STAT1 acts as a tumor promoter for leukemia development

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Summary

The tumor suppressor STAT1 is considered a key regulator of the surveillance of developing tumors. Here, we describe an unexpected tumor-promoting role for STAT1 in leukemia. *STAT1*$^{-/-}$ mice are partially protected from leukemia development, and *STAT1*$^{-/-}$ tumor cells induce leukemia in *RAG2*$^{-/-}$ and immunocompetent mice with increased latency. The low MHC class I protein levels of *STAT1*$^{-/-}$ tumor cells enable efficient NK cell lysis and account for the enhanced tumor clearance. Strikingly, *STAT1*$^{-/-}$ tumor cells acquire increased MHC class I expression upon leukemia progression. These findings define STAT1 as a tumor promoter in leukemia development. Furthermore, we describe the upregulation of MHC class I expression as a general mechanism that allows for the escape of hematopoietic malignancies from immune surveillance.

Introduction

The JAK (*Janus* kinase)-STAT (signal transducer and activator of transcription) signaling pathway mediates a variety of important cellular functions in the hematopoietic system. Ligand binding on the cognate receptor leads to activation of receptor-associated JAKs, which subsequently tyrosine-phosphorylate and activate STAT proteins. After formation of dimers, STAT proteins translocate to the nucleus, where they induce or modulate expression of target genes (Bromberg and Darnell, 2000; Levy and Darnell, 2002).

Targeted deletion was instrumental in clarifying the physiological role of STAT1 in vivo (Durbin et al., 1996; Meraz et al., 1996). *STAT1*$^{-/-}$ mice are viable and fertile but are more susceptible to viral and bacterial infections. This phenotype was attributed to a lack of IFN-γ signaling. Originally, the development and distribution of lymphocytes seemed unaffected in *STAT1*$^{-/-}$ animals. Later reports, however, revealed defects in the maturation of T lymphocytes (Fallarino and Gajewski, 1999; Lee et al., 2000b; Refaeli et al., 2002) and documented an impaired cytolytic capacity of NK cells (Lee et al., 2000a). *STAT1*$^{-/-}$ T cells proliferated faster and showed a reduced propensity to undergo apoptosis due to diminished expression levels of caspase 1, 8, and 11. Recently, an increased susceptibility to autoimmune disease was reported in *STAT1*$^{-/-}$ mice due to an impaired function of CD4$^+$/CD25$^+$ regulatory T suppressor cells (Nishibori et al., 2004).

STAT1$^{-/-}$ mice have also been shown to be tumor prone, and STAT1 was therefore classified as a tumor suppressor (Badgwell et al., 2004; Kaplan et al., 1998; Lesinski et al., 2003; Shankaran et al., 2001). One mechanism by which STAT1 suppresses tumor formation is its key role as a transcription factor downstream of type I and type II interferons (IFNs). IFN-α is widely used for the treatment of metastatic melanoma and for certain forms of leukemia, where it can induce disease regression. IFN-γ is a key component of tumor surveillance and protects the host against spontaneously arising tumors (Dalton et al., 1993; Sexl et al., 2003; Street et al., 2002). Accordingly, spontaneously arising and metylcholanthrene (MCA)-induced tumors evolve faster in mice deficient for components of IFN-γ signaling than in wild-type controls (Kaplan et al., 1998). Similarly, spontaneous tumor development is increased in IFN-γ- and perforin-deficient mice, which develop lymphomas and/or sarcomas with a higher frequency than wild-type animals (Smyth et al., 2000; Street et al., 2002). These findings support a model where IFN-γ tightly collaborates with lymphocytes to protect the host from tumor development; as predicted from this concept, tumors arising in immunocompromised *RAG2*$^{-/-}$ mice are easily rejected when transplanted into (syngenic) immunocompetent wild-type hosts (Dunn et al., 2002; Dunn et al., 2004; Ikeda et al., 2002; Shankaran et al., 2001).

Still, the mechanism of tumor editing is only poorly understood. A very recent report implies a so far underestimated

SIGNIFICANCE

So far, STAT1 was classified as a tumor suppressor, and *STAT1*$^{-/-}$ mice were shown to be prone to develop solid tumors. The tumor-suppressing effect of STAT1 was attributed to its role as a key transcription factor downstream of IFN signaling. We now show that STAT1 can accelerate the development of hematopoietic tumors independently of IFN signaling. Moreover, we demonstrate that the upregulation of MHC class I molecules represents a general mechanism to escape tumor surveillance and that low MHC class I expression might be beneficial for leukemia patients. Our study may therefore redirect immune-therapeutic considerations. The loss of STAT1 is observed in human malignancies, and anticancer drugs like fludarabine are known modulators of STAT1 protein expression.

role of the innate immune system for tumor surveillance (Street et al., 2004). Herein, it is shown that rejection of spontaneous B cell lymphoma arising in immunocompromised mice (β2-microglobulin- or perforin-deficient) was mediated by NK and γδ T cells. More importantly, depletion of either $CD4^+$, $CD8^+$, or both populations from wild-type mice did not prevent tumor rejection.

We explored the contribution of the innate immune system to tumor evolution by investigating two types of hematological cancers: B-lymphoid malignancies induced by the v-abl-oncogene and myeloproliferative disorders (MPDs) caused by the TEL/JAK2 oncogene. Both murine models are relevant to clinical disease in people because the oncogenes that drive tumor development have a human counterpart. The oncogenic properties of *v-abl* are based on a fusion of the retroviral *gag* gene with a C-terminal portion of the *c-abl* gene. The resulting fusion product codes for a constitutively active tyrosine kinase located in the cytoplasm (Rosenberg and Witte, 1988; Witte, 1986). Transformation with a replication-deficient retrovirus encoding v-abl transforms fibroblasts and B-lymphoid precursors in vitro and leads to development of primary B cell leukemia and lymphoma in vivo (Abelson and Rabstein, 1970; Palumbo et al., 1990). The inflicted disease develops slowly and allows the immune system to take corrective actions.

MPDs are clonal hematopoietic stem cell malignancies (Spivak, 2004). The TEL/JAK2 oncogene was initially isolated from T cell childhood leukemia and encodes an ETS transcription factor family member fused to the catalytic domain of JAK2 (Lacronique et al., 1997; Peeters et al., 1997). The fusion protein is a constitutively active tyrosine kinase. Transformed bone marrow cells that express TEL-JAK2 induce MPD and lymphoblastic lymphoma (LBL) after transplantation into lethally irradiated wild-type mice (Carron et al., 2000; Schwaller et al., 1998). Very recently, a single-nucleotide mutation in the pseudokinase domain of JAK2 (V617F) was found in ~75%–90% of polycythemia vera (PV) patients and in other MPDs (James et al., 2005; Kralovics et al., 2005; Levine et al., 2005). Again, this mutation leads to the constitutive activation of JAK2. These observations highlight the similarity between the murine model and the manifestation of the disease in people.

Here, we demonstrate, to our knowledge, a previously undescribed role of STAT1 for the development of hematopoietic malignancies. STAT1 promotes leukemia development by maintaining high MHC class I expression. Leukemic cells that express low MHC class I levels need to upregulate MHC class I levels to allow for development of leukemia in vivo. Our findings were recapitulated in two independent experimental systems, i.e., V-abl-induced and TEL-JAK2-induced tumors that arise from distinct progenitor cells. STAT1-mediated regulation of MHC class I is of general relevance to understand the mechanisms that underlie the evolution of tumor cells under the pressure of the immune system.

