

ORIGINAL ARTICLE

Gads (Grb2-related adaptor downstream of Shc) is required for BCR-ABL-mediated lymphoid leukemia

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Philadelphia chromosome-positive leukemias, including chronic myeloid leukemia and B-cell acute lymphoblastic leukemia (B-ALL), are driven by the oncogenic BCR-ABL fusion protein. Animal modeling experiments utilizing retroviral transduction and subsequent bone marrow transplantation have demonstrated that BCR-ABL generates both myeloid and lymphoid disease in mice receiving whole bone marrow transduced with BCR-ABL. Y177 of BCR-ABL is critical to the development of myeloid disease, and phosphorylation of Y177 has been shown to induce GRB2 binding to BCR-ABL, followed by activation of the Ras and phosphoinositide 3 kinase signaling pathways. We show that the GRB2-related adapter protein, GADS, also associates with BCR-ABL, specifically through Y177 and demonstrate that BCR-ABL-driven lymphoid disease requires Gads. BCR-ABL transduction of Gads (–/–) bone marrow results in short latency myeloid disease within 3–4 weeks of transplant, while wild-type mice succumb to both a longer latency lymphoid and myeloid diseases. We report that GADS mediates a unique BCR-ABL complex with SLP-76 in BCR-ABL-positive cell lines and B-ALL patient samples. These data suggest that GADS mediates lymphoid disease downstream of BCR-ABL through the recruitment of specific signaling intermediates.

Leukemia (2013) 27, 1666–1676; doi:10.1038/leu.2013.40

Keywords: chronic myeloid leukemia; BCR-ABL; GADS; GRB2; bone marrow transplantation

INTRODUCTION

BCR-ABL, the product of the Philadelphia (Ph) chromosome, is formed through a reciprocal translocation between chromosomes 9 and 22.^{1,2} When this translocation occurs, the break point cluster region (BCR) gene from chromosome 22 is fused to the tyrosine kinase ABL gene from chromosome 9,^{3,4} forming the fusion protein BCR-ABL, resulting in constitutive activation of the ABL tyrosine kinase.⁵ BCR-ABL is the causative agent in Ph chromosome-positive (Ph⁺) leukemias, including chronic myeloid leukemia (CML)^{3,4} and B-cell acute lymphoblastic leukemia (B-ALL).⁶

The pathogenesis of CML caused by BCR-ABL can be modeled in the murine bone marrow transplant (BMT) assay. BCR-ABL-transduced bone marrow progenitors (from 5-fluorouracil (5-FU)-primed donors) injected into lethally irradiated recipient mice gives rise to fatal myeloproliferative disease (MPD) that resembles human CML within 3–4 weeks.^{7,8} When whole bone marrow from non-5-FU-primed donors is used for transduction and transplantation, a model of BCR-ABL-mediated B-ALL is observed. In the latter model, BMT recipients succumb to a mixture of disease phenotypes, including a B-cell disease that resembles human B-ALL.⁹

Tyrosine (Y) 177 of BCR-ABL is critical to the development of CML-like disease in mice.^{10–12} Phosphorylation of this residue is responsible for the SH2 domain-dependent binding of the adapter protein Grb2 that serves to couple BCR-ABL to the Gab2 adapter protein.¹³ In turn, Gab2 recruitment leads to the activation of the Ras^{14,15} and phosphoinositide 3 kinase¹⁶ signaling pathways required for BCR-ABL-mediated transformation.^{3,4,14,17}

Gads (Grb2-related adapter protein downstream of Shc) is a Grb2 family member and has been shown to interact with

BCR-ABL through *in vitro* studies.^{18,19} Like its family members Grb2 and Grap,²⁰ Gads possess a central SH2 domain, flanked by two SH3 domains.²¹ Although the SH2 domains of all three family members have similar binding specificities,^{11,18,22} the carboxy terminal SH3 domain of Gads possesses unique binding specificity that allows for interaction with the adapter protein SLP-76 (Src homology 2 (SH2) domain-containing leukocyte protein of 76 kDa).²³ Both Gads²⁴ and SLP-76²⁵ are required for normal T-cell development. Upon T-cell receptor (TCR) activation, the Gads-SLP-76 complex is recruited to tyrosine-phosphorylated LAT (linker for activation of T cells) via the SH2 domain of Gads.^{22,26} The formation of this adapter protein complex allows for the aggregation of signaling complexes critical to T-cell activation, including those leading to cytoskeletal changes, interleukin (IL)-2 gene expression and proliferation.²⁷

Previous studies have sought to clarify the molecular events that differentiate Ph⁺ myeloid disease (CML) and lymphoid disease (B-ALL), which have led to the identification of unique activation of SRC family tyrosine kinases in lymphoid disease.²⁸ Compared with CML, Ph⁺ B-ALL has historically been resistant to therapy and associated with poor clinical outcomes.^{29–32} The identification of unique pathways that distinguish the two diseases could provide insight into the identification of novel therapeutic targets to treat Ph⁺ ALL. Due to its essential role in lymphocyte signaling and development and its ability to interact with BCR-ABL through its SH2 domain, we tested whether Gads could serve as a candidate for mediating BCR-ABL-mediated lymphoid disease. Through the use of the murine BMT assay, we determined that Gads is required for BCR-ABL-mediated lymphoid disease but is inconsequential for BCR-ABL-mediated myeloid

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Received 16 November 2012; revised 31 January 2013; accepted 6 February 2013; accepted article preview online 12 February 2013; advance online publication, 8 March 2013

disease. GADS is expressed in a subset of CML cell lines and B-ALL patient samples and we show in these samples that GADS associates with both BCR-ABL and SLP-76. Together these data provide evidence that signaling through Gads is critical to the development of BCR-ABL-mediated lymphoid disease.

MATERIALS AND METHODS

DNA constructs

The Mig210 construct was used to express the p210 isoform of BCR-ABL in BMT experiments. The vesicular stomatitis virus G and pSV ϕ ⁻env⁻ plasmids were used to generate high titer retroviral supernatants.

Animals

Studies were approved by the Animal Care Committee at the Ontario Cancer Institute (OCI) and The Center for Phenogenomics, Toronto, ON, Canada. The generation of Gads(−/−) mice was previously described.²⁴ Gads(−/−) mice were back-crossed to the BALB/c for eight generations. Wild-type BALB/c donor and recipient mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). All donor and recipient mice were used at 6–8 weeks of age.

Generation of retroviral stocks

Calcium phosphate transfections (CalPhos Mammalian Transfection Kit, Clontech, Mountain View, CA, USA) of 293T cells were performed with retrovirus, pSV ϕ ⁻env⁻ packaging construct and vesicular stomatitis virus G envelope vector. Harvested virus was filtered and concentrated by ultracentrifugation at 53 000 *g* for 2 h at 4 °C. Viral pellets were stored at −80 °C.

Ba/F3 cells were transduced with retroviral supernatant for estimation of retroviral titer. Cells were collected 48 h post infection and analyzed by flow cytometry for green fluorescent protein (GFP) expression. The relative viral titer in colony forming units (CFUs)/ml was calculated as the percentage of GFP-positive (GFP⁺) cells multiplied by the number of cells infected and divided by the volume of supernatant. All retroviral stocks were used at titers of 2×10^5 CFUs/ml.

Bone marrow transduction and transplantation

The induction of CML-like and B-ALL-like disease has been described previously.⁹ To model CML-like disease, donor mice were primed with a 200 mg/kg 5-FU (Sigma-Aldrich, St Louis, MO, USA) intraperitoneal injection 4 days before bone marrow harvest. Bone marrow cells were flushed from femurs and tibias with phosphate-buffered saline and plated in a prestimulation cocktail containing murine IL-3, murine IL-6 and murine stem cell factor. Twenty-four hours post plating, trypsinized bone marrow cells, at a concentration of 1×10^6 WBCs/ml, were subjected to two rounds of infection (24 h each) in prestimulation cocktail plus 3 μ g/ml Polybrene and virus.

