

ORIGINAL ARTICLE

Loss of Smad3 in Acute T-Cell Lymphoblastic Leukemia

Lawrence A. Wolfrain, Ph.D., Tania M. Fernandez, Ph.D., Mizuko Mamura, M.D., Ph.D., Walter L. Fuller, B.S., Rajesh Kumar, Ph.D., Diane E. Cole, B.S., Stacey Byfield, Ph.D., Angelina Felici, Ph.D., Kathleen C. Flanders, Ph.D., Thomas M. Walz, M.D., Anita B. Roberts, Ph.D., Peter D. Aplan, M.D., Frank M. Balis, M.D., and John J. Letterio, M.D.*

ABSTRACT

BACKGROUND

From the Laboratory of Cell Regulation and Carcinogenesis (L.A.W., T.M.F., M.M., W.L.F., R.K., S.B., A.F., K.C.F., A.B.R., J.J.L.), the Pediatric Oncology Branch (D.E.C., F.M.B.), and the Genetics Branch (P.D.A.), Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Md.; and the Department of Biomedicine and Surgery, Division of Oncology, Faculty of Health Sciences, University Hospital, Linköping, Sweden (T.M.W.). Address reprint requests to Dr. Letterio at LCRC, CCR, NIH Bldg. 41, Rm. C629, 41 Library Dr., Bethesda, MD 20892-5055, or at letterij@mail.nih.gov.

*Drs. Wolfrain, Fernandez, and Mamura and Mr. Fuller contributed equally to the article.

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The receptors for transforming growth factor β (TGF- β) and their signaling intermediates make up an important tumor-suppressor pathway. The role of one of these intermediates — Smad3 — in the pathogenesis of lymphoid neoplasia is unknown.

METHODS

We measured Smad3 messenger RNA (mRNA) and protein in leukemia cells obtained at diagnosis from 19 children with acute leukemia, including 10 with T-cell acute lymphoblastic leukemia (ALL), 7 with pre-B-cell ALL, and 2 with acute nonlymphoblastic leukemia (ANLL). All nine exons of the *SMAD3* gene (*MADH3*) were sequenced. Mice in which one or both alleles of *Smad3* were inactivated were used to evaluate the role of Smad3 in the response of normal T cells to TGF- β and in the susceptibility to spontaneous leukemogenesis in mice in which both alleles of the tumor suppressor *p27^{Kip1}* were deleted.

RESULTS

Smad3 protein was absent in T-cell ALL but present in pre-B-cell ALL and ANLL. No mutations were found in the *MADH3* gene in T-cell ALL, and Smad3 mRNA was present in T-cell ALL and normal T cells at similar levels. In mice, the loss of one allele for *Smad3* impairs the inhibitory effect of TGF- β on the proliferation of normal T cells and works in tandem with the homozygous inactivation of *p27^{Kip1}* to promote T-cell leukemogenesis.

CONCLUSIONS

Loss of Smad3 protein is a specific feature of pediatric T-cell ALL. A reduction in Smad3 expression and the loss of *p27^{Kip1}* work synergistically to promote T-cell leukemogenesis in mice.

Smad2 AND Smad3 ARE THE PRINCIPAL CYTOSOLIC INTERMEDIATES INVOLVED IN THE TRANSDUCTION OF SIGNALS FROM RECEPTORS FOR MEMBERS OF THE TRANSFORMING GROWTH FACTOR β (TGF- β) SUPERFAMILY OF CYTOKINES. NAMED FOR THEIR ROLES IN INVERTEBRATE DEVELOPMENT, Smad2 AND Smad3 ARE ACTIVATED WHEN TGF- β BINDS TO ITS CELL-SURFACE RECEPTOR AND MOVE FROM THE CYTOSOL TO THE NUCLEUS, WHERE THEY REGULATE GENE TRANSCRIPTION.¹ THE THREE MAMMALIAN TGF- β ISOFORMS (TGF- β 1, β 2, AND β 3) REPRESENT A FAMILY OF CYTOKINES WITH A VARIETY OF EFFECTS, INCLUDING THE SUPPRESSION OF TUMOR GROWTH. LITTLE IS KNOWN, HOWEVER, ABOUT HOW Smad2 AND Smad3 MEDIATE THE TUMOR-SUPPRESSIVE EFFECTS OF TGF- β .² TO TEST THE HYPOTHESIS THAT Smad3 IS A TUMOR-SUPPRESSOR FACTOR IN ACUTE LYMPHOBLASTIC LEUKEMIA (ALL), WE DETERMINED THE LEVEL OF Smad3 IN SPECIMENS OBTAINED AT DIAGNOSIS FROM PATIENTS WITH ACUTE LEUKEMIA ENROLLED IN TRIALS AT THE PEDIATRIC ONCOLOGY BRANCH OF THE NATIONAL CANCER INSTITUTE BETWEEN 1980 AND 1992. WE STUDIED 10 PATIENTS WITH T-CELL ALL (FRENCH-AMERICAN-BRITISH CLASSIFICATION L1 OR L2, Tdt+, CD7+, CD2+), 7 PATIENTS WITH PRE-B-CELL ALL, AND 2 PATIENTS WITH ACUTE NONLYMPHOBLASTIC LEUKEMIA (ANLL). WE ALSO EXAMINED Smad3 IN SPECIMENS FROM THREE ADULTS WITH THE SÉZARY SYNDROME AND FOUR WITH T-CELL LEUKEMIA RELATED TO INFECTION WITH HUMAN T-LYMPHOTROPIC VIRUS 1 (HTLV-1). WE ALSO EXAMINED THE EFFECT OF THE LOSS OF A SINGLE ALLELE OF THE *Smad3* GENE IN MICE WITH SPONTANEOUS T-CELL LEUKEMIA THAT WERE HOMOZYGOUS FOR A DELETION IN *p27^{Kip1}*, A GENE FREQUENTLY ALTERED IN PEDIATRIC ALL.

