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Transformation of hematopoietic cell lines to growth-factor independence and induction of a fatal myelo- and lymphoproliferative disease in mice by retrovirally transduced *TEL/JAK2* fusion genes

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Recent reports have demonstrated fusion of the *TEL* gene on 12p13 to the *JAK2* gene on 9p24 in human leukemias. Three variants have been identified that fuse the TEL pointed (PNT) domain to (i) the JAK2 JH1-kinase domain, (ii) part of and (iii) all of the JH2 pseudokinase domain. We report that all of the human *TEL/JAK2* variants, and a human/mouse chimeric *hTEL/mJAK2(JH1)* fusion gene, transform the interleukin-3 (IL-3)-dependent murine hematopoietic cell line Ba/F3 to IL-3-independent growth. Transformation requires both the TEL PNT domain and JAK2 kinase activity. Furthermore, all *TEL/JAK2* variants strongly activated STAT 5 by phosphotyrosine Western blots and by electrophoretic mobility shift assays (EMSA). Mice ($n = 40$) transplanted with bone marrow infected with the *MSCV* retrovirus containing either the *hTEL/mJAK2(JH1)* fusion or its human counterpart developed a fatal mixed myeloproliferative and T-cell lymphoproliferative disorder with a latency of 2–10 weeks. In contrast, mice transplanted with a *TEL/JAK2* mutant lacking the TEL PNT domain ($n = 10$) or a kinase-inactive *TEL/JAK2(JH1)* mutant ($n = 10$) did not develop the disease. We conclude that all human TEL/JAK2 fusion variants are oncoproteins *in vitro* that strongly activate STAT 5, and cause lethal myelo- and lymphoproliferative syndromes in murine bone marrow transplant models of leukemia.
Keywords: animal model/leukemogenesis/Stat 5/*TEL–JAK*2 fusion gene/transformation

Introduction

Signal transduction from the membrane to the nucleus upon ligand stimulation of a cytokine receptor is mediated primarily by specific tyrosine kinases, such as Janus kinases (JAKs), and latent cytoplasmic transcription factors, such as signal transducers and activators of transcription (STATs) (Ihle *et al.*, 1995; Watanabe and Arai, 1996;

Darnell, 1997). Four JAK family members are known in mammals, JAK1, JAK2, JAK3 and TYK2. JAK kinases have shared structural domains designated as JH segments 1–7. JH1 is the kinase domain located at the C-terminus and is preceded by the JH2 pseudokinase domain, which has been suggested to be an inhibitory domain regulating JAK2 kinase activity (Luo *et al.*, 1997). JAK proteins associate with cytokine receptors, and ligand stimulation of the cytokine receptor and activation of associated JAKs leads to phosphorylation of specific tyrosine residues on the receptor. This allows SH2-containing signaling proteins, including STATs, and adapter proteins to bind. JAK phosphorylation of STAT proteins induces STAT hetero- and homodimer formation and translocation to the nucleus, where the complexes bind to specific DNA response elements and activate transcription of genes. To date six members of the STAT family (STAT 1–4, 5A, 5B, 6) have been identified (Darnell, 1997; Leaman *et al.*, 1996).

The importance of the JAK–STAT pathway in cytokine signaling suggests a potential role in the pathogenesis of hematological malignancies (Watanabe and Arai, 1996). Several lines of evidence support this hypothesis. First, constitutive activation of the JAK–STAT pathway has been demonstrated in *HTLV-1*-transformed T cells (Mignone *et al.*, 1995; Xu *et al.*, 1995). Secondly, a single amino-acid mutation in the *Drosophila melanogaster* JAK (encoded by the *Hopscotch* locus) has been shown to cause leukemia-like hematopoietic defects (Luo *et al.*, 1995, 1997). Thirdly, the transforming oncoproteins v-*abl*, p210(BCR/ABL) and p190(BCR/ABL) induce tyrosine phosphorylation and DNA-binding activity of multiple specific STAT family members (Danial *et al.*, 1995; Carlesso *et al.*, 1996; Ilaria and Van Etten, 1996; Shuai *et al.*, 1996), and constitutively activated STATs have been found in cells from patients with chronic myelomonocytic leukemia (CML), acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML) and chronic lymphocytic leukemia (CLL) (Gouilleux-Gruart *et al.*, 1996; Chai *et al.*, 1997; Frank *et al.*, 1997). Moreover, constitutive activation of the JAK–STAT pathway has been found in malignant cutaneous T-cell disease (mycosis fungoides, Sézary syndrome) (Zhang *et al.*, 1996; Nielsen *et al.*, 1997). Finally, leukemic cells from patients with relapsed ALL have constitutively activated JAK2, and inhibition of JAK2 activity by a specific tyrosine kinase blocker (AG-490) selectively blocks leukemic cell growth *in vitro* and *in vivo* (Meydan *et al.*, 1996). But perhaps the most direct evidence for involvement of JAK family members in leukemogenesis has been the characterization of chromosomal translocations from leukemic cells involving the *JAK2* kinase gene on 9p24 and the *TEL* gene on 12p13.

The *TEL* gene (also known as Ets-translocation variant gene 6, *ETV-6*) was initially cloned as the fusion partner

of platelet-derived growth factor receptor β gene (*PDGFβR*) in CML with t(5;12) (q31;p13) (Golub *et al.*, 1994). *TEL* has subsequently been shown to be fused to several partner genes on different chromosomes including *AML1*(*CBFA2*) in t(12;21) (p13;q22) associated with childhood pre-B ALL (Golub *et al.*, 1995); *ABL* in t(9;12) (q34;p13) in rare cases of childhood ALLs (Papadopolous *et al.*, 1995); *MN1* in t(12;22) (p13;q11) in AML (Buijs *et al.*, 1995); and *MDS/EVI1* in t(3;12) (q26;p13) associated with myeloproliferative disorders (Peeters *et al.*, 1997a). The *TEL* gene on 12p13 codes for a ubiquitously expressed nuclear protein which contains a C-terminal conserved DNA-binding domain of ETS transcription factors (Golub *et al.*, 1994; Poirel *et al.*, 1997). TEL is one of a subset of the ETS family of transcription factors that contains a conserved N-terminal domain which has been variously referred to as the pointed (PNT) domain, based on homology to the *Drosophila* ETS-protein *pointed*, the N-terminal conserved domain (NCR), or the helix–loop–helix domain, based on weak homology to the basic helix–loop–helix domain found in transcription factors such as myc and myo-D (Wasylyk *et al.*, 1993). The PNT domain was shown to be important for transcriptional activity despite the lack of DNA contact, but no other function was known prior to the cloning of the *TEL/PDGFRβ* fusion gene (Rao *et al.*, 1993). Analysis of the TEL fusion proteins in the context of the PNT domain has provided evidence that this domain acts as a self association domain of functional importance. The PNT domain is essential for TEL/AML1-mediated repression of AML1-dependent transcription (Hiebert *et al.*, 1996). Furthermore, oligomerization by the PNT domain leads to constitutive activation of the ABL and PDGFRβ tyrosine kinase domains, which is necessary for cellular transformation (Carroll *et al.*, 1996; Golub *et al.*, 1996; Jousset *et al.*, 1997).

Three distinct *TEL/JAK2* fusions have recently been cloned. The first is a fusion between *TEL* exon 5 and *JAK2* exon 12 from a patient with atypical CML and a complex t(9;12;14) translocation [*TEL/JAK2* (5–12)]; the second is a fusion of *TEL* exon 4 to *JAK2* exon 17 in a patient with pre-B cell ALL and t(9;12) (p24;p13) [*TEL/JAK2* (4–17)]; and the third is a fusion of *TEL* exon 5 to *JAK2* exon 19 in a case of T-cell childhood ALL and t(9;12) (p24;p13) [*TEL/JAK2* (5–19)] (Lachronique *et al.*, 1997; Peeters *et al.*, 1997b).

By analogy with other TEL-tyrosine kinase fusion proteins (TEL/PDGFRβ, TEL/ABL) we hypothesized that the JAK2 tyrosine kinase was constitutively activated by oligomerization mediated by the TEL PNT domain, leading to transformation of hematopoietic cells (Carroll *et al.*, 1996; Golub *et al.*, 1996; Jousset *et al.*, 1997). The transforming properties of *TEL/JAK2* fusion genes were tested in growth-factor-dependent, murine hematopoietic cell lines and *in vivo* by transplant of primary murine bone marrow infected with *TEL/JAK2* retroviral expression vectors into lethally irradiated recipients. Our results show that all TEL/JAK2 fusion variants transform hematopoietic cells to factor-independent growth and that transformation is dependent on the presence of the TEL PNT domain as well as an active JAK2 kinase domain. TEL/JAK2 is localized to the cytoplasm of cells, and is a potent activator of STAT 5. In addition, the TEL/JAK2 variant associated

with human T-ALL induces a rapidly fatal mixed myelo- and T-cell lymphoproliferative disorder in mice reflecting, at least in part, the heterogeneity of the human leukemic phenotypes associated with TEL/JAK2 fusions.

