Adoptive Immunotherapy for Chronic Myeloid Leukemia in a Mouse Model

A dissertation submitted by

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Abstract

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm caused by t(9;22) chromosomal translocation that creates the Philadelphia chromosome. The translocation encodes a constitutively active tyrosine kinase, BCR-ABL1, which turns a normal hematopoietic stem cell into a leukemic stem cell (LSC). BCR-ABL1 does not affect lineage differentiation. Therefore, CML is characterized clinically by the overproduction of maturing cells of the neutrophil lineage.

Although tyrosine kinase inhibitors (TKIs) are the current preferred initial treatment for CML, eradication of the disease is rarely achieved. Low levels of *BCR-ABL1* transcripts, thought to originate from primitive leukemia stem cells, persist in most TKI-treated patients and lead to disease relapse after TKI withdrawal in the majority of patients. Allogeneic hematopoietic stem cell transplantation (alloHSCT) is the only treatment proven to cure CML, through a powerful graft versus leukemia (GvL) effect. For patients who relapse after alloHSCT, infusions of lymphocytes from the allogeneic donor (donor leukocyte infusion or DLI) can re-induce durable remission in most cases, which strongly suggests that, although intrinsically resistant to conventional chemoradiotherapy or TKI treatment, the LSCs are susceptible to immunological targeting. It is not fully understood at the cellular level how DLI eradicates CML stem cells. Using the retroviral bone marrow transduction/transplantation mouse model of CML, we have established an immunotherapy model that combines the use of TKI treatment and DLI, and is able to eradicate CML-like leukemia in >80% of recipients. Using this platform, we aim to define the contributions of different effector cell types

within DLI, and to identify the tumor-killing mechanisms that may provide insights in separating graft-versus-leukemia (GvL) from graft-versus-host (GvHD) responses.

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List of Abbreviations:

AlloHSCT: allogeneic hematopoietic stem cell transplantation

Allo-SPL: allogeneic splenocytes

BCR: breakpoint cluster region

Abl: Abelson, or v-abl Abelson murine leukemia viral oncogene homolog

APC: antigen presenting cell

BM: bone marrow

BMT: bone marrow transplantation

CAR: chimeric antigen receptor

CCyR: complete cytogenetic remission

CyR: cytogenetic remission

CML: chronic myeloid leukemia

CMR: complete molecular remission

CTL: cytotoxic T lymphocyte

DC: dendritic cell

DLI: donor leukocyte infusion

ECMV: encephalomyocarditis virus

G6PD: glucose-6-phosphate dehydrogenase

GFP: green fluorescent protein

GM-CSF: granulocyte-macrophage colony stimulating factor

GMP: granulocyte-macrophage progenitor, or good manufacturing practice

GvL: graft versus leukemia

GvHD: graft versus host disease

GWAS: genome-wide association study

HR: hematological remission

HSC: hematopoietic stem cell

IM: imatinib mesylate

IRES: internal ribosomal entry site

KIR: killer cell immunoglobulin-like receptor

Lin^-: lineage markers negative

LSC: leukemic stem cell

LSK: $Lin^- Sca\text{-}1^+ c\text{-}Kit^+$

LTR: long terminal repeat

MHC: major histocompatibility complex

miHA: minor histocompatibility antigen

MPN: myeloproliferative neoplasm

MR: molecular remission

MSCV: murine stem cell virus

NK: natural killer

NOD/SCID: nonobese severe combined immuno-deficient

PR-3: proteinase-3

Rag: recombination activating gene

SNP: single nucleotide polymorphism

SRY: sex-determining region Y

TAA: tumor associated antigen

TCD: T cell-depleted

TKI: tyrosine kinase inhibitor

Treg: regulatory T cell

TRM: treatment-related mortality

WT-1: Wilm's tumor antigen-1

5-FU: 5-fluorouracil

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Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm (MPN) caused by BCR-ABL1, the product of the Philadelphia chromosome (Figure 1) (2). CML has a triphasic course beginning with the chronic phase, characterized by leukocytosis and splenomegaly, with general preservation of myeloid cell differentiation. Unless treated by alloHSCT or by TKI therapy, patients inevitably progress to an accelerated phase where more leukemic blasts are found in the bone marrow (BM), and patients may start to show symptoms of infection, and become tired or have bruises easily due to a general malfunction of hematopoiesis. Without proper treatment, the disease inevitably transforms into a terminal blast crisis phase within months, which resembles acute leukemia where over 20% of leukemic blasts can be found in the blood and BM. Patients in blast crisis have a very poor prognosis and short survival.

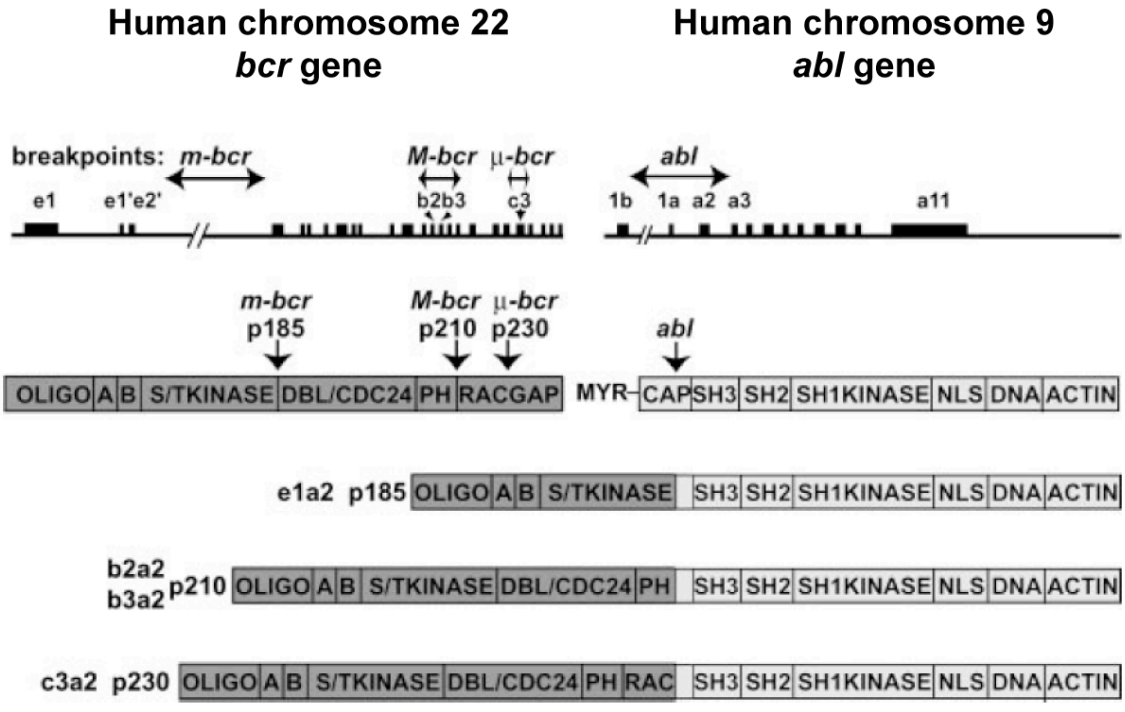


Figure 1. Genetic translocation events of *BCR* and *ABL1* genes. The genetic abnormality of CML is caused by the reciprocal chromosomal translocation between exon b2 or b3 of *BCR* gene and exon a2 of *ABL1* gene; the *b2a2* or *b3a2* fusion product generates the chimeric onco-protein of 210 kDa (p210). Depending on the location of breakpoints occurred in *BCR*, other forms of chimeric proteins, p185 and p230, can be generated when exon e1- or c3-fragment of *BCR* fuses with *ABL1*-a2. Different from p210, which accounts for CML and some B-acute lymphoblastic leukemia (B-ALL), p185 and p230 are primarily associated with B-ALL and an indolent neutropenic form of CML respectively.

BCR protein domains include Oligo: oligomerization domain, S/TKINASE: serine/threonine kinase domain, DBL/CDC24: Dbl homology domain, PH: Pleckstrin homology domain, RACGAP: Rac GTPase domain; ABL1 protein domains include MYR: myristoylation signal, CAP: CAP hydrophobic residues, SH3: Src homology domain, SH1: Src homology tyrosine kinase domain, NLS: nuclear localization signal, DNA: DNA binding sites, and ACTIN: F and G actin binding sites. (Wong and Witte, 2004. (3))

CML is a clonal disease originating from the hematopoietic stem cell (HSC)

It is now clear that CML is a monoclonal disease arising at the HSC level. The initial clonal evidence came from studies analyzing the X-linked glucose-6-phosphate dehydrogenase (G6PD) isoforms in different lineages of cells isolated from heterozygous female CML patients. It was found that, while two isoforms were expressed in fibroblasts, only one form could be detected in granulocytes and erythrocytes (4, 5). It was later found that, in addition to the myeloid lineages, the leukemia-initiating cell of CML could also differentiate into the lymphoid lineage, as demonstrated by the presence of *BCR-ABL1* in B lymphoblastic cell lines derived from CML patients (6). Furthermore, when HSC-enriched $CD34^+$ cells from patients with chronic phase CML were transplanted into irradiated nonobese severe combined immuno-deficient (NOD/SCID) mice, the primitive cell population engrafted and persisted for up to months in the recipients (7). These results suggested that the initial *BCR-ABL1* transformation occurred in a primitive multi-potential cell with the ability to self-renewal.

In mice, CML-like MPN can be induced by transplanting *BCR-ABL1*$^+$ leukemic BM to syngeneic recipients (8). The CML-like disease can also be serially transferred to the secondary recipients, showing the leukemia-initiating cells have self-renewal capacity (9). Leukemic cells of erythroid, lymphoid, or myeloid lineages isolated from individual diseased mice had the identical pattern of proviral integration, demonstrating the multi-potentiality of the leukemia-initiating cells (10). Lastly, transplanting the LSK BM population (Lin^- $Sca\text{-}1^+$ $c\text{-}Kit^+$, which represents a highly enriched HSC population) induced CML-like disease in the recipients (11). Together, these data showed that CML is a clonal disease that originates from HSC.

Treatment options for CML

Depending on detection methods and the corresponding sensitivities, responses to therapy in CML are categorized into three major levels: hematological remission (HR), which means having normal peripheral blood counts; cytogenetic remission (CyR), which means having no Ph chromosome metaphases detected by cytogenetic analysis, or, in some cases, by fluorescent in situ hybridization; and complete molecular remission (CMR), which means having no *BCR-ABL1* transcripts detected by RT-PCR, which is the most sensitive detection method (12). In response to TKI therapy, HR occurs first, which is followed by CyR and MR sequentially (13).

- **Chemotherapy**

Historically, CML was treated with myelosuppressive agents such as busulfan and hydroxyurea. Although these drugs could induce HR in 50-80 % of patients, chemotherapy seldom achieved a CyR and could not prevent disease progression (14), and hence must be considered as palliative therapies.

- **Biological therapy**

In the 1980s, interferon-α was identified as the first agent capable of inducing cytogenetic responses in CML, and rapidly became the front line treatment for CML, as it was subsequently demonstrated to significantly prolong the survival rate when compared to conventional chemotherapy (15). Although its mechanism of action is not fully understood, IFN-α induced complete HR in at least 80% and stable CyR in 20-25% of the patients. However, since IFN-α was generally not well tolerated and complete CyR was achieved in only a minority of patients, interferon was eventually replaced by the more effective ABL1 tyrosine kinase inhibitor (TKI) imatinib (14).

- **Tyrosine kinase inhibitors (TKIs)**

Through rational drug design and high throughput screening methods, a panel of small molecular-based compounds that inhibited ABL1 kinase activity were identified. The use of TKIs has ushered in a new era of CML therapy. Since its approval by the FDA in 2001, imatinib mesylate (IM) has replaced IFN-α and become the new standard of care for initial treatment of CML patients in chronic phase. The effect of imatinib at 400 mg per day was significant, in that over 74% of patients in chronic phase disease achieved complete CyR (16). However, complete remissions at the molecular level (CMR) were rarely achieved, and a multicenter study showed that 60% of the patients who were in stable complete CMR for at least 2 years relapsed within one year after imatinib discontinuation (17), indicating that imatinib alone is not curative in most CML patients, possibly because of failure to eradicate the leukemic stem cells. Acquired resistance to imatinib therapy is also a problem in CML, where the most common cause for relapse is the acquisition of point mutations in *BCR-ABL1* that cause resistance to the inhibitor. Subsequently, second-generation TKIs were developed that maintain activity against these mutant forms of BCR-ABL1, but the potency of these novel TKIs to target the CML stem cell remains limited, and their long-term efficacy awaits further evaluation (18-22). This, in turn, has rekindled the interest in studying alternative therapies that could effectively eradicate residual disease in CML.

- **Allogeneic stem cell transplantation (alloHSCT)**

Although alloHSCT has been less frequently used to treat chronic phase CML today, it is still a therapeutic option for patients who are either intolerant or resistant to multiple TKIs, or for patients with more advanced forms of disease (23). The greatest limitation of alloHSCT/DLI therapy is the risk of causing GvHD. In addition to the use of immune suppressants, GvHD can be minimized by approaches such as depleting T cell effectors, escalating DLI dose gradually from a relatively low initial dosage, or by $CD8^+$ T cell depletion of DLI; however, these approaches have only achieved moderate success to date. An overview of previous attempts and developing strategies aiming to separate GvHD from GvL is given in the following section.

Strategies to reduce GvHD

- **T cell depletion**

The initial approach to treating hematopoietic malignancies by alloHSCT was to induce GvL by using myeloablative conditioning regimens, i.e., a high dose of chemoradiotherapy that depletes the host marrow, and to restore functional hematopoiesis by administering allogeneic BM. Currently, the paradigm has shifted to eliminating leukemia by activating allogeneic effector cells and reducing treatment toxicity by employing non-myeloablative regimens (24). To our knowledge so far, GvHD cannot be successfully separated from GvL effect and remains a major cause of morbidity and mortality after alloHSCT. Attempts to reduce GvHD by depleting T cells within BM grafts led to more relapses and graft failure, indicating that T cells are the major

component mediating both processes (25-27). Of all the hematologic malignancies, CML is the most sensitive to immunotherapy (28, 29). The profound GvL effect in CML was best illustrated by the demonstration that administration of allogeneic donor lymphocyte infusions (DLI) could re-induce durable remissions in the majority of CML patients relapsing after alloHSCT (28). It is known that DLI is more effective in treating early or mild relapses, as it was shown that DLI could re-induce remissions in 75% of patients with molecular or cytogenetic level of relapse, but was effective in only 12-33% in patients with more aggressive form of relapse (24). The result indicates that the effectiveness of immunotherapy in CML correlates inversely with disease burden, and hence GvL is more effective when employed early following relapse (30, 31). Conversely, DLI was complicated by concurrent graft-versus-host disease (GVHD), and also by graft rejection and failure of hematopoiesis, as it was shown that severe GvHD occurred in 41% (28) to 60% (29) of patient cohorts receiving DLI after alloHSCT. With the advance of knowledge regarding the high resolution HLA-matching techniques, GvHD mortality remains similar, with 42% treatment related mortality (TRM) observed in matched unrelated donor-recipient pairs (32).

- **DLI dose escalation**

Several strategies have been developed to prevent or reduce the severity of GvHD caused by DLI treatment. One approach is to reduce the initial T cell dose within DLI and gradually escalate the dosage until GvL occurs. It was shown that neither GvHD nor GvL was observed by infusing DLI containing less than 5×10^6 T cells/Kg body weight, and that GvL could be preferably induced at the dose of 1×10^7 T cells/Kg; however, frequency of GvHD increased when higher initial doses of T cells were administered

(33). This approach was further confirmed by a multicenter retrospective analysis, where CML patients treated with DLI containing lower initial T cell dose showed less GvHD and better overall survival while retaining their GvL responses, whereas the patient cohorts treated with DLI containing higher initial T cell dose showed comparable GvL but had significantly higher TRM (34).

- **$CD8^+$ T cell depletion**

In mice, depletion of $CD8^+$ T cells showed reduced GvHD in some, but not all, MHC matched, minor histocompatibility antigen (miHA)-mismatched transplant models (35-38). In humans, it was found that the onset of GvHD correlates to increased immune reconstitution of circulating $CD8^+$ T cells in matched sibling transplants (39), indicating the important role of $CD8^+$ T cell in the pathogenesis of GvHD. However, the strategy of removing the $CD8^+$ T cell subset from DLI achieved only moderate success (40, 41). With recent advances in immunomagnetic-based cell separation techniques that enable routine generation of CD8-depleted DLI under good manufacturing practice (GMP) conditions, GvHD remains the major complication of this treatment modality (42, 43).

- **Reduced-intensity conditioning regimens & blockage of danger signal**

Shortly after alloHSCT, the host milieu contains a variety of inflammatory cytokines, such as IFN-γ, IL-1 and TNF-α, which are released during the intensive conditioning regimen (44, 45). This, in turn, activates host antigen-presenting cells (APCs), recruits donor T cells from BM graft to the damaged tissues and causes GvHD (46, 47). In contrast to a myeloablative regimen, a reduced-intensity conditioning regimen uses a lower radiation dose in combination with less toxic chemotherapeutic agents and immunosuppression to minimize tissue injury while establishing donor-

derived hematopoiesis. Although this approach decreases the toxicity of the transplant procedure and reduces severity of GvHD, the lower TRM is largely offset by the higher relapse rate. Hence, the overall survival of reduced intensity conditioning transplants is similar to standard myeloablative transplants (48-51). Additionally, it was recently reported that ATP released from the injured or necrotic cells in GvHD-affected individual functions as a danger signal that up-regulates the expression of costimulatory molecules on dendritic cells (DCs) in human subjects and experimental GvHD models (52). Antagonizing the ATP receptor, $P2X_7R$, reduces the severity of GvHD while preserving GvL. Further more, it was shown that $P2X_7R$, deficiency in host DC confers protection against GvHD, whereas the control arm with wildtype DC died of GvHD (52). This report supports the previous finding that host APCs are required for GvHD induction (53), and provides a new target for GvHD prevention without generalized immunosuppression.

- **Gene therapy**

An attractive strategy to avoid severe GvHD after alloHSCT/DLI therapy is to introduce a controllable suicide gene into donor T cells. GvHD caused by *herpes simplex viral thymidine kinase (HSV-TK)*-transduced DLI (TK-DLI) has been shown to be effectively controlled by ganciclovir-induced elimination of T cells in patients with relapsed-phase disease after alloHSCT (54). The HSV-TK/ganciclovir system is the most commonly used model that has been shown to suppress established GvHD, while retaining GvL responses, in several HLA-matched clinical transplants (54-56). This indicates that DLI-mediated GvL and GvHD have different kinetics, and that these two processes might be separated by eliminating host-reactive donor T cells after GvL is

achieved. However, the immunogenicity of the viral protein could induce host rejection to the TK-DLI (54, 57, 58), and could further increases the frequency of secondary graft failure (57). To circumvent the issue of immunizing the host with foreign products, changing the retroviral vector using endogenous proteins such as the truncated NGF receptor diminishes host immune-recognition (56, 59). Additionally, other suicide models using endogenous genes such as *CD20*/rituximab (60), *Fas*/dimerizer (61), or *Caspase-9*/dimerizer (62) could further be studied as the alternatives to the HSV-TK/ganciclovir system. Nevertheless, the greatest concern using this genetically modified therapy lies in the danger of insertional mutagenesis and secondary malignancy that is associated with the retroviral gene transfer (63). Hence, such a strategy remains controversial and has to be carried out with great care and intense monitoring.

- **Mesenchymal stem cells (MSC)**

MSCs are a heterogeneous group of non-hematopoietic cells that have the ability to self-renew and differentiate into mesenchymal lineages (64, 65). MSCs can be found within BM, fetal liver, and umbilical cord (66), and possess immune-suppressive properties toward T cell, B cell, NK cell and monocyte-derived DC (65). The clinical results exploiting the use of MSCs in treating steroid-resistant acute GvHD remains inconclusive. In a large-scale, multicenter phase II study, administration of MSCs to patients with steroid-resistant GvHD showed an overall response rate of 70% (67). Interestingly, the response rate was not associated with the source of MSC, as there was no difference between treatment groups receiving MSCs from HLA-identical siblings, haplo-identical, or HLA-mismatched donors (67). Conversely, a large-scale, double blinded, and placebo controlled phase III trial conducted by a pharmaceutical firm

showed that there was no difference between the MSC-treated and the placebo groups (http://investor.osiris.com/secfiling.cfm?filingID=1104659-09-53523). Nevertheless, according to the two trials, no adverse effect associated with MSC-based adoptive immunotherapy was reported. Further efforts charactering the biological properties and subpopulations within MSCs are needed to better address the therapeutic potential of this approach.

- **Genome-wide mapping for novel miHAs**

In HLA-matched transplants, GvL is primarily mediated through immune-recognition against miHAs, as the disease relapses more frequently in the identical-twin transplants when compared to the HLA-matched sibling transplants (68). Minor histocompatibility antigens are variants of non-HLA genes that could trigger immune responses between individuals (69). For example, an increased risk of GvHD was observed in female→male transplants, where multiple male-specific gene products induced allo-immune responses by the female donors (70-72). In the context of allelic differences, miHA disparity due to host-unique single nucleotide polymorphisms (SNPs) are considered genetic mismatches to the donor and could lead to GvHD (73). In high-resolution HLA-matched transplants, TRM of HLA-matched sibling transplants was 34% and 42% in matched unrelated transplants (32). This suggests that, in addition to the classical HLA typing approach, matching miHAs might further improve the transplant outcome by reducing the severity of GvHD. With the major advances during the past decade in human genome project, high-density genotyping, gene-chip development, and bioinformatics, increasing numbers of miHAs associated with GvHD risks were identified.

Through retrospective analysis of SNPs of the *NOD2/CARD15* gene, of which high-risk alleles are associated with an increased incidence of Crohn disease, a major impact of *NOD2/CARD15* SNP mismatches on GvHD was also found in patients receiving alloHSCT (74). To identify new alleles associated with GvHD, a genome-wide association study (GWAS) survey scanned and compared each SNP disparity among HLA-A, B, DRB1 and DQB1-matched unrelated transplant pairs (75). In this study, GWAS was performed basing on the allelic mismatch of each SNP locus, rather than comparing single mismatched SNP genotype, between the donor-recipient pair. This analysis revealed GvHD association contributed by a combination of more than one SNP genotype. Through this approach, four additional loci were identified when the analysis was restricted to DQB1*0501, Cw*0102, B85201 and Cw*1202-matched transplant pairs (75).

Recently, another class of miHA-mismatch influencing the outcome of alloHSCT was characterized. Through genome-wide survey of structural polymorphism, six common gene deletions associated with protein-coding sequence removal and GvHD-affecting tissue expression were identified (76). Among those, the homozygous deletion of *UGT2B17* gene in the donor results in increased incidence of acute GvHD in *UGT2B17*-sufficient recipients, whereas the association was not found in the donor $^{(+)}$ recipient $^{(-)}$-*UGT2B17* mismatches. This finding suggests that the immune system of *UGT2B17* $^{(-)}$ donor sees the gene products as foreign proteins and hence causes GvHD (76). It was shown by another group that UGT2B17-specific $CD8^+$ T cell responses of *UGT2B17* $^{(-)}$ donors could be detected in recipients expressing at least one allele of *UGT2B17* (77). Together, these results show that the genome-wide matching approach is

a useful tool to identify unknown miHA targets that are associated with GvHD and might improve the overall safety for alloHSCT.

Strategies to enhance GvL

Although often associated with increased relapse rate, anti-leukemia responses were evident in identical-twin transplants (68), T cell-depleted transplants (27) and in patients in long-term remission without overt GvHD (78); these results suggest that GvHD and GvL are dissociable processes. In addition to T cells, the role of NK cells in mediating tumor rejection has been an intense area of interest in the field recently, for their potential of eradicating tumors without causing GvHD (79-81). Rationales for NK-based immunotherapy will be addressed later in Chapter 2 and Future directions.

- **Tumor-associated antigens (TAAs)**

To separate GvHD and GvL, a primary focus in the field has been finding the TAAs, i.e., neo-antigens. There are only a few TAAs identified in CML: the b3a2 neo-peptide derived from *BCR-ABL1* junctional sequence (82, 83); proteinase-3 (PR-3), which resides within the azurophilic granules of normal myeloid progenitors and is over-expressed in CML cells (84); Wilm's tumor antigen-1 (WT-1), a transcription factor highly expressed in leukemic cells and other solid tumors (85); and the recently identified CML-28, an exosome component involved in 3'RNA processing (86) and CML-66, an immunogenic antigen preferentially expressed in myeloid progenitors with unknown function (87). The strategic benefit of finding relevant TAAs is the potential to eliminate leukemic cells through the endogenous immune system without requiring an allogeneic transplantation; as a result, there is no risk of GvHD. In retrospective studies, CML

patients who had previous exposure to IFN-α therapy prior to switching to imatinib showed better chances to achieve CCyR or CMR (16, 88, 89). Although IFN-α has long been used to treat CML, the exact mechanisms of action are not fully understood (88). Interestingly, it was shown that, in HLA-A0201^{+} CML patients who were in major or complete CyR, IFN-α treatment was associated with increased transcription of PR-1, a peptide derivative of PR-3, and the induction of PR-1-specific CTL, whereas the association was low in patients treated with imatinib (90). This indicates an immunological mechanism through which some patients might achieve durable remissions.