METHODS

PATIENTS AND CELL COLLECTION

After obtaining written informed consent from all patients and before treatment, we used cytopheresis to collect cells, which we then froze in medium with 10 percent dimethyl sulfoxide. Samples of leukemia cells used in the study were collected during the period between 1980 and 1990. The diagnosis of childhood ALL or ANLL was made by means of morphologic assessment and immunohistochemical staining of the bone marrow.³ The immunologic subtype of the cells was subsequently determined by flow cytometry with the use of monoclonal antibodies. Adults with the Sézary syndrome were enrolled in clinical trials at the Medicine Branch of the National Cancer Institute,⁴ and leukemia-cell specimens were kindly provided by Dr. Susan Bates.

Adults with HTLV-1-associated T-cell leukemia were enrolled in clinical trials at the Metabolism Branch of the National Cancer Institute, and leukemia-cell specimens were kindly provided by Dr. Thomas Waldmann.

WESTERN BLOTTING

Cellular protein lysates were prepared from cryopreserved specimens from children with leukemia and from peripheral-blood cells of two healthy control subjects. For normal lymphocytes, lymphocyte-rich fractions were collected from healthy donors by leukapheresis and countercurrent centrifugal elutriation according to National Institutes of Health guidelines for human subjects. T cells were purified from lymphocyte-rich elutriation fractions by means of T-cell isolation columns (Miltenyi Pan T-cell purification kit) and were either cultured or immediately processed for extraction of protein. Cells were centrifuged and washed twice with cold phosphate-buffered saline and lysed in radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors, sodium orthovanadate, and sodium fluoride. Lysates were clarified by centrifugation, and protein levels determined with the use of a bicinchoninic acid protein assay (Pierce). Then, 50 μ g of total cellular protein was separated on 8 percent TRIS-glycine (sodium dodecyl sulfate) gels and transferred onto nitrocellulose membranes. Membranes were blocked in TRIS-buffered saline with the use of 5 percent milk and 2 percent bovine serum albumin. The membranes were incubated overnight with 1 μ g of a rabbit polyclonal antibody against Smad3 (51-1500, Zymed Laboratories) or a mouse monoclonal antibody against Smad2 (BD Signal Transduction Laboratories), washed three times, incubated for one hour in the appropriate peroxidase-conjugated secondary antibody (Amersham Biosciences), and developed with a Super Signal-enhanced chemiluminescence kit (Pierce).

DETERMINATION OF FREE AND CYCLIN D3-ASSOCIATED *p27^{Kip1}*

In brief, for the determination of free and cyclin D3-associated *p27^{Kip1}*, leukemia cells and purified peripheral-blood T cells were each lysed in RIPA buffer containing protease inhibitors (Complete Mini protease inhibitor cocktail tablets, Roche) plus 0.1 mM sodium orthovanadate and 1 mM sodium fluoride. Then, 50 μ g of protein in 500 μ l of lysis buffer was immunoprecipitated overnight