Results

Human TEL fused to the kinase domain of murine Jak2 transforms hematopoietic cells to growth-factor independence

Previous reports have demonstrated that upon ligand-mediated dimerization, extracellular cytokine receptor domains (EGF, CD16) are able to induce phosphorylation and activation of a fused JAK2 tyrosine kinase domain (Sakai *et al.*, 1995; Nakamura *et al.*, 1996). We hypothesized that the TEL PNT domain, when fused to the JAK2 tyrosine kinase domain, might act as a dimerization interface leading to activation of the fused tyrosine kinase as demonstrated for TEL/PDGFRβ and TEL/ABL (Carroll *et al.*, 1996; Jousset *et al.*, 1997). A chimeric cDNA construct containing the sequence encoding for the TEL PNT domain (exon 1–5) fused to the murine *JAK2*-kinase domain (JH1) was prepared (Figure 1). The protein encoded by this fusion gene was tyrosine phosphorylated and localized to the cytoplasm when overexpressed in COS-7 cells (Figure 2). These data suggest that, like other TEL-tyrosine kinase fusions, the TEL PNT domain acts as a self-association interface leading to constitutive activation of the JAK2-kinase domain. The TEL/JAK2 fusion protein was tested for its ability to confer factor-independent growth to hematopoietic cells. Three cDNA constructs were prepared in the retroviral expression vector *MSCV-neo*: *hTEL/mJAK2*, *hTELΔPNT/mJAK2* lacking the TEL PNT domain, and *hTEL/mJAK2-KI* containing two point mutations in the type VIII kinase motif leading to a kinase deficiency (Zhuang *et al.*, 1994; Carroll *et al.*, 1996). Murine hematopoietic Ba/F3 cells that are dependent on interleukin-3 (IL-3) for growth were infected with *hTEL/mJAK2*, *hTELΔPNT/mJAK2* and *hTEL/mJAK2-KI*. After 48 h the cells were divided. Half were selected for G418 resistance and half were washed free of IL-3 and replated in growth medium alone. In four independent experiments, IL-3-independent cells infected with *hTEL/mJAK2* were obtained after 2–4 days. No IL-3-independent cells were obtained when Ba/F3 cells were infected with *hTELΔPNT/mJAK2* and *hTEL/mJAK2-KI* or the vector alone (Figure 3A). Immunoprecipitation with an antibody against the murine JAK2–JH1 domain followed by Western blotting confirmed that the hTEL/mJAK2 fusion protein and both mutants were highly expressed (Figure 3B). To determine whether the hTEL/mJAK2 fusion protein was constitutively tyrosine phosphorylated, blots of anti-mJAK2 immunoprecipitates were probed using an anti-phosphotyrosine antibody (4G10). The hTEL/mJAK2 fusion gene product was tyrosine phosphorylated whereas no tyrosine phosphorylation was seen in cells expressing the TEL/JAK2 mutants or the *MSCVneo* vector (Figure 3B). Similar results were obtained when the interleukin-7 (IL-7)-dependent murine hematopoietic cell line IXN/2B was infected with *hTEL/mJAK2* (Park *et al.*, 1990) (data not shown). These results demonstrate that, like other TEL/tyrosine kinase fusion proteins (TEL/PDGFβR, TEL/ABL), fusion of TEL to the JAK2 kinase domain trans-
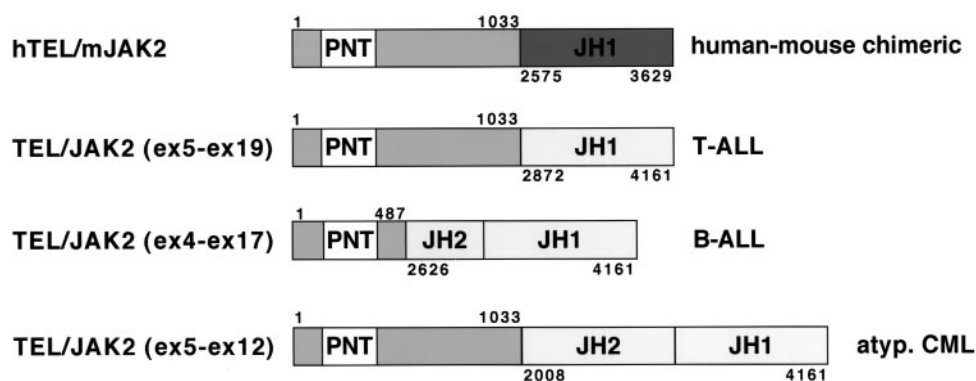
Fig. 1. Schematic representation of *TEL/JAK2* fusion gene variants. A chimeric *TEL/JAK2* fusion between human *TEL* (exons 1–5) to the murine *JAK2-JH1* tyrosine kinase domain, and three human *TEL/JAK2* fusion gene variants as cloned by us and others. The nucleotide positions are numbered according to the cDNAs as deposited in the DDBJ/EMBL/GenBank: *TEL*(*ETV6*), accession No. U11732 (Golub *et al.*, 1994); *mJAK2*, accession No. L16956 (Silvennoinen *et al.*, 1993), *hJak2* accession No. AF005216 (Peeters *et al.*, 1997b).

forms hematopoietic cells, and that transformation is dependent on the TEL PNT domain as well as an active JAK2 tyrosine kinase domain.

Three TEL/JAK2 fusion variants associated with different human leukemia phenotypes transform hematopoietic cells to factor independence

Three different human TEL/JAK2 fusions variants resulting from t(9;12) (p24;p13) have been reported in association with three different leukemia phenotypes (Figure 1). To test the transforming properties of these TEL/JAK2 fusion variants, cDNA constructs were sub-cloned into *MSCV-neo* and Ba/F3 cells were infected. As for the chimeric human–mouse *TEL/JAK2* construct, all three TEL/JAK2 variants associated with human leukemia transformed Ba/F3 cells to factor independence (Figure 3C). In addition, all three fusion proteins were expressed at high levels and constitutively tyrosine-phosphorylated as assessed by immunoprecipitation and Western blotting (Figure 3D). In some experiments a low level of tyrosine phosphorylated endogenous JAK2 was observed, but was not reproducible. These results show that, independent of the presence of all or part of the JAK2 JH2 domain, all three human TEL/JAK2 fusion variants are constitutively tyrosine-phosphorylated and able to transform hemato-poietic cells to growth factor independence.

All three human TEL/JAK2 fusion variants induce tyrosine phosphorylation and DNA-binding activity of STAT 5

Although STAT proteins are targets for phosphorylation by endogenous JAK2, the TEL/JAK2 fusion protein, containing only the JH1-kinase domain and lacking the domains involved in receptor association, may lack speci-ficity for STAT proteins. Activation of STATs was analyzed in Ba/F3 cells transformed to factor-independent growth by TEL/JAK2 fusion proteins. As shown in Figure 4A, all STATs are expressed in hTEL/mJAK2-transformed cells as well as wild-type parental Ba/F3 cells from low (STAT 2, 4) to high levels (STAT 1, 3, 5, 6). An unexplained decreased level of STAT 6 expressed in hTEL/mJAK2 transformed Ba/F3 cells was observed in several experiments. Immunoprecipitation with anti-STAT anti-bodies, followed by anti-phosphotyrosine (4G10) blotting,

showed tyrosine phosphorylation of STAT 5 (Figure 4B). Low levels of STAT 1 and 3 phosphorylation were observed after prolonged exposure (data not shown). Similar to the hTEL/mJAK2 chimeric fusion, increased phosphorylation of STAT 5 was detected in Ba/F3 cells transformed with all three human TEL/JAK2 fusion vari-ants (Figure 5A). Phosphorylated STAT 1 could only be detected with anti-phospho-STAT 1 antibodies but not with anti-phosphotyrosine antibodies, suggesting low levels of tyrosine phosphorylation of STAT 1.

Induction of STAT DNA-binding activity by TEL/JAK2 fusion proteins was assessed by electrophoretic mobility shift assays (EMSA) using a γ-activated-sequence (GAS) oligoprobe derived from the FcγRI promoter as previously described (Ilaria *et al.*, 1996). Nuclear extracts of Ba/F3 cells expressing BCR/ABL and TEL/JAK2 demonstrated high levels of constitutive GAS DNA-binding activity which was impaired by addition of free phosphotyrosine, and eliminated by a cold competitor GAS oligonucleotide (Ilaria *et al.*, 1996; data not shown). To identify the STATs responsible for the GAS-binding activity, specific anti-STAT antibodies were used to supershift the DNA–protein complexes. As demonstrated in Figure 5B, incubation of nuclear extracts from Ba/F3 cells transformed by the human–mouse and the three human TEL/JAK2 fusion variants with anti-STAT 5 antibody resulted in a complete supershift of the GAS DNA-binding activity. Antibodies to STAT 1 resulted in a small decrease in GAS DNA-binding activity. No decrease in GAS DNA-binding, or supershifting was observed when the extracts were incubated with anti-STAT 3 or anti-STAT 6 antibodies (data not shown). These results demonstrate prominent activation of STAT 5 DNA-binding activity in TEL/JAK2-transformed cells, with less activation of STAT 1, consistent with the tyrosine phosphorylation profile.

