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ORIGINAL ARTICLE

Expression of CCL9/MIP-1γ is repressed by BCR/ABL and its restoration suppresses *in vivo* leukemogenesis of 32D-BCR/ABL cells

G Iotti[1,2,3], G Ferrari-Amorotti[1], C Rosafio[2], F Corradini[2], MR Lidonnici[1], M Ronchetti[1], M Bardini[1], Y Zhang[4], R Martinez[4], F Blasi[3] and B Calabretta[1]

[1]*Department of Microbiology and Immunology, Kimmel Cancer Center, Thomas Jefferson Medical College, Philadelphia, PA, USA;* [2]*Department of Biomedical Sciences, Universitá di Modena e Reggio Emilia, Modena, Italy;* [3]*IFOM, FIRC Institute of Molecular Oncology, Milano, Italy and* [4]*Wyeth Genetics Institute, Cambridge, MA, USA*

Transformation of hematopoietic cells by the BCR/ABL oncogene is caused by perturbation of signal transduction pathways leading to altered patterns of gene expression and activity. By oligonucleotide microarray hybridization of polysomal RNA of untreated and STI571-treated 32D-BCR/ABL cells, we identified the β-chemokine CCL9 as a gene regulated by BCR/ABL in a tyrosine kinase-dependent manner. BCR/ABL repressed CCL9 expression at the transcriptional level by mechanisms involving suppression of p38 MAP kinase, and modulation of the activity of CDP/cut and C/EBPα, two transcription regulators of myeloid differentiation. However, repression of C/EBP-dependent transcription did not prevent the induction of CCL9 expression by STI571, suggesting that C/EBPα is involved in maintaining rather than in inducing CCL9 expression. Restoration of CCL9 expression in 32D-BCR/ABL cells had no effect on the *in vitro* proliferation of these cells, but reduced their leukemogenic potential *in vivo*, possibly by recruitment of CD3-positive immune cells. Together, these findings suggest that downregulation of chemokine expression may be involved in BCR/ABL-dependent leukemogenesis by altering the relationship between transformed cells and the microenvironment.
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Introduction

Chronic myelogenous leukemia (CML) is a clonal myeloproliferative disorder characterized by the accumulation of mature and immature myeloid cells in peripheral blood, bone marrow and extramedullary sites (Kantarjian *et al*., 1993). Typical features of CML cells are increased proliferation, reduced susceptibility to apoptosis, and altered interaction with the bone marrow microenvironment and migration (Sawyers, 1999). CML is consistently associated with a reciprocal translocation of the long arms of chromosomes 9 and 22, which generates the BCR/ABL fusion gene. This is translated in the $p210^{BCR/ABL}$ oncoprotein (Ben-Neriah *et al*., 1986) which functions as a constitutively active tyrosine kinase (Konopka *et al*., 1984). Expression of $p210^{BCR/ABL}$ is necessary and sufficient for transformation of hematopoietic cells *in vitro* (McLaughlin *et al*., 1987; Daley and Baltimore, 1988), to generate a CML-like disease in mice (Daley *et al*., 1990; Elefanty *et al*., 1990), and for disease maintenance (Druker *et al*., 2001; Kantarjian *et al*., 2002).

Transformation of hematopoietic cells by $p210^{BCR/ABL}$ involves the assembly of multiprotein complexes and the phosphorylation of several substrates, leading to the activation of signal transduction pathways, which generate proliferative and antiapoptotic signals (Sattler and Griffin, 2003). There is also evidence that $p210^{BCR/ABL}$ affects gene expression patterns, both at the transcriptional and post-transcriptional level (Deininger *et al*., 2000). By oligonucleotide microarray hybridization, we assessed relative mRNA frequency in untreated and Imatinib mesylate (STI571)-treated BCR/ABL-expressing myeloid precursor 32Dcl3 cells. The gene more upregulated following STI571 treatment was CCL9, a member of the β-chemokine family also called MIP-1γ, scya9, MRP-2 or CCF18 (Hara *et al*., 1995; Poltorak *et al*., 1995; Youn *et al*., 1995; Mohamadzadeh *et al*., 1996). CCL9 shares 45 and 24% sequence identity with CCL6 and CCL3, respectively. CCL9 is constitutively expressed in many tissues, including bone marrow (Poltorak *et al*., 1995) and is secreted by Langerhans' cells, dendritic cells, macrophages and myeloid cell lines (Hara *et al*., 1995; Youn *et al*., 1995; Mohamadzadeh *et al*., 1996). It induces chemotaxis of $CD4^+$, $CD8^+$ T cells and monocytes (Hara *et al*., 1995) and was reported to suppress colony formation of murine bone marrow myeloid progenitors (Youn *et al*., 1995).

We show here that CCL9 transcription is markedly repressed by $p210^{BCR/ABL}$ in 32Dcl3 and primary bone

Correspondence: B Calabretta, Department of Microbiology and Immunology, Kimmel Cancer Center, Thomas Jefferson Medical College, 233, S. 10th St. Philadelphia, PA 19107, USA.
E-mail: B_Calabretta@mail.jci.tju.edu
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marrow cells by mechanisms involving p38 MAP Kinase inhibition, CCAAT Displacement Protein (CDP)/cut-dependent transcription repression, and block of CCAAT/Enhancer binding protein (C/EBP)-regulated expression. Furthermore, restoration of CCL9 expression reduces the leukemogenic potential of BCR/ABL-expressing cells *in vivo* indirectly by recruitment of immune cells.

Results

CCL9 expression is downregulated by BCR/ABL

To identify genes potentially regulated by BCR/ABL at the translational level, an Affymatrix array was probed with monosome- and polysome-associated (thus efficiently translated) RNA from untreated and STI571-treated 32D-BCR/ABL cells (Guerzoni *et al*., 2006). Expression of the CCL9 gene (a member of the β-chemokine family) increased 23-fold in polysome-associated RNA of STI571-treated cells.

