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# Blocking programmed cell death 1 in combination with adoptive cytotoxic T-cell transfer eradicates chronic myelogenous leukemia stem cells

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In chronic myelogenous leukemia (CML), quiescent, self-renewing leukemia stem cells (LSCs) are resistant against chemotherapy and specific tyrosine kinase inhibitors (TKIs).<sup>1,2</sup> During treatment, LSCs remain in the bone marrow (BM) and cause disease relapse after drug discontinuation.<sup>3,4</sup> Moreover, persisting LSCs may acquire BCR-ABL1 mutations that interfere with TKI binding or accumulate genetic alterations that lead to blast crisis. T-cell-based immunotherapy has been shown to be efficacious in solid tumors and in leukemia<sup>5</sup> and allogeneic stem cell transplantation and donor lymphocyte infusions (DLIs) are the only curative treatments in CML.<sup>6</sup> However, LSCs evolved several mechanisms to escape cytotoxic CD8<sup>+</sup> T cells (CTLs)-mediated immune attack.<sup>4</sup> We recently demonstrated that interferon gamma (IFN $\gamma$ ) secreted by adoptively transferred leukemia-specific CTLs in a situation with high leukemia load even increased the numbers of LSCs while more differentiated leukemia cells were efficiently eliminated.<sup>7</sup> Why LSCs are selectively resistant against elimination by CTLs is unknown. One major immune escape mechanism of tumor cells is the dysregulation of T-cell inhibitory pathways, so-called immune checkpoints.<sup>1</sup> Indeed, CML cells express T-cell inhibitory ligands such as programmed cell death ligand 1 (PD-L1), light and herpes virus entry mediator and high expression of PD-L1 on CD34<sup>+</sup> CML cells is a negative prognostic factor.<sup>7–10</sup> Here, we addressed the expression of PD-L1 in the differentiation hierarchy of BM CML stem/progenitor cell subsets.<sup>4,11,12</sup> CML was induced by retroviral transduction of BM from 5-fluorouracil-treated H8 transgenic mice<sup>13</sup> with BCR-ABL1-GFP, followed by transfer to sublethally irradiated C57BL/6 (BL/6) recipient mice (H8 CML).<sup>7</sup> In this setting, BCR-ABL1-GFP<sup>+</sup> leukemia cells express the glycoprotein epitope gp33 of lymphocytic choriomenigitis virus (LCMV) under the control of a major histocompatibility complex-I (MHC-I) promoter as model leukemia antigen. Nineteen days after transplantation, BM BCR-ABL1-GFP<sup>+</sup> granulocyte-monocyte progenitors (GMPs), common-myeloid progenitors (CMPs), multipotent progenitor 2 (MPP2s), MPP1s, short-term (ST)- and long-term (LT)-LSCs expressed PD-L1 (Figures 1a and b). Interestingly, LSC subsets (MPPs, ST- and LT-LSCs) expressed higher levels of PD-L1 than leukemia progenitors (GMPs and CMPs), with highest expression on LT-LSCs (Figure 1a and data not shown). To determine PD-L1 expression after adoptive CTL transfer (adTf), H8 CML mice were treated with LCMV-gp33-specific TCR transgenic (p14) effector CTLs. CTL transfer resulted in an upregulation of PD-L1 on CML stem/progenitor cells (Figures 1a and b). PD-L1 was more upregulated on LSC subsets than on leukemia progenitors, with a mean fluorescence intensity increase of ~4-fold in LT-LSCs (Figure 1b). Treatment with IFN $\gamma$ -deficient CTLs (p14xIFN $\gamma$ <sup>-/-</sup>) did not increase PD-L1 expression compared with untreated controls, indicating that PD-L1 upregulation was mediated by CTL-secreted IFN $\gamma$  (Figure 1b). In accordance with our findings, IFN $\gamma$  has been shown to induce

