3)	2.19 +/- 0.17*	(n=4)
Supernatant (1/6)	1.83 +/- 0.33	(n=3)
Supernatant (1/15)	1.39 +/- 0.02	(n=4)
rIL-6 (10 U/ml)	1.85 +/- 0.48	(n=2)
rIL-6 (1 U/ml)	1.69 +/- 0.37	(n=2)
Supernatant (1/3)+anti-IL-6 (1/100)	1.76 +/- 0.16	(n=2)
Supernatant (1/3)+anti-IL-6 (1/1000)	1.86 +/- 0.12	(n=2)
Supernatant (1/6)+anti-IL-6 (1/100)	1.55 +/- 0.18	(n=2)
Supernatant (1/3)+anti-IL-6 (1/100)+rIL-6 (200 U/ml)	2.17 +/- 0.22	(n=2)

Incorporation of [^{3}H] Thymidine was investigated as described in Materials and Methods. Each experimental value represents the mean of four replicate wells. Data are the mean of two to four independent experiments, as indicated.
* $P < 0.01$.

Table 2. Regulation of the KU812 cell line spontaneous differentiation by IL-6

	Positive cells (%)		
	KU812 F	KU812 F +anti-IL-6	KU812 F +anti-IL-6 +IL-6
Toluidine staining	14	0	6
Benzidine reaction	28.4	17.8	25.9
Antibody/Specificity			
CLB/Glycophorin A	68	29	31
ß globin chain	40	29	43
γ globin chain	62	36	54
TAB/GPIIb	12	12.3	ND
C17/GPIIIa	12	10.8	14.7

The KU812 F cells were grown for 5 days in different conditions including control medium, anti-IL-6 antibody neutralising 50 U/ml with or without rIL-6 (50 U/ml). The neutralisation and the reversion of the neutralisation in the supernatants were controled by using IL-6 bioassay. Data are the mean of different experiments.

ND: Not determined.

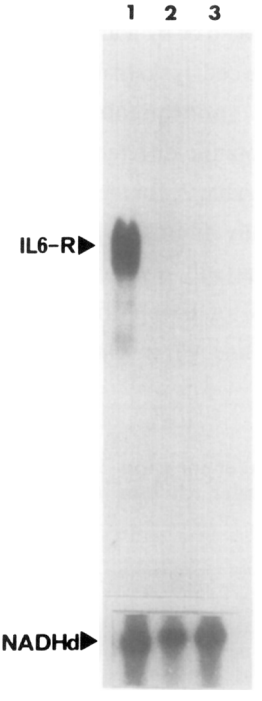

Fig. 3. Loss of IL-6-R mRNA expression in the undifferentiated KU812 F cell line. The KU812 F cell line was cultured for four months until the cells lost their spontaneous differentiation. Poly $(A)^+$ mRNA was then isolated and was analysed by Northern blot procedure (5 µg per lane). U937 cell line (lane 1), K562 cell line (lane 2) and undifferentiated KU812 F cell line (lane 3). The filter was first hybridized with IL-6-R RNA probe, dehybridized and reprobed with NADHd control probe. Exposure times were five days for IL-6-R hybridization and 5 h for NADHd probe. No specific signal for the presence of IL-6-R mRNA was found with longer exposure time (data not shown).

hemoglobinized cells. In most experiments, the percentage of benzidine-positive cells was decreased by 30% to 50% following culture in the presence of anti-IL6 and this effect was reversed by the addition of rIL-6. We then studied by immunofluorescence the expression of erythroid differentiation markers in KU812 F cells in response to antibody against IL-6. After 5-day culture in the presence of anti-IL6, the percentage of cells stained by antibodies to β and γ globin chains was 29% and 36% compared to 40% and 62% respectively in the untreated cell population and this effect of anti-IL-6 on the expression of β and γ globin chains was reversed by adding rIL-6. The effect of the antibody on the other erythroid differentiation markers was less pronounced. In one of the three experiments performed it diminished by 50% the expression of GPA but this neutralisation was not reversed by rIL-6. The expression of GPIV, an early marker of erythroid differentiation, was not modified by the antibody or the addition of rIL-6 nor was the expression of megakaryocytic markers GPIIb, and IIIa.