Results

STAT1$^{-/-}$ B cell precursors are susceptible to transformation with v-abl

The transforming activity of *v-abl* includes the ability to abrogate growth factor requirements of B cell progenitors (Palumbo et al., 1990). Lymphocytes from *STAT1*$^{-/-}$ mice have previously been shown to undergo increased proliferation and have prolonged survival in vitro (Lee et al., 2000b). We therefore assessed the consequences of STAT1 deficiency on growth factor-independent proliferation of fetal liver cells after oncogenic transformation. *STAT1*$^{-/-}$ fetal liver cells were transformed with *v-abl* more efficiently and gave rise to increased numbers of growth factor-independent $CD43^+/CD19^+/B220^+$ (pro-B) cell colonies (Figures S1A–S1C in the Supplemental Data available with this article online). The size of colonies in methylcellulose did not vary between *STAT1*$^{+/-}$ and *STAT1*$^{-/-}$ cells, indicating that transformed *STAT1*$^{-/-}$ cells did not have any proliferative advantage (Figure S1D). Because STAT1 has recently been implicated in apoptosis (Agrawal et al., 2002; Lee et al., 2000b), we analyzed cell death in response to serum starvation and UV irradiation. Three independently derived cell lines from *STAT1*$^{+/-}$ and *STAT1*$^{-/-}$ fetal liver cell suspensions were analyzed and displayed no significant differences ($p = 0.26$ in Figure S2A and $p = 0.6$ in Figure S2B). In some cell types, STAT1 was also shown to inhibit cell proliferation by increasing the levels of cyclin-dependent kinase inhibitors p21 and p27 (Lee et al., 2000b; Yu and Jove, 2004). As already indicated by the equal size of the transformed colonies, *v-abl*-transformed *STAT1*$^{+/-}$ and *STAT1*$^{-/-}$ pro-B cells did not vary in their proliferative capacity ($p = 0.49$; Figure S2C). Immunoblots for cell cycle components confirmed equal protein expression of cyclin D2, cyclin E, cyclin A, and the cell cycle inhibitors p53, p16, p19, p21, and p27 in *STAT1*$^{+/-}$ and *STAT1*$^{-/-}$ cell lines (Figures S2D and S2E). Taken together, our data lead us to conclude that STAT1 counteracts the initial transformation process but has no appreciable impact on cell proliferation or survival of transformed pro-B cells.

Lack of STAT1 decreases leukemia formation in immunodeficient mice

To address the role of STAT1 for the formation of B-lymphoid leukemia in vivo, six independently derived *v-abl*-transformed *STAT1*$^{+/-}$ and six *STAT1*$^{-/-}$ cell lines were injected intravenously into *RAG2*$^{-/-}$ mice. Three different concentrations of each transformed cell line were injected (10^6, 10^5, and 5×10^4 cells/mouse; Figures 1A–1C). All *RAG2*$^{-/-}$ mice that had received *STAT1*$^{+/-}$ leukemic cells succumbed to the disease and displayed the classical signs of leukemia: high numbers of B-lymphoid cells were detected in the peripheral blood, and leukemic cells infiltrated liver, spleen, and bone marrow (data not shown). The latency of disease correlated with the number of cells injected. Within 22 days, 10^6 cells induced leukemia; 10^5 cells induced leukemia within 34 days, and 5×10^4 cells induced leukemia within 46 days.

The correlation between injected cell number and disease latency was more evident when transformed *STAT1*$^{-/-}$ pro-B cells were injected. Whereas 10^6 *STAT1*$^{-/-}$ cells induced leukemia with the same latency as *STAT1*$^{+/-}$ cells ($p = 0.76$), differences emerged upon injection of 10^5 cells (Figure 1B). Most interestingly, some *RAG2*$^{-/-}$ mice even survived the challenge with *STAT1*$^{-/-}$ leukemic cells and remained disease-free for more than 12 months (Figures 1B and 1C). Injection of 10^5 *STAT1*$^{-/-}$ cells resulted in disease onset in 80% of the injected *RAG2*$^{-/-}$ mice with a disease latency of 47 days ($p = 0.04$). This difference was even more pronounced when cell numbers were lowered to 5×10^4 cells (Figure 1C). In this case, 40% of the injected *RAG2*$^{-/-}$ mice remained healthy for more than 10 months ($p = 0.03$). Mice that had developed leukemia upon injection of *STAT1*$^{-/-}$ cells showed a profound decrease in infiltration of

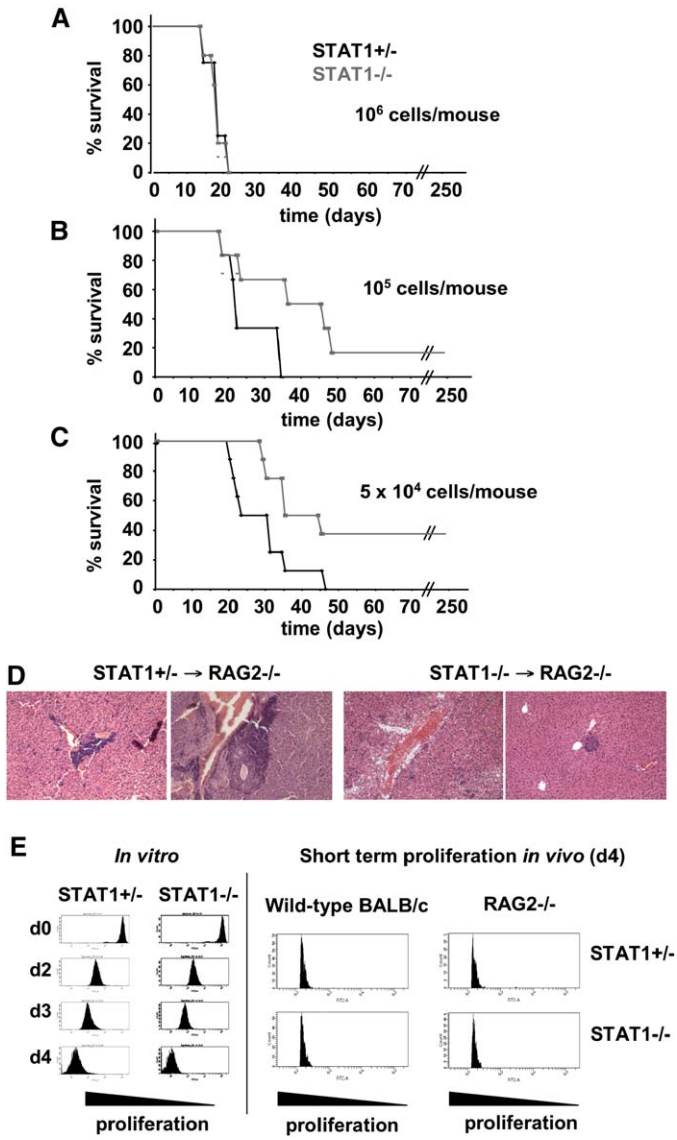

Figure 1. Lack of STAT1 decreases leukemia formation in *RAG2*$^{-/-}$ mice

A–C: In total, six individual cell lines (*STAT1*$^{+/-}$ and *STAT1*$^{-/-}$) were injected at different doses into *RAG2*$^{-/-}$ mice. Leukemia development was observed for more than 250 days. Three different cell numbers (10^6 cells [**A**], 10^5 cells [**B**], and 5×10^4 cells [**C**]) of each genotype were injected into *RAG2*$^{-/-}$ mice (n = 6 for each genotype/experiment in **A** and **B**; n = 8 for each genotype in **C**).

D: Hematoxylin/eosin-stained histological sections of infiltrated livers from *RAG2*$^{-/-}$ mice, which had received *STAT1*$^{+/-}$ (left panel) or *STAT1*$^{-/-}$ cell lines (right panel; magnification: ×100). Two representative examples are depicted for each genotype.

E: Short-term proliferation of CFSE-labeled *STAT1*$^{+/-}$ or *STAT1*$^{-/-}$ leukemic cells was assessed in vitro (left panel), and wild-type BALB/c and *RAG2*$^{-/-}$ mice were assessed 4 days after injection (right panel).

spleen and liver compared to mice that had received *STAT1*$^{+/-}$ cells (Figure 1D and data not shown). The bone marrow represents the initial site of disease development. To test whether *STAT1*$^{-/-}$ cells have difficulties in homing to the bone marrow, we labeled leukemic cells with the fluorescent dye CFSE and injected these into *RAG2*$^{-/-}$ as well as into wild-type recipient mice. The fluorescence intensity decreases with each cell division, since CFSE is equally distributed between the daughter cells. Four days after the injection of tumor cells, the bone marrow was analyzed for the presence of CFSE$^+$ cells. As depicted in Figure 1E, *STAT1*$^{+/-}$ and *STAT1*$^{-/-}$ cells were present at comparable numbers in *RAG2*$^{-/-}$ (0.099% ± 0.005% and 0.111% ± 0.006%; p = 0.8) and in wild-type mice (0.111% ± 0.019% and 0.104% ± 0.004%; p = 0.75). Importantly, the fluorescence intensity that was recorded ex vivo did not differ in *STAT1*$^{+/-}$ and *STAT1*$^{-/-}$ cells. This observation confirmed again that the proliferative capacity of *STAT1*$^{-/-}$ cells was not affected.

