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c-CBL is not required for leukemia induction by *Bcr-Abl* in mice

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Bcr-Abl tyrosine kinase activity is essential for the pathogenesis of chronic myeloid leukemia (CML). A number of _Bcr-Abl_ substrates have been identified, but it is not clear which of these substrates are required for _Bcr-Abl_ to transform cells. The multifunctional protein c-Cbl is one of the most prominently tyrosine-phosphorylated proteins in _Bcr-Abl_-expressing cells. Using cell lines and mice with homozygous disruption of the _c-CBL_ locus, we investigated the role of this protein for _Bcr-Abl_-driven transformation. We find that although _c-Cbl_$^{-/-}$ fibroblast cell lines show a deficit in _Bcr-Abl_ transformation compared to wild-type (Wt) cells, this deficit was less pronounced in _c-Cbl_$^{-/-}$ B cells derived from murine bone marrow. Most importantly, in a transplantation model of CML, _Bcr-Abl_ was capable of inducing fatal leukemia in mice in the absence of c-Cbl protein. Our results indicate that c-Cbl is dispensable for _Bcr-Abl_-induced leukemogenesis in mice.
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Introduction

More than 90% of patients with chronic myelogenous leukemia (CML) harbor a Philadelphia chromosome (Ph). The latter is the product of a balanced translocation between the long arms of chromosomes 9 and 22 (Rowley, 1973; Kurzrock *et al*., 1988) that fuses sequences on chromosome 22, in a region known as the breakpoint cluster region (*BCR*), with *ABL* sequences translocated from chromosome 9. As a result, a *BCR-ABL* fusion gene is formed, which gives rise to a *Bcr-Abl* protein (Rowley, 1973; Bartram *et al*., 1983; Heisterkamp *et al*., 1983). Owing to diversity in the location of breakpoints in chromosome 22, several different *Bcr-Abl* fusion proteins exist (Melo, 1996). In 90–95% of patients with Ph-positive CML, the breakpoint in chromosome 22 is located within a region referred to as the major breakpoint cluster (M-*BCR)*, resulting in the formation of a 210 kDa protein (p210$^{Bcr\text{-}Abl}$) (deKlein *et al*., 1986; Gale and Goldman, 1988). A second breakpoint occurs 5′ of M-*BCR*, in a region referred to as the minor breakpoint cluster region (m-*BCR*) (Chan *et al*., 1987), which yields a transcript encoding p185$^{Bcr\text{-}Abl}$ (Feinstein *et al*., 1987; Hermans *et al*., 1987; Clark *et al*., 1988). In the literature, this *Bcr-Abl* isoform is known as p185$^{Bc\text{-}Abl}$ or p190$^{Bcr\text{-}Abl}$. It is mostly found in patients with Ph-positive acute lymphoblastic leukemia. Rare CML patients express a p230$^{Bcr\text{-}Abl}$ protein, where the breakpoint is located toward the 3′ end of the *BCR* gene.

All *Bcr-Abl* fusion proteins have elevated kinase activity compared to c-Abl (Sefton *et al*., 1981; Konopka *et al*., 1984). *Bcr-Abl* has been shown to transform fibroblasts (Lugo and Witte, 1989), factor-dependent hematopoietic cells (Daley and Baltimore, 1988; Hariharan *et al*., 1988), and primary bone marrow (BM) B-cell progenitors *in vitro* (McLaughlin *et al*., 1987, 1989; Young and Witte, 1988; Kelliher *et al*., 1993). *In vivo*, transplantation of murine BM infected with p210$^{Bcr\text{-}Abl}$ or p185$^{Bcr\text{-}Abl}$ retroviruses induces a CML-like myeloproliferative disorder in syngeneic recipients (Daley *et al*., 1990; Elefanty *et al*., 1990; Kelliher *et al*., 1990; Zhang and Ren, 1998).

Since the tyrosine kinase activity of *Bcr-Abl* has been shown to be essential for its oncogenic potential both *in vitro* and *in vivo* (Lugo *et al*., 1990; Zhang and Ren, 1998), much effort has been directed at determining which of its substrates are required for leukemogenesis. A number of *Bcr-Abl* substrates have been identified, including c-Abl, c-Cbl, Crkl, PI3-kinase, p62-Dok, Ras-GAP, Paxillin, and Shc (Sattler and Salgia, 1997). *Bcr-Abl* has also been shown to form complexes with several of these substrates including Crkl, Shc, c-Cbl, p62-Dok, and phosphatidylinositol-3 kinase (PI-3K) (Gotoh and Broxmeyer, 1997; Raitano *et al*., 1997; Sattler and Salgia, 1997; Sawyers, 1997). The result of all these interactions is the activation of cell signaling pathways including the MAP kinase (MAPK), PI-3K, and

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Jak/STAT pathways (Gotoh and Broxmeyer, 1997; Raitano *et al*., 1997; Sattler and Salgia, 1997; Sawyers, 1997). In the complicated network of interactions that results, the role and relative importance of individual components is difficult to determine.

Diverse functions have been ascribed to c-Cbl in normal cells. The protein is transiently tyrosine-phosphorylated in response to a variety of external stimuli, resulting in the activation of various signaling pathways associated with cellular proliferation or activation (Donovan *et al*., 1994; Fukazawa *et al*., 1995; Galisteo *et al*., 1995; Odai *et al*., 1995; Tanaka *et al*., 1995). More recently, the N-terminus of c-Cbl was reported to have E3 ubiquitin ligase function (Joazeiro *et al*., 1999; Fang *et al*., 2001). The substrates include receptor tyrosine kinases (Rao *et al*., 2002 for review).

c-Cbl is one of the most prominently tyrosine-phosphorylated proteins in *Bcr-Abl*-transformed cells (Andoniou *et al*., 1994). In its phosphorylated form, c-Cbl binds directly to the SH2 domain of *Bcr-Abl*, or indirectly via a protein complex that includes Crkl (Sattler *et al*., 1996; Bhat *et al*., 1997). Tyrosine-phosphorylated Cbl can also bind directly to the SH2 domain of the 85 kDa subunit of PI-3K (Beitz *et al*., 1999), thus linking Bcr-Abl to the PI-3K pathway. Not surprisingly, a significant increase in Cbl-associated PI-3K activity has been observed in Bcr-Abl-expressing cells (Jain *et al*., 1997). In addition, tyrosine-phosphorylated Cbl has been implicated in activation of the MAPK pathway in Bcr-Abl-expressing cells (Jain *et al*., 1997).