Transduced bone marrow cells were collected, washed and resuspended in phosphate-buffered saline. Following lethal irradiation (9 Gy), each recipient mouse received 0.5×10^6 white blood cells through intravenous injection. CML-like and B-ALL disease was induced in a similar manner, with the exception that donors were not primed with 5-FU, and 10 ng/ml rmlL-7 was added to the prestimulation and infection cocktails. Each irradiated recipient mouse received 1×10^6 white blood cells through intravenous injection.

Analysis of diseased mice

Transplant recipient mice were monitored closely for signs of disease as evidenced by cachexia, peripheral white blood counts and GFP expression. GFP expression in the peripheral blood was measured by flow cytometry. Hematopoietic tissues were harvested at killing. Peripheral blood smears and bone marrow cytopins were stained with May-Grünwald and Giemsa (both from EMD Chemicals Inc., Gibbstown, NJ, USA), while the spleen, liver and lung sections were stained with hematoxylin (Sigma-Aldrich) and eosin (Sigma-Aldrich). Spleen single-cell suspensions were created by manual force through wire mesh, and erythrocytes were lysed.

Cell culture

All human CML cell lines and Ba/F3 BCR-ABL cell lines were maintained in RPMI-complete media (RPMI 1640 containing 10% vol/vol fetal calf serum and 50 μ M β -mercaptoethanol). Ba/F3 cells were maintained in RPMI

complete media plus IL-3. 293T cells were expanded in Iscove's Modified Dulbecco's Media plus antibiotics supplemented with 10% fetal calf serum.

Preparation of GST (glutathione S-transferase)-fusion proteins

Gads and Grb2 recombinant GST-fusion proteins were expressed and purified as described previously.³³ Fusion proteins were quantified by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) followed by Coomassie staining and compared with bovine serum albumin standards.

For *in vitro* pull-down experiments, 2 mg of BaF3 BCR-ABL and BaF3 BCR-ABL Y177F cell lysate was incubated overnight at 4 °C with equal amounts of each GST-fusion protein coupled to glutathione sepharose 4B beads. The beads were washed three times with 20 mM TrisHCl (pH 8.0), 100 mM NaCl, 0.5 mM EDTA, 0.5% (w/v) Nonidet P40 lysis buffer and resuspended in SDS-Laemmli sample buffer. Proteins were resolved via SDS-PAGE and transferred to PVDF membranes for further western blotting analysis.

Antibodies

Polyclonal anti-Gads antibody was prepared as described previously.¹⁸ Anti-SLP-76 polyclonal antisera was provided by Dr Gary Koretzky, University of Pennsylvania. The polyclonal anti-Grb2 antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Monoclonal anti-phosphotyrosine antibody (4G10) was Millipore (Billerica, MA, USA). Mouse monoclonal c-Abl (8E9) was obtained from BD Biosciences (San Jose, CA, USA). Secondary antibodies, including horseradish peroxidase (HRP)-labeled Protein A, HRP-linked mouse immunoglobulin G (from sheep) and Donkey anti-sheep-HRP secondary were obtained from GE Healthcare (Piscataway, NJ, USA).

Immunoprecipitation and immunoblotting

Lysates from CML and BaF3 cells were prepared as previously described.³⁴ Samples were resolved by SDS-PAGE and transferred to PVDF transfer membrane for western blotting.

For the detection of proteins via western blotting, membranes were blocked for 1 h. Primary antibodies were diluted in TBST and membranes were incubated for a minimum of 1 h at room temperature. Membranes were then washed three times in TBST and incubated at room temperature for 1 h with the appropriate HRP-conjugated secondary antibody. Membranes were again washed three times in TBST and developed using Western Lightning Chemiluminescence Reagent Plus (ECL; PerkinElmer, Waltham, MA, USA) detection.

Flow cytometry

Cells were blocked with anti-mouse CD16/CD32 (2.4G2; BD Biosciences) and stained with a combination of fluorescently labeled primary antibodies. All flow cytometry antibodies were acquired from BD Biosciences, including: phycoerythrin (PE)-conjugated anti-mouse Thy-1.2 (53-2.1), CD4 (H129.19), TER-119, B220 (RA3-6B2) and Gr-1 (RB6-8C5); PerCP-Cy5.5-conjugated rat monoclonal CD8a (53-6.7) and CD11b/Mac-1 (M1/70). For analysis of SLAM markers, lineage-negative cells were removed by using a MACS lineage cell depletion kit (Miltenyi Biotec, Auburn, CA, USA). CD48 and CD150 antibodies were added and detected as described below.

Flow cytometry data were collected on a FACSCalibur machine (BD Biosciences) and analyzed with FlowJo software (Ashland, OR, USA).

Patient samples

Peripheral blood and bone marrow samples were obtained at the time of diagnosis following informed consent as approved by the University Health Network Research Ethics Board. The diagnosis of ALL was made by hematopathologists not involved in the direct care of the patients. Conventional bone marrow cytogenetics analysis was performed in the clinical cytogenetics laboratory of the University Health Network. Blast cells were enriched by Ficol-Hypaque centrifugation and cryopreserved at −150 °C in 10% dimethyl sulfoxide, 40% fetal bovine serum and alpha Minimum Essential Medium at a concentration of $1\text{--}5 \times 10^7$ cells/ml. Frozen cells were thawed quickly at 37 °C, then washed once with HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)/Hanks buffer containing protease inhibitors. Samples were lysed on ice for 1 h using Triton-X-100 lysis buffer as described above. Cleared lysate was then used in immunoprecipitation and immunoblotting experiments, as described above.

RESULTS

Gads is required for BCR-ABL-mediated lymphoid disease

Bone marrow transplantation experiments were performed utilizing wild-type and Gads (−/−) donor bone marrow. Following infection with BCR-ABL, transduced bone marrow was transplanted into lethally irradiated recipient wild-type recipient mice. Mice transplanted with BCR-ABL-transduced wild-type bone marrow cells succumbed to either a CML-like (myeloid) or B-ALL-like (lymphoid) disease over a period of 19–69 days (Figure 1, Table 1), as previously reported.⁹ By contrast, littermates that were transplanted with Gads −/− bone marrow expressing BCR-ABL exclusively succumbed to a short latency CML-like (myeloid) disease within 35 days (Figure 1, Table 1). None of the recipient mice that were transplanted with BCR-ABL-transduced Gads(−/−) bone marrow developed lymphoid disease.

Flow cytometric analysis of the spleen further confirmed that the CML-like disease in both wild-type and Gads(−/−) donors was due to myeloid expansion as indicated by a large number of GFP-expressing Gr-1 and Mac-1-positive cells (Figure 2). In recipient mice identified as having a B-ALL-like disease derived from wild-type bone marrow expressing BCR-ABL, flow cytometric analysis of spleen cells revealed a large number of GFP-positive B cells, which were positive for B220 and/or CD19 (Figure 2).

Leukemic cells were observed in peripheral blood smears isolated from both wild-type and Gads(−/−) mice (Figure 3a). Recipient mice transplanted with BCR-ABL-positive wild-type bone marrow expressing BCR-ABL had increased white blood cells and elevated neutrophil numbers on differential counting (Figure 3b). Similarly, Gads(−/−) mice expressing BCR-ABL displayed an elevated total white blood cell count with a differential shift towards neutrophils (Figure 3b). Increased blast cells were observed in bone marrow smears from wild-type animals with B-ALL-like disease (Figure 3a). These mice also had a lower peripheral blood white blood cell count, with a differential shift towards blast cells (Figure 3b).

