Tumorigenesis and Aberrant Signaling in Transgenic Mice Expressing the Human Herpesvirus-8 K1 Gene

Om Prakash, Zhen-Ya Tang, Xiaochang Peng, Roy Coleman, Javed Gill, Gist Farr, Felipe Samaniego

Background: **The K1 gene of human herpesvirus-8 (HHV-8; also known as Kaposi's sarcoma-associated herpesvirus) encodes a transmembrane signaling protein that elicits cellular activation events. To evaluate the potential role of K1 in HHV-8-associated pathogenesis, we produced transgenic mice expressing the HHV-8 K1 gene under the transcriptional control of the simian virus 40 promoter.** ***Methods:*** **Three independent heterozygous transgenic K1 mouse lines were generated from founder mice. Mouse splenic and thymic lymphocytes and tumor tissues were analyzed for the expression of cytokines involved in inflammatory and immune responses, including tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6), basic fibroblast growth factor (bFGF), and interleukin 12 (IL-12); for the activation of the transcription factors nuclear factor-κB (NF-κB) and the B cell-specific transcription factor Oct-2; and for the activation of the Src and Syk family kinases, components of B-cell receptor-induced signal-transduction pathways.** ***Results:*** **Expression of bFGF was increased in K1-transgenic mice as compared with nontransgenic mice, whereas expression of TNF-α and IL-6 did not differ using reverse transcriptase–polymerase chain reaction. K1-transgenic mice showed substantially less serum IL-12 induction than did nontransgenic mice when challenged with a lipopolysaccharide. B lymphocytes from K1-transgenic mice but not from nontransgenic mice showed constitutive activation of NF-κB and Oct-2. K1 expression in human B lymphocytes stimulated NF-κB-dependent promoter activity. B lymphocytes from K1-transgenic mice also showed increased phosphorylation of Lyn, a Src family tyrosine kinase, and enhanced Lyn activity. Tumors in K1-transgenic mice showed features indicative of a spindle-cell sarcomatoid tumor and a malignant plasmablastic lymphoma. The pattern of cytokine, transcription factor, and Lyn kinase activity in the lymphoma was similar to that in B lymphocytes from K1-transgenic mice.** ***Conclusion:*** **K1 may be involved in the activation of NF-κB signaling. The enhanced NF-κB activity in nonmalignant lymphocytes of K1 mice and its persistence in lymphoma tumors of these mice suggest that the K1 mouse may be a model of premalignancy. [J Natl Cancer Inst 2002;94: 926–35]**

Human herpesvirus 8 (HHV-8, also known as Kaposi's sarcoma (KS)-associated herpesvirus or KSHV) is a recently identified gamma-2 herpesvirus related to three other tumorigenic viruses: herpesvirus saimiri (HVS), Epstein-Barr virus (EBV), and the murine gamma herpesvirus 68 *(1–3).* HHV-8 has been implicated in the pathogenesis of all epidemiologic forms of KS (classic, African endemic, post transplantation, and acquired immunodeficiency syndrome [AIDS]-associated) and certain lymphoproliferative disorders, such as primary effusion lymphomas (PELs) and multicentric Castleman's disease [*(4,5);* reviewed in *(6)*]. The HHV-8 genome encodes more than 85 open reading frames (ORFs), including 66 with sequence similarity to those in HVS and 15 (K1 to K15) that are unique to HHV-8 *(2).* Several viral homologs of cellular genes have been identified in HHV-8; these include genes encoding interleukin 6 (IL-6, ORF K2), cyclin D (ORF 72), bcl-2 (ORF 16), interferon regulatory factor (ORF K9), three β chemokines (ORFs K6, K4, and K4.1), and the G protein-coupled receptor (ORF 74), which is most homologous to the human receptors for interleukin 8 [reviewed in *(7)* and *(8)*]. These cellular homologs may contribute to the pathogenicity of HHV-8 [reviewed in *(7)*].

Among the HHV-8-unique K ORFs, K1 is a 46-kDa transmembrane glycoprotein related to the immunoglobulin receptor family and contains a sequence that resembles the immunoreceptor tyrosine-based activation motif (ITAM) *(9).* ITAM motifs are found in the cytoplasmic domains of B-lymphocyte, T-lymphocyte, and certain other receptors, and are able to couple extracellular signals in response to ligand–receptor interactions to multiple intracellular signaling; this leads to cellular responses, including proliferation, differentiation, and death [reviewed in *(10)*]. However, K1 is unique in its ability to transduce signals even in the absence of exogenous cross-linking ligands and to orchestrate the expression of an array of transcription factors involved in cellular activation that may ultimately lead to growth deregulation *(11,12).* This capability is further supported by studies of Lee et al. *(13),* showing that K1 expression transforms rodent fibroblasts *in vitro* and induces lymphomas *in vivo* in common marmosets infected with recombinant HVS in which the saimiri transformation protein (STP) oncogene had been replaced with the K1 gene.

K1 is expressed at low levels in chronically infected PEL cells and at higher levels in the early lytic viral life cycle *(9,14).* To assess the biologic consequences of K1 expression *in vivo,* we generated transgenic mice that expressed the HHV-8 K1 gene in their tissues under the transcriptional control of simian virus 40 (SV40) promoter.

K1-mediated cellular responses in these mice were investigated by analyzing the tissues and cells for aberrant expression of cytokines involved in inflammatory and immune responses and for the activation of Oct-2, a B-cell-specific transcription factor *(15),* and nuclear factor-κB (NF-κB), a potent inducer of

Affiliations of authors: O. Prakash, Z.-Y. Tang, X. Peng, R. Coleman (Laboratory of Molecular Oncology), J. Gill, G. Farr (Department of Pathology), Ochsner Clinic Foundation, New Orleans, LA; F. Samaniego, Department of Lymphoma/Myeloma and Clinical Cancer Prevention, The University of Texas M. D. Anderson Cancer Center, Houston.

Correspondence to: Om Prakash, Ph.D., Laboratory of Molecular Oncology, Ochsner Clinic Foundation, 1516 Jefferson Hwy., New Orleans, LA 70121 (e-mail: oprakash@ochsner.org).

See "Notes" following "References."

genes involved in inflammatory responses and cellular growth regulation *(16,17)*. In B lymphocytes, B-cell receptor stimulation is accompanied by phosphorylation of receptor ITAMs and activation of the Src and Syk family of tyrosine kinases *(18,19)*. Thus, we also examined K1 mice for the activation of Src and Syk kinases. These findings may eventually help in the development of targeted therapies.

Materials and Methods

Production of Transgenic Mice

The K1 ORF derived from the DNA of HHV-8-infected BC-3 lymphoma cells was cloned in the pSG5 vector (Stratagene, La Jolla, CA) and was expressed under the transcriptional control of SV40 promoter *(12)*. The 2.2-kb fragment containing the K1 ORF expression cassette was obtained by digesting the plasmid DNA with *Sal*I and microinjecting it into one-cell embryos obtained from F_1 hybrids of C57BL/6J and CBA/J mice. The injected embryos were then implanted into pseudopregnant CD-1 female mice as previously described *(20)*. Offspring were screened for the incorporation of the transgene into the mouse genome by Southern blot analysis of genomic DNA extracted from tail biopsy specimens. Heterozygous lines from the founder mice were established by backcrossing transgenic mice with K1-negative F_1 mice. The K1-positive progeny in the heterozygous lines showed expression of K1 in all tissues tested (liver, kidney, heart, brain, spleen, thymus, lung, lymph nodes) by reverse transcription–polymerase chain reaction (RT–PCR). Animal care and use was in accordance with the Ochsner Clinic Foundation Animal Care and Use Committee guidelines.