Although overexpression of c-Cbl in cells alone does not induce cellular transformation, mutations that result in the constitutive phosphorylation of c-Cbl activate its transforming potential. For instance, when 60% of the protein is deleted as in v-Cbl, the protein can transform B-lymphoid cells and fibroblasts (Mushinski *et al*., 1994). v-Cbl is the naturally occurring viral oncoprotein of the CAS NS-1 retrovirus that induces B-cell lymphoma and myeloid leukemia in mice (de Jong *et al*., 1995). An internal deletion of 17 amino acids in c-Cbl also creates a mutant with transforming potential (Andoniou *et al*., 1994). It is predicted that the constitutive activation of c-Cbl by mutation or phosphorylation by oncogenic tyrosine kinases such as *Bcr-Abl* contributes to oncogenesis (de Jong *et al*., 1995).

The aim of the present study was to investigate the relevance of c-Cbl for *Bcr-Abl* function, by using cell lines and mice that lack the c-Cbl protein.

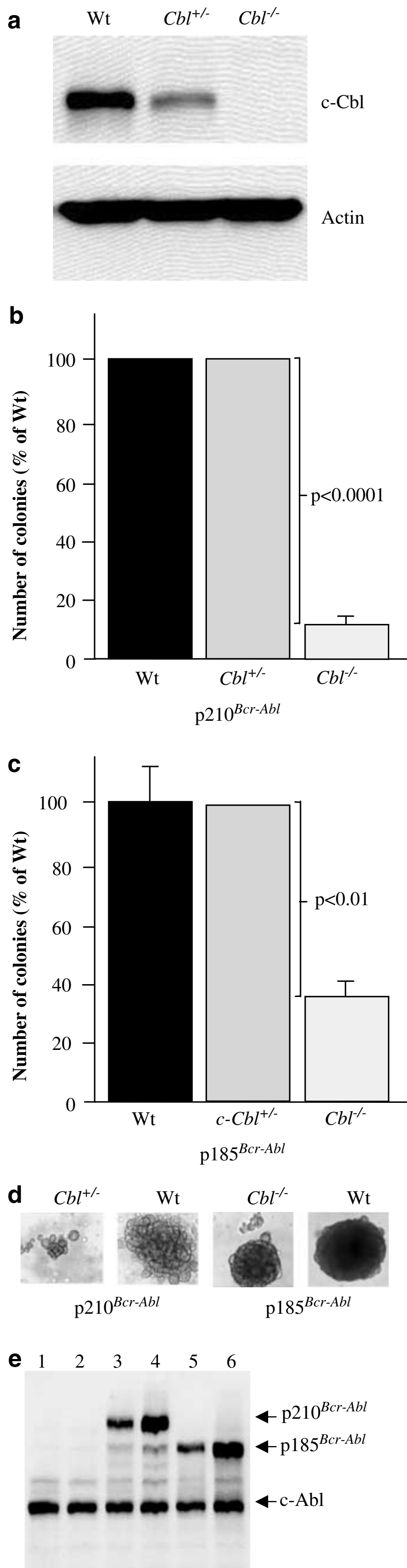
Results

The role of c-Cbl in transformation by Bcr-Abl in vitro

Wt, c-$Cbl^{+/-}$, and c-$Cbl^{-/-}$ fibroblast cell lines were generated from day 13.5 old mouse embryos. Immunoblotting of cell lysates confirmed the absence of c-Cbl protein in c-$Cbl^{-/-}$ cells and decreased the levels of *c-Cbl* protein in c-$Cbl^{+/-}$ compared with Wt murine embryo fibroblasts (MEFs; Figure 1a). Wt, c-$Cbl^{+/-}$, and c-$Cbl^{-/-}$ cells were infected with the same stock of $p210^{Bcr\text{-}Abl}$, $p185^{Bcr\text{-}Abl}$, or control retrovirus, and incubated for 48 h at 37°C. Prior to seeding the cells in soft agar, the percentage of green fluorescent protein (GFP)-expressing cells was determined for each cell line by FACS, and was found to be similar for Wt, c-$Cbl^{+/-}$, and c-$Cbl^{-/-}$ cells (10–20%, data not shown), suggesting that c-*CBL* inactivation does not affect retroviral infection efficiency in fibroblasts. Approximately 1×10^5 GFP-positive cells were seeded in soft agar, and the cells were incubated for 2–3 weeks at 37°C. Neither Wt, c-$Cbl^{+/-}$ nor c-$Cbl^{-/-}$ fibroblasts infected with control retrovirus generated transformed colonies in the soft-agar assay, and only single cells were observed after incubation for 3 weeks (data not shown). Transformed cells capable of anchorage-independent growth formed colonies in the soft-agar assay. Colonies were visible after 2–3 weeks on dishes seeded with Wt, c-$Cbl^{+/-}$, and c-$Cbl^{-/-}$ cells expressing $p210^{Bcr\text{-}Abl}$ or $p185^{Bcr\text{-}Abl}$. However, the number of c-$Cbl^{-/-}$ colonies was significantly reduced compared to Wt and c-$Cbl^{+/-}$ cells (Figure 1b, c). Specifically, the number of colonies formed by c-$Cbl^{-/-}$ $p185^{Bcr\text{-}Abl}$- and c-$Cbl^{-/-}$ $p210^{Bcr\text{-}Abl}$-expressing cells was reduced by over twofold ($P<0.01$; Figure 1c) and 10-fold ($P<0.0001$; Figure 1b), respectively. Furthermore, we observed that the colonies formed by c-$Cbl^{-/-}$ cells were comparatively smaller in diameter than those formed by Wt cells (Figure 1d). Since Wt and c-$Cbl^{-/-}$ parental cells grew at similar rates in normal culture conditions (data not shown), it is unlikely that the differences observed between c-$Cbl^{-/-}$ and Wt cells in the soft agar assay are due to an intrinsic growth defect in c-$Cbl^{-/-}$ cells. Immunoblotting of lysates prepared from MEFs transduced by either $p210^{Bcr\text{-}Abl}$ or $p185^{Bcr\text{-}Abl}$ confirmed that *Bcr-Abl* protein was expressed in all cell lines analysed in the soft agar assay (Figure 1e). Interestingly, we observed that although colony formation by *c-*$Cbl^{-/-}$ cells is reduced compared to Wt cells, *c-*$Cbl^{-/-}$ cells expressed greater levels of Bcr-Abl, the reasons for which are unclear. It is possible that increased Bcr-Abl protein expression in *c-*$Cbl^{-/-}$ cells served to exaggerate the number of colonies formed by these cells in the soft agar assay. Thus, the dependence of Bcr-Abl on *c-Cbl* in the transformation of fibroblasts may be greater than observed.