All mice that developed disease after transplant of BCR-ABL-transduced bone marrow developed splenomegaly and hepatomegaly due to extramedullary hematopoiesis (Figure 4). Splenic architecture was disrupted, and distinction between white and red pulp was lost (Figure 4, top panels). Tumor cells (stained dark purple, in contrast to normal hepatocytes) infiltrated the liver, particularly the sinusoids (Figure 4, middle panels). Lung hemorrhages, stained pink, were present in all the diseased mice (Figure 4, bottom panels).

Gads is dispensable for BCR-ABL-mediated myeloid disease

Human CML is modeled in mice by infecting bone marrow cells from donor mice primed with 5-FU, with a retrovirus expressing BCR-ABL. BCR-ABL-transduced bone marrow cells from 5-FU-primed donors, both wild-type and Gads(−/−), were used to transplant lethally irradiated recipient mice. All recipient mice succumbed to a CML-like disease, within 3–4 weeks of transplant. There were no differences in disease phenotype or latency (Supplementary Figure S1, Supplementary Table S1). Flow cytometry confirmed comparable expression of Gr-1 and Mac-1 in wild-type and Gads −/− splenocytes, confirming MPD (Supplementary Figure S2). Differential counts were comparable between both mouse models (Supplementary Figure S3). Qualitative analysis of peripheral blood and bone marrow fractions isolated from wild-type and Gads(−/−) mice revealed similar number of myeloid cells in both the fractions (Supplementary Figure S3). Extramedullary hematopoiesis was observed in the spleen and liver, and hemorrhages were observed again in the lungs (Supplementary Figure S4).

CD48⁺CD150[−] and common lymphoid progenitors (CLP) are increased in Gads-deficient mice

One explanation for the requirement of Gads to mediate lymphoid disease in our model could be due to alterations in the stem cell

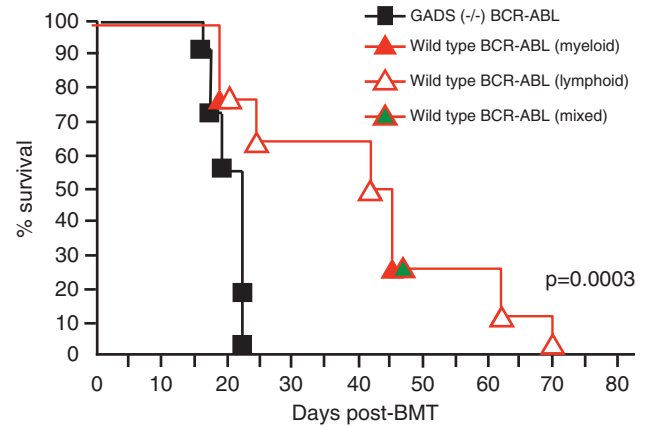


Figure 1. Gads is required for lymphoid disease induced by BCR-ABL. BCR-ABL-transduced bone marrow from non-5-FU donors was transplanted into wild-type recipient mice. Recipients of Gads −/− marrow showed reduced survival in comparison to recipients of WT marrow. The difference in survival is significant ($P=0.004$), as determined by log-rank analysis.

Table 1. Summary of latency and disease phenotypes in BMT recipients (non-5-FU-treated donors)

Donor bone marrow	Latency (days)	Disease phenotype
Wild type	19	Myeloid
Wild type	19	Lymphoid
Wild type	25	Lymphoid
Wild type	42	Lymphoid
Wild type	45	Myeloid
Wild type	65	Lymphoid
Wild type	69	Lymphoid
Gads(−/−)	18	Myeloid
Gads(−/−)	19	Myeloid
Gads(−/−)	19	Myeloid
Gads(−/−)	20	Myeloid
Gads(−/−)	20	Myeloid
Gads(−/−)	21	Myeloid
Gads(−/−)	21	Myeloid
Gads(−/−)	21	Myeloid
Gads(−/−)	24	Myeloid
Gads(−/−)	24	Myeloid

Moribund transplant recipients were killed and relevant tissues were collected and analyzed via flow cytometry. Diagnosis of myeloid or lymphoid disease is based on the presence of Gr-1 and Mac-1 (myeloid) or B220 and CD19 (lymphoid) cell surface markers.

pool of Gads(−/−) donor mice. Therefore, we analyzed expression of Kit⁺Sca-1⁺Lin[−] and CD48[−]CD150⁺ cells. Increased numbers of Kit⁺Sca-1⁺Lin[−] cells were found in Gads-deficient mice when compared with wild-type littermates (Figure 5a). Statistically significant changes were observed in Kit⁺Sca-1⁺Lin[−] and Sca-1⁺Lin[−] cells (Figure 5b). We show that Gads −/− mice have elevated CD48[−]CD150⁺ cells³⁵ when compared with wild-type littermates (Figure 5c and d).

These data led us to investigate CLP, common myeloid progenitors (CMP) and granulocyte-macrophage progenitors (GMP) in wild-type and Gads-deficient mice (Figure 5). Similarly, CLP are also increased in Gads-deficient mice (Figures 6a and c), whereas no significant differences were observed in CMP or GMP (Figures 6b and c). These data suggest that the failure to observe lymphoid disease in recipient mice transplanted with Gads-deficient bone marrow expressing BCR-ABL is not due to a lack of hematopoietic stem cells and CLP.