Isolation of B and T Lymphocytes

Single-cell suspensions of tissues (thymus and spleen) from 6- to 8-month-old mice were prepared in phosphate-buffered saline (PBS). Erythrocytes were lysed, and PBS-washed cell suspensions were incubated at 4 °C for 1 hour with Mouse pan B (B220) and Mouse pan T (Thy 1.2) magnetizable polystyrene beads (Dynal, Inc., Lake Success, NY). The nonbinding cells were pipetted off, and the rosetted cells, isolated by the use of a magnetic device, were washed three times with PBS and used immediately or stored frozen as rosettes at –70 °C until use.

RNA Extraction and RT–PCR for Quantification of K1 and Cytokine Messenger RNAs from Mouse Tissues and Cells

Total cellular RNA from liquid nitrogen-frozen tissues was extracted by use of the RNeasy Mini Kit (Qiagen, Valencia, CA), according to the supplier's instructions. To eliminate DNA contamination, extracted RNA was treated with ribonuclease-free deoxyribonuclease. The final preparation was quantified spectrophotometrically, examined for the presence of 18S and 28S ribosomal RNA bands, and stored at –70 °C until use.

Reverse transcription of the RNA (500 ng) was performed in a final volume of 20 μL containing 50 m*M* Tris–HCl, 75 m*M* KCl, 3 m*M* $MgCl_2$, 10 m*M* dithiothreitol, 0.5 μ*M* each of the deoxynucleotide triphosphates, 200 ng of random primers (Promega Corp., Madison, WI), and 200 U of reverse transcriptase (Life Technologies, Inc. [GIBCO BRL], Rockville, MD). The reaction mixture was incubated at 42 °C for 1 hour and then heated at 94 °C for 10 minutes to inactivate the enzyme. One microliter of this reverse-transcribed mixture was used for PCR amplification of the complementary DNA in a 50 μL reaction volume.

The primers for the PCR amplification of the mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH); NF-κB (p50); all of the mouse cytokines except basic fibroblast growth factor (bFGF), interleukin 12 (p35) (IL-12 [p35]), and IL-12 (p40); and their positive-control PCR products were obtained from BD Biosciences Clontech (Palo Alto, CA). The primer and oligoprobe sequences for bFGF, IL-12 (p35), and IL-12 (p40) were obtained from the published sequence and were as follows: bFGF (GenBank, Accession No. E02544), 5′-GGCTTCTTCTTCCTGCGCATCCAC-3′, 5′-CTGCCACTTCGTTTCAGTGC-3′, and 5′-TGGCTTCTAAATGTGTTACGGATGAGTGTT-3′; IL-12 (p35) (GenBank, Accession No. M86672), 5′-AAGACATCACACGGGACCAAACCA-3′, 5′-CGCAGAGTCTCGCCATTATGATTC-3′, and 5′-CTGCCTGCCCCCACAGAAGACGTCT-3′; IL-12 (p40) (GenBank, Accession No. MN008352), 5′-CCACTCACATCTGCTGCTCCACAAG-3′ and 5′-CACCAAATTACTCCGGACGGTTCAC-3′.

The primer and oligoprobe sequences for K1 were obtained from the sequence analysis (GenBank, Accession No. AF170531) of the K1 ORF cloned in the pSG5 vector *(12)* and were as follows: 5′-ATGTTCCTGTATGTTGTCTGC-3′, 5′-ACTAAGCTATTAGTACTGAATGT-3′, and 5′-CTTTGTATTGTTCTACCTCTGGAAA-3′. The PCR amplification was carried out for 30 cycles by use of PerkinElmer (Foster City, CA) PCR reagents in a 50 μL volume and a PerkinElmer DNA Thermal Cycler Model 9700, according to the PCR protocol provided by BD Biosciences Clontech. To ascertain that equal amounts of RNA were used in each RT–PCR reaction, the same reverse transcription mixture was used for concurrent amplification of GAPDH.

The amplified RT–PCR products were detected by Southern blot analysis of 25 μL (5 μL for GAPDH) of the final reaction mixture by use of the ^{32}P-labeled positive internal PCR products or oligoprobes.

Electrophoretic Mobility Shift Assays

The nuclear NF-κB and Oct-2 activities in the splenic B lymphocytes of K1-transgenic and nontransgenic mice were determined by electrophoretic mobility shift assays (EMSA). Nuclear extracts were prepared and EMSA were performed as described previously with minor modifications *(21)*. Briefly, single-cell suspensions of thymic or splenic mouse tissues, or isolated B and T lymphocytes, were gently homogenized at 4 °C for 10 minutes in cell lysis buffer containing 10 m*M* HEPES (pH 7.6), 60 m*M* KCl, 1 m*M* EDTA, 0.75% (v/v) Nonidet P-40, 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 1m*M* dithiothreitol, and protease inhibitor cocktail (Sigma, St. Louis, MO). After homogenization, the lysates were centrifuged at 5000*g* at 4 °C for 10 minutes to collect nuclear pellets. The pellets were resuspended in nuclear extraction buffer containing 20 m*M* Tris–HCl (pH 8.0), 420 m*M* NaCl, 0.2 m*M* EDTA, 1 m*M* PMSF, 25% (v/v) glycerol, and protease inhibitor cocktail (Sigma). After incubation at 4 °C for 30 minutes, the extracts were centrifuged at 12 000*g* at 4 °C for 20 minutes, and the supernatants containing nuclear extracts were stored at –70 °C until use.

For EMSA, 10 μg of nuclear extracts were preincubated with 50 μg/mL poly (dI-dC) in a reaction mixture containing 10 m*M* Tris–HCl (pH 7.5), 250 m*M* NaCl, 0.5 m*M* EDTA, 1 m*M* $MgCl_2$, and 4% (v/v) glycerol at 25 °C for 10 minutes. The ^{32}P-labeled oligonucleotides (1×10^3 counts per minute [cpm])

were added to a final volume of 10 μL, and incubation was continued for an additional 20 minutes. Competition assays were performed by incubating extracts with a 50- to 100-fold excess of unlabeled competitor oligonucleotides for 10 minutes before the addition of probe. Samples were analyzed on 6% polyacrylamide DNA retardation gels (Invitrogen Corp., Carlsbad, CA.) The gels were dried and then exposed to BioMax MR films (Kodak, Rochester, NY).