To further explore the role of c-Cbl for *Bcr-Abl*-induced transformation, we assessed the susceptibility of c-$Cbl^{-/-}$ B-lymphoid cells to transformation by *Bcr-Abl*. BM cells were isolated from c-$Cbl^{-/-}$, c-$Cbl^{+/-}$, and Wt mice, and transduced with $p210^{Bcr\text{-}Abl}$, $p185^{Bcr\text{-}Abl}$ or control retrovirus, and cultured under conditions that favor the outgrowth of transformed B-lymphoid cells (Whitlock–Witte cultures; McLaughlin *et al*. (1987); see Materials and methods). The transduction efficiency of Wt, c-$Cbl^{+/-}$, and c-$Cbl^{-/-}$ BM cells was equivalent, as approximately 20% of cells from Wt and mutant BM cultures were GFP positive by fluorescence microscopy.

When similar numbers of $p210^{Bcr\text{-}Abl}$-transduced Wt and c-$Cbl^{-/-}$ BM cells were plated, significantly fewer B-lymphoid cells were observed in c-$Cbl^{-/-}$ cultures at day 16 post-transduction as compared to Wt cultures



($P = 0.033$, representative of two independent experiments performed in duplicate; Figure 2a). In contrast, no significant reduction in cell number was observed in c-$Cbl^{-/-}$ cultures expressing p185$^{Bcr-Abl}$ (Figure 2b). This suggests that c-Cbl plays a role in the transformation of B-lymphoid cells by p210$^{Bcr-Abl}$ but not by p185$^{Bcr-Abl}$. c-$Cbl^{+/-}$ cells, which show reduced amounts of c-Cbl protein compared to Wt cells (data not shown), were also deficient in p210$^{Bcr-Abl}$ transformation, suggesting a titration effect of c-Cbl on p210$^{Bcr-Abl}$-induced transformation (Figure 2b). Although c-$Cbl^{+/-}$ cultures transduced with p210$^{Bcr-Abl}$ produced fewer B-lymphoid cells than Wt, this difference was not statistically significant ($P = 0.19$). To gain further insight into the reason for the transformation defect observed in *c-Cbl*$^{-/-}$ cells, we explored the activation of signaling pathways downstream of p210$^{Bcr-Abl}$ in these cells. Initially, we immunoblotted B-lymphoid cell lysates for expression of p210$^{Bcr-Abl}$, to determine whether the transformation defect was a consequence of decreased p210$^{Bcr-Abl}$ expression in *c-Cbl*$^{-/-}$ cells compared to Wt cells. Similar to MEFs, the level of Bcr-Abl was increased in *c-Cbl*$^{-/-}$ B-lymphoid cells (Figure 2c), although, to a lesser extent. Therefore, it is possible that the dependence of Bcr-Abl on *c-Cbl* B-lymphoid cell transformation may be greater than that observed.

Since tyrosine-phosphorylated Cbl has been implicated in the activation of both the PI-3K and MAPK pathways in Bcr-Abl-expressing cells (Jain *et al*., 1997), we sought to determine whether the proliferation defect observed in *c-Cbl*$^{-/-}$ B-lymphoid cells expressing p210$^{Bcr-Abl}$ is a result of diminished PI-3K or MAPK activity. To this end, the levels of phosphorylated Akt (p-Akt) and phosphorylated p44/p42 MAPKs (Erk1 and Erk2) in Wt and *c-Cbl*$^{-/-}$ B-lymphoid cells were determined by immunoblotting, as a measure of PI-3K and MAPK activity, respectively. We found that p-Akt and p-Erk1 and p-Erk2 levels were similar in Wt and c-$Cbl^{-/-}$ cell lysates (Figure 2d), which suggests that the

Figure 1 c-Cbl plays a role in *Bcr-Abl* transformation of fibroblasts. (**a**) Immunoblotting of c-$Cbl^{-/-}$, c-$Cbl^{+/-}$, and Wt MEF protein lysates using an anti-c-Cbl antibody. Lysates were immunoblotted for actin to control for protein loading. Note the absence of c-Cbl protein in c-$Cbl^{-/-}$ cells and decreased levels of c-Cbl protein in c-$Cbl^{+/-}$ compared to Wt MEFs. c-$Cbl^{-/-}$, c-$Cbl^{+/-}$, and Wt MEFs were transduced with (**b**) p210$^{Bcr-Abl}$, or (**c**) p185$^{Bcr-Abl}$ retrovirus, incubated for 48 h at 37°C, and then 10^4–10^5 GFP-positive cells were seeded in soft agar in six-well tissue culture dishes. Colonies were counted 2–3 weeks later. Assay results from two independent experiments performed in duplicate are expressed as the percentage of Wt transformation for both p210$^{Bcr-Abl}$ and p185$^{Bcr-Abl}$-expressing cells. (**d**) Photomicrographs ($\times$200 magnification) of colonies formed by c-$Cbl^{-/-}$ and Wt cells expressing p210$^{Bcr-Abl}$ and p185$^{Bcr-Abl}$. Note the smaller colony size formed by c-$Cbl^{-/-}$ cells compared to Wt cells. Results shown are representative of two separate experiments performed in duplicate. (**e**) Immunoblotting of p210$^{Bcr-Abl}$ and p185$^{Bcr-Abl}$ proteins expressed in c-$Cbl^{-/-}$ and Wt MEFs using an anti-Abl antibody (8E9). Lanes 1–2: Wt and c-$Cbl^{-/-}$ MEFs, respectively, transduced with control retrovirus; lanes 3–4: Wt and c-$Cbl^{-/-}$ MEFs, respectively, transduced with p210$^{Bcr-Abl}$ retrovirus: lanes 5 and 6: Wt and c-$Cbl^{-/-}$ MEFs, respectively, transduced with p185$^{Bcr-Abl}$ retrovirus

proliferation defect observed in *c-Cbl*$^{-/-}$ cells is not due to a reduction in PI-3K or MAPK activity in these cells.