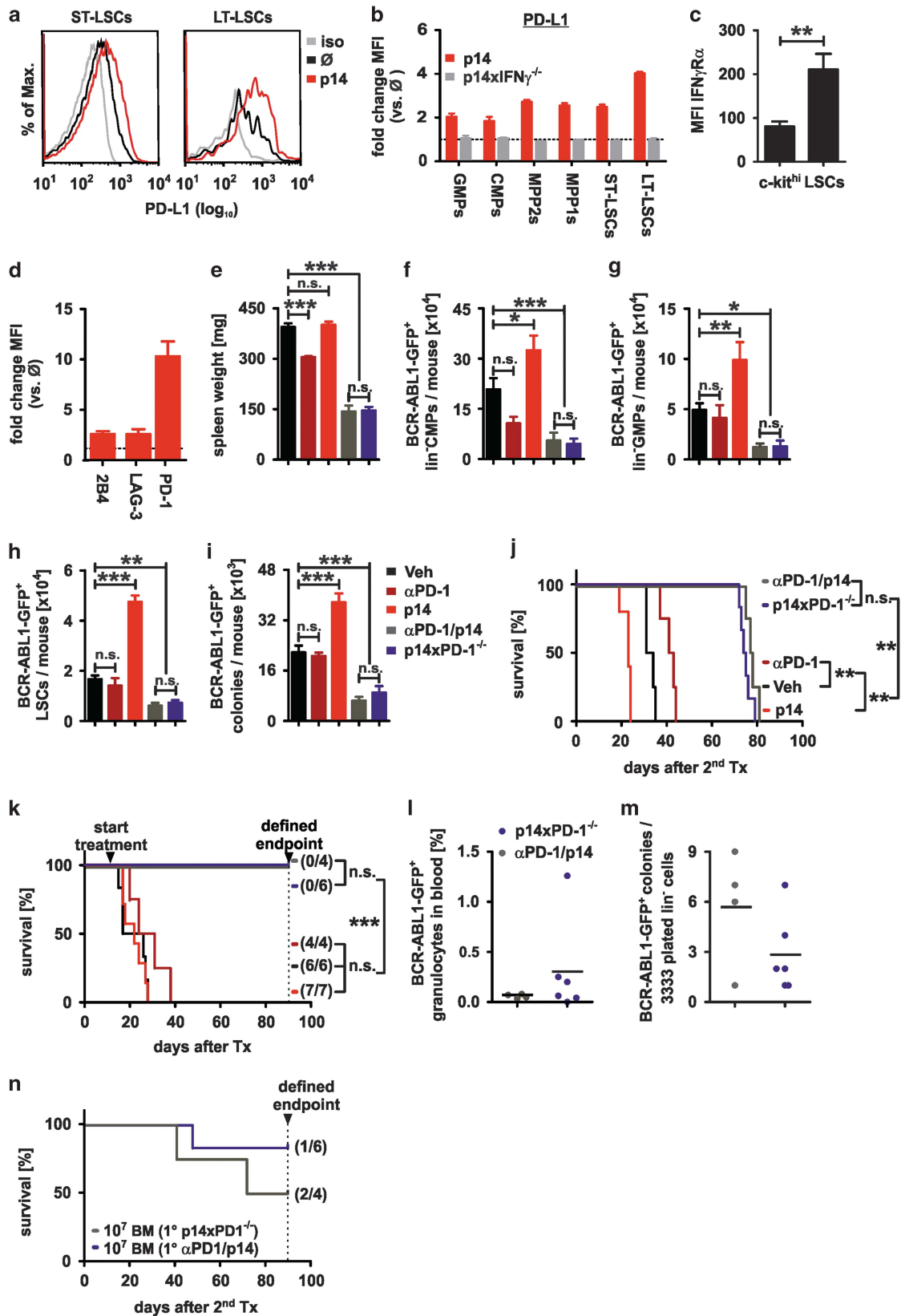
PD-L1 expression on different types of cancer including leukemia.<sup>1</sup> One reason for the observed differences in PD-L1 expression on LSC subsets and leukemia progenitors after transfer of p14 CTLs may be a different sensitivity to IFN $\gamma$ . Therefore, we examined IFN $\gamma$  receptor (IFN $\gamma$ R) expression on total LSCs (lin<sup>-</sup>c-kit<sup>hi</sup>Sca-1<sup>+</sup>) and leukemia progenitors (lin<sup>-</sup>c-kit<sup>+</sup>Sca-1<sup>-</sup>). LSCs expressed higher levels of IFN $\gamma$ R than leukemia progenitors (Figure 1c).