The NF-κB oligonucleotides were purchased from Promega. The double-stranded (only single strand shown) oligonucleotide for Oct-2 was obtained from the published sequence *(22)* and was as follows: 5′-CGTCTCATGCGATGCAAATCACTTGAGATC-3′. The mutated oligonucleotide used for competition was generated by introducing mutations in the consensus octamer site (underlined) and were as follows: ATAATAAT. Nuclear extracts from phorbol 12-myristate 13-acetate (PMA)-treated (100 ng/mL for 16 hours) Jurkat cells showing NF-κB binding activity were used as a positive control.

Western Blot and Immunoprecipitation Analysis of B and T Lymphocytes for the Activation of Protein Tyrosine Kinases

Mouse tissues (spleen, thymus, omentum, or tumor) or isolated B and T lymphocytes were lysed by homogenization in lysis buffer containing 50 m*M* Tris–HCl (pH 7.5), 150 m*M* NaCl, 3 m*M* $MgCl_2$, 0.1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 m*M* PMSF, and 10 μg/mL protein inhibitor cocktail (Sigma). Lysates were centrifuged at 12 000*g* at 4 °C for 10 minutes to collect cell-free supernatants. Proteins (100 μg) were resolved on 12% SDS–polyacrylamide gels by electrophoresis and transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). The blots were blocked with 3% nonfat milk in PBS for 1 hour at room temperature. Phosphorylated Lyn, Syk, and tyrosyl proteins were detected by using anti-Lyn (H-6) and anti-p-Tyr (PY99) monoclonal and anti-Syk (LR) rabbit polyclonal as the primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and horseradish peroxidase-linked sheep anti-mouse immunoglobulin G (IgG) and anti-rabbit IgG (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) as the secondary antibodies. The ECL PLUS chemiluminescent system (Amersham Pharmacia Biotech, Inc.) was used for visualization of the reacted proteins on the blots.

Tyrosyl phosphorylation of Lyn was detected by subjecting Lyn immunoprecipitated with agarose-conjugated anti-Lyn (H-6) antibody from extracts containing 500 μg of protein to electrophoresis and immunoblot analysis, as described above, by using mouse monoclonal anti-p-Tyr antibody.

In Vitro Kinase Assay

Lyn was immunoprecipitated from omentum, spleen, or tumor lysates containing 500 μg of protein as described above and washed twice in kinase buffer (50 m*M* HEPES [pH 7.5], 0.1 m*M* EDTA, 0.15% Brij 35). The washed immunoprecipitates were incubated in 20 μL kinase buffer containing 10 μg of the tyrosine kinase substrate Raytide (Oncogene Research Products, Cambridge, MA), 75 μ*M* ATP, 15 m*M* $MgCl_2$, and 4 μCi [γ-^{32}P]ATP (DuPont-NEN, Boston, MA) and incubated for 30 minutes at 30 °C. The reactions were stopped by adding 120 μL of 10% phosphoric acid, and the radioactivity incorporated into the substrate was estimated by a filter assay.

Transfection and Luciferase Assays

To test whether constitutively activated NF-κB in K1-transgenic mice is functionally active, we carried out transfection assays in Raji cells, a human Burkitt's lymphoma cell line (American Type Culture Collection, Manassas, VA), with a K1 expression plasmid (pSG5-K1) and an NF-κB reporter construct in which the firefly luciferase gene is under the transcriptional control of a synthetic promoter containing five tandem binding sites for NF-κB (Stratagene). The cells were propagated in RPMI-1640 (Irvine Scientific, Irvine, CA) supplemented with 10% fetal bovine serum (FBS), 2 m*M* L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Approximately 2.5 million cells were transfected with 5 μg of NF-κB–luciferase reporter construct and with 10 μg of pSG5-K1 or pSG5 alone by electroporation (Gene Pulser II; Bio-Rad Laboratories) at 280 V and 960 μF in 250 μL of complete medium, resuspended in 6 mL of medium, and incubated for 48 hours. Cells were harvested and lysed in lysis buffer (Promega), and lysates containing equivalent amounts of protein were assayed for luciferase activity by using the Luciferase Assay System (Promega) and a luminometer (Turner Designs, Sunnyvale, CA). Transfection efficiencies were normalized by cotransfection with a reporter plasmid containing chloramphenicol acetyltransferase gene under the transcriptional control of the cytomegalovirus promoter (CMV–CAT). Assays were performed in triplicate, and the means of each with upper and lower 95% confidence intervals (CIs) were plotted.

IL-12 Assay

IL-12 in 50-μL serum samples from mice pretreated with or without lipopolysaccharide (LPS) were quantified by enzyme-linked immunosorbent assay (ELISA) using the Quantikine M mouse IL-12 p70 kit (R&D Systems, Minneapolis, MN). The sensitivity of the assay was 2.5 pg/mL. Animals challenged with LPS (*Escherichia coli* 055:B6 [Sigma]) received 1 mg/kg intraperitoneally 2 hours before blood collection.

Flow Cytometric Analysis of Lymphoma Cells

The immunophenotypic profile of cells from a lymphoma lesion observed in a K1 transgenic mouse was determined by flow cytometry (FACSvantage SE, BD Biosciences, Mountain View, CA). A single-cell suspension of tumor subtransplants maintained in nude mice (BALB/c nu/nu) was treated with red cell lysing reagent (Sigma) to remove red blood cells. A total of 1×10^6 cells were stained with fluorescein isothiocyanate-conjugated antibodies (PharMingen, San Diego, CA) according to the manufacturer's instructions. The cells were suspended in PBS and analyzed. The antibodies targeted CD3, CD4, CD8, CD19, CD34, CD38, CD45, CD45R/B220, CD79, CD138, λ light chain and κ light chain immunoglobulins.

Histologic and Immunohistochemical Evaluation of Tumor Tissues

For histologic examination, tissues were fixed in 10% buffered formalin and embedded in paraffin. Five-micrometer sections were stained with hematoxylin and eosin. Expression of cell surface markers (CD3, CD19, and CD45) in primary tumors or tumor transplants in nude mice (BALB/c nu/nu) was assessed

by immunoperoxidase staining of the 5-μm frozen sections fixed in acetone. Sections were treated sequentially with primary and biotinylated secondary antibodies (PharMingen) and subsequently processed using a Vectastain kit (Vector Laboratories, Inc., Burlingame, CA).

Statistical Analysis

The results are presented as means with 95% CIs. Comparisons between groups were made by use of Student's *t* test, where appropriate. Two-sided *P* values <.05 were considered to be statistically significant.

Results

NF-κB (p50) and Cytokine mRNA Expression in B and T Cells Derived From the Tissues of K1-Transgenic Mice

To better understand the cellular effects of the HHV-8 K1 gene, we generated three independent heterozygous lines of mice that expressed the K1 gene in most tissues under the transcriptional control of the SV40 promoter as determined by semiquantitative RT–PCR (data not shown). We examined the effects of K1 on the expression of transcription factors and cytokine genes by comparing the mRNA levels of NF-κB p50 and cytokines in the B and T lymphocytes isolated from the thymus and spleen tissues of nontransgenic and K1-transgenic mice. NF-κB p50 is one of the subunits of the NF-κB transcription factor. In two randomly selected K1-transgenic mice from one of the lines, the NF-κB p50-related transcripts and bFGF transcripts were substantially elevated in the splenic and thymic B and T lymphocytes compared with those from the nontransgenic animals (Fig. 1). We also found that IL-12 p35 transcripts were markedly decreased in the K1-expressing B lymphocytes compared with the expression in B lymphocytes from nontransgenic mice (Fig. 1). In contrast, consistent with the earlier reports that IL-12 is not expressed in T lymphocytes *(23),* no IL-12 p35 transcripts were observed in T cells. IL-12 is a 70-kDa (p70) heterodimeric cytokine composed of two polypeptide chains, p35 and p40, encoded by separate genes and is a potent inducer of T helper cells type 1 (Th1) responses *(24,25).* No differences in the expression of IL-2, IL-4, IL-6, IL-10, granulocyte–macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α) or TNF-β were observed between the K1-transgenic and nontransgenic mice.