STAT1$^{-/-}$ tumor cells express low levels of MHC class I

Tumor development is a permanent battle of the evolving tumor and the immune system. It is evident that the injection of low numbers of tumor cells allows the immune system to take corrective action, whereas high cell numbers are likely to overrun the immunological defense mechanisms. Therefore, we reasoned that tumor surveillance accounted for the increase in survival of *RAG2*$^{-/-}$ mice. *RAG2*$^{-/-}$ mice lack functional T/B, NKT, and γδT cells and rely on NK cells for tumor surveillance (Shinkai et al., 1992). The presence of MHC class I molecules is one of the central mechanisms that allow NK cells to recognize their targets. Cells bearing low levels of MHC class I are readily recognized as "non-self" and eliminated by NK-mediated cytotoxicity (Cerwenka and Lanier, 2001; Colucci et al., 2003; Karre et al., 1986; Lee et al., 1999). Hence, we tested the expression levels of MHC class I (H-2D^d) on the surface of six individual *STAT1*$^{+/-}$ and six individual *STAT1*$^{-/-}$ cell lines. These in vitro-transformed *STAT1*$^{-/-}$ pro-B cells consistently showed drastically reduced MHC class I surface expression compared to in vitro-transformed *STAT1*$^{+/-}$ cells (Figure 2A). In contrast to transformed *STAT1*$^{-/-}$ cells, *v-abl*-transformed *IFN-γ*$^{-/-}$ pro-B cells did not show any alterations in MHC class I surface expression, indicating that the decreased expression is a cell-autonomous feature of transformed *STAT1*$^{-/-}$ B-lymphoid cells and independent of an autocrine IFN loop. In addition, we incubated freshly prepared fetal liver cells with high concentrations of v-abl retrovirus. Infection with the retrovirus did not enhance the MHC class I expression (Figure 2B).

Thus, the findings summarized are consistent with the interpretation that neither the retroviral infection nor an autocrine IFN-γ loop does per se elevate MHC class I expression and that changes in MHC class I expression reflected cell autonomous properties of the tumor cells. The regulation of MHC class I by STAT1 has also been documented by others (Kamiya et al., 2004; Lee et al., 1999; Lieberman et al., 2004). However, the experiments did not prove that the NK cell compartment was required to account for the differences in outcome observed in animals challenged with STAT1-expressing and STAT1-deficient cells (Figures 1A–1C). To address this issue, we injected *STAT1*$^{+/-}$ and *STAT1*$^{-/-}$ tumor cells at low numbers (5×10^4 cells each) into *RAG2*$^{-/-}\gamma c^{-/-}$ mice, which lack all effector cells of the immune system, including NK cells (Goldman et al., 1998). We did not observe any differences in latency, survival, or disease morphology in livers, spleens, lymph nodes, or bone marrow from mice bearing either *STAT1*$^{+/-}$ or *STAT1*$^{-/-}$ leukemia (Figure 2C; p = not significant and data not shown).

Raising MHC class I levels restores the leukemogenicity of *STAT1*$^{-/-}$ cells

We reasoned that the decreased incidence and increased latency of leukemia induced by *STAT1*$^{-/-}$ cells is a direct

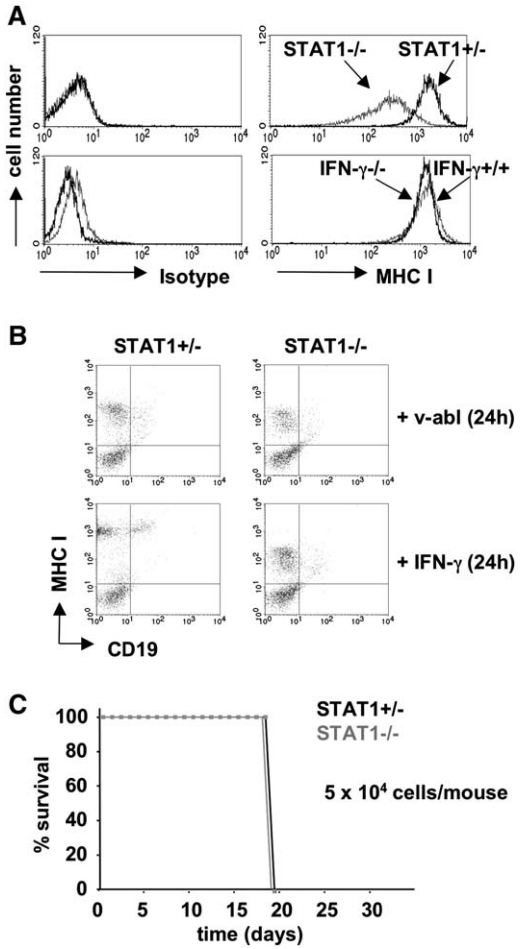

Figure 2. v-abl-transformed *STAT1*$^{-/-}$ cell lines express low levels of MHC class I

A: v-abl-transformed *STAT1*$^{+/-}$ and *STAT1*$^{-/-}$ as well as *IFN-γ*$^{+/+}$ and *IFN-γ*$^{-/-}$ cell lines were stained with isotype control (left panels) and α-H-2D^{d} (right panels).

B: *STAT1*$^{+/-}$ and *STAT1*$^{-/-}$ fetal liver cells were prepared and stained with antibodies against CD19 and MHC class I (H-2D^{d}). Prior to staining, the cells were infected with v-abl retrovirus (upper panel) or stimulated with IFN-γ (10 ng/ml, lower panel) for 24 hr.

C: Kaplan-Meier plot representing the survival of *RAG2*$^{-/-}$γc$^{-/-}$ mice (n = 5 each) injected with either *STAT1*$^{+/-}$ or *STAT1*$^{-/-}$ cells (v-abl-transformed, 5 × 10^{4} cells/mouse).

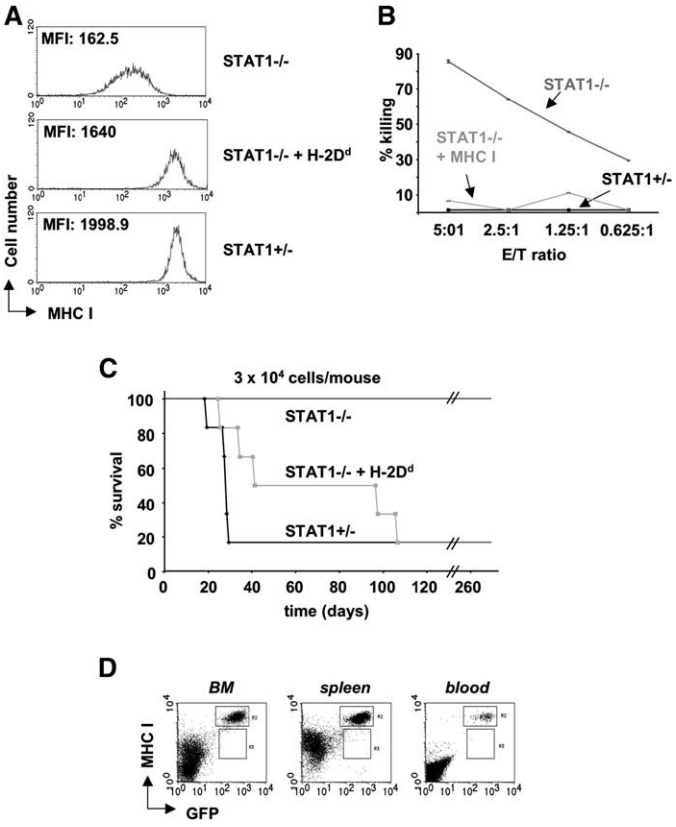

Figure 3. Overexpression of MHC class I in *STAT1*$^{-/-}$ cell lines increases the incidence and shortens the latency of leukemia

A: pMSCV-H-2D^{d}-IRESeGFP was transduced into a *STAT1*$^{-/-}$ cell line, and its expression was compared to the parental *STAT1*$^{-/-}$ and a *STAT1*$^{+/-}$ cell line infected with the empty retroviral construct by flow cytometry. The mean fluorescence intensity (MFI) of MHC class I is indicated in the panel. In parallel, cytotoxicity assays (**B**) were performed using wild-type NK cells as effectors. Data represent mean ± SD. Subsequently, cell lines were injected into *RAG2*$^{-/-}$ mice (n = 6 for each group), and leukemia development was observed over a time period of 300 days (**C**). Flow cytometry analysis of mice that had developed leukemia showed an infiltration of bone marrow (BM), spleen, and blood with tumor cells that were GFP^{+} and expressed high levels of MHC class I (**D**).