T-cell inhibitory surface receptors such as PD-1, natural killer cell antigen 2B4 and lymphocyte activation gene-3 (LAG-3) are commonly upregulated on CD8<sup>+</sup> T cells upon activation and are frequently overexpressed on tumor-infiltrating T cells.<sup>1</sup> We therefore assessed the expression of these receptors on BM CD8<sup>+</sup> T cells after adTf of p14 CTLs. BM-infiltrating leukemia-specific CTLs expressed high levels of PD-1 while 2B4 and LAG-3 expression was only marginal (Figure 1d).

These results indicate that PD-L1 is constitutively expressed on LSCs and leukemia progenitors, is further upregulated in response to effector CTL-secreted IFN $\gamma$  and may protect LSCs from CTL-mediated elimination.

To test this, we analyzed whether genetic or therapeutic blockade of PD-1 signaling during p14 CTL transfer allows eradicating LSCs *in vivo*. Seventeen days after leukemia induction, H8 CML mice with comparable leukemia burden (109 ± 13 BCR-ABL1-GFP<sup>+</sup>Gr-1<sup>+</sup> granulocytes/ $\mu$ l blood) were randomized to control treatment with vehicle (Veh), p14 CTLs (p14), a blocking  $\alpha$ PD-1 monoclonal antibody (mAb;  $\alpha$ PD-1, clone RMP1-14), treatment with PD-1-deficient p14 CTLs (p14xPD-1<sup>-/-</sup>) or p14 CTLs in combination with  $\alpha$ PD-1 ( $\alpha$ PD-1/p14). p14xPD-1<sup>-/-</sup> and  $\alpha$ PD-1/p14 treatments reduced leukemia burden as indicated by smaller spleen size and lower numbers of leukemia progenitors in the BM compared with Veh,  $\alpha$ PD-1 and p14 CTL-treated groups (Figures 1e–g). As reported previously,<sup>7</sup> p14 CTL transfer increased LSC numbers (Figures 1h and i). Importantly, PD-1 blocking during adTf of p14 CTLs significantly reduced LSCs and resulted in fewer BCR-ABL1-GFP<sup>+</sup> methylcellulose colonies from lin<sup>-</sup> BM cells (Figures 1h and i). In CML, only LT-LSCs are able to propagate the disease *in vivo*, whereas other LSC subsets and more differentiated progenitors are able to form colonies *in vitro*.<sup>12</sup> To investigate if the reduced numbers of LSCs as defined immunophenotypically by FACS or functionally *in vitro* actually accounted for reduced numbers of leukemia-initiating cells *in vivo*, we secondarily transplanted BM cells from H8 CML mice of all treatment groups into lethally irradiated BL/6 mice. Animals transplanted with BM from p14 CTL-treated CML mice succumbed significantly faster to the disease than mice after transplantation of Veh-treated control CML BM.<sup>7</sup> In contrast, mice transplanted with BM from  $\alpha$ PD-1-treated H8 CML mice survived significantly longer than controls, but the prolongation of survival was marginal. Importantly, only PD-1 blockade during transfer of p14 CTLs in primary CML mice substantially prolonged the survival of secondary recipient mice, indicating that this treatment indeed targets leukemia-initiating cells (Figure 1j). However, LSCs were not eliminated completely within 2 days after treatment (Figures 1h–j).