Serum IL-12 p70 Levels in K1-Transgenic Mice

To determine whether the defect in IL-12 p35 expression in the B cells of K1 mice was accompanied by impaired production of IL-12 p70 heterodimer, we assayed serum IL-12 p70 levels in 15 nontransgenic and 20 transgenic mice by ELISA. As shown in Fig. 2, the serum IL-12 levels were low but comparable in nontransgenic and K1-transgenic mice (compare lanes 1 and 2), but when we compared IL-12 production in LPS-treated mice, K1-transgenic mice produced only about 20% as much IL-12 heterodimer as the nontransgenic mice (compare lanes 3 and 4). LPS treatment did not produce significant ($P = .09$) increases in IL-12 production in K1-transgenic mice compared with that in the nontransgenic mice (compare lanes 2 and 4). Our results suggested that the defect in IL-12 production in K1-transgenic mice was directly or indirectly related to K1 expression in these mice.

Activation of NF-κB and B-Lymphocyte-Specific Transcription Factor Oct-2 in K1-Transgenic Mice

Because NF-κB p50 subunit mRNA levels were increased in the B and T cells of K1 mice, we examined the nuclear NF-κB activity in splenic B lymphocytes by EMSA. As shown in Fig. 3, A, the nuclear extracts from K1-expressing B lymphocytes showed intense NF-κB DNA-binding activity compared with undetectable levels in the extracts from nontransgenic mice (compare lanes 1 and 2 with lanes 3 and 4). Data shown are representative of six mice in each group. An identical shifting pattern was also observed when nuclear extracts from PMA-treated Jurkat cells were used for EMSA (lane 7). A 100-fold excess of unlabeled NF-κB oligonucleotides effectively abrogated the binding activity (lanes 5, 6, and 8), indicating that the protein–DNA complex was NF-κB specific. The results sug-

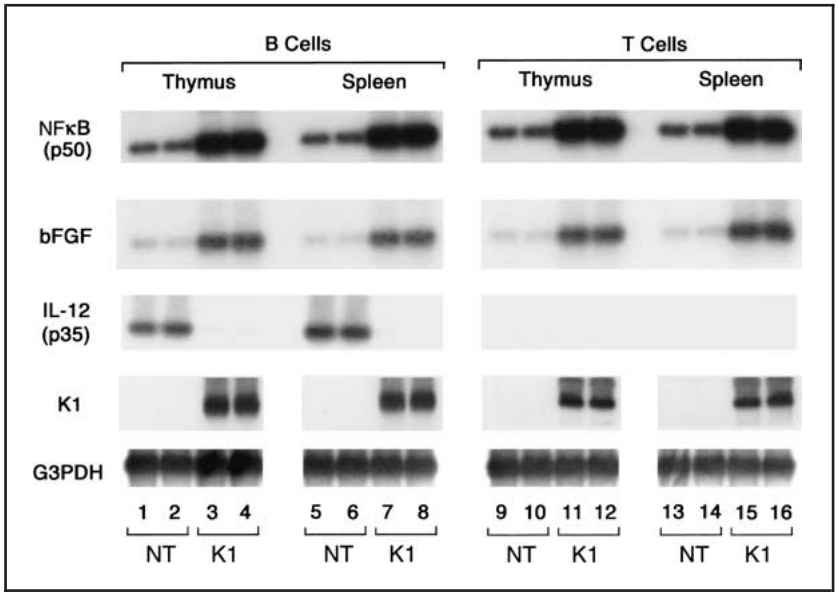

Fig. 1. Expression of the genes for human herpesvirus 8 (HHV-8) K1 protein, the p50 subunit of nuclear factor-κB [NF-κB (p50)], basic fibroblast growth factor (bFGF), and the p35 subunit of interleukin 12 [IL-12 (p35)] in B and T cells from mouse thymus and spleen tissues. Samples from K1-nontransgenic (NT) mice are shown in **lanes 1**, **2**, **5**, **6**, **9**, **10**, **13**, and **14**; samples from K1-transgenic (K1) mice are shown in **lanes 3**, **4**, **7**, **8**, **11**, **12**, **15**, and **16**. Semiquantitative reverse transcription–polymerase chain reaction analysis was carried out as described in the "Materials and Methods" section. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression was used as an internal control.

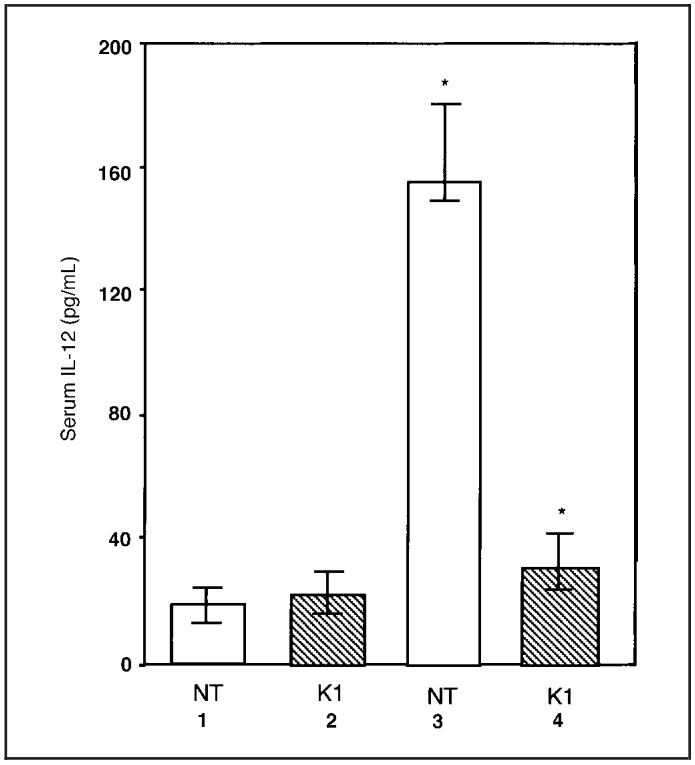

Fig. 2. Lipopolysaccharide (LPS)-stimulated production of interleukin 12 (p70) [IL-12 (p70)] in K1-transgenic mice. IL-12 (p70) concentrations in serum samples (50 μL) of nontransgenic mice (NT) (n = 15) and K1-transgenic mice (n = 20) were measured by enzyme-linked immunosorbent assay (ELISA) as described in the "Materials and Methods" section before (**bars 1** and **2**) and after (**bars 3** and **4**) mice were treated with LPS. Data are presented as the mean with **error bars** indicating 95% confidence intervals (CIs). * There were statistically significant differences in serum IL-12 levels between NT mice (**bar 1**) and LPS-treated NT mice (**bar 3**) (P<.001) and between NT mice (**bar 1**) and LPS-treated K1-transgenic mice (**bar 4**) (P = .023).

gested that K1 expression leads to constitutive activation of the NF-κB transcription factor.

