

Impairment of Natural Killer Functions by Interleukin 6 Increases Lymphoblastoid Cell Tumorigenicity in Athymic Mice

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Abstract

Expression of the human IL-6 gene in EBV-immortalized normal human B lymphocytes following retroviral-mediated transduction rendered these cells highly tumorigenic in athymic mice. The tumors were lymphomas composed of the originally inoculated human lymphoblastoid cells. Co-injection of IL-6 expressing EBV-immortalized cells with IL-6 nonexpressing control cells resulted in increased tumorigenicity of the IL-6 nonexpressing cells. The lymphoblastoid cells expressing IL-6 were indistinguishable from parental cell lines in morphology and in a variety of cell surface characteristics, and did not exhibit growth advantage over parental cell lines in vitro, such that increased tumorigenicity is unlikely to depend upon a direct oncogenic effect of IL-6 on the B cells. Rather, at high concentrations, IL-6 markedly inhibits human lymphoblastoid cell killing by IL-2-activated murine splenocytes in vitro, suggesting that IL-6-related tumorigenicity might depend upon IL-6 inhibiting cytotoxicity at the tumor site. Thus, production of IL-6 by tumor cells that results in natural killer cell dysfunctions illustrates a novel mechanism of tumor cell escape from immune surveillance. (*J. Clin. Invest.* 1991. 88:239-247.) **Key words:** cytokine • gene transfer • Epstein-Barr virus • lymphoma • cytotoxicity

Introduction

Malignancies of EBV-infected B cells such as X-linked lymphoproliferative disease (1), AIDS-associated lymphomas (2, 3), and post-transplant lymphomas (4) represent unique model systems for which a number of pathogenetic steps have been suggested (5, 6). In vitro, B lymphocytes can be immortalized by EBV into long-term cell lines (7). In vivo, B cells naturally infected with EBV are long-lived and subjected to immunoregulatory controls to prevent their expansion (6, 7). In the presence of severe immunodeficiency, EBV-infected B cells can proliferate in vivo, giving rise to polyclonal or oligoclonal malignancies.

IL-6, a multifunctional cytokine produced in monocytes, fibroblasts, and other cell types (for a review, see reference 8), has been implicated in the pathogenesis of several human cancers, including Kaposi sarcoma (9), cardiac myxoma (10), Castleman's disease (11), multiple myeloma (12, 13), non-Hodgkin's B cell lymphomas (14), and B cell chronic lympho-

cytic leukemia (15). Recently, IL-6 has been identified as an important factor in the establishment and maintenance of EBV-immortalized B cells (16, 17). These virally infected cells secrete low levels of IL-6 in the culture supernatant (18), express surface receptors for IL-6 (19), and can use this cytokine as an autocrine growth factor (18).

To explore a possible role of IL-6 in the multistep process of B cell tumorigenesis, we have stably expressed the human IL-6 gene into EBV-immortalized B cells and examined the ability of these cells to form tumors in athymic mice. Unlike in vitro studies that rely upon the contribution of a limited number of cell types, this in vivo approach allows one to register the participation of the diverse cell types that may be affected by IL-6 and may contribute or prevent tumor formation. This is particularly relevant in the case of IL-6 that is emerging as one of the most pleiotropic of cytokines with a broad range of biological activities (for a review, see reference 8). While parenteral administration of the cytokine together with EBV-immortalized cells has the potential of affecting all IL-6-responsive targets, this approach is compromised by the relatively short half-life of IL-6 (20), and the potential need to achieve sustained effective levels locally. Accordingly, we have used an alternative strategy of expressing the IL-6 gene in the EBV-immortalized cells. Using this approach, high concentrations of IL-6 may be achieved within the local environment during the entire tumor-immune effector cell interaction. The present experiments show that IL-6-expressing lymphoblastoid cell lines are highly tumorigenic in vivo, and this effect is likely due to IL-6-induced dysfunction of natural killer (NK)¹ functions.

Methods

Plasmid construction. The nucleotide sequence containing the open reading frame for human IL-6 was isolated as the Hind III and Bam I (0.67 kb) fragment from the cDNA clone p β -2-22 (21; a gift from P. B. Sehgal, The Rockefeller University, New York, NY), followed by T4 polymerase repair, and blunt-end ligation of Bam HI linkers (Gibco Laboratories, Grand Island, NY). The insert was Bam HI restricted and inserted at the Bam HI site of the vector pZip-NEO-SV(x)1 (22, 23). Proper orientation was verified by restriction endonuclease analysis.

Transfection and infection. Subconfluent PA317.3 cells (No. CRL9078; American Type Culture Collection, Rockville, MD) that contain the packaging-signal-defective AM-MLV amphotropic helper virus (24) were transfected with 10–20 μ g of linearized pZip-NEOSV(x)1 or pZip-NEOSV(x)1-IL-6 plasmid DNA in 60-mm petri dishes using lipofection (Gibco Laboratories). After 48–72 h the cells were replated in culture medium consisting of DME (Gibco Laboratories) with 10% FCS (Rehatuin; Interger Co., Purchase, NY) and 600 μ g/ml of Geneticin (Gibco Laboratories). After a 3-wk incubation, neomycin-resistant colonies were picked and assayed for expression of IL-

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Received for publication 7 November 1990 and in revised form 18 January 1991.

The Journal of Clinical Investigation, Inc.
Volume 88, July 1991, 239–247

1. *Abbreviations used in this paper:* LAK, lymphokine-activated killer; NK, natural killer.

6, using the IL-6-dependent murine hybridoma cell line B9 (25; a gift of Dr. R. Nordan, National Institutes of Health, Bethesda, MD).

Culture supernatants of human IL-6-transfected PA317.3 cells producing the pZip-NEO-SV(x)1-IL-6 retroviruses were used to infect two EBV-induced lymphoblastoid cell lines, VDS-0 and TB. Neomycin-resistant VDS-0 and TB cell clones were selected in RPMI-1640 medium (Gibco Laboratories) supplemented with 10% FCS (Rehauin), 2 mM L-glutamine (Gibco Laboratories), and 1 mg/ml geneticin (Gibco Laboratories). Neomycin-resistant VDS-0 and TB cell clones were assayed for IL-6 production using the IL-6-dependent cell line B9.