- **Vaccination approach**

Most TAA-related research can be divided into two major categories: one is to inject *in vitro*-raised, TAA-specific T cell clones or *ex vivo*-stimulated autologous T cells into patients or diseased mice (91, 92), and another is to elicit anti-tumor immune responses through immunization. It was shown that repetitive immunizations of five b3a2 peptides with QS21 plus granulocyte macrophage colony stimulating factor (GM-CSF) as adjuvant, alongside with the pre-existing IFN-α or TKI therapies, led to CCyR in most patients and CMR in some of the cases (93). This was the first trial to observe clinical responses through the approach of b3a2 peptides immunization. However, it is not clear whether remissions can be induced without the co-administration of imatinib or IFN-α treatment- answering this question will require a prospective randomized clinical trial.

CTL clones specific to HLA-A*0201-restricted PR-3 peptides can be generated from peripheral blood of healthy individuals. These clones were able to lyse human myeloid leukemic cell lines (94) and specifically inhibit the colony forming ability of

primary CML progenitor cells without affecting the corresponding normal population (84). CTLs specific to HLA-A*0201-restricted WT-1 peptides were also shown capable to discriminate between the leukemic and normal $CD34^+$ primitive marrows (95). Clinical trials immunizing leukemia patients with natural or heteroclitic WT-1 peptides, or with combined PR-3 + WT-1 peptides in HLA-matched patients showed a clear correlation between increased frequency of peptide-specific CTLs and tumor reduction (96-98). However, a major problem commonly seen with the immunization approach is that CTLs showing promising *in vitro* killing responses do not always behave the same when administered *in vivo*, indicating the presence of negative regulation within the host (99) and potential tumor escape mechanisms (100). Additionally, clonal deletion of high-avidity peptide-specific CTL was also observed in several recent vaccination trials that failed to show immune-protection (101, 102). A clinical report has followed the evolution and localization of a predominant WT-1-specific CTL clone in a leukemia patient receiving peptide vaccination (103). The quantity and functionality of the peptide-specific CTL clone were analyzed by quantitative RT-PCR for its clonotypic TCR-Vβ usage and intracellular IFN-γ staining, respectively. Early post-immunization, expansion of the CTL clone was associated with complete remission. However, the clone was gradually deleted and this was associated with disease relapse. Reappearance of the clone with impaired functionality was detected (103), indicating the presence of leukemia tolerating mechanisms such as receptor editing. The involvement of $CD4^+CD25^+$, $FoxP3^+$ regulatory T cell (Treg) in mediating tumor tolerance has been suggested in both mice and human vaccine studies (104-106). It was shown that Treg was depleted early post-immunization, and the recovery of Treg correlates with the loss of peptide-specific CTL

responses in the vaccinated patients (102). This indicates that systemic Treg-depletion may enhance the GvL efficacy of TAA-associated immunization approach.

- **Confining GvHD to the hematopoietic system**

Compared to anti-TAA reactions, anti-allogeneic responses are far more robust, but at the cost of losing specificity for tumor cells. A feasible option to enhance GvL is to selectively induce allo-responses by targeting miHAs with restricted expression in hematopoietic lineages such as the HLA-A2-restricted HA-1, HA-2 (107). It was shown that DLI from HA-1/HA-2 $^{(-)}$ donors could induce specific CTL responses associated with complete remission in HA-1/HA-2 $^{(+)}$ patients with relapsed leukemia, and that CTL clones derived from the patients displayed selective *in vitro* cytotoxicity against recipient-stem and progenitor cells (108). To look for more miHAs with specific expression pattern, the genome-wide searching approaches will be extremely useful in identifying potential targets for immunotherapy.

It was shown in a murine tandem transplant model of acute leukemia that host APC were required for GvHD induction, as the chimeric recipients repopulated with class I MHC-deficient APC were resistant to GvHD caused by DLI administration at time of alloHSCT (53). However, further studies using this tandem transplant model investigating APC's role in orchestrating GvL demonstrated that the anti-tumor response was predominated by host APC, suggesting that eliminating host APC in order to prevent GvHD might lead to loss of GvL (109). The tight linkage of GvHD and GvL became relatively loose when the timing of DLI administration was delayed to a few weeks post-transplant, when susceptibility to GvHD decreased while distinct GvL responses could still be induced (110-114). Several mechanisms have been postulated for the waning

GvHD susceptibility during the post-transplant period. The delayed timing of DLI avoids the immediate cytokine storm generated by the transplant conditioning regimen (115), and the decreased proportion of host-APC after allogeneic reconstitution might reduce the frequency of alloreactive T cell priming (53). In addition, the transient appearance of regulatory T cells and immature myeloid suppressor cells in the post-transplant period may also contribute to tolerance induction (116-120).

Why is CML sensitive to immunotherapy?

For reasons not fully understood, CML is the most susceptible hematologic malignancy to immunotherapy. The sensitivity of CML to immune-recognition is best demonstrated in the context of alloHSCT/DLI therapy (24). The myeloablative conditioning regimens effectively dampens the host endogenous immunity, and this in turn favors the establishment of donor chimerism and GvHD or GvL reactions mediated by allogeneic T cells present within the BM graft. Early post-transplant, donor T cells have a transient opportunity to interact with residual host APCs, such as DC, macrophage and B cell, that survived the myeloablative regimen (53). The brief interaction between donor T cell and host APC has been shown to be critical in inducing GvHD or GvL responses (53, 109, 121). In CML, it has been shown that primary leukemic cells could express and present BCR-ABL1-derived peptide on class I HLA molecules, and that low level of BCR-ABL1-specific CTL could be detected through tetramer binding assay (83). Therefore, it is plausible that the myeloid leukemic cells can serve as the host APC and prime the allogeneic T cells during the transient post-transplant window. Additionally, DC within the tumor-affected tissue usually expresses an immature phenotype that

prevents effective T cell priming and renders tumor-tolerance through mechanisms involving regulatory T cell function (122, 123). An early report showed that primary leukemic DC, when stimulated with a combination of cytokines, including GM-CSF, could induce *in vitro* CML-specific responses by autologous CTL (124). The strategy of enhancing DC maturation might provide a potential rationale for the success of a b3a2-immunization trial, in which GM-CSF was used as a component of the adjuvant (93).

Another possibility explaining the sensitivity of CML to immunotherapy is that the LSC compartment in chronic phase CML phenotypically resembles normal HSC and is not expanded, whereas the LSCs in blast crisis phase CML resemble granulocyte-macrophage progenitors (GMPs) and are expanded (125). From the aspect that immunotherapy is most effective when the disease burden is low, CML might be relatively vulnerable to such strategy due to its slow-progressing kinetics and low LSC frequency, as compared to the blast crisis phase disease or other leukemias.

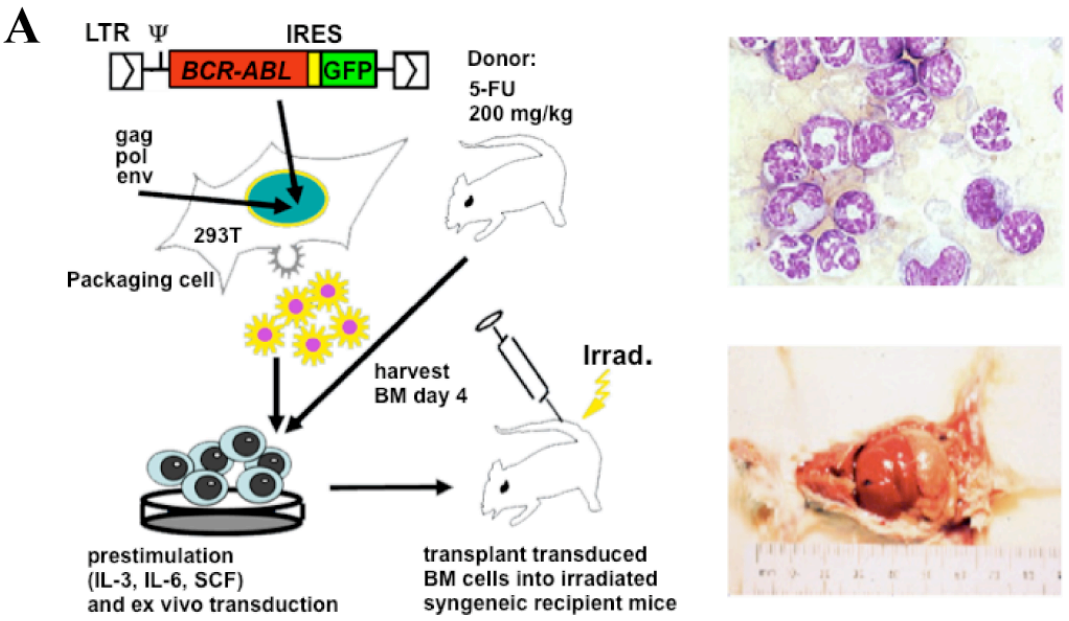
Together, these findings suggest that the LSC in CML is sensitive to immunological targeting, and that improvement in this aspect might provide insights for developing long-term cure. However, since the introduction of DLI therapy for relapsing CML in 1990, the transplant field has not moved as far as the TKI field. Part of the reason for this is a lack of suitable preclinical animal models, as the cellular dynamics of the post-transplant period are highly regulated and complicated. It is becoming clear by now that TKI therapy has a limited ability to eradicate residual disease in CML. This points to the need for additional strategies to eliminate residual CML stem cells and lead to permanent cure, and one potential approach is immunotherapy (126, 127).

To improve the efficacy and reduce the toxicity of current transplantation immunotherapy for CML, our laboratory focuses on a retrovirally-induced CML transplant model (8, 128). Unlike the monoclonal disease observed in humans, the retroviral transplant system typically induces polyclonal CML-like disease in recipient mice. Under a modified transplant protocol, where limiting numbers of leukemic-initiating cells are transplanted, the recipients mice develop oligo- or monoclonal CML-like disease that has a relatively prolonged latent period, but is otherwise identical to the polyclonal disease. Hence, the difference of disease clonality simply reflects the number of cycling HSC present during retroviral transduction and subsequent transplantation, but does not alter the fundamental pathophysiology of CML in the mouse model. Furthermore, modeling CML disease in a transplant setting facilitates the administration of allogeneic BM and DLI to the leukemic recipients, which resembles the clinical scenario of relapsing CML patients treated with DLI.

The retroviral BM transduction/transplantation mouse model of CML

The retrovirally-induced CML transplant mouse model was first reported by Daley *et al.* and was later optimized by Pear *et al.* to achieve efficient MPN disease induction (8, 128). Briefly, the donor marrows were transduced with MIG-p210, the MSCV-based retrovirus carrying a bi-cistronic construct of *BCR-ABL1* and *GFP*, and were transplanted into lethally irradiated syngeneic mice. The engrafted recipients began to show leukemic cell repopulation within two weeks after transplantation, and without treatment, all recipients succumbed to CML-like disease within 30 days after the transplant. Unlike other methods, this approach models the complete leukemogenesis

from chronic to blast-crisis phases (9, 128), and recapitulates many of the disease phenotypes found in humans, such as peripheral leukocytosis with mostly polymorphonuclear neutrophils and metamyelocytes. Enhanced cell infiltration to spleen and liver led to hepatosplenomegaly, in which majority of the cells were positive for Mac-1 and Gr-1 myeloid markers (128); lastly, neutrophil infiltration to the lungs caused pulmonary hemorrhage, which appeared to be the cause of death.


A
LTR Ψ
IRES
BCR-ABL
GFP
Donor:
5-FU
200 mg/kg
gag
pol
env
293T
Packaging cell
harvest
BM day 4
Irrad.
prestimulation
(IL-3, IL-6, SCF)
and ex vivo transduction
transplant transduced
BM cells into irradiated
syngeneic recipient mice

B

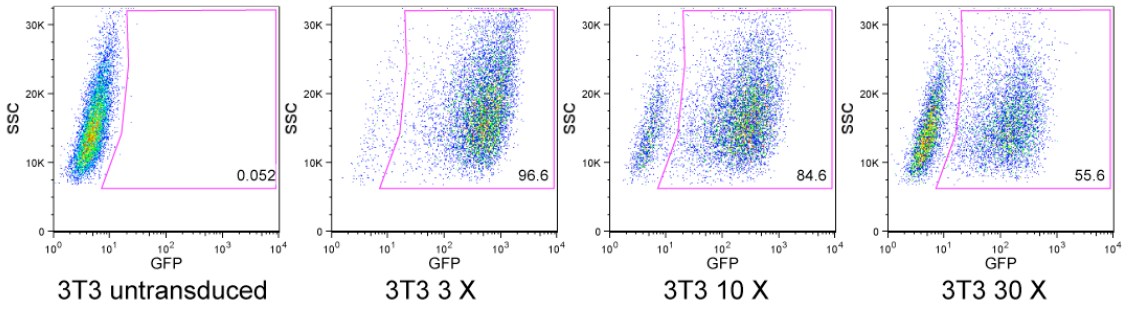

SSC
GFP
0.052
96.6
84.6
55.6
3T3 untransduced
3T3 3 X
3T3 10 X
3T3 30 X

Figure 2. Induction of CML-like disease in mice. (A) Schematic diagram of the retrovirally-induced CML transplant model. Donor Balb/c mice are primed with a chemotherapeutic agent, 5-fluorouricil (5-FU), four days before BM harvesting. The 5-FU-marrows are then subjected to ex vivo retroviral transduction for two days before being transplanted into irradiated syngeneic Balb/c recipients. The MIG-p210 retrovirus contains the bicistronic *BCR-ABL1/GFP* construct linked by an internal-ribosomal-entry-site (IRES) sequence, a packaging signal (Ψ), and two MSCV long terminal repeats (LTRs) at the flanks. MIG-p210 retrovirus was produced by transfecting both MIG-p210 vector and a packaging EcoPac construct (providing gag/pol/env functions) into a 293T packaging cell line. The method induces highly aggressive CML in transplant recipients that causes mortality within about 30 days. Upper right: a cytospin picture showing myeloid-expansion of mostly neutrophils in peripheral blood of a recipient mouse; lower right: a picture showing severe hepatosplenomegaly of a representative CML mouse at time of death. (Schematic diagram courtesy of Rick Van Etten.) (B) Representative viral titering result using 3T3 mouse fibroblasts in vitro transduction assay. In brief, 3T3 cells were transduced by serial dilutions of MIG-p210 retroviral stock (dilution factors: 1:3, 1:10, and 1:30) and the efficiency of transduction measured by flow cytometric detection of GFP expression (*x*-axis).

Objectives of the study

The major objective of the work presented in this thesis is to understand the cellular mechanisms through which allogeneic immune cells eradicate BCR-ABL1-expressing leukemic cells in CML. When patients relapse after alloHSCT, donor leukocyte infusions (DLI) can re-induce durable remission in most cases. This strongly suggests that, although intrinsically resistant to conventional chemoradiotherapy or to TKI treatment, the LSC in CML are susceptible to immunological targeting. Using the retrovirally-induced CML model, we aimed to establish a workable murine model that will allow us to investigate the contributions of different immune cell types within the DLI population to the GvL effect, and to dissect the leukemia stem cell-killing mechanisms that may provide insights in separating GvL and GvHD responses.

Chapter 1. Establishing a model of adoptive immunotherapy of CML-like disease in mice

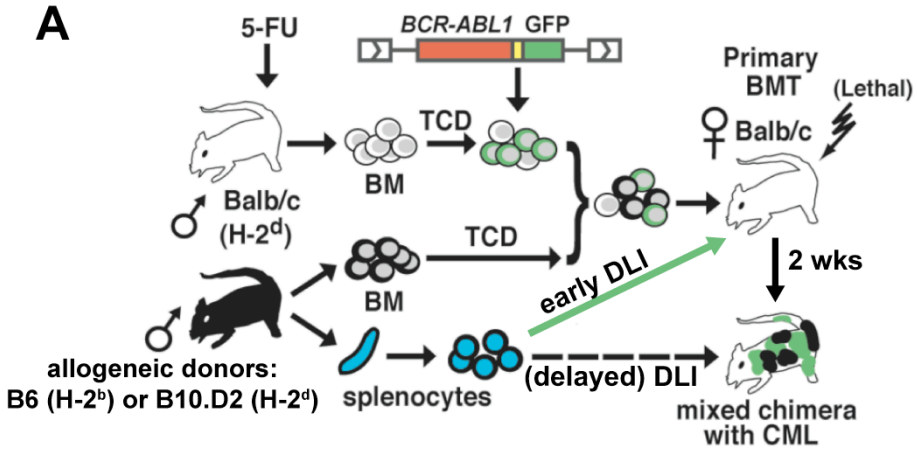
The AlloHSCT/DLI Model

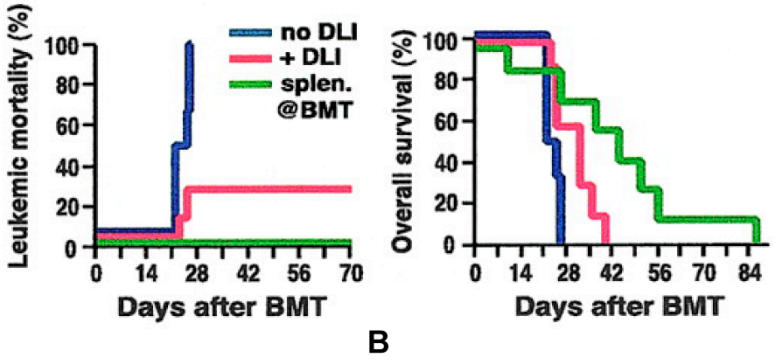
Using the retroviral BM transduction/transplantation model of CML described above, our laboratory has previously established an adoptive immunotherapy model in order to study mechanisms of DLI-mediated GvL effect. To model CML patients who relapse after alloHSCT, we co-transplanted T cell-depleted allogeneic BM cells (TCD-allo-BM) together with *BCR-ABL1*-transduced BM into lethally irradiated syngeneic Balb/c hosts. The recipients become mixed chimeras in which normal allogeneic and leukemic BM-derived cells co-exist in the marrow and circulation, resembling CML patients with cytogenetic and hematologic relapse following alloHSCT. Without intervention, the leukemia expands and inevitably causes death within 30 days. Immunotherapy, in the form of DLI from the allogeneic donor, can be delivered to recipient chimeras at the same time as the initial BM graft ("early" DLI) or later, following engraftment ("delayed" DLI; Figure 3A). When MHC-mismatched C57Bl/6 (B6) mice (MHC haplotype H-2^b) are used as the allogeneic donors, multiple infusions of B6 splenocytes beginning at d14 post-transplant converted recipients to full allogeneic chimerism and resulted in eradication of CML-like leukemia in the majority of recipients, but at the expense of severe and fatal GvHD (Figure 3B). When MHC-matched, miHA-mismatched B10.D2 allogeneic donors were used, delayed DLI did not cause significant GvHD, but was ineffective at controlling leukemia or prolonging survival (Figure 3C). These results, previous published by Krause and Van Etten (129), demonstrated that allo-

reactivity across MHC boundary resulted in a powerful GvL effect but at the expense of fatal GvHD. However, in the MHC-matched setting, which is more relevant to human CML treated by alloHSCT, the disparity across miHA alone was not sufficient to result in meaningful GvL effects under these conditions in the murine model system (129).

Since HLA-matched alloHSCT usually induces durable remissions clinically (over 75% in chronic phase patients, (30)), it suggests that anti-miHA immune responses are able to eradicate minimal residual disease by targeting the leukemic stem cells. In the murine model, we and others have shown that early DLI, administered at time of transplant, abrogated leukemogenesis completely in the MHC-mismatched (Figure 3, green curve) or MHC-matched, MiHA-mismatched (1) setting. These results demonstrated that miHA-mismatched DLI is clearly competent in eradicating leukemic stem cells; however, the same modality failed to effectively treat disease when administered 14 days post-transplant. From a retrospective study analyzing overall survival of CML patients receiving alloHSCT/DLI therapy, the best responses to immunotherapy were observed when DLI was administered at early stage of relapse, in the setting of minimal leukemic burden (EMBT-95 survey, (24)). Hence, we hypothesized that miHA-mismatched DLI failed to treat established leukemia because the rapid leukemic progression overwhelms the GvL effect of DLI, and, as a corollary, that anti-leukemic response in an MHC-matched, miHA-mismatched transplant setting might be modeled if the aggressiveness of the CML-like leukemia could be reduced. To accomplish this in the retrovirally-induced CML model, we first tested whether the leukemia could be attenuated by reducing the number of leukemic stem cells at time of

transplant. Subsequently, we attempted to prolong survival and the treatment window for immunotherapy by combining DLI with imatinib treatment.


A
5-FU
BCR-ABL1 GFP
Primary BMT
(Lethal)
Balb/c
TCD
BM
Balb/c (H-2d)
TCD
BM
2 wks
early DLI
allogeneic donors:
B6 (H-2b) or B10.D2 (H-2d)
splenocytes
(delayed) DLI
mixed chimera with CML


B
Leukemic mortality (%)
no DLI
+ DLI
splen. @BMT
Days after BMT
Overall survival (%)
Days after BMT

B

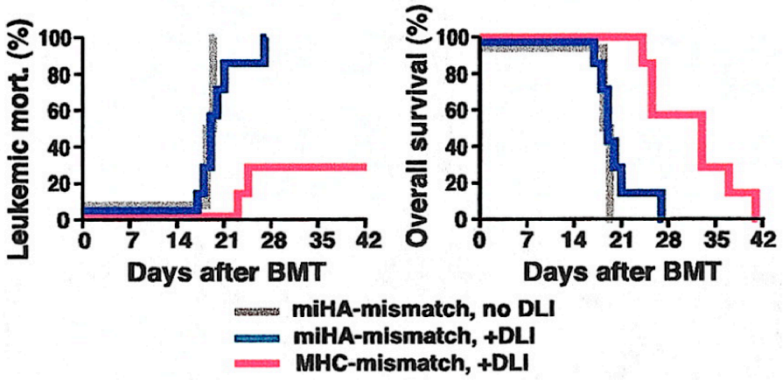

C
Leukemic mort. (%)
Days after BMT
Overall survival (%)
Days after BMT
miHA-mismatch, no DLI
miHA-mismatch, +DLI
MHC-mismatch, +DLI

Figure 3. GvL mediated by delayed DLI is more pronounced in MHC-mismatched transplant pairs. (A) Schematic diagram of the retrovirally-induced CML transplant model. Same as the retrovirally-induced model, CML was induced by transplanting MIG-210-transduced 5-FU marrows to lethally irradiated syngeneic Balb/c recipients. To model relapse-phase CML after alloHSCT, T cell depleted (TCD) BMs from MHC-mismatched B6 donors, or miHA-mismatched B10.D2 donors, were co-transplanted with leukemic BMs. Following two weeks of cell repopulation, the recipients will become mixed chimeras bearing both allogeneic and leukemic BM-derived cells in circulation. (B & C) Leukemic mortality (left panels) and overall survival curves (right panels) of recipients treated with MHC-mismatched B6-DLI (B) and MHC-matched, miHA-mismatched B10.D2-DLI (C), figures reproduced from ref. (129)**.** Without treatment, the recipients will die of CML within 30 days, shown as "no DLI" curves in both panels. DLI treatment was performed by repetitive infusions of splenocytes of corresponding marrow-donors two weeks after the transplant, the result are shown by "+DLI" curves in both panels. In the male-to-female transplant setting, donor-derived cells can be tracked by male-specific *sry* gene. Of note, early administration of B6-DLI at time of transplant (green arrow, panel A) abolished leukemogenesis completely (spleen @ BMT, panel B), demonstrating the susceptibility of leukemic stem cells to immune targeting (Krause & Van Etten, 2004, (129)). The mechanism of GvL mediated by early DLI treatment will be further discussed in Chapter 2.

Attenuation of CML-like leukemia in mixed chimeras by reducing leukemia stem cell dose

In humans, CML is a monoclonal disease that is associated with a long latent period. The aggressiveness of murine CML is primarily due to the polyclonal nature of the leukemia (10), and a previous study suggested that the CML-like disease could be converted into a more indolent oligo- to monoclonal disease by transplanting reduced numbers of lineage-depleted *BCR-ABL1*-transduced BM cells (130). In this strategy, lineage depletion is essential to eliminate precursor cells for *BCR-ABL1*-induced B-cell acute lymphoblastic leukemia (B-ALL), which have the characteristics of early lymphoid progenitors and can cause death of recipients from lymphoid leukemia (10, 11).