The JNK pathway is activated in Bcr-Abl-transformed B-lymphoid cells, and has been linked to an antiapoptotic pathway from p210$^{Bcr\text{-}Abl}$ to Bcl-2 (Hess *et al*., 2002). Elevated expression of Bcl-2 also occurs in 32D cells expressing constitutively active Cbl (Hamilton *et al*., 2001). To determine whether *c-Cbl* plays a role in JNK activation and subsequent induction of Bcl-2 expression by p210$^{Bcr\text{-}Abl}$, we analysed the levels of phosphorylated JNK (p-JNK) and Bcl-2 in B-lymphoid cells. We found that there was approximately a twofold reduction in the levels of p-JNK in *c-Cbl*$^{-/-}$ B-lymphoid cells compared to Wt cells (Figure 2d). However, levels of Bcl-2 in Wt and *c-Cbl*$^{-/-}$ cells were similar (Figure 2d), which suggests that the transformation defect observed in *c-Cbl*$^{-/-}$ cells was not due to decreased Bcl-2 expression.

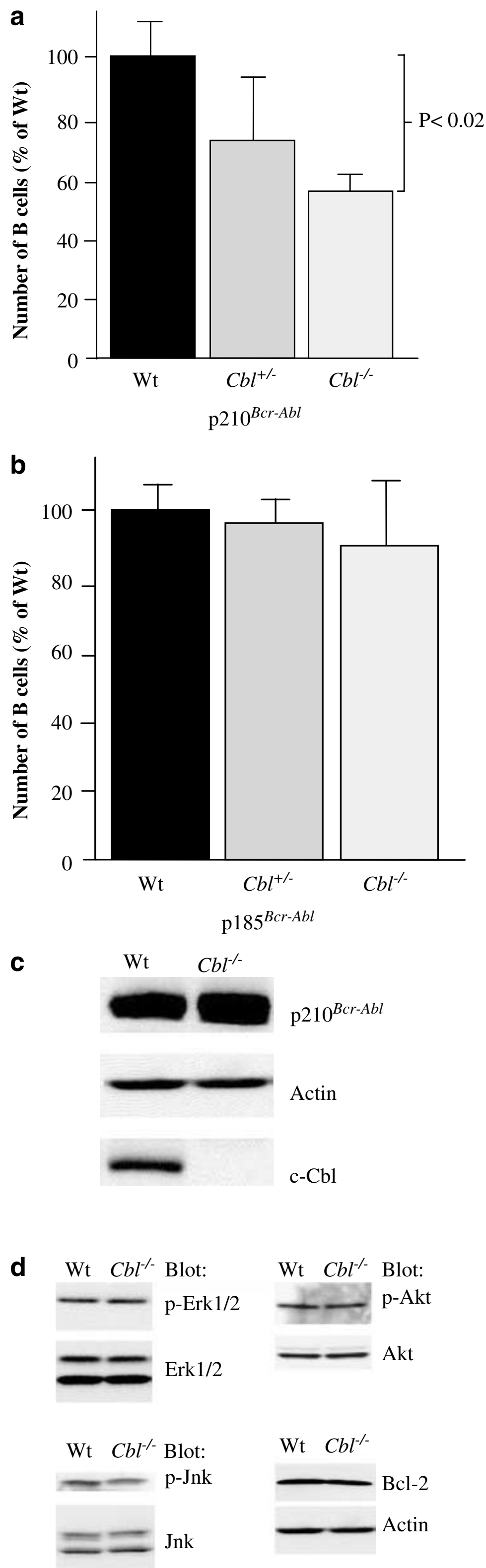


Figure 2 c-Cbl contributes to, but is not required for lymphoid transformation by *Bcr-Abl*. (**a**, **b**) BM cells from Wt, c-*Cbl*$^{+/-}$, and c-*Cbl*$^{-/-}$ mice were transduced with p210$^{Bcr\text{-}Abl}$ or p185$^{Bcr\text{-}Abl}$ retrovirus, and cultured under conditions that favor the outgrowth of transformed B-lymphoid cells (Whitlock–Witte cultures). B-lymphoid cells were counted at 16 days post-transduction. Each bar represents counts from two separate experiments performed in duplicate. Note that significantly fewer cells grew in c-*Cbl*$^{-/-}$ cultures transduced by p210$^{Bcr\text{-}Abl}$ ($P<0.02$) than in Wt cultures. (**c**) Immunoblotting of p210$^{Bcr\text{-}Abl}$ protein expressed in Wt and c-*Cbl*$^{-/-}$ B-lymphoid cells using an anti-Abl antibody. Lysates were also immunoblotted with anti-c-Cbl and anti-actin antibodies to confirm c-Cbl status and to control for protein loading. (**d**) Activation of the PI-3K, MAPK, and JNK pathways in Wt and c-*Cbl*$^{-/-}$ B-lymphoid cells transduced by p210$^{Bcr\text{-}Abl}$. Cell lsyates were immunoblotted with antibodies recognizing phosphorylated and total Akt and p44/42 MAPK (Erk1 and Erk2) as a measure of PI-3K and MAPK activation, respectively. Activation of the JNK pathway was determined by immunoblotting protein lysates with antibodies recognizing phosphorylated and total JNK and Bcl-2 protein levels

Cbl is not required for Bcr-Abl-*induced leukemogenesis*

Our findings indicate that c-Cbl contributes to transformation by p210$^{Bcr\text{-}Abl}$ and p185$^{Bcr\text{-}Abl}$ *in vitro*; however, this does not necessarily imply a role *in vivo*. To further evaluate the role of c-Cbl in *Bcr-Abl*-induced leukemogenesis *in vivo*, we assessed the ability of *Bcr-Abl* to induce leukemia in a c-*Cbl*$^{-/-}$ background, using the murine BM transduction/transplantation assay. To determine whether c-Cbl is required for the induction of leukemia by *Bcr-Abl*, we injected c-*Cbl*$^{-/-}$ BM cells transduced with p210$^{Bcr\text{-}Abl}$ or p185$^{Bcr\text{-}Abl}$ retrovirus into lethally irradiated recipient c-*Cbl*$^{-/-}$ mice.

Of the 11 mice that received c-*Cbl*$^{-/-}$ BM transduced with either p210$^{Bcr\text{-}Abl}$ or p185$^{Bcr\text{-}Abl}$, 10 developed a fatal leukemia (Figure 3; Table 1). There was no significant difference in survival between recipients of c-*Cbl*$^{-/-}$ versus Wt BM transduced with either p210$^{Bcr\text{-}Abl}$ or p185$^{Bcr\text{-}Abl}$. Mice in both groups receiving marrows cells transduced with p185$^{Bcr\text{-}Abl}$ succumbed to a fatal leukemia by day 16 post-BM transplantation. Mice injected with *c-Cbl*$^{-/-}$ marrow transduced with p210$^{Bcr\text{-}Abl}$ retrovirus showed a median survival of 21 days compared to 18 days for mice receiving Wt marrow. The difference in the length of survival between recipients of c-*Cbl*$^{-/-}$ versus Wt BM transduced with

p210$^{Bcr-Abl}$ was statistically insignificant (Figure 3; Table 1).