Radiolabeling and Immunoprecipitation. Lymphoblastoid cells (10^6 cells/ml, total volume 2 ml) were incubated for 18–24 h in methionine-free MEM (Gibco Laboratories) supplemented with 10% dialyzed FCS (Gibco Laboratories) and 200 μ Ci/ml [35 S]methionine (1055 Ci/mmol; New England Nuclear, Boston, MA). Aliquots (1 ml) of cell-free culture supernatants that had been clarified by 30 min centrifugation (13,000 g) were incubated with a rabbit antiserum to human IL-6 (Genzyme Corp., Boston, MA) at a final dilution of 1:100. After a 2-h incubation at 4°C, the samples were processed for immunoprecipitation as previously described (26), suspended in 50 μ l gel sample buffer (0.125 M Tris, pH 6.8, 4% SDS, 10% 2-mercaptoethanol, and 10% glycerol), boiled for 10 min, centrifuged, and then electrophoresed through a 12.5% polyacrylamide gel containing 0.1% SDS (27). Gels were fixed for 30 min in 25% methanol and 10% glacial acetic acid, treated with Enlightening (New England Nuclear), dried, and exposed to Kodak XAR-2 film at –80°C.

Assay for IL-6 bioactivity. The murine hybridoma cell line B9 (a gift of Dr. R. Nordan, National Institutes of Health) was used in a standard assay for IL-6 bioactivity (25). 1 U of IL-6 bioactivity in this assay is defined as the activity inducing half-maximal proliferation of B9 cells. An IL-6 concentration of 1 U/ml corresponds to ~20 pg of *Escherichia coli*-derived human IL-6 used throughout as a laboratory standard (a gift of Genetics Institute, Boston, MA) and to 5.5 U/ml of the interim NIH reference IL-6 standard (preparation 88/414).

Cell proliferation assay. Parental cell lines VDS-0 and TB, and IL-6-transduced clones VDS-0 and TB were plated at 50–100,000 cells/ml in triplicate flat-bottomed microtiter wells (0.2 ml/well; Costar, Cambridge, MA) in culture medium consisting of either Opti-MEM (Gibco Laboratories), or RPMI-1640 medium supplemented with 10% heat-inactivated FCS and 2 mM glutamine, and incubated at 37°C in a humidified atmosphere containing 5% CO₂. Cultures were pulsed with [3 H]thymidine (0.5 μ Ci/well) during the final 4.5 h of a 3-d culture. Results are expressed as mean cpm \pm SEM.

Anti-IL-6 antibodies and neutralization assays. A rabbit antiserum to highly purified natural murine IL-6 (28) was purified with protein A-Sepharose (a gift of Dr. R. Nordan, National Institutes of Health). A mouse Mab to *E. coli*-derived human IL-6 (CLB IL-6/8, reference 29) was used after protein G purification of hybridoma culture supernatant (a gift of L. A. Aarden, Central Laboratory of the Netherlands, Red Cross Blood Transfusion Service, Amsterdam, The Netherlands). Mouse IL-6 purified from culture supernatants of the murine macrophage P388D1 cell line (30) was a gift of Dr. R. Nordan, National Institutes of Health. Highly purified *E. coli*-derived recombinant human IL-6 was a gift of Genetics Institute.

Anti-mouse or anti-human IL-6 antibody (10 μ g/ml and 5 μ g/ml, respectively) was incubated (37°C for 1 h) with either IL-6 (mouse or human) or test culture supernatants at varying dilutions in flat-bottomed microtiter plates (final volume 0.1 ml). After incubation, B9 cells (3×10^3 cells in 0.1 ml) were added to each well. Culture medium consisted of RPMI-1640 (Gibco Laboratories) supplemented with 10% FCS (Reheis) and 10^{-4} M 2-mercaptoethanol. [3 H]Thymidine incorporation was measured during the last 18 h of a 3-d culture.

Animal studies. 4–6-wk-old female outbred *nu/nu* mice (Harlan-Sprague-Dawley, Madison, WI) maintained in pathogen-limited conditions were used throughout for these experiments. Exponentially growing lymphoblastoid cell lines with a viability greater than 95% were injected (10^7 cells in 0.2 ml RPMI-1640 medium containing 10% FCS) subcutaneously in the right abdominal quadrant through a 25-

gauge needle on plastic 1-ml syringes. In some experiments, an IL-6 virus-infected cell line was co-injected subcutaneously with a control cell line (5×10^6 cells of each type in 0.2 ml), essentially as described (31). Animals injected with either cell line alone served as controls. All animals were observed every 2–3 d and tumor size was estimated in mm² as the product of two-dimensional caliper measurements (longest perpendicular length and width).

Histology evaluation. Representative samples of tumor tissue were fixed in 10% neutral buffered-formalin (Media Kitchen, NIH, Bethesda, MD), blocked in paraffin, sectioned at 4 μ M, and stained with hematoxylin and eosin.

Expression of cell surface determinants. Exponentially growing lymphoblastoid cell lines (10^7 cells/ml in PBS containing 5% FCS) were incubated for 30 min at 4°C with control reagents and mouse MAbs. These included anti-CD23 (anti-Leu-20; Becton Dickinson & Co., Mountain View, CA), anti-CD10 (J5, Calla; Coulter Electronics Inc., Hialeah, FL), anti-CD11a (CLB-CD11a; Janssen Biochimica Turnhoutseneg, Beerse, Belgium), anti-CD18 (CLB-CD18, Janssen Biochimica), anti-CD20 (anti-Leu-16, Becton Dickinson & Co.), anti-transferrin receptor (Becton Dickinson & Co.), anti HLA-DR (Becton Dickinson & Co.), and an anti-human Ig goat IgG antiserum specific for heavy and light chains (Cappel Laboratories, Malvern, PA). When appropriate, the cells were further incubated (30 min at 4°C) with an affinity-purified, fluorescein-labeled, goat anti-mouse Ig reagent (Becton Dickinson & Co.). After washing, the cells were analyzed on a FACScan (Becton Dickinson & Co.). For each determination, 10^4 cells were examined.

Histocompatibility typing for HLA-DR was performed by the microcytotoxicity assay, as described (32).