We tested the utility of this approach by performing limiting dilution repopulation of irradiated syngeneic Balb/c recipient mice with *BCR-ABL1*-transduced BM without allogeneic donor cells. We observed a gradual prolongation of survival associated with decreased transplanted cell dose that was optimal (median survival 40 days) at around a dose of $1x10^4$ cells per recipient, whereas recipients of lower cell doses failed to engraft efficiently with leukemia (Figure 4A). The increased survival correlated with a gradual reduction in the clonality of the leukemia as assessed by Southern blotting, down to one or two clones per recipient (Figure 4B). Hence, repopulation of mice with one or a few leukemic stem cells attenuates the severity of the CML-like leukemia and prolongs survival of recipients. We then tested the effect of co-transplanting different ratios of *BCR-ABL1*-transduced, lineage markers-depleted (Lin^-), syngeneic (Balb/c) cells and allogeneic (B10.D2) TCD BM cells on chimerism and survival. The results indicated that a mixture of $1x10^4$ Lin^- *BCR-ABL1*-transduced BM (containing 1-2 leukemic stem cells,

Figure 4B) and a 100-fold excess of TCD allogeneic HSC (based on an abundance of 1 HSC per 10^5 normal BM cells) was associated with a median survival of over 40 days (Figure 4C), and yielded excellent allogeneic chimerism over a period of 14-50 days post-transplant (Figure 4D).

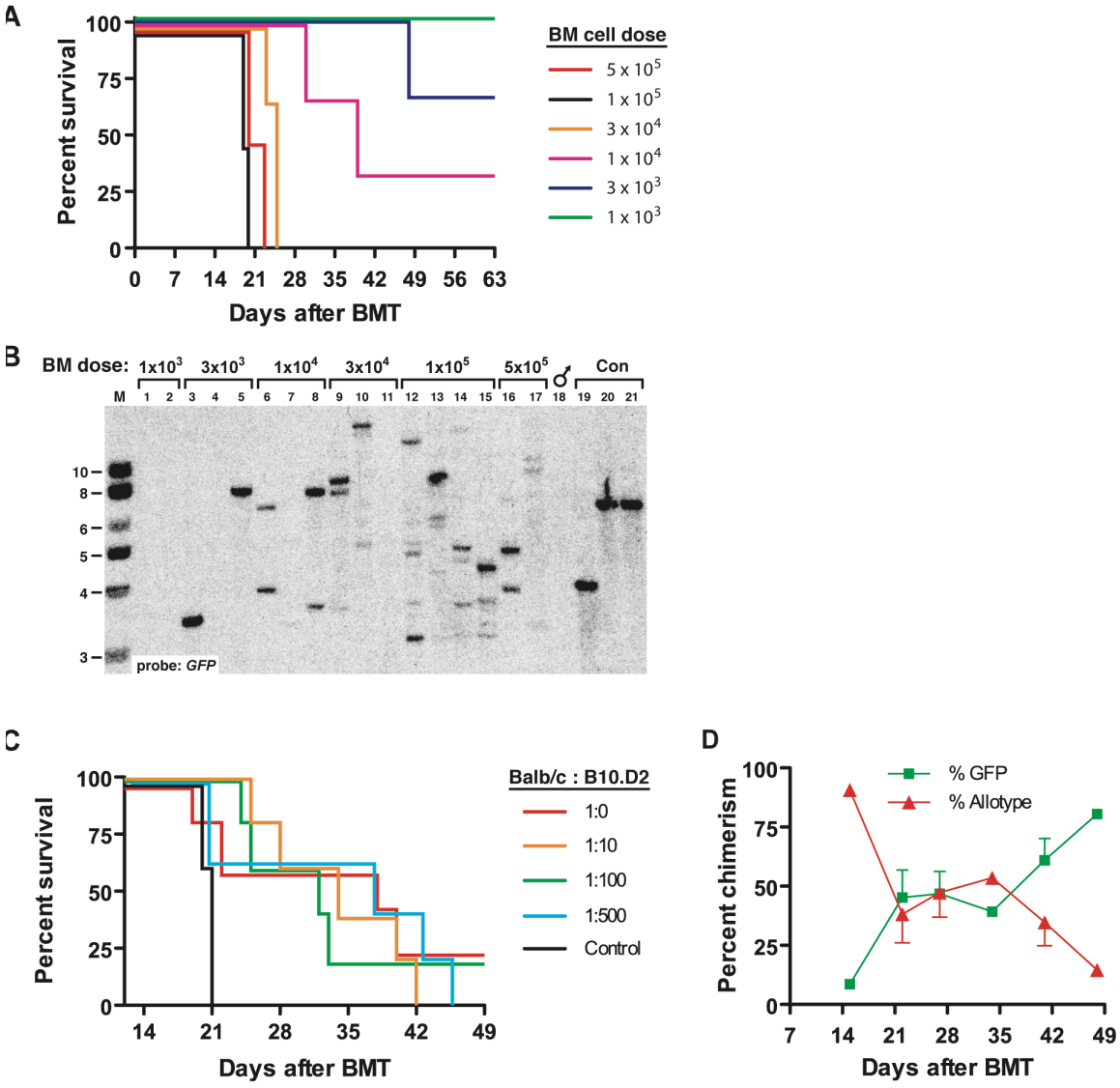


Figure 4. Prolonged survival of allogeneic chimeras engrafted with limiting numbers of leukemic stem cells. (A) Kaplan-Meier survival curve of Balb/c recipients of *BCR-ABL1*-transduced BM from 5-FU-treated syngeneic donors injected with the indicated numbers of total BM cells. (B) Southern blot analysis of BM genomic DNA from the cohorts in panel A. Lanes 1 and 2 were from recipients of $1x10^3$ *BCR-ABL1*-transduced BM cells, lanes 3-5 received $3x10^3$ transduced cells, lanes 6-8 received $1x10^4$ transduced cells, lanes 9-11 received $3x10^4$ transduced cells, lanes 12-15 received $1x10^5$ transduced cells, while lanes 16 and 17 received $5x10^5$ transduced cells. Lanes 19-21 were from control cell lines each containing a single provirus. Note that reduction in BM cell dose to $\leq 1x10^4$ cells results in repopulation with 1-2 leukemic stem cells. (C) Survival curve of mixed allogeneic chimeras repopulated with limiting numbers of leukemic stem cells, together with increasing numbers of TCD allogeneic BM cells. The numbers represent the ratio of leukemic stem cells in $1x10^4$ *BCR-ABL1*-transduced BM cells (approximately 2) to the number of allogeneic HSC (assuming 1 HSC per 10^5 BM cells). Control mice received $3x10^5$ syngeneic *BCR-ABL1*-transduced BM cells only. (D) Percentage of circulating allogeneic cells (red) and GFP^+ cells (green) vs. time after BMT for the cohort that received the 1:100 mixture of *BCR-ABL1*-transduced and allogeneic BM from panel C. Note the high level of allogeneic chimerism that persists until leukemic progression at 7 weeks. The GFP^+ population between d21 and d42 contained some cells that were $allotype^+$ due to phagocytosis of GFP^+ leukemic cells by allogeneic macrophages (129).

Combined therapy with delayed DLI and imatinib results in prolonged leukemia-free survival in mixed allogeneic chimeras with *BCR-ABL1*-induced CML-like disease

A complementary strategy to extend the time period available for immunotherapy in the CML model is treatment of recipient chimeras with an Abl kinase inhibitor such as imatinib mesylate, which significantly prolongs the survival of mice reconstituted with larger numbers of *BCR-ABL1*-transduced cells (131), and has been used in combination with DLI in CML patients relapsing after alloHSCT (132). To test this approach, we generated mice with mixed allogeneic chimerism (B10.D2→Balb/c) and CML using the conditions defined in Figure 4D, and treated cohorts either with delayed DLI (allogeneic splenocytes) alone, imatinib at an intermediate dose (~30 mg/kg once daily by oral gavage), or with both modalities (Figure 5). Treatment with imatinib alone prolonged survival but did not eradicate the leukemia, as the mice relapsed and succumbed to CML-like disease when the drug was discontinued (Figure 5, dotted line). By contrast, the addition of delayed DLI to imatinib treatment resulted in prolonged survival relative to sham-treated control mice and led to probable cure when kinase inhibitor therapy was discontinued. Significantly, although the majority of mice treated with DLI only succumbed to CML-like disease in a similar kinetics as the control, a small portion of the group reached long-term survival with normal blood counts and no GFP^+ cells in circulation (Figure 5B & C). These results show that miHA-mismatched DLI therapy can be better modeled by inducing the attenuated form of CML. Of note, the therapeutic efficiency improved when imatinib was added to the DLI treatment, further supporting our hypothesis that miHA-mismatched DLI therapy can be modeled in mice when disease

burden is low. In addition, under the current conditioning regimen, all treated mice showed no overt evidence of GvHD.

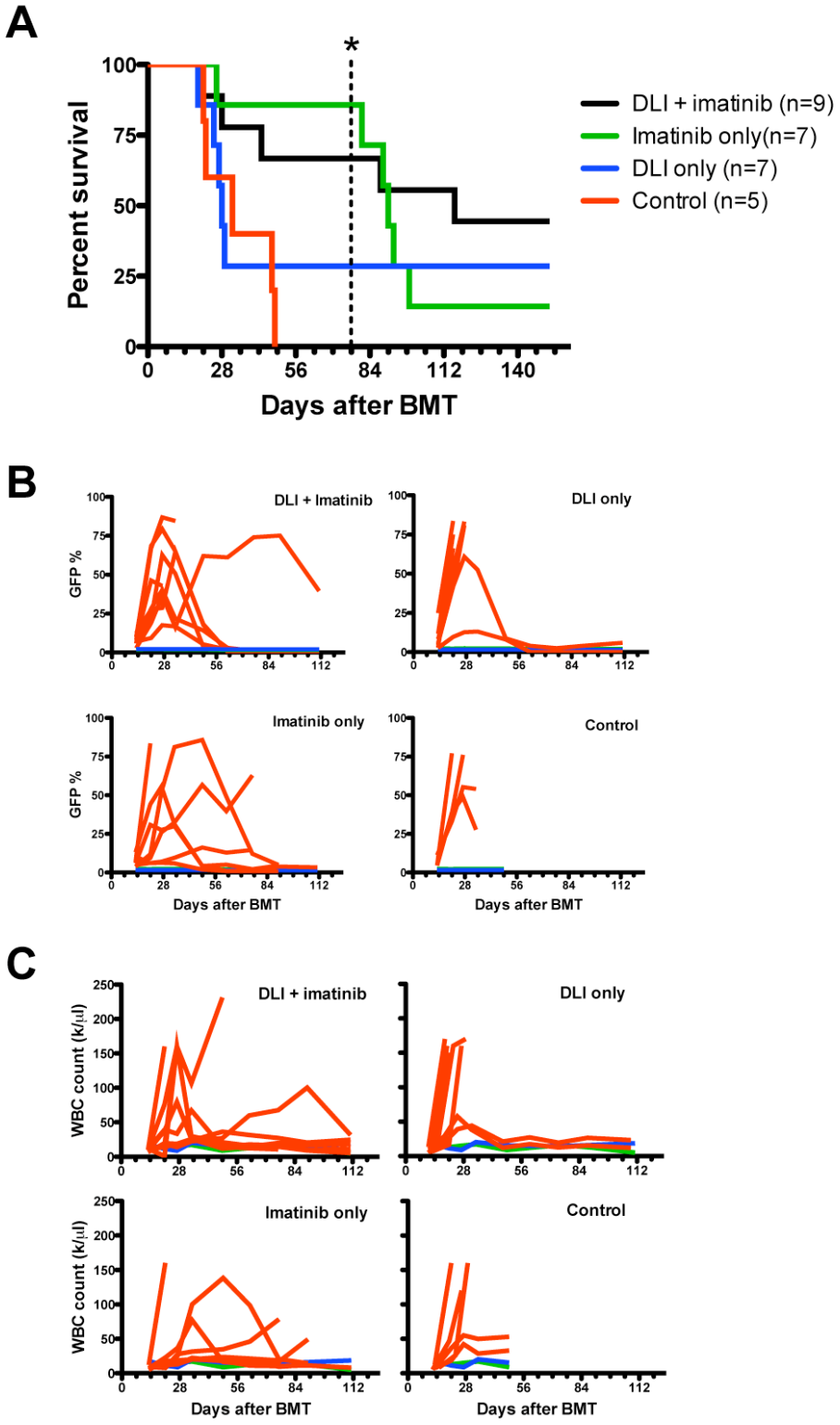


Figure 5. Combined therapy with imatinib and delayed DLI in MHC-matched/miHA-mismatched chimeras with *BCR-ABL1*-induced CML-like disease. (A) Survival curve for cohorts of Balb/c recipients of *BCR-ABL1*-transduced Balb/c marrow ($1x10^4$ cells) mixed with 100-fold excess of MHC-matched allogeneic stem cells (from B10.D2 donors). All recipients developed mixed hematopoietic chimerism with CML-like leukemia (GFP^+ myeloid cells) at d14 after transplant. Beginning at d14, mice were treated with repeated weekly infusions of allogeneic (B10.D2) splenocytes (DLI: 3 x 10^7 cells per treatment, 8 infusions per recipient), treated with low-dose imatinib (30 mg/kg once daily by oral gavage), or a combination of the two (DLI + imatinib). Imatinib treatment was discontinued at d77 (indicated by the dotted line). The survival of the DLI + imatinib cohort was significantly longer than of the Control arm (P=0.0294, Mantel-Cox test), while the difference in survival of mice receiving DLI + imatinib versus either therapy alone did not reach statistical significance. (B and C) Percentage of GFP^+ leukemic cells in circulation (B) and peripheral white blood cell count (C) of recipients in the cohorts in panel (A) over the treatment course. Values from normal Balb/c and B10.D2 control mice are shown in green and blue, respectively. Note the absence of leukemic cells and low blood count in most of the survivors in DLI + imatinib and some mice in the DLI only group.

In subsequent experiments, we increased the dose of imatinib to 100 mg/kg/day (near the maximum tolerated dose in mice (133)) and increased the conditioning radiation dose from ~670 cGy to ~750 cGy. Under these conditions, disease eradication and long-term survival was reproducibly achieved in over 80% of recipients treated with imatinib and delayed DLI; the efficacy of DLI only treatment was also improved, with to up to 50% of recipients achieving long-term survival. In accordance with our previous results, about half of mice treated with DLI only succumbed to CML-like leukemia at the same initial rate as the control (untreated) cohort, but reached a steady disease-free plateau several weeks after the initial DLI treatment (Figure 6A). A prolonged time is also necessary for the therapeutic effect of DLI in CML patients, where the time required to achieve molecular remission varies from 3 months to more than one year after DLI treatment (24). Our data suggests that addition of imatinib to DLI might have enhanced treatment efficacy by sustaining the cohort until DLI took effect. At cellular level, flow cytometric analysis of peripheral blood demonstrated that elimination of circulating GFP^+ leukemic cells in the majority of recipients of combined DLI + imatinib therapy after three weeks of treatment, with conversion to full allogeneic chimerism (Figure 6B). At genomic level, southern blot analysis confirmed the eradiation of CML stem cells in these recipients, with replacement by male HSC of allogeneic origin (Figure 6C). Together, these results demonstrate that combination treatment of leukemic mixed chimeras with delayed DLI and kinase inhibitor therapy yield potent GvL effects in a scenario that is relevant to allografted CML patients.

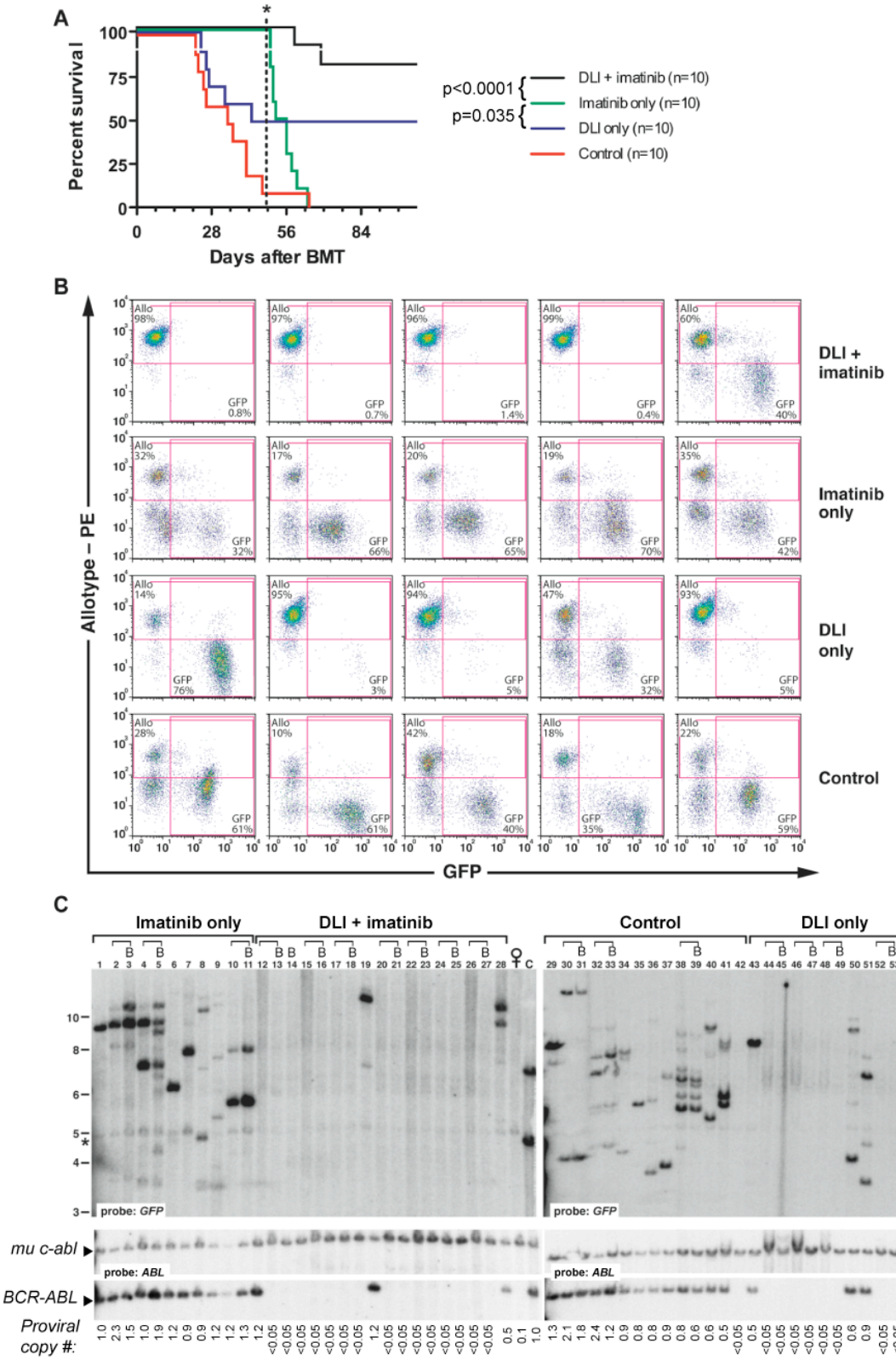

A
Percent survival
100
75
50
25
0
0
28
56
84
Days after BMT
*
p<0.0001
p=0.035
DLI + imatinib (n=10)
Imatinib only (n=10)
DLI only (n=10)
Control (n=10)
B
Allotype – PE
GFP
DLI + imatinib
Imatinib only
DLI only
Control
C
Imatinib only
DLI + imatinib
Control
DLI only
probe: GFP
probe: ABL
mu c-abl
BCR-ABL
Proviral copy #:

Figure 6. Delayed DLI and imatinib therapy lead to prolonged leukemia-free survival in MHC-matched/miHA-mismatched chimeras with *BCR-ABL1*-induced CML-like disease. (A) Survival curve for cohorts of Balb/c recipients of *BCR-ABL1*-transduced Balb/c marrow ($1x10^4$ cells) mixed with 100-fold excess of MHC-matched allogeneic stem cells (from B10.D2 donors). All recipients developed mixed hematopoietic chimerism with CML-like leukemia (GFP^+ myeloid cells) at d14 after transplant. Beginning at d14, mice were treated with repeated weekly infusions of allogeneic (B10.D2) splenocytes (2×10^7 cells per treatment, total of 5 infusions per recipient), treated with high-dose imatinib (100 mg/kg once daily by oral gavage), or a combination of the two (DLI + imatinib). Imatinib treatment was discontinued at d49 (indicated by the dotted line). Both the combined DLI + imatinib and DLI only treatment resulted in superior survival compared to imatinib alone ($P<0.0001$ and $p=0.035$ respectively, Mantel-Cox test), while the survival difference between the two treatment cohorts was of borderline significance ($P=0.072$). In this particular experiment, the Control and Imatinib only cohorts received infusions of syngeneic (Balb/c) splenocytes at the same cell dose and schedule, which did not result in any measurable GvL effects in other experiments (data not shown). (B) Flow cytometric analysis of peripheral blood leukocytes from five representative mice each from the cohorts in (A), analyzed at five weeks post-transplantation (following three DLI doses). Allogeneic chimerism (*y*-axis) was detected by a polymorphism in β2-microglobulin as described in Materials and Methods, while GFP^+ cells (*x*-axis) represent BCR-ABL1-expressing leukemic cells. The percentage of $allotype^+$ and GFP^+ cells in each plot is indicated. Note the eradication of leukemia in the majority of recipients treated with DLI + imatinib, contrasted with

persistent leukemia in control mice or those treated with imatinib alone. (C) Southern blot analysis of genomic DNA of the cohorts in panel A, harvested at d120 post-BMT. The blot was hybridized with a ^{32}P-labeled *GFP* probe that detects each retroviral integration site among genome (top), and an *ABL* probe that detects both murine *c-Abl (mu c-abl)* and *BCR-ABL1* (middle and bottom panels, respectively). Each lane represents the splenic or BM (B) sample of a recipient; DNA acquired from the same mouse was marked by bracket on the top. Lanes 1-11 are from recipients of syngeneic Balb/c splenocyte control + imatinib (imatinib only), lanes 12-28 are from recipients of DLI + imatinib, lanes 29-42 are from recipient of syngeneic BALB/c splenocyte treatment (control), lanes 43-55 are from recipients of DLI only treatment; DNA from normal female Balb/c mice, and from a control cell line (C) containing a mixture of two proviral clones are on the right of each blot. Proviral copy number was determined using NIH ImageQuant program and was normalized against value of control (C). Note the eradication of disease in both splenic and BM samples of DLI + imatinib and DLI only cohorts. The band indicated by the asterisk is a background band.

Chapter 2. Cellular mechanisms of miHA-mismatched delayed DLI therapy

The GvL effect of delayed DLI is predominantly mediated by $CD4^-$ splenocytes

To determine the T cell subsets responsible for the GvL effect of delayed DLI, we fractionated splenocytes by selectively depleting $CD4^+$ or $CD8^+$ cells as described previously (129). The resulting depleted splenocyte populations, when reanalyzed by FACS, had less than 0.5% of the original $CD4^+$ or $CD8^+$ T cell content (Figure 7A). The GvL activity of the CD4- and CD8-depleted splenocytes was compared to that of the same relative dose of total unfractionated splenocytes, using four DLI treatments of MHC-matched/miHA-mismatched leukemic chimeras, generated by the transplant conditions described in Figure 6. Under these conditions, administration of DLI with unfractionated splenocytes, in combination with imatinib at 100 mg/kg/day, resulted in eradication of leukemia and cure in 100% of recipients (Figure 7B). Interestingly, depletion of $CD4^+$ T cells had little impact on the GvL activity of delayed DLI, as the majority of recipients also cleared their GFP^+ leukemia and converted to full allogeneic chimerism (Figure 7B and C). By contrast, depletion of $CD8^+$ T cells profoundly impaired the anti-leukemic effect of delayed DLI (Figure 7B), with the majority of recipients failing to show disease clearance (Figure 7C). Additionally, an independent experiment where the leukemic mixed chimeras were treated with positively selected $CD8^+$ T cell-DLI showed a similar trend of survival benefit (Figure 7D). These results differ fundamentally from early DLI when T cells are administered at the time of initial transplantation of *BCR-ABL1*-transduced stem cells, where there are approximately equal

anti-leukemic contributions from both $CD4^+$ and $CD8^+$ T-cells (1, 129). Hence, our results have demonstrated that allogeneic T lymphocytes employed different GvL mechanisms when administered at a delayed time following allografting. Of note, most recipients of CD8-depleted DLI had evidence of clinical graft-versus-host disease, with weight loss (Figure 7E), skin changes, and diarrhea (data not shown). The result suggests that in MHC-matched, miHA-mismatched chimeras with CML-like leukemia, $CD8^+$ T cells mediate GvL predominantly, while $CD4^+$ cells cause GvHD.

