

# Cytotoxic T cells induce proliferation of chronic myeloid leukemia stem cells by secreting interferon- $\gamma$

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**Chronic myeloid leukemia (CML) is a clonal myeloproliferative neoplasia arising from the oncogenic break point cluster region/Abelson murine leukemia viral oncogene homolog 1 translocation in hematopoietic stem cells (HSCs), resulting in a leukemia stem cell (LSC). Curing CML depends on the eradication of LSCs. Unfortunately, LSCs are resistant to current treatment strategies. The host's immune system is thought to contribute to disease control, and several immunotherapy strategies are under investigation. However, the interaction of the immune system with LSCs is poorly defined. In the present study, we use a murine CML model to show that LSCs express major histocompatibility complex (MHC) and co-stimulatory molecules and are recognized and killed by leukemia-specific CD8<sup>+</sup> effector CTLs in vitro. In contrast, therapeutic infusions of effector CTLs into CML mice in vivo failed to eradicate LSCs but, paradoxically, increased LSC numbers. LSC proliferation and differentiation was induced by CTL-secreted IFN- $\gamma$ . Effector CTLs were only able to eliminate LSCs in a situation with minimal leukemia load where CTL-secreted IFN- $\gamma$  levels were low. In addition, IFN- $\gamma$  increased proliferation and colony formation of CD34<sup>+</sup> stem/progenitor cells from CML patients in vitro. Our study reveals a novel mechanism by which the immune system contributes to leukemia progression and may be important to improve T cell-based immunotherapy against leukemia.**

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Abbreviations used: AAD, aminoactinomycin D; alloSCT, allogeneic stem cell transplantation; BCR/ABL, break point cluster region/Abelson murine leukemia viral oncogene homolog 1; CML, chronic myeloid leukemia; CMP, common myeloid progenitor; DLI, donor lymphocyte infusion; GMP, granulocyte-macrophage progenitor; HSC, hematopoietic stem cell; LCMV, lymphocytic choriomeningitis virus; LMPP, leukemia MPP; LSC, leukemia stem cell; LSK, lin<sup>-</sup>Sca-1<sup>+</sup>c-kit<sup>hi</sup>; MPP, multipotent progenitor cell; rh, recombinant human; rm, recombinant murine; TKI, tyrosine kinase inhibitor.

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm that arises from break point cluster region/Abelson murine leukemia viral oncogene homolog 1 (BCR/ABL)-transformed hematopoietic stem (HSCs) or early progenitor cells known as leukemia stem cells (LSCs; Kavalierchik et al., 2008). LSCs have been first characterized as the tumor-initiating cells in acute myeloid leukemia (Lapidot et al., 1994) and have also been defined in other hematopoietic neoplasms since then (Cox et al., 2004; Matsui et al., 2004).

BCR/ABL-specific tyrosine kinase inhibitors (TKIs) such as Imatinib mesylate (Gleevec) have revolutionized the therapy of CML (Druker et al., 2001a,b; Baccarani et al., 2006). Nevertheless, LSCs seem resistant to TKIs and traditional chemotherapy (Weiden et al., 1979; Deininger et al., 2000; Savona and Talpaz, 2008) and CML inevitably progresses to incurable

acute leukemia (Faderl et al., 1999). Quiescent, self-renewing LSCs remain in the BM and are responsible for refractoriness and relapse of CML after treatment (Hughes et al., 2003). Therefore, novel cytotoxic agents that selectively target LSCs are under investigation (Jin et al., 2006; Guzman et al., 2007; Neviani et al., 2007; Ito et al., 2008; Bellodi et al., 2009; Majeti et al., 2009; Wang et al., 2010; Schürch et al., 2012).

Another promising approach in the treatment of CML is immunotherapy. In fact, currently, the only curative treatment for CML remains allogeneic stem cell transplantation (alloSCT). The graft-versus-leukemia effect of alloSCT is most likely executed by donor CD8<sup>+</sup> effector CTLs specific for minor histocompatibility

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antigens (Weiden et al., 1979; Kolb et al., 1990; Gale et al., 1994; Druker et al., 2002). Patients who receive T cell-depleted alloSCT grafts have a higher risk of disease relapse, and donor lymphocyte infusions are able to induce complete remission after relapse (Thomas et al., 1979; Horowitz et al., 1990; Kolb et al., 1995; Sehn et al., 1999). Furthermore, endogenous CTLs directed against leukemia antigens have been detected in the peripheral blood of chronic phase CML patients (Molldrem et al., 2000; Butt et al., 2005). Several proteins may potentially act as potent leukemia-specific antigens for T cells, including BCR/ABL, Wilms' tumor 1 protein (WT1), and proteinase 3 (Pr3; Van Driessche et al., 2005). Peptides from the junctional region of BCR/ABL are not present in healthy individuals and therefore are leukemia-specific. Yotnda et al. (1998) identified a BCR/ABL junctional nonapeptide that binds to human leukocyte antigen (HLA)-A2.1 and elicits specific CTL responses *in vitro* and *in vivo*. Additional studies confirmed and extended the finding of immunogenic BCR/ABL junction peptides (Bocchia et al., 1996; Clark et al., 2001).

CTLs have been shown to kill CML target cells *in vitro* via Fas-receptor triggering (Selleri and Maciejewski, 2000). In a BCR/ABL-induced murine CML model, we have shown that CD8<sup>+</sup> T cells crucially contribute to disease control *in vivo*. However, programmed death ligand 1 (PD-L1) expression by the malignant cells induced T cell dysfunction leading to disease progression (Mumprecht et al., 2009b).

Despite these advances in the understanding of the immunosurveillance of CML and the development of immunotherapy strategies, the interaction of effector CTLs with the disease-originating LSCs has not been analyzed so far. In the present study, we analyzed the immunogenicity of LSCs *in vitro* and *in vivo* using the glycoprotein of lymphocytic choriomeningitis virus (LCMV) as model leukemia antigen. LSCs expressed MHC and co-stimulatory molecules. LSCs isolated from CD8<sup>+</sup> T cell-depleted CML mice were more immunogenic than LSCs from control CML mice, indicating that CD8<sup>+</sup> T cells interact with LSCs *in vivo* and select for low immunogenic variants. To analyze whether LSCs can be recognized and lysed by activated leukemia-specific effector CTLs, we treated CML mice with large numbers of specific T cells. Although these effector CTLs efficiently lysed LSCs *in vitro*, adoptive immunotherapy *in vivo* failed to eradicate LSCs but paradoxically increased LSC numbers. LSC proliferation was induced by CTL-secreted IFN- $\gamma$ . Specific effector CTLs secreted high amounts of IFN- $\gamma$  when transferred to CML mice in advanced disease stage with high antigen load, but not after transfer to CML mice in early stage of disease. Importantly, our results were confirmed in a humanized CML model using HLA-A2.1 transgenic mice and a BCR/ABL b3a2 junctional peptide. In addition, IFN- $\gamma$  induced the proliferation of primary CD34<sup>+</sup> stem/progenitor cells from newly diagnosed CML patients.

## RESULTS

### Immunogenicity of LSCs *in vitro*

To establish a CML-like disease, BM cells were retrovirally transduced with BCR/ABL-GFP, followed by transfer to

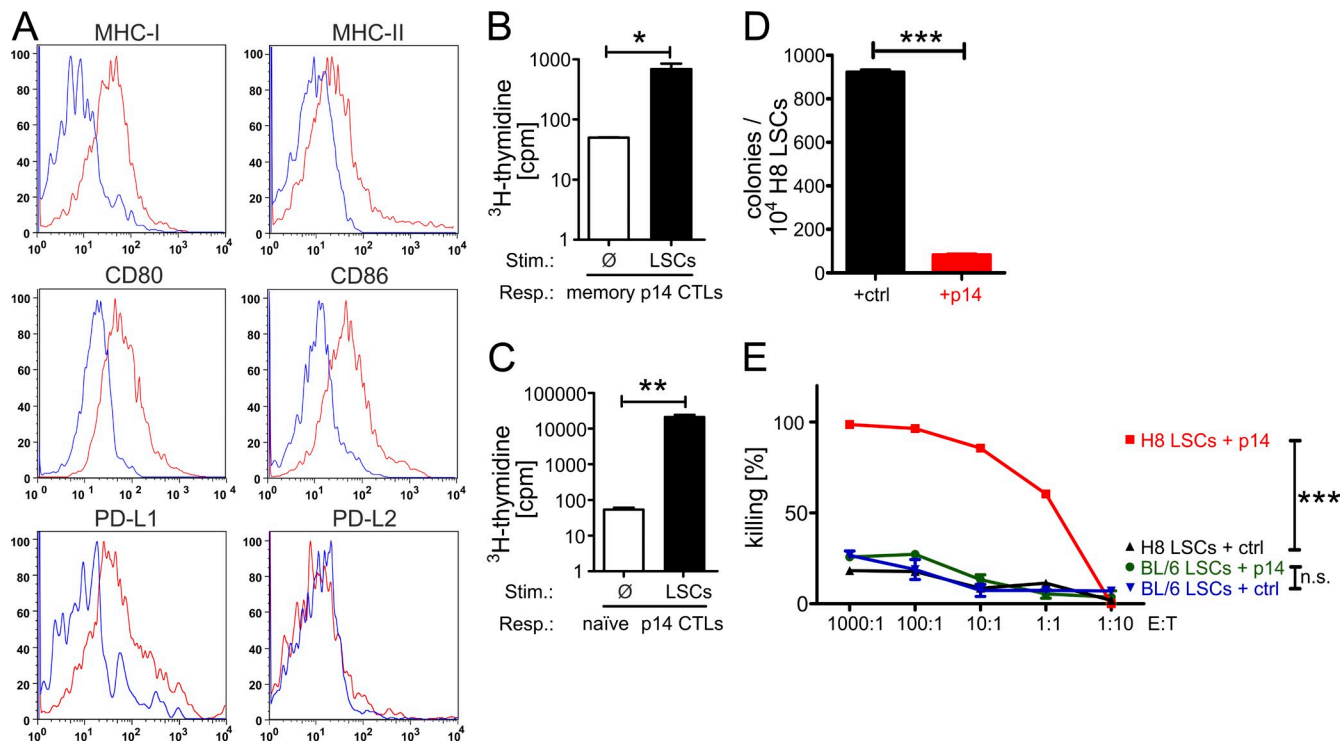
sublethally irradiated (6.5 Gy) C57BL/6 (BL/6) recipient mice (Mumprecht et al., 2009a). As shown before (Mumprecht et al., 2009b), at this reduced irradiation dose the adaptive immune system, including CD8<sup>+</sup> T cells, CD4<sup>+</sup> T cells, and B cells, originates from the recipient mouse (Mumprecht et al., 2009b), allowing us to generate CML in a BL/6 host with a largely intact immune system. To analyze the interaction of CTLs with defined antigen specificity with LSCs, we used donor BM of H8 transgenic mice (Ehl et al., 1998) that ubiquitously express the LCMV glycoprotein epitope gp33 under the control of an MHC class I promoter to generate a CML in BL/6 mice (H8 CML). In this setting, BCR/ABL-GFP-expressing cells coexpress the LCMV-gp33 antigen.

20 d after transplantation, H8 CML mice were sacrificed and BM was isolated. H8 LSCs were defined as BCR/ABL-GFP<sup>+</sup>, lineage negative (lin<sup>-</sup>), Sca-1<sup>+</sup>, c-kit<sup>hi</sup> (Neering et al., 2007) and analyzed for expression of MHC class I and II and co-stimulatory molecules by FACS (Fig. 1 A). MHC-I, MHC-II, CD80, and CD86 were all strongly expressed on H8 LSCs. In addition, LSCs expressed PD-L1 but not PD-L2. We next sought to determine the capacity of H8 LSCs to activate naive or reactivate memory LCMV-gp33-specific, TCR transgenic CD8<sup>+</sup> T cells (p14 CTLs; Pircher et al., 1989) in a standard <sup>3</sup>H-thymidine incorporation assay. In the absence of additional cytokines, H8 LSCs induced proliferation not only in memory, but also in naive p14 CTLs (Fig. 1 B and C). These data indicate that LSCs possess the molecular repertoire to interact with T cells and are able to induce proliferation of naive and memory CTLs.