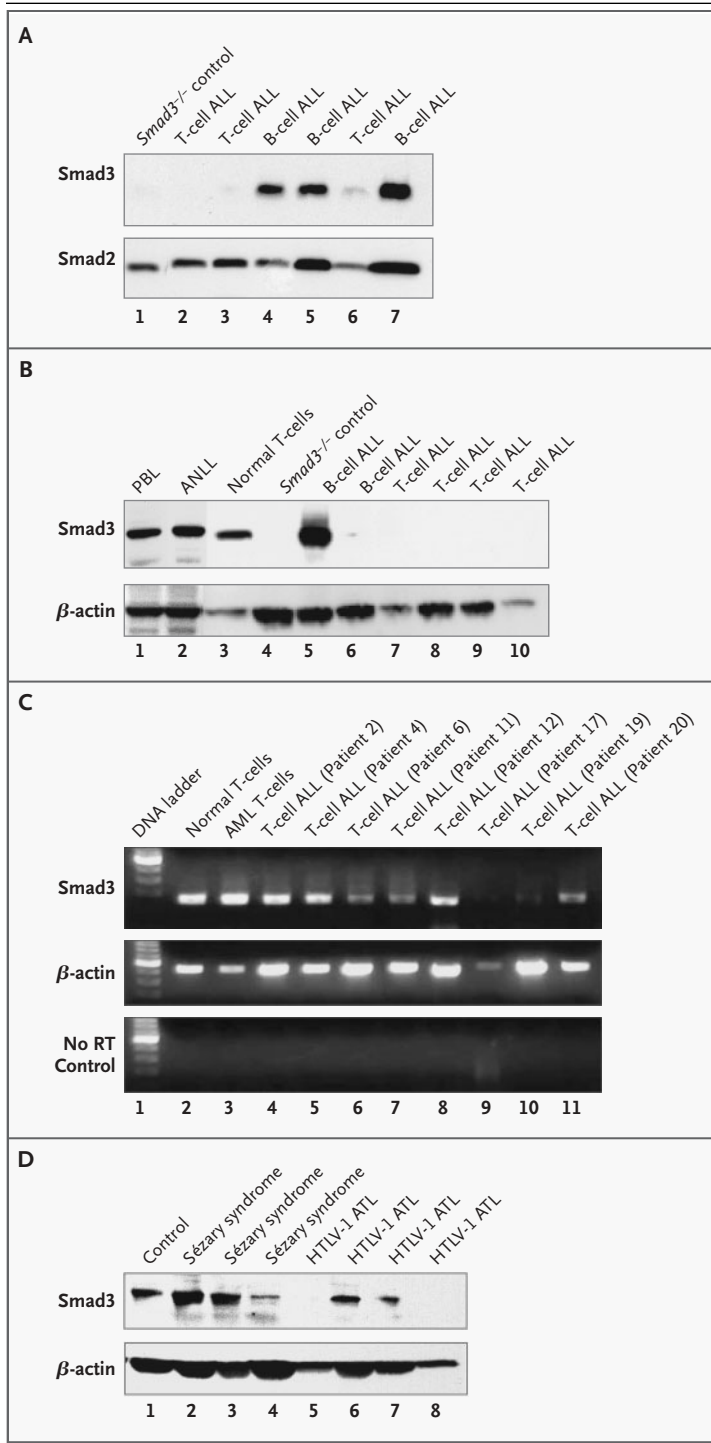


Figure 1. Levels of Smad3 in T-Cell and Pre-B-Cell ALL.

Cryopreserved leukemia samples were processed. In Panel A, total cell extracts were resolved by sodium dodecyl sulfate–polyacrylamide-gel electrophoresis and transferred for Western blot analysis of either Smad3 or Smad2. Smad3 is present in pre-B-cell specimens but is absent or markedly reduced in T-cell ALL specimens. Panel B shows the expression of Smad3 and β-actin by a second set of ALL samples, ANLL (a myeloid leukemia), normal peripheral-blood lymphocytes (PBL), and normal T cells. Smad3 is present in lysates of PBL, T cells purified from PBL, pre-B cell ALL, and ANLL, but not T-cell ALL. The investigator running the gels was unaware of the immunophenotype of the cells. Panel C shows the level of expression of Smad3 and β-actin mRNA by T-cell ALL specimens from eight children, control T cells, and one specimen of acute myelogenous leukemia (AML). Panel D shows the level of expression of Smad3 and β-actin by specimens from three adults with the Sézary syndrome and four adults with HTLV-1-associated T-cell leukemia (ATL).

against p27^{Kip1} (sc-1641AC, Santa Cruz Biotech) at 4°C for six hours. This latter fraction represented free p27^{Kip1}, which was not bound to cyclin D3. The cyclin D3–bound immune complexes were washed three times with 500 μl of RIPA buffer, and the pellet was resuspended in 30 μl of gel-loading buffer and then heated at 95°C for five minutes to remove bound proteins from the agarose beads. The p27^{Kip1} immune complexes were treated in a similar manner. Both the cyclin D3–associated and free p27^{Kip1} fractions were resolved by means of sodium dodecyl sulfate–polyacrylamide-gel electrophoresis on a TRIS–Glycine Novex gel (4 to 20 percent), blotted onto nitrocellulose, and incubated overnight at 4°C with a rabbit polyclonal antibody against human p27^{Kip1} (sc-528, Santa Cruz Biotech) at a dilution of 1:500 in TRIS-buffered saline containing 4 percent fat-free milk. Donkey antirabbit IgG conjugated with horseradish peroxidase (Amersham) was used to detect bound antibody against p27^{Kip1} by means of chemiluminescence (Pierce Super Signal West Pico system).