Rapid development of a mixed myelo- and lymphoproliferative disease in mice transplanted with TEL/JAK2 retrovirally transduced bone marrow

To assess the consequences of ectopic expression of a gene involved in normal or malignant hematopoiesis *in vivo*, lethally irradiated mice were reconstituted with bone marrow infected with retroviral expression vectors
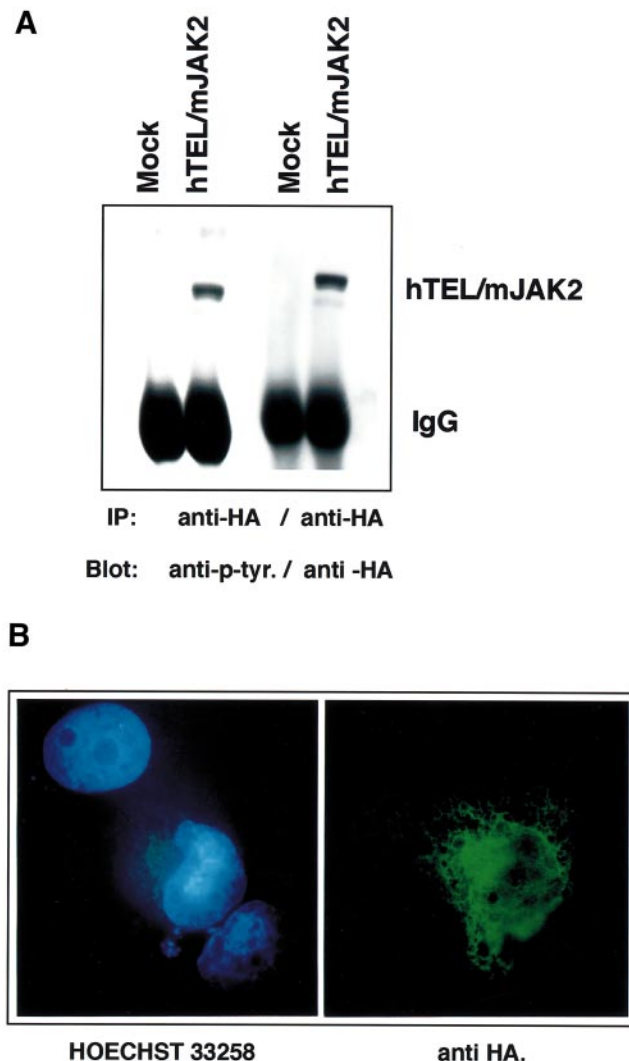
A

IP: anti-HA / anti-HA

Blot: anti-p-tyr. / anti -HA

B

HOECHST 33258   anti HA.

Fig. 2. Characterization of the chimeric human–mouse TEL/JAK2 (JH1) fusion protein. (**A**) *pcDNA3-HA-hTEL/mJAK2* (10 μg) was transfected into COS-7 and whole cell lysates prepared. Protein (1 μg) was precipitated with an anti-HA antibody and the blot then probed with either the anti-HA or anti-phosphptyrosine antibody (4G10) showing expression and tyrosine-phosphorylation of the hTEL/mJAK2 fusion protein. (**B**) Immunolocalization of hTEL/mJAK2 fusion protein in COS-7 cells. The right panel shows cytoplasmic immunofluoresence staining for hTEL/mJAK2 (5–19) with anti-HA-antibody coupled with a FITC-labeled secondary antibody. The left panel shows staining of the nuclei of the same cells using HOECHST 33258 dye.

containing the gene of interest (Johnson *et al.*, 1989; Daley *et al.*, 1990; Elefanty *et al.*, 1990, 1992; Hawely *et al.*, 1992, Gishizky *et al.*, 1993; Kuefer *et al.,* 1997). To characterize the transforming potential of TEL/JAK2 in an animal model, murine bone marrow cells were infected with the retroviral expression vector *MSCV* containing the cDNA coding for the *in vitro*-characterized chimeric human–mouse *TEL/JAK2* and the human counterpart [*TEL/JAK2* (5–19)] fusion gene. *MSCV* has been previously used to induce transformation of either lymphoid or myeloid lineage cells (Hawely *et al.*, 1992; Lavau *et al.*, 1997). To increase the efficiency of the retroviral gene transfer, infection of primary murine bone marrow cells was performed on fibronectin-fragment (CH-296, Retronectin™) coated plates (Hanenberg *et al.*, 1996; Kuefer *et al.*, 1997). Forty mice were transplanted with

MSCV-TEL/JAK2 [30 *hTEL/mJAK2*; 10 *TEL/JAK2* (5–19)] infected bone marrow in three separate experiments with independently prepared viral supernatants. All animals died of disease within 4–10 weeks post transplant as shown in a Kaplan–Meier survival plot (Figure 6).

Pathological examination was performed on 37 animals. Seven out of seven animals from the first transplant series (1.1–1.10) developed a myeloproliferative disorder, with enlarged spleen and expansion of the red pulp by maturing myeloid elements; elevated white blood cells (WBC: $9–180 \times 10^6$/ml) with mature and immature myeloid progenitors seen in blood smears, and variable degrees of extramedullary hematopoiesis involving mostly the liver, as well as lymph nodes and adrenal medulla (Table I and Figure 7A–D). The lungs showed areas of chronic hemorrhage accompanied by aggregates of macrophages (Figure 7G). In three of these seven animals with the myeloproliferative disorder, enlarged lymph nodes containing a high-grade lymphoid neoplasm were found in the abdomen as well as in cervical lymph nodes. All eight animals from the second transplant series (2.2–2.10) showed extensive involvement of a high-grade lymphoid neoplasm (Figure 7E) concomitant with signs of myeloproliferative disease at variable levels (WBC: $5–175 \times 10^6$/ml). In one animal (2.8), immature lymphoid cells were also observed in the blood smear. In addition, lymphoid blasts were mixed with maturing myeloid elements on some tumor specimens from the same animal (Figure 7E–F). The lymphoproliferation was locally destructive, infiltrating and replacing normal pancreas, kidney and liver (Figure 8H).

To confirm the presence of *MSCV-TEL/JAK2* provirus in affected tissues, DNA was isolated from various organs and digested with *Kpn*I to release the entire 5.5 kb provirus. Southern blot analysis of DNA from animal 2.5 using a *Neo* probe showed provirus in blood, bone marrow, spleen, thymus and lymph nodes as well as lungs and liver, reflecting the pattern of widespread disease seen on histopathological examination (Figure 8A). This blot is representative of DNA analysis of 10 *TEL/JAK2* transplanted animals. The presence of tumor masses containing lymphoid blasts as well as myeloid precursors raised the question whether these masses are collision tumors comprised of a mixture of myeloid and lymphoid clones, or a single clone with bi-phenotypic differentiation potential. To address clonality of the neoplastic cells, DNA was digested with *Eco*RI, which has a single site within in the *MSCVneo-TEL/JAK2* provirus DNA. Southern blot analysis showed the presence of several distinct bands in all the organs of mouse 2.5 (not shown). Mice of the second BMT (2.2, 2.4, 2.5) all showed enlarged spleens with an increase of myeloid cells and enlarged lymph nodes that contained mostly lymphoid blasts with some masses containing myeloid precursors. Therefore, provirus integration was analyzed in spleen and lymph nodes from these animals. As demonstrated in Figure 8B, several distinct bands were observed in all samples analyzed. The different provirus integration pattern with partial overlap in spleen versus lymph nodes supports the presence of a mixture of myeloid and lymphoid clones responsible for the disease. In addition, the variation in intensity of the observed bands compared with the DL-1 cell line containing a single copy of *BCR/ABL-neo* provirus
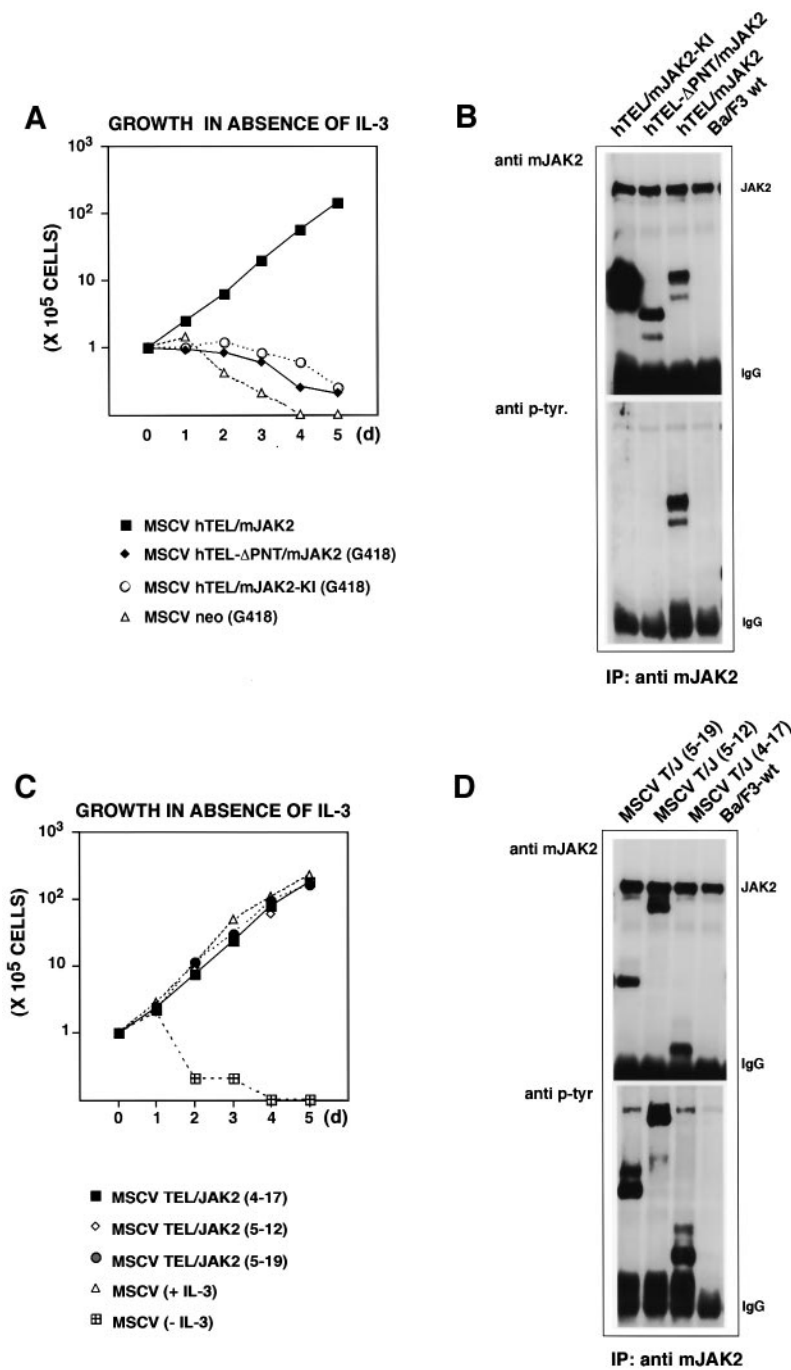
Fig. 3. Transformation of Ba/F3 cells to IL-3-independent growth by hTEL/mJAK2 is dependent on the hTEL PNT domain and an active mJAK2 JH1 tyrosine kinase domain. All three different human TEL/JAK2 fusion protein variants (4–17, 5–12, 5–19) transform Ba/F3 cells to IL-3 independent growth. (**A**) Ba/F3 cells (1×10^6) were infected with retroviral expression vectors (*MSCVneo*) encoding either wild-type *hTEL/JAK2*; a mutant lacking the TEL PNT domain (*hTELΔPNT/mJAK2*); or a mutant containing an inactive mJAK2 JH1-kinase domain (*hTEL/mJAK2-KI*). After 48 h, the cells transduced with *hTEL/mJAK2* were washed free of IL-3, and cultivated until the cells reached logarithmic growth. Cells transduced with the mutants were selected in medium containing IL-3 and G-418. After selection, the cells were washed free of IL-3, plated in duplicate to 1×10^5 cells, and counted daily. The figure represents three independent experiments. (**B**) All transduced constructs are expressed in Ba/F3 cells but only the wild-type hTEL/mJAK2 fusion protein is tyrosine phosphorylated. Total cell lysates were prepared using lysis buffer containing 1% Triton X-100. Lysates (1 mg input protein) were immunoprecipitated with a rabbit anti-JAK2(JH1) antibody, separated on an 8% SDS–PAGE, blotted and probed with an anti-phosphotyrosine antibody (4G10). The blot was then stripped and reprobed with the rabbit anti-JAK2(JH1) antibody initially used for IP. TEL fusion proteins appear as doublets due to two closely spaced start sites in the *TEL* cDNA. All three human TEL/JAK2 fusion variants transform Ba/F3 cells to factor-independent growth (**C**). The fusion proteins are expressed and tyrosine phosphorylated (**D**). Experiments shown in (C) and (D) were performed exactly as described in (A) and (B), respectively.