To test whether effector CTLs are able to kill LSCs *in vitro*, we isolated LSCs from H8 CML and BL/6 CML mice by FACS. LSCs were co-incubated with either naive cells (ctrl) or p14 effector cells (p14) overnight, followed by colony-forming assays. Co-incubation of H8 LSCs with p14 effector splenocytes resulted in significantly fewer colonies as compared with control naive splenocytes (Fig. 1 D). A titration of the effector/target ratio revealed an efficient lysis of H8 LSCs by MACS-purified p14 effector CD8<sup>+</sup> CTLs *in vitro*. In contrast, p14 effector CD8<sup>+</sup> CTLs did not lyse BL/6 LSCs (Fig. 1 E). These experiments revealed that LSCs are recognized and killed by activated CTLs *in vitro* and that this killing is antigen specific.

### Immunoediting of LSCs by endogenous CD8<sup>+</sup> T cells *in vivo*

We next wanted to address the question of whether specific CTLs actually interact with LSCs *in vivo*. To this end, as an indirect parameter, we analyzed the immunogenicity of LSCs in the presence or absence of CD8<sup>+</sup> T cells. We generated H8 CML mice and treated them *i.p.* with depleting anti-CD8 monoclonal antibody (mAb), starting 12 d after transplantation. This treatment depletes CD8<sup>+</sup> T cells from peripheral blood to below detection limits of FACS (not depicted) and leads to rapid CML progression (Mumprecht et al., 2010). Control H8 CML mice were left untreated. 8 d later, we isolated LSCs from both groups of mice and performed a <sup>3</sup>H-thymidine assay using naive p14 CD8<sup>+</sup> T cells



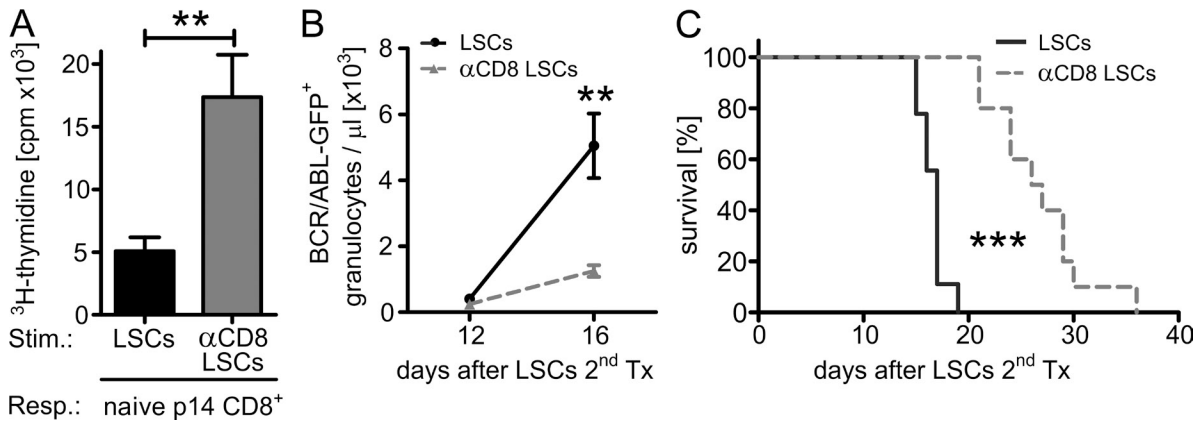
**Figure 1. Immunogenicity of CML LSCs in vitro.** (A) Expression of MHC class I, MHC class II, CD80, CD86, PD-L1, and PD-L2 on H8 CML LSCs. 1 representative plot of 3–10 is shown. (B and C)  $^3\text{H}$ -thymidine assays. (B)  $3 \times 10^3$  FACS-purified, irradiated (10 Gy) LSC stimulators were co-incubated with  $1.5 \times 10^4$  MACS-purified CD8<sup>+</sup> T cell responders from BL/6 mice infected with LCMV 6 wk earlier. One representative experiment out of two is shown. (C)  $3 \times 10^3$  FACS-purified, irradiated (10 Gy) LSC stimulators were co-incubated with  $1.5 \times 10^4$  MACS-purified CD8<sup>+</sup> T cell responders from naive p14 TCR transgenic animals. One representative experiment out of five is shown. (D–E) In vitro killing assay. (D)  $10^4$  H8 LSCs FACS-purified from H8 CML mice at day 18 after transplantation were co-incubated with  $2 \times 10^4$  naive BL/6 splenocytes (ctrl) or p14 effector splenocytes (p14) overnight, followed by transfer into methylcellulose. Colonies were enumerated 7 d later. One representative experiment out of two is shown. (E)  $10^4$  FACS-purified H8 or BL/6 LSCs from H8 or BL/6 CML mice (20 d after CML induction) were co-incubated with 0,  $10^3$ ,  $10^4$ ,  $10^5$ ,  $10^6$ , or  $10^7$  MACS-purified naive CD8<sup>+</sup> T cells (ctrl) or p14 effector CD8<sup>+</sup> T cells (p14) overnight, followed by transfer into methylcellulose in duplicates. Colonies were enumerated 7 d later. Percent killing was calculated in relation to culture condition without CD8<sup>+</sup> T cells (0). Data are displayed as mean  $\pm$  SEM. Statistics: Student's *t* test (B–D), two-way ANOVA (E). \*,  $P < 0.05$ ; \*\*,  $P < 0.005$ ; \*\*\*,  $P < 0.0001$ . Isotype controls, blue lines; stainings, red lines. E:T, effector/target ratio; LSCs, leukemia stem cells; Resp., responders; Stim., stimulators.

as responders (Fig. 2 A). Interestingly, LSCs isolated from CD8-depleted H8 CML mice ( $\alpha\text{CD8}$  LSCs) induced a more efficient expansion of naive p14 responder CD8<sup>+</sup> T cells than LSCs from control H8 CML mice (LSCs).

To determine the immunogenicity of LSCs from CD8-depleted hosts in vivo, we secondarily transplanted  $2 \times 10^4$  LSCs or  $\alpha\text{CD8}$  LSCs to irradiated (6.5 Gy) BL/6 recipients. 12 d after transplantation, secondary recipients had similar numbers of BCR/ABL-GFP<sup>+</sup> granulocytes in peripheral blood, indicating similar engraftment of LSCs from both groups (Fig. 2 B). However, secondary CML originating from  $\alpha\text{CD8}$  LSCs progressed much slower than secondary CML originating from control LSCs. This was reflected in a slower increase of BCR/ABL-GFP<sup>+</sup> granulocytes in blood (Fig. 2 B) and a significantly prolonged survival of secondary recipients (Fig. 2 C). These experiments indicate that CD8<sup>+</sup> T cells interact with LSCs in vivo. This interaction leads to a selective pressure to develop poorly immunogenic LSCs, a process called immunoediting (Schreiber et al., 2011).

### CTL killing of LSCs in vivo is dependent on antigen/leukemia load

Our experiments thus far suggest that specific effector CTLs should be very efficient in the elimination of LSCs in vivo and therefore can be developed as a therapeutic strategy. To test the role of specific effector CTLs in a therapeutic setting with minimal leukemia load, we injected FACS-purified H8 LSCs into irradiated (6.5 Gy) secondary BL/6 recipient mice and adoptively transferred p14 effector CTLs 18 h later (Fig. 3 A). 2 d later, the numbers of BCR/ABL-GFP<sup>+</sup> cells and LSCs in the BM were analyzed. In concordance with our in vitro experiments, the adoptive transfer of leukemia-specific CTLs drastically reduced the number of BCR/ABL-GFP<sup>+</sup> cells and LSCs in the BM of p14 effector CTL-treated mice (Fig. 3, B and C). In line with these findings, primary H8 CML mice treated 7 d after CML induction survived, whereas untreated controls died after  $\sim 3$  wk (Fig. 3 D). These experiments indicate that LSCs can be recognized and killed by adoptively transferred specific CTLs in vivo.



**Figure 2. Immunoediting of LSCs by endogenous CD8<sup>+</sup> T cells in vivo.** (A) <sup>3</sup>H-thymidine assay.  $3 \times 10^3$  FACS-purified, irradiated (10 Gy) LSC stimulators from H8 CML mice (LSCs) or from CD8-depleted H8 CML mice ( $\alpha$ CD8 LSCs) were co-incubated with  $1.5 \times 10^4$  MACS-purified CD8<sup>+</sup> T cell responders from naive p14 TCR transgenic animals. Pooled data from two independent experiments are shown. (B and C)  $2 \times 10^4$  FACS-purified LSCs (solid line,  $n = 9$ ) or  $\alpha$ CD8 LSCs (dotted line,  $n = 10$ ) were secondarily transplanted into irradiated (6.5 Gy) BL/6 recipient mice. (B) Numbers of BCR/ABL-GFP<sup>+</sup> granulocytes/ $\mu$ l blood and (C) Kaplan-Meier-survival curves resulting from secondary transplantations. One representative out of three independent experiments is shown. Data are displayed as mean  $\pm$  SEM. Statistics: Student's *t* test (A), two-way ANOVA (B), and log-rank test (C). \*\*,  $P < 0.005$ ; \*\*\*,  $P < 0.0001$ . Resp., responders; Stim., stimulators.

To more closely model the clinical situation of leukemia, we treated H8 CML mice with p14 effector CTLs 20 d after primary leukemia transplantation. At this time point, the frequency of Gr-1<sup>+</sup> BCR/ABL-GFP<sup>+</sup> granulocytes in the peripheral blood was  $43 \pm 9\%$  (not depicted), indicating a high leukemia load. Injections of p14 effector CTLs significantly reduced total BM cells compared with untreated mice (Fig. 3 F). Surprisingly, when analyzing the lineage-negative fraction and LSCs in these mice, we found a drastic increase in total LSC numbers (Figs. 3 E, 3G) in p14 effector CTL-treated H8 CML mice. This increase in LSCs was accompanied by a similar increase in host BCR/ABL-GFP<sup>-</sup>, lin<sup>-</sup>, Sca-1<sup>+</sup>, c-kit<sup>hi</sup> (LSK) cells (Fig. 3, H and I) suggesting that a soluble factor secreted by effector CTLs, rather than a direct cell-cell interaction, is responsible for the observed effect. However, treatment with p14 T cells at this late stage of disease did not improve survival (Fig. 3 J).

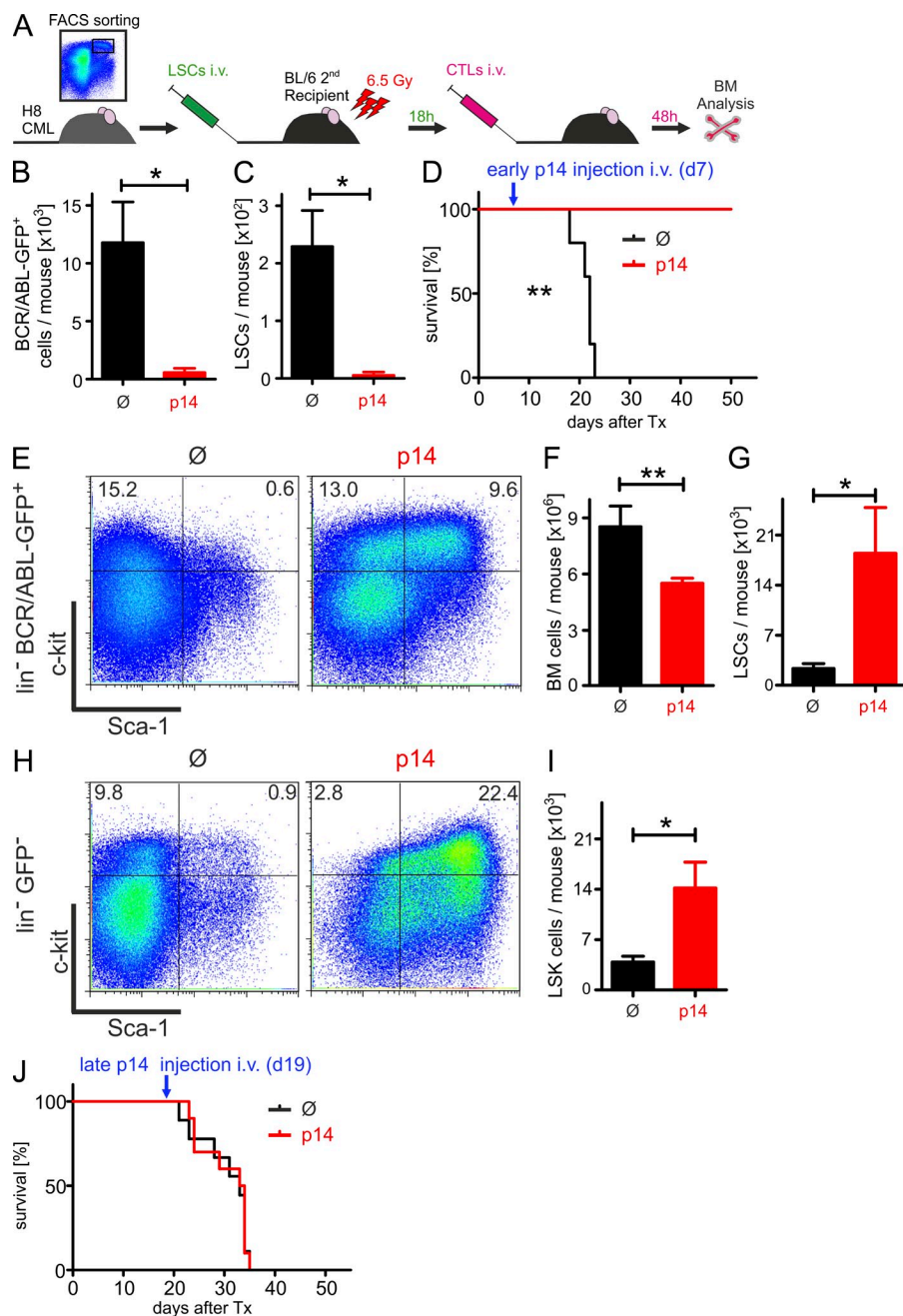
### IFN- $\gamma$ enhances proliferation of LSCs and leukemia progenitor cells in vivo