By Northern blot hybridization, CCL9 expression was significantly lower in 32D-BCR/ABL than in parental cells and was re-induced by STI571 treatment in STI571-sensitive but not in STI571-resistant cells (Figure 1a). The pattern of CCL9 protein expression was identical to that of CCL9 mRNA (Figure 1a). The findings are specific as STI571 treatment suppressed BCR/ABL auto-phosphorylation in STI571-sensitive but not in STI571-resistant cells (Figure 1a). The effect of BCR/ABL on CCL9 expression was dependent on constitutive ABL kinase activity because CCL9 expression was suppressed in 32Dcl3 cells expressing TEL/ABL, a tyrosine-phosphorylated cytoskeletal oncoprotein that, like BCR/ABL, transforms 32Dcl3 cells (Okuda *et al*., 1996) (Figure 1b), but was abundant in v-SRC-transformed 32Dcl3 cells (Figure 1c).

We also analysed CCL9 protein levels in untreated and STI571-treated MigRI $p210^{BCR/ABL}$-transduced Lin^- $Sca\text{-}1^+$ Kit^+ mouse bone marrow cells and FDCP-mix BCR/ABL cells. As shown in Figure 1d, STI571 treatment enhanced expression of CCL9 in both cell cultures.

BCR/ABL activity blocks CCL9 transcription

The observation that CCL9 levels were upregulated in RNA from STI571-treated 32D-BCR/ABL cells (Figure 1a) is inconsistent with a purely translational mechanism of regulation and suggests that enhanced transcription may be the predominant mechanism underlying the increased CCL9 expression. Thus, we carried out reverse transcriptase–polymerase chain reaction (RT–PCR) on DNase-I-treated nuclear RNA using primers within the second and the third intron of the gene. Nuclear pre-mRNA CCL9 transcripts (detected by Southern blot hybridization with a ^{32}P-labeled oligomer within the amplified PCR product) were almost undetectable in untreated 32D-BCR/ABL cells but became clearly detectable after treatment with STI571 (Supplementary Figure S1). Actinomycin D

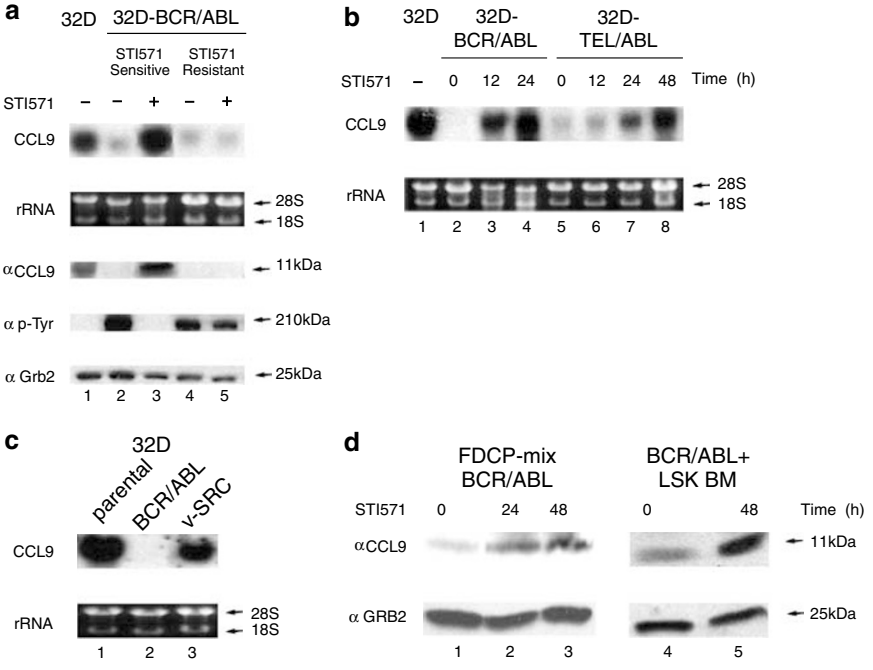

Figure 1 Effects of BCR/ABL on CCL9 expression. (**a**) CCL9 expression in parental and in 32D-BCR/ABL cells. rRNA and GRB2 levels were monitored as control for RNA and protein loading, respectively. Levels of tyrosine-phosphorylated BCR/ABL are shown as control of STI571 treatment efficacy. (**b**) CCL9 mRNA levels in parental, BCR/ABL-expressing and TEL/ABL-expressing 32Dcl3 cells. (**c**) CCL9 mRNA levels in parental, BCR/ABL- and v-SRC-expressing 32Dcl3 cells. (**d**) CCL9 protein levels in untreated and STI571-treated FDCP-mix BCR/ABL cells and BCR/ABL-expressing $Lin^-Sca\text{-}1^+$ Kit^+ cells. Levels of GRB2 were monitored as a control for protein loading.

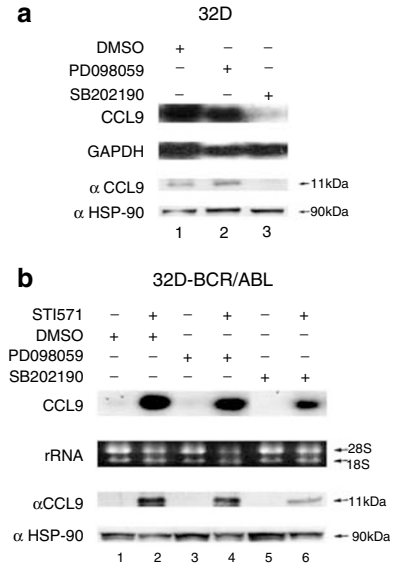

Figure 2 Effect of p38 MAP kinase on CCL9 expression. (**a**) CCL9 expression in 32Dcl3 cells treated with PD098059 (50 μM), SB202190 (10 μM) or vehicle only. GAPDH and HSP90 levels were monitored as control for RNA and protein levels, respectively; (**b**) CCL9 expression in 32D-BCR/ABL cells pre-incubated 1 h with PD098059, SB202190 or vehicle only and, where indicated, treated with STI571 (1 μM, 12 h). rRNA and HSP90 levels were monitored as control of RNA and protein loading, respectively.

completely blocked STI571-stimulated CCL9 mRNA expression (Supplementary Figure S1), consistent with the involvement of transcription repression in BCR/ABL-dependent downregulation of CCL9 levels.

p38 MAP kinase inhibition blocks STI571-dependent CCL9 induction

To identify the BCR/ABL-dependent transduction pathway(s) responsible for CCL9 downregulation, parental and BCR/ABL expressing 32Dcl3 cells were treated with pharmacologic inhibitors of pathways known to be regulated by BCR/ABL. Pharmacologic block of p38 MAP kinase activity by the selective inhibitor SB202190 reduced CCL9 expression in parental 32Dcl3 cells (Figure 2a) and partially blocked the STI571-dependent CCL9 induction in 32D-BCR/ABL cells (Figure 2b). Similar results were obtained with SB203580, another selective p38 inhibitor (data not shown), while the selective MAP kinase/ERK kinase (MEK) inhibitor PD098059 had no effect (Figure 2a and b) at a concentration that blocked MAPK/Erk1/2 phosphorylation in IL-3-treated 32Dcl3 cells (not shown).