To determine whether K1-induced NF-κB stimulated NF-κB-dependent promoters, we coexpressed K1 (pSG5-K1) and NF-κB–luciferase reporter constructs in Raji cells. Cells transfected with K1 plasmid DNA showed nearly 12-fold higher luciferase activity compared with the control empty vector (Fig. 3, B). Moreover, the NF-κB activity varied proportionately with that in the concentration of the K1 plasmid (data not shown). These results indicate that K1 expression leads to the activation of functionally active NF-κB.

Earlier studies have shown that NF-κB plays an important role in the transcriptional activation of the Oct-2 gene *(26)*. Because NF-κB nuclear activity was increased in the splenic B cells from K1 mice (Fig. 3, A), we examined the possibility that Oct-2 DNA binding was also increased in these cells. As shown in Fig. 4, the nuclear extracts from B cells of K1 mice had substantially higher Oct-2 DNA binding activity than did nuclear extracts from B cells of nontransgenic mice (compare lanes 1 and 2 with lanes 3 and 4). The data are representative of six transgenic mice. Excess unlabeled oligonucleotides, but not mutant oligonucleotides, abrogated Oct-2 DNA binding (compare lanes 5 and 6 with lanes 7 and 8). The results of the EMSA suggest that K1 expression also leads to constitutive activation of Oct-2.

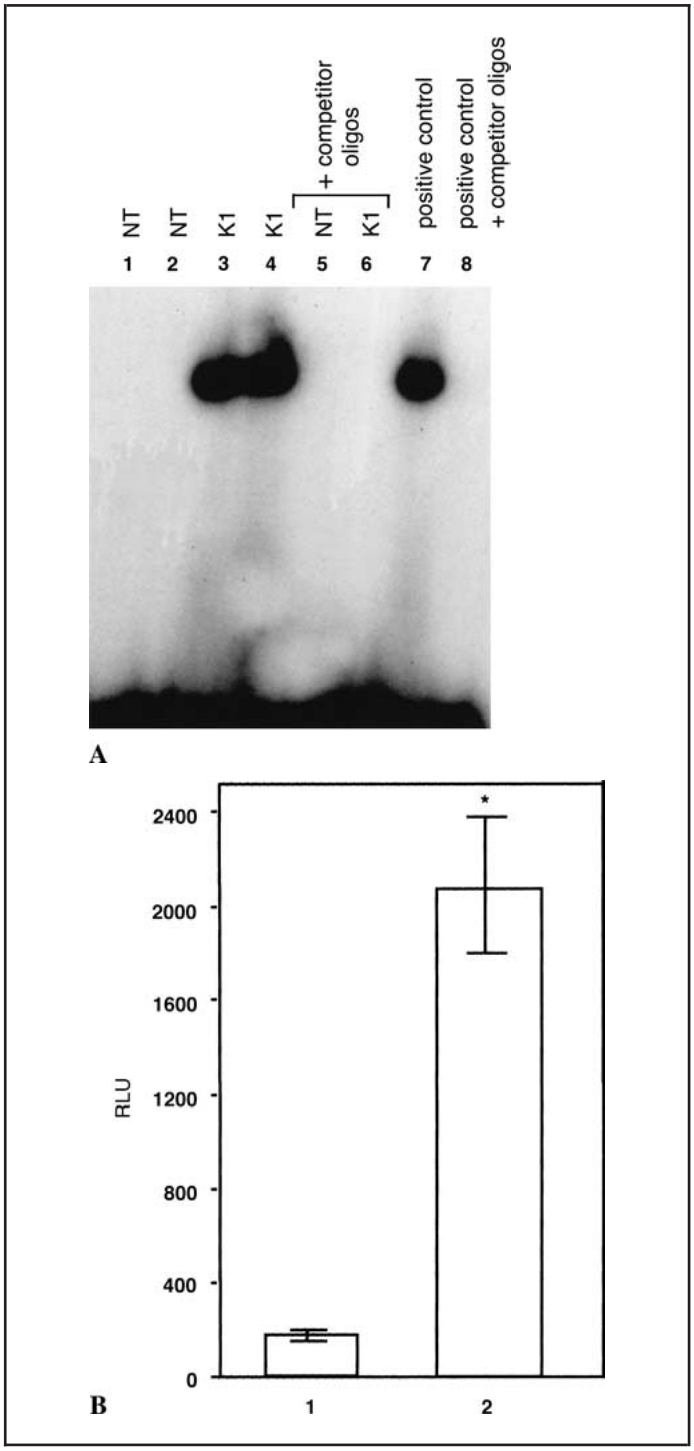

Fig. 3. A) Electrophoretic mobility shift assays of nuclear proteins from splenic B cells of nontransgenic (NT) and K1-transgenic (K1) mice using radiolabeled NF-κB-specific probe. The assays were performed as described in the "Materials and Methods" section. A 100-fold excess of unlabeled NF-κB oligonucleotides was used for competition. Nuclear extracts from phorbol myristate acetate-treated Jurkat cells showing NF-κB binding activity were used as a positive control. **B**) Human herpesvirus 8 (HHV-8) K1-induced activation of NF-κB promoter activity in human Raji B cells. Raji cells were transfected with pSG5-K1 and NF-κB luciferase reporter constructs, and the transfected cells were assayed for luciferase activity as described in the "Materials and Methods" section. Luciferase values are expressed as relative light units (RLU) and were normalized to the chloramphenicol acetyltransferase (CAT) activity of cotransfected CMV-CAT. The results shown are averages of three independent experiments; **error bars** indicate 95% confidence intervals (CIs). * There were statistically significant differences in luciferase activities in cells vector-transfected (1) or transfected (2) with K1 expression plasmid (P = .005).