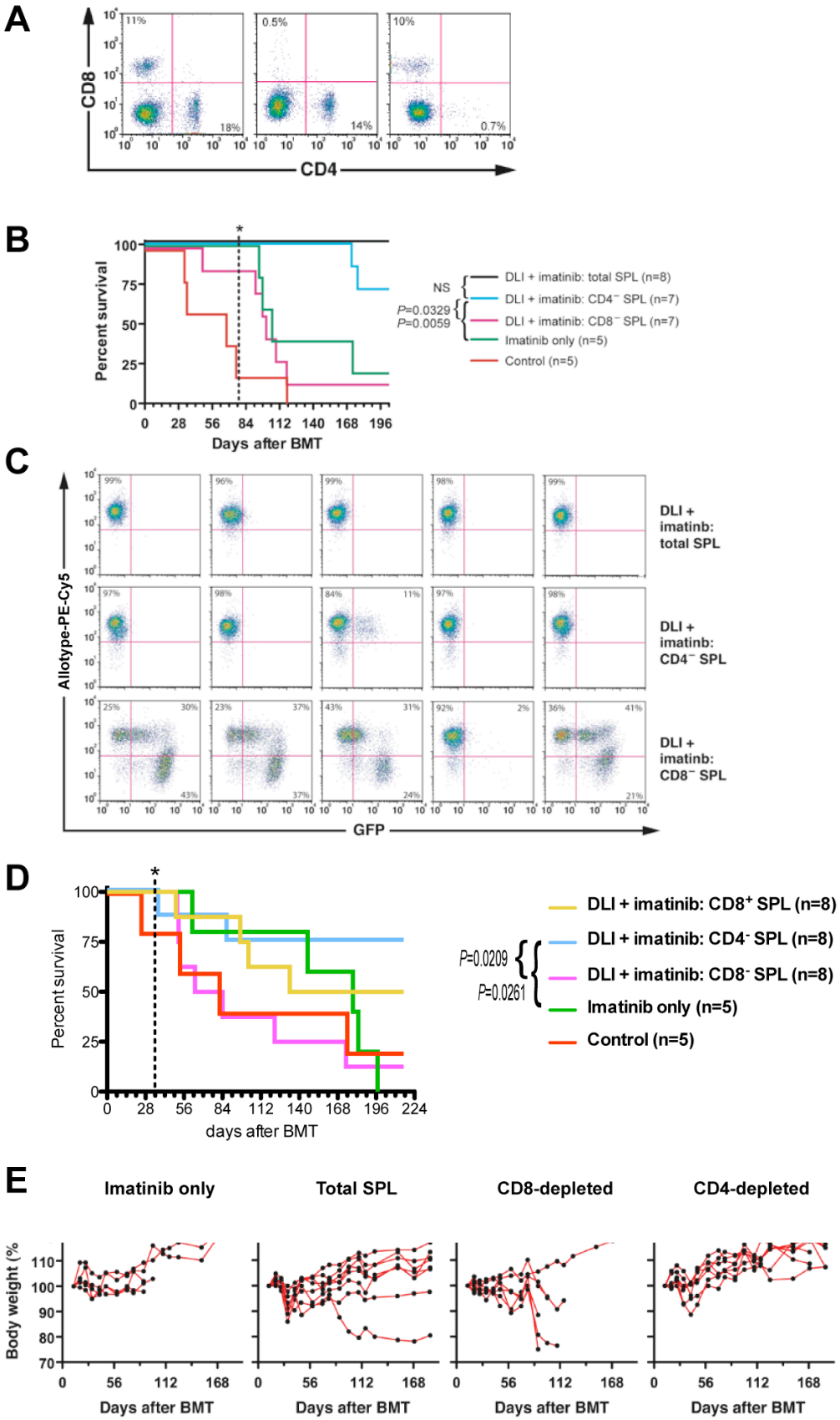

A
CD8
CD4
11%
18%
0.5%
14%
10%
0.7%
B
Percent survival
Days after BMT
NS
P=0.0329
P=0.0059
DLI + imatinib: total SPL (n=8)
DLI + imatinib: CD4⁻ SPL (n=7)
DLI + imatinib: CD8⁻ SPL (n=7)
Imatinib only (n=5)
Control (n=5)
C
Allotype-PE-Cy5
GFP
DLI + imatinb: total SPL
DLI + imatinb: CD4⁻ SPL
DLI + imatinb: CD8⁻ SPL
D
Percent survival
days after BMT
P=0.0209
P=0.0261
DLI + imatinib: CD8+ SPL (n=8)
DLI + imatinib: CD4- SPL (n=8)
DLI + imatinib: CD8- SPL (n=8)
Imatinib only (n=5)
Control (n=5)
E
Imatinib only
Total SPL
CD8-depleted
CD4-depleted
Body weight (%
Days after BMT

Figure 7. $CD8^+$ T cells mediate GvL against CML-like leukemia. (A) Flow cytometric analysis of splenocyte populations following selective depletion of $CD4^+$ (middle panel) or $CD8^+$ (right panel) cells. (B) Survival curve for B10.D2→Balb/c mixed allogeneic chimeras with CML who were untreated (red curve), treated with imatinib alone (100 mg/kg/day, green curve) or in combination with weekly DLI (total of 4 infusions beginning at d14 post-BMT) of unfractionated (black curve), CD4-depleted (blue curve) or CD8-depleted splenocytes (pink curve). Imatinib treatment was stopped at d77 (dotted line). Recipients treated with CD4-depleted DLI had significantly superior survival, as compared to recipients treated with CD8-depleted DLI + imatinib (P=0.0329), or recipients treated with imatinib alone (P=0.0059, Mantel-Cox tests); while the difference of recipients treated with total splenocyte DLI + imatinib vs. CD4-depleted DLI + imatinib, or of recipients treated with CD8-depleted DLI + imatinib vs. imatinib only, was not statistically significant. (C) FACS analysis of leukemia burden (GFP^+, x-axis) and allogeneic chimerism (β2-microglobulin b allele, y-axis) at d49 (two weeks following the last DLI) in five representative recipients of DLI with total (top row), CD4-depleted (middle row) or CD8-depleted (bottom row) splenocytes. Note the eradication of GFP^+ cells and full allogeneic chimerism in most recipients of CD4-depleted splenocytes, but persistent leukemia in recipients treated with CD8-depleted splenocytes. (D) Survival curve for an independent transplant cohort of B10.D2→Balb/c mixed chimeras with CML who were treated with the combined regimen of imatinib and fractionated DLI. In addition to the CD4 or CD8 T cell-depleted splenocytes, a group of mixed chimeras were treated with positively selected $CD8^+$ T cell-DLI (deep yellow curve). Imatinib treatment was stopped at d35 post-transplant (dotted line). Note that recipients treated with CD4-

depleted DLI had significantly superior survival, as compared to recipients treated with CD8-depleted DLI + imatinib (P=0.0209), or recipients treated with imatinib alone (P=0.0261). Although recipients treated with $CD8^+$ splenocytes showed prolonged survival, the difference between this cohort and recipients treated with imatinib only was not statistically significant (P=0.2886). The compromised GvL was likely due to an increased rate of cell death after positive selection. (E) Graph of body weight versus time for the cohorts presented in Figure 7B. Recipients treated with CD8-depleted DLI + imatinib showed obvious weight loss, whereas majority of mice treated with total SPL DLI + imatinib, CD4-depleted DLI + imatinib, and imatinib alone showed gained weight overtime.

Non-T cells do not make a major contribution to GvL against CML-like leukemia in MHC-matched/miHA-mismatched chimeras

Our results demonstrate that delayed DLI with allogeneic splenocytes can cure mice with CML-like leukemia. The splenocyte population used for DLI is a heterogeneous mix of $CD4^+$ T-cells (~20%), $CD8^+$ T-cells (~10%), $B220^+$ B cells (~50%), $CD11b^+$ myeloid cells (~5%), and $NK1.1^+$ NK cells (<5%) (Figure 9A). To determine whether non-T cells can mediate GvL in this model, we depleted splenocytes of T cells using anti-CD5 mAb, and treated MHC-matched/miHA-mismatched leukemic chimeras with DLI and/or imatinib, as described earlier. CD5 is a cell surface receptor highly expressed on mature T cells and peritoneal B-1 lymphocytes that functions as a negative regulator for antigen receptor-mediated signaling, and was commonly used as a T cell marker before the development of anti-CD3 mAb.

As previously shown, we observed significant prolongation of survival and clearance of GFP^+ leukemic cells in recipients treated with DLI + imatinib, with a few cases of GvHD. By contrast, majority of the recipients treated with CD5-depleted DLI ($CD5^-$ SPL) + imatinib or with imatinib alone succumbed to CML-like leukemia soon after cessation of TKI treatment at d35 post-transplant (Figure 8A). While one recipient of $CD5^-$ DLI exhibited prolonged survival, this mouse had persistent leukemia at autopsy when euthanized at end of the trial. There were ~1% of residual $CD5^+$ T cells in the splenocyte population following CD5 depletion (Figure 8C). To exclude the possibility that this level of residual T cells confers a significant GvL effect, we added back $CD5^+$ T cells to the $CD5^-$ DLI population at the 1% level ($CD5^{low}$ SPL), which led to a slightly prolonged survival that was not significantly different from $CD5^-$ DLI cohort. By contrast, addition of $CD5^+$ T cells to the $CD5^-$ DLI population to the relative 30% level characteristic of unfractionated splenocytes ($CD5^{hi}$ SPL) resulted in robust GvL that was as potent as in the unfractionated DLI + imatinib cohort (Figure 8A). Serial flow cytometric analysis of peripheral blood leukocytes showed that either imatinib alone, or the combination of imatinib and $CD5^-$ / $CD5^{low}$ DLI was ineffective in eradicating disease in these recipients, while combined therapy with imatinib and either unfractionated DLI or $CD5^{hi}$ SPL showed conversion to full allogeneic chimerism and clearance of leukemia (Figure 8B). These results demonstrate that non-T cells do not make a major contribution to the GvL effect of allogeneic splenocyte infusions under these experimental conditions.

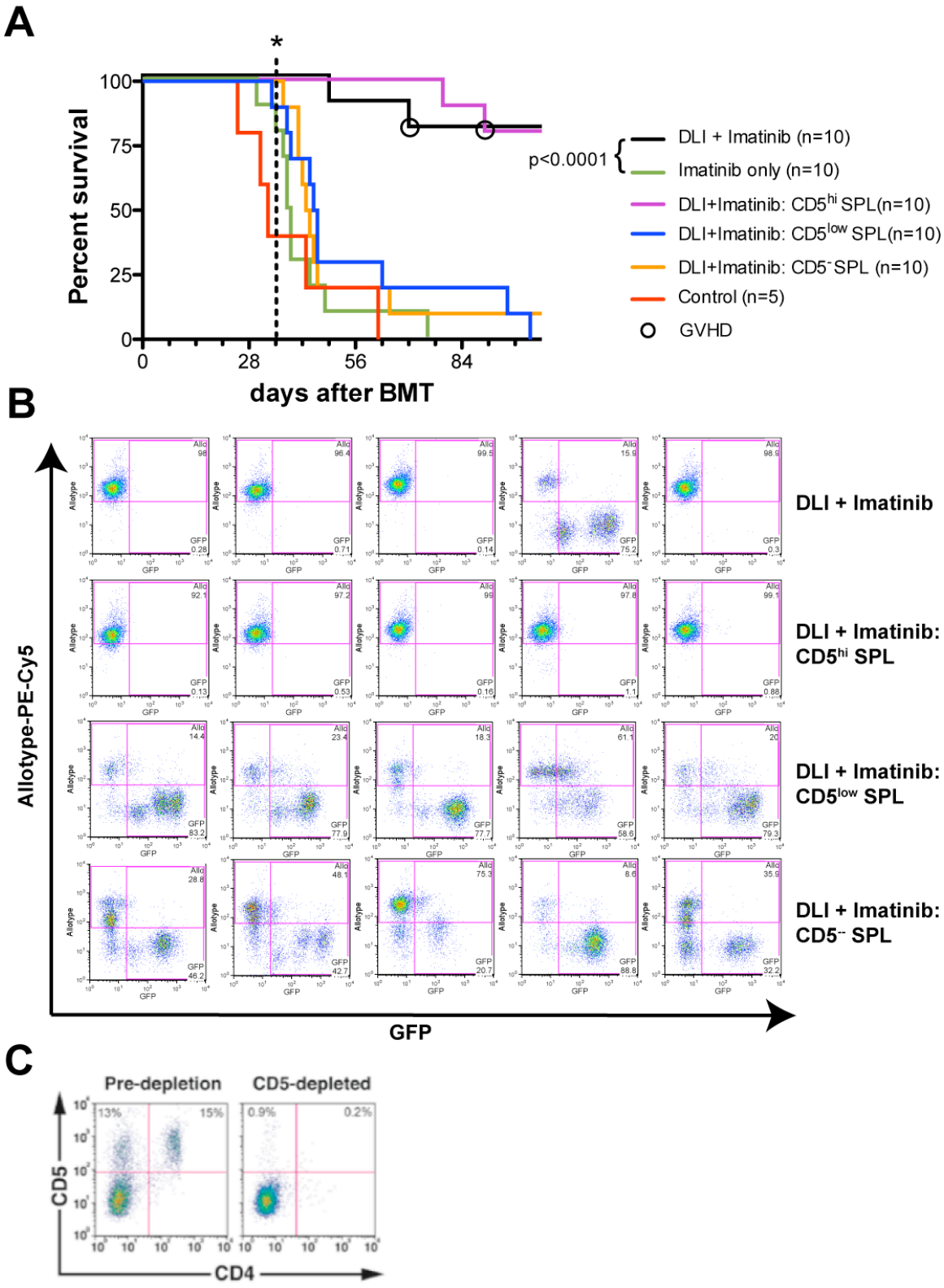

A
Percent survival
days after BMT
p<0.0001
DLI + Imatinib (n=10)
Imatinib only (n=10)
DLI+Imatinib: CD5hi SPL(n=10)
DLI+Imatinib: CD5low SPL(n=10)
DLI+Imatinib: CD5- SPL (n=10)
Control (n=5)
GVHD
B
Allotype-PE-Cy5
GFP
DLI + Imatinib
DLI + Imatinib: CD5hi SPL
DLI + Imatinib: CD5low SPL
DLI + Imatinib: CD5-- SPL
C
Pre-depletion
CD5-depleted
CD5
CD4

Figure 8. $CD5^-$ splenocytes do not contribute to GvL against CML-like leukemia. (A) Survival curve for cohorts of Balb/c recipients of *BCR-ABL1*-transduced Balb/c marrow ($1x10^4$ cells) mixed with 10^7 TCD MHC-matched allogeneic stem cells from B10.D2 donors. All recipients developed mixed hematopoietic chimerism with CML-like leukemia (GFP^+ myeloid cells) at d14 after transplant. Beginning at d14, mice were treated with imatinib (100 mg/kg) + unfractionated DLI (3×10^7 total splenocytes per treatment, total of 4 infusions per recipient), imatinib + $CD5^-$ SPL (2.1×10^7 CD5-depleted splenocytes per treatment), imatinib + $CD5^{hi}$ SPL (2.1×10^7 CD5-depleted splenocytes plus 0.9×10^7 $CD5^+$ T cells per treatment), imatinib + $CD5^{low}$ SPL (2.1×10^7 CD5-depleted splenocytes plus 2.1×10^6 $CD5^+$ T cells per treatment), or imatinib + syngeneic Balb/c splenocytes (3×10^7 unfractionated SPL per treatment, the imatinib only curve). The numbers of $CD5^+$ cells were adjusted such that $CD5^{hi}$ SPL contained an equal number of T cells to the unfractionated DLI arm, and $CD5^{low}$ SPL contained the extra 1% of $CD5^+$ cells as compared to the CD5-depleted SPL arm. Both imatinib and DLI treatments were discontinued at d35, as indicated by the dotted line. The addition of delayed DLI to imatinib resulted in superior survival compared to imatinib alone ($P<0.0001$, Mantel-Cox test), while the survival differences between cohorts receiving $CD5^-$ SPL, $CD5^{low}$ SPL, or syngeneic Balb/c SPL only (Control) were not statistically significant. Note that the addition of $CD5^{hi}$ SPL to imatinib also induced highly significant GvL. (B) Flow cytometric analysis of peripheral blood leukocytes from five representative mice each from the cohorts in (A), analyzed at d43 post-transplantation (following a complete course of combined treatment). Allogeneic chimerism (*y*-axis) was detected by a polymorphism in β2-microglobulin, and GFP^+ cells (*x*-axis) represent BCR-

ABL1-expressing leukemic cells. The percentage of allotype$^+$ and GFP$^+$ cells in each plot is indicated. Note the eradication of leukemia in the majority of recipients treated with DLI + imatinib and CD5^{hi} SPL + imatinib, contrasted with persistent leukemia in other groups. (C) Flow cytometric analysis of splenocyte populations following selective depletion of CD5$^+$ cells by biotinylated anti-CD5 mAb. Percentages of CD4$^+$ (anti-CD4-FITC, x-axis) and CD5$^+$ (streptavidin-PE-Cy5, y-axis) cells before and after depletion are indicated.

NK cell-enriched DLI from miHA-mismatched B10.D2 donors attenuates CML-like leukemia but does not induce durable GvL against CML-like leukemia

Natural killer (NK) cells have long been recognized as an important part of the host innate immune response against cancer. Each NK cell possesses a panel of activating receptors recognizing ligands expressed under transformational stress, and another panel of inhibitory receptors recognizing autologous class I MHC molecules. In general, the balance between activating and inhibitory signals controls the activation status of an NK cell (134). Several lines of evidence have suggested that NK cells contribute to the GvL response in CML. It was shown that an infusion of killer cell immunoglobulin-like receptor (KIR)-mismatched NK cells eradicated advanced human leukemia in NOD/SCID mice (135). Additionally, early NK recovery correlated with less relapse and GvHD in myeloid leukemia patients (136) and that expression of stimulatory KIR or its ligand was associated with more frequent leukemia-free survival (137, 138). These results suggest that altering the balance between activating and inhibitory KIR signals could induce clinical effects in myeloid leukemias, hence we decided to investigate

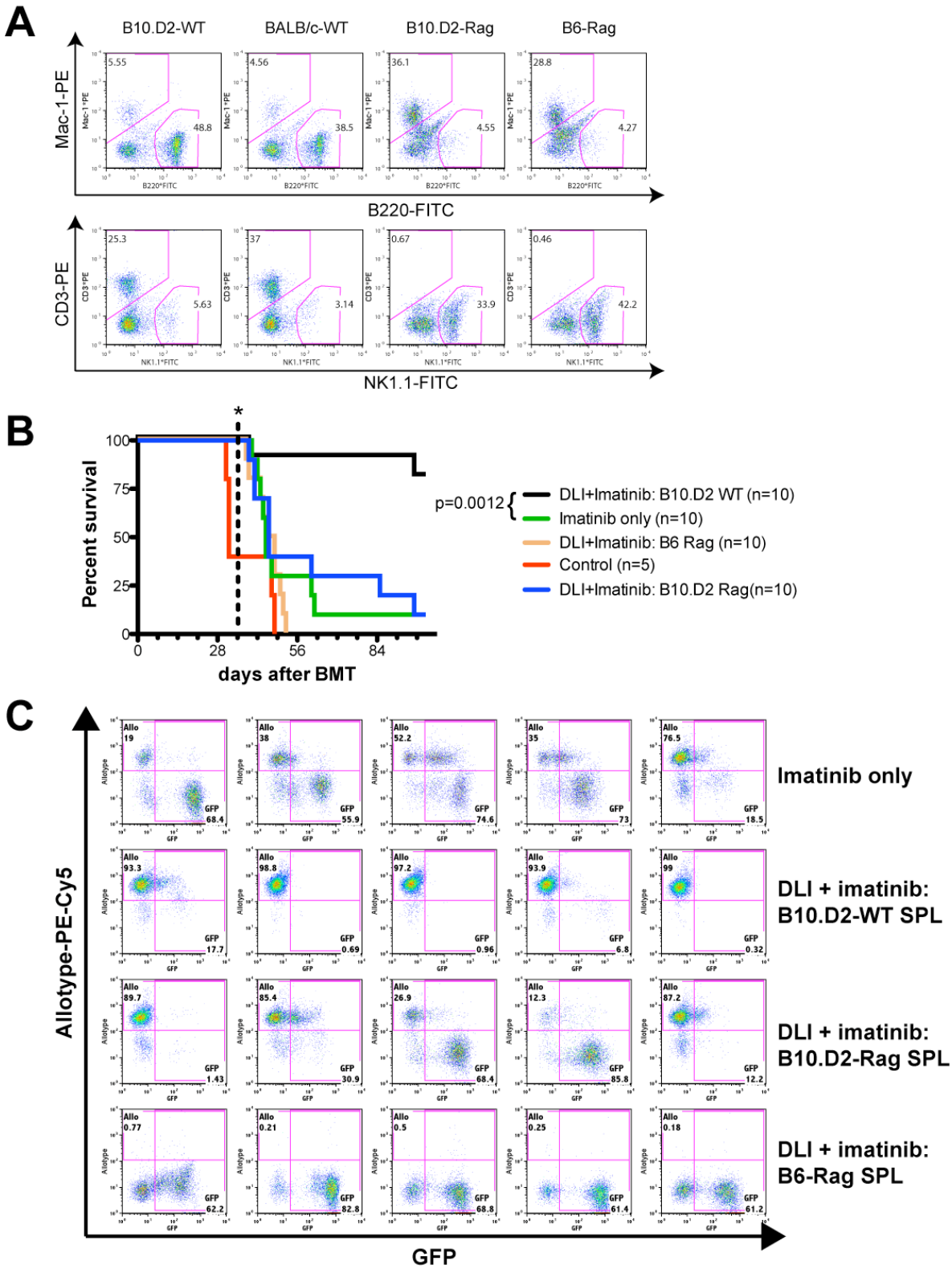
whether miHA-mismatched NK cells might play a role in GvL against CML-like leukemia in the transplant model.

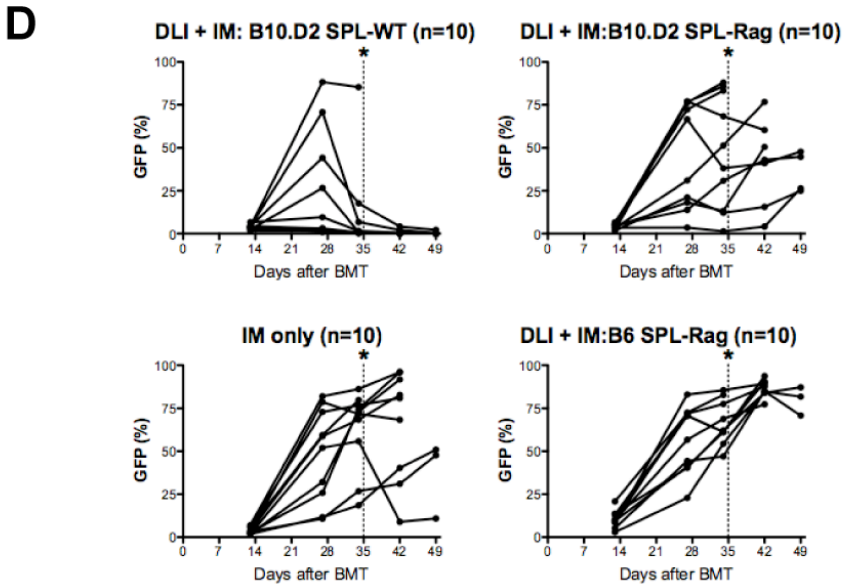
In order to obtain a population of NK cells that had no contamination by T lymphocytes, we used mice with homozygous null mutations in the *Recombinase activating gene-2* (*Rag2*) as the source of DLI. As expected, the spleens of *Rag2*-deficient mice exhibited no $CD3^+$ T cells and were highly enriched in $NK1.1^+$ NK cell and Mac-1^+ myeloid cells; the minor population of $B220^+$ cells are not functionally mature (Figure 9A). To determine whether NK cells mediate GvL, leukemic mixed chimeras, generated by transplant conditions defined in Figure 6, were treated with combined therapy with imatinib + B10.D2-wild-type splenocytes (B10.D2-WT SPL), imatinib + B10.D2-Rag SPL, imatinib + syngeneic Balb/c SPL (imatinib only), or syngeneic Balb/c SPL alone (Control). In addition, to define the potential GvL effect of MHC-mismatched NK cells, a parallel cohort receiving the same dose of leukemic BM and MHC-mismatched B6-Rag BM graft at time of transplant was treated with B6-Rag splenocyte DLI.