consequence of low expression of MHC class I, which allows for more efficient NK cell recognition. To test this, we overexpressed MHC class I (H-2D^{d}-IRES-GFP) in *STAT1*$^{-/-}$ leukemic cells (Figure 3A). As expected, *STAT1*$^{-/-}$ cells overexpressing H-2D^{d} could not be lysed efficiently by NK cells in an in vitro cytotoxicity assay (Figure 3B; $p < 0.001$ for *STAT1*$^{-/-}$ versus *STAT1*$^{+/-}$ and *STAT1*$^{-/-}$ versus *STAT1*$^{-/-}$ + MHC I; p = not significant for *STAT1*$^{+/-}$ versus *STAT1*$^{-/-}$ + MHC I). We selected one independently derived *STAT1*$^{-/-}$ cell line that expressed levels of H-2D^{d} comparable to *STAT1*$^{+/-}$ control cells. Forced expression of MHC class I did not alter cell proliferation or the response to proapoptotic stimuli (data not shown). Differences in survival are most readily detected if *RAG2*$^{-/-}$ mice are challenged with a low burden of tumor cells (Figure 1C). We therefore lowered the number of injected cells to 3 × 10^{4} cells/mouse. This burden of the parental *STAT1*$^{-/-}$ cell line was unable to inflict leukemia in *RAG2*$^{-/-}$ mice. In contrast, the cell line stably expressing MHC class I induced disease in 5/6 *RAG2*$^{-/-}$ mice, with a mean survival of 61.6 days. Transformed *STAT1*$^{+/-}$ cells were included as controls and also induced disease in 5/6 mice with a mean survival of 27.1 days (Figure 3C; p = 0.001). A second independently performed experiment using the same cell lines was repeated in *STAT1*$^{-/-}$ mice and confirmed this observation (Figure S3; p = 0.001). Analysis of the diseased animals revealed that the bone marrow, spleen, and blood were densely infiltrated with leukemic cells expressing high levels of H-2D^{d}, and these B cells were also positive for GFP (Figure 3D). These experiments define MHC class I expression on leukemic cells as a key determinant for the NK-mediated tumor surveillance.

Unrejected *STAT1*$^{-/-}$ leukemic cells have an "edited" phenotype

Tumor cells can evade the selective pressure of the adaptive immune system by downregulating MHC class I expression on their cell surface (Garcia-Lora et al., 2003; Karre et al., 1986;

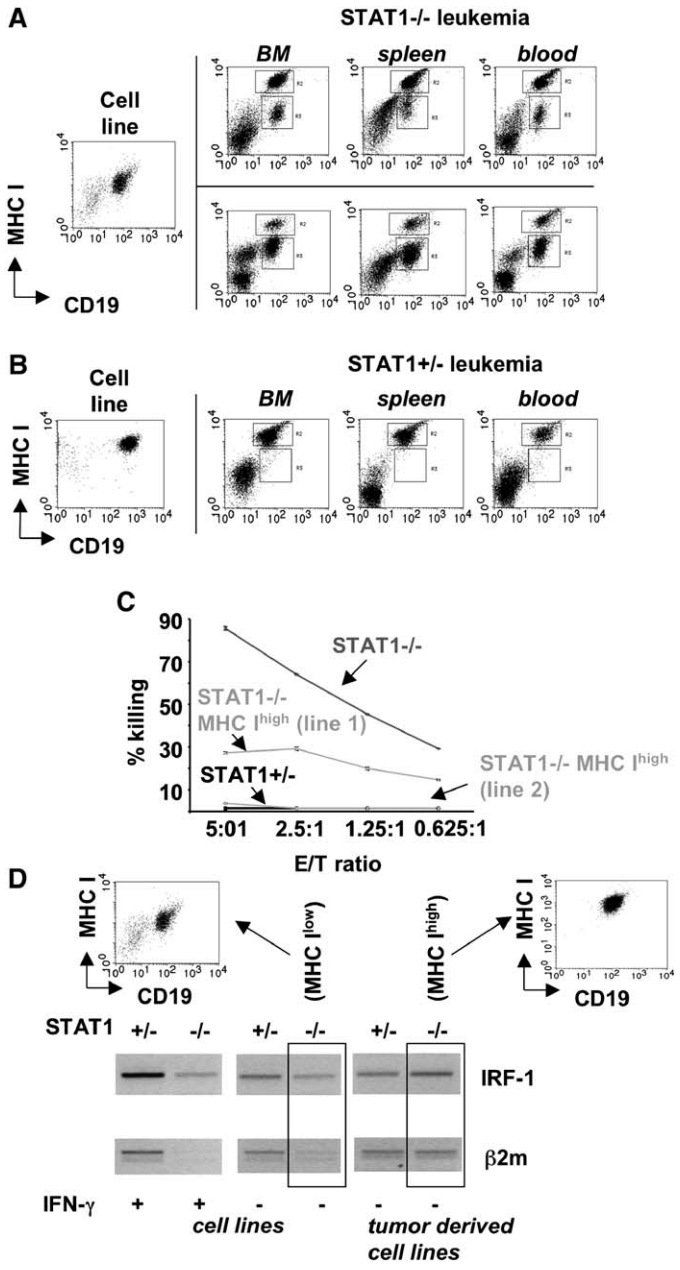

Figure 4. MHC class I upregulation in *STAT1*$^{-/-}$ tumor cells occurs as a consequence of immunoediting

A and B: V-abl-transformed *STAT1*$^{-/-}$ (**A**) and *STAT1*$^{+/-}$ (**B**) cell lines (5×10^4 cells/mouse) were injected into six *RAG2*$^{-/-}$ mice each. Tumor development was observed for 6 months. Expression of CD19 and MHC class I was assessed in the cell lines by flow cytometry prior to injection (left panels), as well as in bone marrow (BM), spleen, and blood of mice that had developed leukemia (right panels). Two representative examples for *STAT1*$^{-/-}$-induced leukemia (**A**) and one representative example for *STAT1*$^{+/-}$-induced leukemia (**B**) are depicted.

C: Cytotoxicity assays were performed using wild-type NK cells and two ex vivo derived *STAT1*$^{-/-}$ cell lines with high MHC class I expression, and *STAT1*$^{+/-}$ and *STAT1*$^{-/-}$ cell lines as control. Data represent mean ± SD.

D: Analysis of transcriptional expression of IRF-1 and β2-microglobulin (β2m) genes by semiquantitative RT-PCR. One representative *STAT1*$^{+/-}$ and *STAT1*$^{-/-}$ cell line and their corresponding tumor-derived cell lines were analyzed for the expression of IRF-1 and β2m mRNA (middle and right panel). As a control for expression of IRF-1 and β2m, cells were stimulated with 10 ng/ml IFN-γ for 2 hr (left panel). MHC class I^{low} corresponds to the in vitro derived *STAT1*$^{-/-}$ parental cell line, whereas MHC class I^{high} corresponds to the tumor-derived *STAT1*$^{-/-}$ cell line.

Mocikat et al., 2003; Smyth et al., 2002). We speculated that the opposite might occur in tumors that are predominantly eradicated by NK cells: tumors may escape the immune system by upregulating MHC class I molecules on their cell surface. This conjecture would also explain why, in *RAG2*$^{-/-}$ mice, *STAT1*$^{-/-}$ cells are not entirely cleared by the immune system and eventually succeed in inducing leukemia.

This hypothesis was verified by using the following approach: four individual *STAT1*$^{-/-}$ and three individual *STAT1*$^{+/-}$ cell lines (10^5 cells each) were injected into *RAG2*$^{-/-}$ mice (n = 12 each). The MHC class I expression of these cells was determined prior to injection and compared to the MHC class I levels on leukemic cells arising in bone marrow, spleen, and peripheral blood from diseased *RAG2*$^{-/-}$ mice.