C/EBPα induction restores CCL9 mRNA levels in BCR/ABL-expressing cells

BCR/ABL blocks granulocytic differentiation by inhibiting the expression of C/EBPα (Perrotti *et al*., 2002; Schuster *et al*., 2003) and restoration of C/EBPα expression in BCR/ABL-expressing cells induces granulocytic differentiation (Perrotti *et al*., 2002; Tavor *et al*., 2003; Ferrari-Amorotti *et al*., 2006). Thus, we

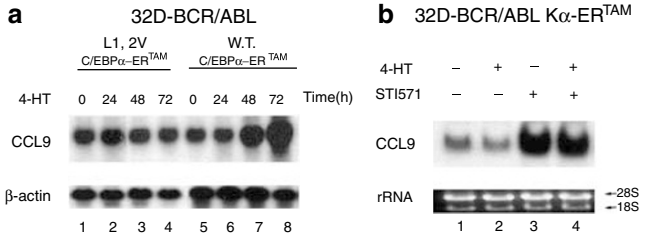

Figure 3 Effect of C/EBP activity on CCL9 expression. CCL9 mRNA levels in: (**a**) 4-HT-treated 32D-BCR/ABL cells transduced with MigRI L1, 2V C/EBPα-ER^{TAM} or MigRI WT C/EBPα-ER^{TAM}. Levels of β-actin were monitored as control for RNA loading; (**b**) 32D-BCR/ABL cells transduced with pBabePuro Kα-ER^{TAM}. Cells were pre-treated or not for 12 h with 4-HT (200 nM) and then exposed or not to STI571 (1 μM) for additional 12 h. rRNA was monitored as control for RNA loading.

sought to establish whether C/EBPα ectopic expression increases CCL9 levels in 32D-BCR/ABL cells, using tamoxifen-regulated C/EBPα chimeric proteins. Activation of WT C/EBPα increased CCL9 expression in 32D-BCR/ABL cells (Figure 3a, lanes 5–8), whereas activation of L1, 2V C/EBPα-ER^{TAM}, a leucine-zipper mutant unable to bind DNA and activate transcription (Friedman *et al*., 1989), had no effect (Figure 3a, lanes 1–4). Compared to the increase of CCL9 expression induced by STI571, the effect of C/EBPα was delayed, suggesting that CCL9 is not a direct target of C/EBPα.

This was further confirmed using 32D-BCR/ABL cells retrovirally transduced with pBabePuro Kα-ER^{TAM} in which the C/EBPα DNA-binding domain is linked to both the Krüppel-associated box (KRAB) transrepression domain and the estrogen receptor (ER)^{TAM} ligand-binding domain. When activated by 4-HT treatment, this fusion protein suppresses the transactivation activity of C/EBP family members (Wang and Friedman, 2002). STI571-dependent CCL9 induction was not prevented by blocking C/EBP activity (Figure 3b), suggesting that other factors function as immediate regulators of CCL9 transcription in BCR/ABL-expressing cells.

CDP/cut is downregulated in STI571-treated BCR/ABL expressing cells and its repression is associated with increased CCL9 levels

CDP/cut is a highly conserved transcription factor which negatively regulates gene expression by binding to CCAAT boxes and displacing positively acting transcription factors (Barberis *et al*., 1987) and by histone deacetylase-dependent active repression (Nepveu, 2001). CDP/cut is expressed at highest levels in undifferentiated myeloid cells and is downregulated with differentiation. CDP/cut represses the expression of secondary granule protein genes in myeloid progenitors (Lawson *et al*., 1998).

In the microarray analysis of RNA from untreated and STI571-treated 32D-BCR/ABL cells, CDP/cut expression in polysomal RNA was markedly decreased (~20-fold) after STI571 treatment; in untreated cells,

CDP/cut mRNA levels were more abundant in polysome-associated than in total RNA, suggesting active translation.

By Western blot analysis, CDP/cut expression was markedly and rapidly downregulated after STI571 treatment of 32D-BCR/ABL cells (Figure 4a), a pattern opposite to that of CCL9 (Figure 4a), but no changes were observed in lysates of STI571-resistant and kinase-deficient (K1172R) 32D-BCR/ABL cells (Figure 4b).