Loss of IL-6-R in the undifferentiated KU812 cell line. After several passages (usually more than 30), KU812 F cells lose the ability to differentiate spontaneously and benzidine- and toluidine-blue-positive cells are no longer detectable. We investigated whether the IL-6 autocrine loop persisted at this stage and found that IL-6 transcripts were detectable by Northern blotting at the same level as in the first passages of the cell line (data not shown). An IL-6 activity was also observed in the culture supernatant (20-50 U/ml). In contrast, IL-6-R was no longer detectable either by binding studies at 4°C or 37°C (data not shown) or by Northern blotting (Fig.3).

DISCUSSION

IL-6 is a cytokine with pleiomorphic effects. On hematopoietic cells, it was first demonstrated to act synergistically with interleukin-3 on pluripotent stem cells (3). However, recent evidence suggests that IL-6 also acts later in differentiation as a colony-stimulating factor for the granulo-monocytic lineages (4, 5). IL-6 has quite opposite effects on the proliferation of leukemic cells. In some of them, it can stimulate their growth, whereas in others it has inhibitory effects which could be associated with differentiation, as in the murine M1 and WEHI 3B cell lines and the human U937 and HL60 cell lines (19-21, 16). Numerous studies have demonstrated that autocrine stimulation is more frequently involved in the proliferative advantage of growth for leukemic cells than previously thought. For example, it has been shown that IL-6 is an autocrine growth factor for myeloma cells (22).

The KU812 cell line has the important characteristic of being able to undergo spontaneous terminal differentiation towards the erythroid and basophilic lineages and preliminary results have suggested that its growth and differentiation are regulated by an autocrine mechanism, since both are dependent upon cell concentration. In the present study, we have shown that IL-6 is detectable in the supernatant of KU812 F cells and that IL-6-R is synthesized by KU812 F cells. Blocking experiments showed that IL-6 had a modest effect on the proliferation of KU812 F cells and was a differentiation-inducing factor for this cell line. Indeed, the antibody markedly diminished the percentage of erythroid (benzidine-positive) cells, whereas its effect was weak on earlier markers of erythroid differentiation such as GPA

or GPIV. Neutralization of endogenous IL-6 also led to the disappearance of basophilic (toluidine blue-positive) cells. We demonstrated that, when the cell line lost its capacity to spontaneously differentiate, no more IL-6-R were detectable. We suggest that this loss of differentiation is associated with the disappearance of the IL-6 autocrine loop. It is noteworthy that other erythroid cell lines such as K562 which are blocked in their differentiation, synthesize IL-6 but do not express IL-6-R (Navarro, S. *et al.*, manuscript submitted).

IL-6 was shown to permit the maturation of B-cells to plasmocytes, to be a colony-stimulating factor for the granulo-monocytic lineages, to induce the differentiation of cytolytic T-cells, and recently to act on late stages of megakaryocytic differentiation (17, 4, 23, 24). The present results suggest that IL-6 also acts on late stages of erythroid and basophilic differentiation and we propose that IL-6 acts as a late regulator of differentiation for most, if not all, hematopoietic cell lineages.

ACKNOWLEDGMENTS: We are grateful to Dr. L. Edelman for FA6-152; Dr. C. Gahmberg for the anti-GPA antiserum; Dr. R.P. McEver for Tab; Dr. L.H. Stanker for the monoclonal antibody against β globin chain; Dr. P.A.T. Tetteroo for C17, CLB-Ery 1 and CLB-Ery 3; Dr. S. Clark (Genetics Institute, MA) for rIL-6; Drs. T. Kishimoto and T. Hirano (Osaka, Japan) for IL-6-R probe, sheep polyclonal antibodies against IL-6 and rIL-6; Drs. C. Vaquero and J. Sanceau (Paris, France), Dr. N. Modjtahedi (Villejuif, France), and Dr. Van Snick (Brussels, Belgium) for providing IL-6 probe, NADHd probe and 7TD1 cells respectively and Drs. P. Orsolini and R. Skoda (Cytotec, Switzerland) for induced FS-4 cells. We thank J.M. Massé for photographic assistance; Dr. A. Katz (Créteil, France) for performing cytofluorometry experiments and Dr. D. Mason for improving the English manuscript. This work was supported in part by grants from La Ligue Nationale Française contre le Cancer and the Association pour la Recherche sur le Cancer. S. Navarro is a student of the Institute of Postgraduate Biomedical Studies (IFSBM).