(Figure 8B) further supports oligoclonality of the disease, rather than a single clone with potential for myeloid and lymphoid maturation (Daley *et al.*, 1990).

Flow-cytometric immunophenotypic analysis was performed on single-cell suspensions from the spleens of affected animals. In some animals, where histopathology
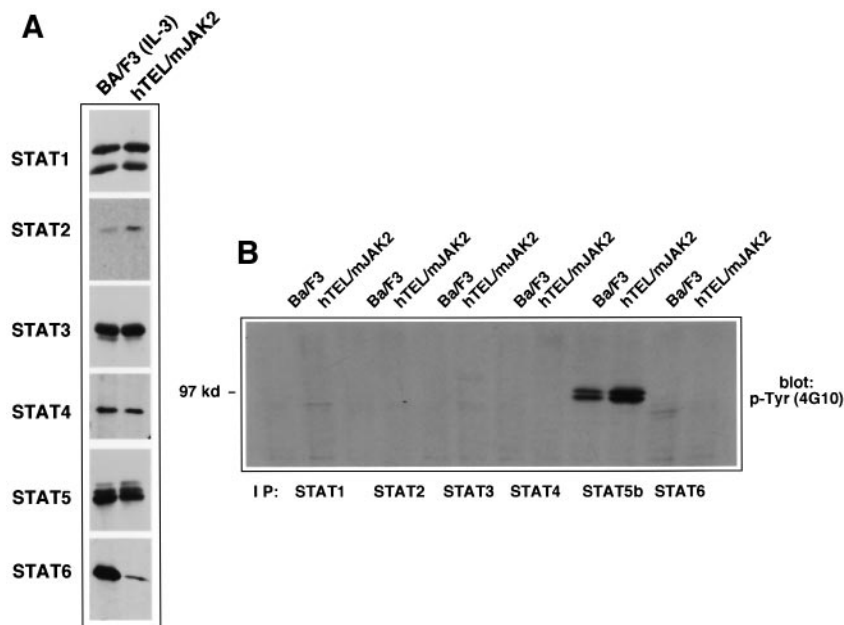
Fig. 4. Expression and tyrosine phosphorylation of STAT family members in hTEL/mJAK2 transformed Ba/F3 cells. (**A**) Total cell lysates (1 mg) were immunoprecipitated, separated on 7% SDS–PAGE, and the blots probed with the same anti-STAT antibody used for IP. (**B**) Analysis of STAT protein tyrosine phosphorylation in *hTEL/mJAK2* transformed Ba/F3 cells (–IL-3) compared with wild-type cells (+IL-3). Blots were prepared as described in (A) and probed with an anti-phosphotyrosine antibody (4G10). Transformation of Ba/F3 cells to IL-3-independent growth by *hTEL/mJAK2* is associated with high levels of phosphorylated STAT5, and low levels of phospho-STAT 1 and phospho-STAT 3.

revealed predominantly myeloid expansion (e.g. animal 1.2; Figure 9A), the spleen contained a large number of Gr-1+ and Mac-1+ granulocytes (45% compared with <5% in spleens from normal BALB/c mice). In mice in which lymphoproliferative disease predominated (e.g. animal 2.4), the number of Gr-1+ and Mac-1+ cells was not increased. Most of the lymphoid cells expressed intermediate to high levels of CD3, indicating that they were T cells. Only a small percentage of the splenocytes displayed an immunophenotype typical of mature splenic B lymphocytes (B220+ and CD3–). Many of the CD3+ cells expressed B220 at levels below that seen on normal B cells. Analysis of CD4 and CD8 staining by the same splenocytes demonstrated a mixture of CD4+, CD8+, and CD4+CD8+ T cells. T cells double positive for CD4 and CD8 represent immature T cells normally confined to the thymus; their presence in the spleens of these mice is consistent with a neoplastic proliferation of T cells.

To identify cells containing the retroviral expression vector, 10 mice were transplanted with a TEL/JAK2 retroviral expression construct variant (*MSCV-TEL/JAK2-IRES EGFP*) that contains an expression cassette for the enhanced green fluorescent protein (EGFP) driven from an internal ribosome entry site (IRES) derived from mRNA of the encephalomyocarditis virus (ECMV) (Kim *et al.*, 1992; Gurtu *et al.*, 1996; Persons *et al.*, 1997). Fluorescence of the abnormal cells was examined to identify cells expressing the retroviral construct. A distinct population of cells with EGFP fluorescence (Figure 9B) was seen in the lymph nodes of a mouse in which the EGFP-containing construct was used (animal 3.18 with lymphadenopathy and 18% EGFP+ cells). In contrast, cells with this level of fluorescence were essentially absent from the lymph node of animal 2.4 (which also had lymphadenopathy, but

did not have the IRES–EGFP cassette as part of the retroviral construct). Similarly, 9% of the spleen cells from animal 3.1 displayed sufficient levels of EGFP fluorescence to define a subset of cells that was essentially absent from the spleen of animal 1.2 which also had myeloproliferative disease. Using regions consisting only of cells with clear EGFP fluorescence, we used immunophenotyping to show that the EGFP+ cells in animal 3.18 were nearly all T lymphocytes positive for CD8 that included a subset with a high level of CD3. The same gating strategy was used to show that 77% of the EGFP+ spleen cells in the spleen of animal 3.1 expressed the granulocyte marker Gr-1 (Figure 9C). TEL/JAK2 protein expression was further confirmed by immunohistochemical analysis of tumor tissue as well as of total protein lysates from lymph nodes, bone marrow and spleens of animals with disease (data not shown).

In control BMT experiments, 10 animals each were transplanted with either the *MSCVneo-hTEL-ΔPNT/mJAK2* or *MSCVneo-hTEL/mJAK2-KI* constructs which do not transform cell lines to factor-independent growth (Figure 2). None of these 20 animals developed any signs of disease post transplant (>20 weeks), and two animals that were analyzed by histopathology showed no microscopic evidence of disease (Figure 6, Table I; data not shown).