As depicted in Figure 4A, in all mice (n = 12) that had received *STAT1*$^{-/-}$ transformed cells, a cell population emerged that was CD19$^+$ and that expressed increased levels of MHC class I. This cell population was detected in peripheral blood, bone marrow, and spleen of the diseased *RAG2*$^{-/-}$ mice. In vitro cytotoxicity assays confirmed that the cells expressing high levels of MHC class I were either not killed or killed in fewer numbers by purified NK cells when compared to the parental cell line (Figure 4C, $p < 0.001$ for *STAT1*$^{-/-}$ versus *STAT1*$^{+/-}$, *STAT1*$^{-/-}$ versus *STAT1*$^{-/-}$ MHC I^{high} line 1, and *STAT1*$^{-/-}$ versus *STAT1*$^{-/-}$ MHC I^{high} line 2; $p = 0.01$ for *STAT1*$^{+/-}$ versus *STAT1*$^{-/-}$ MHC I^{high} line 1; p = not significant for *STAT1*$^{+/-}$ versus *STAT1*$^{-/-}$ MHC I^{high} line 2). In contrast, none of the transplanted *RAG2*$^{-/-}$ mice (n = 12) that had received *STAT1*$^{+/-}$ tumor cells displayed this phenomenon (Figure 4B). Most importantly, if the tumor-derived *STAT1*$^{-/-}$ cells were propagated in vitro, they retained the expression of high levels of MHC class I even after long cultivation periods (>2 months; Figure 4D). This allowed us to explore the underlying molecular mechanisms, which resulted in an increased MHC class I expression. We concentrated on the proposed regulatory factors implicated in the regulation of gene expression of the MHC class I protein complex and on the processing mechanism leading to their surface expression (Gobin et al., 2003; Hobart et al., 1997; Lee et al., 1999). When compared to their parental cell lines, MHC class I^{high} cells consistently expressed higher levels of the transcription factor IRF-1 and β_2-microglobulin (β2m) (Figure 4D). In contrast, we did not observe any alterations in the levels of LMP-2, LMP-7, TAP-1, CIITA, or SOCS-1 mRNAs (data not shown).

Taken together, these experiments prove that selective pressure of the innate immune system enables the upregulation of MHC class I expression by increasing the IRF-1 and β_2-microglobulin mRNA expression. The importance of this phenomenon is underlined by the fact that it occurred in all (12/12) *RAG2*$^{-/-}$ mice investigated.

STAT1$^{-/-}$ mice are partially protected from leukemia formation

Previous experiments revealed a reduced cytotoxic capacity of *STAT1*$^{-/-}$ NK cells (Lee et al., 2000a). It was therefore questionable whether the impaired cytotoxic ability of *STAT1*$^{-/-}$ NK cells

sufficed to kill leukemic cells and whether that sufficiently eradicated tumor cells arising in *STAT1*$^{-/-}$ mice (which bear *STAT1*$^{-/-}$ NK cells). These questions were addressed by the following experiments: we first confirmed that *STAT1*$^{-/-}$ cells have an impaired cytotoxic capacity by using purified and in vitro amplified NK cells as effectors and YAC-1 cells as target cells (data not shown). YAC-1 cells represent optimal target cells because of their low MHC class I levels. However, they may behave differently from transformed B-lymphoid cells. Therefore, we used v-abl-transformed cell lines as targets. *STAT1*$^{+/-}$ and *STAT1*$^{-/-}$ NK cells were tested for their ability to lyse *STAT1*$^{+/-}$ or *STAT1*$^{-/-}$ tumor cells in any possible combination. *STAT1*$^{+/-}$ tumor cells were not killed by NK cells, irrespective of their genotype (Figure 5A; $p < 0.001$ for *STAT1*$^{+/-}$ NK cells killing *STAT1*$^{-/-}$ versus *STAT1*$^{+/-}$ NK cells killing *STAT1*$^{+/-}$ targets and for *STAT1*$^{-/-}$ NK cells killing *STAT1*$^{-/-}$ versus *STAT1*$^{-/-}$ NK cells killing *STAT1*$^{+/-}$ targets; p = not significant for *STAT1*$^{+/-}$ targets versus *STAT1*$^{+/-}$ or *STAT1*$^{-/-}$ NK cells). In contrast, *STAT1*$^{-/-}$ tumor cells were efficiently recognized and lysed by NK cells of both genotypes. *STAT1*$^{+/-}$ NK cells efficiently eradicated *STAT1*$^{-/-}$ leukemic cells, but they were unable to eradicate leukemic cells of their own genotype. However, in spite of their reduced cytotoxic capacity, *STAT1*$^{-/-}$ NK cells were still able to recognize and eliminate *STAT1*$^{-/-}$ tumor cell targets (Figure 5A).

Infection of newborn BALB/c mice with a retrovirus encoding for v-abl results in onset of B-lymphoid leukemia/lymphoma within weeks. The inflicted disease is mono- or oligoclonal and evolves slowly, allowing the immune system to take corrective action. To ask whether the absence of STAT1 in the tumor or the absence of STAT1 in NK cells contributed to leukemia development in vivo, we performed infections in wild-type and *STAT1*$^{-/-}$ mice. To ensure appropriate littermate controls, *STAT1*$^{+/-}$ and *STAT1*$^{-/-}$ mice were intercrossed and the offspring injected with retrovirus within 24 hr after birth. All 11 *STAT1*$^{+/-}$ mice (100%) died from leukemia within 5 weeks, whereas 6/28 (21%) of the injected *STAT1*$^{-/-}$ mice remained healthy and disease-free for more than 8 months despite the oncogenic challenge (Figure 5B; $p < 0.001$). Twenty-two out of twenty-eight *STAT1*$^{-/-}$ animals developed leukemia, and in these animals the disease latency was increased and the signs of disease were mitigated (decreased white blood cell counts, reduced tumor cell infiltrations in liver, spleen, and bone marrow) compared to those in *STAT1*$^{+/-}$ littermate controls (Figure 5B).

Our model predicts that the leukemia that eventually arises in *STAT1*$^{-/-}$ mice is accompanied by an upregulation of MHC class I during the development of disease. As expected, MHC class I surface expression on leukemic cells infiltrating bone marrow, spleen, and forming tumors was highly increased compared to nonleukemic (CD19-negative) cells of the same tissue (Figure 5D).

STAT1$^{-/-}$ animals have been repeatedly used to study the effects of IFN-γ in tumor surveillance (Kaplan et al., 1998; Shankaran et al., 2001). However, transformed *IFN-*$\gamma^{-/-}$ and *STAT1*$^{-/-}$ B-lymphoid cells differ substantially in MHC class I expression. Thus, infection of newborn *IFN-*$\gamma^{-/-}$ mice with v-abl retrovirus is predicted to result in an opposite phenotype, that is, aggravated disease. As depicted in Figure 5E, 40% of the wild-type control C57BL/6 mice remained disease-free (this is the expected result for this genetic background), whereas

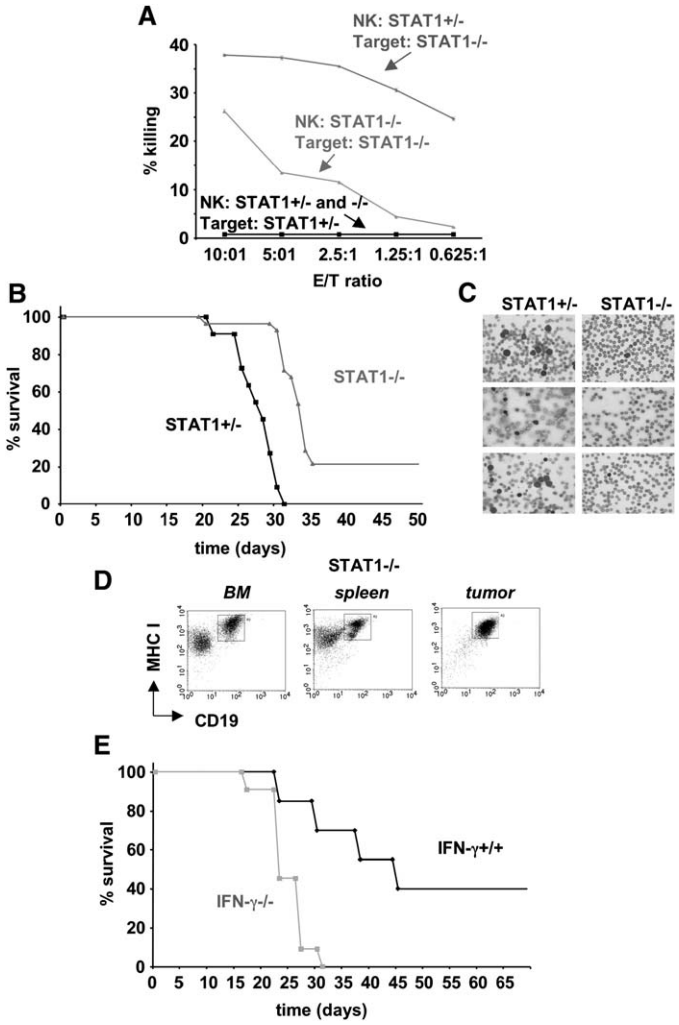

Figure 5. Decreased leukemia formation in *STAT1*$^{-/-}$ mice after V-abl challenge

A: Cytotoxicity assays were performed with purified NK cells from *STAT1*$^{+/-}$ and *STAT1*$^{-/-}$ mice as effector cells and *STAT1*$^{+/-}$ and *STAT1*$^{-/-}$ v-abl-transformed cell lines as target population. Data represent mean ± SD.