The disease in all mice was characterized by massive expansion of leukemic cells in spleen, liver, and peripheral blood (Figure 4a; Table 1). Immunohistochemical staining of liver and spleen sections with the antimyeloid marker antibody Ly-6 revealed extensive infiltration by maturing myeloid cells with total disruption of the splenic architecture, accompanied by atypical megakaryocytes and erythroid progenitors (Figure 4a, b). The liver sinusoids were also heavily infiltrated with maturing myeloid cells (Figure 4; Table 1). All recipient mice showed elevated white blood cell (WBC) counts compared to control mice receiving BM transduced by control retrovirus (Table 1). This CML-like disease phenotype has been seen previously in mice receiving 5-FU-treated BM transduced by p210$^{Bcr-Abl}$ or p185$^{Bcr-Abl}$ (Zhang and Ren, 1998; Li *et al*., 1999, 2001).

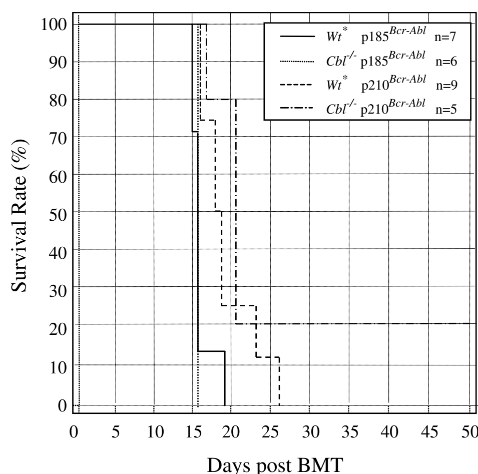


Figure 3 *c-Cbl* is not required for leukemia induction by *Bcr-Abl* in mice. c-*Cbl*$^{-/-}$ BM or BM pooled from c-*Cbl*$^{+/-}$ and Wt mice (referred to as Wt* in the figure) were transduced with either p210$^{Bcr-Abl}$ or p185$^{Bcr-Abl}$ and injected into recipient mice. Survival curves were generated by Kaplan–Meier survival analysis using data collected from one representative experiment. The number of recipients in each arm is shown in parentheses. The majority of mice in all groups developed a fatal leukemia by day 26 post-BM transplantation (BMT). One mouse injected with c-*Cbl*$^{-/-}$ marrow transduced by p210$^{Bcr-Abl}$ showed extended survival. Survival curves were compared using the log-rank test. There was no significant difference in survival between the recipients of c-*Cbl*$^{-/-}$ BM or marrow expressing c-Cbl transduced with either p210$^{Bcr-Abl}$ or p185$^{Bcr-Abl}$ ($P = 0.23$ and 0.83, respectively)

One mouse injected with c-*Cbl*$^{-/-}$ BM transduced by p210$^{Bcr-Abl}$ showed extended survival compared to other mice. This animal was killed at day 50, and pathological examination revealed splenomegaly and elevated WBC counts (Table 1; data not shown) but no hepatomegaly (Table 1). Hematoxylin and eosin (H&E) staining showed a normal liver architecture, whereas extensive leukemic cell accumulation was observed in the spleen (data not shown).

Discussion

c-CBL is a proto-oncogene related to *v-CBL*, the viral oncogene of the CAS NS-1 retrovirus that induces B-cell lymphoma and myeloid leukemia in mice (de Jong *et al*., 1995). It is one of the most heavily tyrosine-phosphorylated proteins in *Bcr-Abl*-expressing cells, and has been implicated in *Bcr-Abl*-driven transformation.

In the present study, we used c-*Cbl*$^{-/-}$ cells and mice to explore the requirement of this protein for *Bcr-Abl* function. We found that both p210$^{Bcr-Abl}$ and p185$^{Bcr-Abl}$ showed a diminished capacity to transform c-*Cbl*$^{-/-}$ MEFs compared to Wt cells. The number of transformed colonies generated by c-*Cbl*$^{-/-}$ cells expressing p185$^{Bcr-Abl}$ or p210$^{Bcr-Abl}$ was reduced two- and 10-fold, respectively. In addition to a reduction in the number of colonies, the colonies that formed were smaller than those formed by Wt cells.

We also found that both p210$^{Bcr-Abl}$ and p185$^{Bcr-Abl}$ were able to induce the outgrowth of B-lymphoid cells in c-*Cbl*$^{-/-}$ BM cultures. However, similar to c-*Cbl*$^{-/-}$ MEFs, significantly fewer B-lymphoid cells grew in c-*Cbl*$^{-/-}$ cultures transduced by p210$^{Bcr-Abl}$ compared to Wt cultures. This transformation defect was not observed in cells expressing p185$^{Bcr-Abl}$. Taken together, our *in vitro* data indicate that, although c-*Cbl* is not an absolute requirement for p210$^{Bcr-Abl}$ or p185$^{Bcr-Abl}$ transformation *in vitro*, it does contribute to transformation. This is particularly true for fibroblasts and more pronounced in the case of p210$^{Bcr-Abl}$. The difference in the transforming ability of p185$^{Bcr-Abl}$ and p210$^{Bcr-Abl}$ in *c-Cbl*$^{-/-}$ cells might reflect the increased kinase activity of p185$^{Bcr-Abl}$ compared to p210$^{Bcr-Abl}$ (Lugo *et al*., 1990; Ilaria and Van

Table 1 Summary of bone marrow transduction and transplantation experiments

Donor genotype	*Construct*	*Median survival in days (range)*	*Median spleen weight in grams (range)*	*Median liver weight in grams (range)*	*Mean WBC count ($\times 10^3/\mu l$)*	*Lung hemorrhages*[a]
Wt	GFP-Control	NA[b]	0.05 (0.02–0.08)	0.95 (0.87–1.34)	0.7	0/4
Wt	p185$^{Bcr-Abl}$	15–18 (16)	0.52 (0.45–0.60)	1.95 (1.60–2.30)	30	6/6
c-Cbl$^{-/-}$	p185$^{Bcr-Abl}$	16 (16)	0.50 (0.48–0.60)	1.99 (1.70–2.10)	4	6/6
Wt	p210$^{Bcr-Abl}$	16–26 (18)	0.40 (0.30–0.68)	1.80 (1.30–2.38)	13	5/7
c-Cbl$^{-/-}$	p210$^{Bcr-Abl}$	17–50[c] (21)	0.65 (0.37c–0.78)	1.30 (0.86c –2.16)	76	2/5