Taken together, these results show that a *TEL/JAK2* fusion gene is capable of inducing a reproducible and rapidly fatal, oligoclonal, mixed myelo- and T-cell lymphoproliferative disorder in mice. The characteristics of the disease with the presence of a mixture of lymphoid and myeloid clones is consistent with the observed association of *TEL/JAK2* fusion genes with human myeloid (e.g.
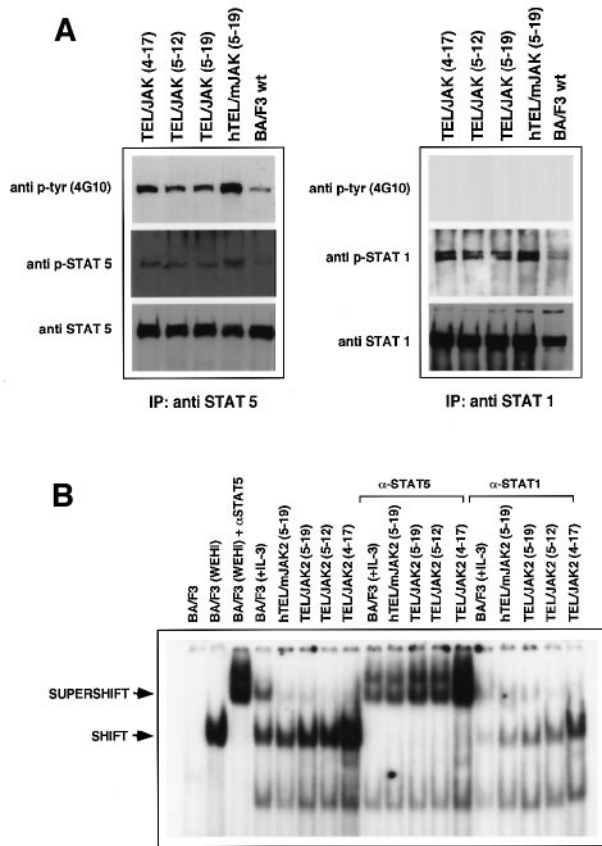
Fig. 5. (**A**) Transformation of Ba/F3 cells by three TEL/JAK2 fusion gene variants associated with human leukemia is associated with increased levels of STAT5 and STAT1 tyrosine phosphorylation. Total cell lysates (1 mg) were immunoprecipitated with anti-STAT 5 and anti-STAT 1 antibodies, respectively, separated on 8% SDS–PAGE, blotted and probed for phosphotyrosine (4G10), then stripped and sequentially reprobed with anti-phospho-STAT and anti-STAT antibody. No increase in phospho-STAT 1 was detected in several experiments while 4G10 probing, but seen when probed with anti-phospho-STAT1. (**B**) Induction of STAT GAS DNA binding activity by TEL/JAK2. Nuclear extracts from parental, hTEL/mJAK2 and TEL/JAK2 (4–17, 5–12 and 5–19) Ba/F3 cells were analyzed by EMSA using ^{32}P-labeled FcγRI-derived GAS probe. No baseline GAS DNA-binding was observed in nuclear extracts from parental Ba/F3 growing in the absence of IL-3 for 12 h. WEHI (cellular IL-3 source) stimulated Ba/F3 cells produced a completely supershifted complex when anti-STAT5 antibodies were added. Adding of 10–100× cold competitor GAS probe fully abrogated the complex (data not shown). Baseline GAS DNA-binding complexes from parental Ba/F3 cells (an unexplained higher molecular weight band was observed in some experiments) and TEL/JAK2 transformed cells were completely supershifted when anti-STAT 5 antibodies were added to the reaction. Adding of anti-STAT 1 antibodies showed a decrease in baseline GAS DNA-binding but no significant supershift. Adding of anti-STAT 2, STAT 3 or STAT 6 showed no effect on GAS DNA complexes (data not shown).

CML-like disease) and lymphoid (e.g. B-ALL and T-ALL) leukemias.

Discussion

The recent characterization of the breakpoint of the translocation (9;12) (p24;p13) identified three different TEL/JAK2 fusion variants, each of which is associated with a different leukemic phenotype (Lachronique *et al.*, 1997; Peeters *et al.*, 1997b). Here we demonstrate the trans-
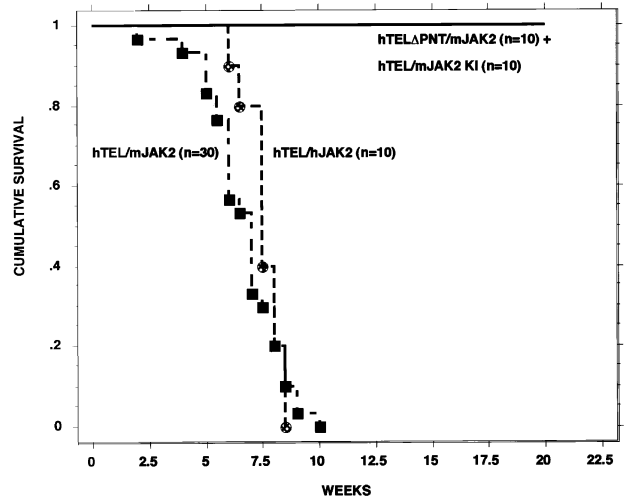

Fig. 6. Comparative survival analysis of TEL/JAK2 bone marrow transplanted mice (Kaplan–Meier). Mice transplanted with bone marrow transduced with *hTEL/mJAK2* (*n* = 30), or *hTEL/hJAK2* (5–19) (*n* = 10) die of disease within 10 weeks after transplant. None of the mice transplanted with bone marrow transduced with the non-transforming mutants (*hTELΔPNT/mJAK2*, *n* = 10; *hTEL/mJAK2-KI*, *n* = 10) died of disease after 30 weeks post-transplant.

forming properties of TEL/JAK2 fusion proteins *in vitro* and *in vivo*. Murine hematopoietic cells with myeloid and lymphoid phenotypes (Ba/F3, Ixn/2B) are transformed to factor-independent growth by expression of a chimeric fusion gene containing exon 1–5 of human TEL fused to the murine JH1–JAK2 kinase domain. Mutants either lacking the TEL PNT domain or containing an inactivating mutation in the JAK2-kinase domain demonstrate that transformation is dependent on the TEL PNT domain as well as an active JAK2-kinase domain (Lachronique *et al.*, 1997). These data, together with the previous characterization of other transforming TEL-tyrosine kinase fusions (*TEL-PDGFR*β*, TEL-ABL*), further emphasize the importance of the TEL PNT domain as a self-association motif leading to constitutive activation of the tyrosine kinase partner (Carroll *et al.*, 1996; Golub *et al.*, 1996; Jousset *et al.*, 1997). There is increasing evidence that ligand-independent oligomerization, or activation through point mutations, is a general mechanism for oncogenic activation of receptor and nonreceptor tyrosine kinases in leukemias as well as in solid tumors. In addition to the TEL PNT domain, the BCR coiled-coil domain (BCR/ABL), the leucine-zipper (TPR-MET, TPR-TRK, HIP-PDGFRβ) and the zinc-finger motifs (FGR1-ZNF198) may serve as dimerization/oligomerization interfaces (Mc Whirther and Wang, 1991; Rodrigues and Park, 1994; Ross *et al.*, 1998; Xiao *et al.*, 1998).

Comparison of the *in vitro* transformation properties of the *TEL/JAK2* fusion variants showed no difference in induction or kinetics of factor-independent growth in Ba/F3 cells. In contrast with the TEL/JAK2 (5–19) fusion, which contains only the JAK2 JH1–kinase domain, TEL/JAK2 (5–12) and TEL/JAK2 (4–17) fusion variants contain all of, and part of the JH2 pseudo-kinase domain, respectively (Figure 1). The role of the JH2 domain, which has similarities to known tyrosine kinase domains but does not contain certain amino acid residues that are required for catalytic activity, has not been characterized
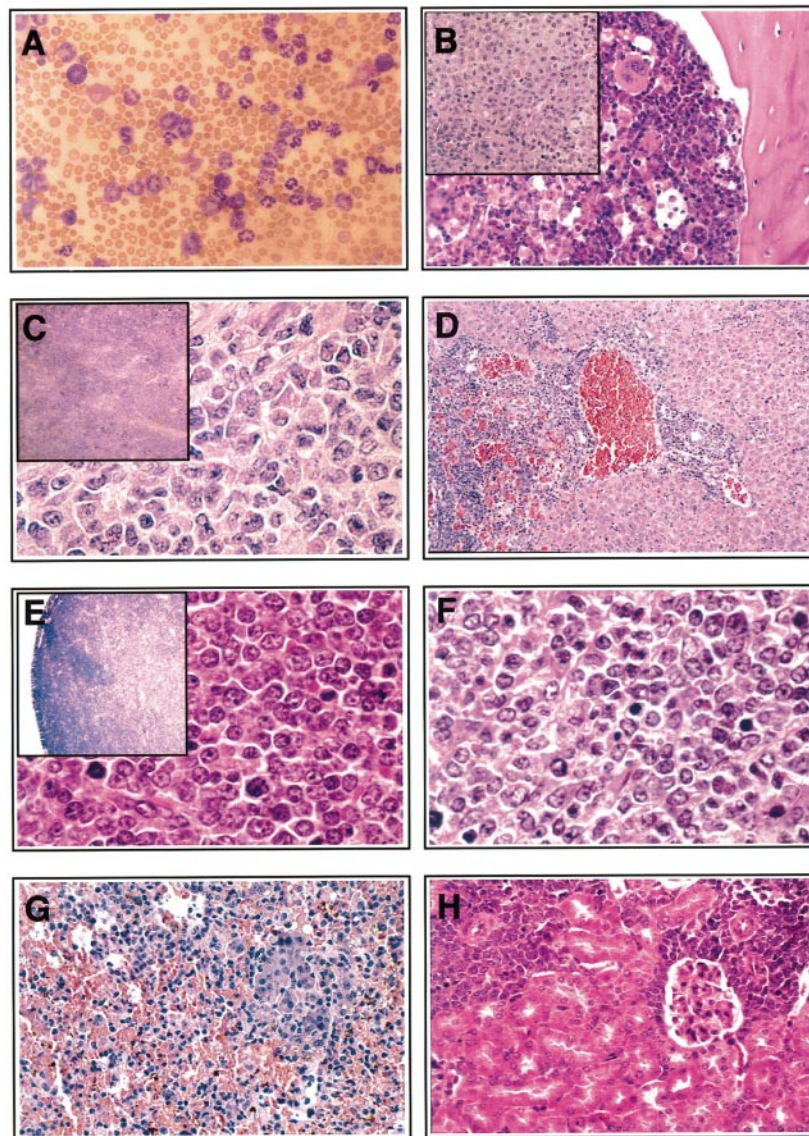
Fig. 7. Histopathological characterization of mice transplanted with hTEL/mJAK2 transduced bone marrow. [Peripheral blood smear (A): Wright-Giemsa stain; paraffin-embedded sections (B-H): hematoxylin and eosin stain]. (**A**) Peripheral blood smear from animal 3.1 showing marked leukocytosis ($10^6/mm^3$) consisting predominantly of mature and immature myeloid cells. (**B**) Bone marrow from animal 1.2. The marrow is 100% cellular (no fat) and sinuses are effaced, with most of the cellularity being comprised of maturing myeloid forms and megakaryocytes. Inset shows a sheet-like focus of cells with folded nuclear contours and abundant cytoplasm, an appearance consistent with these cells being macrophages. (**C**) Spleen from animal 1.2. Inset shows expansion of red pulp by sheets of maturing myeloid forms. (**D**) Liver from animal 1.5 showing extensive perivenular and sinusoidal extramedullary hematopoiesis. (**E–F**) Enlarged abdominal lymph node from animal 2.8 (inset). In some areas (E), the nodal architecture was diffusely effaced by a homogeneous population of intermediate-sized blasts; several mitotic figures and an apoptotic cell are present in this field. Other areas of the same node (**F**) showed a mixed population of blasts and maturing myeloid forms. (**G**) Lung from animal 1.2, showing extensive extravasation of erythrocytes and clusters of pigment-laden macrophages, consistent with acute and chronic pulmonary hemorrhage. (**H**) Kidney from animal 2.5, showing interstitial and periglomerular infiltration by lymphoid blast forms.