B: Kaplan-Meier plot indicating survival of the littermate progeny from a *STAT1*$^{+/-}$ × *STAT1*$^{-/-}$ intercross infected with v-abl retrovirus. Survivors were followed up over a period of 8 months for leukemia development (n = 11 for *STAT1*$^{+/-}$ and n = 28 for *STAT1*$^{-/-}$).

C: Representative hematoxylin/eosin-stained blood smears from three mice of each group are shown (magnification: ×100).

D: Expression of CD19 and MHC class I was assessed by flow cytometry in bone marrow (BM), spleen, and a tumor from one representative *STAT1*$^{-/-}$ mouse, which rapidly developed leukemia. An upregulation is observed for MHC class I in the leukemic cells compared to nonleukemic cells.

E: Kaplan-Meier plot of *IFN-*$\gamma^{+/+}$ and *IFN-*$\gamma^{-/-}$ mice after *v-abl* retrovirus challenge (n = 13 for *IFN-*$\gamma^{+/+}$ and n = 11 for *IFN-*$\gamma^{-/-}$).

100% of the *IFN-*$\gamma^{-/-}$ mice succumbed to a rapidly progressing leukemia within 4 weeks ($p < 0.0001$). We want to stress that the disease latency of *STAT1*$^{-/-}$ and *IFN-*$\gamma^{-/-}$ animals cannot be directly compared because the animals differ in their genetic background (see Experimental Procedures). These experiments confirm the important role of IFN-γ for tumor surveillance. More importantly, we stress that STAT1 and IFN-γ deficiency cannot be superimposed in the case of tumors predominantly eradicated by NK cells.

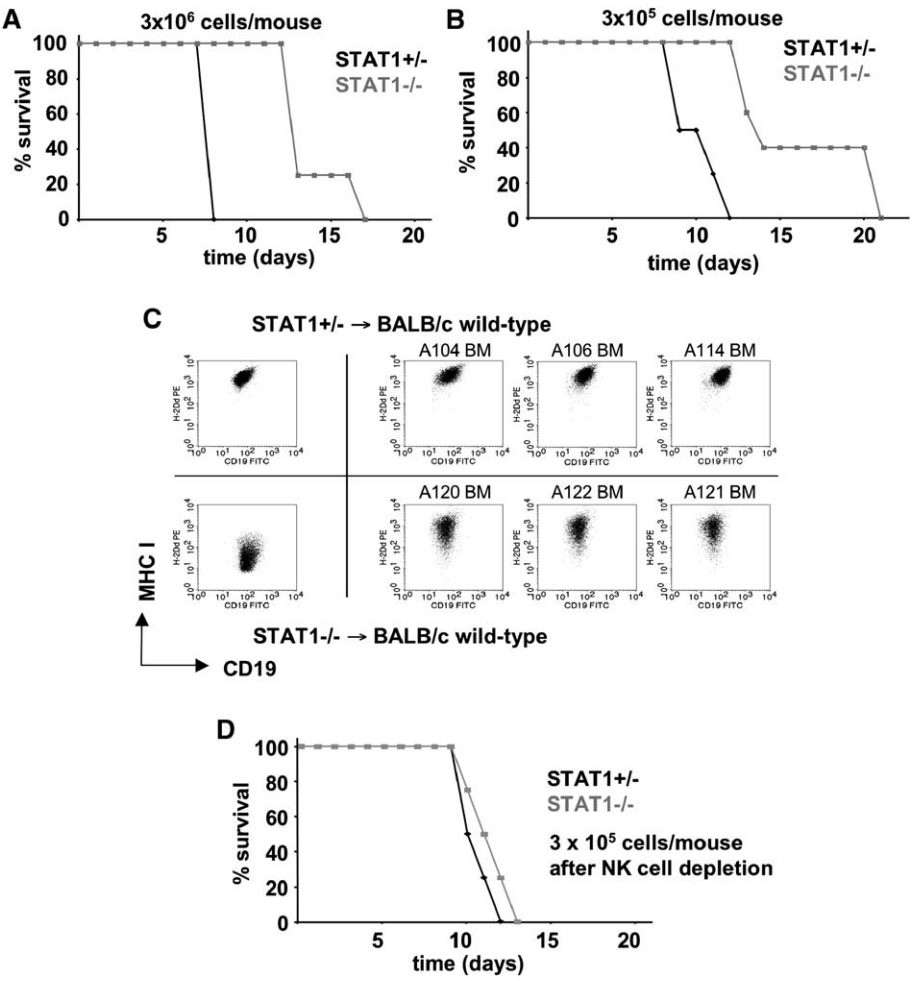

Figure 6. Low tumorigenicity of *STAT1*$^{-/-}$ leukemic cells in immunocompetent mice

A and B: Kaplan-Meier plots indicating survival of immunocompetent BALB/c mice bearing either *STAT1*$^{+/-}$ or *STAT1*$^{-/-}$ leukemia. Two different cell numbers (3×10^6 [**A**] cells and 3×10^5 [**B**] cells of each genotype) were injected into wild-type BALB/c mice (n = 10 per genotype and cell amount used).

C: Flow-cytometric analysis of CD19 and MHC class I expression in the cell lines used for injection (left panel) and in the bone marrow (BM) from three representative mice each that had developed leukemia upon injection of *STAT1*$^{+/-}$ and *STAT1*$^{-/-}$ cells (numbers on top of the scatter plot identify the individual mouse).

D: Kaplan-Meier plots of immunocompetent BALB/c mice injected with either *STAT1*$^{+/-}$ or *STAT1*$^{-/-}$ leukemic cells after depletion of NK cells with α-asialo GM1.

Loss of STAT1 delays disease progression in immunocompetent animals and in TEL-JAK2 leukemic mice

Our data show the central role of STAT1 during leukemia development in maintaining high expression levels of MHC class I. However, both *RAG2*$^{-/-}$ and *STAT1*$^{-/-}$ mice do have an impaired immune system. We therefore investigated disease progression inflicted by *STAT1*$^{-/-}$ and *STAT1*$^{+/-}$ leukemic cells in immunocompetent hosts (BALB/c animals). This experimental setup mimics the situation in human patients, where a loss of STAT1 can be found in leukemic cells (Irish et al., 2004; Landolfo et al., 2000; Sun et al., 1998).

We transplanted *STAT1*$^{+/+}$ or *STAT1*$^{-/-}$ v-abl-transformed cells at different numbers into syngeneic immunocompetent BALB/c (wild-type) mice. As depicted in Figures 6A and 6B, wild-type mice that had received *STAT1*$^{-/-}$ tumor cells showed an increased latency of disease—irrespective of the injected cell number ($p < 0.01$ and $p < 0.005$). *STAT1*$^{-/-}$ leukemic cells were isolated from the diseased mice and again showed a significant increase in MHC class I expression (Figure 6C). Depletion of NK cells by repeated injections of α-asialo GM1 antibody in wild-type mice abolished the differences in disease latency (Figure 6D; p = not significant). Together, these experiments prove that STAT1 also plays a crucial role as a tumor promoter in the context of an intact immune system by interfering with NK cell-mediated tumor lysis.