Generation of IL-2-activated killer cells and cytotoxicity assay. Splenocytes, obtained from normal outbred female nude mice, 4–8 wk old, and suspended in culture medium consisting of a 1:1 mixture of RPMI-1640 medium (Gibco Laboratories) and EHAA medium (enriched Eagle's medium, Biofluids Inc., Rockville, MD) supplemented with 10% FCS (Reheis), 2 mM L-glutamine (Gibco Laboratories), 10^{-4} M 2-mercaptoethanol (Gibco Laboratories), and 5 μ g/ml gentamycin (Sigma Chemical Co., St. Louis, MO), were cultured at 2×10^6 cells/ml in 24-well tissue culture plates (Linbro, Flow Laboratories, Inc., McLean, VA), 2 ml per well, with either IL-2 (500 U/ml; a gift of Cetus Corp., Emeryville, CA), or Cos-7-derived IL-6 alone (31; 10^{-8} U/ml) or a combination of IL-2 and IL-6. After a 3-d culture, splenocytes were harvested and washed twice in tissue culture medium. Then viable cells were counted and tested for cytotoxicity, as described (33). Lymphoblastoid cells (VDS-0 and TB) and murine T cell leukemia YAC-1 cells (ATCC T1B 160) were used as 51 Cr-labeled target cells. The percent specific cytotoxicity was calculated as: $100 \times [(\text{experimental release} - \text{spontaneous release}) / (\text{total release} - \text{spontaneous release})]$.

Results

Generation of lymphoblastoid cell lines expressing high level IL-6. Two lymphoblastoid cell lines obtained by EBV (B95-8) immortalization of normal peripheral blood B lymphocytes were selected for these studies. VDS-0 is monoclonal and does not secrete immunoglobulin (34), while TB is polyclonal and secretes IgM, IgG, and IgA. Because EBV-immortalized B cells have been shown to express low levels of IL-6, we selected lines that most differed in the levels of endogenous IL-6 secreted (19).

Complementary DNA sequences spanning the entire human IL-6 coding region were cloned into the retroviral vector depicted in Fig. 1 (top panel) (22, 23). The plasmid pZip-NEO-SV(x)-1-IL6 was transfected into the amphotropic retroviral packaging cell line PA317.3 (24), and stably transfected cells were selected as a source of IL-6-encoding virus. Virus-con-

pZip-Neo SV(X)I-IL-6

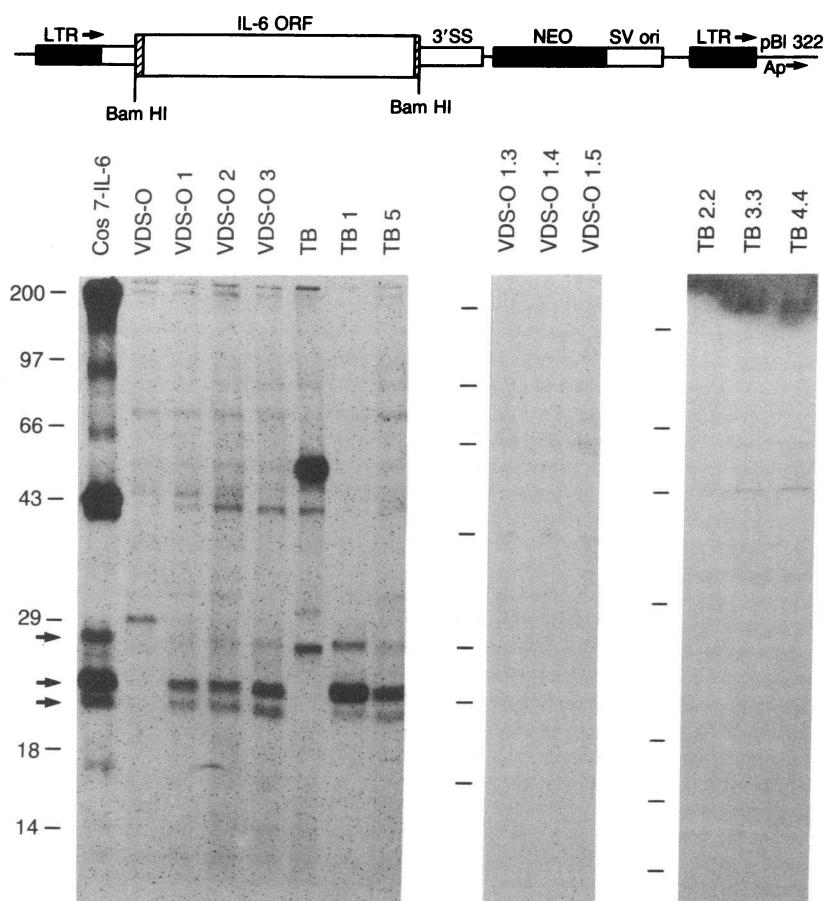


Figure 1. (Top) Diagrammatic representation of the IL-6 retroviral vector used for transfection of the retroviral packaging cell line PA317. The open reading frame (ORF) for human IL-6, isolated as a Hind III and Bam I fragment (0.67 kb) from the cDNA clone p- β -2-22 (21), was inserted at the Bam HI site of the vector pZip-Neo-SV(x)1 (22). (Bottom) Immunoprecipitable IL-6 detected in the culture supernatant of IL-6 virus-infected lymphoblastoid cells. 35 S-Labeled supernatants of either IL-6-transfected Cos 7 cells (26) or the indicated lymphoblastoid cell lines were immunoprecipitated with an anti-human IL-6 rabbit serum. Immunoprecipitates were boiled and electrophoresed through a 12.5% polyacrylamide gel containing 0.1% SDS. Molecular weight standards are indicated at the left, and IL-6-related bands are indicated by arrows.

taining supernatants from these cells were used to infect the VDS-0 and TB lymphoblastoid cell lines. Five neomycin-resistant, IL-6-producing cell clones were derived. As shown in Table I, while the two parent and vector control virus-infected (neomycin-resistance gene only) cell lines produced little or no IL-6, high level IL-6 secretion was observed in the IL-6 virus-infected cell lines. Immunoprecipitation of 35 S-labeled culture supernatants from the IL-6 virus-infected cell lines revealed the presence of material that reacted specifically with a polyclonal rabbit antiserum to human IL-6 and had electrophoretic mobility similar to that of IL-6 secreted by Cos7 cells stably transfected with the human IL-6 gene (26) (Fig. 1, bottom panel). Under the conditions used, little or no immunoprecipitable IL-6 was detected in the supernatant of parental or virus control-infected cell lines (Fig. 1, bottom panel).