[a]Number of mice in which lung hemorrhages were observed. [b]Not applicable. [c]Values obtained for mouse, which did not develop fatal leukemia by day 26

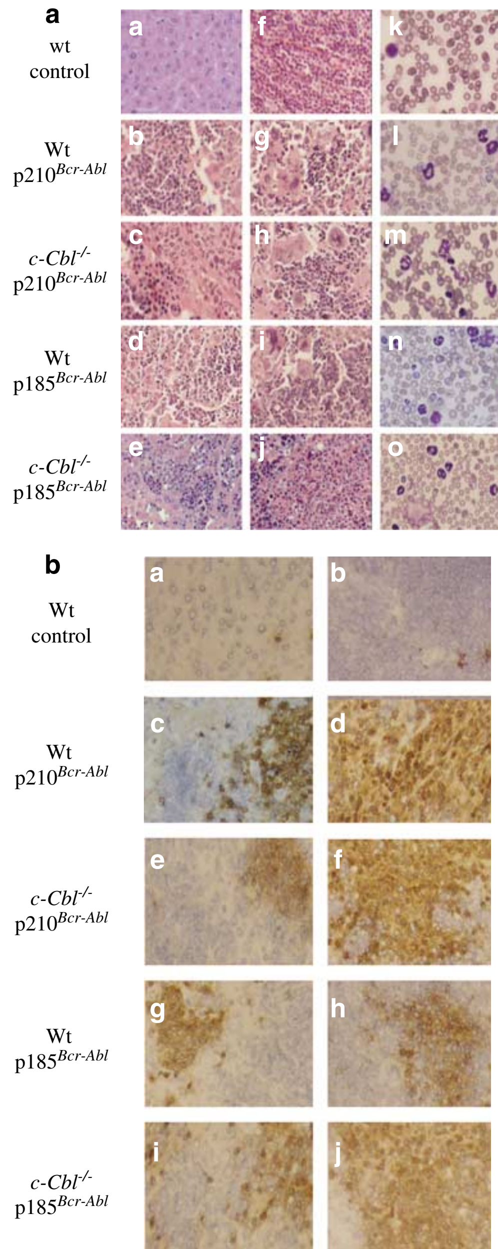


Figure 4 Leukemia in tissues of mice receiving BM transduced by p210$^{Bcr\text{-}Abl}$ or p185$^{Bcr\text{-}Abl}$. The donor BM genotype and *Bcr-Abl* expression construct are shown to the left of the figure. (**a**) Photomicrographs of H&E-stained sections (magnification $\times 200$) of liver (**a**–**e**) and spleen (**f**–**j**), and Wright–Giemsa stained smears (magnification $\times 400$) of peripheral blood (**k**–**o**), from mice that developed fatal leukemia in the BM transduction/transplantation experiment. Spleens demonstrate complete disruption of architecture by infiltrating myeloid precursors, erythroid precursors, and megakaryocytes. Livers exhibit sinusoidal infiltration with maturing neutrophils, extramedullary erythropoiesis, and perivascular collections of neutrophils and macrophages. (**b**) Immunohistochemical staining of liver (**a**–**e**) and spleen (**f**–**j**) with antimyeloid marker antibody Ly-6. Note the massive infiltration of liver and spleen by maturing myeloid cells in mice injected with c-*Cbl*$^{-/-}$ and Wt marrow transduced by *Bcr-Abl*

Etten, 1995). Another explanation is that in addition to signaling through pathways common to p210$^{Bcr\text{-}Abl}$, p185$^{Bcr\text{-}Abl}$ also signals through additional pathways not activated by p210$^{Bcr\text{-}Abl}$. Although the majority of studies of p185$^{Bcr\text{-}Abl}$ and p210$^{Bcr\text{-}Abl}$ to date have found similar sets of tyrosine-phosphorylated proteins in transformed cells (Okuda *et al*., 1996), one exception is STAT6, a transcription factor normally activated by IL-4 and implicated in lymphoid proliferative responses (Kaplan *et al*., 1996), which is preferentially tyrosine phosphorylated and activated by p185$^{Bcr\text{-}Abl}$ but not p210$^{Bcr\text{-}Abl}$ (Ilaria and Van Etten, 1996).

In contrast to the *in vitro* studies, we found that c-Cbl is dispensable for *Bcr-Abl*-induced leukemogenesis *in vivo*. Recipient mice injected with c-*Cbl*$^{-/-}$ BM cells transduced by either p210$^{Bcr\text{-}Abl}$ or p185$^{Bcr\text{-}Abl}$ developed a fatal leukemia within 26 days of BM transplantation. There was no significant difference in survival between recipients of c-*Cbl*$^{-/-}$ versus Wt BM transduced with either p210$^{Bcr\text{-}Abl}$ or p185$^{Bcr\text{-}Abl}$ (Figure 3; Table 1). The disease in all mice was characterized by massive expansion of hematopoietic cells in the spleen, liver, and peripheral blood (Figure 4a). Immunostaining of liver and spleen samples revealed extensive infiltration of maturing myeloid cells (Figure 4b) in all mice. In a previous study, it was shown that the myeloproliferative disease induced by *Bcr-Abl* in Wt C57Bl/6 mice is distinct from that observed in Balb/*c* mice (Li *et al*., 2001). In this earlier report, only 50% of C57Bl/6-recipient mice injected with BM transduced with p210$^{Bcr\text{-}Abl}$ developed a myeloproliferative disease (Li *et al*., 2001). Some animals developed a B-lymphoid leukemia or macrophage tumors (Li *et al*., 2001). Those mice that developed a myeloproliferative disease exhibited a prolonged latency, 39 days compared to 22 days for Balb/*c* mice (Li *et al*., 2001).

We reasoned that one explanation for the low induction frequency and lengthened latency of myeloproliferative disease in C57/B6 mice observed in these studies is due to the low transduction rate of multipotent hematopoietic progenitor cells. To address this issue, we modified the existing protocol in two ways. Firstly, we administered increased levels of 5-FU to donor mice from 200 to 300 mg/kg per donor mouse, to further enrich the hematopoietic progenitor cell population in these mice. Secondly, we increased the amount of viral supernatant used to transduce BM cells by fivefold. It is likely that these experimental modifications contributed to the observed increased efficiency of myeloproliferative disease development in C57/B6 mice in the present study. All mice, with one exception, developed a similar CML-like myeloproliferative disease by day 26 following BMT. The exception was a single mouse that received Cbl null BM transduced by p210$^{Bcr\text{-}Abl}$. Pathological examination at day 50 revealed splenomegaly and elevated WBC counts but no hepatomegaly. H&E staining of the enlarged spleen showed marked infiltrates of leukemic cells similar to other diseased mice. One simple explanation for the extended survival of this mouse is that it received less transduced BM than others in the group. Lateral tail-vein injections can be problematic in C57/B6 mice due to poor visibility of the tail vein and infiltration of BM cells into the tissue surrounding the vein rather than into the vein itself can occur.