REFERENCES

1. Nakasawa, M., Mitjavila, M.T., Debili, N., Casadevall, N., Mayeux, P., Rouyer-Fessard, P., Dubart, A., Romeo, P.H., Beuzard, Y., Kishi, K., Breton-Gorius, J., and Vainchenker, W. (1989) Blood 73, 2003-2013.
2. Kishimoto, T. (1989) Blood. 74, 1-10.
3. Ikebuchi, K., Wong, G.G., Clark, S.C., Ihle, J.N., Hirai, Y., and Ogawa, M. (1987) Proc. Natl. Acad Sci. USA 84, 9035-9039.
4. Wong, G.G., Witek-Giannotti, J.S., Temple, P.A., Kriz, R., Ferenz, C., Hewick, R.M., Clark, S.C., Ikebuchi, K., and Ogawa, M. (1988) J. Immunol. 140, 3040-3044.
5. Caracciolo, D., ClarK, S.C., and Rovera, G. (1989) Blood 73, 666-670.
6. Friedman-Einat, M., Revel, M., and Kimchi, A.(1982) Mol. Cell. Biol. 2, 1472-1480.
7. Singer, D., Cooper, M., Maniatis, G., Marks, P., and Rifkind, R. (1974) Proc. Natl. Acad. Sci. USA 71, 2668-2670.
8. Edelman, P., Vinci, G., Villeval, J-L., Vainchenker, W., Henri, A., Miglierina, R., Rouger, P., Reviron, J., Breton-Gorius, J., Sureau, C., and Edelman, L. (1986) Blood 67, 56-63.
9. Lansdorp, P., Bauman, J., Bos, M., Von dem Borne, A., Oosterhof, F., Van Mourik, P., Tetteroo, P., and Zeijlemaker, W. (1986) Leuk. Res. 10, 155-165.
10. Stanker, L., Branscomb, E., Vanderlaan, M., and Jensen, R. (1986) J. Immunol. 136, 4174-4180
11. Henri, A., Testa, U., Tonthat, H., Riou, J., Titeux, M., Vainchenker, W., Feuilhade, F., Galacteros, F., and Rochant, H. (1980) Am. J. Hemat. 9, 161-170.
12. McEver, R., Bennett, E., and Martin, M. (1983) J. Biol. Chem. 258, 5269-5275.
13. Tetteroo, P., Lansdorp, M., Leeksma, O., and von dem Borne, A. (1983) Br. J. Haemat. 55, 509-522.

14. Van Snick, J., Cayphas, S., Vink, A., Uyttenhove, C., Coulie, P.G., Rubira, M.R., and Simpson, J. (1986) Proc. Natl. Acad.Sci. USA 83, 9679-9683.
15. Mosmann, T. (1983) J. Immunol. Methods 65, 55-63.
16. Navarro, S., Debili, N., Bernaudin, J-F., Vainchenker, W., and Doly, J. (1989) J. Immunol. 142, 4339-4345.
17. Hirano, T., Yasukawa, K., Harada, H., Taga, T., Watanabe, Y., Matsuda, T., Kashiwamura, S.I., Nakajima, K., Koyama, K., Iwamatsu, A., Tsunasawa, S., Sakiyama, F., Matsui, H., Takahara, Y., Taniguchi, T., and Kishimoto, T. (1986) Nature 324, 73-76.
18. Yamasaki, K., Taga, T., Hirata, Y., Yawata, H., Kawanishi, Y., Seed, B., Taniguchi, T., Hirano, T., and Kishimoto, T. (1988) Science 241, 825-828.
19. Chen, L., Novick, D., Rubinstein, M., and Revel, M. (1988) FEBS Lett. 239, 299-304.
20. Chen, L., Mory, Y., Zilberstein, A., and Revel, M. (1988) Proc. Natl. Acad. Sci. USA 85, 8037-8041.
21. Chiu, C.P., and Lee, F. (1989) J. Immunol. 142, 1909-1915.
22. Kawano, M., Hirano, T., Matsuda, T., Taga, T., Horii, Y., Iwato, K., Asaoku, H., Tang, B., Tanabe, O., Tanaka, H., Kuramoto, A., and Kishimoto, T. (1988) Nature 332, 83-85.
23. Takai, Y., Wong, G.G., Clark, S.C., Burakoff, S.J., and Herrmann, S.H. (1988) J. Immunol. 140, 508-512.
24. Ishibashi, T., Kimura, H., Uchida, T., Kariyone, S., Friese, P., and Burstein, S.A. (1989) Proc. Natl. Acad. Sci. USA 86, 5953-5957.