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To assess the therapeutic role of PD-1 blockade during adTf in more detail, H8 CML mice ( $161 \pm 16$  BCR-ABL1-GFP<sup>+</sup>Gr-1<sup>+</sup> granulocytes/ $\mu$ l blood) were treated as described and disease progression and survival were monitored. Transfer of p14 CTLs or treatment with  $\alpha$ PD-1 mAb alone did not improve survival. In contrast, PD-1 blockade during CTL transfer led to long-term survival of all H8 CML mice (Figure 1k). These data suggest that LSCs were either eliminated or effectively controlled by this treatment.

Therefore, we next analyzed if the surviving mice had eliminated LSCs 73 days after adTf of CTLs. Some mice harbored residual disease as indicated by low percentages of BCR-ABL1-GFP<sup>+</sup>Gr-1<sup>+</sup> granulocytes in peripheral blood (Figure 1l). In addition, lin<sup>-</sup> BM cells from surviving animals still formed few BCR-ABL1-GFP<sup>+</sup> colonies (Figure 1m). To functionally investigate whether leukemia-initiating cells had persisted, we transferred BM cells from surviving mice into lethally irradiated secondary BL/6 recipients. Only 3 out of 10 secondary recipients developed a CML, whereas the other 7 survived long term without signs of leukemia, as analyzed by FACS of peripheral blood (Figure 1n and data not shown). These results indicate that one single transfer of leukemia-specific effector CTLs in combination with genetic deletion or mAb blocking of PD-1 eliminated the disease-initiating LSCs in a majority of animals treated at a late stage of the disease.

LCMV-gp33 was used in the first experiments as a model leukemia antigen that allows an in-depth characterization of T-cell responses. To analyze whether our findings are of relevance in a more physiological setting, we made use of an MHC-I mismatch model (Balb/c  $\times$  BL/6 F1-generation, CB/6) that has been used to analyze the effect of human leukocyte antigen-mismatched DLIs.<sup>14</sup> MHC-I in BL/6 mice consists of H2-K<sup>b</sup>/D<sup>b</sup>; Balb/c mice express H2-K<sup>d</sup>/D<sup>d</sup>. CB/6 mice consequently express H2-K<sup>bd</sup>/D<sup>bd</sup>. CB/6 BM was retrovirally transduced with BCR-ABL1-GFP before transfer into sublethally irradiated CB/6 recipient mice (CB/6 CML). In this setting, the leukemia cells as well as the endogenous non-malignant cells express H2-K<sup>bd</sup>/D<sup>bd</sup>.<sup>15</sup> CB/6 CML mice were then treated 11 days after CML induction with Veh, MACS-purified naive CD8<sup>+</sup> T cells from BL/6 mice (CD8, H2-K<sup>b</sup>/D<sup>b</sup>), a blocking  $\alpha$ PD-1 mAb ( $\alpha$ PD-1) or BL/6 CD8<sup>+</sup> T cells and  $\alpha$ PD-1 mAb in combination ( $\alpha$ PD-1/CD8). Treatment effects on the different LSC subsets in CB/6 CML mice were analyzed 10 days later. CD8 transfer increased numbers of LSC subsets in BM compared with Veh-treated controls (Figures 2a–g), indicating that similarly to the transfer of gp33-specific effector CTLs (Figures 1h and i),<sup>7</sup>