in detail. Luo *et al.* (1997) demonstrated hyperactivation of *Drosophila* and mammalian JAK–STAT pathway by a mutation in the Jak JH2–kinase domain. However, by analysis of deletion mutants, these authors suggest that at least in *Drosophila*, the JH2 domain may not serve solely as a negative regulatory element (Luo *et al.*, 1997). Additional evidence for functional importance of the JAK2 JH2 domain has been demonstrated by expression of CD16 (dimerization motif)-CD7-JAK2 chimeric proteins in factor-dependent Ba/F3 cells. Stably transfected clones with a CD16-CD7-JAK2 JH1 domain induced *bcl*-2 and *pim*-1 gene expression and delayed cell death whereas a fusion including the JAK2 JH2 and JH1 did not, although

both proteins were constitutively phosphorylated (Sakai and Kraft, 1997). However, there were no detectable differences in *in vitro* transformation ability of the three TEL/JAK2 variants, suggesting that the JH2 domain does not influence transformation in this context.

Ba/F3 cells transformed to factor-independent growth by TEL/JAK2 fusion variants show an increase in tyrosine phosphorylation of STAT 5. EMSA analysis shows that there is also increased STAT 5 DNA-binding activity compared with wild-type cells, and these data are similar to those reported by Lachronique *et al.* (1997). Recent work in our laboratory has further demonstrated that transformation by other TEL-tyrosine kinase fusions (TEL/
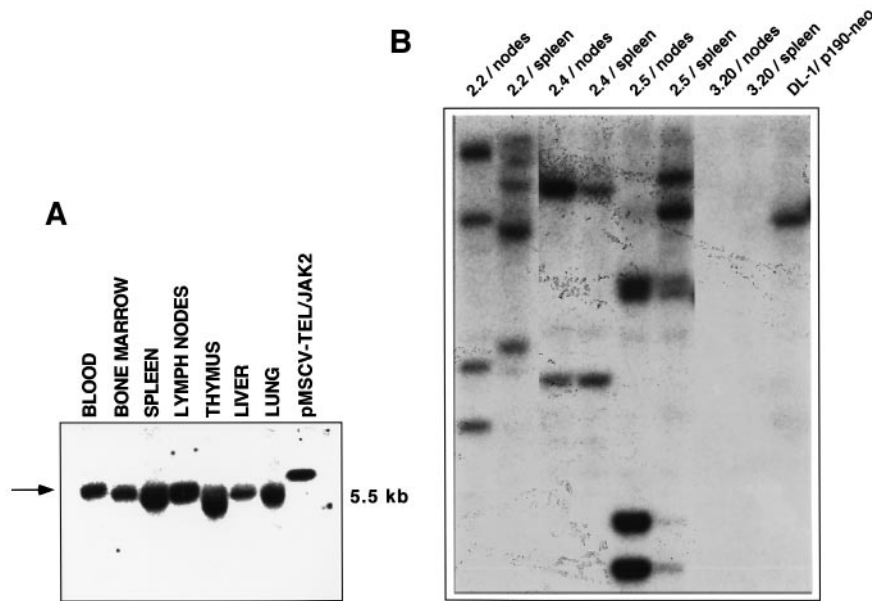
Fig. 8. Integration of *MSCV-TEL/JAK2* retrovirus into DNA of transplanted mice. (**A**) Southern blot analysis of DNA (10 μg) from various organs digested with *Kpn*I releasing the entire *LTR-MSCVneo-TEL/JAK2-LTR* provirus (5.5 kb) and probed with an 1.3 kb *Neo* probe. [A slight size difference observed in the plasmid control lane is based on rearrangements in the *3′-LTR* of the retroviral expression plasmid after cotransfection with *pIK6* packaging plasmid (Finer *et al.*, 1994)]. The blot shown in (A) derives from animal 2.5 and is representative of 10 animals analyzed. (**B**) Southern blot analysis of DNA (10 μg) digested with *Eco*RI cutting once in the *MSCV-TEL/JAK2* provirus and probed with the *Neo* probe. All DNA samples from spleens (containing myeloid progenitors) and lymph nodes (containing mostly lymphoid blasts with some myeloid progenitors in some samples) from affected animals show several bands. The pattern is almost identical (animal 2.4), partly overlapping (2.2) or almost completely different in spleen versus lymphnode DNA. DNA from a cell line containing a single copy of *BCR-ABL-neo* provirus (DL-1 Daley *et al.*, 1990) is used as a positive control. Samples from animal 3.20, containing *MSCV-TEL/JAK-EGFP* (without neo-cassette) serve as negative control.

Table I. Summary of bone marrow transplant experiments with *TEL/JAK2* and related mutants

	#tpl./#anal.	Latency (weeks)	WBC (10^6/ml)	Spleen weight (mg)	Histopathology
#1					
hTEL/mJAK2	10/7	5.5 (2–8)	72 (9–180)	590 (220–760)	MPD: 7/7 LBL: 3/7
#2					
hTELΔ PNT/mJAK2	5/1	n.s. ≥24	3	70	normal
hTEL/mJAK2-KI	5/1	n.s. ≥24	5	100	normal
#3					
hTEL/mJAK2	10/9	6 (6–8.5)	93 (8–350)	547 (250–690)	MPD: 6/7 LBL: 7/7
TEL/JAK2 (5–19)	10/10	7.5 (6–8.5)	167 (18–1000)	578 (300–850)	MPD: 6/7 LBL: 6/7
hTELΔPNT/mJAK2	5/0	n.s. ≥20	n.d.	n.d.	n.d.
hTEL/mJAK2-KI	5/0	n.s. ≥20	n.d.	n.d.	n.d.

WBC, white blood count; MPD, myeloproliferative disease; LBL, lymphoblastic lymphoma; n.s., no symptoms; n.d., not determined.

PDGFRβ, TEL/ABL) is also associated with increased STAT 5 activity (M.Carroll and D.G.Gilliland, in preparation). These findings, together with several reports that have shown activation of the JAK–STAT pathway in BCR/ABL transformed cells, suggest that specific activation of STAT 5 is a common theme in cellular transformation by tyrosine kinase fusion proteins (Carlesso *et al.*, 1996; Ilaria *et al.*, 1996; Shuai *et al.*, 1996). STAT 5 was originally characterized as a transcription factor mediating prolactin response in mammary epithelial cells. Further studies have demonstrated activation of STAT 5 by variety of hormones and cytokines (e.g. IL-2, IL-3, IL-7, GM-CSF, EPO, TPO, EGF amongst others). Moreover, it has been demonstrated that STAT 5 activity is involved in regulation of IL-3-induced proliferation of hematopoietic cells (Mui *et al.*, 1996). However, a large number of studies have shown that oncogenic tyrosine kinase fusion genes may mediate their function through activation of

several signaling pathways. The first and best characterized hematological tyrosine kinase fusion, BCR/ABL, associated with CML, activates multiple downstream effector molecules (Cortez *et al.*, 1995, 1996). In addition to activation of the JAK–STAT pathway, BCR/ABL activates RAS and MAP kinase which appears to be required but not sufficient for cellular transformation (Goga *et al.*, 1995; Raitano *et al.*, 1995). Moreover, transformation by BCR/ABL also involves PI3-kinase activation of Akt kinase as a downstream target (Skorski *et al.*, 1997). Based on these studies, it remains to be elucidated whether other pathways, in addition to STAT activation, are activated in TEL-tyrosine kinase mediated cell transformation.