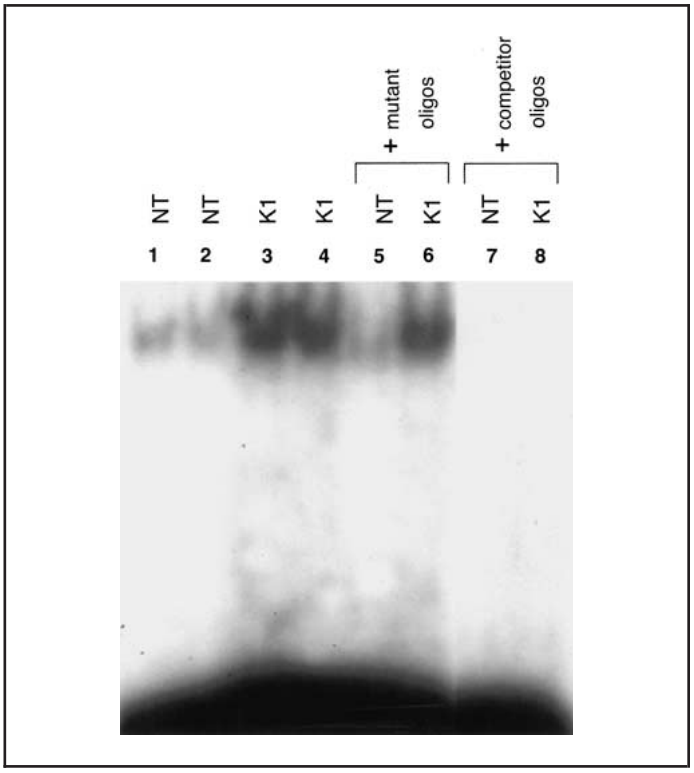

Fig. 4. Electrophoretic mobility shift assays of nuclear proteins from splenic B cells of nontransgenic (NT) and K1-transgenic (K1) mice using radiolabeled Oct-2-specific probe. The assays were performed as described in the "Materials and Methods" section. A 100-fold excess of unlabeled Oct-2-specific oligonucleotides or mutant oligonucleotides was used for competition to confirm the specificity of the binding complex.

Protein Tyrosine Kinase Activation in the B Lymphocytes of K1-Transgenic Mice

Lyn kinase, a member of the Src family, is known to play a critical role in ligand-induced signal transduction events in B-lineage lymphoid cells *(18,27–29).* We examined by immunoblot assay the effects of K1 expression in the thymic and splenic B lymphocytes from K1 mice to detect the levels of phosphorylated Lyn with an antiphosphotyrosine antibody (Fig. 5, A). The data are representative of five transgenic mice. Although the levels of total Lyn in the cells from nontransgenic and K1-transgenic mice were comparable, the level of tyrosine-phosphorylated Lyn was markedly increased in the cells from K1 mice (compare lanes 1 and 2 with lanes 3 and 4, and compare lanes 5 and 6 with lanes 7 and 8). Although K1 signaling has been shown to activate Syk kinase *in vitro (11),* we did not observe Syk activation in the B lymphocytes from K1 mice.

To further evaluate Lyn kinase activation in K1-transgenic mice, we determined the *in vitro* activity of Lyn immunoprecipitated from the splenic extracts of K1-transgenic and nontransgenic mice. Equivalent amounts of immunoprecipitates were incubated with the tyrosine kinase substrate Raytide in kinase buffer containing [γ-^{32}P]ATP, and the radioactivity incorporated into the substrate was estimated by filter assay. As shown in Fig. 5, B (lanes 1–4), the catalytic activity of immunoprecipitated Lyn kinase from the splenic extracts of two K1 mice was approximately threefold higher than the activity from the splenic extracts of two nontransgenic mice. Taken together, these results suggest that Lyn kinase activation was associated with K1 expression in the B cells from K1 mice.

Characterization of Tumors in K1 Mice

The C57BL/6J hybrid lines of male and female mice maintained in our mouse facility show no evidence of lymphoma or sarcoma up to 24 months of age (our unpublished observations). However, at about 14 months of age, two of the 13 female K1 mice in our study developed large tumor masses (3.5–4.5 cm in diameter). In one mouse, the tumor was localized in the submaxillary region. Histologic examination of the tumor cells by light microscopy showed heterogeneity in size and shape of cells and nuclei as well as a fine chromatin pattern, with a few cells showing single, prominent, centrally placed nucleoli (Fig. 6, A). Several cells also showed abundant cytoplasm and eccentric nuclei resembling plasma cells. A preliminary immunophenotypic characterization of the lymphoma-derived cell line by flow cytometric analysis showed that most cells were CD45 positive, suggesting a hematolymphoid origin (Table 1). A large percentage (87%) of cells also showed CD34 expression, a stem cell marker. About 71% of the cells expressed the plasma cell marker CD138, which suggested differentiation arrest at preterminal B-cell or plasma-cell stage. However, another plasma cell marker, CD38, was not detectable. Furthermore, the analysis showed no significant expression of T-cell markers (CD3, CD4, CD8) or B-cell markers (CD19, B220, CD79, κ and γ light chains). Results of the morphologic and flow cytometric analyses suggested that the tumor was probably a plasmablastic malignant lymphoma.

The tumor in the other mouse occurred in the omentum. Preliminary histologic examination by light microscopy suggested that it was a spindle-cell sarcomatoid tumor (Fig. 6, B). Immunohistochemical staining with anti-CD3, anti-CD19, and anti-CD45 antibodies was negative (data not shown), suggesting that the tumor was not of hematolymphoid origin.

K1-Mediated Cellular Effects in Tumor Cells

To understand the potential role of K1 in the development of tumors in K1-transgenic mice, we examined whether Lyn kinase activation and the pattern of transcription factor and cytokine expression in the tumors resembled that observed in K1-expressing lymphocytes from K1-transgenic mice. Lyn kinase was activated in both of the tumors, as revealed by enhanced phosphorylation and enhanced *in vitro* catalytic activity (Fig. 7). In fact, the catalytic activity in both of the tumors, the lymphoma in particular, was substantially higher than the activity in the splenic B lymphocytes of K1 mice. These observations suggested that Lyn kinase activation might play a role in tumor induction in K1 mice. NF-κB p50-related transcripts were substantially elevated in both the tumors compared with transcripts in the control tissues, omentum, and spleen from nontransgenic mice (data not shown). The bFGF transcripts were modestly increased in tumors relative to control tissues. Although IL-12 p35 expression was markedly decreased in both tumors, the decrease in the lymphoma was much greater than the high expression level in the normal spleen (data not shown).

Discussion

In this study, we provide evidence that HHV-8 K1 has transforming potential *in vivo.* Earlier studies have shown that K1 can functionally substitute for STP in HVS for the immortalization