IFN- $\gamma$  is a major effector cytokine secreted by effector CTLs (Farrar and Schreiber, 1993). In addition, recent studies have shown that interferons have the capacity to activate HSCs (Essers et al., 2009; Baldrige et al., 2010). Therefore, we analyzed the concentration of IFN- $\gamma$  in the sera of p14 effector CTL-treated CML mice. H8 LSCs were transplanted into secondary BL/6 recipient mice and p14 effector CTLs were adoptively transferred either 18 h or 16 d after LSC transplantation. 2 d after adoptive transfer of p14 effector CTLs, serum IFN- $\gamma$  levels were analyzed. IFN- $\gamma$  concentrations were below the detection limit in sera of untreated CML mice and of CML mice that were treated with p14 effector CTLs 18 h after H8 LSC transplantation (Fig. 4 A). In contrast, in CML animals with an established leukemia 16 d after secondary

transplantation, adoptively transferred p14 effector CTLs produced IFN- $\gamma$ , resulting in detectable serum concentrations of IFN- $\gamma$  (Fig. 4 A). The level of IFN- $\gamma$  increased in dependence of the number of transferred p14 effector CTLs (Fig. 4 B). Intracellular stainings revealed that BM-infiltrating CTLs produced IFN- $\gamma$  in CML mice (Fig. 4 C). Moreover, the IFN- $\gamma$  receptor was expressed on H8 LSCs, suggesting that LSCs are able to respond to IFN- $\gamma$  (Fig. 4 D).

To investigate the effect of IFN- $\gamma$  on leukemia development and the behavior of LSCs, we injected recombinant murine (rm)-IFN- $\gamma$  or vehicle i.p. into BL/6 CML mice on 2 consecutive days, starting 17 d after CML induction. IFN- $\gamma$  treatment stabilized the number of Gr-1<sup>+</sup> BCR/ABL-GFP<sup>+</sup> granulocytes in the peripheral blood (before IFN- $\gamma$ ,  $164 \pm 28/\mu$ l; after IFN- $\gamma$ ,  $264 \pm 38/\mu$ l; ns), whereas in vehicle-treated mice, Gr-1<sup>+</sup> BCR/ABL-GFP<sup>+</sup> granulocyte numbers significantly increased during this time period (before vehicle:  $194 \pm 69/\mu$ l; after vehicle:  $879 \pm 166/\mu$ l;  $P = 0.005$ , Fig. 4 E). In contrast, FACS analysis of BM after IFN- $\gamma$  treatment (day 19) revealed a fivefold increase in LSC numbers compared with vehicle-treated mice (Fig. 4 F), a finding that was confirmed by colony forming assays of lin<sup>-</sup> cells (Fig. 4 G). Similar results with IFN- $\gamma$  treatment were obtained in H8 CML mice expressing the LCMV-gp33 as a model tumor antigen (not depicted).

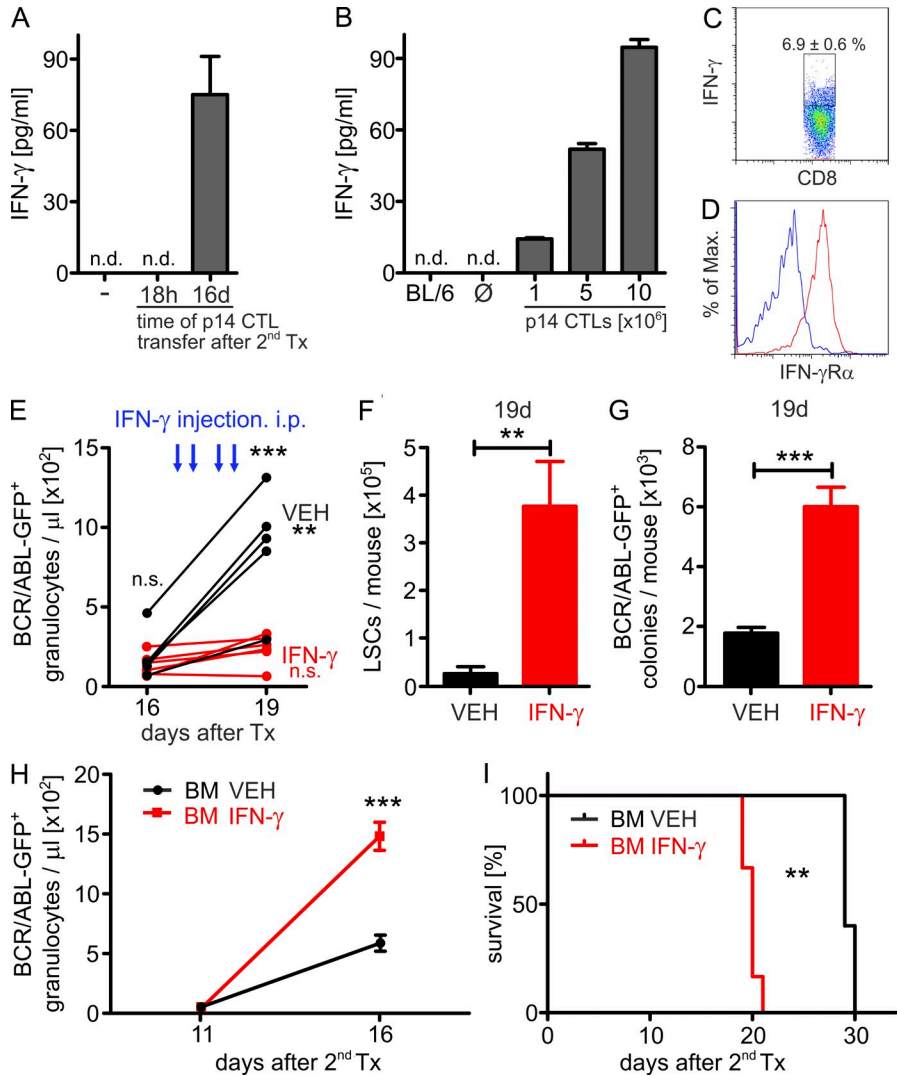
To verify that the increase in LSCs detected in FACS analysis and in methylcellulose cultures in vitro actually represents an increase in cells that can induce leukemia in vivo, we secondarily transplanted  $3 \times 10^6$  BM cells from vehicle- and IFN- $\gamma$ -treated CML mice (day 19) into sublethally irradiated (4.5 Gy) BL/6 mice. Mice that received BM from IFN- $\gamma$ -treated leukemia mice developed a more severe course of the disease and died significantly faster than control mice (Fig. 4, H and I).



LSCs are a heterogeneous population consisting of long-term and short-term LSCs and leukemia multipotent progenitor cells (MPPs; Li et al., 2012). We therefore determined the LSC subpopulation preferentially responding to rm-IFN- $\gamma$  (Fig. 5 A). IFN- $\gamma$  increased numbers of primitive long-term and short-term LSCs, as well as early myeloid leukemia MPPs (LMPPs) in BM (Fig. 5, B–E). In contrast, there was no difference in lymphoid-primed LMPPs (Fig. 5 F). IFN- $\gamma$  increased leukemia c-kit<sup>hi</sup> cells by enhancing their proliferation (Fig. 5, G and H); cell survival was not affected (Fig. 5, I–J). In addition, we analyzed more differentiated

leukemia common myeloid progenitors (CMPs) and leukemia granulocyte-macrophage progenitors (GMPs). Upon IFN- $\gamma$  treatment, leukemia CMP numbers increased as a result of proliferation (Fig. 5, K and L). Interestingly, although IFN- $\gamma$  increased proliferation of leukemia GMPs, their absolute numbers in BM were not increased (Fig. 5, M and N). This suggests that leukemia GMPs rapidly differentiate into more mature myeloid cells.

In summary, these experiments indicate that IFN- $\gamma$  increases the numbers of LSCs and leukemia progenitor cells by enhancing their proliferation.



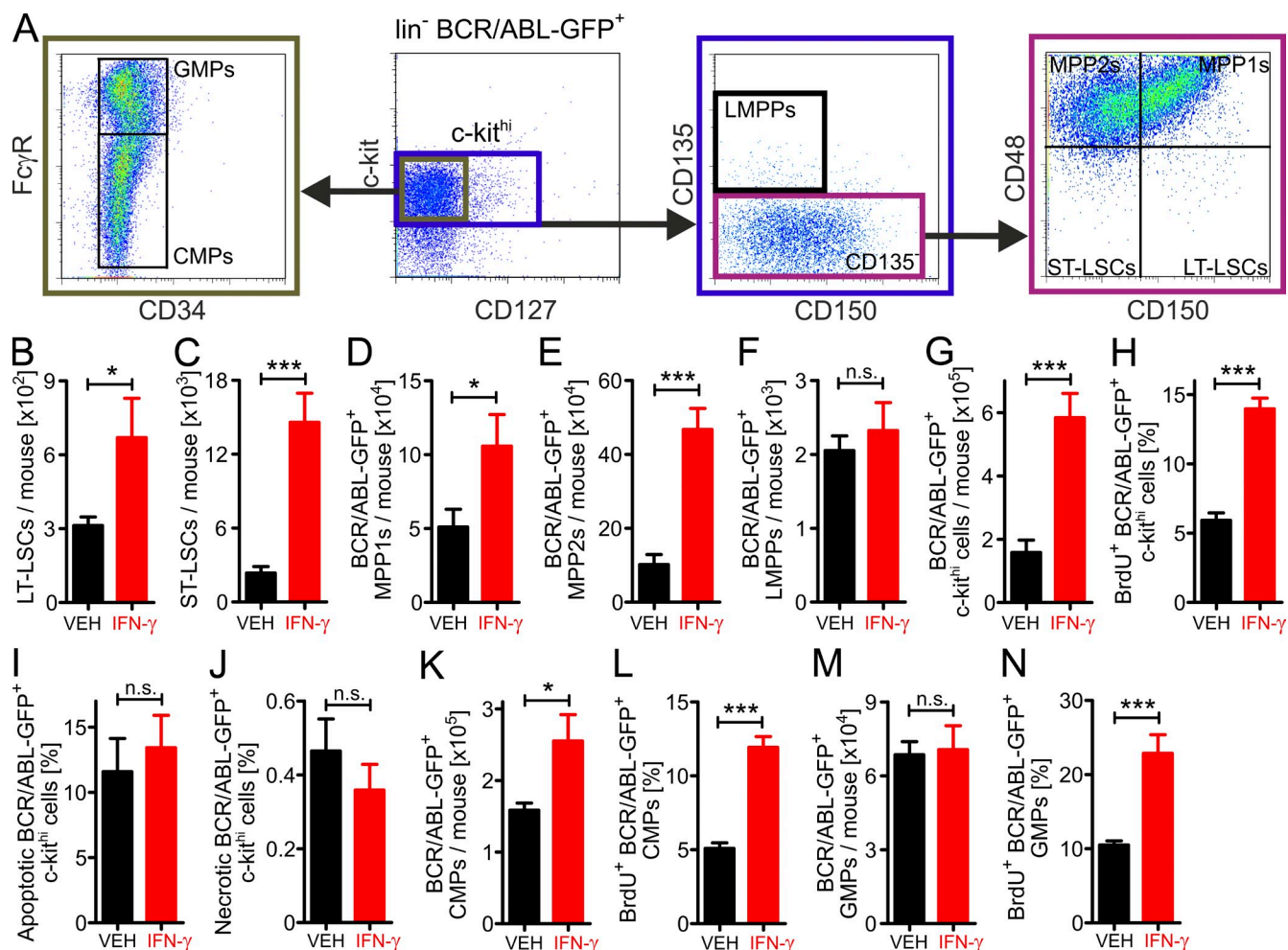
**Figure 4. IFN- $\gamma$  increases LSC numbers in CML.** (A and B) IFN- $\gamma$  serum levels of CML mice 48 h after p14 CTL treatment. (A)  $2 \times 10^4$  H8 LSCs were FACS-purified 20 d after primary transplantation and were transferred i.v. into irradiated (6.5 Gy) secondary BL/6 hosts. 18 h or 16 d later, mice were either left untreated ( $\emptyset$ ,  $n = 10$ , 18h) or treated with  $3 \times 10^6$  FACS-purified p14 CTLs i.v. (18h:  $n = 10$ ; 16d:  $n = 5$ ). (B) H8 CML mice were treated with titrated numbers of MACS-purified p14 CTLs ( $10^6$ ,  $5 \times 10^6$  or  $10 \times 10^6$  cells) 16 d after primary transplantation or were left untreated ( $n = 6-7$  mice per group). (C) IFN- $\gamma$  production of BM-infiltrating CTLs in BL/6 hosts that were treated 16 d after secondary LSC transplantation ( $n = 5$ ). (D) Expression of IFN- $\gamma$  receptor  $\alpha$  chain (IFN- $\gamma$ R $\alpha$ ) on H8 CML LSCs. One representative plot of 5 is shown. (E-I) BL/6 CML mice were treated with either vehicle (VEH,  $n = 5$ ) or  $2.5 \times 10^5$  U of rm-IFN- $\gamma$  ( $n = 6$ ) i.p. twice daily on two consecutive days (days 17 and 18 after primary transplantation). (E) Numbers of BCR/ABL-GFP $^+$  granulocytes/ $\mu$ l blood and (F) LSC numbers were determined on day 19 after transplantation. (G) Equal numbers of lin $^-$  cells were plated in methylcellulose and BCR/ABL-GFP $^+$  colonies were enumerated 7 d later by fluorescence microscopy. (H-I)  $3 \times 10^6$  BM cells from vehicle-treated (black line,  $n = 5$ ) or IFN- $\gamma$ -treated (red line,  $n = 6$ ) BL/6 CML mice were secondarily transplanted into irradiated (4.5 Gy) BL/6 mice. (H) Numbers of BCR/ABL $^+$  granulocytes/ $\mu$ l blood and (I) Kaplan-Meier survival curves resulting from secondary transplantations. Data are displayed as mean  $\pm$  SEM. Statistics: Student's  $t$  test (F and G), two-way ANOVA (E and H), log-rank test (I). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.0005$ .