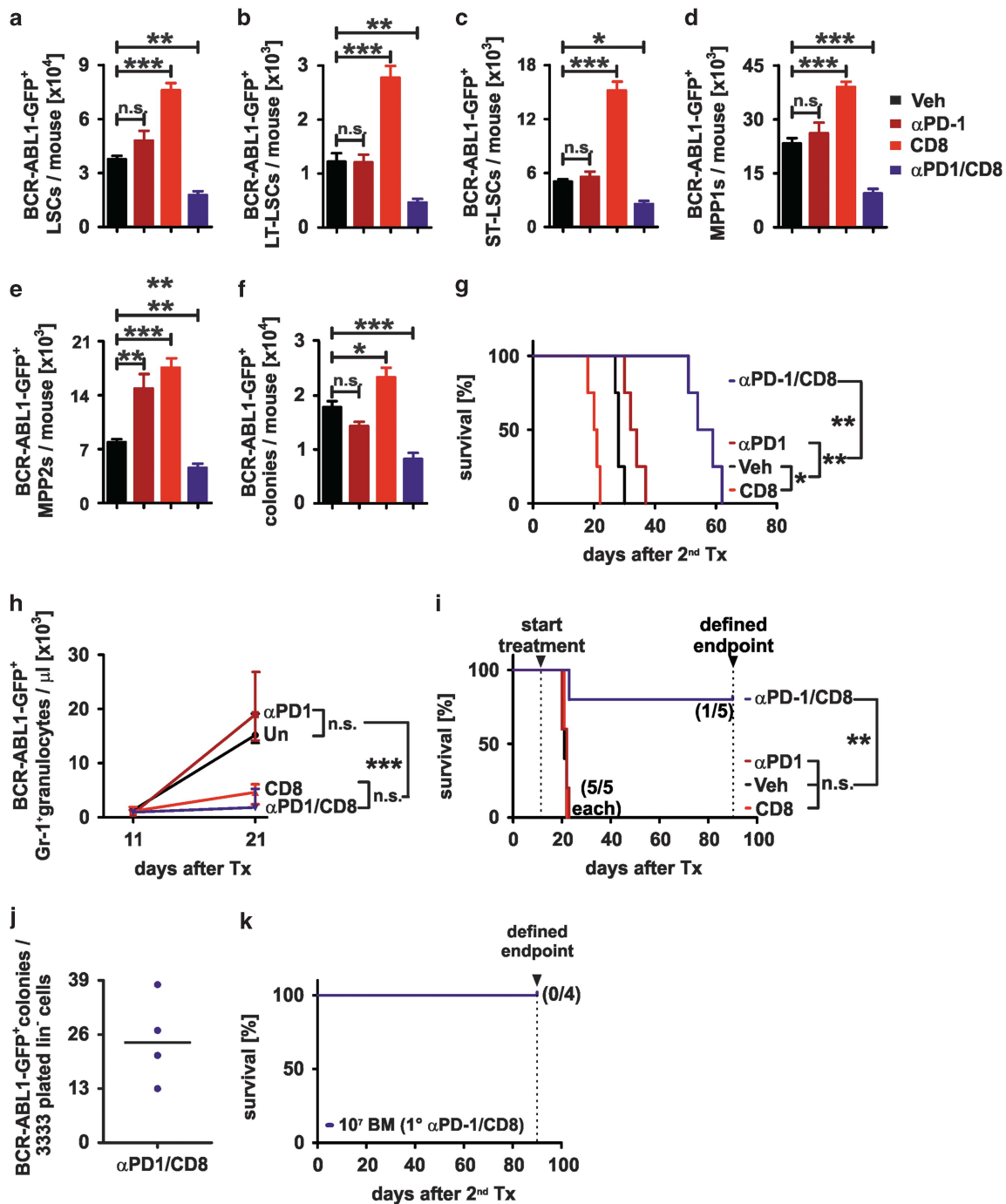
allogeneic CD8<sup>+</sup> T cells induce expansion of LSCs. In contrast,  $\alpha$ PD-1/CD8 treatment significantly reduced the numbers of all LSC subsets (Figures 2a–e). This finding was confirmed functionally by assessing BCR-ABL1-GFP<sup>+</sup> colony formation (Figure 2f) as well as by secondary transplantations (Figure 2g).

Furthermore, CD8 and  $\alpha$ PD-1/CD8 treatment reduced leukemia burden in the peripheral blood compared with Veh- and  $\alpha$ PD-1-treated mice (Figure 2h). CD8 or  $\alpha$ PD-1 treatment alone did not result in a survival advantage when compared with Veh-treated controls. In contrast, the majority of CB/6 CML mice that received  $\alpha$ PD-1/CD8 combination treatment survived long term (Figure 2i).