Murine primary bone marrow was infected with a retroviral expression vector containing the *TEL/JAK2* (5–19) fusion gene variant and transplanted into lethally irradiated syngeneic recipients to address transformation of hematopoietic cells by *TEL/JAK2 in vivo*. This technique
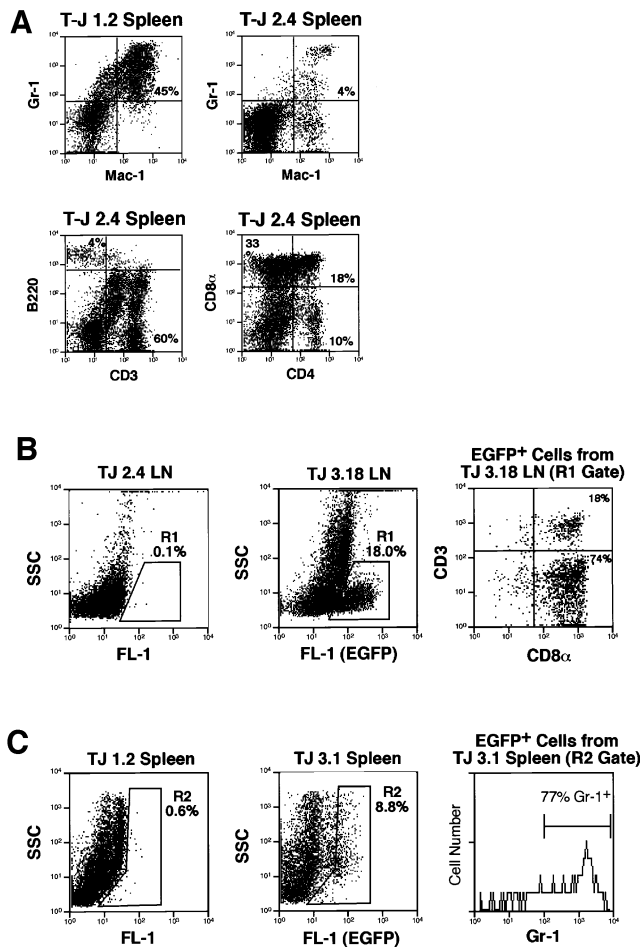
Fig. 9. Immunophenotypic analysis of TEL/JAK2 bone marrow transplant. (**A**) Spleen cell suspensions were obtained from mice 1.2 and 2.4 transplanted with bone marrow transduced with *MSCV-neo/ hTEL/mJAK2* (Table I). The cells were stained with FITC-conjugated anti-Mac-1 and biotinylated anti-Gr-1 followed by streptavidin-PE, demonstrating an expansion of Gr-1+, Mac-1+ granulocytes in 1.2 spleen and a normal percentage of granulocytes in the 2.4 spleen. Staining of 2.4 splenocytes with FITC-conjugated anti-CD3 together with PE-conjugated anti-B220 or FITC-conjugated anti-CD4 with PE-conjugated CD8α demonstrated a predominance of CD3+ T cells which included CD4+, CD8α+, and CD4+CD8α+ subsets). (**B**) Dot plots of FL-1 fluorescence versus side scatter (SSC) were analyzed for unstained lymph node cells from mouse 3.18 (*MSCV-EGFP-hTEL/ hJAK2*) and mouse 2.4 (*MSCV-neo-hTEL/mJAK2*), both of which had lymphoproliferative disease. A population of cells with increased FL-1 fluorescence representing EGFP expression was identified only in cells from mouse 3.18. The cells in this region (18% of total cells) were further analyzed by staining with PE-conjugated anti-CD8α and biotin-conjugated anti-CD3 followed by streptavidin-Tricolor (detected in FL3 channel) revealing expression of CD8α by almost all of the EGFP+ cells and CD3 by a subset of these CD8α+ cells. (**C**) A similar method was used to study spleen cells from mice with myeloproliferative disease. Cells in the spleen of mouse 3.1 (*MSCV-EGFP-hTEL/hJAK2*) displayed EGFP expression which was not seen in spleen cells from mouse 1.2 (*MSCV-neo-hTLEL/mJAK2*). Cells from mouse 3.1 that were positive for EGFP expression, 77% were positive for Gr-1 by staining with biotinylated anti-Gr-1 followed by strepatavidin-PE.

was first used to characterize the *BCR/ABL* fusion gene and has become an important approach for *in vivo* characterization of oncogenic fusion genes, as well as regulatory genes important in hematopoiesis (Elefanty *et al.*, 1990, 1992; Daley *et al.*, 1990; Hawely *et al.*, 1992). *TEL/JAK2*-transplanted animals rapidly develop a fatal oligoclonal

mixed myelo- and T-cell lymphoproliferative disease. This finding is similar to other oncogenic fusion-gene murine transplant models, as rapidly fatal oligoclonal disease has also been observed in animals transplanted with *BCR/ABL*-infected marrow cells (Gishizky *et al.*, 1993). In other studies, involvement of several lineages has also been described, but multiple phenotypically distinct tumors in the same animal have been traced back to a single infected multipotent precursor cell in most cases. (Elephanty *et al.*, 1990, 1992). In certain respects, the disease generated by the *TEL/JAK2* transplant model is similar to the phenotype observed after transplant of bone marrow infected with a retrovirus that contains genes coding for growth factors associated with the myeloid lineage; overexpression of GM-CSF, G-CSF, as well as IL-3, induce a oligoclonal myeloproliferative syndrome, with a fatal outcome in the case of IL-3 and GM-CSF (Chang *et al.*, 1989a,b; Johnson *et al.*, 1989). These similarities might be explained by the fact that activation of the JAK2 kinase has been shown to play an important role in signaling of these cytokines (Silvennoinen *et al.*, 1993; Ihle *et al.*, 1995). The early onset myelo- and lymphoproliferative syndrome in *TEL/JAK2*-transplanted mice suggests that the disorder may originate from more differentiated progenitor cells with limited replication capacity that have undergone oligoclonal expansion. However, the use of Retronectin™ in these experiments may also influence the development of oligoclonal disease. Retronectin™ is comprised of specific proteolytic fragments of fibronectin, and is a potent enhancer of retrovirally mediated gene transfer in long-term repopulating murine hematopoietic cells and in human CD34+ cells (Hanenberg *et al.*, 1996). Clonal lymphoid disease has been observed in mice transplanted with bone marrow infected with low titer of *MSCV* retrovirus encoding for *NPM-ALK*, a tyrosine kinase fusion gene associated with human lymphomas, using Retronectin™ as an enhancing agent for gene transfer (Kuefer *et al.*, 1997). Therefore, it is also possible that the use of high-titer viral supernatants together with RetronectinTM as a gene transfer enhancing agent may promote oligoclonal disease. Experiments with titration of the viral supernatants and variation of transplanted cell numbers will be required to address this question.

Development of T-cell lymphoproliferative disease in mice transplanted with TEL/JAK2 (5–19), originally cloned from a patient with T-ALL is of interest, as JAK1 and JAK3 (but not JAK2) have been shown to be critical for mediation of a proliferative signal by IL-2 in T cells (Nelson *et al.*, 1994). However, activation of JAK1 and JAK3 leads to tyrosine phosphorylation of STAT 5, as well as to a lesser extent STAT 1 and STAT 3 (Gilmour *et al.*, 1995; Johnston *et al.*, 1995). These data would suggest that activation of STAT 5 may induce T-cell expansion in *TEL/JAK2* bone marrow transplants. Additional bone marrow transplant experiments with the other TEL/JAK2 fusion variants, TEL/JAK2 (4–17) and TEL/JAK2 (5–12) (associated with B-ALL and atypical CML, respectively) will be required to determine whether the phenotype associations seen in humans can be recapitulated in the animal model.

This animal model of TEL/JAK2-induced hematopoietic malignancy offers a rapid and reliable tool to test the

requirement for specific signaling molecules in transformation by TEL/JAK2 and other TEL/tyrosine fusions. It also provides a model system to test new therapeutic approaches, such as JAK-specific tyrosine inhibitors (e.g. tyrphostin AG 490; Meydan *et al.*, 1996), naturally occurring dominant negative variants of STAT 5 (Wang *et al.*, 1996; Azam *et al.*, 1997), STAT decoy oligonucleotides (Boccaccio *et al.*, 1998), and endogenous JAK–STAT inhibitors (Endo *et al.*, 1997; Naka *et al.*, 1997; Starr *et al.*, 1997). Characterization of transformation by *TEL/JAK2* fusion genes provides further insight into the role of the JAK–STAT pathway in human leukemogenesis.

Materials and methods

Cell lines

Murine Ba/F3 cells (a gift from A.D'Andrea, Dana Farber Cancer Institute, Boston, MA) were maintained in RPMI-1640 medium with 10% fetal bovine serum (FBS) and 0.5–1 ng/ml of recombinant IL-3 (R&D Systems, Minneapolis, MN) in a 5% CO_2 incubator at 37°C. Murine IXN/2B cells (Park *et al.*, 1990; kind gift from Immunex, Seattle, WA) were kept in IL-7-containing IMDM medium. COS-7 and 293-T cells were maintained in DMEM with 10% FBS.