**Adoptively transferred CTLs increase the proliferation of leukemia progenitor cells via IFN- $\gamma$**

To analyze if the observed increase of LSCs in CML mice after p14 effector CTL-treatment is indeed caused by IFN- $\gamma$  secretion from CTLs, treatment protocols early and late after LSC transfer were repeated using IFN- $\gamma$ -deficient p14 effector CTLs. First, secondary recipients were treated 18 h after LSC transplantation, either with IFN- $\gamma$ -competent (p14) or -deficient (p14xIFN- $\gamma^{-/-}$ ) p14 effector CTLs. 2 d later, numbers of BCR/ABL-GFP $^+$  cells and LSCs were analyzed in the BM. P14 and p14xIFN- $\gamma^{-/-}$  effector CTLs were equally able to eradicate BCR/ABL-GFP $^+$  cells and LSCs at this early time point after LSC transfer (Fig. 6, A and B). In vitro cytotoxicity assays revealed that target cell lysis by p14 and p14xIFN- $\gamma^{-/-}$  CTLs could be inhibited by addition of the granzyme B inhibitor Z-aminoactinomycin D (AAD)-CMK, but not by blocking the FAS-FASL interaction using mAb (Fig. 6 C). Importantly, additional injection of rm-IFN- $\gamma$  in

the same experimental setting prevented the rejection of leukemia cells by p14 effector CTLs and increased LSC numbers. In addition,  $3 \times 10^6$  BM cells from untreated, p14-treated, or p14+rm-IFN- $\gamma$ -treated H8 CML mice were transplanted into sublethally (6.5 Gy) irradiated tertiary BL/6 recipient mice. Mice that received p14-treated BM did not develop leukemia, therefore they survived (Fig. 6, D and E). In contrast, mice receiving untreated BM or p14+rm-IFN- $\gamma$ -treated BM developed leukemia and died from classical symptoms of leukemia (Fig. 6, D and E). These experiments indicate that IFN- $\gamma$  increases LSC numbers and prevents the elimination of LSCs by CTLs. The increase of leukemia cells in the BM in response to IFN- $\gamma$  is in contrast to the reduction of malignant granulocytes after IFN- $\gamma$  treatment in the circulation (Fig. 4 E), indicating that the effect on mature granulocytes and on leukemia stem/progenitor cells is different.

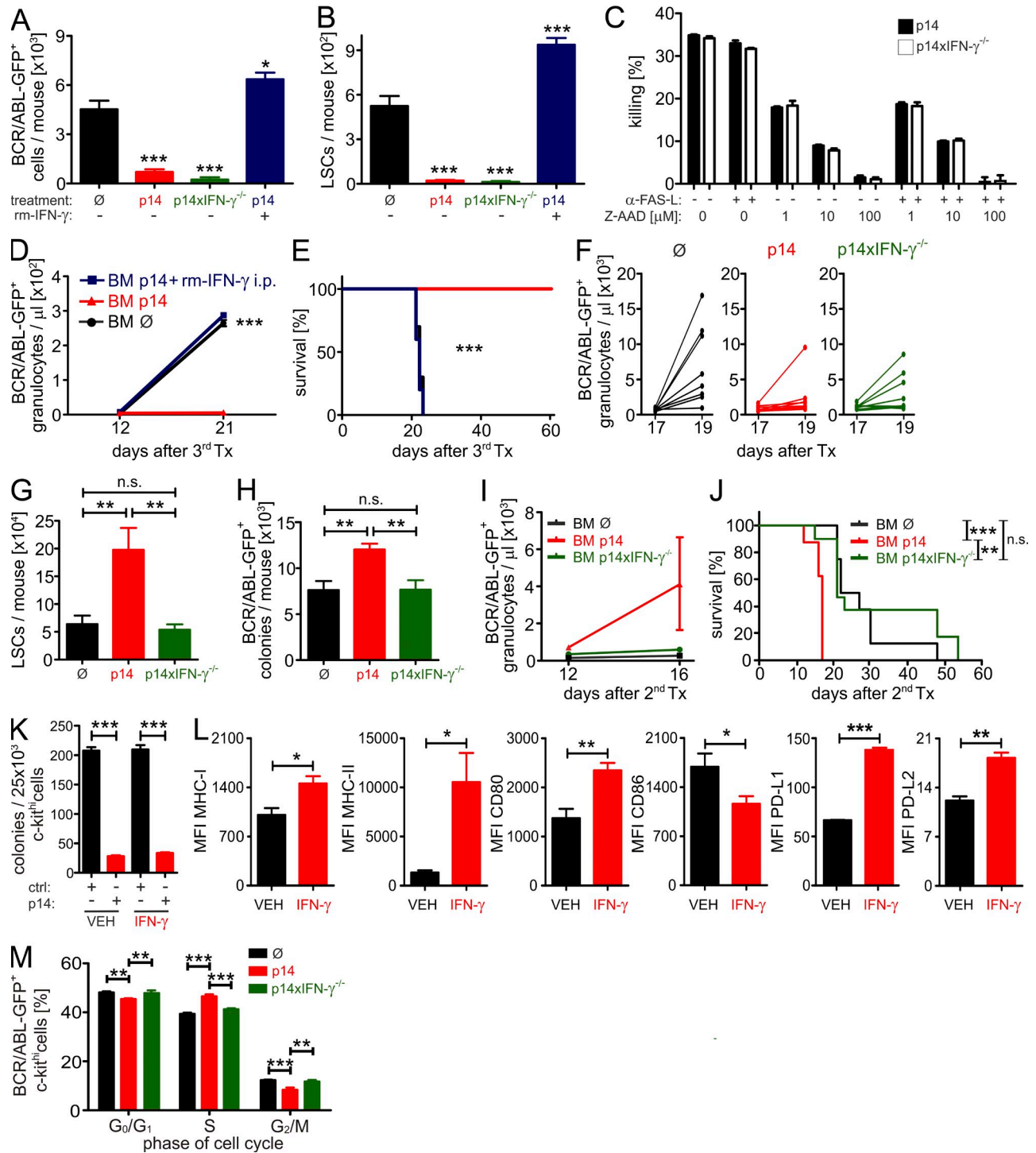
Next, we adoptively transferred p14 and p14xIFN- $\gamma^{-/-}$  effector CTLs to primary H8 CML mice in an advanced



**Figure 5. IFN- $\gamma$  enhances the proliferation of early LSCs and leukemia progenitor cells in vivo.** (A) Gating strategy to identify early LSCs and leukemia progenitor cells in the BM of CML mice. (B–N) BL/6 CML mice were treated with either vehicle (VEH,  $n = 7$ ) or  $2.5 \times 10^5$  U of rm-IFN- $\gamma$  ( $n = 5$ ) i.p. twice daily on two consecutive days (days 17 and 18 after primary transplantation). On the same days, mice also received BrdU (0.8 mg/ml in drinking water and 1 mg i.p./day). 1 d later,  $lin^{-} BCR/ABL-GFP^{+}$  BM cells were analyzed for LSCs and leukemia progenitor cells. (B) Long-term LSCs ( $c-kit^{hi}CD135^{-}CD48^{-}CD150^{+}$ ). (C) Short-term LSCs ( $c-kit^{hi}CD135^{-}CD48^{+}CD150^{-}$ ). (D) Leukemia MPP1s ( $c-kit^{hi}CD135^{-}CD48^{+}CD150^{+}$ ). (E) Leukemia MPP2s ( $c-kit^{hi}CD135^{-}CD48^{+}CD150^{-}$ ). (F) Leukemia LMPPs ( $c-kit^{hi}CD135^{+}CD150^{-}$ ). (G) Numbers, (H) proliferation, and (I–J) viability of  $c-kit^{hi}$  leukemia progenitor cells. Numbers (K) and proliferation (L) of leukemia CMPs ( $c-kit^{hi}CD127^{-}CD34^{+}Fc\gamma R^{-}$ ). Numbers (M) and proliferation (N) of leukemia GMPs ( $c-kit^{hi}CD127^{-}CD34^{+}Fc\gamma R^{+}$ ). Data are displayed as mean  $\pm$  SEM. Statistics: (B–N) Student's  $t$  test. \*,  $P < 0.05$ ; \*\*\*,  $P < 0.0005$ . Apoptotic cells, Annexin V $^{+}$  cells; necrotic cells, Annexin V $^{+}$ -AAD $^{+}$  cells. CMPs, common myeloid progenitors; GMPs, granulocyte-macrophage progenitors; LMPPs, lymphoid-primed multipotent progenitors; LT, long-term; MPPs, multipotent progenitors; ST, short-term.