Surviving mice still harbored some residual disease as indicated by BCR-ABL1-GFP<sup>+</sup> methylcellulose colony formation of lin<sup>-</sup> BM (Figure 2j). However, although capable of colony formation, these residual leukemia cells were unable to induce CML after transplantation of BM into lethally irradiated secondary recipients (Figure 2k), indicating that the leukemia-initiating cells had been eliminated.

T-cell therapy is very efficacious in a setting with low leukemia load. This has been documented by clinical data in CML but also experimentally in our CML model.<sup>6,7</sup> However, in a setting of high leukemia load without prior cytoreduction, LT-LSCs upregulate PD-L1 in response to IFN $\gamma$  secreted by leukemia-specific or allogeneic CTLs during adTf and thereby escape CTL-mediated killing. Together with the increased proliferation of LSCs induced by CTL-secreted IFN $\gamma$ <sup>7</sup> this results in increased LSC numbers and represents a major obstacle for T-cell immunotherapy of CML. We now show that blocking PD-1 in combination with T-cell immunotherapy efficiently eliminates the disease-initiating LSCs even in a therapeutic setting with high leukemia load. In contrast,  $\alpha$ PD-1 treatment alone was largely inefficacious. This may be because of the fact that at later stages of disease, the majority of leukemia-specific T cells have been exhausted and deleted.<sup>8</sup> Numerous leukemia-associated antigens (LAAs) are expressed by human CML cells that may be used for adoptive immunotherapy.<sup>16</sup> However, not all of these antigens are expressed by LSCs. Stem-cell enriched cells from CML patients preferentially express the LAAs Wilms tumor protein (WT-1) and PRAME, but not proteinase 3, survivin or human telomerase (hTert).<sup>16</sup> Therefore, WT-1 and PRAME may represent promising therapeutic targets for CTL-based immunotherapy in combination with  $\alpha$ PD-1-treatment to eliminate LSCs.