Tumorigenicity of high level IL-6 gene-expressing lymphoblastoid cell lines. EBV-immortalized lymphoblastoid cell lines are capable of long-term proliferation in tissue culture but generally fail to generate tumors when injected subcutaneously in athymic mice (35). As shown in Table II, parental and control virus-infected lymphoblastoid cells only rarely caused tumors in nude mice. Tumors developed in only 6 of 80 animals (7.5%). In contrast, a high proportion (68%) of the mice inoculated with the IL-6 virus-infected lymphoblastoid cell lines developed tumors in parallel experiments.

It should be noted (Table II) that the mean time of first tumor observation after inoculation was shorter for tumors

induced by IL-6-expressing cell lines as opposed to the controls. For example, the rare tumors derived from injection of parental VDS-0 line and virus-control VDS-0 1.5 line appeared

Table I. IL-6 Secretion in Lymphoblastoid Cell Lines

Cell line	Infecting virus	IL-6 activity*
		U/ml/48 h
VDS-0	None	18.4
VDS-0 1.3	pZip-Neo-SV(x)1	2.8
VDS-0 1.4	pZip-Neo-SV(x)1	3.75
VDS-0 1.5	pZip-Neo-SV(x)1	3.2
VDS-0 1	pZip-Neo-SV(x)1-IL6	7,600
VDS-0 2	pZip-Neo-SV(x)1-IL6	826
VDS-0 3	pZip-Neo-SV(x)1-IL6	6750
TB	None	1.2
TB 2.2	pZip-Neo(x)1	0
TB 3.3	pZip-Neo(x)1	0
TB 4.4	pZip-Neo(x)1	0
TB 1	pZip-Neo-SV(x)1-IL6	40,562
TB 5	pZip-Neo-SV(x)1-IL6	16,750

* IL-6 activity was determined in a standard B9 culture assay and is expressed as units per milliliter of culture supernatant conditioned by 0.5×10^6 cells for 48 h. Each data point corresponds to the mean of at least three determinations.

Table II. Tumorigenicity of EBV-induced Lymphoblastoid Cell Lines in Athymic Mice

Cell line	Tumor incidence	Mean time of first observation	Mean maximum tumor size
		<i>d</i>	<i>mm</i> ²
VDS-0 parent	3/20	32	218
VDS-0 1.3 control	0/7	—	—
VDS-0 1.4 control	0/5	—	—
VDS-0 1.5 control	1/8	36	39
VDS-0 1 IL-6	9/15	21	42
VDS-0 2 IL-6	12/15	25	140
VDS-0 3 IL-6	12/15	23	168
TB parent	1/20	48	103
TB 2.2 control	0/6	—	—
TB 3.3 control	0/6	—	—
TB 4.4 control	0/8	—	—
TB 1 IL-6	9/15	33	27
TB 5 IL-6	9/15	27	45

Outbred 4–6-wk-old female athymic mice were inoculated subcutaneously in one site with 10^7 cells from the indicated cell lines and observed for 15 wk for appearance of tumors $> 5 \text{ mm}^2$ in surface area. Data are compiled from four separate experiments. Tumor incidence is expressed as the fraction of mice with tumor per number of mice injected. Tumor size is expressed in surface area and reflects the product of two-dimensional caliper measurements.

at a mean of 33 d after inoculation and the more frequent tumors derived from injection of IL-6 virus-infected VDS-0 lines appeared at a mean of 23 d after inoculation. In contrast, it should be noted that the mean maximum size reached by these tumors was similar in the two groups receiving either control (120 mm^2) or IL-6 virus-infected lines (84 mm^2).

Variation in IL-6 production among the different IL-6 virus-infected cell lines allows us to examine a possible correlation between the level of tumorigenicity and level of IL-6 produced by each line. The results of such analysis (Table III) show that neither tumor incidence nor time of first tumor occurrence, correlated directly with the levels of IL-6 produced by the IL-6 virus-infected lines. A similar conclusion was reached from experiments in which two IL-6-transfected cell lines, TB 1, a high IL-6 producer, and VDS-0 2, a low IL-6 producer, were injected subcutaneously at doses of 5, 2.5, and 1.25×10^6 cells per animal. The observation that IL-6 virus-infected lines are significantly more tumorigenic than control lymphoblastoid lines together with the observation that the level of IL-6 secreted by these lines fails to correlate with tumorigenicity suggests that there might be a critical level of IL-6 sufficient for high level tumorigenicity.

Mixed tumor transplantation assays demonstrate cell transfer of IL-6-associated tumorigenicity. To test further whether IL-6 is responsible for increased tumorigenicity in EBV-immortalized cells, mixed tumor transplantation assays were performed (31). Initially, IL-6 virus-infected lines were inoculated subcutaneously in nude mice contralateral to control cells. Six of 10 mice injected with the IL-6 virus-infected line VDS-0 3 (10^7 cells/site) developed a tumor at the site of injection, but no animal developed a tumor at the contralateral site of injection of parent TB line (10^7 cells/site). Since serum IL-6 levels were found to be within normal limits ($\leq 5\text{--}10 \text{ U/}$

ml) in tumor-bearing mice, we can draw no conclusions as to the systemic effects of IL-6 on B cell tumorigenicity.

In subsequent experiments, we simultaneously injected the IL-6 virus-infected lymphoblastoid cells together with control cells at the same site. When the parent TB cell line (5×10^6 cell) was inoculated together with the IL-6 virus-infected VDS-0 1 line (5×10^6 cell) in one subcutaneous site, 6 of 10 animals developed a tumor. Histocompatibility typing of the tumor cells within 2 wk from removal revealed four of these tumors to be composed of a mixed cell population, where both HLA-DR types of TB (2, 6) and VDS-0 (1, 7) lines were represented. The two remaining tumors only expressed VDS-0-related histocompatibility HLA-DR antigens. We had previously determined that HLA-DR typing of purposely mixed VDS-0 and TB cell lines can readily detect either HLA-DR type when at least 20% of the cells in the mixture derive from each of the cell lines. To quantitate further the cellular composition of the mixed tumors we took advantage of the observation that VDS-0 and TB cell lines differ in surface Ig expression. As shown in Table IV, 38–62% of the human HLA-DR-expressing tumor-derived cells were surface Ig⁺, demonstrating their derivation from the IL-6-producing VDS-0 1 cell line. Most of the remaining human HLA-DR⁺ cells were surface Ig⁺, demonstrating their derivation from the parental TB cell line. These findings demonstrate that tumorigenicity of IL-6 virus-infected lines can be transferred to other cells, and strongly suggests that IL-6 is responsible for these effects.