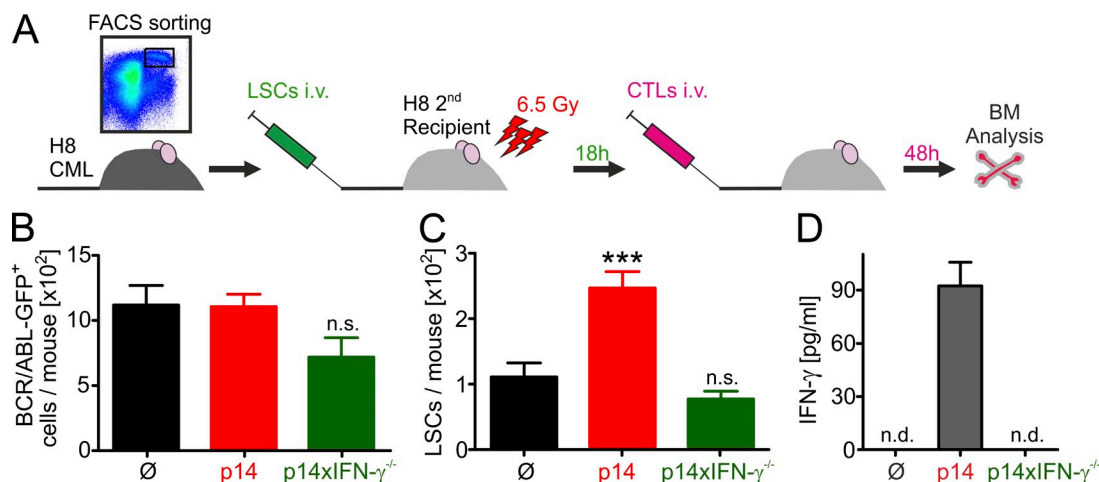
stage of the disease 17 d after transplantation. Control H8 CML mice were left untreated. Before adoptive transfer, numbers of Gr-1 $^{+}$  BCR/ABL-GFP $^{+}$  granulocytes in the peripheral blood were comparable (untreated,  $728 \pm 59/\mu l$ ; p14-treated,  $719 \pm 143/\mu l$ ; p14x IFN- $\gamma^{-/-}$ -treated,  $977 \pm 139/\mu l$ ; ns). Blood and BM were analyzed 2 d after adoptive transfer. In untreated mice, peripheral blood BCR/ABL-GFP $^{+}$  granulocyte numbers rapidly increased (Fig. 6 F). In contrast, p14 effector CTLs and, to a lesser extent, p14xIFN- $\gamma^{-/-}$  effector CTLs reduced the increase of BCR/ABL-GFP $^{+}$  granulocytes after these 2 d (Fig. 6 F). Strikingly, we found a marked increase in total LSC numbers in p14-treated, but not p14xIFN- $\gamma^{-/-}$ -treated CML mice (Fig. 6 G). This finding

was confirmed by performing colony forming assays from  $lin^{-}$  BM and assessing the numbers of BCR/ABL-GFP $^{+}$  colonies by fluorescence microscopy (Fig. 6 H). CML mice treated at this late stage of disease died rapidly a few days after treatment (Fig. 3 J), thus the effect on CML disease cannot be analyzed directly. To further confirm the increase in malignant progenitors in the BM of p14-treated mice, we secondarily transplanted  $3 \times 10^6$  BM cells from untreated, p14-treated, or p14xIFN- $\gamma^{-/-}$ -treated H8 CML mice into sublethally irradiated (6.5 Gy) BL/6 mice. Monitoring secondary CML by repetitive bleeding revealed that mice receiving p14-treated BM developed CML and succumbed to lethal disease by day 18 after transplantation (Fig. 6, I and J).



**Figure 6. CTL-secreted IFN-γ increases LSC numbers.** (A)  $2 \times 10^4$  H8 LSCs were FACS-purified 20 d after transplantation and were transferred i.v. into irradiated (6.5 Gy) secondary BL/6 recipients. 18 h later, mice were either left untreated ( $\emptyset$ ,  $n = 11$ ) or treated with  $3 \times 10^6$  FACS-purified p14 ( $n = 12$ ) or p14xIFN- $\gamma^{-/-}$  ( $n = 3$ ) CTLs i.v. One group of mice received p14 CTLs and was treated with  $2.5 \times 10^5$  U of rm-IFN- $\gamma$  twice daily for two consecutive days ( $n = 5$ ). After 48 h, BM was harvested and analyzed for BCR/ABL-GFP<sup>+</sup> (A) cells and LSCs (B) by FACS. Pooled data from two independent experiments are shown. (C) Duplicates of bulk BM cells from H8 CML mice were either incubated alone or were co-cultured with CTLs at a ratio of 5:1 with or without 10  $\mu$ g/ml anti-FAS-ligand, in the presence or absence of the indicated concentrations of the granzyme B inhibitor Z-AAD-CMK. After 4 h, the killing of BCR/ABL-GFP<sup>+</sup> cells was determined. (D and E)  $3 \times 10^6$  BM cells from p14-treated (red line,  $n = 5$ ), p14+rm-IFN- $\gamma$ -treated (blue line,  $n = 5$ ), or untreated (black line,  $n = 10$ ) H8 CML mice were tertiarily transplanted into irradiated (6.5 Gy) BL/6 mice. Numbers of BCR/ABL-GFP<sup>+</sup> granulocytes/ $\mu$ l blood (D) and Kaplan-Meier survival curves (E) resulting from tertiary transplantations are shown. (F) BCR/ABL-GFP<sup>+</sup> granulocytes in blood of primary H8 CML mice either left





**Figure 7. CML treatment with CTLs specific for a ubiquitously expressed antigen increases LSC numbers.** (A) Experimental design.  $2 \times 10^4$  H8 LSCs were FACS-purified 20 d after transplantation and were transferred i.v. into irradiated (6.5 Gy) secondary H8 hosts. 18 h later, mice were either left untreated ( $\emptyset$ ,  $n = 15$ ) or treated with  $3 \times 10^6$  FACS-purified p14 ( $n = 16$ ) or p14xIFN- $\gamma^{-/-}$  ( $n = 11$ ) CTLs i.v. After 48 h, BM was harvested and analyzed for BCR/ABL-GFP<sup>+</sup> cells (B) and LSCs (C) by FACS. (D) In one experiment, serum IFN- $\gamma$  levels were determined 48 h after CTL transfer ( $n = 4$ –7 mice per group). (B and C) Pooled data from four independent experiments are shown. Data are displayed as mean  $\pm$  SEM. Statistics: one-way ANOVA (B and C). \*\*\*,  $P < 0.0001$ .

In contrast, mice receiving untreated BM or p14xIFN- $\gamma^{-/-}$ -treated BM developed CML substantially slower and survived significantly longer (Fig. 6, I and J).

We next sought to analyze how IFN- $\gamma$  protects LSCs from CTL-mediated lysis, or even increases their numbers. Therefore, we analyzed the lysis of H8 LSCs from vehicle- or IFN- $\gamma$ -treated H8 CML mice in vitro. We isolated BCR/ABL-GFP<sup>+</sup> c-kit<sup>hi</sup> cells by FACS and co-incubated them directly ex vivo either with naive BL/6 or p14 effector CD8<sup>+</sup> T cells overnight, followed by colony-forming assays (Fig. 6 K). These experiments revealed that BCR/ABL-GFP<sup>+</sup> c-kit<sup>hi</sup> cells from vehicle- and IFN- $\gamma$ -treated CML mice were similarly killed by effector CTLs. In addition, the mean fluorescence intensities of MHC-I, MHC-II, CD80, and PD-L1 and PD-L2 were significantly up-regulated on BCR/ABL-GFP<sup>+</sup> c-kit<sup>hi</sup> cells from IFN- $\gamma$ -treated CML mice (Fig. 6 L).

A similar lysis of IFN- $\gamma$ -treated and control LSCs excludes the possibility that IFN- $\gamma$  prevents CTL-mediated lysis of LSCs. Therefore, the observed IFN- $\gamma$ -dependent increase in LSC numbers might be caused by an increase in LSC proliferation. To investigate whether adoptively transferred p14 effector CTLs induce proliferation of myeloid

leukemia progenitor cells, we purified lin<sup>-</sup> BCR/ABL-GFP<sup>+</sup> c-kit<sup>hi</sup> cells by FACS and performed an analysis of the cell cycle using DAPI staining. BCR/ABL-GFP<sup>+</sup> c-kit<sup>hi</sup> cells from p14-treated H8 CML mice were highly significantly more in S-phase than BCR/ABL-GFP<sup>+</sup> c-kit<sup>hi</sup> cells from untreated or p14xIFN- $\gamma^{-/-}$ -treated mice (Fig. 6 M), indicating that CTL-secreted IFN- $\gamma$  increases the proliferation of leukemia progenitor cells. A similar increase of cells in S-phase was observed for nonmalignant c-kit<sup>hi</sup> in these mice (not depicted).

The experiments so far suggested that the increased IFN- $\gamma$  production after CTL transfer to a CML mouse with high leukemia load but not with low leukemia load is caused by differences in antigen load. To confirm this experimentally, we transferred H8 LSCs into sublethally irradiated (6.5 Gy) H8 recipient mice and analyzed the effects of specific CTL therapy 18 h after adoptive transfer (Fig. 7 A).

In this experimental setup, the leukemia load is low, but all host cells express gp33 on MHC-I. Transfer of p14 and p14xIFN- $\gamma^{-/-}$  effector CTLs had no or only a marginal effect on the numbers of BCR/ABL-GFP<sup>+</sup> cells in the BM (Fig. 7 B). In contrast, LSC numbers increased in p14-treated

untreated ( $\emptyset$ ) or treated with  $3 \times 10^6$  FACS-purified p14 CTLs or p14xIFN- $\gamma^{-/-}$  CTLs ( $n = 8$  each). LSC numbers (G) and BCR/ABL-GFP<sup>+</sup> colonies per mouse (H) 2 d after adoptive transfer. (I–J)  $3 \times 10^6$  BM cells from primary H8 CML mice left untreated (black line,  $n = 8$ ) or treated with p14 CTLs (red line,  $n = 8$ ) or p14xIFN- $\gamma^{-/-}$  CTLs (green line,  $n = 11$ ) were secondarily transplanted into irradiated (6.5 Gy) BL/6 mice. (I) Numbers of BCR/ABL<sup>+</sup> granulocytes/ $\mu$ l blood and (J) Kaplan-Meier survival curves resulting from secondary transplantations. (K) H8 CML mice were treated with either vehicle (VEH,  $n = 5$ ) or  $2.5 \times 10^5$  U of rm-IFN- $\gamma$  ( $n = 5$ ) i.p. twice daily on two consecutive days (d17 and d18 after primary transplantation).  $2.5 \times 10^4$  FACS-purified H8 BCR/ABL-GFP<sup>+</sup> c-kit<sup>hi</sup> cells from VEH- or IFN- $\gamma$ -treated H8 CML mice were co-incubated with  $2.5 \times 10^4$  naive BL/6 CD8<sup>+</sup> T cells (ctrl) or p14 CTLs (p14) overnight, followed by transfer into methylcellulose in duplicates. Colonies were enumerated 7 d later. (L) Mean fluorescent intensity (MFI) of MHC class I, MHC class II, CD80, CD86, PD-L1 and PD-L2 on LSCs from VEH- or IFN- $\gamma$ -treated H8 CML mice. (M) Cell cycle analysis by DAPI stainings of total c-kit<sup>hi</sup> cells from primary H8 CML mice left untreated ( $\emptyset$ ,  $n = 6$ ) or treated with p14 ( $n = 5$ ) or p14xIFN- $\gamma^{-/-}$  ( $n = 5$ ) CTLs. Data are displayed as mean  $\pm$  SEM. Statistics: one-way ANOVA (A, B, G–H, and M), two-way ANOVA (D and I), Student's  $t$  test (K and L), and log-rank test (E and J). \*,  $P < 0.05$ ; \*\*,  $P < 0.005$ ; \*\*\*,  $P < 0.0005$ .