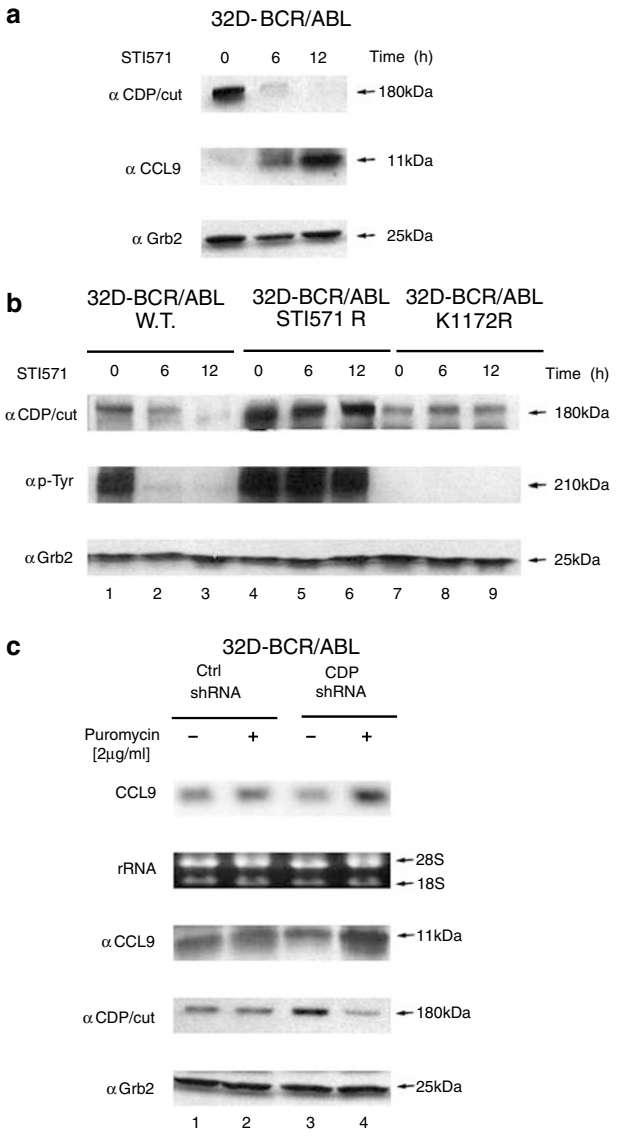

Figure 4 Effect of CDP/cut on CCL9 expression. Blots show expression of: (**a**) CDP/cut and CCL9 in STI571-treated 32D BCR/ABL cells; (**b**) CDP/cut in STI571-treated parental and STI571-resistant 32D BCR/ABL cells and in 32Dcl3 cells expressing the kinase-deficient K1172R BCR/ABL. Tyrosine-phosphorylated BCR/ABL levels are shown as control of the STI571 treatment; (**c**) CCL9 mRNA and protein in 32D BCR/ABL cells transduced with pSRP CDP shRNA or control shRNA. mRNA and protein levels were monitored 2 days after puromycin selection. The nitrocellulose filter was blotted with an anti-CDP antibody to control efficiency of RNA interference-induced protein down-regulation. (**a**, **b**) GRB2 expression was monitored as control for protein loading. (**c**) rRNA and GRB2 levels are shown as control for RNA and protein loading, respectively.

To investigate whether CDP/cut is involved in BCR/ABL-dependent CCL9 downregulation, 32D-BCR/ABL cells were transduced with a retroviral vector expressing a short hairpin RNA targeting CDP/cut or a scrambled control shRNA. After puromycin selection, RNA and whole cell extracts were tested for CDP/cut and CCL9 expression. Maximum CDP/cut downregulation was detected at 48 h, a time point at which CCL9 expression was also increased (Figure 4c).

CCL9 overexpression had no effect on the proliferation of 32D-BCR/ABL cells

To investigate the biologic activity of CCL9 in BCR/ABL expressing cells, 32D-BCR/ABL cells were transduced with MigRI or MigRI CCL9-HA. Ectopic expression and secretion of the CCL9 protein were confirmed by anti-HA Western blotting (Figure 5a).

As CCL9 reportedly suppressed colony formation of normal murine bone marrow progenitors (Youn *et al*., 1995), we investigated if CCL9 constitutive expression by 32D-BCR/ABL cells had any effect on their proliferation, but found no effect by liquid culture (Figure 5b) or methylcellulose colony formation assays (Figure 5c). Proliferation of 32D-BCR/ABL cells was also unaffected by incubation with recombinant CCL9 (not shown).

CCL9-overexpressing cells have a reduced leukemogenic potential in vivo

To test the effects of CCL9 overexpression *in vivo*, syngenic immunocompetent mice (C3H/HeJ) were injected subcutaneously (s.c.) or intravenously (i.v.) with 32D-BCR/ABL cells transduced with MigRI or MigRI CCL9.

For the s.c. injections, the same animal was inoculated with CCL9-producing cells in one flank and with control cells in the contralateral one. Tumors derived from cells overexpressing CCL9 were smaller in size than controls (41% weight reduction, Figure 6a).

As syngenic mice develop an aggressive leukemia when injected i.v. with 32D-BCR/ABL cells, disease progression of mice injected with CCL9-expressing cells or with control cells was compared. First, we analysed the colony-forming potential of peripheral blood leukocytes harvested (at day 14) from mice injected with 32D-BCR/ABL cells. Peripheral blood cells (10^4) were plated in methylcellulose in the absence of cytokines to allow only BCR/ABL-expressing cells to form colonies. Compared to controls, peripheral blood cells of mice injected with CCL9-expressing 32D-BCR/ABL cells formed fewer colonies (14-fold reduction) (Figure 6b). Mice injected with CCL9-expressing cells survived longer than controls (median survival of 31 vs 20.5 days) (Figure 6c). To assess whether the impaired leukemogenic potential of CCL9-expressing cells reflected intrinsic properties of the cells or was due to interaction with immunocompetent cells, we injected immunodeficient severe combined immunodeficiency (SCID) mice with MigRI-transduced or CCL9-expressing 32D-BCR/ABL cells. In this experiment, survival of