ANALYSIS OF Smad3 MESSENGER RNA

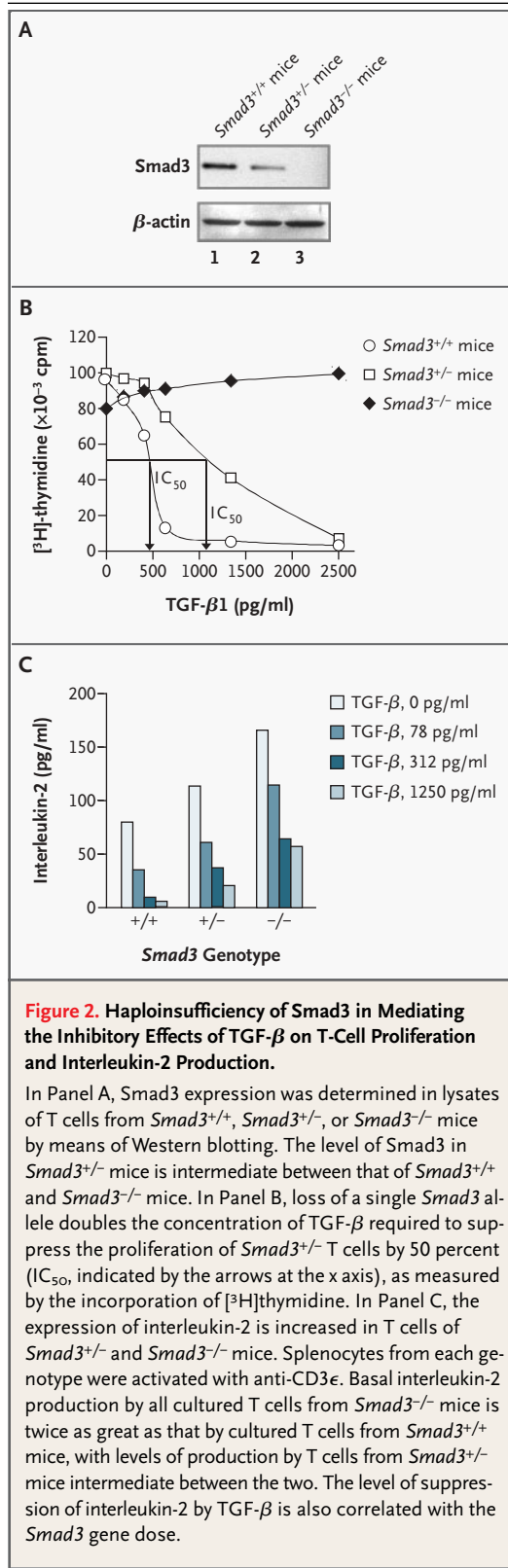
RNA was purified from leukemia-cell specimens and from freshly isolated normal peripheral-blood T cells with the use of Trizol reagent (Invitrogen), and any contaminating genomic DNA was removed by digestion with RQ1 DNase (Promega). The reverse-transcription–polymerase-chain-reaction (RT-PCR) assay was carried out with the use of In-

with gentle rocking at 4°C with 25 μl of agarose-conjugated antibody against cyclin D3 antibody (sc-6283AC, Santa Cruz Biotech). Supernatants containing unbound proteins were immunoprecipitated with 50 μl of agarose-conjugated antibody

vitrogen's SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase according to the manufacturer's instructions; 700 ng of RNA was used. The annealing temperature was 56°C. PCR was also carried out in the absence of reverse transcription and involved 700 ng of RNA, Platinum Taq Supermix (Invitrogen), and Smad3 primers. The identity of the RT-PCR product was confirmed as being Smad3 by direct sequencing of the gel-purified DNA. The following primers were used for the RT-PCR of the human Smad3 messenger RNA (mRNA): 5'ACCATCCCCAGTCCCTGGATGGCC3' (forward primer) and 5'AACCTCGCCGGGATCTCTGTGTGGCGT3' (reverse primer); the PCR product size is 253 bp. The human β -actin PCR primers were purchased from R&D Systems (primer pair RDP-38-025); the PCR product is 528 bp.

SEQUENCING OF THE MADH3 GENE

To prepare genomic DNA, leukemia cells were digested in 300 μ l of lysis buffer (200 mM sodium chloride; 40 mM TRIS, pH 8.0; 20 mM EDTA; 0.5 percent sodium dodecyl sulfate; 0.5 percent β -mercaptoethanol; and 2 mg of fresh proteinase K per milliliter), and DNA was precipitated from lysates by the addition of 600 μ l of ice-cold ethanol, then rinsed, and dissolved in water. The following primers were used for PCR amplification and sequencing of human *MADH3* exons: exon 1, 5'CGAAGTTGGGCGACCGCG3' (forward) and 5'CTCTCCCTCTTCCCATCTCCAGC3' (reverse), yielding a 420-bp PCR fragment; exon 2, 5'CAATCACATTTCCCTCTCTTTCTG3' (forward) and 5'CAGCATACTGGTGTCTCTAC3' (reverse), yielding a 246-bp fragment; exon 3, 5'GTCTTTGCAAAGGTGTC-TC3' (forward) and 5'CTGCTAATCAGTTAAGAATAAG3' (reverse), yielding a 190-bp product; exon 4, 5'CAGGCCAAGAATCTTTGTGAAG3' (forward) and 5'AAACCTGGCATATGGTTGTCTTTC3' (reverse), yielding a 319-bp product; exon 5, 5'GAGATTATAATCCCTCTGAAATGC3' (forward) and 5'CTGCATTCCTGTTGACATTG3' (reverse), yielding a 305-bp product; exon 6, 5'CATTGTGTGTGAGCAAAG3' (forward) and 5'CACCTCCAGATGACAACGCAATC3' (reverse), yielding a 315-bp product; exon 7, 5'CTGTTTCTGTGTTTTTGGCAG3' (forward) and CTTGGCCTCTCTCTGATCTTGTG3' (reverse), yielding a 306-bp product; exon 8, 5'AGATGGGTTCAAGGGGAGGGACTG3' (forward) and 5'TTTCTGCTCTGACGCTAGGGCTG3' (reverse), yielding a 467-bp product; and exon 9,