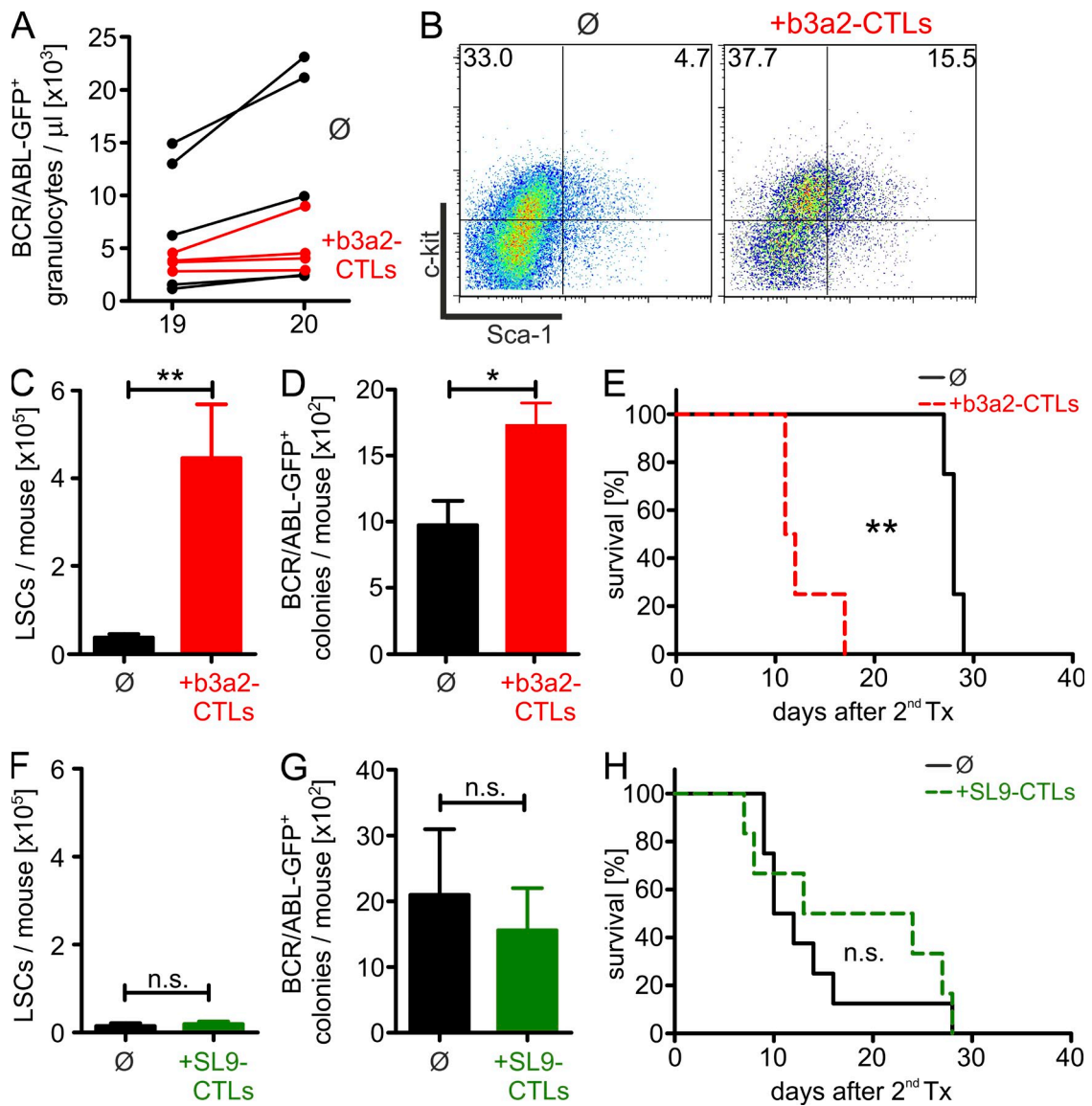
mice (Fig. 7 C). This increase in LSC numbers is attributed to CTL-secreted IFN- $\gamma$  as indicated by elevated IFN- $\gamma$  serum levels (Fig. 7 D). Treatment with p14xIFN- $\gamma^{-/-}$  effector CTLs neither increased LSC numbers nor IFN- $\gamma$  serum levels (Fig. 7 C, D).

In summary, our experiments indicate that CTLs are able to eradicate LSCs in vitro and in vivo in a setting with minimal leukemia load. In contrast, LSC numbers increase because of CTL-secreted IFN- $\gamma$  if T cell therapy is applied in

a situation with high leukemia load or in a situation where the targeted antigen is not leukemia-specific but widely expressed in other tissues.

**Adoptive immunotherapy using BCR/ABL junctional peptide-specific CTLs increases LSC numbers in a humanized CML model**

The use of the LCMV-gp33 as a model tumor antigen has the advantage that TCR transgenic mice are available and that the



**Figure 8. CTL treatment paradoxically increases LSCs in a humanized CML model.** HLA-A2tg CML mice 19 d after transplantation were either left untreated ( $\emptyset$ ,  $n = 5$ ) or treated with BCR/ABL b3a2 junction peptide-specific HLA-A2tg CD8<sup>+</sup> T cells (+b3a2-CTLs,  $n = 4$ ). In a similar, independent experiment, mice were left untreated ( $\emptyset$ ,  $n = 8$ ) or treated with HIV SL9 peptide-specific HLA-A2tg CD8<sup>+</sup> T cells (+SL9-CTLs,  $n = 6$ ). 1 d later, mice were bled and sacrificed for analysis. (A) Numbers of BCR/ABL-GFP<sup>+</sup> granulocytes/ $\mu$ l blood before and 1 d after adoptive transfer of b3a2-CTLs. (B) BM was lineage depleted and analyzed by FACS for the expression of c-kit and Sca-1 after adoptive transfer of b3a2-CTLs. One representative plot of four untreated and five b3a2-CTL-treated HLA-A2tg CML mice is shown. (C and F) LSC numbers per mouse 1 d after adoptive transfer. (D and G) Equal numbers of lin<sup>-</sup> cells were plated in methylcellulose and BCR/ABL-GFP<sup>+</sup> colonies were enumerated 7 d later by fluorescence microscopy. (E and H)  $3 \times 10^6$  BM cells from HLA-A2tg CML mice left untreated ( $n = 4-8$ ), treated with BCR/ABL b3a2-junction peptide-specific HLA-A2tg CTLs ( $n = 4$ ), or treated with HIV SL9 peptide-specific HLA-A2tg CD8<sup>+</sup> T cells (+SL9-CTLs,  $n = 6$ ) were secondarily transplanted into irradiated (6.5 Gy) HLA-A2tg mice. Kaplan-Meier survival curves resulting from secondary transplantations are shown. Data are displayed as mean  $\pm$  SEM. Statistics: Student's  $t$  test (C, D, F, and G) and log-rank test (E and H). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

expression of the antigen can be clearly controlled using transgenic animals. However, results may differ if a relevant leukemia antigen is targeted. Therefore, we made use of  $\beta$ 2-microglobulin ( $\beta$ 2m)/HLA-A2.1 monochain transgenic, H2-D<sup>b</sup>/ $\beta$ 2m double knock-out mice (HLA-A2tg mice; Pascolo et al., 1997). HLA-A2tg mice were repeatedly immunized with autologous DCs pulsed with a HLA-A2.1-binding junctional fusion oncoprotein (SSKALQRPV, b3a2) derived from BCR/ABL (Yotnda et al., 1998) to induce b3a2-specific CTLs. Spleens were harvested after 14 d, and CD8<sup>+</sup> T cells were purified, cultured in vitro, and were stimulated (weekly) with autologous, b3a2 peptide-pulsed DCs to select for b3a2-specific T cell lines. After 3 wk of restimulation, MACS-purified b3a2-specific CTLs were injected into HLA-A2tg CML mice, and control mice were left untreated. Again, we observed a block in the increase of BCR/ABL-GFP<sup>+</sup> granulocytes in the peripheral blood after adoptive T cell therapy (Fig. 8 A). FACS analysis of BM revealed that LSCs increased in frequency (Fig. 8 B) and in absolute numbers (Fig. 8 C) after b3a2-specific CTL treatment, which was confirmed by colony-forming assays of lin<sup>-</sup> cells (Fig. 8 D). Most importantly, secondary transplantations of  $3 \times 10^6$  BM cells from CTL-treated HLA-A2tg CML mice into sublethally irradiated (6.5 Gy) HLA-A2tg mice led to rapid death of secondary recipients, whereas secondary recipients receiving BM from untreated HLA-A2tg CML mice survived substantially longer (Fig. 7 E).

To analyze if the effects on LSCs after CTL treatment require antigen specificity for the leukemia antigen, we performed a control experiment using CTLs specific for the HLA-A2.1-binding HIV peptide (SLYNTVATL, SL9; Brander et al., 1998). The number of LSCs in BM (Fig. 8 F) and BCR/ABL-GFP<sup>+</sup> colony-forming capacity of lin<sup>-</sup> cells (Fig. 8 G) was not affected by SL9-specific CTL treatment. In addition, we observed similar survival in mice secondarily transplanted with BM from untreated or SL9 CTL-treated primary HLA-A2tg CML (Fig. 8 H).

Therefore, BCR/ABL fusion protein-specific effector CTLs specifically increase the numbers of LSCs and confirm the results obtained with LCMV-gp33 as a model leukemia antigen.

### IFN- $\gamma$ increases proliferation and colony formation of primary CD34<sup>+</sup> stem/progenitor cells from CML patients

To extend our findings to primary human CML stem/progenitor cells, we stained the IFN- $\gamma$  receptor on CD34<sup>+</sup> cells from newly diagnosed CML patients (initial leukocyte count,  $169.9 \pm 93.2 \times 10^9$ /liter). The IFN- $\gamma$  receptor was expressed on CD34<sup>+</sup> stem/progenitor cells in peripheral blood and BM, suggesting that CML stem cells are able to respond to IFN- $\gamma$  (Fig. 9 A). Recombinant human (rh)-IFN- $\gamma$  enhanced the proliferation of FACS-purified CD34<sup>+</sup> stem/progenitor cells from CML patients in liquid cultures in vitro, resulting in increased BrdU incorporation and elevated cell numbers after 7 d of culture (Fig. 9, B and C). Cell death was not affected by rh-IFN- $\gamma$  (Fig. 9, D and E). Furthermore, rh-IFN- $\gamma$  increased the colony-forming capacity of CD34<sup>+</sup> stem/progenitor cells from CML patients (Fig. 9 F).

In summary, these data indicate that IFN- $\gamma$  increases the numbers of primary CD34<sup>+</sup> stem/progenitor cells from blood and BM from newly diagnosed CML patients by enhancing their proliferation.

### DISCUSSION

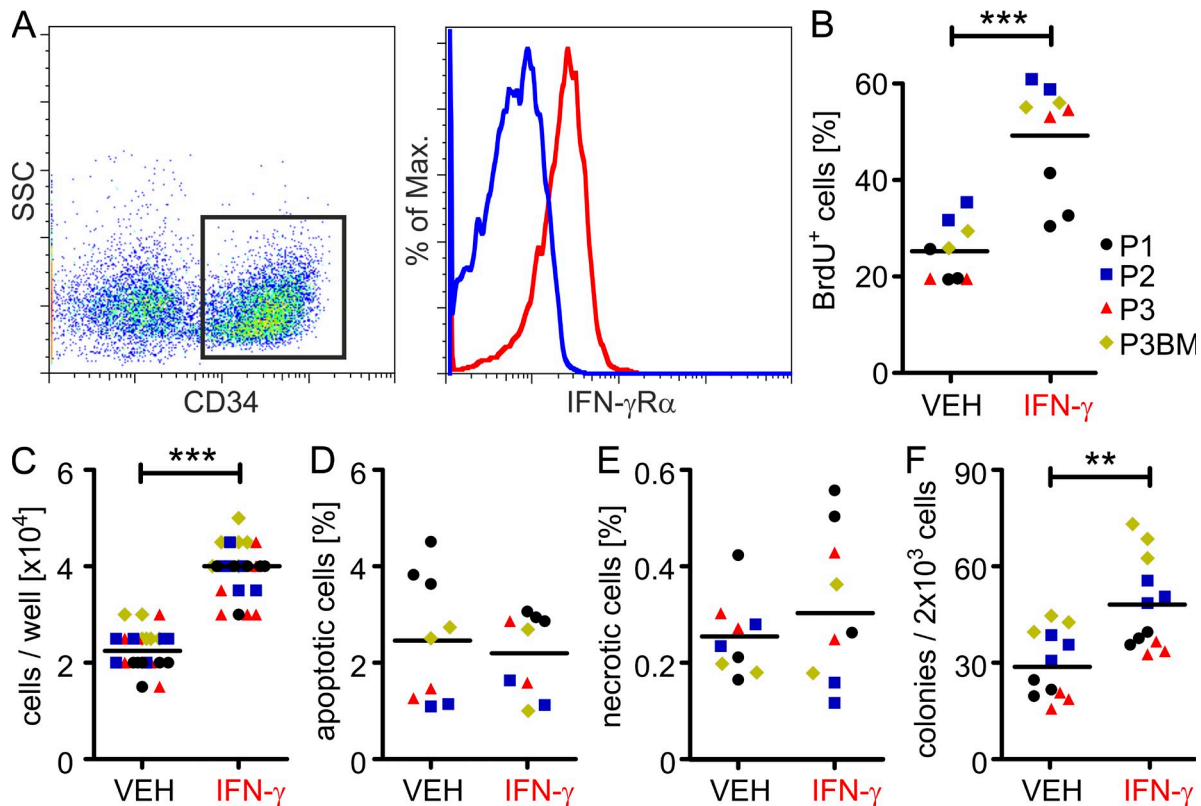
The long-term success of a leukemia treatment depends on its effect on LSCs. Unfortunately, LSCs are selectively resistant against most current treatment strategies, including chemotherapy and TKIs (Weiden et al., 1979; Deininger et al., 2000; Savona and Talpaz, 2008). In contrast, the susceptibility of LSCs to immune therapies is less clear, and it is suspected that T cells and NK cells are able to eliminate residual tumor cells after cytoreduction in the BM (Kurz et al., 2010). However, although LSCs were identified more than a decade ago, the interaction of the adaptive immune system with LSCs is poorly defined. Therefore, we addressed the interaction of CTLs with LSCs in vitro and in vivo. Surprisingly, LSCs expressed high levels of MHC class I and II, as well as costimulatory molecules. Therefore, they acted very efficiently as antigen-presenting cells and were able to induce the proliferation of naive CTLs even in the absence of additional costimulatory molecules or cytokines. Similarly, effector CTLs efficiently eliminated LSCs in vitro. These characteristics suggest that a T cell-mediated therapy could efficiently target LSCs also in vivo. However, immunotherapy with identically activated CTLs did not eliminate, and in fact increased, the number of LSCs in vivo.