Despite the large number of NK cells present in the DLI population (~ 4 x 10^6 NK cells per treatment for a total of 4 infusions), there was no significant prolongation in survival of any of the DLI-treated cohorts, except for the cohort treated with imatinib + B10.D2-WT SPL (P=0.012, Mantel-Cox test), where 90% of recipients achieved long-term, leukemia-free survival (Figure 9B). By contrast, most of the mice receiving Rag-deficient splenocytes died of leukemia soon after cessation of treatment at d35 post-transplant (Figure 9B). Interestingly, flow cytometric analysis showed that a few mice of the imatinib + B10.D2-Rag SPL cohort had superior levels of allogeneic chimerism and

reduced percentages of circulating GFP^+ cells (Figure 9C and D); however, the effect appeared to be transient and was rapidly supplanted by leukemic expansion after treatment was discontinued. All three long-term survivors treated with imatinib + B10.D2-Rag SPL eventually relapsed with time. Among these recipients, one succumbed to a combination of CML and histiocytic sarcoma by d85 post-transplant, and the other two had persistent myeloid leukemia detectable by flow cytometry (data not shown). The weak tumor suppressing response mediated by MHC-matched, miHA-mismatched B10.D2 Rag DLI suggests the presence of activating NK ligands on leukemic cells; however, this might be balanced by inhibitory KIR receptor signals mediated by MHC-I ligands expressed on the leukemic cells. By contrast, GvL mediated by T cell in the imatinib + B10.D2-WT SPL cohort was more robust and durable.

Our data reflects the distinct nature of the two lymphoid cell types. Different from T cells, NK cells are generally considered not capable of executing clonal expansion and forming memory responses. Although NK cell immunotherapy reduced the leukemic burden and prolonged survival in some recipients, sustaining such an effect might require constant boosting of DLI cells, as indicated by the observation that one week after the previous cell infusion, the percentage of circulating NK^+ cell was only slightly higher than majority of the imatinib only cohort (Figure 9E). Disappointingly, we did not observe any significant GvL effect in the cohort treated with imatinib + MHC-mismatched B6-Rag SPL. However, flow cytometric analysis of peripheral chimerism showed that these recipients had very low levels of allogeneic chimerism at d13 post-transplant (data not shown), so that it is possible that the B6 Rag-deficient splenocytes were rejected in these recipients.


A
B10.D2-WT
BALB/c-WT
B10.D2-Rag
B6-Rag
Mac-1-PE
B220-FITC
5.55
48.8
4.56
38.5
36.1
4.55
28.8
4.27
CD3-PE
NK1.1-FITC
25.3
5.63
37
3.14
0.67
33.9
0.46
42.2
B
Percent survival
days after BMT
p=0.0012
DLI+Imatinib: B10.D2 WT (n=10)
Imatinib only (n=10)
DLI+Imatinib: B6 Rag (n=10)
Control (n=5)
DLI+Imatinib: B10.D2 Rag(n=10)
C
Allotype-PE-Cy5
GFP
Imatinib only
DLI + imatinib: B10.D2-WT SPL
DLI + imatinib: B10.D2-Rag SPL
DLI + imatinib: B6-Rag SPL


D
DLI + IM: B10.D2 SPL-WT (n=10)
DLI + IM:B10.D2 SPL-Rag (n=10)
IM only (n=10)
DLI + IM:B6 SPL-Rag (n=10)
GFP (%)
Days after BMT

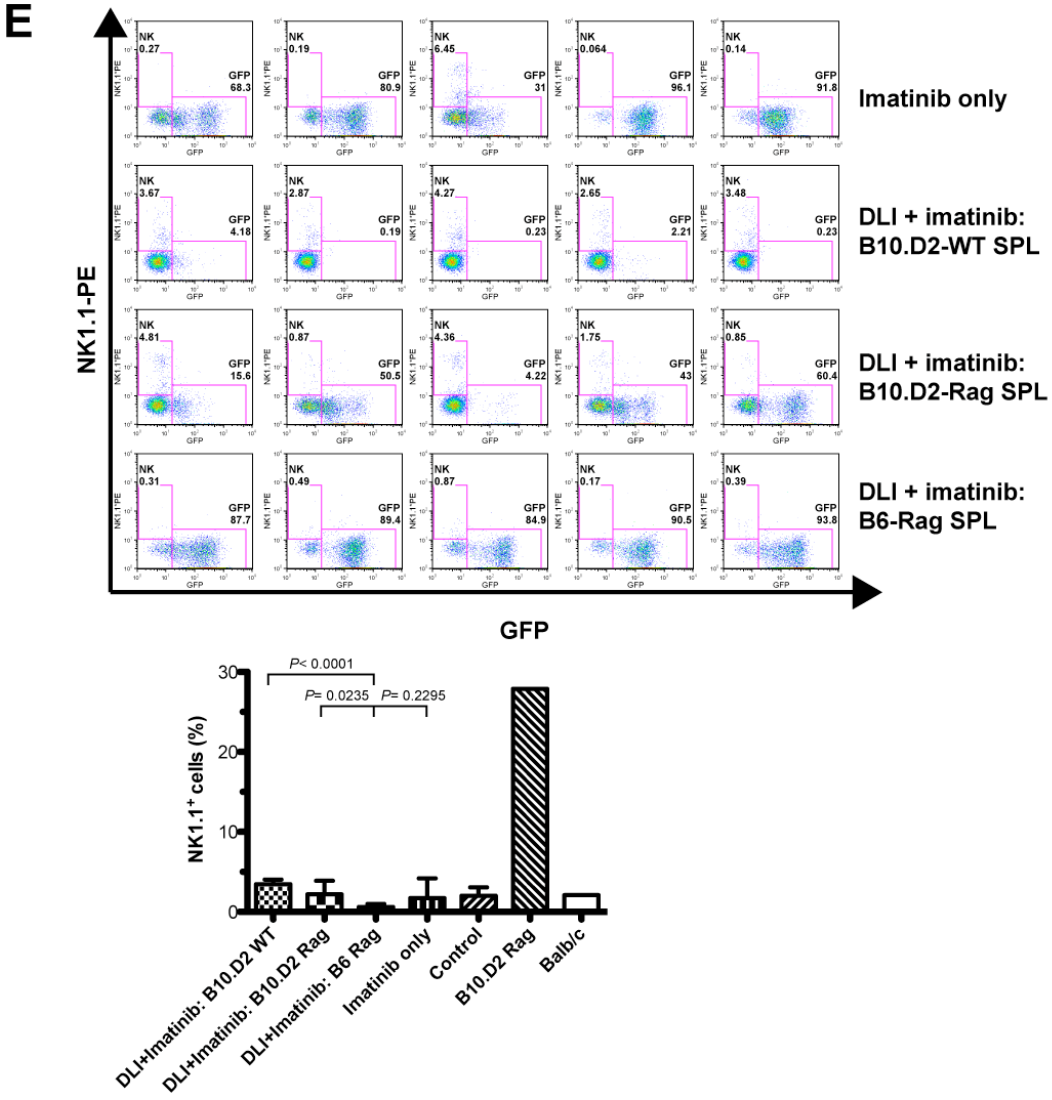

E
NK1.1-PE
GFP
Imatinib only
DLI + imatinib: B10.D2-WT SPL
DLI + imatinib: B10.D2-Rag SPL
DLI + imatinib: B6-Rag SPL
P< 0.0001
P= 0.0235
P= 0.2295
NK1.1+ cells (%)
DLI+Imatinib: B10.D2 WT
DLI+Imatinib: B10.D2 Rag
DLI+Imatinib: B6 Rag
Imatinib only
Control
B10.D2 Rag
Balb/c

Figure 9. DLI with miHA-mismatched NK cell-enriched splenocytes from Rag2-deficient donors attenuates CML-like leukemia but does not significantly prolong survival. (A) Flow cytometric analysis showing cellular composition of splenocytes of wild-type B10.D2, wild-type Balb/c, Rag2-deficient B10.D2 and Rag2-deficient B6 mice. Percentages of B cells ($B220^+$), myeloid cells ($Mac\text{-}1^+$), T cells ($CD3^+$) and NK cells ($NK1.1^+$) are as indicated. Note that Rag2-deficient splenocytes consist of NK cells and myeloid populations, but contains no T cells. (B) Kaplan-Meier survival curve of chimeric leukemic Balb/c recipients of *BCR-ABL1*-transduced Balb/c marrow mixed with TCD-MHC-matched allogeneic stem cells from B10.D2 donors. All mice were treated by the same protocol of combined therapy as described in Figure 8. Beginning at d14, mice were treated with imatinib and weekly infusions of allogeneic splenocytes from wild-type B10.D2 allogeneic donors (B10.D2-WT, 3×10^7 cells per treatment for a total of 4 infusions), *Rag2*-deficient B10.D2 donors (B10.D2-Rag, $8\text{-}10 \times 10^6$ cells per treatment), or the *Rag2*-deficient MHC-mismatched B6 donors (B6-Rag, $8\text{-}10 \times 10^6$ per treatment). Both treatments were discontinued at d35, as indicated by the dotted line. Note that, except for the recipients treated with imatinib + B10.D2-WT DLI (P=0.012, Mantel-Cox test), there was no significant difference among other cohorts. (C) Flow cytometric analysis of peripheral blood of five representative leukemic chimeras, analyzed at d34 post-transplant (following 3 doses of DLI treatment). The percentage of allogeneic chimerism (y-axis) and GFP^+ leukemic cells (x-axis) in each plot is indicated. Note the relative reduction of GFP^+ cells and high degree of allogeneic chimerism in some of the B10.D2 Rag SPL cohort. (D) Percentage of GFP^+ peripheral blood cells of individual recipient of each cohort over a 50-day follow-up period. Note the relatively

low percentage of leukemic cells in some of the cohorts receiving B10.D2-Rag splenocytes, which subsequently increased after treatment withdrawal, as shown by the dotted line in each plot. (E) Flow cytometric analysis of peripheral blood of five representative leukemic chimeras, analyzed at d42 post-transplant (one week after treatment withdrawal). Percentage of GFP^+ cells (x-axis) and residual allogeneic NK cells (NK1.1-PE, y-axis) of each plot is indicated. Of note, NK1.1 is an activating receptor expressed on NK and NKT cells in mice of B6 origin, including the B10.D2 strain used in the present study; the Balb/c strain does not express this antigen. Bottom panel shows the quantitative difference of circulating $NK1.1^+$ cells of the cohorts presented above. Note that cohorts treated with B10.D2 wild-type or Rag2-deficient splenocytes had significantly higher level of NK cells than the cohort treated with B6 Rag2-deficient splenocytes; the difference between the B6 Rag2-deficient treated group and imatinib only group was not significant.

Adoptive immunotherapy for CML can also be modeled in a second MHC-matched miHA-mismatched transplant setting, C3.SW (H-2^b) →B6 (H-2^b)

In order to facilitate the study of GvL effector mechanisms, we tested whether our GvL model could be reproduced in another MHC-matched miHA-mismatched transplant pair, C3.SW→B6, because many mouse strains with mutations in genes affecting immune cell functions are available only in the B6 (H-2^b) background. The C3.SW strain was created by breeding the H-2^b haplotype of an outbred Swiss stock onto the C3H background. To distinguish allogeneic cells in the H-2^b transplant setting, we used congenic B6 recipients that expresses the CD45.1 allelic variant of the protein tyrosine phosphatase type C receptor, as opposed to the common CD45.2 variant expressed in C3.SW allogeneic donors.

Despite different doses of conditioning radiation, we found that the transplant conditions defined for the B10.D2→Balb/c strain paring (Figure 4) of 1 x 10^4 lineage-depleted *BCR-ABL1*-transduced 5FU-B6 marrow and 1 x 10^7 T cell-depleted C3.SW marrow generated excellent allogeneic chimerism (~80%) in B6 recipients at d13 post-transplant, assessed by flow cytometry using a mAb against CD45.2 (Figure 10A). Our result demonstrated that, when recipients were conditioned with 900 cGy radiation, combined immunotherapy with DLI + imatinib mediated significant GvL effects in the C3.SW→B6 transplant pairing, with conversion to full allogeneic chimerism and no evidence of leukemic cells in 60% (3 of 5) of the recipients (Figure 10B). Mice receiving a higher conditioning radiation dose (1000 cGy), although showing evident GvL, had higher incidence of fatal GvHD (Figure 10C), whereas the cohort receiving the lower radiation dose did not show significant GvHD (data not shown).

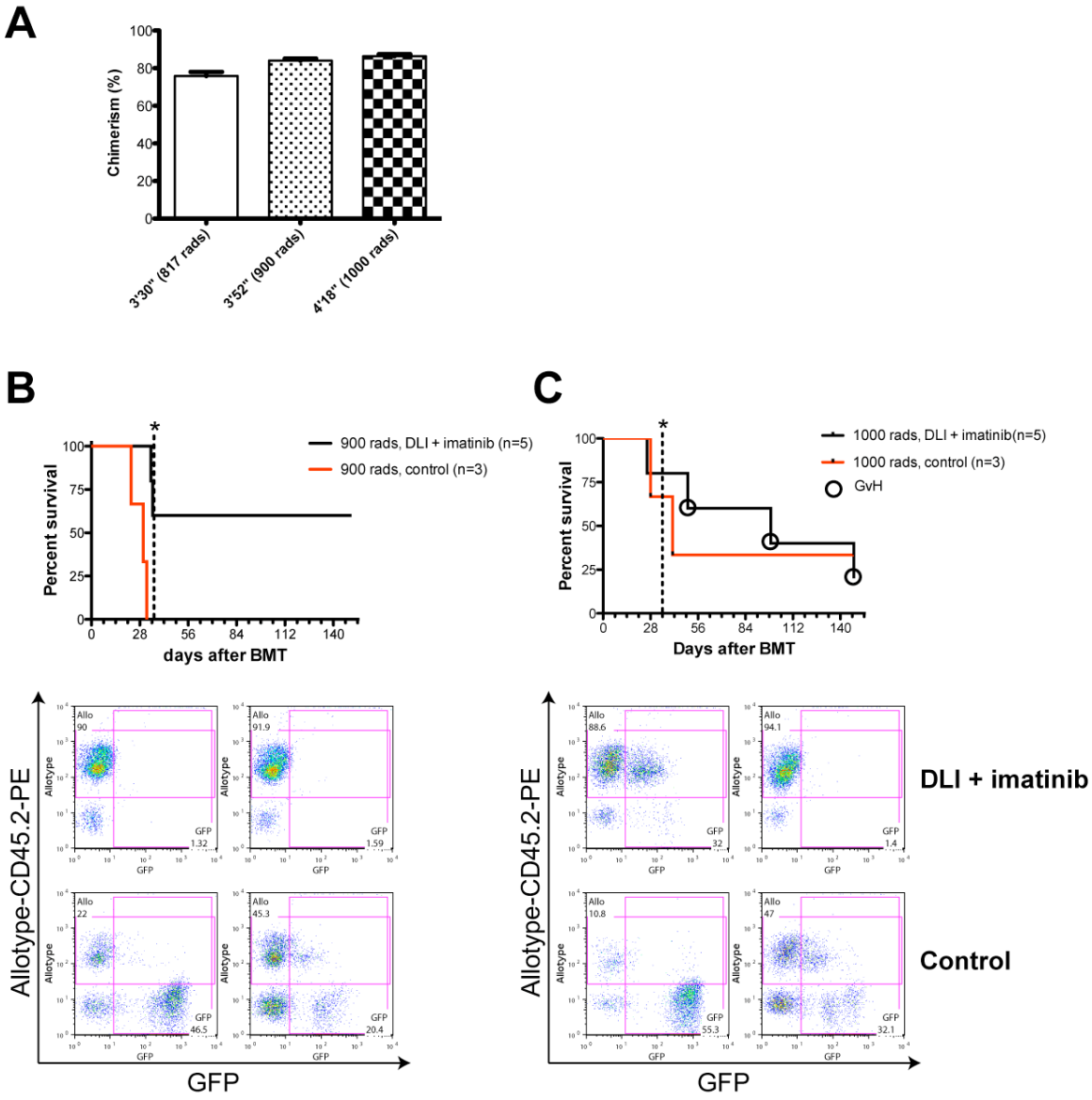

A
Chimerism (%)
100
80
60
40
20
0
3'30'' (817 rads)
3'52'' (900 rads)
4'18'' (1000 rads)
B
Percent survival
100
75
50
25
0
0
28
56
84
112
140
days after BMT
900 rads, DLI + imatinib (n=5)
900 rads, control (n=3)
*
C
Percent survival
Days after BMT
1000 rads, DLI + imatinib(n=5)
1000 rads, control (n=3)
GvH
Allotype-CD45.2-PE
GFP
Allo
90
GFP
1.32
Allo
91.9
GFP
1.59
Allo
22
GFP
46.5
Allo
45.3
GFP
20.4
Allo
88.6
GFP
32
Allo
94.1
GFP
1.4
Allo
10.8
GFP
55.3
Allo
47
GFP
32.1
DLI + imatinib
Control

Figure 10. Combined DLI + imatinib immunotherapy can also be modeled in MHC-matched miHA-mismatched C3.SW (H-2^{b})→B6 (H-2^{b}) transplant pairs.

(A) Comparison of allogeneic chimerism among cohorts receiving different doses of conditioning radiation, assessed at d13 post-transplant. Note the percentage of allogeneic chimerism (*x*-axis) was slightly lower in the cohort receiving 817 cGy. (B and C) Kaplan-Meier survival curve (top panels) and flow cytometric analyses (bottom panels) of percentage of GFP^{+} (*x*-axis) and allotype^{+} (*y*-axis) blood cells of the C3.SW→B6 transplant cohorts, assessed at d27 post-transplant. (B) Cohort receiving a lower dose of conditioning radiation (900 cGy). Each recipient was transplanted with 1 x 10^{4} lineage-depleted *BCR-ABL1*-transduced 5FU-BM of B6 (CD45.1^{+}) and 1 x 10^{7} T cell-depleted allogeneic C3.SW (CD45.2^{+}) BM cells. Allogeneic chimerism was assessed by staining with a mAb against CD45.2. Starting from d14 post-transplant, recipients were either treated with the combined DLI + imatinib treatment, using the same protocol as previously described in Figure 6, or was left untreated (control). The combined treatment was discontinued at d35 post-transplant, as indicated by the dotted line. (C) Cohort receiving a higher dose of conditioning radiation (1000 cGy). This transplant condition was less favorable, as it was associated with a high frequency of fatal GvHD (circles).

Allogeneic splenocytes mediate GvL against MHC-I-deficient leukemic (stem) cells: possible role for miHA-mismatched NK cells?

Our previous results (Figure 7) demonstrate that the majority of the GvL effect of delayed DLI is mediated by $CD8^+$ cells, with little or no contribution by $CD4^+$ lymphocytes. For cytolytic function, $CD8^+$ lymphocytes require cognate interactions with MHC class I receptors on their target cells. By contrast, $CD4^+$ lymphocytes can mediate anti-tumor effects either directly, through cytolysis mediated through MHC class II receptors on target cells (139), or indirectly, through interactions with APCs presenting miHAs. To define the role of MHC class I and class II interactions in the GvL response to CML-like leukemia, we took the approach of generating leukemia in HSC from B6 mutant donors that lacked either MHC-I (β2 microglobulin-deficient) or MHC-II (I-A β chain-deficient) expression. Importantly, previous work from the laboratory of Warren Shlomchik has shown that *BCR-ABL1* can efficiently induce fatal CML in BM lacking MHC-I (140) and MHC-II (1).

We transplanted *BCR-ABL1*-transduced MHC-I-deficient, MHC-II-deficient, or wild-type B6 BM under the C3.SW→B6 transplant conditions defined in Figure 10 and treated the cohorts with DLI (unfractionated splenocytes, wild-type donors) + imatinib. As expected, for the cohorts transplanted with wild-type *BCR-ABL1*-transduced BM, combined treatment with DLI (from the allogeneic C3.SW donor) and imatinib produced significant GvL, with prolonged survival and clearance of leukemia (Figure 11A and B, DLI + IM: WT p210-BM), while the majority of recipients treated with syngeneic B6 splenocyte DLI + imatinib (Figure 11A and B, IM only) succumbed to CML-like leukemia after treatment was discontinued at d35 post-transplant. To our surprise, the

cohorts transplanted with MHC-I-deficient leukemic BM and treated with combined therapy showed significant prolongation of survival, and completely cleared the leukemia in peripheral blood as assessed by flow cytometry (Figure 11A and B, DLI + IM: I-KO p210-BM). As predicted, given the minimal role for $CD4^+$ lymphocytes in the GvL response to CML, combined DLI + imatinib therapy cleared the leukemia in the majority of recipients transplanted with MHC-II deficient *BCR-ABL1*-transduced BM (DLI + IM: II-KO p210-BM, Figure 11A and B), although several recipients (3/10) developed fatal GvHD, which made the overall survival of this cohort not significantly different from that of the IM only cohort. However, the combined therapy was equally efficient, in terms of leukemia survival, between the II-KO p210-BM and WT p210-BM cohorts.

Our data has previously shown that miHA-mismatched $CD8^+$ T cells, the activation of which depends on its T cell receptor recognizing target peptide presented by MHC-I molecules, are the main splenocyte effectors for GvL (Figure 7). The strong GvL response observed against MHC-I-deficient leukemia targets suggests that, in addition to $CD8^+$ T cell, miHA-mismatched NK cell might efficiently eradicate leukemic cells, including the leukemic stem cells. Mixed leukemic chimeras treated with combined therapy of CD4-depleted DLI showed superior survival, as compared to the cohort treated with purified $CD8^+$ DLI (Figure 7D). The difference between the two treatment groups indicates that NK cell might also contribute to GvL. Additionally, in the mixed leukemic chimeras treated with allogeneic splenocytes from the *Rag2*-deficient donors, increased percentage of circulating $NK1.1^+$ cells in the recipients has been found to correlate with reduced leukemic burden before the combined treatment was discontinued (Figure 9E and data not shown). Along with the partial response that over 40% of the recipients

treated with *Rag2*-deficient DLI displayed prolonged survival over 8 weeks, these observations indicate that NK cell might share a role in the anti-leukemia response mediated by miHA-mismatched DLI.

It has long been appreciated that NK cells express inhibitory receptors that normally keep the cell in a resting status through interacting with autologous class I MHC-I molecules (141). NK cell become activated when the target cells fail to express sufficient amount of class I MHC; i.e. "the missing self" hypothesis (142). Therefore, NK cell could mediate graft rejection when the target cells are deficient in MHC-I expression (143). The major discrepancy between the results of the cohorts transplanted with wildtype leukemia and treated with *Rag2*-deficient DLI (Figure 9), and the cohorts transplanted with MHC-I KO leukemia and treated with wildtype DLI (Figure 11) suggests that the inhibitory signals triggered by MHC-I molecules may offset the activating signal transduced by putative activating NK ligands expressed by leukemic (stem) cells. We are currently attempting to exploit the potential of using leukemia-specific NK clones in our transplant model by expressing a single-chain Fv-anchored chimeric antigen receptor (CAR) directed against an antigen on CML stem cells (144) in NK cells, which should bypass the MHC-I mediated inhibitory barrier and directly activate NK cells when engaging the target.

An alternative interpretation for the robust GvL observed in the cohorts transplanted with MHC-I KO leukemia is that the anti-leukemia effect might be the consequence of engraftment blocking mediated by the host endogenous NK cells present at time of transplantation. It has been shown that, through strategy of *in vivo* NK depletion in the host, CML can be induced by transplanting the leukemic MHC-I KO BM

to lethally irradiated syngeneic recipients (145). To determine whether host residual NK cells eliminate MHC-I KO BM and hence block the leukemic engraftment of LSCs, the experiment needs to be repeated with pre-transplant administration of NK-depleting antibody in the cohorts receiving MHC-I KO leukemia. To consolidate our previous finding that GvL of delayed DLI was predominantly mediated by miHA-mismatched $CD8^+$ T cell, the cohort transplanted with MHC-I KO leukemia should be treated with purified $CD8^+$ T cell DLI from the miHA-mismatched donors. Loss of GvL is expected in this treatment group, whereas GvL in the control cohort transplanted with wildtype leukemia and treated with $CD8^+$ T cell DLI should remain intact.

Another plausible effector mediating GvL in our transplant model is the NKT cell, which is a heterogeneous class of CD1d-restricted T cells sharing NK properties (146). The involvement of NKT cell can be further addressed by using the CD1d-deficient leukemic targets in an independent delayed DLI experiment.

As previously shown in Figure 7E, GvHD in the CML immunotherapy model was primarily induced by miHA-mismatched $CD4^+$ T cells. The data in Figure 11 suggest that the GvHD observed in the II-KO p210-BM cohort was likely caused by host-attacking $CD4^+$ T cells in the splenocyte population, as the non-hematopoietic tissues such as keratinocytes and bowel epithelial cells in these mixed leukemic chimeras still express class II MHC molecules.