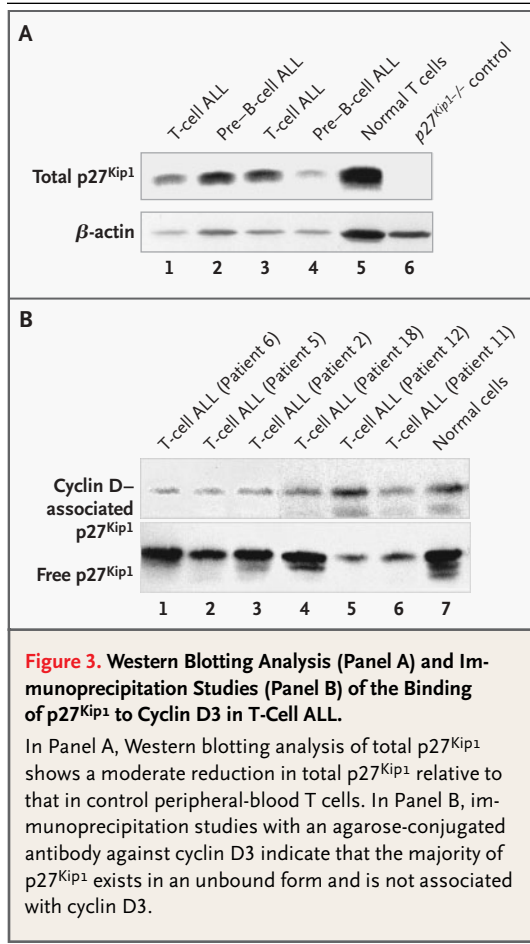


Figure 3. Western Blotting Analysis (Panel A) and Immunoprecipitation Studies (Panel B) of the Binding of p27^{Kip1} to Cyclin D3 in T-Cell ALL.

In Panel A, Western blotting analysis of total p27^{Kip1} shows a moderate reduction in total p27^{Kip1} relative to that in control peripheral-blood T cells. In Panel B, immunoprecipitation studies with an agarose-conjugated antibody against cyclin D3 indicate that the majority of p27^{Kip1} exists in an unbound form and is not associated with cyclin D3.

5'GGTAGAGGAGTTTGGCCGGGTAGTT3' (forward) and 5'TGGGGCCAAAGGTAAATGTGTT3' (reverse), yielding a 521-bp product.

PROLIFERATION ASSAYS AND INTERLEUKIN-2 MEASUREMENTS

Splenocytes from *Smad3*^{+/+}, *Smad3*^{+/-}, and *Smad3*^{-/-} mice were depleted of red cells with the use of ACK Lysis Buffer (Quality Biological) and plated at 5 × 10⁴ cells per well (round-bottom 96-well plate) in a final volume of 200 μl of RPMI complete medium (RPMI supplemented with 10 percent heat-inactivated fetal-calf serum, 100 U of penicillin G per milliliter, 100 U of streptomycin per milliliter, 0.25 μg of Fungizone per milliliter, and 50 μM 2-mercaptoethanol). Cells were stimulated for 60 hours with plate-bound anti-CD3ε (2 μg per milliliter) in the absence or presence of recombinant human TGF-β1 (5 ng per milliliter), pulsed with 1 μCi of tritiated thymidine per well for 12 hours, and harvested with the use of a Brandel cell harvester. The amount of