LSCs retain many characteristics of HSCs and reside in and probably depend on the HSC niche in the BM. For example, blocking CD44 suppresses both AML and CML progression and induces differentiation, probably by disrupting the interaction between LSCs and the BM niche (Jin et al., 2006; Krause et al., 2006). BM niche cells express membrane-bound and secreted factors that regulate HSC quiescence, differentiation, and migration (Kiel and Morrison, 2008; Wilson et al., 2007). Therefore, the microenvironment in the HSC niche might be protective for LSCs in vivo and prevent CTL-mediated lysis. Two experiments argue against this possibility. First, CTLs were able to eliminate LSCs 18 h after transfer in vivo, a time period that is sufficient for HSCs and LSCs to migrate to the niche (Colvin et al., 2007; Bhattacharya et al., 2009; Xie et al., 2009; Capron et al., 2010). Second, CD8<sup>+</sup> T cell depletion in CML mice increased the immunogenicity of LSCs, as documented by a more efficient stimulation of naive T cells in vitro and a more efficient rejection in secondary transplantation experiments. This strongly suggests that LSCs in vivo are accessible to effector CTLs and that effector CTLs select for low immunogenic variants. For solid tumors, it has been demonstrated that in equilibrium, the immune system is able to control occult tumor cells over a prolonged period of time while selecting for lower immunogenic variants that ultimately escape immune surveillance and lead to tumor progression (Schreiber et al., 2011). Our data suggest that LSCs undergo a similar immunoeediting

process. In the experimental conditions chosen in our murine CML model, progression and death occur after 3–4 wk. In contrast, in human CML, the chronic phase lasts 3–5 yr before outgrowth of blasts that have acquired additional mutations (Sawyers, 1999).

Type I IFNs (IFN- $\alpha$  and - $\beta$ ) and type II IFN (IFN- $\gamma$ ) are important cytokines in the control of infections (Kurz et al., 2010). As the elimination of an infectious pathogen requires a concerted action of different elements of the immune system it is not surprising that IFNs have direct effects on hematopoiesis. IFN- $\alpha$  activates dormant HSCs to enter proliferation. In contrast, chronic activation of the IFN- $\alpha$  pathway in HSC impairs their function, probably as a result of exhaustion (Essers et al., 2009). Before the introduction of TKIs, IFN- $\alpha$  was used as standard therapy for CML (Sawyers, 1999). In addition, T cell-derived cytokines have been described to influence hematopoiesis (Metcalf, 2008). IFN- $\gamma$  induces apoptosis of erythroid progenitors and BM cultures in vitro (Means, 1995; Wang et al., 1995) and may be a central cytokine in the induction of acquired aplasia (Young, 2006) and in anemia of chronic disease (Weiss and Goodnough, 2005). In contrast, in combination

with SCF and IL-3, IFN- $\gamma$  stimulates differentiation of the granulocyte-monocyte and erythropoietic lineages in vitro (Kurz et al., 2010). More recently, IFN- $\gamma$  was recognized as important mediator of infection-induced myelopoiesis (MacNamara et al., 2011). In a murine model of malaria infection, IFN- $\gamma$  induced a unique myelolymphoid progenitor population that gave rise to mostly myeloid cells (Belyaev et al., 2010). Other studies have shown that IFN- $\gamma$  directly induces the expansion of  $\text{lin}^- \text{Sca-1}^+ \text{c-kit}^+$  cells (Zhao et al., 2010) and that IFN- $\gamma$ -induced genes are critical for HSC survival and maintenance (Baldrige et al., 2010). We now documented a comparable effect of IFN- $\gamma$  on LSCs. IFN- $\gamma$  induced proliferation of leukemia progenitor cells in our CML model as well as in primary CD34 $^+$  CML stem/progenitor cells from newly diagnosed patients. In the murine model, an increase in disease-initiating LSCs was documented in a secondary transplantation experiment where CML progression was faster after transplantation of IFN- $\gamma$ -treated LSCs. In addition, IFN- $\gamma$  treatment up-regulated the inhibitory molecules PD-L1 and PD-L2 on LSCs, probably contributing to LSC immune escape in vivo (Zou and Chen, 2008). Thus, IFN- $\gamma$  evolutionary evolved as



**Figure 9.** IFN- $\gamma$  increases proliferation and colony formation of primary CD34 $^+$  stem/progenitor cells from CML patients. (A) Expression of IFN- $\gamma$  receptor  $\alpha$  chain (IFN- $\gamma$ R $\alpha$ ) on CD34 $^+$  stem/progenitor cells from newly diagnosed CML patients. One representative plot of four is shown. (B–E)  $10^4$  FACS-sorted CD34 $^+$  cells from the peripheral blood or BM were cultured in the presence or absence of rh-IFN- $\gamma$  for 7 d in sextuplicates. At day 7 of culture, BrdU incorporation (B), cell numbers (C), apoptosis (D), and necrosis (E) were assessed in duplicates or triplicates. (F)  $2 \times 10^3$  FACS-sorted CD34 $^+$  cells were cultured in methylcellulose in triplicates and colony-forming capacity was determined after 14 d. Data are displayed as mean  $\pm$  SEM. Statistics: Student's *t* test. \*\*,  $P < 0.005$ ; \*\*\*,  $P < 0.0001$ . Apoptotic cells, Annexin V $^+$  cells; necrotic cells, Annexin V $^+$ -AAD $^+$  cells. P, Patient; VEH, vehicle.

an important regulator of infection- or stress-induced myelopoiesis. However, the same IFN- $\gamma$ -mediated effects on LSCs induce their proliferation and disease progression.

Therefore, the adoptive transfer of specific CTLs affected leukemia development in different ways. It eliminated leukemia granulocytes, and probably LSCs as well, and selected for low immunogenic LSC variants. Alternatively, CTL-secreted IFN- $\gamma$  established a cytokine environment that facilitated leukemia progression. It is well documented that effector mechanisms of the immune system contribute to tumor control as well as to tumor progression (Schreiber et al., 2011). Whether effector CTLs, in addition to their tumor-protective role, may also enhance tumor growth, is less clear. In addition to IFN- $\gamma$ , we recently showed that T cells contribute to leukemia progression via the co-stimulatory CD70-CD27 interaction (Schürch et al., 2012).

The expansion of the LSC population clearly depended on IFN- $\gamma$ , whereas the lytic effect on leukemia cells was independent of IFN- $\gamma$  and mediated by perforin/granzyme B. The level of IFN- $\gamma$  produced after adoptive T cell transfer depended on the antigen-load in the leukemia mouse. We used the LCMV-gp33 antigen as leukemia antigen to be able to control for the expression of the antigen. When gp33 was expressed also on nonleukemic host cells, or when the leukemia load was high and gp33-expressing cells were abundant, the transferred cells were restimulated *in vivo* to produce high amounts of IFN- $\gamma$ . Similarly, adoptive transfer of a BCR/ABL fusion protein-specific CTL line induced the expansion of LSCs in the humanized CML model, confirming that our findings are not specific for LCMV gp33. In addition, IFN- $\gamma$  induced cell-cycling and expansion of primary human CD34<sup>+</sup> CML stem/progenitor cells, indicating the relevance of this pathway for human disease.

In contrast, in a situation with a low leukemia load resembling minimal residual disease, restimulation of the transferred CTLs did not lead to IFN- $\gamma$  levels sufficient to induce LSC expansion. This fits well with clinical observations. Although recent studies showed that chimeric antigen receptor-modified T cells are able to eliminate up to 1 kg of leukemia mass (Porter et al., 2011), it is generally accepted that immunotherapy is most efficacious in the presence of a low leukemia load (Carlens et al., 2001; Raiola et al., 2003). Therefore, most immunotherapies including alloSCT and donor lymphocyte infusions are applied after induction chemotherapy (Kolb et al., 1990; Kolb et al., 1995; Weiden et al., 1979). Independent of the leukemia load, the expression of the target antigen is crucial for the efficacy of adoptive T cell therapy. Our results reveal that even in a situation with a low leukemia load, the transfer of T cells that target an antigen that is widely expressed in the body leads to restimulation of the transferred T cells and secretion of high amounts IFN- $\gamma$ . This leads to proliferation of LSCs and precludes the success of the therapy.

Our study now documents that LSCs are immunogenic and can be targeted by specific CTLs, provided that the target antigen is leukemia specific and that the immunotherapy is applied in a situation with a low leukemia load, probably after cytoreduction.

## MATERIALS AND METHODS

**Mice.** C57BL/6J (BL/6) mice were obtained from RCC Ltd. CD45.1<sup>+</sup> mice p14TCR transgenic mice (Pircher et al., 1989) specific for the LCMV glycoprotein epitope 33–41 (gp33), H8 transgenic mice (Ehl et al., 1998) ubiquitously expressing LCMV-gp33 under the control of an MHC class I promoter, IFN- $\gamma$ -deficient mice (Dalton et al., 1993), and  $\beta$ 2m/HLA-A2.1 monochain transgenic, H2-D<sup>b</sup>/ $\beta$ 2m double knock-out mice (HLA-A2tg mice; Pascolo et al., 1997) were obtained from the Institute for Laboratory Animals (Zürich, Switzerland). Animal experiments were approved by the local experimental animal committee of the Canton of Bern and performed according to Swiss laws for animal protection.

**Patients.** Peripheral blood samples and one BM aspirate from untreated, newly diagnosed CML patients ( $n = 3$ ; age,  $54.6 \pm 19.4$  yr) were obtained at the University Hospital of Bern, Switzerland, in 2011 and 2012. All CML patients possessed a BCR/ABL translocation as tested by molecular analysis from the blood. Analysis of blood and BM samples was approved by the local ethical committee of the Canton of Bern and all patients gave written, informed consent.

**Virus and peptides.** LCMV strain WE was provided by R.M. Zinkernagel (University of Zürich, Zürich, Switzerland) and propagated on L929 fibroblasts as previously described (Matter et al., 2006; Mumprecht et al., 2006). The HLA-A2.1-binding BCR/ABL b3a2-junction peptide (aa 926–934, SSKALQRPV; Yotnda et al., 1998) was purchased from Innovative Peptide Solutions. The MHC-II-specific LCMV glycoprotein peptide p13 (aa 61–80, GLNGPDIYKGVYQFKSVFED) was purchased from NeoMPS SA. The HIV peptide SL9 (aa 77–85, SLYNTVATL; Brander et al., 1998) was provided by D.Yerly (University of Bern, Bern, Switzerland).

**Antibodies.**  $\alpha$ H2-D<sup>b</sup>-PE,  $\alpha$ I-A/I-E-PE,  $\alpha$ c-kit-PE and -PE-Cy7,  $\alpha$ Scal-1-perimidin-chlorophyll-protein (PerCP)-Cy5.5 and -allophycocyanin (APC),  $\alpha$ Ly6G-PE,  $\alpha$ Gr1-PE and -APC;  $\alpha$ CD80-PE,  $\alpha$ CD86-APC,  $\alpha$ IFN- $\gamma$ -PE,  $\alpha$ IFN- $\gamma$ -R $\alpha$ -PE,  $\alpha$ IFN- $\gamma$ -R $\beta$ -PE, Armenian hamster-IgG-PE,  $\alpha$ PD-L1-PE,  $\alpha$ CD127-PE, and -APC were obtained from eBioscience. CD11c-FITC,  $\alpha$ I-A/I-E-APC-Cy7,  $\alpha$ c-kit-APC-Alexa750,  $\alpha$ CD80-Pacific Blue, mouse-IgG2b-PE, rat-IgG2a-APC, rat-IgG2b-APC-Cy7, Armenian hamster-IgG-Pacific Blue,  $\alpha$ CD3 $\epsilon$ -biotin,  $\alpha$ CD19-biotin,  $\alpha$ Gr1-biotin,  $\alpha$ Ter119-biotin,  $\alpha$ PD-L2-PE,  $\alpha$ -human-IFN- $\gamma$ -R $\alpha$ -PE, LEAF-purified anti-FAS-ligand, LEAF-purified Armenian hamster-IgG isotype control, and Annexin V-Pacific Blue were purchased from BioLegend. Annexin V-FITC was obtained from ImmunoTools. 7-Aminoactinomycin D (AAD),  $\alpha$ BrdU-APC (3D4), and the corresponding isotype control (MOPC21) were obtained from BD.  $\alpha$ CD8 (2.43) was purchased from BioXCell, and IgG from rat serum was purchased from Sigma-Aldrich.