Natural history, histology, and IL-6 production by tumor cells.

The IL-6 virus-infected lines displayed a characteristic pattern of tumorigenicity in nude mice. As shown above (Table II), all tumors arose within 2 mo of inoculation at the site of injection, and reached a maximum size of 240 mm^2 . Within 6–8 weeks from first appearance, some of these tumors, particularly the large tumors, underwent ulceration followed by total necrosis often associated with wasting and death of the animal; other tumors gradually decreased in size and eventually disappeared. In most cases, the tumors formed well-circumscribed spherical masses with at least some involvement of the overlying skin and underlying connective tissue and muscle, but local spread was limited and there was no evidence of metastasis.

Histologically (Fig. 2), rapidly growing tumors appeared as malignant lymphomas composed of sheets of cells without ar-

Table III. Relationship between Levels of IL-6 Production and Tumor Development

Cell line	IL-6 level*	Tumor incidence [†]	Mean time of first observation	Mean maximum size [‡]
	<i>U/ml</i>		<i>d</i>	<i>mm</i> ²
VDS-0 2	Low (826)	12/15	35	140
VDS-0 1	Intermediate (7,600)	9/15	21	42
TB 1	High (40,562)	9/15	33	27

* IL-6 levels are expressed as B9 U/ml supernatant conditioned by 0.5×10^6 cells in 48 h.

[†] Tumor incidence is expressed as number of animals with tumor per number of animals injected. Each animal received 10^7 cells subcutaneously in one site.

[‡] Tumor size is expressed in mm^2 and reflects the product of two-dimensional caliper measurements.

Table IV. Cells Surface Ig⁺ or Ig⁻ Expressing Human HLA-DR

Mixed tumors*	Human HLA-DR positive cells [†]	
	Ig ⁻	Ig ⁺
1	38	58
2	45	47
3	47	41
4	62	34

* Single cell suspensions of tumors derived from subcutaneous injection of the IL-6-secreting VDS-O 1 line (5×10^6 cells) and the IL-6 nonsecreting TB cell line (5×10^6) were incubated at 4°C with a rhodamine-conjugated monoclonal antihuman HLA-DR reagent (Becton Dickinson & Co.) and a fluorescein-conjugated polyclonal goat antiserum to human Ig (Cappel Laboratories).

[†] Percent cells surface Ig⁺ or Ig⁻ expressing human HLA-DR.

chitectural arrangement with little connective tissue and little inflammatory cells, except at the tumor margins. These consisted of lymphocytes and histiocytes. Most of the tumors were composed of medium-sized, uniform cells, reminiscent of Burkett's lymphoma (36). In some cases, there was a range in cell size with larger cells with vesicular nucleoli and prominent nucleoli.

Regressing tumors had prominent tumor necrosis, particularly toward the center of the tumor mass, and some infiltration with histiocytes both within and at the periphery of the tumor. It is important to note that three tumors from mice injected with control lines were indistinguishable microscopically from tumors observed in mice injected with IL-6 virus-infected lines.

With the goal of further defining their phenotype, tumors were fragmented into single cell suspensions and cultured in vitro. Two continuous cell lines were derived from separate mice injected with the parent VDS-0 line (t VDS-0 a and b); one from a mouse injected with the virus control line VDS-0 1.5 (t VDS-0 1.5); and five lines were derived each from separate mice injected with the IL-6 virus-infected lines VDS-0 1, 2, and 3, and TB 1 and 5.

Table V. IL-6 Secretion by Tumor-derived Cells

Tumor-derived cells/cell lines	IL-6 bioactivity*	
	Immediately after initiation of culture [‡]	After 12-15 passages in vitro [§]
	U/ml/48 h	
tVDS-0 a	29,050	980
tVDS-0 b	51,187	6,224
tVDS-0 1.4	15,160	528
tVDS-0 1	9,587	6,620
tVDS-0 2	15,500	1,743
tVDS-0 3	28,293	8,216
tTB 1	57,887	49,600
tTB 5	13,436	13,443

* IL-6 bioactivity was determined in a standard B9 culture assay and is expressed as U/ml of culture supernatant conditioned with tumor-derived cells for 48 h.

[‡] After removal from the animal, tumors were fractionated into single cell suspensions and incubated at 2.0×10^6 cells/ml.

[§] Tumor-derived lymphoblastoid cell lines that had been passaged in culture 12-15 times were cultured at 0.5×10^6 cells/ml.

All these tumor-derived lines were indistinguishable microscopically from those originally injected in the animals and expressed the HLA type of VDS-0 or TB cell lines (not shown). In addition, all cell lines derived from tumor-bearing animals inoculated with virus-infected cell lines were neomycin-resistant, while the two cell lines from the parent VDS-0 cell lines were neomycin-sensitive. Together, these data demonstrate that the tumors derive from in vivo growth of the injected human lymphoblastoid cells.

As shown in Table V, culture medium conditioned for 48 h with tumor-derived cells just removed from the animal contained high levels of IL-6 bioactivity, generally higher than the IL-6 levels detected in the culture supernatant of the lines giving rise to the tumors (Table II). This difference could not be explained solely on the basis of cell culture input. In addition,

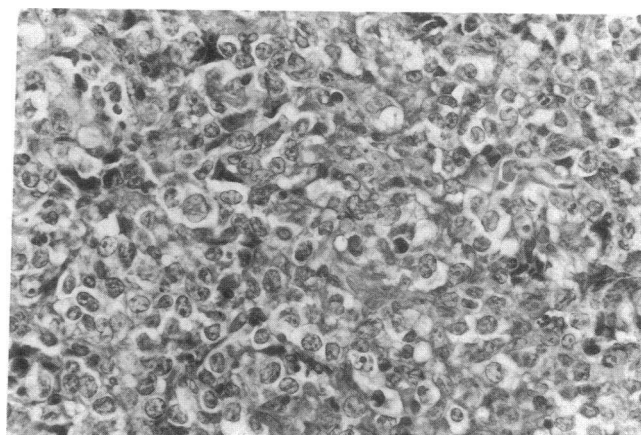
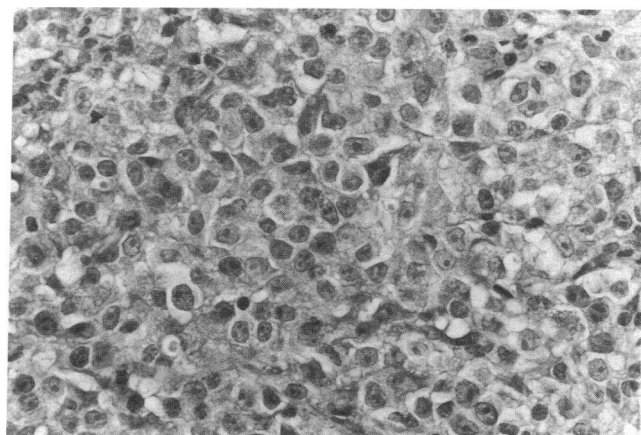