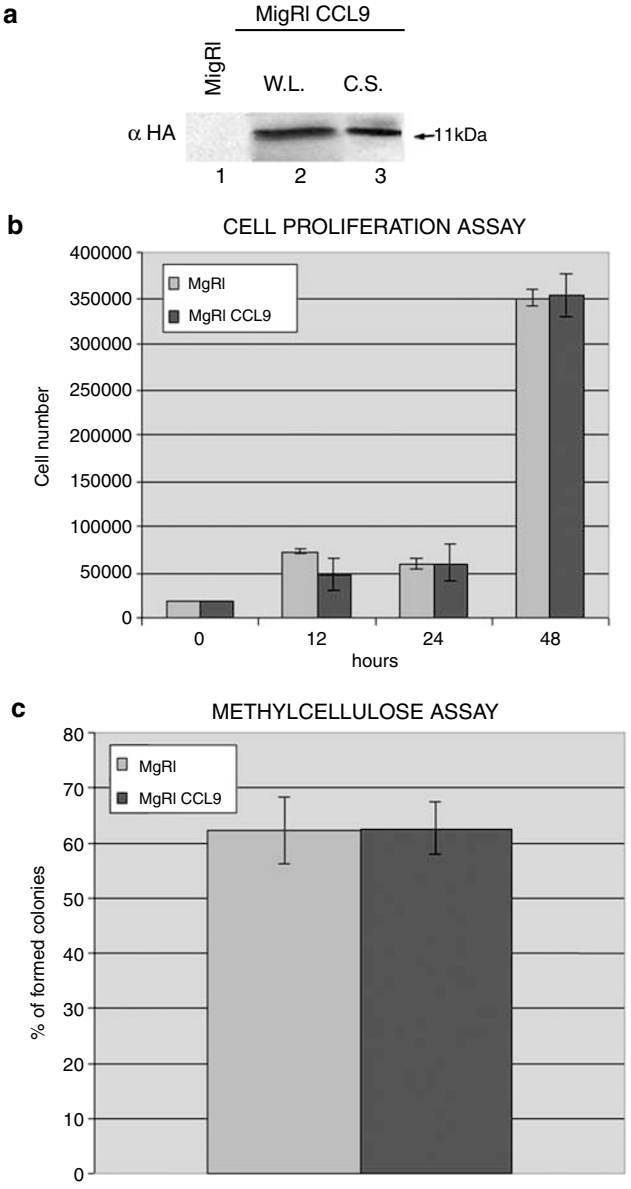

Figure 5 Effect of CCL9 on proliferation of 32D-BCR/ABL cells. (**a**) CCL9 expression in MigRI or MigRI CCL9-transduced 32D-BCR/ABL cell extracts. Cell lysate (from 3×10^5 cells) and supernatant (33 μl collected after 72 h of culture), were loaded in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). (**b**) Proliferation of 32D-BCR/ABL cells transduced with MigRI or MigRI CCL9. Representative of three experiments, each performed in triplicate. (**c**) Methylcellulose colony formation from 32D-BCR/ABL cells transduced with MigRI or MigRI CCL9. Representative of two experiments, each performed in duplicate.

the mice was identical (Figure 6d), suggesting that CCL9 ectopic expression might suppress leukemogenesis by recruitment of immunocompetent cells.

CCL9 overexpression alters bone marrow and spleen microenvironment in leukemic mice

To assess whether CCL9 had chemotactic activity, mononuclear marrow cells from IL-2-treated C3H/HeJ mice were exposed to supernatant derived from MigRI or MigRI CCL9-transduced 32D-BCR/ABL cells. After 4 h, cells migrated in wells containing supernatant of CCL9-expressing cells were more numerous than controls (1.5-fold increase, Figure 7a). Recombinant murine CCL9 (10 nM) used as positive control caused a 4.3-fold increase in cell migration. Wells containing supernatant from CCL9-expressing cells showed also a 4.6-fold increase in the number of cells adhered to the lower surface of the filter, as revealed by crystal violet staining and microscopy (data not shown).

Based on these findings, we assessed the cellular composition of bone marrow and spleen in mice injected with MigRI-transduced or CCL9-expressing 32D-BCR/ABL cells. In the bone marrow, there was a slight (1.93 ± 0.45 vs 1.60 ± 0.36), but not statistically significant, increase in the percentage of CD3+ cells in mice injected with CCL9-expressing 32D-BCR/ABL cells. Instead, spleens of mice injected with CCL9 32D-BCR/ABL cells contained more CD3+ T cells than controls (38.89 ± 2.07 vs 24.73 ± 2.40) (Figure 7b). As the number of CD3+ T cells in spleens of non-injected mice was $\sim$30% and the proportion of GFP+ cells was similar ($<$10%) in spleens of mice injected with MigRI or CCL9-expressing 32D-BCR/ABL cells, there might be increased T-cell recruitment in spleens of mice injected with CCL9-expressing 32D-BCR/ABL cells. On the contrary, proportions of $CD11b^+$ or $CD49b^+$ cells were identical (data not shown).

To further assess the role of enhanced T-cell recruitment in the reduced leukemogenic potential of CCL9-expressing cells, 32D-BCR/ABL cells were co-cultured with splenocytes (recovered by FACS sorting of GFP-negative cells) from mice previously injected with MigRI or MigRI CCL9-transduced 32D-BCR/ABL cells at a 1:100 ratio in the presence of rhIL-2 to facilitate T-lymphocyte proliferation and activation. 32D-BCR/ABL (GFP-positive) cells were counted after 5 or 6 days. In all experiments, 32D-BCR/ABL cells cultured with GFP-negative cells from spleens of mice injected with CCL9-expressing 32D-BCR/ABL cells proliferated less (threefold reduction) than cells cultured with splenocytes of control mice (Figure 7c). Proliferation of 32D-BCR/ABL cells cultured in the absence of splenocytes or with splenic cells obtained from naïf (uninjected) mice was not different from that of cells cultured with splenocytes of control mice (Figure 7c and data not shown).

We also assessed proliferation of 32D-BCR/ABL cells cultured with T-lymphocyte-depleted splenocytes; in this condition, proliferation of CCL9-expressing 32D-BCR/ABL cells was higher than that of cells cultured in the presence of the whole splenic population (1.7-fold increase), while no difference was detected with cells from control or naïf mice (Figure 7c). Moreover, no differences were detected when proliferation of 32Dcl3 cells was assessed in this assay (Figure 7d). To identify a possible mechanism for the effects of splenocytes from mice injected with CCL9-expressing 32D-BCR/ABL cells, we tested IFN-γ production on the extracts used for the cell proliferation experiments. Compared to controls, increased production (even if not statistically