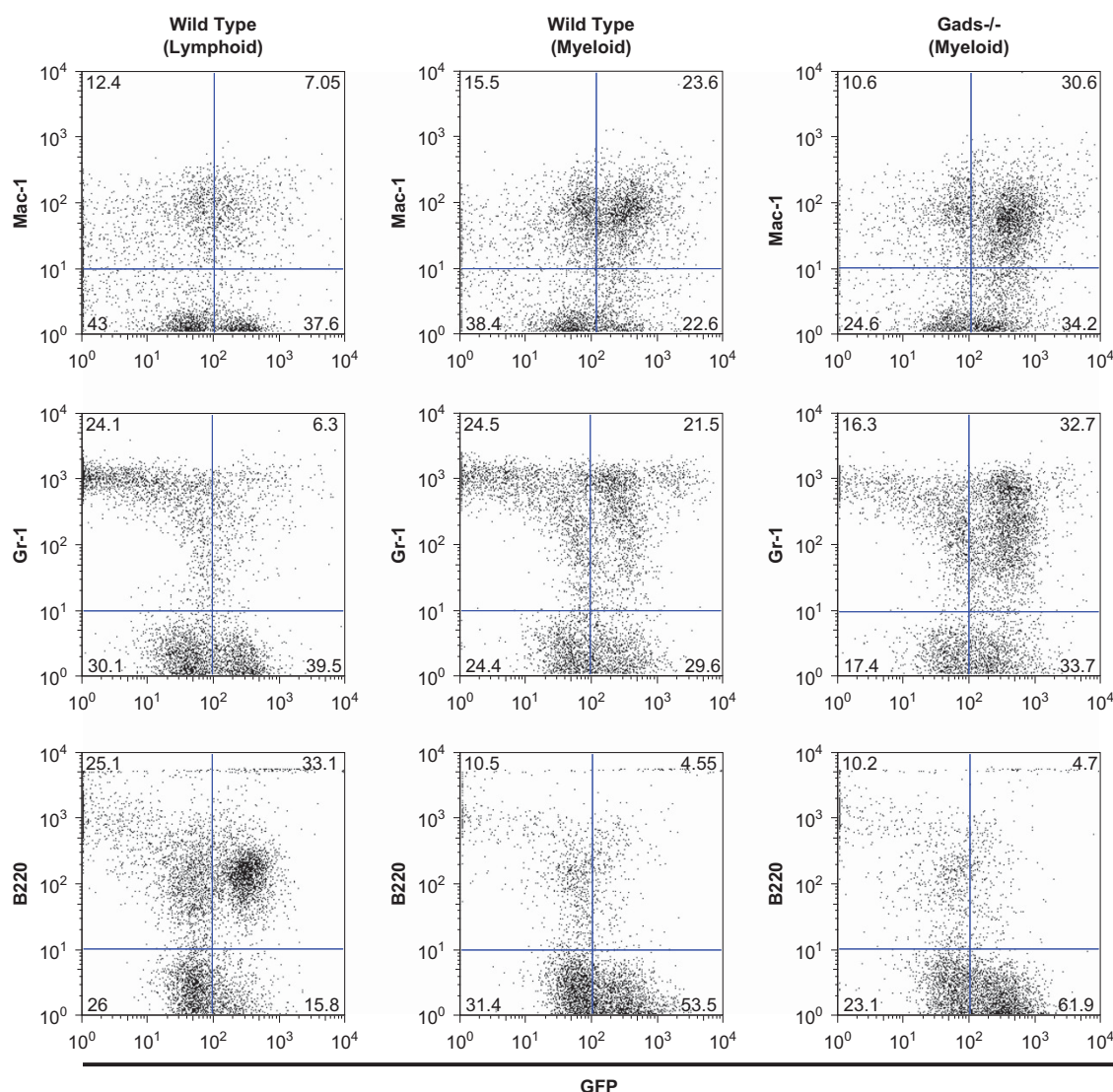


Figure 2. BCR-ABL induces a mixed disease phenotype in recipients of non-5-FU-treated donor cells. Spleen cells from transplant recipients were analyzed by flow cytometry to determine the forward and side scatter profiles and the proportions of GFP-expressing B220 + , CD19 + , Gr-1 + and Mac-1 + cells. Wild-type recipients were diagnosed as either lymphoid (B220 + and CD19 +) or myeloid (Gr-1 + and Mac-1 +) whereas Gads-deficient recipients were diagnosed exclusively as myeloid.

Gads is expressed in CML cell lines

To identify potential signaling pathways that depend on the presence of GADS, we examined several Ph⁺ human leukemia cell lines for Gads expression. Western blot analysis of CML-T1,³⁶ K562,³⁷ EM2,³⁸ EM3,³⁸ LAMA-84³⁹ and MC3⁴⁰ cell lysates confirmed the presence of BCR-ABL as a 210-kDa phosphoprotein. Of the lines tested, all expressed GRB2 (not shown), while CML-T1 and LAMA-84 showed the greatest expression of GADS protein (Figure 7a).

BCR-ABL Y177 recruits Gads

Gads and Grb2 both have central SH2 domains with similar binding specificities.¹⁸ The SH2 domain of Grb2 is known to interact specifically with phosphorylated Y177 of BCR-ABL.^{10–12} Given a role for Gads in BCR-ABL-mediated lymphoid disease, we hypothesized that pY177 mediates an interaction between Gads and BCR-ABL.

BaF/3 cells expressing BCR-ABL or BCR-ABL with a tyrosine-to-phenylalanine mutation at Y177 (Y177F) were used in an *in vitro* pull-down experiment with GST-Gads fusion proteins

(Figure 7b). Gads binds to BCR-ABL in BaF/3 cell lysates. Presence of the Y177F mutation abolishes this interaction, which supports the prediction that BCR-ABL Y177 can recruit both Gads and Grb2.

GADS is tyrosine phosphorylated downstream of BCR-ABL

Although GADS has not previously been reported to be tyrosine phosphorylated, we noted the presence of a prominent band at the same molecular weight as GADS. To further investigate whether GADS is tyrosine phosphorylated in the CML-T1 cell line, anti-phosphotyrosine immunoprecipitates were performed and blotted with anti-GADS antibody (Figure 7c). GADS phosphorylation was blocked by incubation with Dasatinib, demonstrating that GADS is a target of ABL and/or SRC kinases.

GADS forms a unique signaling complex with BCR-ABL

Previous studies have shown that GRB2 is required to couple BCR-ABL to Ras and PI3K signaling pathways, which are critical in the development of Ph⁺ leukemias. Although experiments have