Figure 2. Sections of tumors from nude mice showing the histology of representative lymphoblastoid cell line-induced tumors. (Left) A lymphoma composed of medium-sized, uniform cells, reminiscent of Burkitt's lymphoma. Note the scarcity of connective tissue and of inflammatory cells. (Right) a lymphoma composed of cells of varying size, with larger cells displaying vesicular nuclei and prominent nucleoli. Both tumors derived from animals injected with the IL-6 virus-infected cell line VDS-0 3. Hematoxylin & eosin stain, $\times 430$.

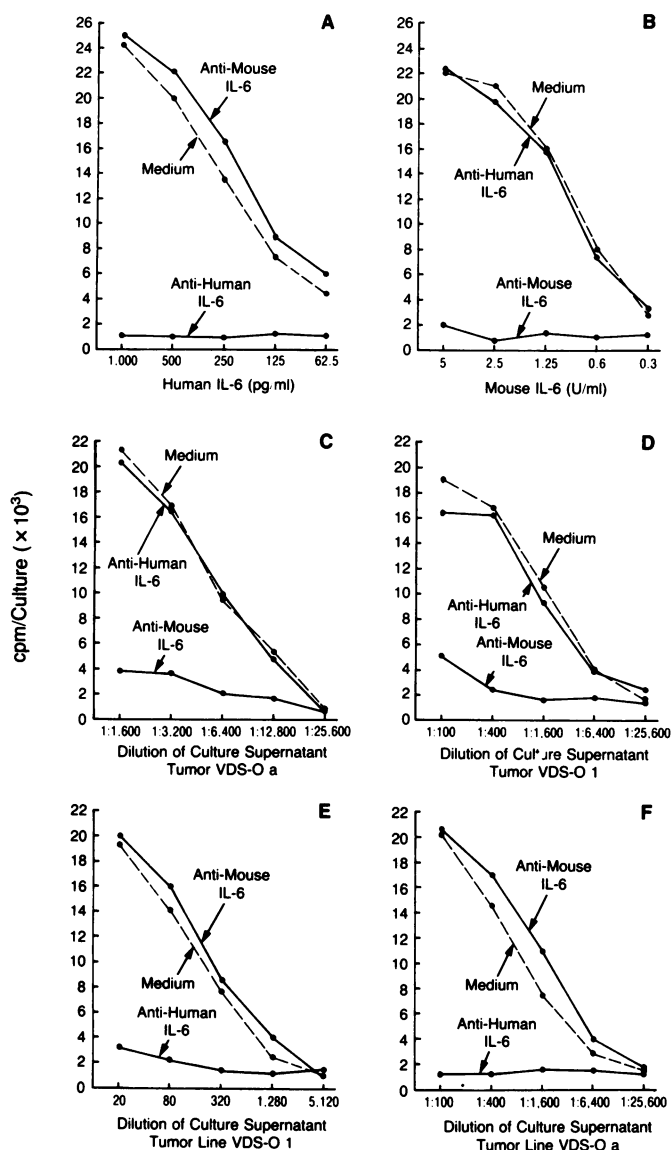


Figure 3. Detection of murine and human IL-6 in the culture supernatant of tumor-derived cells. Recombinant human (A) or mouse (B) IL-6 at the indicated concentration, was incubated for 1 h at 37°C with either medium or a MAb to human IL-6 (5 µg/ml) or with a rabbit IgG anti-mouse IL-6 (10 µg/ml) in a total volume of 0.1 ml. Culture medium conditioned for 48 h with cells from either fresh tumor VDS-0a (C), or fresh tumor VDS-0 1 (D), or tumor-derived cell lines VDS-0a (E), or VDS-0 1 (F) were incubated (1 h, 37°C) at the indicated dilutions with either a MAb to human IL-6 (5 µg/ml) or rabbit IgG anti-mouse IL-6 (10 µg/ml); total culture volume, 0.1 ml. After incubation, B9 cells (3×10^3 in 0.1 ml) were added to each well and incubation was continued for 3 d. [³H]Thymidine was added during the final 18 h of culture. Results are expressed as mean cpm of triplicate cultures.

much of this bioactivity derived from murine IL-6 in the culture supernatant, because it was largely neutralized by a heteroantiserum to mouse IL-6 (28) and only to a small degree by a MAb to human IL-6 (29). Results for all tumor-conditioned media were similar, and representative neutralizations are shown in Fig. 3, C and D. It should be noted that the anti-IL-6 reagents were specific for human or mouse IL-6 (Fig. 3, A

and B). Detection of mouse IL-6 in the culture supernatants suggests the presence of reactive mouse cells secreting high levels of IL-6 mixed in with the tumor cells of human origin.

After at least 12 passages in continuous cell culture, all tumor-derived cell lines continued to produce IL-6 bioactivity, in four of eight cases at substantially lower levels (≥ 8 -fold) than immediately after transfer to in vitro culture (Table V). In each case, all of this IL-6 bioactivity was neutralized by a MAb to human IL-6 (29), indicative of its human nature (representative neutralizations are shown in Fig. 3, E and F). This is likely due to a loss, with passage in culture, of nonproliferating IL-6-secreting murine cells, and overgrowth of human IL-6-secreting lymphoblastoid cells. It should be noted that the three cell lines originated from tumor-bearing animals inoculated with the parent cell line VDS-0 and the virus control line VDS-0 1.4 (tVDS-0a, tVDS-0b, and tVDS-0 1.4) produced substantially higher levels of human IL-6 than the lines originally inoculated in the animal (Table II). This finding suggests the occurrence of selection in vivo for high level IL-6-producing clones.