**Figure 1.** (a,b and d) H8 CML mice were either left untreated ( $\emptyset$ ,  $n=7$ ) or treated with  $5 \times 10^6$  MACS-purified IFN $\gamma$ -competent (p14,  $n=7$ ) or -deficient (p14xIFN $\gamma$ <sup>-/-</sup>,  $n=3$ ) effector CTLs 17 days after CML induction. Two days later, PD-L1 expression in lin<sup>-</sup>BCR-ABL1-GFP<sup>+</sup> BM CMPs (c-kit<sup>hi</sup>CD127<sup>+</sup>CD34<sup>+</sup>FcyR<sup>+</sup>), GMPs (c-kit<sup>hi</sup>CD127<sup>+</sup>CD34<sup>+</sup>FcyR<sup>+</sup>), MPP2s (c-kit<sup>hi</sup>CD135<sup>+</sup>CD48<sup>+</sup>CD150<sup>-</sup>), MPP1s (c-kit<sup>hi</sup>CD135<sup>+</sup>CD48<sup>+</sup>CD150<sup>+</sup>), ST-LSCs (c-kit<sup>hi</sup>CD135<sup>+</sup>CD48<sup>+</sup>CD150<sup>-</sup>) and LT-LSCs (c-kit<sup>hi</sup>CD135<sup>+</sup>CD48<sup>+</sup>CD150<sup>+</sup>) was determined by FACS. (a) Representative FACS plot of PD-L1 expression on ST- and LT-LSCs from untreated or p14-treated H8 CML mice. (b) Fold change of MFIs of PD-L1 on LSC subsets versus no treatment ( $n=3-7$  mice per group). Dotted line represents untreated H8 CML mice. (c) IFN $\gamma$ R $\alpha$  chain on CML progenitors (lin<sup>-</sup>c-kit<sup>+</sup>Sca-1<sup>-</sup>) and LSCs (lin<sup>-</sup>c-kit<sup>+</sup>Sca-1<sup>+</sup>) of  $n=6$  mice is shown. (d) Primary H8 CML mice were treated with p14 CTLs as described in a starting 17 days after transplantation. Two days later the expression of indicated inhibitory markers on CD8<sup>+</sup> T cells in the BM was analyzed. Values represent the fold change in MFI versus untreated controls ( $n=4-5$  mice per group). (e) Spleen weights and numbers of (f) CMPs, (g) GMPs and (h) LSCs were determined in lin<sup>-</sup>BCR-ABL1-GFP<sup>+</sup> BM of H8 CML mice treated with rat-IgG (Veh, 200  $\mu$ g intraperitoneally every third day),  $3 \times 10^6$  FACS-purified p14 effector CTLs (p14), PD-1-deficient p14 effector CTLs (p14xPD-1<sup>-/-</sup>), anti-PD-1 mAb ( $\alpha$ PD-1, 200  $\mu$ g intraperitoneally every third day, clone: RMP1-14) alone or in combination with effector p14 T cells ( $\alpha$ PD-1/p14) starting 17 days after CML induction. (i) Equal numbers of lin<sup>-</sup> cells were plated in methylcellulose and BCR-ABL1-GFP<sup>+</sup> colonies were enumerated 7 days later by inverted fluorescence microscopy. (j)  $5 \times 10^6$  BM cells from H8 CML mice were transplanted into lethally irradiated ( $2 \times 6.5$  Gy) recipient mice and survival was monitored. Pooled data from two independent experiments with  $n=6-9$  mice per group are shown. (k) Kaplan–Meier survival curves of H8 CML mice treated as described in e–h. Pooled data from two independent experiments with  $n=4-7$  mice per group are shown. (l) Frequency of BCR-ABL1-GFP<sup>+</sup> granulocytes in peripheral blood and (m) BCR-ABL1-GFP<sup>+</sup> colonies in lin<sup>-</sup> BM from H8 CML mice that were alive 90 days after CML induction. (n) Survival of lethally irradiated ( $2 \times 6.5$  Gy) secondary recipients that were injected with  $1 \times 10^7$  BM cells from surviving  $\alpha$ PD-1/p14- and p14xPD-1<sup>-/-</sup>-treated primary CML mice ( $n=4-6$  mice per group). (k, n) Numbers of mice that succumbed to CML of total transplanted mice are indicated. Data are displayed as mean  $\pm$  s.e.m. Statistics: (c) Student's *t*-test, (e–i) one-way analysis of variance, (j and k) log-rank test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .



**Figure 2.** (a–g) CB/6 CML mice ( $n = 4$  mice per group) were treated either with rat-IgG (Veh, 200  $\mu$ g intraperitoneally every third day),  $8 \times 10^6$  MACS-purified CD8<sup>+</sup> T cells from BL/6 mice (CD8),  $\alpha$ PD-1 mAb ( $\alpha$ PD-1, 200  $\mu$ g intraperitoneally every third day) alone or in combination with BL/6 CD8<sup>+</sup> T cells ( $\alpha$ PD-1/CD8) starting 11 days after CML induction. Ten days later, lin<sup>-</sup>BCR-ABL1-GFP<sup>+</sup> BM was analyzed for (a) LSCs (c-kit<sup>hi</sup>Sca-1<sup>+</sup>), (b) LT-LSCs (c-kit<sup>hi</sup>CD135<sup>+</sup>CD48<sup>+</sup>CD150<sup>+</sup>), (c) ST-LSCs (c-kit<sup>hi</sup>CD135<sup>+</sup>CD48<sup>+</sup>CD150<sup>+</sup>), (d) leukemia MPP1s (c-kit<sup>hi</sup>CD135<sup>+</sup>CD48<sup>+</sup>CD150<sup>+</sup>) and (e) leukemia MPP2s (c-kit<sup>hi</sup>CD135<sup>+</sup>CD48<sup>+</sup>CD150<sup>+</sup>). (f) Equal numbers of total lin<sup>-</sup> cells were plated in methylcellulose and BCR-ABL1-GFP<sup>+</sup> colonies were enumerated 7 days later by inverted fluorescence microscopy. (g) Primary CB/6 CML mice were treated as described in a starting 11 days after transplantation. Ten days later,  $5 \times 10^6$  BM cells were transplanted into lethally irradiated ( $2 \times 6.5$  Gy) recipient mice ( $n = 4$  mice per group) and survival was monitored. (h and i) Primary CB/6 CML mice were treated as described in a starting 11 days after transplantation ( $n = 5$  per group). (h) Numbers of BCR-ABL1-GFP<sup>+</sup> granulocytes/ $\mu$ l blood and (i) Kaplan–Meier survival curves ( $n = 5$  mice per group). (j) BCR-ABL1-GFP<sup>+</sup> colonies in lin<sup>-</sup> BM and (k) survival of lethally irradiated ( $2 \times 6.5$  Gy) secondary recipients ( $n = 4$  mice) that received  $1 \times 10^7$  BM cells from  $\alpha$ PD-1/CD8-treated primary CML mice that were alive 90 days after CML induction. (i, k) Numbers of mice that succumbed to CML of total transplanted mice are indicated. Data are displayed as mean  $\pm$  s.e.m. Statistics: (a–f) one-way analysis of variance (ANOVA), (g, i) log-rank test, (h) two-way ANOVA. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .



## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## AUTHOR CONTRIBUTIONS

CR designed and performed experiments, analyzed data and wrote the manuscript; TG, A-LH and CMS performed experiments and analyzed data; AFO designed experiments, analyzed data and wrote the manuscript. All authors revised the manuscript and approved the final version.

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# Cellular origin of prognostic chromosomal aberrations in AML patients

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Acute myeloid leukemia (AML) represents an aggressive cancer entity, whose malignant cells respond abnormally to regulatory stimuli and have lost the ability to differentiate and become fully mature blood cells.<sup>1,2</sup> AML evolves through accumulation of independent genetic aberrations, including chromosomal structural rearrangements and single nucleotide variants (SNVs). Conventional AML diagnostics and recent seminal next-generation sequencing (NGS) studies have identified more than 200 recurrent genetic aberrations presenting in various combinations in individual patients.<sup>1,3–7</sup> Significantly, many of these aberrations occur in normal hematopoietic stem and progenitor cells (HSCs/HPCs) before definitive leukemic transformation through additional acquisition of a few (that is, mostly 1 or 2) leukemia-promoting driver aberrations.<sup>5</sup> NGS studies on sorted bone marrow (BM) populations of AML patients with a normal karyotype have demonstrated the presence of prognostic driver aberrations (that is, *NPM1*, *FLT3-ITD* and *FLT3-TKD*) in committed HPCs but not in

multipotent HSCs.<sup>8–10</sup> However, the HSC populations lacking the prognostic driver aberrations contained preleukemic clones harboring a series of recurrent molecular aberrations that were present in the fully transformed committed HPCs together with the prognostic driver aberration.<sup>8,10,11</sup> Adding to this vast heterogeneity and complexity of AML genomes and their clonal evolution, a recent study of a murine AML model demonstrated that t(9;11) AML originating from HSCs responded poorly to *in vivo* chemotherapy treatment as compared with t(9;11) AML originating from HPCs.<sup>12</sup>

Hence, recent advances in genetics support that AML is initiated and sustained by a few aberrations that, in concert with their cellular origin, define the leukemic phenotype of AML patients and ultimately clinical outcome following conventional chemotherapy.

In the present study we examined the variegation among AML patients with prognostic chromosomal driver aberrations with respect to (i) the cellular composition of their HSC/HPC compartment, (ii) the cellular origin of their 'driver' aberrations and (iii) the expression of aberrant transcriptional programs as compared with normal.

For this, we applied an integrative experimental strategy combining flow cytometry-based immunophenotyping, as well as