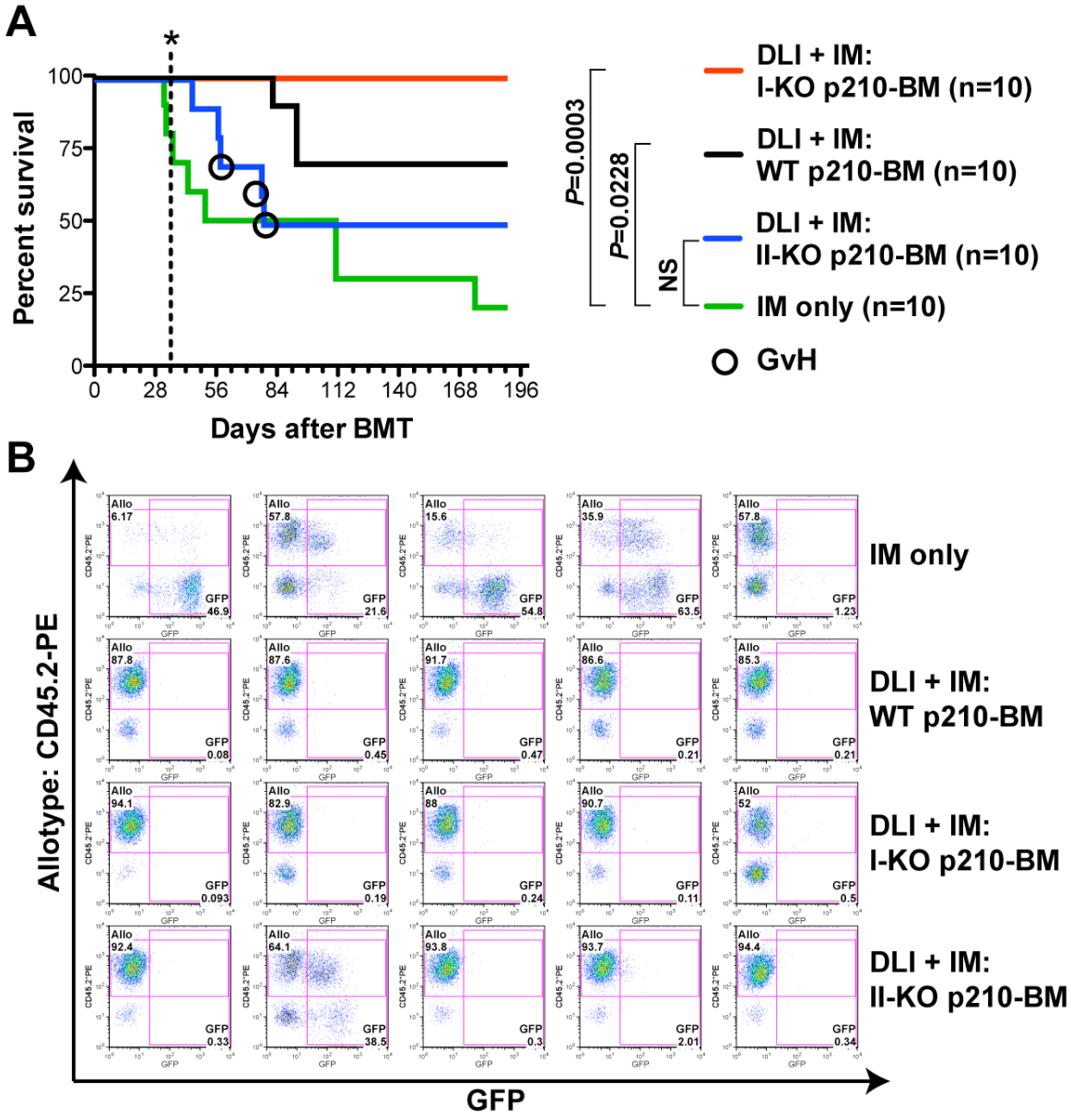

A
Percent survival
100
75
50
25
0
0
28
56
84
112
140
168
196
Days after BMT
*
P=0.0003
P=0.0228
NS
DLI + IM:
I-KO p210-BM (n=10)
DLI + IM:
WT p210-BM (n=10)
DLI + IM:
II-KO p210-BM (n=10)
IM only (n=10)
GvH
B
Allotype: CD45.2-PE
GFP
IM only
DLI + IM:
WT p210-BM
DLI + IM:
I-KO p210-BM
DLI + IM:
II-KO p210-BM

Figure 11. Combined DLI + imatinib treatment showed significant GvL in the transplant cohorts receiving MHC-I deficient *BCR-ABL1*-transduced BM.

(A) Kaplan-Meier survival curve of the cohorts receiving *BCR-ABL1*-transduced marrows of MHC-I-deficient (I-KO p210-BM), MHC-II-deficient (II-KO p210-BM), or wild-type (WT p210-BM) syngeneic B6 donors. Combined therapy of whole splenocyte-DLI of C3.SW allogeneic donors and imatinib (IM) was administered during the course of d14-35 post-transplant. Time of treatment cessation was shown by the dotted line, and individual mice that developed lethal GvHD was marked by open circle. Note that 100% of recipients transplanted with MHC-I-deficient p210-BM exhibited long-term leukemia-free survival, suggesting that potent GvL might be mediated by NK cells. (B) Flow cytometric analysis of percentage of GFP^+ (*x*-axis) and $allotype^+$ (CD45.2-PE, *y*-axis) peripheral blood cells of five representative leukemic chimeras, assessed at d37 post-transplant. Note the eradication of leukemic cells and nearly full allogeneic chimerism in recipients transplanted with MHC-I-deficient p210-BM.

miHA-mismatched DLI-T cell recognized leukemic (stem) cells through mechanism of allogeneic targeting

Our results have demonstrated that allogeneic splenocytes, principally $CD8^+$ lymphocytes with a potential contribution from NK cells, mediate GvL against established *BCR-ABL1*-induced CML-like leukemia in our MHC-matched, miHA-mismatched mouse model. However, we do not know whether the relevant antigens involved in this GvL response are tumor-specific, or instead are allo-specific miHAs. It is clear that neither BCR-ABL1 nor GFP are sufficient to invoke a GvL response, as DLI with syngeneic Balb/c splenocytes did not result in any protection against CML-like leukemia (Figure 6). To investigate mechanistically whether the GvL effect of allogeneic DLI was mediated by an immune response directed specifically against BCR-ABL1-expressing target cells or against miHA shared by normal HSC, we serially transplanted BM from B10.D2→Balb/c DLI-treated cohorts to immunocompetent syngeneic Balb/c female recipients (Figure 12A), whose immune system would reject all allogeneic cells and allow repopulation only by syngeneic HSC. Because untransduced Balb/c HSC are co-transplanted with *BCR-ABL1*-transduced HSC to generate the primary chimeric recipients, we reasoned that these normal (male) Balb/c HSC might persist in DLI-treated chimeras if the GvL effect was specific to leukemia stem cells. Hence, we assessed these secondary recipients for the development of CML-like leukemia and the presence of donor-derived male hematopoiesis, using a PCR assay for the *Sry* gene in BM genomic DNA (Figure 12C).

While CML-like leukemia could be efficiently transplanted to secondary recipients from primary chimeras treated with syngeneic DLI alone (Figure 12B,

Control), imatinib treatment alone decreased the efficiency of secondary transplantation of CML. However, all secondary recipients receiving leukemia-free marrows of primary chimeras treated with either DLI + imatinib or DLI alone maintained their disease-free status (Figure 12B). To detect male cells within these secondary transplant cohorts, we employed a PCR assay amplifying the male-specific *Sry* sequence that could detect a minimum of 3% male DNA within a background of female DNA (Figure 12C). The results showed that, except for a mouse receiving marrows from a leukemia^{+} primary recipient (lanes #41-42), *Sry* was not detectable in any secondary cohorts receiving marrow from primary recipients treated with DLI + imatinib or DLI only (Figure 12D). By contrast, *Sry* was readily detected in primary recipients of BM from male syngeneic donors (M-Balb/c→F-Balb/c, lanes #83-86), or from secondary recipients of leukemia^{+} BM that developed disease (lanes #4-5 and #12-14). The absence of detectable *Sry* sequences in Balb/c recipients of male BM from allogeneic donors (M-B10.D2→F-Balb/c, lanes #79-82) demonstrated that, while allowing long-term repopulation by syngeneic male HSC, these female Balb/c recipients were immunocompetent to reject allogeneic cells. *Sry* was also detectable in splenic DNA of all primary recipients, as these mice were either leukemia-bearing or full allogeneic chimeras with male B10.D2 hematopoiesis. Overall, these results demonstrate that normal syngeneic (Balb/c-derived) HSC are not present in DLI-treated primary chimeras, suggesting that the anti-leukemia effect of DLI is directed against the entire pool of miHA-mismatched HSC, regardless of BCR-ABL1 transformation status.

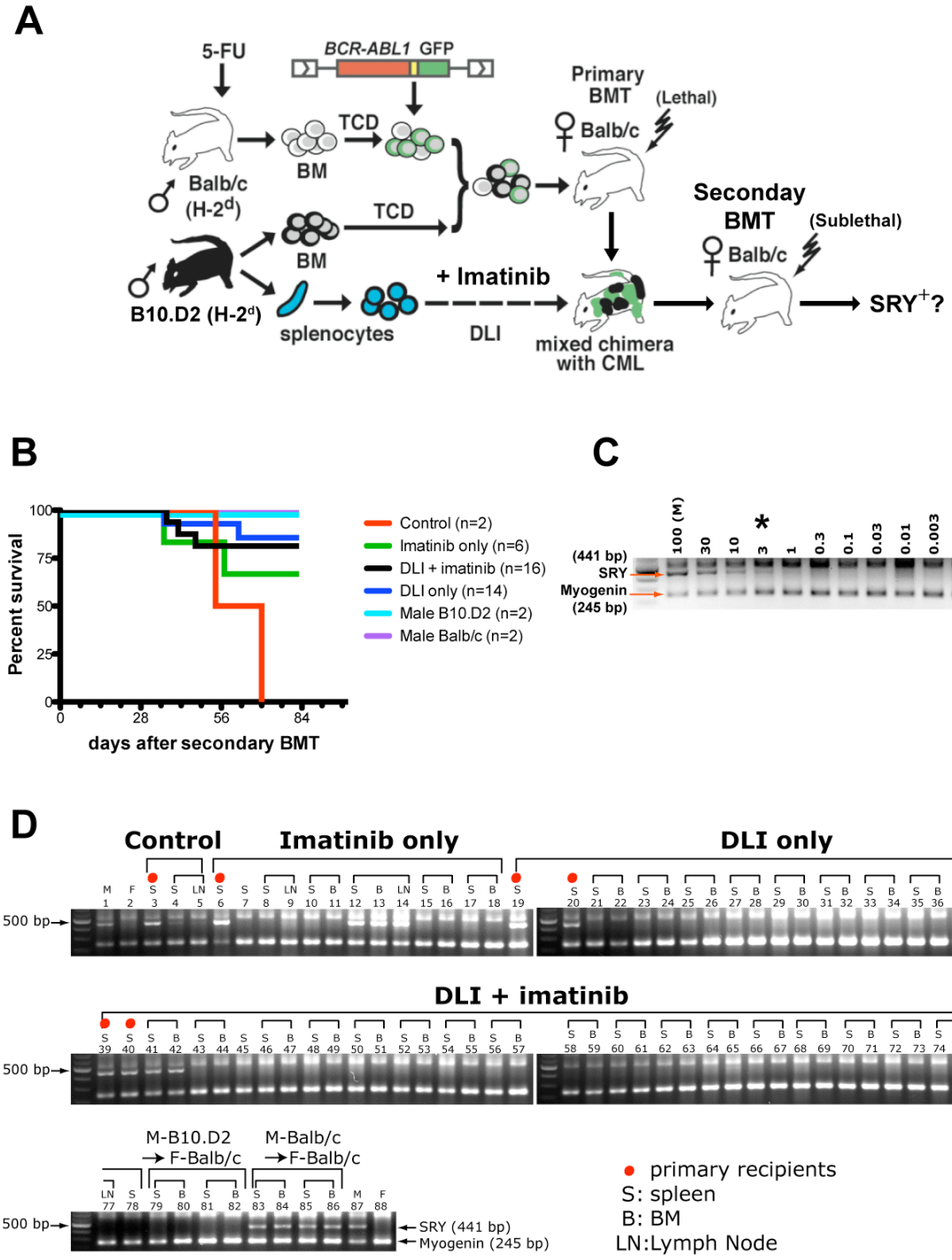

A
5-FU
BCR-ABL1 GFP
Primary BMT
(Lethal)
Balb/c
TCD
BM
Balb/c (H-2d)
TCD
BM
Seconday BMT
(Sublethal)
Balb/c
+ Imatinib
B10.D2 (H-2d)
splenocytes
DLI
mixed chimera with CML
SRY+?
B
Percent survival
100
75
50
25
0
0
28
56
84
days after secondary BMT
Control (n=2)
Imatinib only (n=6)
DLI + imatinib (n=16)
DLI only (n=14)
Male B10.D2 (n=2)
Male Balb/c (n=2)
C
100 (M)
30
10
3
1
0.3
0.1
0.03
0.01
0.003
0.001
0 (F)
(441 bp) SRY
Myogenin (245 bp)
D
Control
Imatinib only
DLI only
DLI + imatinib
500 bp
M-B10.D2 → F-Balb/c
M-Balb/c → F-Balb/c
SRY (441 bp)
Myogenin (245 bp)
primary recipients
S: spleen
B: BM
LN:Lymph Node

Figure 12. GvL was mediated by DLI recognition against allogeneic miHA

(A) Schematic diagram of the secondary transplantation experiment. BM of the primary chimeras treated with combined B10.D2 splenocyte infusion + imatinib (DLI + imatinib), DLI only, syngeneic Balb/c splenocyte infusions + imatinib (Imatinib only) or Balb/c splenocytes alone (Control) were transplanted to sublethally (450 cGy) irradiated syngeneic Balb/c female recipients. The secondary transplant was performed either at time of death of individual leukemic primary recipient, or at over 100 days post-primary BMT. Both transplants were carried out in the male→female direction, and the readout of the serial transplant experiment was to detect the presence of male cells by *Sry* PCR in BM genomic DNA of the female secondary recipients, whose immune system would reject allogeneic cells derived from T cell-depleted B10.D2 BM (black circled marrow cells) at time of primary BMT, but allow syngeneic repopulation from either *BCR-ABL1*$^{+}$ (green-circled marrow cells) or non-*BCR-ABL1*-transduced (white-circled marrow cells) male BM cells. The Kaplan Meier survival curve, flow cytometric analysis of leukemogenesis and chimerism, and quantification of leukemic clones of the primary transplant cohort are shown in Figure 6. (B) Kaplan-Meier curve of secondary recipients of primary chimeras treated with syngeneic Balb/c splenocytes (Control), imatinib and Balb/c splenocytes (Imatinib only), allogeneic B10.D2 splenocytes (DLI only), or imatinib and B10.D2 splenocytes (DLI + imatinib). (C) Sensitivity of the *Sry* PCR assay. Male Balb/c spleen DNA was subjected to serial dilutions into female Balb/c DNA; the percentage of male content per reaction is indicated. The PCR assay has a sensitivity of ~3%, as indicated by asterisk. The *Sry* amplification product is 441 bp, and the 245 bp myogenin band served as the internal control for each amplification reaction; the band

above the *Sry* band is a non-specific product. (D) Genomic DNA samples of the secondary transplant cohorts were collected either at time of death for leukemia^{+} mice, or at the end of experiment (d80+ post secondary BMT) for the disease-free recipients. Male-specific *Sry* sequences were PCR amplified from splenic (S), BM (B), or lymph node (LN) genomic DNA samples of secondary transplant recipients of primary chimeras from different treatment cohorts as indicated; samples acquired from the same secondary recipient are marked by brackets on top of the lanes. The 441 bp *Sry* band can be detected in both spleen and BM samples of sublethally irradiated female Balb/c mice transplanted with syngeneic Balb/c male marrows (M-Balb/c→F-Balb/c), but not in the group receiving allogeneic B10.D2 male marrows (M-B10.D2→F-Balb/c). Male (M) and female (F) Balb/c splenic DNAs are shown as controls; the 245 bp myogenin band serves as the internal control for each reaction. While the *Sry* band can be clearly detected in primary recipients that had CML-like leukemia or full-donor chimerism, as marked by red dots, most of the secondary recipients were *Sry*-negative, demonstrating that DLI treatment eradicated Balb/c-derived cells without discriminating based on the leukemic status of each cell. Of note, lanes 41 & 42 shows the splenic and BM DNA samples from a leukemic secondary recipient of a primary chimera treated with DLI + imatinib that had persistent leukemia.

Future directions: separating GvHD from GvL

- **Signaling transduction pathways**

In addition to the cellular mechanisms, it has been shown that differential death receptor signals predetermine the outcome of having GvHD or GvL: administration of DLI splenocytes from the autoimmune-prone *gld* mice (deficient in Fas ligand) to mixed leukemic chimeras preserved GvL with limited GvHD; whereas injecting perforin-deficient-DLI cells showed intact GvHD with diminished GvL activity (147). The result showed that GvHD was mainly mediated through Fas-Fas ligand interaction and GvL was primarily mediated through perforin pathway; this also suggests that selective blockage of death receptor-mediated pathways has great clinical implications (148).

A substantial amount of transplant literature in the past had also supported the idea that selective usage of death receptor signaling pathways plays an important role in orchestrating GvHD or GvL processes in the host (147, 149-152). With the combined therapeutic model in hand, we became interested in testing the role of several major death pathways, such as Fas-Fas ligand and TNFR-TNF interactions, in GvL by using receptor-deficient mice as the source of leukemic BMs. Fas-deficient B6 mice can be easily purchased; on the other hand, there are 2 forms of TNFR, p75 and p55, and both are known to mediate death signals. Dr. Warren Shlomchik has kindly agreed to provide B6 mice lacking both TNFR I and II, and we plan to utilize these mice to test the involvement of TNF pathways in the GvL effect in our model.

- **NK-based immunotherapy**

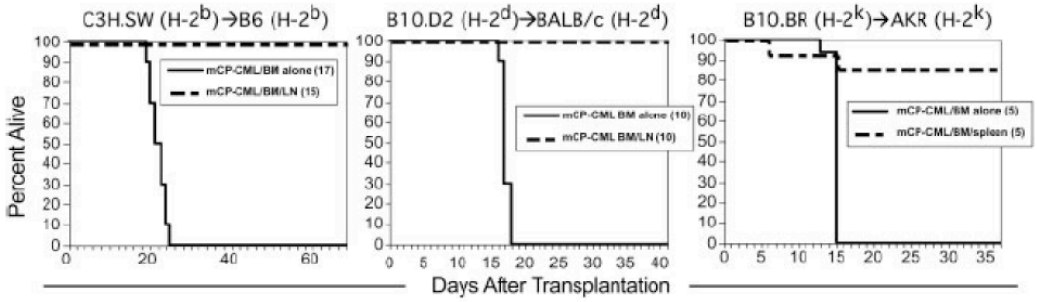
As previously mentioned, the activation status of NK cell is controlled by the balance between activating and inhibitory signals transduced through interactions with activating ligands or self-class I MHC molecules, respectively. The absence of MHC-I expression on hematopoietic cells leads to NK rejection (153); see also Figure 11. However, the absence of class I MHC molecules does not always lead to NK activation; for instance, NK cells are unable to reject MHC-I deficient tissues such as skin grafts (154), presumably because non-hematopoietic tissues lack the expression of activating ligand. Hence, the insight of studying the NK-based immunotherapy is being able to induce GvL without the risk of causing GvHD. A recent study has identified IL-1 receptor associated protein (IL1RAP), a co-receptor for IL-1, as a biomarker expressed on the $CD34^{+}38^{-}$ leukemic stem cell but not on corresponding normal population (144). Generating an IL1RAP-CAR-expressing NK clone is an interesting approach to cure the CML with minimal GvHD.

CHAPTER 3. Mechanism of GvL mediated by early DLI

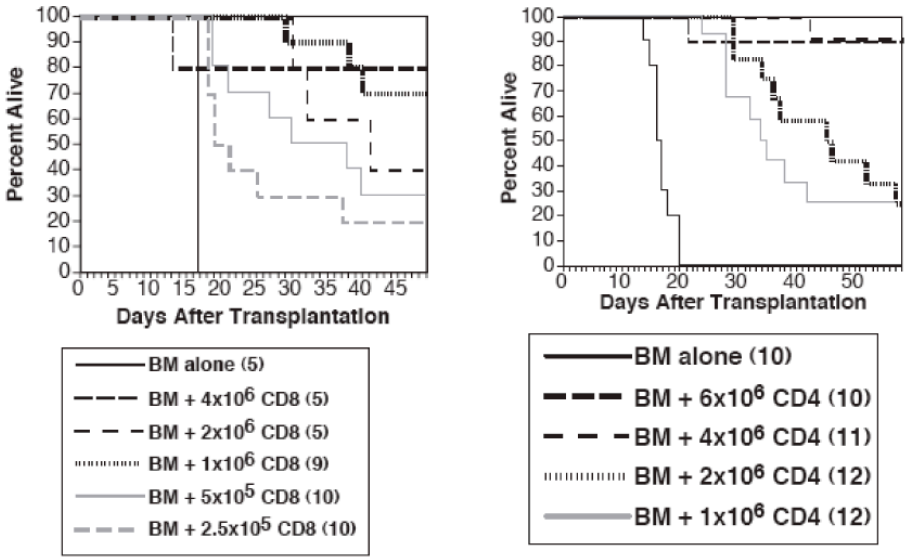
Using the retroviral BM transduction/transplant CML model, we (129) and others (1) have previously reported an immunotherapy model in which DLI treatment administered at the same time at transplantation of *BCR-ABL1*-transduced and allogeneic BMs ("early" DLI, green arrow in Figure 3A) was effective at preventing death from CML-leukemia and in maintaining leukemia-free status months after the transplant (Figure 3B and Figure 13A). In both scenarios, circulating leukocytes expressing the retroviral reporter gene, either GFP (Figure 14) or truncated nerve growth factor receptor (1), can be detected shortly after transplantation, but this likely reflects contributions from transduced committed progenitors rather than from hematopoietic stem cells. In the early DLI model, because the same transplant procedure is used to both initiate the leukemia and deliver the immunotherapy, this may be quite different from the treatment of established leukemia. Mechanistic studies treating leukemic chimeras with purified $CD4^+$ or $CD8^+$ T cells administered at the time of initial transplant (early DLI) showed that both T cell subsets contributed equally to GvL (Figure 13B). By contrast, when DLI was given following engraftment (delayed DLI), GvL was mediated principally by $CD8^+$ T cells, whereas the role of $CD4^+$ T cells was relatively minor (Figure 7). This strongly suggests that early and delayed DLI have fundamental differences in the mechanisms of conferring leukemia-free survival. In addition, it has long been appreciated that introduction of allogeneic lymphocytes at the time of BMT tends to drive hematopoietic engraftment towards the allogeneic donor (155), raising the possibility that introduction

of T cells at the time of transplant might prevent leukemia by blocking the initial engraftment of the leukemic stem cells.

A



B



Matte *et al.*, 2004 *(1)*

Figure 13. GvL mediated by early DLI is dependent on both $CD4^+$ and $CD8^+$ T lymphocytes (figure reproduced from Matte *et al.* (1)). (A) Kaplan-Meier survival curves demonstrating that early DLI immunotherapy could be modeled in three different MHC-matched, miHA-mismatched donor-recipient pairs. In this transplant model, syngeneic BM was transduced with a bicistronic retrovirus expressing BCR-ABL1 and the truncated nerve growth factor receptor. DLI cells derived from lymph nodes (left and middle panels) or splenocytes (right panel) of the indicated allogeneic strains were co-transplanted with the *BCR-ABL1*-transduced and T cell depleted allogeneic BM mixture into irradiated syngeneic recipients. GvHD was not observed under these conditions. (B) B6 recipients were irradiated and reconstituted with the leukemic BM, T cell-depleted allogeneic BM, and titrated doses of $CD8^+$ (left panel) or $CD4^+$ (right panel) allogeneic T cells administered as early DLI treatment (C3.SW→B6 strain pairing). The results showed that, when administered at time of the transplant, both cell types could mediate GvL.