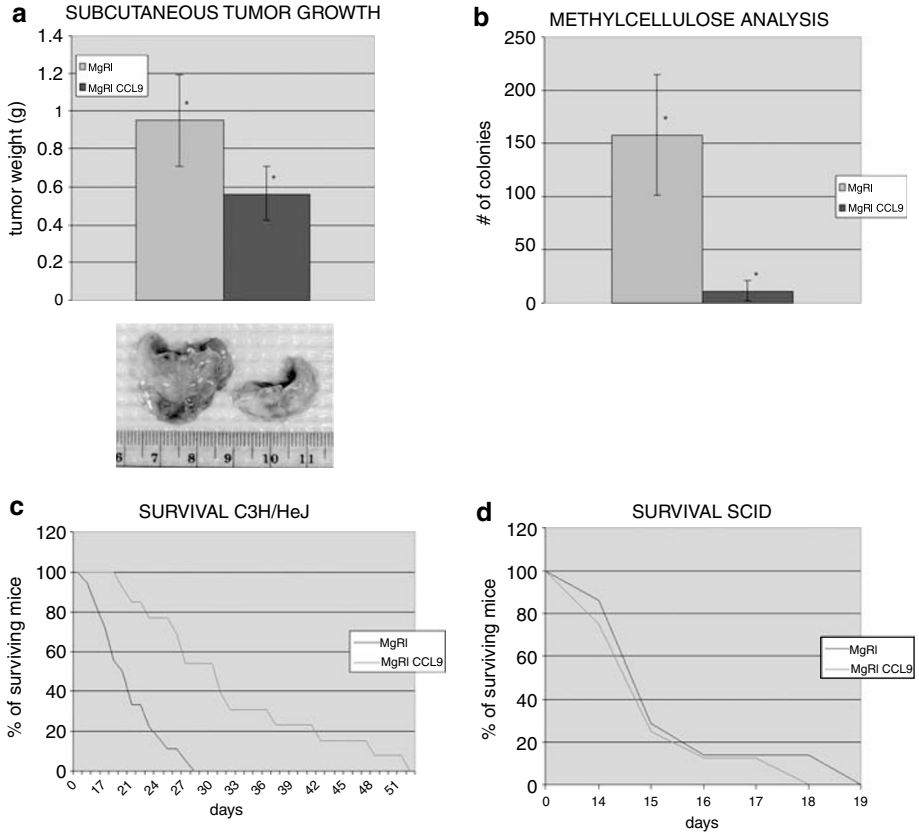

Figure 6 Effects of CCL9 on BCR/ABL leukemogenesis. (**a**) S.c. tumors in mice injected with MigRI (right flank) or MigRI CCL9-transduced (left flank) 32D-BCR/ABL cells. Tumors were excised and analysed after 14 days. Mean weight ± s.e.m. is shown, $n = 10$. $^*P = 0.033$; paired *t*-test. (**b**) Methylcellulose colonies from peripheral blood cells (10^4) of mice injected 14 days earlier with MigRI or MigRI CCL9-transduced 3×10^5 32D-BCR/ABL cells. Values indicate mean ± s.e.m. colony number from cells of 10 mice/group; $^*P = 0.031$. (**c**) Survival of C3H/HeJ mice injected with MigRI or MigRI CCL9-transduced 32D-BCR/ABL cells. X axis, time-to-death postinjection (days); Y axis, percent survival of mice; Mann–Whitney test (variable is time-to-death). $P < 0.001$, $n = 15$/group. (**d**) Survival of SCID mice injected with MigRI-transduced or CCL9-expressing 32D-BCR/ABL cells. $P = 0.519$, $n = 8$/group.

significant) of this cytokine was detected in splenocyte lysate from mice injected with CCL9-expressing 32D-BCR/ABL (Supplementary Figure S2).

Together, these data suggest that the splenic microenvironment of mice injected with CCL9-expressing 32D-BCR/ABL cells is enriched in cells that negatively influence their proliferation.

Discussion

By microarray analysis of untreated and STI571-treated polysomal RNA of 32D-BCR/ABL cells, we found that CCL9 chemokine mRNA levels were markedly increased after STI571 treatment. The effect of BCR/ABL on CCL9 expression was dependent on ABL tyrosine kinase activity, since CCL9 levels were also low in TEL-ABL-transformed cells, but were abundant in v-SRC-transformed cells and did not increase upon STI571 treatment of STI571-resistant 32D-BCR/ABL cells. The effect of STI571 was not limited to 32D-BCR/ABL cells, as it induced also an increased expression of CCL9 in primary $p210^{BCR/ABL}$-transduced Lin^- $Sca\text{-}1^+$ Kit^+ cells and in FDCP-mix BCR/ABL cells.

The microarray experiments were designed to identify translationally regulated genes. However, CCL9 mRNA expression was upregulated by STI571 in total RNA, its nuclear RNA levels were markedly enhanced by STI571 treatment and pre-incubation of STI571-treated 32D-BCR/ABL cells with actinomycin D prevented STI571-dependent mRNA upregulation, all suggesting transcription regulation.

Pharmacologic inhibition of p38 MAP kinase activity suppressed CCL9 expression in parental 32Dcl3 cells and partially reverted STI571-dependent induction of CCL9 in BCR/ABL-expressing cells. This is consistent with the observation that BCR/ABL inhibits p38 MAP kinase (Wong *et al*., 2003) and that some of the effects of STI571 treatment in BCR/ABL-expressing cells depend on reactivation of this pathway (Parmar *et al*., 2004). Thus, STI571 may affect gene expression by reactivating pathways repressed by BCR/ABL. As v-SRC also represses the p38 MAK kinase (Johnson *et al*., 2000) and yet CCL9 levels are abundant in v-SRC-transformed 32Dcl3 cells, inhibition of the p38 MAP kinase, *per se*, might be insufficient to repress CCL9 expression and BCR/ABL-dependent regulation of other factors might be also required. The 3′ untranslated region of