Our *in vitro* data suggest that Cbl does contribute to p210$^{Bcr-Abl}$ transformation. Bcr-Abl protein expression is also elevated in c-*Cbl*$^{-/-}$ cells *in vitro*, which suggests that the dependence of Bcr-Abl on Cbl for *in vitro* transformation may be greater than observed. In the BM transduction/transplantation model, mice injected with *c-Cbl*$^{-/-}$ marrow transduced with p210$^{Bcr-Abl}$ retrovirus showed a median survival of 21 days compared to 18 days for mice receiving Wt marrow. It is possible that if Bcr-Abl expression was elevated in *c-Cbl*$^{-/-}$ cells compared to Wt cells, as was observed *in vitro*, the survival time for animals receiving *c-Cbl*$^{-/-}$ bone cells may be shortened.

Although a prolongation of latency cannot be ruled out with certainty, our data indicate that Bcr-Abl induces leukemia in the absence of Cbl. Other studies of proteins that have been strongly implicated in *Bcr-Abl* function such as STAT5a/b (Sexl *et al*., 2000), Dok-1 (Di Cristofano *et al*., 2001), Crkl (Hemmeryckx *et al*., 2002), IL-3, and GM-CSF (Li *et al*., 2001) also failed to demonstrate the requirement of these proteins for *Bcr-Abl*-induced leukemia. One explanation for the observed redundancy of c-Cbl in *Bcr-Abl* function is that another protein(s) could functionally compensate for c-Cbl. One candidate is Cbl-b, which shows a 50% protein sequence similarity with c-Cbl (Keane *et al*., 1995), and, similar to c-Cbl, has been identified as a potential ubiquitin ligase (Joazeiro *et al*., 1999; Fang *et al*., 2001). A recent study showed that expression of *Cbl-b* is downregulated in *Bcr-Abl*-expressing cells (Sattler *et al*., 2002). Whether Cbl-b is also downregulated in c-*Cbl*$^{-/-}$ null cells expressing *Bcr-Abl* is unknown.

However, since *Bcr-Abl* interacts with multiple signaling proteins and consequently activates a number of signaling cascades, it is likely that other proteins can substitute for the function(s) performed by c-Cbl in *Bcr-Abl*-induced transformation.

Until now, all studies that used knockout mice failed to identify effector molecules that are essential for mediating the transforming capacity of *Bcr-Abl*. Frequently, these data were in contrast with previous results that were generated within different experimental systems, such as fibroblast transformation assays. Our study is in line with these observations. While there is an impairment of *in vitro* transformation, leukemogenesis in the *c-Cbl*$^{-/-}$ animals is identical to Wt. With respect to individual features of the *Bcr-Abl* protein, there is an analogous situation. For example, tyrosine 177, the Grb-2-binding site, is essential for fibroblast transformation but not for factor-independent growth (Pendergast *et al*., 1993; Cortez *et al*., 1995; Goga *et al*., 1995). Two important conclusions should be drawn. Firstly, *in vitro* assays have limited power for predicting the importance of a given molecule for transformation *in vivo*. Secondly, the signaling network operated by *Bcr-Abl* exhibits extensive redundancy. Thus, targeting single components for the therapy of *BCR-ABL*-positive leukemias may not be successful. It remains to be seen if removing more than one downstream effector will be capable of abrogating transformation by *Bcr-Abl*.

Materials and methods

Mouse strains

All animal studies were approved by the Animal Use and Care Committees (AUCC) of the Oregon Health & Science University. Wt, *Cbl*$^{+/-}$, and *Cbl*$^{-/-}$ mice (Naramura *et al*., 1998) were generously provided by Dr Hua Gu at the National Institutes of Health, Bethesda, MD, USA. Genotyping was performed by PCR according to previously published protocols (Naramura *et al*., 1998). All mice were of a C57B1/6 background.

Plasmids

An *Eco*RI p185$^{Bcr-Abl}$ fragment was subcloned into the *Eco*RI site of the retroviral transducing vector containing the GFP gene, MSCV-IRES-gfp (Zhang and Ren, 1998), to generate MSCV-p185^{Bc-Abl}-IRES-GFP. The MSCV-p210^{Bc-Abl}-IRES-GFP construct has been previously described (Zhang and Ren, 1998).

Antibodies

The rabbit polyclonal anti-c-Cbl antibody C-15 (Santa Cruz Biotechnology Inc.), rabbit polyclonal anti-phospho-Akt^{Ser473} antibody (Cell Signaling Technology), rabbit polyclonal anti-Akt antibody (Cell Signaling Technology), mouse monoclonal anti-phospho-JNK antibody (Santa Cruz Biotechnology Inc.), rabbit polyclonal anti-JNK antibody (Santa Cruz Biotechnology Inc.), mouse monoclonal phospho-p44/42 MAP kinase$^{Thr202/Tyr204}$ antibody E10 (Cell Signaling Technology), rabbit polyclonal p44/p42 MAPK antibody (Cell Signaling Technology), mouse monoclonal antiactin antibody (Oncogene Research Products), mouse monoclonal anti-Bcl-2 antibody (Transduction Laboratories), and the mouse monoclonal anti-c-Abl antibody 8E9 (kindly provided by Jean YJ Wang, UCSD, La Jolla, CA, USA) were used at a dilution of 1 : 1000 for immunoblotting. *C-Cbl*, Akt, p44/p42 MAPK, Bcl-2, JNK, and *Bcr-Abl* proteins were detected using a horse-radish peroxidase-conjugated anti-rabbit or anti-mouse antibody, respectively (Vector Laboratories) at a dilution of 1 : 5000. Actin protein was detected using a horse-radish peroxidase-conjugated anti-mouse antibody (Dako), and used at a dilution of 1 : 2000. Both primary and secondary antibodies were diluted in 5% nonfat dry milk in TBS-T. Proteins were visualized by chemiluminescence (Supersignal; Pierce, USA). Rat anti-mouse Ly-6G monoclonal antibody (BD Pharmingen) was used at a dilution of 1 : 40 for immunohistochemical staining of tissue sections.