V-abl-induced B-lymphoid leukemia may represent a specific challenge to the immune system, and the observations summarized above may therefore be of limited importance. In order to document that they reflect a more general mechanism of tumor escape in hematopoietic malignancies, we chose MPD induced by constitutively active JAK2 as a second independent leukemia model. Bone marrow cells of wild-type and *STAT1*$^{-/-}$ BALB/c mice were infected with a retrovirus encoding the TEL/JAK2 fusion protein (Schwaller et al., 1998). Infection rates were controlled by FACS and were found to be comparable (around 5%) at the time point of injection. The cells were transplanted into five wild-type recipients each. All mice were analyzed 3 months after injection. At this time point, the first mice that had been injected with *STAT1*$^{+/-}$ bone marrow were obviously sick and displayed elevated red blood cell counts accompanied by thrombocytosis, granulocytosis, and splenomegaly (James et al., 2005; Kralovics et al., 2005; Levine et al., 2005). The hematocrit values as well as the numbers of granulocytes were significantly lower in mice that had been injected with STAT1-deficient cells than in those injected with STAT1-expressing cells (Figure 7A; $p < 0.001$ and data not shown). MPD is characterized by aberrations in early hematopoietic progenitors. As indicated in the upper panels of Figure 7B, erythroid precursor cell numbers (P2/P3/P4/P5 regions) were elevated 3-fold in mice that had been injected with TEL/JAK2-infected *STAT1*$^{+/-}$ bone marrow compared to TEL/JAK2-infected *STAT1*$^{-/-}$ bone marrow

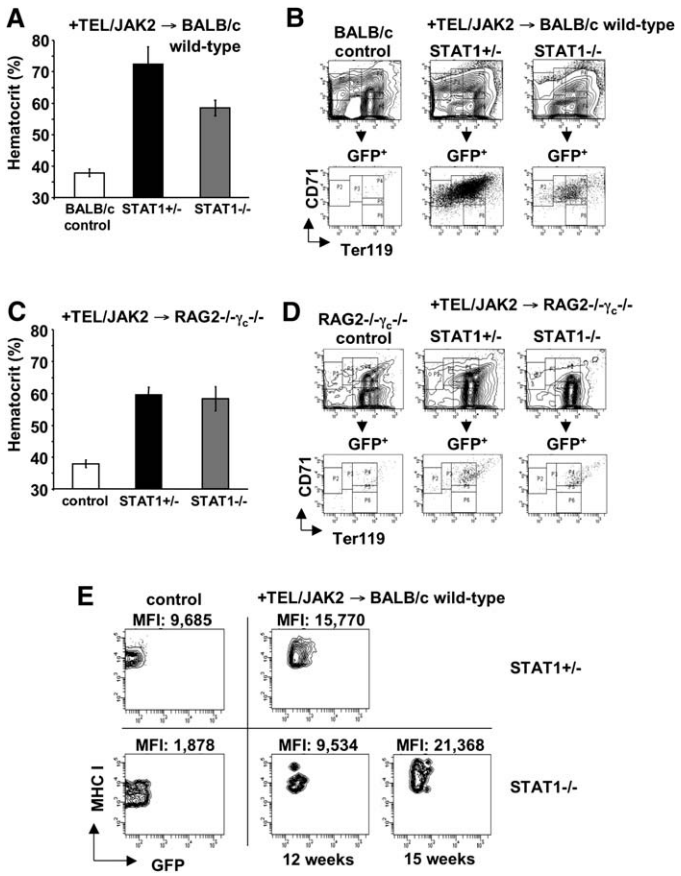

Figure 7. Low tumorigenicity of STAT1-deficient TEL/JAK2-induced MPD in immunocompetent mice

Mean hematocrit values from peripheral blood of immunocompetent (**A**) and immunodeficient (**C**) mice injected with TEL/JAK2-transduced *STAT1*$^{+/-}$ or *STAT1*$^{-/-}$ bone marrow cells and mice injected with PBS were analyzed after 3 months (n = 10/genotype/cell amount). Data represent mean ± SD. Flow-cytometric analysis of the erythroid compartment (CD71 and TER119) in the spleens from one representative PBS-injected, one *STAT1*$^{+/-}$-injected, and one *STAT1*$^{-/-}$-injected immunocompetent (**B**) and immunodeficient (**D**) mouse (upper panel). Distribution of GFP$^+$ cells in different stages of erythroid development is indicated in the lower panel. **E:** Flow-cytometric analysis of MHC class I expression and its mean fluorescence intensity (MFI). MHC class I expression in the spleen of wild-type mice prior to infection with the TEL/JAK2 oncogene (left panels). Representative examples of GFP$^+$ erythroid precursor cells were isolated from spleens 3 months after transplantation (right panels). Tumor editing is documented by the increase in MHC class I expression, which is confined to the *STAT1*$^{-/-}$ cells and progressive with time (bottom row, panels labeled 12 and 15 weeks).

(20.5% ± 1.1% versus 8.2% ± 0.4%, respectively; $p < 0.01$). This difference was even more pronounced with respect to the amount of GFP$^+$ cells. Whereas 31.4% ± 5.1% of erythroid precursor cells were GFP$^+$ in mice injected with wild-type TEL/JAK2-infected cells, only 3.7% ± 2.1% GFP$^+$ cells were observed in mice injected with *STAT1*$^{-/-}$ TEL/JAK2-infected cells ($p < 0.001$; Figure 7B, lower panels). This experiment was repeated using *RAG2*$^{-/-}\gamma c^{-/-}$ mice as recipients. In this setting, no differences in disease latency or hematocrit values were detected (Figure 7C), and the total amounts of erythroid precursors (20.2% ± 4.7% and 21.7% ± 7.0% in regions P2–P5 for *STAT1*$^{+/-}$ and *STAT1*$^{-/-}$ cells, respectively; p = not significant) and GFP$^+$ cells (8.95% ± 2.35% and 7.65% ± 3.65% in regions P2–P5 for *STAT1*$^{+/-}$ and *STAT1*$^{-/-}$ cells, respectively; p = not significant) in the spleens were comparable (Figure 7D). This experiment rules out that the lack of STAT1 within the tumor cells accounts for the differences observed in wild-type mice.

Again, in wild-type mice, the *STAT1*$^{-/-}$ GFP$^+$ erythroid precursor cells expressed significantly higher MHC class I levels compared to noninfected cells, indicating that the cells had undergone an "editing" process (Figure 7E) as observed for v-abl-induced B-lymphoid leukemia. The tumor editing of MHC class I was first observed 3 months after injection of *STAT1*$^{-/-}$ cells and was more pronounced 3 weeks thereafter (Figure 7E, middle and right panels). Thus, this experiment faithfully recapitulated the observations made in v-abl-induced B-lymphoid leukemia and confirms the hitherto unappreciated tumor-promoting role of STAT1 in hematopoietic malignancies.

Discussion

The absence of STAT1 is generally thought to be detrimental because it allows for more rapid tumor progression (Badgwell et al., 2004; Lee et al., 2000b; Lesinski et al., 2003; Nishibori et al., 2004; Refaeli et al., 2002). Thus, the presence of STAT1 protects against tumor development, and therefore the molecule is considered a tumor suppressor. The tumor-suppressing effect of STAT1 is consistent with the in vitro effects on v-abl-induced transformation; *STAT1*$^{-/-}$ cells gave rise to increased numbers of growth factor-independent colonies. This observation is in strong contrast with leukemia development in the mouse. We show here that the presence of STAT1 accelerates leukemia progression in vivo. This paradox can only be understood if immune surveillance is taken into account. Transformed *STAT1*$^{-/-}$ cells were efficiently eliminated in immunodeficient *RAG2*$^{-/-}$ mice by NK cells. The role for NK cell-mediated tumor surveillance was further substantiated in NK cell depletion studies that abolished the differences between *STAT1*$^{-/-}$ and *STAT1*$^{+/-}$ leukemic cells and accelerated disease onset. *STAT1*$^{-/-}$ mice were also more resistant to challenge with the v-abl oncogene, succumbed to the disease with a decreased incidence, and had an increased chance of survival. Moreover, using models for leukemia and MPD, we documented that the presence of STAT1 in a tumor was also detrimental in the context of an intact immune system.

Our observations demonstrate that tumor progression and tumor evolution can only be understood if the tumor is studied within the context of an intact organism. It has been recently appreciated that STAT1 supports expression of MHC class I molecules (Kamiya et al., 2004; Lee et al., 1999; Lieberman et al., 2004). Quite predictably, we observed that loss of STAT1 resulted in low expression of MHC class I molecules. Several arguments support the conclusion that there is a relation between MHC class I levels on the tumor cells and their ability to evade immunosurveillance by NK cells. (1) If MHC class I levels were raised using a viral promoter, STAT1-deficient tumor cells were rendered resistant to immunosurveillance. These cells were not cleared by the innate immune system of *RAG2*$^{-/-}$ mice and gave rise to a leukemic disease that progressed almost as rapidly as that induced by STAT1-expressing cells. This observation is consistent with the proposed model that B-lymphoid malignancies are predominantly under surveillance by NK cells (Cerwenka et al., 2000; Stoiber et al., 2004; Street et al., 2004). (2) The natural course of the disease allowed for escape of STAT1-deficient tumor cells from immunosurveillance.