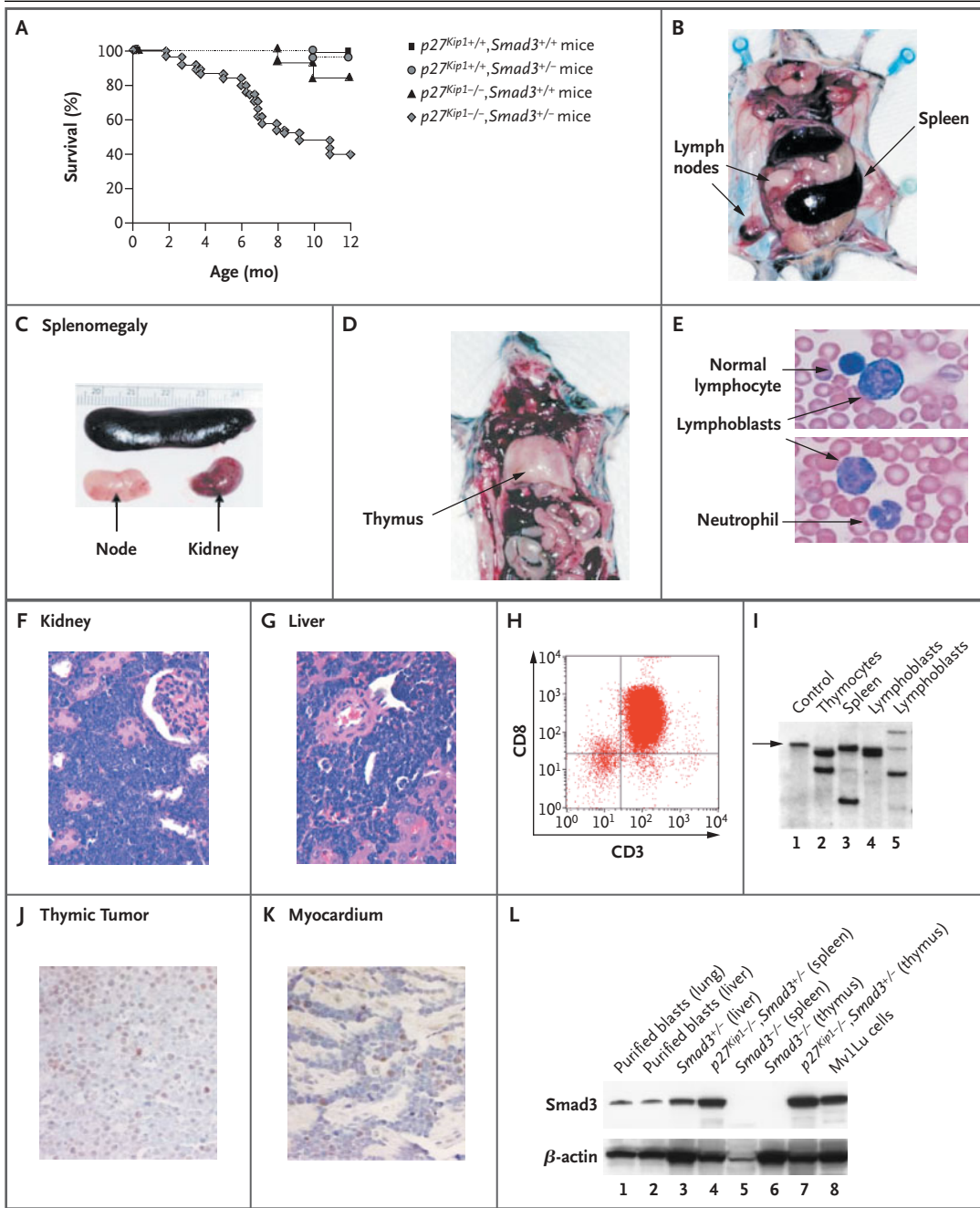
Figure 4 (facing page). Spontaneous T-Cell Leukemia in p27^{Kip1}^{-/-}, Smad3^{+/-} Mice.

In Panel A, survival curves show decreased survival for p27^{Kip1}^{-/-}, Smad3^{+/-} mice, as compared with p27^{Kip1}^{-/-}, Smad3^{+/+} mice, p27^{Kip1}^{+/+}, Smad3^{+/-} mice, and p27^{Kip1}^{27+/+}, Smad^{+/+} mice. There were 50 mice per group. Panels B, C, and D show enlarged, leukemia-infiltrated lymphoid organs and soft tissues obtained at gross autopsy. A spleen as large as 5 cm (Panel C) was observed. Peripheral-blood smears demonstrate the presence of circulating lymphoblasts (Panel E). Staining with hematoxylin and eosin shows extensive infiltration of leukemia cells (blue) into soft tissues. Panel F shows lymphoblasts surrounding a normal glomerulus in the kidney, and Panel G lymphoblasts surrounding a blood vessel in the liver. Cytometric analysis shows a T-cell phenotype, with surface markers positive for CD3 and CD8 (Panel H). In Panel I, T-cell receptor (TCR) β gene rearrangements indicate clonal expansion of malignant T cells in p27^{Kip1}^{-/-}, Smad3^{+/-} mice. DNA from thymocytes (lane 2) or spleen (lane 3) of affected p27^{Kip1}^{-/-}, Smad3^{+/-} mice was analyzed by Southern blot hybridization to a TCR Cβ2 probe. Appearance of one or more rearranged fragments, distinct from the germ-line configuration (indicated by the arrow in lane 1), suggests a clonal process, similar to that observed in leukemic lymphoblasts in *SCL-LMO1* transgenic mice (lanes 4 and 5).⁹ Smad3-positive (brown) leukemic blasts are evident in a thymic tumor (Panel J) and infiltrating myocardium (Panel K). Western blot analysis shows Smad3 expression in the thymus and spleen of affected p27^{Kip1}^{-/-}, Smad3^{+/-} mice (Panel L). Smad3 expression by lymphoblasts is documented in lymphoid organs (lanes 4 and 7) and in leukemia cells purified by magnetic-bead separation from the parenchyma of infiltrated lung and liver (lanes 1 and 2, respectively). Specificity of the Smad3 antibody is indicated by the band detected in TGF-β-responsive Mv1Lu cells (lane 8), but absent in the spleen and thymus of *Smad3*^{-/-} mice (lanes 5 and 6, respectively).

incorporated [³H]thymidine was measured by scintillation spectrophotometry (1450 Microbeta liquid scintillation counter, Perkin Elmer). In a parallel series of experiments involving the same conditions, cell-culture supernatants were removed after 48 hours and levels of interleukin-2 were measured with the use of a Quantikine M sandwich enzyme-linked immunoassay kit according to the manufacturer's instructions (R&D Systems).