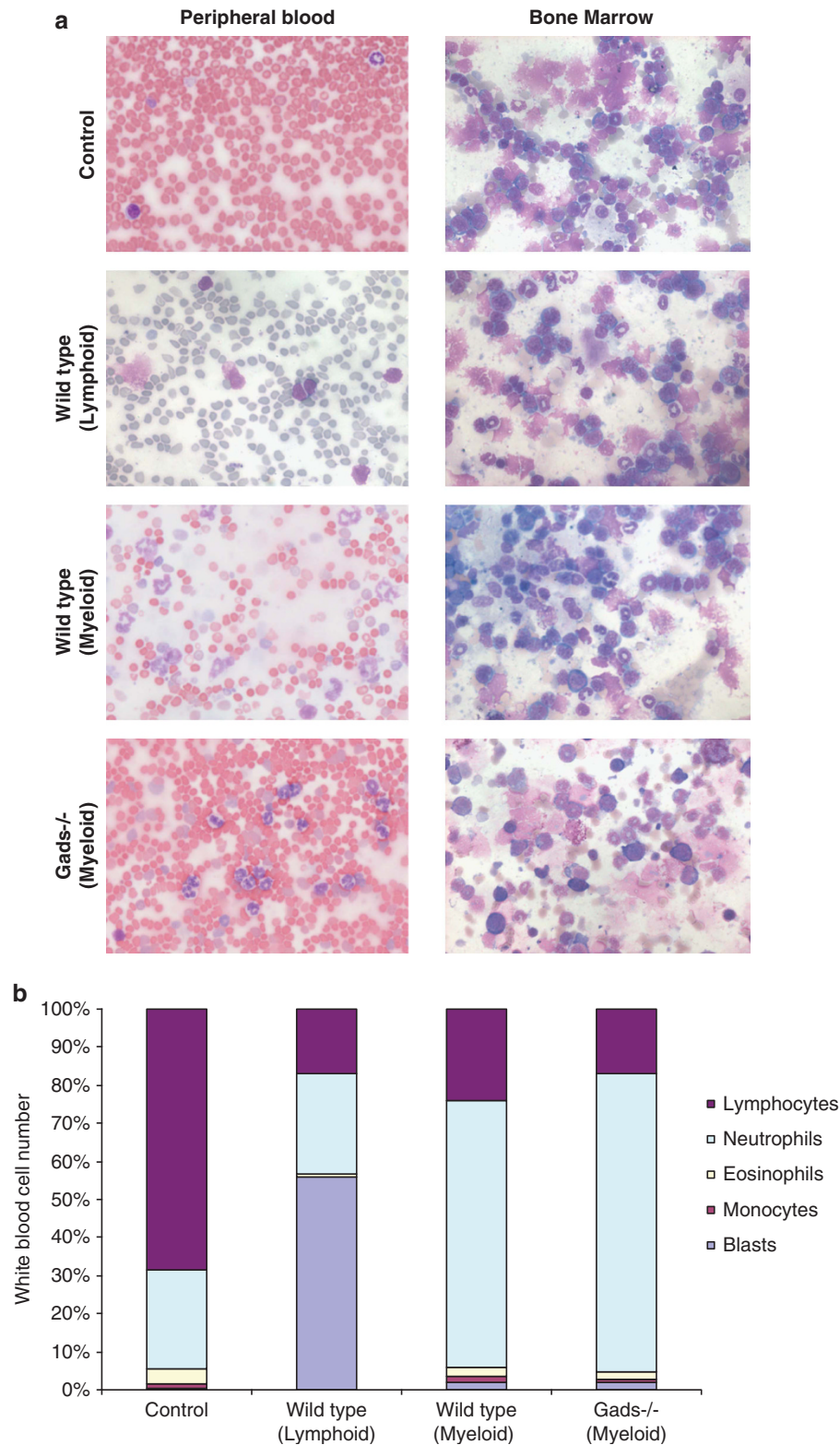


Figure 3. Disease phenotypes are confirmed via histopathology and differential counting. **(a)** Histopathological analysis of peripheral blood and bone marrow was visualized by microscopic evaluation of slides stained with May-Grünwald and Giemsa. Original magnification $\times 400$. **(b)** Differential counts were recorded for multiple animals (wild type ($n = 5$); Gads^{-/-} ($n = 8$); for control non-transplanted mice ($n = 4$)) through counts of at least 200 total white blood cells in randomly selected sections of the peripheral blood smear. Average count of each compartment is reported.

shown that ectopically expressed GADS can bind to BCR-ABL, the specific role of GADS downstream of BCR-ABL has not been determined.

Endogenous GADS, GRB2 or SLP-76 was immunoprecipitated from CML-T1 cells lysates, and associated proteins were detected by western blotting with specific antibodies (Figure 7d). BCR-ABL,

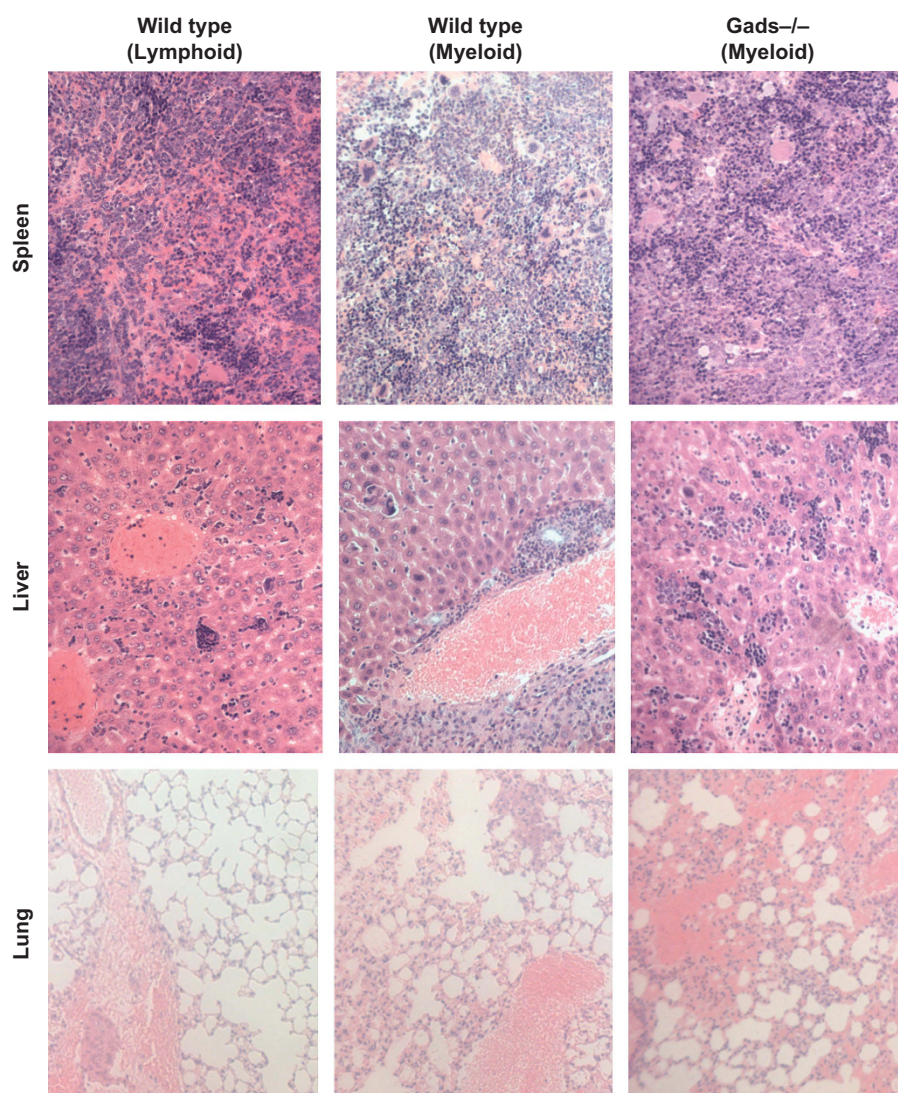


Figure 4. Extramedullary hematopoiesis is found in bone marrow transplant recipients via histopathology. Spleen, liver and lung sections from transplant recipients were stained with hematoxylin and eosin and visualized by light microscopy. Original magnification ($\times 200$).

detected with an anti-ABL antibody, was present in GRB2, GADS and SLP-76 immunoprecipitations. GAB2 also co-precipitated with all three proteins, though to a much lesser degree with SLP-76. There was reciprocal association of SLP-76 and GADS in immunoprecipitation experiments; however, GRB2 did not associate with SLP-76. These data suggest that similar to GRB2, GADS is capable of coupling BCR-ABL to GAB2-dependent pathways but that it also recruits SLP-76 to the complex, potentially activating unique signaling pathways.

GADS forms a complex with SLP-76 in primary B-ALL patient samples

Our biochemical analysis of the CML-T1 cell line suggests that GADS may have a role signaling downstream of BCR-ABL, and the outcome of bone marrow transplantation experiments demonstrate that Gads is necessary for the development of BCR-ABL-mediated lymphoid disease. To determine whether GADS and SLP76 proteins are expressed in BCR-ABL-positive lymphoid cells from patients, we obtained viably frozen cells from Ph⁺ adult B-ALL patients treated at Princess Margaret Hospital. Lysates from B-ALL patient samples were analyzed for GRB2, GADS and SLP-76 expression by western blot (Figure 8a). Although GRB2 was

uniformly expressed in all the B-ALL samples, GADS and SLP-76 were expressed in a subset. GADS immunoprecipitations were performed on those samples that were positive for GADS protein and were blotted with anti-SLP-76. GADS was observed to form a complex with SLP-76 in a subset of primary B-ALL patient samples (Figure 8b).

DISCUSSION

Several studies have shown that BCR-ABL Y177 is required for leukemogenesis.^{10–12} As Y177 falls into a consensus binding motif for SH2-dependent binding of Grb2, it has been assumed that Grb2 mediates the recruitment of Gab2¹³ and subsequent activation of Erk and Akt signaling pathways. However, Grb2-deficient mice are embryonic lethal,⁴¹ precluding a murine bone marrow transplant study using BCR-ABL-transduced bone marrow from Grb2-deficient mice. Therefore, the role of Grb2 or other SH2 domain-containing adapters in BCR-ABL-mediated leukemogenesis had not been well defined.