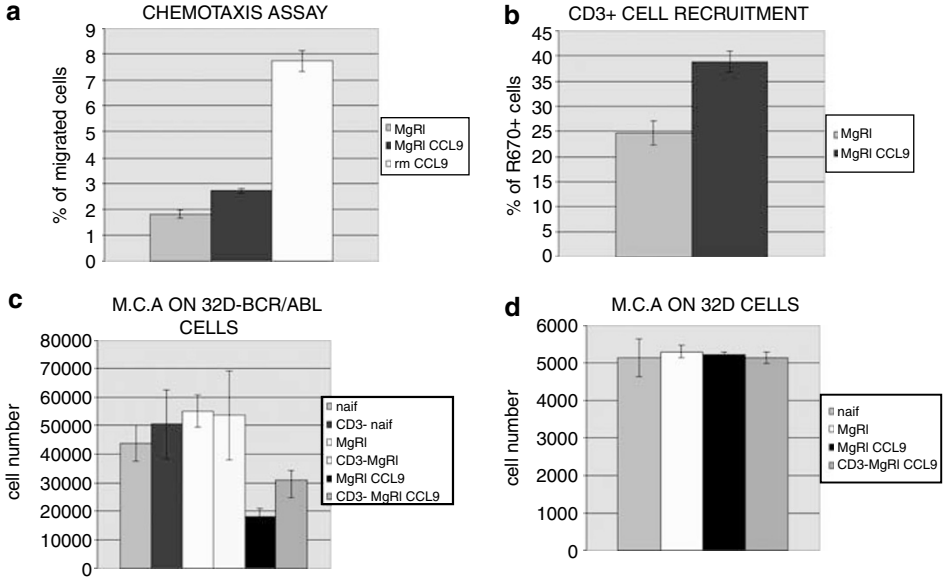

Figure 7 Effect of CCL9 on chemotaxis and T-cell recruitment. (**a**) Chemotaxis assay on murine mononuclear marrow cells from rhIL-2-treated mice. Cells in chemotaxis chambers were exposed to culture supernatants from MigRI or MigRI CCL9-transduced 32D-BCR/ABL cells. Positive control wells contained culture supernatant from MigRI-transduced 32D-BCR/ABL cells supplemented with 10 nM CCL9. Data (in quadruplicate) are representative of two experiments. Results are expressed as the percent of plated cells migrated to the lower chamber. $*P = 0.002$ MigRI CCL9 vs MigRI; $P < 0.001$ recombinant CCL9 vs MigRI; (**b**) CD3 immunostaining of splenocytes of mice injected with MigRI or MigRI CCL9-transduced 3×10^5 32D-BCR/ABL cells. Spleens were recovered and analysed 14 days after injection. Values indicate mean ± s.e.m. CD3 positivity of splenocytes of three mice/group. $*P = 0.011$. Proliferation of 32D-BCR/ABL (**c**) or 32Dcl3 cells (**d**) cultured with splenocytes from naïf mice or mice injected with MigRI or MigRI CCL9-transduced 32D-BCR/ABL cells. Splenocytes were recovered 14 days after injection, GFP-negative and (when indicated) GFP-negative/CD3-negative cells were sorted and cultured in multiwell with MigRI-transduced 32D-BCR/ABL or 32Dcl3 cells (starting ratio 100:1) in the presence of rhIL-2 (50 U/ml). Number of GFP-positive 32D-BCR/ABL or 32Dcl3 cells was then assessed. Representative of three experiments. Values indicate mean ± s.e.m. GFP-positive cells from five wells/group. $P < 0.001$ MigRI CCL9 vs MigRI; $P = 0.005$ MigRI CCL9 vs naïf; $P = 0.015$ MigRI CCL9 vs CD3-MigRI CCL9; not significant CD3-MigRI CCL9 vs CD3-MigRI and CD3-MigRI CCL9 vs CD3-naïf.

CCL9 mRNA contains potentially destabilizing AU-rich stretches and p38 MAP kinase regulates the stability of various mRNAs (Dean *et al*., 2004). However, it is unlikely that this kinase regulates CCL9 mRNA stability because CCL9 mRNA was stable (half-life ~8 h) in untreated 32Dcl3 cells and its half-life was unaffected by p38 MAP kinase inhibition (not shown). Together, these data suggest that the effects of the p38 MAP kinase on CCL9 expression depend on the expression/activity of transcription factor(s) regulated in a BCR/ABL-dependent manner in myeloid cells. Two of these factors might be C/EBPα and CDP/cut. C/EBPα expression is repressed in BCR/ABL-expressing cell lines and in CML-BC primary cells and its ectopic expression restores myeloid differentiation (Perrotti *et al*., 2002; Tavor *et al*., 2003). Activation of wild-type C/EBPα, but not of a leucine zipper mutant defective in transcription regulation, led to enhanced CCL9 expression. However, the slow kinetics of CCL9 induction and the inability of a conditional C/EBP-specific transcription repressor (Kα-ER^{TAM}) to prevent STI571-dependent induction of CCL9 expression suggest that C/EBP-regulated transcription is not the primary mechanism controlling CCL9 expression. Perhaps, C/EBPα-driven granulocytic differentiation is accompanied by upregulation of CCL9, but other factors have a role in the BCR/ABL-dependent inhibition of CCL9 transcription and their effects are rapidly relieved by BCR/ABL kinase inhibition. CDP/cut is a transcription repressor (via C/EBP-dependent and -independent mechanisms) of myeloid differentiation through its effects on neutrophil secondary granule protein genes, which are positive targets of C/EBPα and C/EBPε (Khanna-Gupta *et al*., 2003). Its expression is highest in myeloid progenitors and its DNA-binding activity decreases during differentiation (Khanna-Gupta *et al*., 2003). CDP/cut expression is abundant in BCR/ABL-expressing murine and human cell lines (Figure 4 and unpublished data), and is rapidly downregulated by BCR/ABL tyrosine kinase inhibition. The functional link between CDP/cut and CCL9 expression was demonstrated by showing that CCL9 mRNA levels increased upon CDP/cut down-regulation. Thus, the constitutive expression of CCL9 in myeloid precursor cells may depend on C/EBP-regulated transcription, whereas the rapid block of CCL9 transcription in 32D-BCR/ABL cells may depend, in part, on CDP/cut transcription repression that, based on its kinetics, is likely to involve C/EBP-independent mechanisms.

To gain insight on the possible consequences of CCL9 repression for BCR/ABL-dependent leukemogenesis, we expressed CCL9 in 32D-BCR/ABL cells and assessed proliferation and leukemogenic potential of these cells. Although CCL9 was reported to inhibit colony formation of normal myeloid progenitors (Youn *et al*., 1995), 32D-BCR/ABL cells ectopically expressing CCL9 proliferated as well as MigRI-32D-BCR/ABL cells and proliferation of these cells was unaffected by incubation with recombinant CCL9 protein. However, s.c. or i.v. injection of CCL9-expressing 32D-BCR/ABL cells in immunocompetent syngenic mice generated solid tumors of smaller size and a slower leukemic disease than control cells.

The effects of CCL9 ectopic expression on *in vivo* leukemogenesis by 32D-BCR/ABL cells are best explained by an alteration of the marrow and spleen microenvironment negatively influencing proliferation and infiltration of leukemic cells. Indeed, the proportion of CD3+ T lymphocytes was increased in the spleen of mice injected with CCL9-expressing 32D-BCR/ABL cells, suggesting that CD3+ T lymphocytes were recruited by CCL9-producing 32D-BCR/ABL cells and suppressed tissue infiltration and spreading of these cells. Consistent with this interpretation, CCL9 expression had no effect on leukemogenesis and survival of immunodeficient mice and splenocytes from mice injected with CCL9-expressing 32D-BCR/ABL cells suppressed the proliferation of co-cultured 32D-BCR/ABL cells. Depletion of $CD3^+$ cells from splenocyte cultures of mice injected with CCL9-expressing 32D-BCR/ABL cells significantly reduced the proliferation inhibitory effect on 32D-BCR/ABL (but not on parental 32Dcl3) cells, suggesting that the effect of CCL9 is, in part, due to T-cell activation. Together, these data point to mechanisms of transformation by $p210^{BCR/ABL}$, which do not depend only on the autonomous properties of transformed cells, but also on the relationship of these cells with the microenvironment. The role of other chemokines and chemokine receptors in CML is under active investigation: CML progenitors fail to respond to CCL3 and CCL2 (Eaves *et al*., 1993; Wark *et al*., 1998), and levels and/or activity of chemokine receptors (Salgia *et al*., 1999; Ptasznik *et al*., 2002; Geay *et al*., 2005; Jongen-Lavrencic *et al*., 2005) are downregulated in $p210^{BCR/ABL}$-expressing cells, changes potentially involved in the abnormal trafficking of CML progenitors and in their release from the bone marrow. Our findings indicate that the production of some chemokines may also be impaired in $p210^{BCR/ABL}$-expressing cells and suggest that restoration of chemokine expression negatively affects leukemogenesis.