IMMUNOHISTOCHEMISTRY

Smad proteins were stained on 5-μm sections of formalin-fixed, paraffin-embedded mouse tissues (heart, kidney, and lymph nodes) as previously described, with the use of the Optimax Plus 2.0 Automated Cell Staining System with research software



(BioGenex).⁵ Sections were incubated for two hours with the primary antibodies listed above at a concentration of 4 μ g of IgG per milliliter in TRIS-buffered saline with 1 percent bovine serum albumin. Rabbit or goat IgG (4 μ g per milliliter) was used as a negative control. Antigen-antibody complexes were detected with the use of the Vectastain Elite avidin-biotin complex peroxidase kit (Vector Laborato-

ries) according to the manufacturer's instructions. Carazzi hematoxylin was used as the counterstain.

RESULTS

LOSS OF Smad3 IN T-CELL ALL

We examined the Smad3 content by means of Western blotting in leukemia cells obtained from

children at diagnosis. Smad3 protein was readily detected in two of two ANLL specimens and in six of seven specimens of pre-B-cell ALL, with low levels detected in the remaining specimen of pre-B-cell ALL (Fig. 1). Smad3 was also present in freshly isolated peripheral-blood lymphocytes and purified T cells from control subjects (Fig. 1B), but absent or barely detectable in all 10 specimens from patients with T-cell ALL (Fig. 1A and 1B). In contrast, Smad2, the other Smad that becomes activated by the TGF- β receptor, was present at normal levels in all 20 specimens (shown for 6 samples in Fig. 1A).

To evaluate the mechanism underlying the loss of Smad3 protein in T-cell ALL, we used RT-PCR to measure the level of Smad3 mRNA in specimens from 8 of 10 children. Smad3 mRNA was present in all eight specimens, and the identity of these PCR products was confirmed as Smad3 by means of direct-sequencing analysis of DNA purified from the gel (Fig. 1C). There was no reduction in the level of Smad3 mRNA in the specimens of T-cell ALL, as compared with levels in normal T cells and in ANLL specimens. To determine whether the loss of Smad3 protein was the consequence of alterations in the *MADH3* gene, which encodes Smad3, we sequenced all nine exons of the gene, using genomic DNA from normal T cells as a reference. We found no evidence of deletions, point mutations, or other alterations of *MADH3* in any of the T-cell ALL specimens. We also performed Western blot analysis of Smad3 in specimens from four adults with HTLV-1-associated T-cell leukemia and three adults with the Sézary syndrome. Smad3 protein was readily detected in five of the seven specimens (Fig. 1D).

LEVELS OF Smad3 AND T-CELL RESPONSES TO TGF- β IN MICE

The above data suggest that the level of Smad3 protein expressed by T cells might be an important determinant in the suppression of T-cell leukemogenesis. We hypothesized that a reduction in Smad3 would also impair the response of normal T cells to TGF- β . To address this question, we examined the relation between the level of Smad3 and the suppression of T-cell proliferation and interleukin-2 production by TGF- β in mice in which one or both alleles of the *Smad3* gene were inactivated by homologous recombination.⁶ The experiment compared the ability of TGF- β to suppress the proliferative response and interleukin-2 production by T cells from *Smad3*^{+/+}, *Smad3*^{+/-}, and *Smad3*^{-/-} mice. The

level of Smad3 protein in T cells from such mice was directly correlated with the gene dose: levels in *Smad3*^{+/-} mice were approximately half those in *Smad3*^{+/+} mice (Fig. 2A), and the proliferation of *Smad3*^{-/-} T cells was not inhibited by TGF- β 1 (Fig. 2B).⁶ The concentration of TGF- β required to suppress the proliferation of T cells from *Smad3*^{+/-} mice by 50 percent (IC₅₀) was exactly twice the IC₅₀ required for wild-type T cells (Fig. 2B). As for interleukin-2, basal production in cultured T cells from *Smad3*^{-/-} mice was twice that in cultures of T cells from *Smad3*^{+/+} mice, with an intermediate value in cultures of T cells from *Smad3*^{+/-} mice (Fig. 2C). Culturing T cells from *Smad3*^{+/-} mice with TGF- β at the IC₅₀ depicted in Figure 2B reduced the level of interleukin-2 by a factor of 74, as compared with a factor of 7.4 for T cells from *Smad3*^{+/-} mice and 3 for T cells from *Smad3*^{-/-} mice.