The hematopoietic adapter protein Gads is critical during T-cell development. Thymocytes from Gads (–/–) mice have impaired positive selection and proliferation due to defects in pre-TCR signaling.²⁴ The carboxy terminal SH3 domain of Gads alone

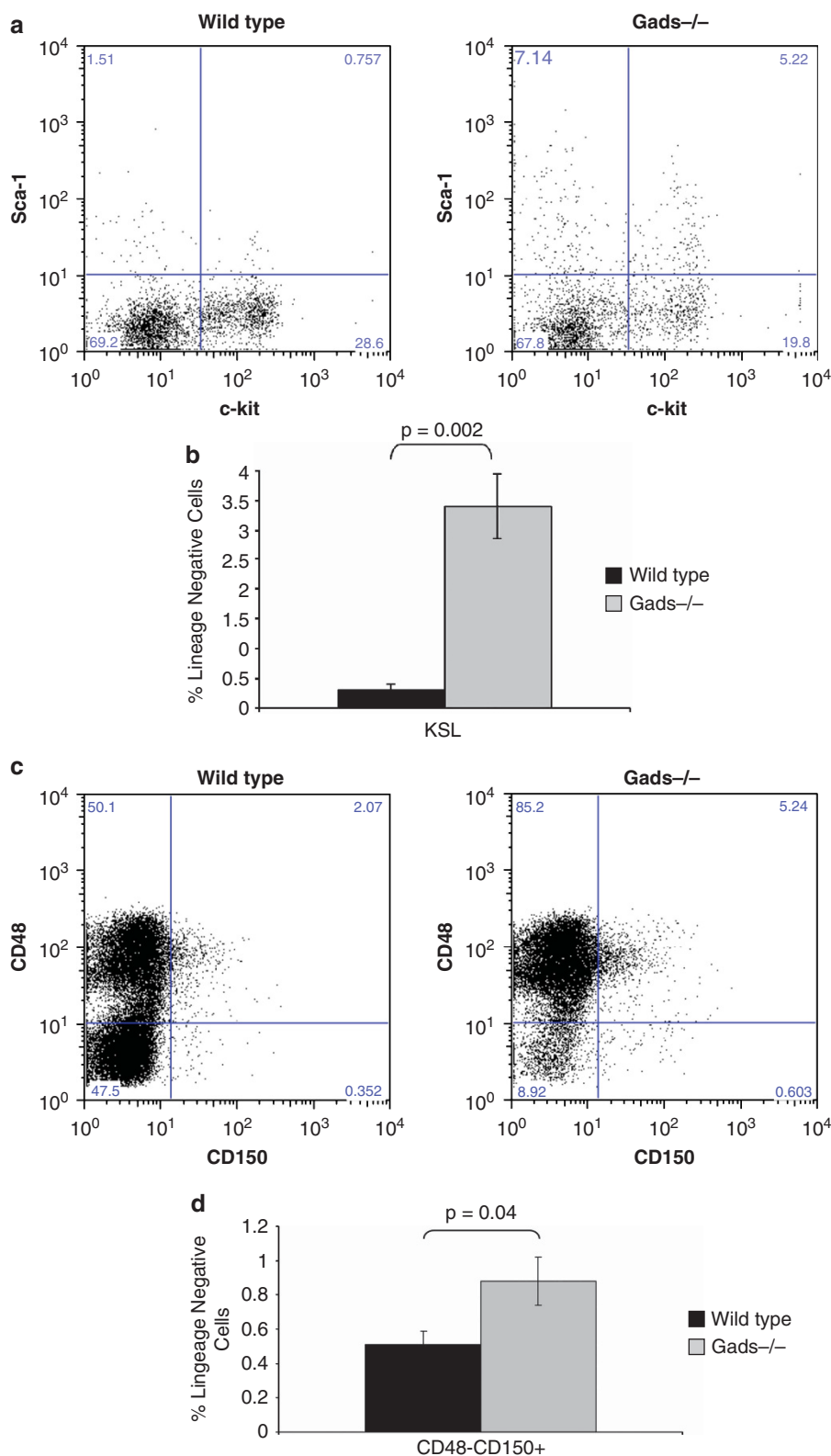


Figure 5. Stem cell progenitors are increased in Gads-deficient bone marrow. **(a)** Bone marrow cells were collected from 8-week-old wild-type ($n = 6$) and Gads-deficient mice ($n = 6$). Red blood cells were lysed and the remaining white blood cells were lineage depleted and incubated with fluorescent antibodies specific for cell surface markers kit (K), Sca-1 (S) and Lineage negative (Lin). Flow cytometry was used to enumerate specific cell types, representative samples are shown. **(b)** Data are presented as the average percentage of positive (lineage depleted) cells. Error bars represent standard error and significant differences, as measured by *T* test are noted. **(c)** Bone marrow cells were collected from 8-week-old, sex- and age-matched wild-type ($n = 9$) and Gads-deficient ($n = 12$) mice. Red blood cells were lysed and the remaining white blood cells were lineage depleted, then incubated with fluorescent antibodies specific for cell surface markers (CD48 and CD150). Flow cytometry was used to enumerate specific cell types. **(d)** Data are presented as the average percentage of positive (lineage depleted) cells. Error bars represent standard error and significant differences, as measured by *T* test, are indicated.