DNA constructs and retroviral vectors

The chimeric *hTEL/mJAK2* cDNA was constructed by PCR by linking *TEL* (nt 1–1033) to murine *JAK2-JH1* (nt 2575–3629, Silvenoinen *et al.*, 1993) using the unique *Avr*II site between mJAK2 JH2 and JH1 domains, sequenced and expressed by *in vitro* transcription/translation using a rabbit reticulocyte lysate kit (TNT system; Promega, Madison, WI). For expression in COS-7 cells, the cDNA was subcloned into *HA-TELpcDNA3* (Carroll *et al.*, 1996). The *hTELΔPNT/mJAK2* cDNA lacking the TEL PNT domain was generated by cutting within the *TEL* cDNA at *Bsp*MI and *Eco*NI sites, blunting with mung bean nuclease and religating, leading to deletion of nucleotides 195–348 of the TEL gene (Carroll *et al.*, 1996). The *hTEL*/kinase deficient *mJAK2* fusion mutant was generated by mutation of two conserved amino acids (Trp→Gly; Glu→Ala) in the type VIII kinase motif using site-directed mutagenesis (Site Directed Mutagenesis Kit, Stratagene, La Jolla, CA) as described previously (Zhuang *et al.*, 1994). Human *TEL/JAK2* (*TEL* exon 5- *JAK2* exon 19) was constructed similarly to *hTEL/mJAK2* using a unique *Stu*I site near the breakpoint into the human *JAK2* cDNA (Peeters *et al.*, 1997b; Dalal *et al.*, 1998). The human *TEL/JAK2* (*TEL* exon 4-*JAK2* exon 17) and (*TEL* exon 5 -*JAK2* exon 12) cDNAs were subcloned from *pGreenlantern* into retroviral expression vectors using standard techniques (Figure 1).

Retrovirus production and transfer into hematopoietic cell lines and primary murine bone marrow

For gene transfer into Ba/F3 cells and primary murine bone marrow, cDNA constructs were subcloned into the retroviral expression vector murine stem cell virus (*MSCV*) known to yield high virus titer capable of efficient transduction and expression of genes in hematopoietic cells (Hawely *et al.*, 1994; Lavau *et al.*, 1997). In some experiments a variant (*MSCV* version 2.2, kindly provided by W.Pear, University of Pennsylvania, PA) containing an *IRES* (internal ribosomal entry site)–EGFP expression cassette was used (Parsons *et al.*, 1997). Retroviral stocks were generated by transient cotransfection of 293-T cells with the respective *MSCV* construct together with a packaging construct (*pIK6*, Cell Genesys Inc., Foster City, CA) providing sequences necessary for retrovirus production (Finer *et al.*, 1994). Forty-eight hours post-transfection, virus containing supernatant was harvested, filter sterilized (0.45 μm) and kept at –70°C. For transformation assays as well as estimation of the viral titer, Ba/F3 cells ($0.5–1.0×10^6$) were infected with retrovirus (*MSCV-EGFP*) containing supernatant with 10 μg/ml polybrene. After 48 h, the percentage of infected cells was determined by counting EGFP positive cells (fluorescent microscope or flow cytometry); estimated viral titers of $≥5×10^5$ ml were obtained. For *in vitro* transformation assays, the cells were split, washed twice in phosphate-buffered saline (PBS) and selected in G-418 (1 mg/ml) or put into IL-3-free medium. For infection of primary murine bone marrow 6–8 week old BALB/cBvJ mice (Taconic, Germantown, NY) were primed with 150 mg/kg 5-Fluorouracil (5-FU, Sigma, St Louis, MO) administrated

intraperitoneally beginning 6 days prior to harvest. Two days before transplantation, male donor mice were sacrificed by CO_2 asphyxiation followed by cervical dislocation, the femurs and tibias removed and the bone marrow flushed with medium using sterile techniques. After counting ($3–5×10^6$ cells/mouse) the cells were incubated for 48 h in a cocktail with recombinant murine IL-3 (6 U/ml; Genzyme, Cambridge, MA), recombinant murine stem cell factor (SCF, 5 U/ml; Genzyme, Cambridge, MA), recombinant murine Interleukin-6 (IL-6, 10 000 U/ml) (Peprotech), 20% FBS, 100 U/ml penicillin/streptomycin (Gibco-BRL, Gaithersburg, MD) in RPMI 1640 medium (10 ml) with 1 ml viral supernatant in Retronectin™ (Tanaka-Shuzo, Japan)-coated Petri dishes (Hanenberg *et al.*, 1996). After 24 h viral soup was added again and the cells were incubated for additional 24 h. The cells were then harvested, washed and injected ($0.5–1×10^6$ in 500 μl HBSS) into the tail vein of previously lethally-irradiated ($2×450cGy$) female syngeneic recipient mice. The recipient mice were maintained in microisolator cages with acidified water and autoclaved chow at the animal facility of Harvard Medical School, Boston, MA.

Southern blot analysis

Genomic DNA was isolated form various tissues using a PUREGENE DNA isolation kit (Gentra Systems, Minneapolis, MN) followed by a single phenol–chloroform extraction. DNA (10 μg) was digested with restriction enzymes (10–20 U/μg DNA, Boehringer Mannheim), size-separated on a 1% TBE-agarose gel and blotted to a Nylon membrane (Hybond N+, Amersham, Arlington Heights, IL) by alkaline transfer. The membranes were then hybridized to [^{32}P]dCTP-labeled DNA probes ($1–2×10^6$ c.p.m./ml) in a aqueous hybridization buffer (5× SSC, 10% Dextran sulfate, 5× Denhardt's, 0.1% SDS) at 65°C.

Protein analysis

Parental cells and cells expressing the TEL/JAK2 fusion proteins were lysed in 1% Triton X-100 lysis buffer with protease inhibitor cocktail tablets (Complete Mini™ Boehringer Mannheim), 1 mM sodium vanadate, 10 mM sodium fluoride, 10 mM EDTA, and quantified by the Bradford colorimetric method. Lysates (1 mg input protein) were used for immunoprecipitation with 1 μl of rabbit polyclonal anti-JAK2 antibody directed against the C-terminus (kindly provided by A.Ziemiecki, University of Berne, Switzerland) or 3 μl of anti-STAT antibodies (Trans Cruz anti-STAT 1, STAT 2, STAT 3, STAT 4, STAT 5b, STAT 6; Santa Cruz, CA) at 4°C for 1–12 h. The protein was precipitated with Staph-A or protein-G Sepharose (1:3), washed 3 times with 1% Triton X-100 lysis buffer, separated by PAGE and transferred to a nylon membrane (Millipore) or nitrocellulose membrane (Corning). Immunoblotting was performed with 4G10 (UBI), rabbit anti-mJak2, rabbit anti-TEL-PNT domain, anti-phospho-STAT [PS-1 antibody which detects tyrosine phosphorylated STAT 1 (α and β isoforms) and STAT 5; kindly provided by D.Frank, Dana Farber Cancer Institute, Boston, MA] and anti-STAT antibodies at a dilution of 1:1000–1:2000 in TBS-T for 1 h (Frank *et al.*, 1997). Blots were washed four times in TBS-T, incubated with HRP-conjugated anti-mouse or anti-rabbit antibodies for 30 min and visualized with ECL reagent (Amersham, Arlington Heights, IL). EMSAs were performed as described previously (Ilaria *et al.*, 1996). In brief, nuclear extracts (6 μg) from parental and cells expressing TEL/JAK2 fusion proteins are incubated (20 min) with a ^{32}P-labeled and purified double-stranded oligonucleotide probe recognized by activated STAT protein complexes (GAS based on the FcγRI gene promoter). For supershift analysis, anti-STAT antibodies (Santa Cruz) were added. The DNA–protein complexes were resolved on 4% TAE–polyacrylamide gels and detected by autoradiography. To localize hemagglutinin (HA)-tagged hTEL/mJAK2 in COS-7 cells, 48 h after transfection the cells were fixed in 3% formaldehyde and blocked with 1% dried milk solution in PBS/0.1% Triton X-100 and probed with an anti-HA antiserum (Boehringer Mannheim) followed by an FITC-labeled secondary antibody, briefly stained with HOECHST 33258, and analyzed with an immunofluorescence microscope.

Histopathologic and immunohistochemical analysis of murine tissues

Murine tissues were fixed for 24 h in 10% neutral buffered formalin and embedded in paraffin. Femurs were subjected to an additional decalcification step in RDO (Apex Engineering Products, Plainfield, IL) for 4 h prior to processing. Sections (3 μm) were deparaffinized and stained with hematoxylin and eosin.

Flow-cytometric analysis

Single cell suspensions of bone marrow, spleen and lymph nodes were prepared. Red blood cells were lysed in ammonium chloride solution

(150 mM NH$_4$Cl, 10 mM KHCO$_3$, 0.1 mM EDTA, pH 7.4) for 5 min at room temperature. The cells were washed in PBS with 0.1% NaN$_3$ and 0.1% BSA (staining buffer). To block nonspecific Fc receptor-mediated binding, the cells were preincubated with supernatant from the 2.4G2 hybridoma line (anti-CD16/CD32; cell line obtained from American Type Culture Collection, Rockville, MD) for 20 min on ice. Aliquots of 0.5–1.0×10^6 cells were then stained for 20 min on ice with monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), or biotin. The antibodies used in these experiments included reagents specific for: B220 (CD45R), CD3, CD4, CD8α, Mac-1(CD11b) and Gr-1 (all antibodies purchased from PharMingen, San Diego, CA). The binding of biotinylated primary antibodies was detected using PE-conjugated streptavidin (Immunotech, Westbrook, ME) or Tricolor-conjugated streptavidin (Caltag, South San Francisco, CA). Cells were washed once in staining buffer and multicolor flow-cytometric analysis was done with a FACScan (Becton-Dickinson). A minimum of 10 000 events were acquired and the data was analyzed using CellQuest software (Version 3.1). The results are presented as dot plots showing FITC and PE fluorescence signals of viable cells gated on the basis of forward and side scatter signals. In mice transplanted with bone marrow transduced with an EGFP-containing retroviral construct, dot plots of FL-1 fluorescence versus side-scatter were used to identify the EGFP-positive cells. A region encompassing only the cells expressing EGFP was defined and used to gate the cells for analysis of additional markers (CD3 and CD8α for lymphoid cells and Gr-1 for granulocytes).

Acknowledgements

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