Cell culture and retrovirus preparation

MEFs were established from 13.5-day-old embryos by using standard procedures and culture conditions (Goldman, 1998). MEFs, 293T, and Bosc23 cells (Pear *et al*., 1993) were maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% FBS, 1 U/ml penicillin, and 1 μg/ml streptomycin. MEFs and BM cells for the BM assay were infected with p210$^{Bcr-Abl}$ or p185$^{Bcr-Abl}$ retrovirus generated by transiently transfecting 293T cells with MSCV-p210^{Bc-Abl}-IRES-GFP, MSCV-p185$^{Bcr-Abl}$-IRES-GFP or MSCV-IRES GFP vector lacking *Bcr-Abl* (control retrovirus) together with ψ-Eco-packaging DNA (Muller *et al*., 1991). For the production of p210$^{Bcr-Abl}$, p185$^{Bcr-Abl}$, and control retrovirus for use in the BM transduction/transplantation experiments, Bosc23 cells were transiently transfected with MSCV-p210$^{Bcr-Abl}$-IRES-GFP, MSCV-p185$^{Bcr-Abl}$-IRES-GFP,

or MSCV-IRES-GFP control plasmid, and the viral supernatant harvested at 48 h post-transfection.

In vitro transformation assays

The soft-agar assay was performed as previously described (Gaston *et al*., 2000). Briefly, MEFs were infected with $p210^{Bcr\text{-}Abl}$, $p185^{Bcr\text{-}Abl}$ or control retroviral supernatant containing 8 μg/ml polybrene, and incubated at 37°C for 48 h. Prior to seeding in soft agar, the infection efficiency was determined by measuring the percentage of GFP-positive cells in each cell line. Approximately 1×10^5 GFP-positive cells expressing $p210^{Bcr\text{-}Abl}$ and $p185^{Bcr\text{-}Abl}$ were seeded in soft agar in duplicate six-well tissue culture dishes. Colonies were counted 2–3 weeks later. Soft-agar assays were performed on cells derived from two separate retroviral infections.

The BM assay was performed as described previously (Whitlock-Witte cultures; McLaughlin *et al*., 1987). Briefly, BM cells were isolated from the tibias and femurs of 4-week-old c-$Cbl^{-/-}$, c-$Cbl^{+/-}$, and Wt mice, and infected with $p210^{Bcr\text{-}Abl}$, $p185^{Bcr\text{-}Abl}$ or control retrovirus in lymphoid media (RPMI plus 5% fetal calf serum, 1 U/ml penicillin, 1 μg/ml streptomycin, 1% L-glutamine, and 0.05 mM 2-mercaptoethanol) containing 8 μg/ml polybrene. Approximately 5×10^6 cells were plated at a density of 1×10^6 cells/ml cells per 60 mm dish in duplicate. Cultures were fed every 3 days by adding 2 ml lymphoid media to each culture dish. B-lymphoid cells were counted at day 16 postinfection.

BM transduction/transplantation

BM cell transduction and transplantation was performed as previously described (Zhang and Ren, 1998) with the following exceptions. BM cells were isolated from the tibias and femurs of 10–12-week old c-$Cbl^{-/-}$, c-$Cbl^{+/-}$, and Wt donor mice 4–5 days after intravenous treatment with 300 mg/kg of 5-fluorouracil (5-FU; Sigma). Since the c-$Cbl^{-/-}$ mice were obtained from crosses of homozygous c-$Cbl^{-/-}$ and c-$Cbl^{+/-}$ mice, BM was pooled from both Wt and c-$Cbl^{+/-}$ mice for controls. BM cells (2×10^6 cells) from c-$Cbl^{-/-}$ animals or BM cells pooled from Wt and c-$Cbl^{+/-}$ animals were infected with either $p210^{Bcr\text{-}Abl}$, $p185^{Bcr\text{-}Abl}$ or control retroviral supernatant in DMEM (final concentration of viral supernatant of 35%), containing 1 U/ml penicillin, 1 μg/ml streptomycin, 2 mM L-glutamine, 15% fetal bovine serum, 15% WEHI, 7 ng/ml interleukin-3, 12 ng/ml interleukin-6, 56 ng/ml stem cell factor, and 3 μg/ml polybrene, by two rounds of spinoculation (Pear *et al*., 1998). Following infection, the cells were washed extensively in phosphate-buffered saline (PBS), and 6.25×10^5 cells were injected into the lateral tail vein of recipient mice that had been exposed to two doses of 600 rad whole-body irradiation in a cesium irradiator administered 4 h apart. After transplant, recipients were housed in microisolator cages supplied with acidified (pH 2.0) water supplemented with antibiotics (Septra IV).

Pathological examination of diseased mice

For survival analysis, the death end point was determined either by spontaneous death of the animal or by elective killing of the animal, because of signs of pain or suffering according to established criteria (AUCC Oregon Health & Science University). For pathological analysis, premoribund mice were killed by CO_2 asphyxiation, and the liver and spleens were harvested and analysed histologically. Paraffin-embedded thin sections of liver and spleen were stained with H&E. Peripheral blood smears were stained with Wright/Giemsa stain. For immunohistochemistry, 5-μm cryostat sections of the snap-frozen tissue were prepared and stained with the anti-myeloid marker antibody Ly-6 (BD Pharmingen), according to previously established protocols (Rader *et al*., 2001).

Immunoblotting

Cells were lysed in a buffer containing 20 mM Tris, 1 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 10 μg/ml aprotinin, 1 mM Na_3VO_4, and 1 mM phenylmethylsulfonylfluoride at a concentration of 5×10^7 cells/ml. Lysates were precleared by centrifugation (6000 *g*) for 10 min, and protein concentrations were determined using the Bradford assay (BioRad, Hercules, CA, USA). For immunoblotting, 500 μg of each sample was boiled in SDS sample buffer, and separated by SDS–PAGE. Proteins were transferred onto PVDF (Immobilon-P, Millipore, Bedford, MA, USA) membranes in a buffer containing 25 mM Tris, 192.5 mM glycine, and 20% MeOH for 4 h at 0.55 A. Following transfer, the membrane was blocked with 5% nonfat dry milk in TBS-T (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween-20) for 60 min at 25°C. Membranes were incubated for 2 h at room temperature with the appropriate primary antibody, then washed three times in TBS-T for 15 min each prior to incubation for 1 h at RT in the appropriate secondary antibody.

Statistics

All data are represented as the standard error of the mean. Survival curves were generated by Kaplan–Meier survival analysis and compared using the log-rank test. All other statistical comparisons were performed using the two-tailed *t*-test with a significance level of $P < 0.05$.

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