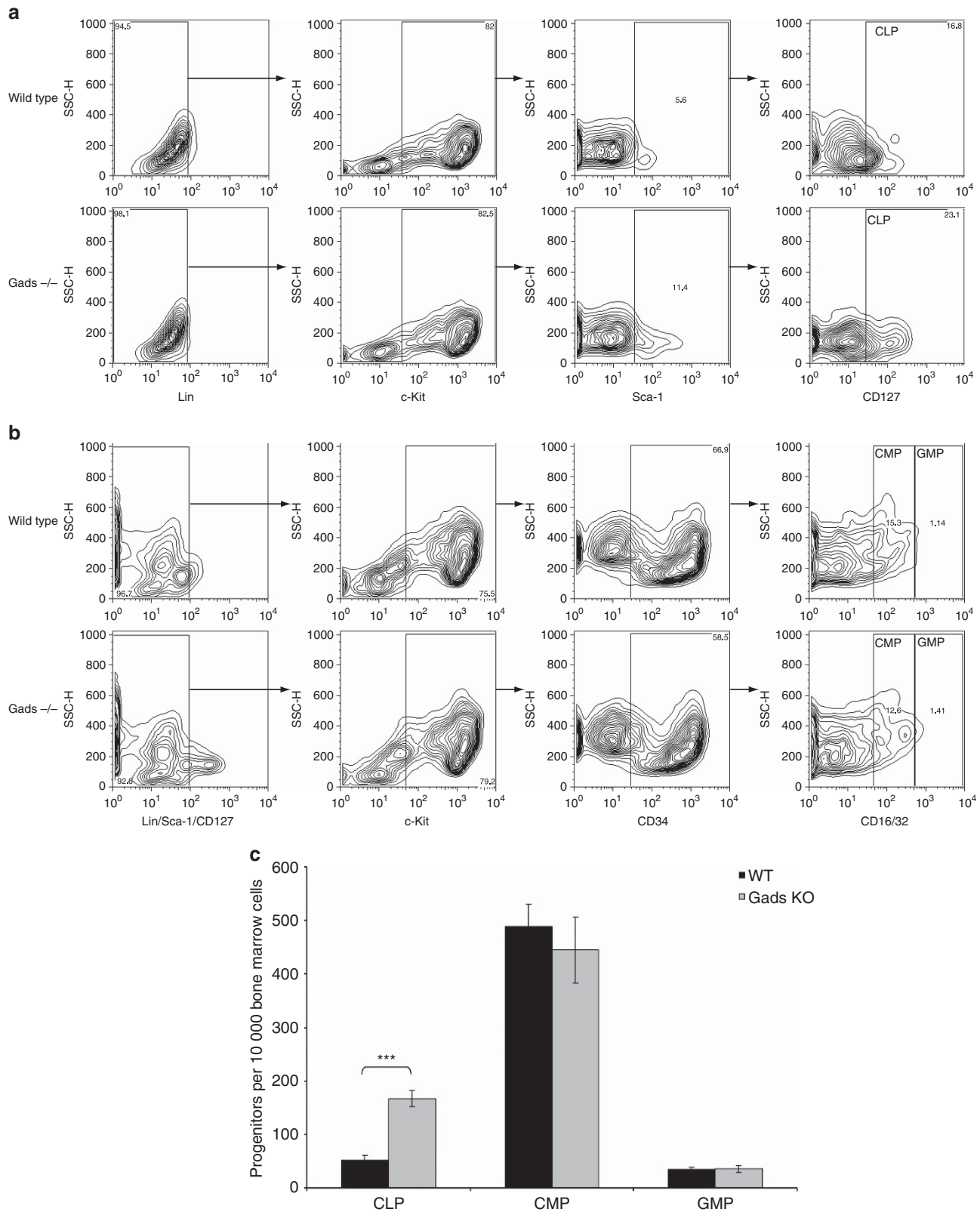


Figure 6. Increased CLP are observed in Gads-deficient bone marrow. Following lineage depletion, cells were incubated with fluorescent antibodies specific for cell surface markers expressed on CLP, CMP and GMP. Flow cytometry was used to enumerate specific cell types. The gating strategy utilized to identify (a) CLP, (b) CMP and (b) GMP is illustrated for representative wild-type and Gads^{-/-} bone marrow. (c) Data are presented as the average number of CLP, CMP or GMP cells per 10 000 lineage-depleted bone marrow cells for wild-type ($n = 12$) and Gads-deficient mice ($n = 12$). Error bars represent standard error and significant differences, as measured by T test, are noted. (***) $P < 0.0001$.

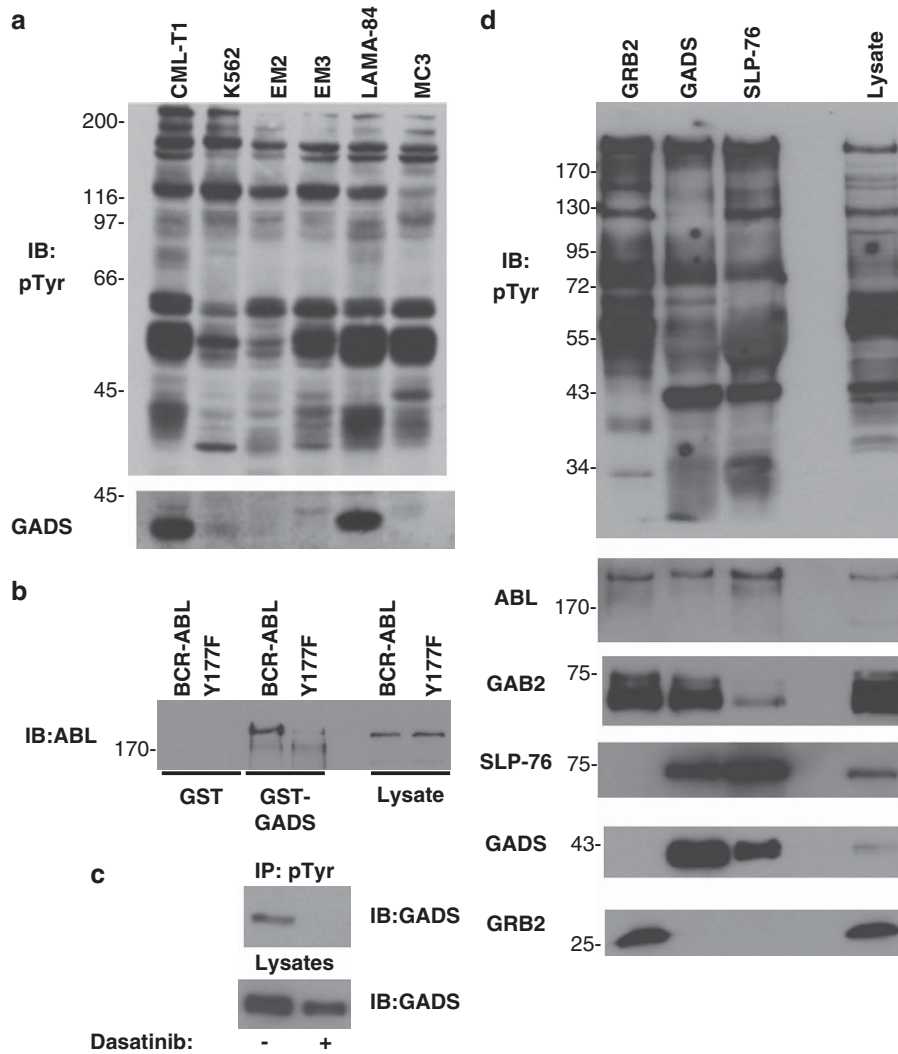


Figure 7. GADS-SLP-76 are recruited to BCR-ABL Y177. **(a)** Several human leukemia cell lines were lysed in SDS-PAGE sample buffer and analyzed for levels of tyrosine-phosphorylated proteins via immunoblotting (IB). The blot was stripped and reprobed for GADS expression. **(b)** Pull-down experiments, with GST-Gads or GST alone, were performed using lysates from BaF/3 cells expressing wild-type BCR-ABL (wild type) or BCR-ABL Y177F (Y177F). The membrane was probed with a peptide-specific Abl antibody. **(c)** CML-T1 cells were incubated in RPMI or RPMI + Dasatinib. Tyrosine phosphorylation of GADS protein was detected by immunoprecipitations performed with the 4G10 anti-phosphotyrosine monoclonal antibody followed by western blot with anti-Gads antibody (upper panel). Lysates were probed with the anti-Gads antibody (lower panel). **(d)** CML-T1 cells were incubated with RPMI media (control), RPMI containing Dasatinib or RPMI containing R406. Lysates were immunoprecipitated with a GADS antibody and western blotting was completed with an anti-phosphotyrosine antibody and reprobed with a peptide-specific GADS antibody. GRB2, GADS and SLP-76 immunoprecipitations were performed on CML-T1 cell lysates and blotted with anti-phosphotyrosine. Proteins were detected by blotting with antibodies raised against ABL, GAB2, SLP-76, GADS and GRB2.

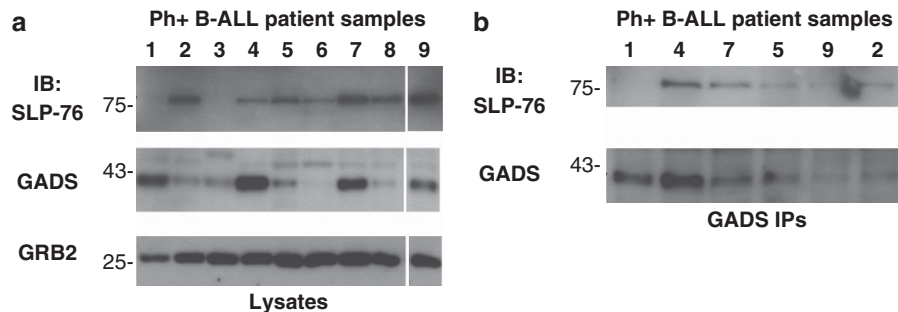


Figure 8. Gads is expressed in a complex with SLP-76 in a subset of Philadelphia chromosome-positive patient samples. **(a)** Lysates from Philadelphia chromosome-positive B-ALL patient samples were analyzed by western blot for expression of SLP-76, GADS and GRB2. **(b)** Anti-GADS immunoprecipitations (IPs) were performed on the B-ALL sample lysates that expressed GADS protein. The presence of SLP-76 protein was detected by western blot analysis.

possesses a unique binding specificity that allows for interaction with the adapter protein SLP-76.²³ The interaction between Gads and SLP-76 ultimately leads to cytoskeletal changes, IL-2 gene expression and proliferation during T-cell activation.²³ Because of the similarities between Gads and Grb2, we hypothesized that in addition to its role in normal hematopoiesis, Gads is a component of the BCR-ABL signaling complex. The differences between Grb2 and Gads, particularly relating to the binding specificity of their SH3 domains, suggest that Gads may be responsible for activating distinct or novel pathways downstream of BCR-ABL.