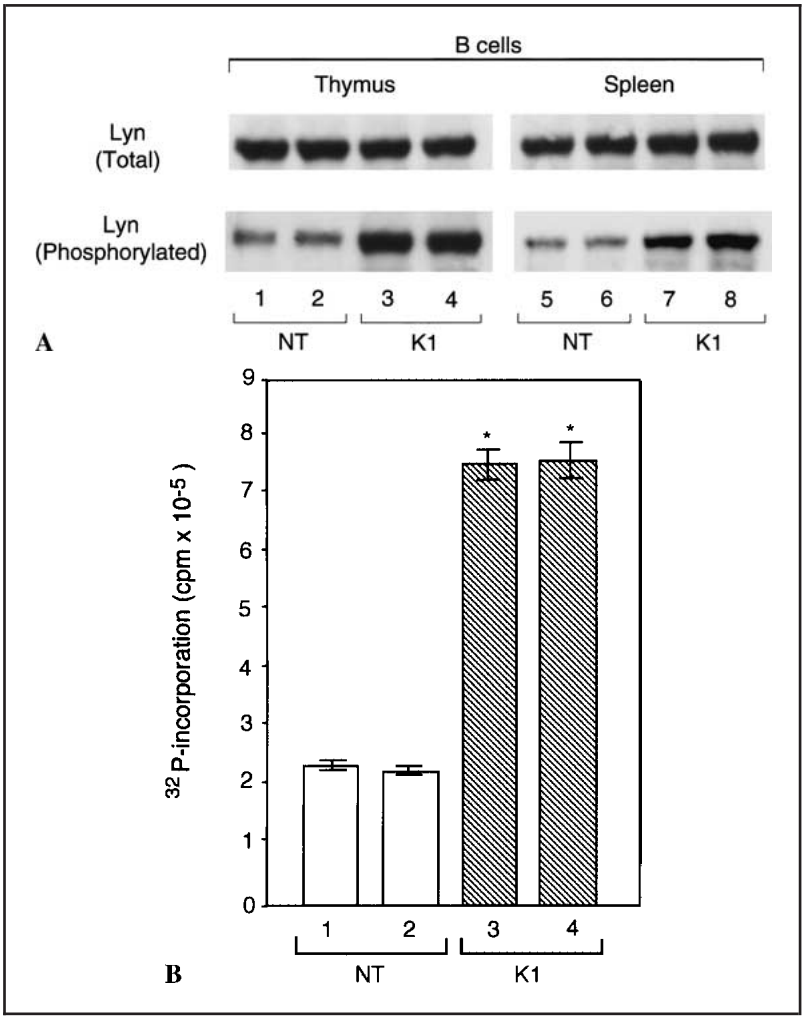

Fig. 5. A) Hyperphosphorylation of Lyn tyrosine kinase in the thymic and splenic B cells of nontransgenic (NT) and K1-transgenic (K1) mice. Detection of total Lyn and tyrosyl phosphorylated proteins was carried out as described in the "Materials and Methods" section. **B**) *In vitro* catalytic activity of Lyn tyrosine kinase in splenic extracts of randomly selected NT and K1 mice. The *in vitro* kinase assays were carried out as described in the "Materials and Methods" section. Data are presented as the mean, with **error bars** indicating 95% confidence intervals (CIs). * There were statistically significant differences in phosphorylation levels between NT (**bars 1** and **2**) and K1 (**bars 3** and **4**) mice ($P<.001$).

of primary T lymphocytes *in vitro* and induction of lymphomas *in vivo (13).* However, these studies are complex because tyrosine kinase-interacting protein (tip), the other major HVS-transforming protein, was still present in the recombinant virus. Even so, the *in vitro* findings that K1 provides constitutive ITAM-based signal transduction and keeps cells in an activated state as seen by constitutive activation of protein kinases, transcription factors, and cytokines, clearly suggest that K1 has oncogenic potential *(9,11,12,30).* The HHV-8 K1 has positional homology with HVS STP, EBV latent membrane protein 1, and Rhesus monkey rhadinovirus R1 transforming genes *(14,31).*

K1 expression in B lymphocytes is known to induce the activation of Syk protein kinase, which leads to a cascade of downstream signaling events including activation of nuclear factor of activated T cells and activator protein 1 transcription factors *(11).* In our transgenic mouse studies, although we failed to detect the activation of Syk, we found that Lyn, a Src proto-oncogene family protein–tyrosine kinase, was substantially activated in the B lymphocytes and tumors of K1-transgenic mice. It is interesting that *in vitro* Lyn activity was further enhanced in the lymphoma cells relative to that in the splenic extracts from K1 mice. Lyn activation was also observed in the sarcomatoid tumor, although to a lesser extent than in the lymphoma cells. This could be attributed to the presence of lymphocytes in the tumor and not to the nonlymphoid tumor cells. Moreover, Lyn is preferentially expressed in B lymphocytes and other hematopoietic cells *(32,33).* Our findings are further supported by a recent *in vitro* study of a murine pro-B cell line suggesting that NF-κB activation can be triggered by Lyn kinase *(34).*

Although further studies are required to identify additional signal-transducing molecules associated with Lyn activation, we found that RNA transcripts of the NF-κB p50 subunit and NF-κB DNA-binding activity were constitutively increased in the K1-expressing lymphocytes from K1-transgenic mice. Even though we did not examine the levels of the NF-κB p65 subunit transcript, its stoichiometric appearance with p50 *(16)* suggests that the native DNA-binding NF-κB complex seen in our EMSA was a functional p50/p65 heterodimer. This possibility was further confirmed by our findings that the K1-induced NF-κB was able to activate the NF-κB promoter in human B lymphocytes.

Other HHV-8 genes are also known to induce signaling pathways that activate NF-κB. For example, the HHV-8 lytic phage gene ORF-74, which is related to the G protein-coupled receptor, has been shown to activate NF-κB in KS and primary endothelial cells as well as in epithelial, monocytic, and T cells *(35,36).* According to an earlier report, HHV-8-infected PEL cells showed constitutive activation of NF-κB and this activation is essential for the survival of the infected cells *(37).* Thus, NF-κB may play a crucial role in HHV-8 pathogenesis by providing a survival advantage to infected cells. Our findings also suggest that K1 might contribute to NF-κB activation in HHV-8-infected PEL cells.

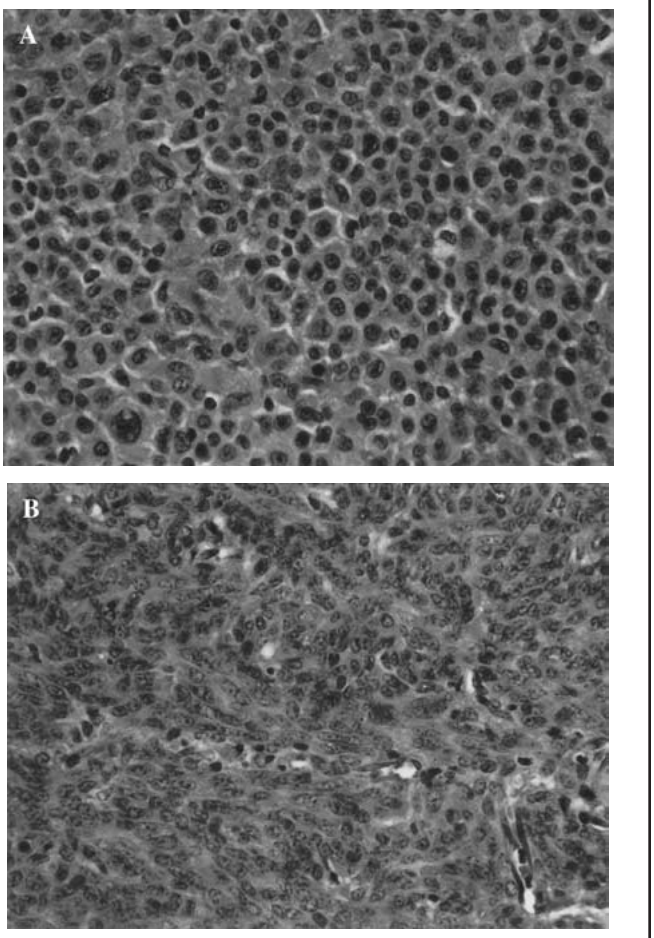

Fig. 6. A) Hematoxylin-and-eosin-stained section of a plasmablastic lymphoma from a K1-transgenic mouse, showing large cell plasmacytoid differentiation. **B**) Hematoxylin-and-eosin-stained section of a sarcomatoid tumor from a K1-transgenic mouse.