**Retroviral particles.** The retroviral vector pMSCV-p210BCR/ABL-IRES-GFP and the packaging vector pIK7 were a gift from J. Schwaller (University of Basel, Basel, Switzerland). Retroviral particles were produced and titrated as previously described (Mumprecht et al., 2009a).

**CML model.** CML was induced and monitored as previously described (Mumprecht et al., 2009a,b). In brief,  $4 \times 10^6$  BM cells of 5-fluorouracil-pretreated BL/6 mice, H8 mice, or HLA-A2tg mice were transduced twice in transplant medium with  $10^5$  retroviral particles through spin infection.  $10^5$ -transduced BM cells were injected *i.v.* into previously sublethally irradiated BL/6 recipient mice (BL/6  $\rightarrow$  BL/6, BL/6 CML; irradiation, 4.5 Gy; H8  $\rightarrow$  BL/6, H8 CML; irradiation, 6.5 Gy) or HLA-A2tg mice (HLA-A2tg  $\rightarrow$  HLA-A2tg, HLA-A2tg CML; irradiation: 6.5 Gy).  $5 \times 10^5$  transduced BM cells were incubated for 3 d in transplant medium, and GFP expression was analyzed by FACS. For secondary whole BM transplantations,  $3 \times 10^6$  BM cells from primary CML mice (18–20 d after primary transplantation) were injected *i.v.* into previously sublethally irradiated (6.5 Gy) secondary recipients.

**Lineage depletion and flow cytometry.** BM lineage depletion was performed using biotinylated antibodies against red cell precursors ( $\alpha$ Ter119), B cells ( $\alpha$ CD19), T cells ( $\alpha$ CD3 $\epsilon$ ), and myeloid cells ( $\alpha$ Gr1), MACS  $\alpha$ -biotin beads, and LS columns (Miltenyi Biotec).

Determination of granulocyte counts/microliter was performed using a VetABC animal blood counter (Medical Solution GmbH) in combination with FACS. All samples were analyzed on a BD LSR II and sorting was performed on a BD FACSAria (BD). Data were analyzed using FlowJo software (Tree Star).

**Cell cycle analysis.** FACS-sorted,  $\text{lin}^-$  BCR/ABL-GFP $^+$ c-kit $^{\text{hi}}$  were pooled and incubated in 1% PFA/PBS overnight at 4°C. Samples were permeabilized with 0.2% Triton X-100 for 30 min at 4°C and labeled with 5  $\mu$ g/ml DAPI (Roche).

**Generation of p14 and p14xIFN- $\gamma^{-/-}$  effector CTLs and memory cells.** 10 $^6$  splenocytes from p14 TCR transgenic (p14) mice or p14xIFN- $\gamma^{-/-}$  mice (all CD45.1 $^+$ ) were adoptively transferred to BL/6 mice. 18 h later, BL/6 mice were infected with 10 $^4$  plaque-forming units of LCMV-WE, and spleens were harvested 6 d later. For experiments using p14 CD8 $^+$  effector T cells exclusively, cells were MACS-purified by  $\alpha$ CD8 beads. 5  $\times$  10 $^6$  p14 CD8 $^+$  effector T cells were injected i.v. into recipient mice. For experiments using p14 and p14xIFN- $\gamma^{-/-}$  CD8 $^+$  effector T cells in vivo, cells were FACS-purified for CD8, CD45.1, V $\alpha$ 2, and V $\beta$ 8.1. 3–5  $\times$  10 $^6$ -purified effector T cells were injected i.v. into recipient mice. For the isolation of memory p14 cells, spleens of BL/6 mice were harvested 6 wk after infection.

**Generation of mature DC from HLA-A2tg mice.** BM was flushed from femurs and tibiae of HLA-A2tg mice using RPMI 1640 medium (Sigma-Aldrich) containing 10% FCS, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin and 2 mM L-glutamine. Erythrocytes were removed using RBC lysis buffer (QIAGEN). 2–3  $\times$  10 $^6$  BM cells were cultured in RPMI 10% FCS supplemented with 50  $\mu$ M 2-mercaptoethanol and 10% (vol/vol) supernatant from a GM-CSF hybridoma cell line in nontissue culture-treated Petri dishes at 37°C and 5% CO $_2$ . After 6 and 8 d, half of the medium was removed and fresh medium was added. After 10 d, cells were transferred to tissue culture Petri dishes and 10 $^4$  U/ml recombinant mouse (rm)-TNF (Prospec) was added to cultures. 2 d later, mature DC were harvested from the supernatant and analyzed by flow cytometry for expression of CD11c, MHC class II, CD80, and CD86.

**Generation of HLA-A2-specific CD8 $^+$  effector cells.** To induce an optimal CTL response in HLA-A2tg mice, the coexpression of both CTL and CD4 helper peptides on DCs was necessary (Bennett et al., 1997). Therefore, mature DCs from HLA-A2tg mice were pulsed with the CTL peptide b3a2 (10 $^{-5}$  M) in combination with the CD4 helper peptide p13 (10 $^{-6}$  M) in RPMI 2% FCS for 90 min at 37°C and 5% CO $_2$ . DCs were washed twice and naive HLA-A2tg mice were vaccinated i.v. with 10 $^6$  DCs at days 0, 2, 10, and 12 (Ludewig et al., 2000). At day 14, spleens from HLA-A2tg mice were harvested and CD8 $^+$  T cells were purified by MACS, and then cultured in IMDM 10% FCS in the presence of 50 U/ml rm-IL-2 (Prospec) and 0.01% 2-mercaptoethanol. Mature DCs from HLA-A2tg mice were pulsed as with the CTL peptide b3a2 (10 $^{-5}$  M) only and added to CD8 $^+$  T cell cultures at a ratio of 1:5 to 1:10 at the beginning of culture. Fresh DCs were added weekly. Cultures were maintained at 37°C and 5% CO $_2$  for 3 wk. 2–3  $\times$  10 $^6$  MACS-purified CD8 $^+$  T cells were injected into HLA-A2tg CML mice.

**Proliferation assay.** 3  $\times$  10 $^3$  FACS-sorted LSCs were irradiated (10 Gy) and co-cultured with 1.5  $\times$  10 $^4$  naive or memory p14 CD8 $^+$  T cells in 96-well V-bottom plates (Corning) for 4 d. For the last 18 h of culture, 0.5  $\mu$ Ci of  $^3$ H-thymidine (PerkinElmer) was added to each well. Next, cells were collected with a Packard Filtermate 196-Harvester (Packard Bioscience) on UniFilter-96, GF/C plates (PerkinElmer). Microscint-0 (PerkinElmer) was

added, and the incorporation of radioactivity was detected on a TopCount microplate scintillation counter (PerkinElmer).

**Colony-forming assays.** 5  $\times$  10 $^3$  MACS-purified lineage-negative cells from CML mice were plated into MethoCult M3134 medium (STEMCELL Technologies) supplemented with 15% FCS, 20% BIT (50 mg/ml BSA in IMDM, 1.44 U/ml rh-insulin [Actrapid; Novo Nordisk], and 250 ng/ml human holo transferrin [Prospec]), 100  $\mu$ M 2-mercaptoethanol, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 2 mM L-glutamine, and 50 ng/ml rm-SCF, 10 ng/ml rm-IL-3, 10 ng/ml rh-IL-6, and 50 ng/ml rm-Flt3-ligand (all from Prospec). BCR/ABL-GFP $^+$  colonies were enumerated after 7 d ( $\geq$ 30 cells/colony) on a DMIL inverted microscope (Leica) equipped with an Intensi-light C-HGFI unit (Nikon).

2  $\times$  10 $^3$  FACS-purified CD34 $^+$  stem/progenitor cells from peripheral blood and BM of CML patients were plated into MethoCult H4435 Enriched medium (STEMCELL Technologies) in the presence or absence of 500 U/ml rh-IFN- $\gamma$  (Prospec). Colonies and cells were enumerated after 14 d ( $\geq$ 30 cells/colony).

**In vitro LSC killing assay.** 10 $^4$  FACS-sorted H8 or BL/6 LSCs were co-cultured overnight in 96-well V-bottom plates (Corning) with titrated numbers of naive cells or p14 effector cells (either total splenocytes or MACS-purified CD8 $^+$  T cells). On the next day, the contents of wells were resuspended and colony assays were performed.

**In vitro BCR/ABL-GFP $^+$  leukemia cell killing assay.** p14 or p14xIFN- $\gamma^{-/-}$  CTLs were generated as described in Generation of p14 and p14xIFN- $\gamma^{-/-}$  effector CTLs and memory cells and were isolated by MACS from spleens and lymph nodes. CTLs were preincubated for 20 min with or without 10  $\mu$ g/ml anti-FAS-ligand (MFL4; BioLegend), in the presence or absence of titrated concentrations of the granzyme B inhibitor Z-AAD-CMK (1, 10, and 100 mM; Enzo Life Sciences). Armenian hamster IgG (HTK888; BioLegend) and water served as negative controls, respectively. Bulk BM cells from H8 CML mice (containing  $\sim$ 20% of BCR/ABL-GFP $^+$  cells) were either incubated alone or were co-cultured with CTLs at a ratio of 5:1 in the presence or absence of the indicated compounds in 24-well tissue culture plates. After 4 h, cells were analyzed by FACS and the killing of BCR/ABL-GFP $^+$  cells was calculated in relation to control conditions.

**In vitro liquid culture of primary human CD34 $^+$  CML stem/progenitor cells.** 10 $^4$  FACS-purified CD34 $^+$  stem/progenitor cells from the peripheral blood and BM of CML patients were cultured in StemSpan SFEM medium (Stem Cell Technologies) supplemented with human cytokines (StemSpan CC100; Stem Cell Technologies) in the presence or absence of 500 U/ml rh-IFN- $\gamma$  in 96-well plates at 37°C and 5% CO $_2$  in sextuplicates. 10  $\mu$ M BrdU (BD) was added to culture for the last 4 h of incubation. Numbers of viable cells were determined by trypan blue staining and BrdU staining was performed according to the manufacturer's instructions (BrdU Flow kit; BD) after 7 d of culture.

**IFN- $\gamma$  treatment of CML mice.** CML mice were treated with 2.5  $\times$  10 $^5$  U of rm-IFN- $\gamma$  (Prospec) i.p. twice daily on two consecutive days, 16–18 d after primary transplantation or 18 h after secondary transplantation (a total of 10 $^6$  U per animal).

**CD8 $^+$  T cell depletion in vivo.** Depletion of CD8 $^+$  T cells was performed by two injections of 75  $\mu$ g  $\alpha$ CD8 mAb (clone 2.43; BioXCell) at days 10 and 11 after transplantation.

**IFN- $\gamma$  determination in mouse serum.** IFN- $\gamma$  protein levels were measured using a multiplexed particle-based flow cytometry cytokine assay (R&D Systems) according to the manufacturer's instructions.

**Analysis of proliferation in vivo using BrdU.** BL/6 CML mice received BrdU (Sigma; 0.8mg/ml in drinking water and 1mg i.p./day) on 2 consecutive

days (days 17 and 18 after primary transplantation). BrdU staining was performed according to the manufacturer's instructions (BrdU Flow kit; BD).

**Statistical analysis.** Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software). Data are represented as mean  $\pm$  SEM. Data were analyzed using one-way ANOVA and Tukey's multiple comparison test or Dunnett's test, Student's *t* test (two-tailed), one-sample *t* test, or two-way ANOVA and Bonferroni post-hoc test (p-value shows interaction). Significance of differences in Kaplan-Meier survival curves was determined using the log-rank test (two-tailed).  $P < 0.05$  was considered significant.

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C. Schürch and C. Riether designed and performed experiments, analyzed data and wrote the manuscript. M.A. Amrein performed experiments and analyzed data. A.F. Ochsenbein designed experiments, wrote the manuscript, and supervised the project.

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