We show that Gads is required for lymphoid disease mediated by BCR-ABL, using a retroviral transduction bone marrow transplant assay. Mice expressing BCR-ABL in Gads-deficient bone marrow develop an aggressive, short latency MPD. These animals display the hallmark features of murine MPD, including elevated expression of Gr-1 and Mac-1, as well as infiltration of leukemic cells into the spleen, liver and lungs of transduced animals.

In comparison to their wild-type counterparts that developed both myeloid and lymphoid disease, mice that received BCR-ABL-transduced Gads(−/−) bone marrow cells exclusively developed myeloid disease, suggesting that Gads has a critical role in the development of lymphoid leukemia downstream of BCR-ABL. Because of the striking difference in disease phenotype and latency between recipients of wild-type and Gads(−/−) mice, we sought to determine a mechanism for the involvement of Gads in signaling downstream of BCR-ABL. We have confirmed that GADS interacts with BCR-ABL and is specifically recruited to Y177, similar to GRB2. The difference between GRB2 and GADS signaling downstream of BCR-ABL appears to lie in the ability of GADS to recruit SLP-76 to BCR-ABL. It is possible that the development of BCR-ABL-mediated lymphoid leukemia involves the formation of a complex that includes BCR-ABL, GADS and SLP-76 and other unique binding partners.

Our work is supported by the fact that reduction of GADS expression *in vitro* in K562 cells leads to the disruption of a complex that includes BCR-ABL, GADS, SLP-76 and Actin.¹⁹ This is of particular interest because GADS mediates cytoskeletal changes in T cells, through SLP-76 binding. SLP-76, in turn, is known to recruit VAV, NCK and adhesion and degranulation-promoting adaptor protein (ADAP).²⁷ This complex has been demonstrated to be critical for Actin rearrangement in T cells.⁴² Because BCR-ABL is also known to interact with Actin,^{43,44} it is possible that in Ph⁺ leukemias the GADS-SLP-76 complex may be involved in the altered cell adhesion and migration associated with leukemia cells. It was previously reported that the Actin-binding domain of BCR-ABL is not required for the development of CML,⁴⁵ utilizing 5-FU-primed donors which models myeloid disease only. Whether the BCR-ABL Actin-binding domain is required for lymphoid disease in a bone marrow transplant model remains to be investigated. However, the requirement of the Actin-binding domain in lymphoid disease is suggested from a transgenic model. p190 BCR-ABL transgenic mice typically develop an aggressive lymphoid leukemia, but disruption of the Actin-binding domain leads to an attenuated leukemia phenotype and increased disease latency.⁴⁶

GADS-SLP76 binding to BCR-ABL could also contribute to RAS activation. In T cells, GADS-dependent recruitment of SLP76 to LAT is required for Phospholipase Cγ1 (PLCG1)-mediated diacylglycerol release,²⁴ leading to recruitment of RAS-GRP and RAS activation.⁴⁷ Whether this pathway is also functional in Ph⁺ ALL remains to be determined.

We demonstrate that GADS is tyrosine phosphorylated downstream of BCR-ABL and that this phosphorylation event is inhibited by pre-treatment with Dasatinib. Mass spectrometry suggests several GADS tyrosines are phosphorylated, including Y45, Y207, Y222 and Y324.⁴⁸ Phosphorylation of Y45 and Y222 are associated with T-cell activation, whereas Y207 is phosphorylated

in the K562 cell line.⁴⁸ BCR-ABL-dependent tyrosine phosphorylation of GRB2 was also documented,⁴⁹ and mass spectrometry has identified Y160 and Y209 as phosphorylation sites in K562 cells.⁴⁸ No studies have identified SH2-dependent interactors with phosphorylated GADS or GRB2.

Expression of BCR-ABL in whole bone marrow leads to MPD, B-cell acute lymphoid leukemia and development of macrophage tumors.⁹ Although GADS appears to usurp T-cell signaling pathways in CML cell lines, we failed to observe T-cell ALL in bone marrow transplantation experiments. T-cell ALL is often associated with long latency disease and/or low titer retroviral preparations.

Evaluation of the stem cell and progenitor populations in Gads-deficient mice revealed that hematopoietic stem cells (defined by Kit⁺Sca-1⁺Lin[−] and CD48⁺CD150⁺ cells) and CLP were enhanced, whereas no differences in CMP or GMP were observed. A previous study illustrated a proliferative defect in Gads-deficient B cells in adoptive transfer experiments.⁵⁰ In addition, elevated numbers of transitional stage 1 (IgM^{hi}CD21[−]CD23[−]) and 2 (IgM^{hi}CD21⁺CD23⁺) as well as follicular B cells (IgM^{lo}CD21⁺CD23⁺) were identified in Gads-deficient mice.⁵⁰ Therefore, the failure to observe lymphoid disease in Gads-deficient bone marrow expressing BCR-ABL was not due to an absence of a lymphoid target cell for infection but more likely due to a block in proliferation.

In contrast to our data modeling lymphoid leukemia, BCR-ABL effectively induced an MPD that resembled human CML in all mice that received either wild-type or Gads(−/−) transduced cells from 5-FU-primed donors. There were no differences in disease phenotype or latency between recipients of wild-type or Gads(−/−) bone marrow. Because the disease phenotype was indistinguishable between recipients of wild-type or Gads(−/−) donor cells, we conclude that Gads is not required for the pathogenesis of BCR-ABL-mediated myeloid disease and/or that Grb2 and Gads have redundant role in myeloid disease development in this model.

Introduction of BCR-ABL Y177F into 5-FU-primed bone marrow resulted in a long latency T-cell ALL, demonstrating the critical importance of Y177 in mediating MPD.^{10–12} Unfortunately, none of these groups examined leukemia induction utilizing whole bone marrow expressing BCR-ABL Y177F to determine whether loss of Y177 affect lymphoid disease development. Our data suggest that Gads has an important role in lymphoid disease development upon expression of BCR-ABL in whole bone marrow and subsequent bone marrow transplantation.

Treatment of Ph⁺ B-ALL faces several challenges. This disease accounts for up to 10% of adolescent ALL and one-fourth of adult ALL.³¹ Patients respond well to chemotherapy with 80% complete remission; however, many patients relapse with median disease-free survival of 10 months and 5-year survival <20%. Therefore, there is an urgent need to identify differences between CML and ALL driven by BCR-ABL. Our data suggest that, in addition to SRC kinase activation,²⁸ GADS adapter protein is central to a lymphocyte-specific signaling pathway critical for the development of B-ALL. Further understanding of this pathway will aid in the development of selective therapeutic agents to specifically treat Ph⁺ B-ALL.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Renu Sarao and Amanda Luck for animal colony management and Mojib Javadi for final figure preparation. This research was supported by operating funds from Leukemia and Lymphoma Society of Canada (to CJM and DLB) and Canadian Institutes of Health Research—MOP#12859 (CJM) and MOP#42428 (DLB).

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Supplementary Information accompanies this paper on the Leukemia website (<http://www.nature.com/leu>)