Phenotype of IL-6 virus-infected lymphoblastoid cell lines. In view of the evidence that lymphoblastoid cell lines expressing high levels of IL-6 are more tumorigenic than control lines, it was of interest to know whether these lines might display a Burkitt's lymphoma-like phenotype (37-43). However, all lymphoblastoid cell lines, regardless of how much IL-6 they produced and how tumorigenic they were, expressed similar levels of the B cell activation antigen CD23, and the cell adhesion molecule CD18 (β chain of LFA-1); none expressed CD10 (data not shown). Furthermore, all the IL-6 virus-infected lines grew in tissue culture as large clumps. Together, these data demonstrate that high level IL-6 expression in EBV-immortalized B lymphocytes does not result in their acquisition of a Burkitt's phenotype.

Given that IL-6 promotes the proliferation of EBV-immortalized cells (16-18), the tumorigenic effect of IL-6 could depend upon IL-6 providing a growth advantage to EBV-immor-

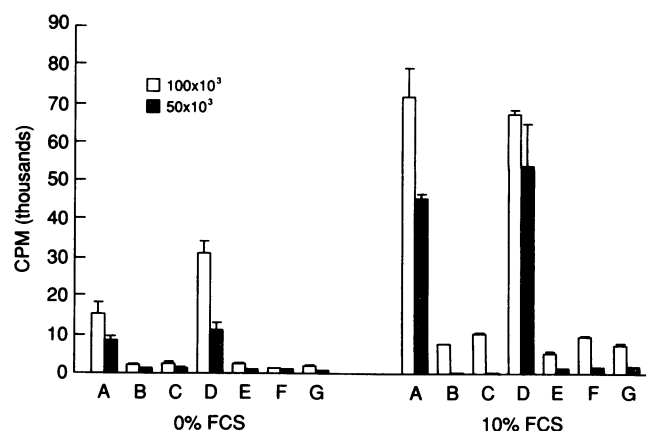


Figure 4. In vitro proliferative responses of parental TB and VDS-0 cell lines and IL-6-expressing TB and VDS-0 clones. TB (A), TB 1 (B), TB 5 (C), VDS-0 (D), VDS-0 1 (E), VDS-0 2 (F), and VDS-0 3 (G) lines were cultured at 100×10^4 (open bar) and 50×10^4 (hatched bar) as described in Methods in either Opti-MEM or RPMI supplemented with 10% FCS. Cell proliferation was measured by [³H]thymidine incorporation during the final 4.5 h of a 3-d incubation. Results are expressed as the mean cpm of triplicate samples \pm SEM.

talized cells. However, in a series of in vitro studies, all IL-6-transfected lines proliferated less vigorously than the parental cell lines either under optimal culture conditions (cell density $1-3 \times 10^5$ /ml and culture medium composed of RPMI with 10% FCS) or under suboptimal culture conditions, including low cell densities ($1-5 \times 10^4$ cells/ml) and/or deficient medium (serum-free culture medium, Opti-MEM) (Fig. 4). We examined whether the inhibited growth seen in IL-6 virus-infected lines was due to direct inhibition of B cell growth by IL-6 secreted at high levels. In three separate experiments, IL-6 at doses of 10^4 , 10^5 , 10^6 , 10^7 , and 10^8 U/ml inhibited the proliferation of VDS-0 cells (10^4 cells/ml in culture medium consisting of RPMI 1640 supplemented with 10% FCS; 3-d culture) by a mean of 38, 42, 67, 78, and 92%, respectively. It is therefore likely that inhibited growth by IL-6-transfected lymphoblastoid cell lines is due to direct inhibition by IL-6 secreted at high levels in the immediate proximity of the target cells. This finding further suggests that increased tumorigenicity of lymphoblastoid cells secreting high levels of IL-6 is not due to IL-6 providing a growth advantage to the cells secreting it.

Potential mechanisms mediating tumorigenicity of EBV-immortalized B cells. Since the above experiments indicated that tumorigenicity of IL-6-expressing lymphoblastoid lines is not readily explained on the basis of a direct effect of IL-6 on the B cells, an alternative possibility is that IL-6 might inhibit host immunity. Although immunocompromised, nude mice can produce antibodies to human cell-associated antigens (44), display high level natural killer function (45), and may develop activated monocytes in response to certain tumor cells (46). In vitro, EBV-activated B cells are sensitive to natural killer activity (47, 48), and activated monocytes secrete tumoricidal molecules such as tumor necrosis factor (49) and IL-1 (50).

To address the possibility that IL-6 might inhibit host immunity, spleen cells from nude mice were cultured for 4 d with or without IL-6 (10^8 U/ml) in the presence of IL-2 (400 U/ml). As shown in Table VI, IL-2-activated spleen cells killed efficiently both the lymphoblastoid cell line VDS-0 and the NK-sensitive YAC cell line. Addition of IL-6 together with IL-2 during preculture resulted in marked reduction of target cell killing at all E/T ratios tested. Because IL-6, at doses of 10^4 – 10^8 U/ml, had no effect both on VDS-0 and YAC-1 target cell

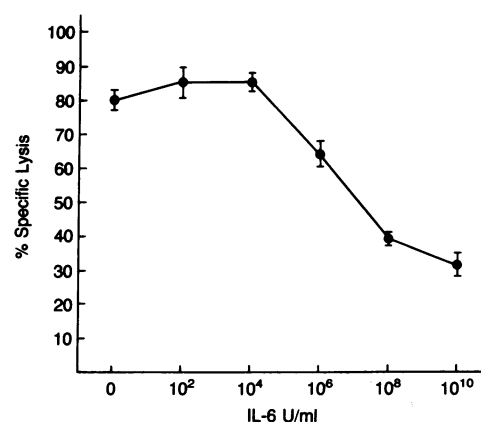


Figure 5. IL-6 inhibition of IL-2-induced killing is dose dependent. Combined splenocytes from three nude mice were preincubated for 4 d in medium supplemented with either IL-2 alone (500 U/ml) or with IL-2 plus IL-6 at varying concentrations (10^2 – 10^{10} U/ml). After incubation, the cells were tested in triplicate for cytotoxic activity against ^{51}Cr -labeled VDS-0 target cells at a ratio of 100:1. The results are expressed as percent mean specific lysis \pm SD.

killing by cells already activated in vitro with IL-2 (not shown) and on the viability of splenocytes cultured with IL-2, the present experiments demonstrate that IL-6 interferes with IL-2 activation of killer cells but is not cytotoxic for these cells. Effective suppression of IL-2-induced killing required a high cytokine concentration (Fig. 5), $>10^8$ U/ml.