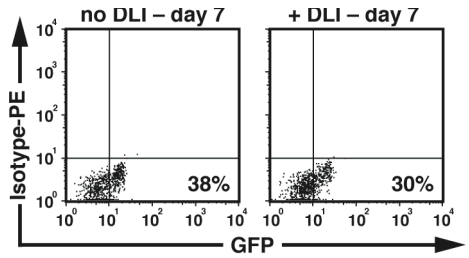


Figure 14. Circulating GFP$^+$ cells are found at day 7 post-BMT in primary recipients regardless of whether they received DLI at the time of transplant. Flow cytometric plots of GFP expression (*x*-axis) in peripheral blood leukocytes obtained 7 days after transplantation of *BCR-ABL1*-transduced syngeneic BM and allogeneic BM, either without (left panel) or with (right panel) allogeneic splenocytes. In samples from control mice, less than 1% of GFP$^-$ leukocytes were outside the lower left quadrant (data not shown).

We reasoned that if leukemic stem cells had engrafted and were present in these primary recipients, it should be possible to "rescue" them from the GvL effect by transplanting them into an immunocompetent syngeneic host, whose immune system would eliminate all allogeneic cells, but still allows syngeneic reconstitution. To test this hypothesis, we performed secondary BM transplants from primary mice that received *BCR-ABL1*-transduced marrows and TCD allogeneic marrows, with or without allogeneic splenocytes (B10.D2→Balb/c strain pairing), into sublethally irradiated (immunocompetent) syngeneic (Balb/c) recipients (Figure 15A). At day 14, day 7, or as early as day 4 following the initial transplant, we could transfer CML-like leukemia into immunocompetent syngeneic secondary recipients from primary mice that did not receive splenocytes, representing *bona fide* evidence of leukemic stem cells in these primary recipients (Figure 15B and Table 1). Blood smears and histopathological examination of lung, liver, and spleen showed massive myeloid expansion of mostly mature neutrophils with segmented or banded nuclei in these recipients (Figure 16A and B); in addition, the presence of GFP^+ leukemic cells in circulation confirmed that these secondary recipients developed CML-like leukemia that originated from the leukemic marrows of primary chimeras (Figure 17A). Flow cytometric analysis also showed that these secondary recipients rapidly lost their allogeneic chimerism, consistent with a host vs. graft effect (Figure 17A and B). By contrast, we were unable to transfer leukemia from any primary mice that received splenocytes at the time of the initial transplant. Secondary recipients of the primary transplant cohorts treated with early DLI showed normal hematopoiesis and normal structures of all tissues examined, and no sign of long-term repopulating GFP^+ cells (Figures 16 and 17A), indicating that leukemic stem cells are not present in the

primary transplant cohorts at these early time points, and have likely failed to engraft at all (Figure 15B and Table 1). Analysis of BM from secondary recipients revealed that, as expected, secondary recipients of BM from primary mice not treated with DLI engrafted with multiple leukemic stem cells of donor (male) origin, while secondary recipients from primary DLI-treated mice had no evidence of detectable provirus or cells of male origin, reconstituting hematopoiesis instead with host-derived (female) stem cells (Figure 15C).

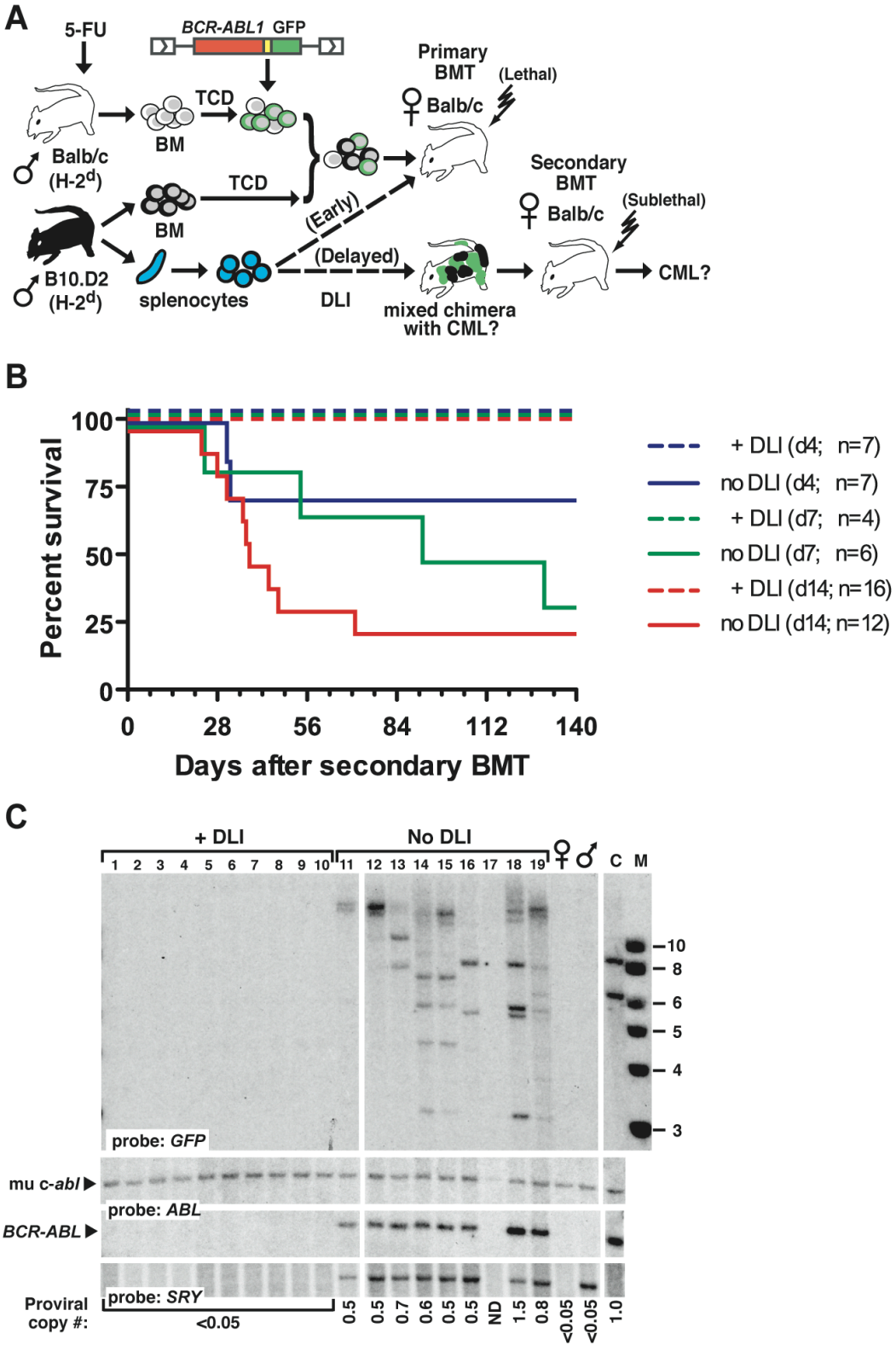

A
5-FU
BCR-ABL1 GFP
Primary BMT
(Lethal)
Balb/c
TCD
BM
Balb/c
(H-2d)
TCD
BM
(Early)
Secondary BMT
Balb/c
(Sublethal)
B10.D2
(H-2d)
splenocytes
(Delayed)
DLI
mixed chimera with CML?
CML?
B
Percent survival
100
75
50
25
0
0
28
56
84
112
140
Days after secondary BMT
+ DLI (d4; n=7)
no DLI (d4; n=7)
+ DLI (d7; n=4)
no DLI (d7; n=6)
+ DLI (d14; n=16)
no DLI (d14; n=12)
C
+ DLI
No DLI
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
C M
10
8
6
5
4
3
probe: GFP
mu c-abl
probe: ABL
BCR-ABL
probe: SRY
Proviral copy #:
<0.05
0.5 0.5 0.7 0.6 0.5 0.5 ND 1.5 0.8 <0.05 <0.05 1.0

Figure 15. Co-transplantation of allogeneic splenocytes with *BCR-ABL1*-transduced marrow blocks the engraftment of leukemic stem cells. (A) Schematic diagram of secondary transplant experiment. Primary Balb/c recipients (H-2^d) were lethally irradiated (700-900 cGy) and received *BCR-ABL1*-transduced syngeneic BM and TCD allogeneic BM from MHC-matched (H-2^d), miHA-mismatched B10.D2 donors, with (dotted line) or without allogeneic splenocytes (donor leukocyte infusion, DLI) administered at the time of transplant (Early) or beginning 2 weeks post-transplant (Late). At different intervals following the primary transplant, recipient BM was transplanted into sublethally (310 cGy) irradiated secondary Balb/c recipients, who were followed for development of CML-like leukemia. (B) Kaplan-Meier curve depicting the time to morbidity or death from CML-like disease of secondary recipients of BM from primary mice that received allogeneic splenocytes at the time of initial transplant (+ DLI; dashed lines) or did not receive allogeneic splenocytes (no DLI; solid lines). The secondary BMT was performed at 14 days (red curves), 7 days (green curves), or 4 days (blue curves) following the primary transplant. Note that in this model, CML-like leukemia is not transplanted with 100% efficiency even from non-chimeric donors (128). (C) Southern blot analysis of genomic DNA from BM of secondary recipients in panel C (d14 cohort) harvested at d56 post-BMT. The blot was hybridized with a *GFP* probe that detects individual leukemic stem cell clones (top panel), an *ABL1* probe that detects murine c-*Abl1* and *BCR-ABL1*, allowing quantitation of proviral DNA content (10) (middle panels), and a *SRY* probe to detect male (donor) sex (bottom panel). Lanes 1-10 are from recipients of BM from DLI-treated primary mice, lanes 11-19 from recipients of primary mice who did not receive DLI; DNA from normal female and male Balb/c mice,

and from a control cell line (C) containing a mixture of two proviral clones are on the right. Note that secondary recipients of BM from primary mice treated with early DLI did not engraft with any male *BCR-ABL1*$^{+}$ stem cells. Similar results were obtained with analysis of the d7 cohort (data not shown).

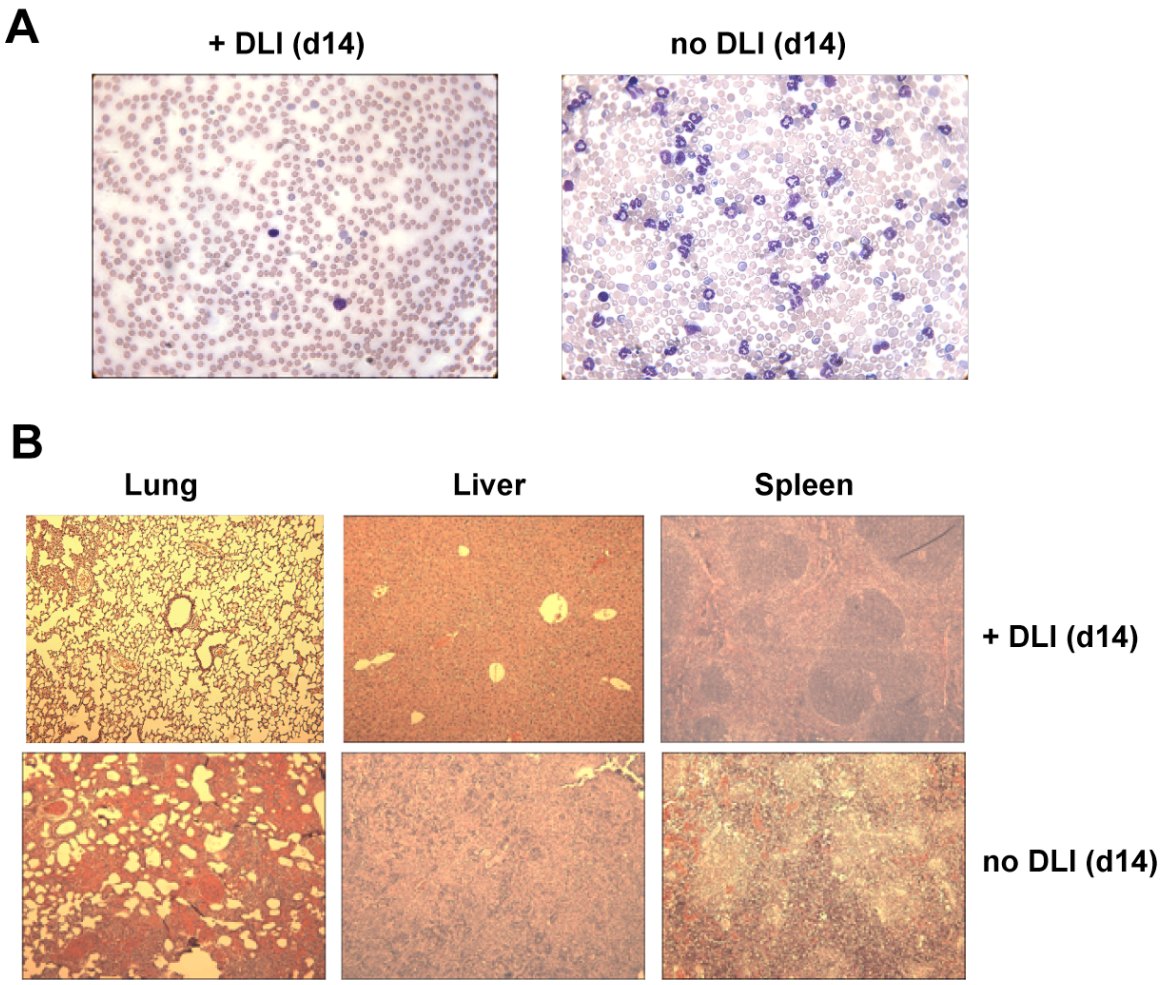
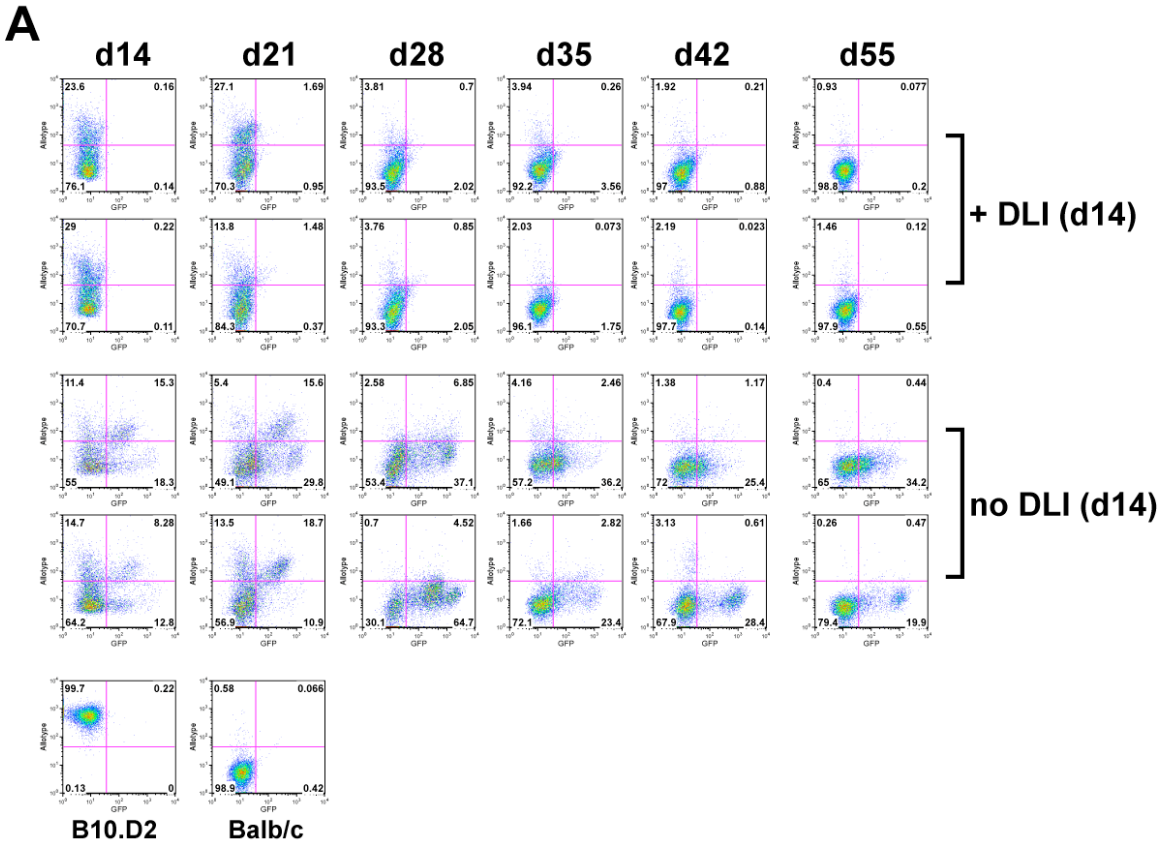


Figure 16. Lack of MPN phenotype in secondary recipients of BM from primary mice treated with early DLI.

(A) Blood smear of representative secondary recipients of primary cohorts treated with (+ DLI) or without early DLI (no DLI) at d35 post-secondary BMT, which was performed at d14 after the primary transplantation. 40x magnification, Wright-Giemsa staining. Note the excess of segmented neutrophils in the "no DLI" cohorts. (B) Histopathological examination (40x magnification, hematoxylin and eosin (H&E) staining) of the indicated tissues of representative secondary recipients, obtained either at time of death, or at the end of trial (d152 post-secondary BMT). In contrast with the severe pulmonary hemorrhage and massive leukemic infiltrations in liver and spleen in the "no DLI" cohort, secondary recipients of primary recipients treated with early DLI showed fine alveolar structures and the morphology of the liver and spleen is normal.


A
d14
d21
d28
d35
d42
d55
+ DLI (d14)
no DLI (d14)
B10.D2
Balb/c

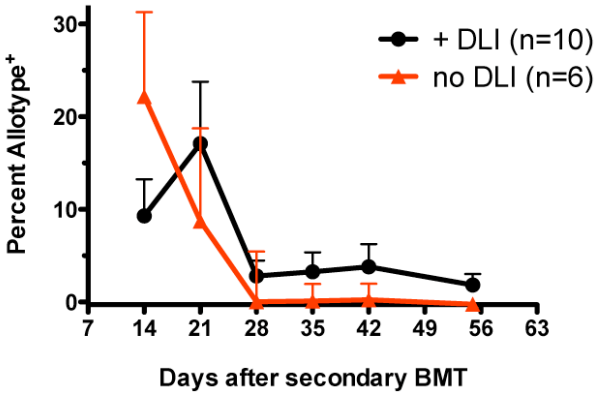

B
+ DLI (n=10)
no DLI (n=6)
Percent Allotype+
Days after secondary BMT

Figure 17. Lack of engrafted leukemic stem cells in recipients of early DLI.

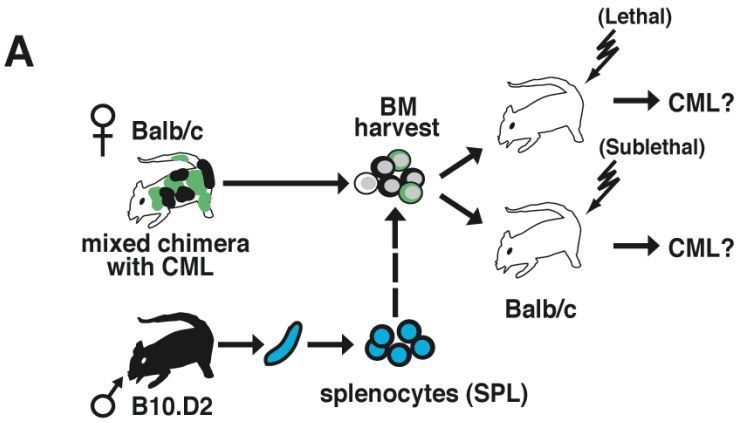
(A) Continuous flow cytometric monitoring of circulating GFP^+ leukemic cell (*x*-axis) and allogeneic chimerism (*y*-axis) of two representative secondary recipients of primary cohorts treated with (+ DLI) or without (no DLI) early DLI, assessed at the indicated times after secondary BMT, which was performed at d14 post-primary transplant. Bottom two panels show the normal blood profiles of allogeneic B10.D2 and syngeneic Balb/c mice. (B) Percentage of allogeneic chimerism in peripheral blood of the secondary recipients of primary recipients treated with (+ DLI, black symbols) or without early DLI (no DLI, red symbols). Allogeneic cells were identified by a polymorphism in β2-microglobulin as described in Materials and methods. Values presented are mean + SD after subtraction of background. The differences between the values are not statistically significant at any time point.

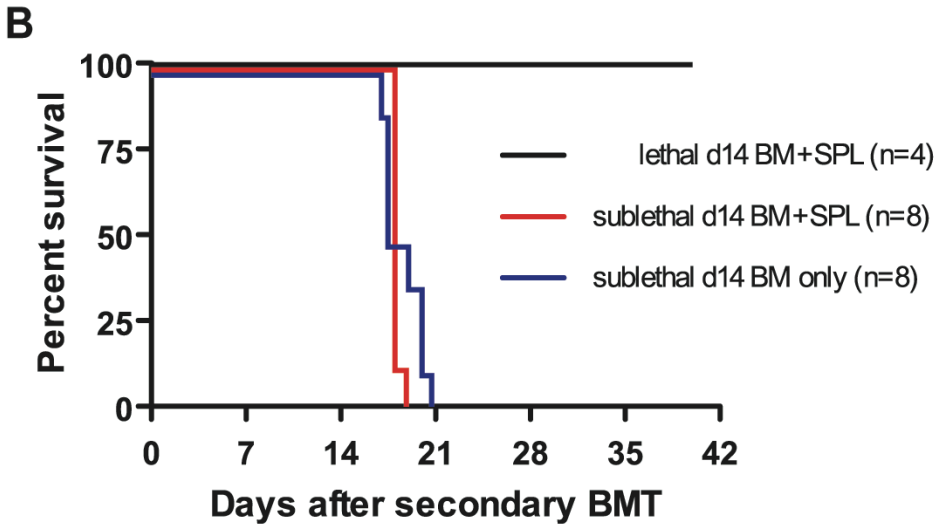
Table 1. Frequency of transplantable CML in primary BMT recipients

	Frequency of transplantable CML*		
	Day after primary transplant		
Primary transplant	Day 14	Day 7	Day 4
No DLI	9/12	4/6	2/7
+ DLI	0/16	0/4	0/7

*No. of secondary recipients developing CML/no. of secondary recipients transplanted; BM from each primary donor was transplanted into two recipients

To exclude the possibility that the allogeneic immune cells present in these primary chimeric donors might have specifically blocked leukemic stem cell engraftment in secondary recipients, we generated mixed chimeras with CML-like disease, harvested their BM at d14, and transplanted the cells into lethally or sublethally irradiated secondary Balb/c recipients with or without allogeneic splenocytes ($1x10^7$; Fig 17A). CML-like leukemia was efficiently transplanted from primary mixed chimeras to sublethally irradiated secondary recipients regardless of whether allogeneic splenocytes were added to the graft, while lethally irradiated recipients failed to develop CML when splenocytes were co-transplanted (Figure 17B and C), engrafting instead with allogeneic HSC. Together, these observations indicate that co-transplantation of allogeneic immune cells can exert a potent anti-leukemia effect by preventing engraftment of leukemic stem cells.


A
(Lethal)
Balb/c
BM
harvest
CML?
(Sublethal)
mixed chimera
with CML
CML?
Balb/c
B10.D2
splenocytes (SPL)


B
100
75
50
25
0
Percent survival
0
7
14
21
28
35
42
Days after secondary BMT
lethal d14 BM+SPL (n=4)
sublethal d14 BM+SPL (n=8)
sublethal d14 BM only (n=8)

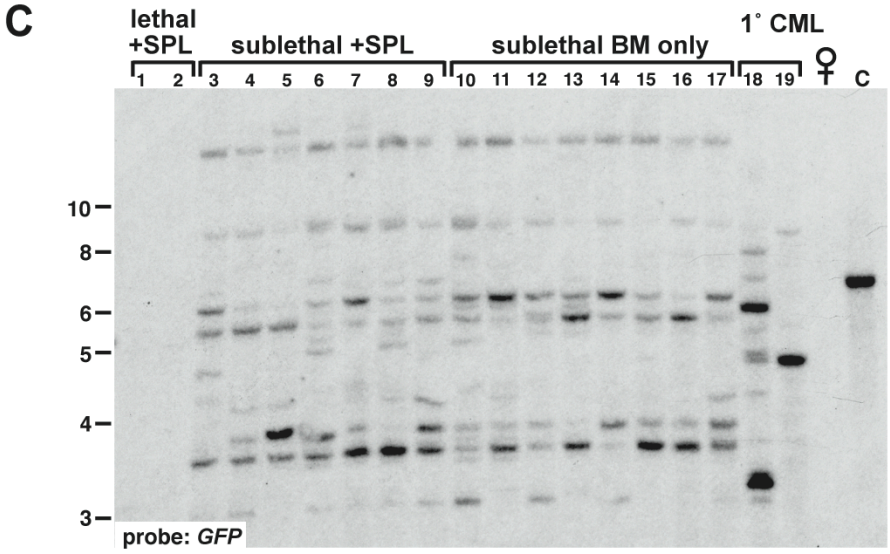

C
lethal
+SPL
sublethal +SPL
sublethal BM only
1˚ CML
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 C
10
8
6
5
4
3
probe: GFP

Figure 18. Allogeneic splenocytes cannot block leukemic stem cell engraftment in sublethally irradiated, immunocompetent recipients. (A) Schematic diagram of secondary transplant experiment. BM was harvested at d14 from an allogeneic mixed chimera with CML-like leukemia, generated by transplantation of a mixture of TCD *BCR-ABL1*-transduced BM and TCD B10.D2 BM into lethally irradiated Balb/c recipients as in Figure 1 A. The BM was transplanted into lethally or sublethally irradiated Balb/c secondary recipients with or without allogeneic splenocytes ($1x10^7$), who were monitored for development of leukemia. (B) Kaplan-Meier survival curve for recipients in panel A. Note that allogeneic splenocytes efficiently prevent the transplantation of leukemia only in lethally irradiated recipients. (C) Southern blot analysis of leukemic stem cells in secondary recipients from panel B. Genomic DNAs from BM of the cohorts in B were blotted and hybridized with a *GFP* probe as in Figure 1C. Lanes 1 and 2 are from lethally irradiated recipients of BM and allogeneic splenocytes (SPL); lanes 3-9 from sublethally irradiated recipients of BM + SPL; lanes 10-17 from sublethally irradiated recipients of BM alone. Note the efficient polyclonal engraftment of sublethally irradiated recipients with leukemic stem cells. Lanes 19 and 20 are from representative primary mice transplanted with *BCR-ABL1*-transduced BM alone; lane 21 from a normal Balb/c female; lane 22 from a control Ba/F3 cell line containing a single provirus.