Smad3 AND p27^{Kip1} IN THE SUPPRESSION OF LEUKEMOGENESIS

Suspension of Smad3 production in *Smad3*^{-/-} mice is not associated with T-cell leukemia,⁶ indicating that the loss of Smad3 alone is insufficient to initiate leukemogenesis. The gene-dose effect for *Smad3* in the response of normal T cells to TGF- β suggested that a reduction in Smad3 would increase the risk of leukemia when associated with alterations in other factors that control the activation, proliferation, or viability of T cells. We selected p27^{Kip1} for analysis because the gene encoding p27^{Kip1} (*CDKN1B*) is located at chromosomal region 12p12, a site frequently altered by deletions and translocations in pediatric ALL.⁷ We examined the level of total p27^{Kip1} protein and the ratio of free to cyclin D-associated p27^{Kip1} protein in patients. In specimens from children with T-cell ALL and pre-B-cell ALL, p27^{Kip1} was present, but at low levels (Fig. 3A). Analysis of cyclin D-associated proteins by immunoprecipitation and Western blotting revealed that most of the p27^{Kip1} was not associated with cyclin D3 (Fig. 3B).

We next developed a mouse model in which the loss of one allele of *Smad3* was combined with a homozygous deletion of p27^{Kip1} by crossing *Smad3*^{+/-} female mice with p27^{Kip1}^{-/-} male mice.⁸ Although p27^{Kip1}^{-/-} mice have an increased number of normal T cells and increased cellularity of lymphoid organs, leukemia does not develop in such mice.⁸ Only 50 percent of the p27^{Kip1}^{-/-}, *Smad3*^{+/-} mice lived beyond six months of age (Fig. 4A). Histologic evaluation of these mice showed lymphocytic in-

filtrates in multiple organs, lymphoid hyperplasia, nephropathy, and cardiomyopathy. T-cell leukemia developed in 10 percent (5 of 50) of these $p27^{Kip1-/-}$, $Smad3^{+/-}$ mice (Fig. 4). The disease was characterized by diffuse lymphadenopathy and splenomegaly (Fig. 4B and 4C), massive thymic enlargement (Fig. 4D), and lymphoblasts (Fig. 4E) in multiple organs (Fig. 4F and 4G). A T-cell immunophenotype of mouse leukemia cells was demonstrated by cytometric analysis (Fig. 4H), and Southern blot analysis of the T-cell-receptor β chain demonstrated that these tumors were clonal (Fig. 4I). *Smad3* was expressed from the retained allele, as evidenced by the detection of Smad3 in thymic tumors (Fig. 4J) and infiltrated organs (Fig. 4K) by immunohistochemistry and in lysates of purified lymphoblasts by Western blotting (Fig. 4L). We could not evaluate the effect of a homozygous deletion of *Smad3* in the $p27^{Kip1-/-}$ mice, since there was a substantial rate of death during embryogenesis in mice with this genotype.

DISCUSSION

We have found direct evidence of a tumor-suppressor function for Smad3 in the T-cell lineage. Although mutations in genes encoding Smad2 (*MADH2*) or Smad4 (*MADH4*) have been found in human tumors,² to our knowledge, no inactivating mutations in the Smad3 gene (*MADH3*) have been reported. We also failed to find mutations in *MADH3* in our patients with T-cell ALL. Our results show that a relative reduction in the level of Smad3 can impair the tumor-suppressor function of the TGF- β

pathway. Thus far we have not been able to determine the mechanism responsible for the loss of expression of Smad3 protein.

Our analysis of $p27^{Kip1-/-}$, $Smad3^{+/-}$ mice clearly indicates that Smad3 can work in tandem with a loss of $p27^{Kip1}$ to promote T-cell leukemogenesis. The importance of the tumor-suppressor activity of $p27^{Kip1}$ in humans is suggested by the association of reduced $p27^{Kip1}$ levels in tumors with a poor prognosis.¹⁰ The inhibitory effect of $p27^{Kip1}$ on cell-cycle progression is also abrogated by multiple mechanisms in lymphoid cancers.¹¹ Point mutations and homozygous deletions of *CDKN1B* (the gene encoding $p27^{Kip1}$) have been found in adult T-cell leukemia.¹² In pediatric ALL, hemizygous deletion of *CDKN1B* has been described as the primary consequence of 12p chromosomal deletions.⁷ Although the production of $p27^{Kip1}$ is associated with arrest of the cell cycle in G_1 in response to TGF- β , $p27^{Kip1}$ is not absolutely required for the halting of the cell cycle, since primary T cells of $p27^{Kip1-/-}$ mice are inhibited by TGF- β .

In summary, our data show that the level of Smad3 is a determinant of the T-cell response to TGF- β and that a reduction in Smad3 can work in tandem with oncogenic events, such as alterations in the retinoblastoma pathway, to promote T-cell leukemogenesis. Most important, these data identify a distinct role for Smad3 in mediating TGF- β signaling in the activation and leukemogenesis of T cells.

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