Discussion

In the present study, we have explored a potential role for IL-6 in B-cell lymphomagenesis. When the IL-6 gene was expressed at high levels in EBV-immortalized normal B lymphocytes these cells became highly tumorigenic in athymic mice. It is unlikely that this effect is the result of a direct action of IL-6 on the B cell because IL-6-expressing lymphoblastoid cell lines were indistinguishable from IL-6 nonexpressing lines in morphology and a number of cell surface characteristics. In addition, the IL-6-expressing cells exhibited no growth advantage

Table VI. IL-6 Inhibits the Activation of IL-2-stimulated Killer Cells

Exp.	Culture conditions*	Percent specific killing							
		VDS-O Cell Targets				YAC Cell Targets			
		100:1	50:1	25:1	12:1	100:1	50:1	25:1	12:1
1	IL-2	81 [‡]	54	38	20	62	58	48	35
	IL-2 + IL-6	28	26	16	7	40	37	28	26
2	IL-2	80	60	49	29	97	81	79	72
	IL-2 + IL-6	39	29	5	4	63	61	48	28
3	IL-2	50	49	33	14	46	44	41	20
	IL-6	2	0	0	0	6	1	0	1
	IL-2 + IL-6	15	14	3	5	28	23	29	16

* Fresh splenocytes from 4–6-wk-old nude mice were incubated (2×10^6 cells/ml, 2 ml/well) with either IL-2 alone (500 U/ml) or IL-6 alone (10^8 U/ml) or IL-2 (500 U/ml) plus IL-6 (10^8 U/ml) for 4 d in complete mouse cell culture medium. After incubation, the cells were tested for killing of ^{51}Cr -labeled VDS-O or YAC-1 cell targets at the indicated ratios. [‡] Results of cytotoxicity assays are expressed as mean percent specific lysis of triplicate culture. Standard deviations were within 15% of the mean.

in vitro over IL-6 nonexpressing cells. Rather, IL-6-associated tumorigenicity in this experimental system is likely to depend upon IL-6 inhibiting host tumor immunity.

In recent studies, introduction of an IL-6 cDNA expression vector in an EBV-immortalized B cell line conferred the line a growth advantage in culture, and made it tumorigenic in nude mice (51). The growth advantage exhibited by the IL-6-transfected cells was attributed to a direct autocrine action of IL-6 on the B cell. In the experiments described here, the IL-6-transfected cells proliferated less vigorously than control cells under a variety of culture conditions. The different results are likely to depend upon the different amounts of IL-6 secreted by the two sets of transfectants. The IL-6 virus-infected cell lines used here produced between 6,500 and 40,000 IL-6 units per milliliter of culture conditioned by 10^6 cells for 48 h (Table I). In contrast, the IL-6-transfected MC3 cells appeared to produce levels of IL-6 lower by at least 20-fold. We have previously reported that IL-6 can stimulate growth in EBV-infected B cells at concentrations of 10–100 U/ml (16–18). However, at concentrations greater than 5,000 U/ml, IL-6 was found to inhibit growth in EBV-immortalized B cells in a dose-dependent manner. Thus, similar to other cytokines, IL-6 may exhibit both stimulatory and inhibitory effects on the same target cells depending upon its concentration.

Since all five IL-6-expressing lines used here exhibited decreased proliferation in vitro when compared with control lines, it is unlikely that increased tumorigenicity is the result of direct B cell stimulation by IL-6. Rather, tumorigenicity may lie upon an IL-6-mediated impairment of tumor cell surveillance. Although immunocompromised, nude mice display high levels of natural killer functions (45), and in vitro, EBV-activated B cells are sensitive to NK activity (47, 48).

Results presented here indicate that IL-6, at high concentrations, inhibits lymphokine-activated killer (LAK) activity by nude mouse splenocytes. LAK cells are believed to derive, for the most part, from lymphokine activation of NK cells (52). Consistent with these results in vitro, the increased tumorigenicity of IL-6-expressing lymphoblastoid cell lines in vivo might depend upon IL-6 inhibiting NK functions at the tumor site. In the human, NK cells are activated by IL-6 at low concentrations (≤ 1 U/ml), indicating that these cells express functional IL-6 receptors (53). At high concentrations, IL-6 might downregulate IL-6 receptor expression, a process reported to occur in monocytes (54).

In addition to displaying high levels of NK activity, nude mice may develop activated monocytes in response to certain tumor cells (46). Monocyte activation at the tumor site was prominent in this study, as evidenced by the high levels of murine IL-6 produced by tumor cell suspensions cultured in vitro. When activated, monocytes may secrete tumoricidal molecules such as tumor necrosis factor (49) and IL-1 (50). Recently, it was reported that IL-6 can downregulate IL-1 β and TNF gene expression in human activated monocytes by acting at the level of transcription (55). If tumor-derived IL-6 is capable of similar actions in vivo, it could effectively inhibit a potentially important cytotoxic function of the monocyte at the tumor site.

Polyclonal and oligoclonal malignancies of EBV-infected B lymphocytes have been reported with increasing frequency in individuals with acquired immunodeficiencies such as AIDS patients (2, 56) and post-transplant recipients (4). The observation that EBV-immortalized B cells secreting high levels of IL-6

have a propensity for giving rise to tumors in immunocompromised hosts raises the possibility that IL-6 may be a factor in human B cell lymphomagenesis. It is worth noting that HIV-1-infected individuals generally display abnormally elevated serum IL-6 levels (57, 58), possibly through a direct action of the virus (58, 59). This abnormality could predispose AIDS patients to the development of EBV-associated malignancies.

Recently, IL-6 has been considered for experimental therapy in cancer patients. The results presented here suggest that this molecule should be used with caution.

Acknowledgments

We thank Dr. E. Jaffe for examining tumor cell biopsies; the NIH Blood Bank for continuous support; Dr. P. B. Sehgal for providing the cDNA clone pBeta-2-22; Dr. R. Nordan for providing the hybridoma B9 cell line, mouse IL-6, and an anti-murine IL-6 antibody; Dr. M. Aarden for providing a MAbs to human IL-6; and Dr. McManaway for helping with animal experiments. We also thank Dr. I. T. Magrath for helpful discussions; Drs. R. Yarchoan and L. Matis for critically reading the manuscript; and Ms. E. Caruso for editorial assistance.

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