The murine chemokine most highly related to CCL9 is CCL6 (Youn *et al*., 1995), whose expression is also downregulated in $p210^{BCR/ABL}$-expressing 32Dcl3 cells (Lane *et al*., 1999). We found that pharmacologic block of $p210^{BCR/ABL}$ by STI571 treatment restored CCL6 expression, but not as efficiently as CCL9 (not shown). Two human chemokines, CCL15 and CCL23, have the highest homology to CCL9, but the similarity is not striking and the pattern of expression is distinct (Berahovich *et al*., 2005). In BCR/ABL-expressing human MO7e cells, CCL15 transcripts were barely detectable and did not change after STI571 treatment (not shown); by contrast, CCL23 mRNA levels increased as early as 6 h after treatment (Supplementary Figure S3). However, it remains to be determined whether CCL23 shares the characteristics of CCL9 in CML cells.

Together, our findings suggest widespread effects of BCR/ABL on chemokine expression that may facilitate hematopoietic tissue infiltration and spreading of BCR/ABL-expressing cells to other organs.

Materials and methods

Plasmids

MigRI WT C/EBPα-ER^{TAM}: This plasmid contains the tamoxifen-regulated ligand-binding domain of the murine ER fused in-frame with C/EBPα (Ferrari-Amorotti *et al*., 2006).

The *MigRI L1, 2V C/EBPα-ER^{TAM}* Plasmid was generated by site-directed mutagenesis of MigRI WT C/EBPα-ER^{TAM}.

The *pBabePuro Kα-ER^{TAM}* plasmid was a kind gift of AD Friedman (John Hopkins University, Baltimore, MD, USA).

pSRP CDP shRNA: To generate this plasmid, a double-strand oligonucleotide targeting nucleotides 139–158 of CDP/cut mRNA was cloned into the *Bam*HI/*Xho*I-digested pSRP vector (gift of Stephen Lessnick, Dana Farber Cancer Center, Boston, MA, USA).

pSRP Ctrl shRNA: This plasmid contains a double-strand oligonucleotide corresponding to scrambled sequences of CDP/cut shRNA without homology to other murine mRNAs.

MigRI CCL9-HA: The CCL9 coding sequence was amplified by RT–PCR from 32Dcl3 RNA using a sense (5′-ATGAAGCCTTTTCATACTGCCC-3′) and an antisense (5′-TTATTGTTTGTAGGTCCGTGGTT-3′) primer. The PCR product was reamplified with a primer containing a 5′-flapping *Xho*I site and a primer containing a 5′-flapping *Eco*RI site flanked by the HA tag and a mutated stop codon and cloned into the *Xho*I/*Eco*RI digested MigRI vector.

MigRI and *MigRI p210 BCR/ABL* were previously described (Pear *et al*., 1998).

Cell lines, retroviral transductions and treatment with chemical inhibitors

32Dcl3 cells and derivative cell lines were cultured as described (Ferrari-Amorotti *et al*., 2006). STI571-resistant 32D-BCR/ABL cells were established by exposing 32D-BCR/ABL cells to increasing concentrations of STI571 (0.1–2 μM) and cultured with 1 μM STI571.

The FDCP-mix BCR/ABL cell line, which expresses a temperature-sensitive BCR/ABL, was cultured as described (Pierce *et al*., 2002) and treated with STI571 (10 μM, at 32°C) for the indicated times. MO7e (gift of AM Gewirtz, University of Pennsylvania, Philadelphia, PA, USA) and MO7e-BCR/ABL cells were cultured in the presence of 10 ng/ml GM-CSF.

Retroviral transductions of 32Dcl3, 32D-BCR/ABL or MO7e cells were performed as described (Perrotti *et al*., 2002).

Culture conditions and retroviral transductions of primary bone marrow cells

Mouse marrow cells were enriched for lineage-negative (Lin-) precursors using the StemSep Mouse Progenitor Enrichment Kit (Stem Cell Technologies Inc., Vancouver, Canada) and cultured 24 h in StemSpan SFEM medium (Stem Cell

Technologies Inc.), supplemented with IL-3 (6 ng/ml), IL-6 (10 ng/ml) and Kit ligand (50 ng/ml). Lin^- $Sca\text{-}1^+$ Kit^+ cells were obtained by FACS sorting using PE-conjugated anti-Sca-1 and APC-conjugated anti-c-kit antibodies (BD Biosciences Pharmingen, San Diego, CA, USA). Cells were transduced with supernatant of MigRI p210 BCR/ABL-transfected Phoenix cells and after sorting cultured in StemSpan SFEM medium supplemented with IL-3, IL-6, and Kit ligand with or without STI571 and processed for Western blot analyses.

Formation of s.c. tumors in mice
C3H/HeJ mice (syngenic with the 32Dcl3 cell line) were injected s.c. with MigRI-transduced 32D-BCR/ABL cells in the right flank and with CCL9-expressing 32D-BCR/ABL cells in the left flank. Mice were killed 14 days post-injection and tumors were excised and analysed.

Analysis of leukemogenesis in mice
C3H/HeJ and SCID mice were injected i.v. with MigRI or MigRI CCL9-transduced 32D BCR/ABL cells (3×10^5/mouse). Colony formation assays were performed 14 days post-injection using peripheral blood withdrawn from 10 mice/group. After erythrocyte hypotonic lysis, cells were plated in methylcellulose (10^4/plate) in the absence of cytokines. Survival of injected mice was also determined.

Chemotaxis assay
Details are available as Supplementary Information.

Immunofluorescence staining
Details are available as Supplementary Information.

Mixed culture assay
Details are available as Supplementary Information.

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