This escape was consistently accompanied by the upregulation of MHC class I levels in all cases where injected *STAT1*$^{-/-}$ tumor cells eventually gave rise to B-lymphoid leukemia and MPD. (3) Finally, tumor evolution was associated with increased levels of β2-microglobulin, the dimeric partner of MHC class I (Gobin et al., 2003; Lee et al., 1999), and their common upstream regulator IRF-1 (Hobart et al., 1997). In all tumors that arose from transformed *STAT1*$^{-/-}$ cells, their MHC class I levels substantially exceeded those seen in the parental cell line and approached the levels observed in STAT1-expressing cells. Taken together, these data are consistent with a model where high MHC class I levels protect the tumor cells from elimination by NK cells (Cerwenka and Lanier, 2001; Colucci et al., 2003; Karre et al., 1986; Mocikat et al., 2003; Smyth et al., 2002).

When the concept of cancer immunoediting was originally introduced, the focus was placed on the evolutionary arms race between the tumor cell and the acquired immune system, that is, T and B cells (Shankaran et al., 2001). Very recently, the theoretical framework was expanded by the observation that lymphomas are subject to immunosurveillance by the innate immune system (Street et al., 2004). In the present work, we demonstrate that the evolutionary pressure exerted by NK cells sculpts and edits the leukemic tumor cells in a predictable manner. Under these conditions, upregulation of MHC class I molecules is the mechanism that allows for escape (Cerwenka and Lanier, 2001; Colucci et al., 2003; Karre et al., 1986; Mocikat et al., 2003; Smyth et al., 2002). Accordingly, in immunocompetent and immunodeficient mice, STAT1-expressing tumor cells gave rise to highly aggressive disease because they could not be cleared by NK cells. Likewise, the evolutionary arms race was readily evident, if STAT1-deficient tumor cells were injected: the tumor cells eventually overcame their defect by bypassing the STAT1-dependent regulation of MHC class I. This upregulation was stable—as seen by the fact that *STAT1*$^{-/-}$ tumors maintained high MHC class I levels after long cultivation periods (ex vivo)—and resulted in a concerted upregulation of β2-microglobulin and IRF-1. Thus, the mechanistic possibilities of a STAT1-independent regulation of MHC class I are diverse and may exceed a simple gain-of-function mutation.

Phenotypically, the disease inflicted by transplanting *STAT1*$^{-/-}$ v-abl-transformed cells into immunodeficient mice was similar to that induced in STAT1-deficient animals. The same effect was also observed in disease phenotypes caused by transplanting either *STAT1*$^{-/-}$ leukemia or *STAT1*$^{-/-}$ MPD in immunocompetent hosts. In other words, irrespective of the model system used, *STAT1*$^{-/-}$ transformed cells either were easily rejected, had increased latencies, developed more moderate forms of malignancies, or gave rise to tumors only after acquiring an "edited" phenotype, which manifested itself in an increase of MHC class I.

The concept of cancer immunoediting is based on the following predictions: the tumor can be eliminated by the immune system, and the tumor cells are sculpted by the immune system and may escape by Darwinian evolution. Our study verifies the predictions of this concept, supporting its general applicability. The role of IFN-γ and its signaling component STAT1, however, has to be reevaluated. While IFN-γ acts like a potent tumor suppressor, this role cannot be generally attributed to STAT1. In fact, the phenotypes observed in IFN-γ- and STAT1-deficient mice are diametrically opposed.

MHC class I-mediated killing by NK cells has been extensively investigated in various tumors. This concept is therefore well documented and understood in considerable detail. Hence, there is substantial circumstantial evidence from this earlier work that is in line with our model that STAT1 acts as a tumor promoter in leukemia and MPDs or, conversely, that a lack of STAT1 and low MHC class I expression protects the organism from hematopoietic malignancies. In light of this finding, our work may provide the basis for novel therapeutic approaches, using concerted inhibition of STAT1 and MHC class I.

Experimental procedures

Animals

BALB/cJ (BALB/c) *STAT1*$^{-/-}$ mice, C57BL/6J (C57BL/6) *IFN-γ*$^{-/-}$ mice, *RAG2*$^{-/-}$ mice, *RAG2*$^{-/-}$γc$^{-/-}$ mice, and wild-type BALB/c mice were maintained at the Biomedical Research Institute, Medical University of Vienna, Research Institute of Molecular Pathology, IMP, Vienna. All animal experiments were carried out in accordance with protocols approved by the Animal Welfare Committee and with the general regulations specified by the Good Scientific Practices guidelines of the Medical University of Vienna.

Mouse experiments

Each transplantation experiment was repeated at least in an additional independent experiment, and the observations were consistently reproducible. In detail, cells from six independently derived *STAT1*$^{+/-}$ and six *STAT1*$^{-/-}$ cell lines were injected into *RAG2*$^{-/-}$ mice (n = 40 in total) and *RAG2*$^{-/-}$γc$^{-/-}$ mice (n = 10 in total). For MHC class I (H-2D^{d}) reconstitution, *STAT1*$^{-/-}$ (parental cell line), *STAT1*$^{-/-}$ + H2D^{d}, and *STAT1*$^{+/-}$ (parental cell line) cells were injected into *RAG2*$^{-/-}$ mice (n = 18 in total), *STAT1*$^{-/-}$ mice (n = 18 in total), and wild-type BALB/c mice (n = 18 in total). For transplantation into immunocompetent mice, *STAT1*$^{+/-}$ and *STAT1*$^{-/-}$ leukemic cell lines were injected at different cell numbers (3×10^6 and 3×10^5) into syngeneic BALB/c recipients (n = 60 in total). For TEL/JAK2-induced experiments, bone marrow from *STAT1*$^{+/-}$ and *STAT1*$^{-/-}$ mice was transduced with pMSCV-TEL/JAK2-IRES-GFP-derived retrovirus and cultured as described earlier (Moriggl et al., 2005) prior to injection into syngeneic BALB/c mice (n = 20 in total) or *RAG2*$^{-/-}$γc$^{-/-}$ mice (n = 12 in total). For NK depletion experiments, wild-type BALB/c mice (n = 8 in total) were treated (i.p.) with α-asialo GM1 (WAKO, Japan) on days −4, 0, and 7 of tumor cell injection. For short-term homing experiments CFSE-labeled *STAT1*$^{+/-}$ and *STAT1*$^{-/-}$ cell lines were injected into wild-type (n = 8) or *RAG2*$^{-/-}$ mice (n = 8) and analyzed 4 days after injection. For newborn infection experiments, mice were injected intraperitoneally with 50 μl of replication incompetent ecotropic retrovirus encoding for v-abl.

Flow cytometry

Single-cell suspensions were preincubated with αCD16/CD32 antibodies (BD/Pharmingen) to prevent nonspecific Fc receptor-mediated binding. Cells (5×10^5) were stained with monoclonal antibodies conjugated with fluorescent markers and analyzed on FACScan and FACScanto (Beckton-Dickinson) with CellQuestPro and Facs Diva software. The antibodies used for lineage determination included the B cell lineage markers B220 (RA3-6B2), CD19 (1D3), and CD43 (1B11); the T cell marker CD3 (17A2); the pan-NK marker (DX5); and the erythroid lineage markers CD71 (C2) and TER119 (Ter119) (all BD/Pharmingen). In addition, an antibody against MHC class I (H-2Dd, clone 34-5-8S, BD/Pharmingen) was used. For cell cycle analysis, the cells were fixed in a buffer containing 0.05 mg/ml propidium iodide, 0.01% Triton X-100, and 0.1% sodium citrate.

Cytotoxicity assay

The YAC-1 cell line and fetal liver-derived *STAT1*$^{+/-}$, *STAT1*$^{-/-}$, and *STAT1*$^{-/-}$ + H-2D^{d} cell lines as well as tumor-derived *STAT1*$^{-/-}$ MHC I^{high} cells were used as target cells. DX5^{+} purified splenocytes were used as effector cells after 10 days of culture in h-IL-2 (5000 U/ml). Lactate dehydrogenase (LDH) release was measured as described using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega).

Statistical analysis

Statistics were carried out using Student's t test and the χ^2 test as appropriate. Kaplan-Meier plots were analyzed for statistical significance using the

log-rank test. Cytotoxicity assays were analyzed by the ANOVA test with Tukey's post hoc test for every E/T ratio.

Additional information regarding analysis of mice, RT-PCRs, tissue culture conditions, transformation, and proliferation assays is available within the Supplemental Data.

Supplemental data

The Supplemental Data include Supplemental Experimental Procedures and four supplemental figures and can be found with this article online at http://www.cancercell.org/cgi/content/full/10/1/77/DC1/.

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