Table 1. Immunophenotypical characterization of the lymphoma observed in a K1-transgenic mouse*

Cell marker	Percentage of positive cells
CD45	98.7
CD19	1.1
CD45R/B220	1.9
CD138	70.8
CD38	1.5
CD34	87.4
CD79	1.1
λ	0.7
κ	4.0
CD3	4.3
CD4	1.0
CD8	1.3
Background	1.0

*The flow cytometric analysis was carried out as described in the "Materials and Methods" section.

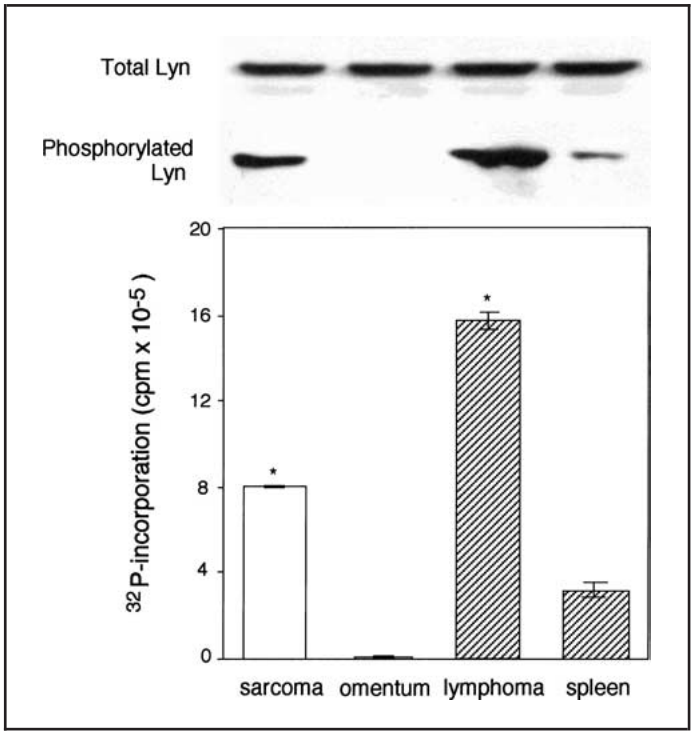

Fig. 7. Hyperphosphorylation and *in vitro* kinase activity of Lyn tyrosine kinase in tumors from K1-transgenic mice and normal tissues of nontransgenic mice. Assays were carried out in triplicate as described in the "Materials and Methods" section. **Error bars** indicate 95% confidence intervals (CIs). * There was a statistically significant difference in ^{32}P incorporation between sarcoma and omentum ($P<.001$) and between lymphoma and spleen ($P<.001$).

The critical role of NF-κB is further supported by constitutive induction of NF-κB-dependent inflammatory cytokines and chemokines such as IL-6, IL-8, GM-CSF, bFGF, and regulated on activation normal T cells expressed and secreted (RANTES) *(35,36).* Of the cytokines that we examined (IL-2, IL-4, IL-6, IL-10, IL-12, GM-CSF, TNF-α, TNF-β, and bFGF), we found elevated expression of bFGF in lymphocytes as well as in the tumors of K1 mice. bFGF is a potent autocrine growth factor for endothelial cells and promotes growth and angiogenesis of AIDS–KS cells *(38).* Because constitutive activation of NF-κB is associated with several types of human cancer including breast, thyroid, colon, melanoma, and T- or B-lymphocytic leukemia *(39–43),* it is reasonable to speculate that K1-induced activation of NF-κB, along with altered expression of various transcription factors and cytokines, could eventually lead to cell activation, inflammation, and transformation.

Recent studies have shown that NF-κB is necessary for transcriptional activation of the Oct-2 gene *(26).* Oct-2 is predominantly a B-lymphocyte lineage transcription factor that is essential for the inducible expression of immunoglobulin genes (μ, κ, and λ) *(44–47).* All human B lymphocytes, all B-cell chronic lymphocytic leukemias, and as many as 80% of B-cell precursor acute lymphoblastic leukemias express Oct-2 *(48,49).* In our study, we found constitutive activation of Oct-2 in B lymphocytes as well as in the B-cell lymphoma in a K1-transgenic mouse. Deregulated expression of NF-κB and Oct-2 has been found in cultured Reed–Sternberg cells, suggesting that activation of these transcription factors may have a role in the pathologic manifestation of Hodgkin's disease *(39).*

IL-12, a 70-kDa heterodimeric cytokine, is produced in response to a variety of infectious agents such as bacteria, parasites, fungi, and viruses *(23).* It was originally discovered as a product of EBV-transformed lymphoblastoid B-cell lines *(50).* The major role of IL-12 is to maintain cell-mediated immunity through the induction of Th1 responses *(23).* IL-12 also has

antitumor and antimetastatic effects as well as antiangiogenic activity *(51–54)*. We found that serum IL-12 levels were severely impaired in K1 mice, suggesting that one of the *in vivo* effects of K1 expression alone or in an HHV-8-infected host, could be to impair the IL-12-related antitumor response and to contribute to HHV-8 pathogenesis. Decreased IL-12 production may also be linked to increased risk of KS in individuals who are dually infected with human immunodeficiency virus (HIV)-1 and HHV-8, because HIV-1 infection is known to impair the expression of IL-12 *(55)*. Although the precise mechanism involved in the decreased production of bioactive IL-12 needs additional studies, our results suggest that K1 might directly or indirectly inhibit the transcriptional activity of p35 (one of the subunits of IL-12 p70) and consequently decrease the synthesis of the bioactive protein.

We found K1-mediated activation of NF-κB in Raji B cells, and it has been observed in human KS SLK cells *(12)*. One of the K1 mice also developed a sarcomatoid tumor that contained elevated levels of phosphorylated Lyn, a target of ITAM proteins. K1 may have a direct or paracrine role similar to that proposed for HHV-8 G protein-coupled receptor-induced angioproliferative diseases in transgenic mice *(56)*. The fact that B lymphocytes are the primary reservoir of HHV-8 in humans and that K1 as well as other lytic genes are expressed in a small subset of cells in PEL and KS lesions provides strong support for this type of mechanism. An earlier report that K1 induces transformation of rodent fibroblasts *(13)* suggests that K1 may affect cell growth by other mechanisms as well. The induction of a lymphoma and a sarcoma in a very small population of K1-transgenic mice ($n = 13$) further supports the proposition that K1 expression *in vivo* has pleiotropic effects.

In conclusion, our *in vivo* study strengthens the evidence for a role of K1 in HHV-8 pathogenesis. Our findings may help to identify novel preventive and therapeutic measures, such as those that target NF-κB activity. Because K1 was expressed in most tissues that we examined, our K1-transgenic mice also provide a valuable model with which to test the oncogenic potential of K1 in a variety of cell types and to investigate cell-specific mechanisms in oncogenesis.

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NOTES

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