Previous studies have shown that the anti-leukemic effect of early DLI in the mouse CML model is mediated by both $CD8^+$ and $CD4^+$ T cells (1). When the dose of $CD8^+$ or $CD4^+$ T cells is decreased, the efficiency of the anti-leukemic effect is also reduced, with an increasing proportion of recipients succumbing to CML-like disease (Figure 13). To determine the effect of graded doses of T cells delivered as early DLI on leukemia stem cell engraftment, we purified $CD4^+$ allogeneic splenocytes and varied the number of $CD4^+$ T cells added to the initial B10.D2→Balb/c graft. When the dose of $CD4^+$ T cells was successively reduced from $6x10^6$ to $0.5x10^6$ $CD4^+$ cells per recipient, there was a decrease in the anti-leukemic effect of early DLI, with an increasing proportion of recipients succumbing to CML-like MPN with successively shorter latency and survival (Figure 18A). However, analysis of the frequency of leukemia-initiating cells in these recipients demonstrated that reduction in the dose of $CD4^+$ T cells correlated with an increasing number of engrafting leukemic stem cells (Figure 18B), from 2-3 proviral clones to the polyclonal (>10 clones) leukemia that is characteristic of the CML-like MPN induced in recipients who are not treated with DLI in this model (Figure 18C; (10, 128)). Together, these results demonstrate that the predominant anti-leukemic mechanism of early DLI is to prevent engraftment of leukemic stem cells in this mouse CML model.

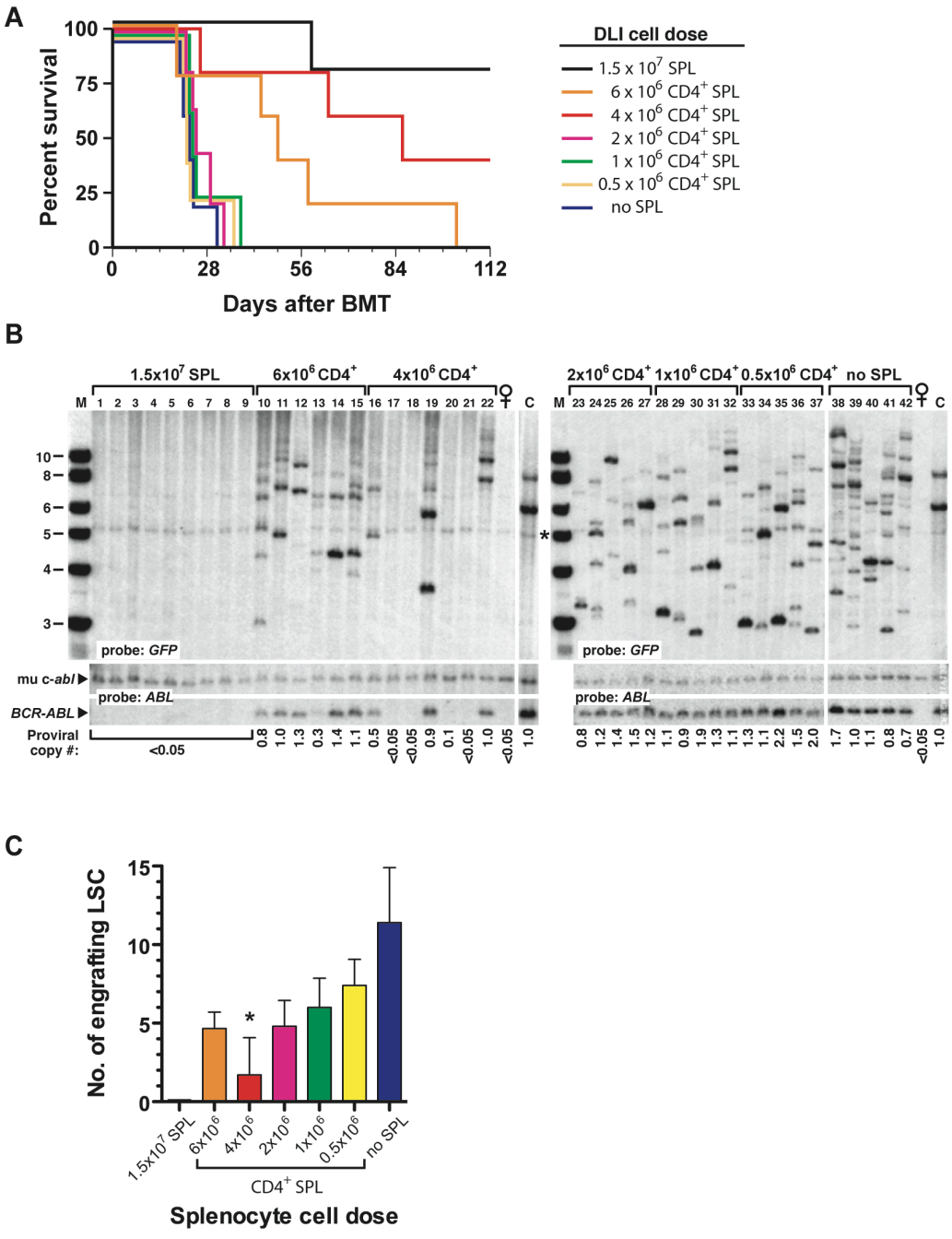

A
Percent survival
100
75
50
25
0
0
28
56
84
112
Days after BMT
DLI cell dose
1.5 x 10^7 SPL
6 x 10^6 CD4+ SPL
4 x 10^6 CD4+ SPL
2 x 10^6 CD4+ SPL
1 x 10^6 CD4+ SPL
0.5 x 10^6 CD4+ SPL
no SPL
B
1.5x10^7 SPL
6x10^6 CD4+
4x10^6 CD4+
2x10^6 CD4+
1x10^6 CD4+
0.5x10^6 CD4+
no SPL
probe: GFP
mu c-abl
BCR-ABL
probe: ABL
Proviral copy #:
<0.05
C
No. of engrafting LSC
15
10
5
0
1.5x10^7 SPL
6x10^6
4x10^6
2x10^6
1x10^6
0.5x10^6
no SPL
CD4+ SPL
Splenocyte cell dose

Figure 19. Reduction in CD4+ T cell dose in early DLI correlates with increased engraftment of leukemic stem cells. (A) Kaplan-Meier survival curve of B10.D2→Balb/c recipients of *BCR-ABL1*-transduced BM treated with early DLI with the indicated dose of purified $CD4^+$ splenocytes (n=5 for each cohort). As controls, recipients received either no DLI (no SPL, blue curve) or DLI with unfractionated splenocytes at the standard dose (black curve). (B) Southern blot analysis of leukemia-initiating cell frequency in genomic DNA of BM from the cohorts in A. The blot was hybridized with a *GFP* probe (top panel) to quantitate the number of engrafting leukemic stem cells, or with an *ABL1* probe (bottom panels) to quantitate proviral DNA content, as in Figure 1 C. Lanes 1-9 received $1x10^7$ total splenocytes, lanes 10-15 received $6x10^6$ $CD4^+$ splenocytes, lanes 16-22 received $4x10^6$ $CD4^+$ splenocytes, lanes 23-27 received $2x10^6$ $CD4^+$ splenocytes, lanes 28-32 received $1x10^6$ $CD4^+$ splenocytes, lanes 33-37 received $0l5x10^6$ $CD4^+$ splenocytes, while lanes 38-42 were not treated with DLI. Note that recipients of $4x10^6$ T-cells had only 1-2 clones per leukemic recipient. The band indicated by the asterisk is a background band. (C) Quantitation of frequency of engrafting leukemic stem cells from the data in panel B. The difference in number of engrafting LSCs between untreated recipients and recipients of $4x10^6$ $CD4^+$ splenocytes was significant (P=0.0002, unpaired t-test).

Conclusions and discussions

Achieving long-term cure by eradicating minimal residual disease is the ultimate goal of every cancer therapy. Since the discovery that CML is caused by the *BCR-ABL1* oncogene, intensive studies aiming to inhibit the constitutively active tyrosine kinase activity have led to the development of a panel of tyrosine kinase inhibitors and have changed the paradigm of CML therapy. However, it has also became clear by now that most patients are not cured by TKI therapy alone, as treatment discontinuation is frequently followed by disease relapse. In addition, alloHSCT is still used to treat other hematopoietic malignancies, where the pathophysiology of the disease is less well defined and targeted therapies are lacking. This has triggered the need to study alternative strategies to cure CML and other blood cancers without long-term administration of medication. For unknown reasons, CML is the most responsive leukemia to immunotherapy. The GvL effect of allogeneic immune cells can be best demonstrated that DLI administration could re-induce durable remissions in most patients who relapsed after their initial transplants (156); however, this came at the cost of severe GvHD and graft failure. Non-myeloablative or reduced-intensity conditioning regimens could induce mixed hematopoietic chimerism in recipients (157, 158), but DLI and conversion to full allogeneic chimerism was still complicated by frequent GvHD. To understand whether GvL and GvHD are separable events and whether there is a preferential cell type in mediating each process, we have used a physiologically relevant model of CML in laboratory mice to investigate the mechanisms of DLI in a MHC-matched, miHA-mismatched transplant setting.

Through the combined effort of reducing the number of leukemic stem cells at time of the transplant and administering imatinib during the treatment course, we successfully induced an attenuated form of CML-like leukemia, and were able to model "delayed" DLI therapy against CML-like leukemia in miHA-mismatched allogeneic chimeras, resembling DLI therapy in patients who relapsed after MHC-identical allogeneic stem cell transplantation. Although allogeneic $CD4^+$ T cells were shown to mediate GvL when administered at time of transplant, in the delayed DLI setting, GvL was predominantly mediated through allogeneic $CD8^+$ T cells in these leukemic mixed chimeras. Conversely, administration of $CD4^+$ T cells correlated with a higher frequency of GvHD. The identities of the target antigens are not clear, but neither BCR-ABL1 nor GFP are major contributors because infusion of splenocytes from syngeneic Balb/c donors was ineffective in mediating a GvL response (Figure 6). Our data detecting male cells in secondary recipients of the primary cohorts treated with delayed DLI has demonstrated that the specific antigens involved in GvL are likely to be minor histocompatibility antigens (Figure 12), and the GvL effect may actually represent a graft-versus-hematolymphoid system reaction. Since the cohorts treated with CD4-depleted T cell-DLI and $CD8^+$ T cell-DLI did not develop severe GvHD (Figure 7 and data not shown), it is likely that the specific miHA-targets have restricted expression on cells of hematopoietic lineages, such as H4 (159), H13 (160), H28 (161) and H60 (162); as it was shown that, in MHC-matched setting, severe GvHD occurs only when multiple miHAs were involved, or when the target miHA had ubiquitous expression (163, 164). Among those, H60, a non-classical class I molecule expressed in the Balb/c but not the B10.D2 strain, was shown to be an activating ligand of NKG2D receptor (165, 166); in

addition, a H60-derived peptide/MHC complex was shown to elicit strong cytotoxic T cell responses in the B6 strain (167). Together, these results indicate that H60, either as a native protein or as a presented peptide, could activate NK cell and $CD8^+$ T cells. Hence it is plausible that H60 played a role in eliciting anti-miHA immune responses in our combined immunotherapy model.

In CML, very little is known about the contribution of NK cells to the GvL effect of alloHSCT and DLI. In allografted CML patients, donor-derived NK cells with cytolytic activity against CML cell lines and primary host CML progenitors can be identified (168), and retrospective analyses suggest that GvL effects of alloHSCT for CML correlate with rapid recovery of donor-derived NK populations (136, 169) and with expression of specific activating NK receptors (137, 138). Our results using Rag2-deficient mice as DLI donors showed that miHA-mismatched NK cell had limited GvL activity (Figure 9). However, miHA-mismatched NK cells were able to effectively eradicate CML induced by MHC-I deficient leukemic BM (Figure 11). This argues that overcoming the inhibitory signals transduced by cognate class I MHC molecules is crucial for NK-based adoptive immunotherapy, and that activated NK cells are competent to eradicate CML-like disease. Similarly, a retrospective study showed that donor KIR ligand disparity was associated with significantly reduced relapse rate and prolonged disease-free survival in myeloid leukemia patients receiving haplo-identical (one haplotype-mismatched) cord blood transplantations, suggesting the clinical potential of applying activated NK cells in treating MPN (170). However, direct evidence of NK participation in adoptive immunotherapy for CML is lacking. Our model of adoptive immunotherapy will be useful in defining the relative roles of T lymphocytes and NK

cells in the GvL effect against established CML, and in defining the mechanisms of leukemic stem cell killing. This should in turn lead to improvements in immunotherapy of CML and other leukemias.

In the present study, we characterized two distinct GvL mechanisms mediated by DLI at early or delayed time points post-transplant. Our results showed that none of the recipients of early DLI treatment was able to transfer CML-like disease to the syngeneic secondary recipients, whereas most of the control cohorts without early DLI co-transplantation were able to adoptively transfer the disease (Figure 15 & Table 1). This demonstrates the absence of leukemic stem cells in the recipients of early DLI, and argues that co-administration of allogeneic lymphocytes at time of disease induction prevents the engraftment of *BCR-ABL1*-transduced stem cells. Although transient circulating leukemic cells could be detected in recipients of early DLI treatment (Figure 14 & (1)), we confirmed the absence of leukemic stem cells in these cohorts by the stringent criterion of secondary transplantation. It is well known that both short-term repopulating cells and committed myeloid progenitors contribute to myelopoiesis following transplantation (171), and it is plausible that the circulating leukemic cells observed in recipients of early DLI are the progeny of such progenitors.

We have also demonstrated that early and delayed DLIs exploit distinct mechanisms to eradicate leukemia. Different from GvL of early DLI, where the cure was mediated through blocking the engraftment of LSCs by both $CD4^+$ and $CD8^+$ T cells, GvL of delayed DLI was mediated through eliminating the engrafted LSCs by allogeneic $CD8^+$ T cells in the mixed leukemic chimeras. Conversely, administration of $CD4^+$ T cells correlated with higher frequency of GvHD (Figure 7). Using the early DLI

approach, it was shown previously that the cognate MHC-interaction was required for optimal GvL mediated by both T cell subsets (1, 140, 145, 172), including the $CD4^+$ memory T cells (172), whereas this interaction was not a prerequisite for GvHD induced by $CD4^+$ T cell (140). In addition, Fas or TNFR I and II expression on the leukemic targets were not required for GvL mediated by $CD8^+$ T cell (1); correspondingly, Fas or TRAIL receptor expression was not required for GvL mediated by $CD4^+$ T cell (1, 172), indicating the redundant use of these pathways.

The specific role of NK cell in mediating GvL has never been directly addressed. Our data of treating the leukemic mixed chimeras with *Rag2*-deficient DLI suggest a potential involvement of NK cell in the anti-leukemia effect (Figure 9). The relatively weak GvL might be the consequence of concurrent inhibitory signals transduced through the cognate class I MHC molecules. In addition, the limited abilities of clonal expansion and memory forming of NK cell might as well dampen its therapeutic potential. Nevertheless, our data of another transplant cohort, where the leukemic cells lack MHC-I expression, indicate that NK cell can also mediate robust GvL when the inhibitory signals are removed (Figure 11). An immediate priority will be to investigate whether the GvL is a direct effect mediated by adoptively transferred allogeneic NK cells, as it is also plausible that the strong GvL effect was the consequence of engraftment blocking mediated by the host residual NK cells. Our results suggest that using strategies that are designed to bypass the inhibitory regulation mediated through MHC-I interactions, such as expressing LSC-specific chimeric receptors on NK cell, is necessary to further exploit the potential use of adoptively transferred NK cells in cancer immunotherapy.

Lastly, the absence of male-derived hematopoietic reconstitution in immune competent secondary hosts suggests that the GvL effect was mediated through allogeneic immune cells targeting both leukemic and non-leukemic host stem cells (Figure 15C, *Sry* Southern blotting). This suggests that the anti-engraftment strategy might be of benefit in the autologous transplant setting, in which leukemia-specific or allo-reactive lymphocyte infusions could prevent the re-engraftment of leukemic stem cells after high dose of chemotherapy. Together, our results demonstrate a potent anti-engraftment effect of allogeneic splenocytes in a transplantation setting that might be exploited as a way to purge the leukemia^{+} BM grafts for autologous transplant patients.

Materials and methods

Mice

In the B10.D2→Balb/c (H-2^d) retrovirally induced CML transplant model, male Balb/cAnNTac mice (H-2^d, Taconic Farms), 6-8 weeks old, were used as the leukemic marrow donors; syngeneic female Balb/cAnNTac mice (H-2^d, Taconic Farms), 7-9 weeks old, were used as the transplant recipients; MHC-matched, miHA-mismatched B10.D2-*Hc*^{1} *H2*^{d} *H2-T18*^{c}/nSnJ males (H-2^d, The Jackson Laboratory), ranging from 6 to 12 weeks of age, were used as the allogeneic BM and DLI donors; male Balb/cAnNTac mice (H-2^d, Taconic Farms), 7-9 weeks old, were used as donors for syngeneic control infusion.

In one of the B10.D2→Balb/c (H-2^d) transplant series investigating the role of miHA-mismatched NK cell infusion in disease cure, the protocol was same as described above, except that the B10.D2-Rag2 deficient mice, 6-10 weeks old, were used as the source of DLI. In the same transplant cohort, we included a parallel arm testing the role of MHC-mismatched NK cell infusion in disease cure. Instead of using the B10.D2 mice, we switched the allogeneic marrow donor to the MHC-mismatched B6.129S7-Rag1^{tm1Mom}/J strain (H-2^b, The Jackson Laboratory), 6-8 weeks old, and used the same strain as the source of NK infusions.

In the C3.SW→B6 (H-2^b) transplant system, male B6.SJL-*Ptprc*^{a}/BoyAiTac mice (H-2^b, Taconic Farm), 6-10 weeks old, were used as the leukemic marrow donors, which carry the Ptprc^a allele (protein-tyrosine phosphatase, receptor type c locus previously known as CD45.1, Ly5.1) from SJL/J strain; syngeneic female B6.SJL-*Ptprc*^{a}/BoyAiTac mice (H-2^b, CD45.1$^+$, Taconic Farm), 6-10 weeks old, were used as the transplant

recipients; MHC-matched, miHA-mismatched C3.SW-H2^{b}/SnJ males (H-2^{b}, CD45.2^{+}, The Jackson Laboratory), ranging from 6 to 10 weeks of age, were used as the allogeneic BM and DLI donors; male C57BL/6NTac (H-2^{b}, CD45.2^{+}, Taconic Farm), 6-8 weeks old, were used as donors for syngeneic control infusion. Additionally, in the transplant testing the efficacy of DLI against MHC-I and MHC-II deficient leukemia, B6.SJL-Ptprc^{a}/BoAiTac-β2m^{tm1Jae} N10, (H-2^{b}, CD45.1^{+}, Taconic Farm) and B6.SJL-Ptprc^{a}/BoAiTac-H2-Ab1^{m1Glm} N13 (H-2^{b}, CD45.1^{+}, Taconic Farm) were used as the leukemic BM donors, respectively.

Bone marrow transduction and transplantation

Replication-defective ecotropic retroviral stocks were generated by transient transfection of 293T cells using p210 BCR-ABL1-MSCV-IRES-GFP and EcoPac packaging constructs; the stocks were tittered by transduction of NIH-3T3 cells (10) (Figure 2). For induction of CML-like leukemia, we collected BM cells from male Balb/c donors, 4d after intravenous administration of 200 mg per kg (body weight) 5-fluorouracil (5-FU), transduced cells with retrovirus, performed either T cell depletion with anti-CD5 biotinylated antibody, or lineage depletion with a panel of biotinylated antibodies against CD5, B220, Gr-1, and Ter-119 (BD Biosciences), with streptavidin-conjugated magnetic microbeads (Miltenyi Biotec), and injected 2x10^{5} T cell-depleted cells, or 1x10^{4} lineages-depleted cells per recipient, intravenously into 700-900 cGy-irradiated female Balb/c mice. To induce donor chimerism, we collected allogeneic BM cells from male B10.D2 donors on the same day of the transplant, depleted T cells and injected ≥ 1x10^{7} cells per recipient with the transduced leukemic graft into irradiated female Balb/c mice. The clinical features and histopathology of *BCR-ABL1*-induced CML-like disease, B

lymphoid leukemia, and histiocytic sarcoma were described previously (173). All mouse experiments were approved by the Institutional Animal Use and Care Committee of Tufts Medical Center.

Secondary transplantations

BM cells of primary chimeras were collected at the indicative time points post-transplant and intravenously injected into sublethally- irradiated (310 cGy) female Balb/c mice. For most of the secondary transplants, BM cells collected form one primary recipient ($\leq 2 \times 10^6$ marrows per primary mouse) were transplanted to two secondary recipients; for the experiment described in figure 18, BM cells of 10 primary recipients were pooled together and transplanted into 20 irradiated secondary mice, with or without the addition of DLI cells at time of secondary transplantation.

Donor leukocyte infusions

For early DLI treatment, splenocytes of allogeneic B10.D2 males were collected and injected intravenously, at the dose of 1.5×10^7 cells per mouse, into recipient at time of transplant. For delayed DLI treatment, B10.D2 splenocytes were either directly injected into the leukemic chimeras at the dose of 3×10^7 per mouse, or were subjected to cell depletions by biotinylated antibodies against CD4, CD8 or CD5 (BD Biosciences) and streptavidin-conjugated magnetic microbeads (Miltenyi Biotec) prior infusions. After depletion, the efficiency of magnetic separation was tested by secondary staining of PE-Cy5-conjugated Streptavidin and analyzed by flow cytometry. Starting at d14 post-transplant, DLI was repetitively administered once per week until eradication of disease; usually 4 to 5 doses of DLI were required for disease clearance. To match equal numbers of acting cells as the whole splenocyte DLI, the fractionated DLIs were infused at the

dose of 2.4 x 10^7 CD4-depleted DLI (80% of whole splenocyte), 2.7 x 10^7 CD8-depleted DLI (90% of whole splenocyte), and 2.1 x 10^7 CD5-depleted DLI (70% of whole splenocyte).

Flow cytometric analysis of chimerism

In the B10.D2→Balb/c (H-2^d) mixed chimeras with CML-like disease, peripheral blood chimerism was detected by flow cytometry with the antibody specific to an allelic variant of β2-microglobulin (β2-$M^{b,c}$, clone S19.8, BD Biosciences) expressed exclusively on cells of C57BL/6 origin, including the B10.D2 allogeneic donors (129). In the C3.SW→B6 (H-2^b) transplant system, chimerism was detected by antibody specific to the common CD45.2 allele (BD Biosciences) expressed on allogeneic cells. In both transplant systems, leukemogenesis was monitored either by detecting GFP expression, or by measuring complete white count using the automatic HEMAVET analyzer.

Southern blot analysis

Genomic DNA samples were collected from recipient mice at time of death/morbidity, phenol-chloroform extracted, Bgl-II (50,000 U/mL, New England Bioabs) digested and transferred to Amersham Hybond nylon membranes (GE Healthcare) after overnight electrophoresis. The blots were hybridized with ^{32}P-α-CTP labeled GFP probe to detect individual proviral integration site, with ^{32}P-α-CTP labeled ABL1 probe to detect both endogenous c-ABL1 and BCR-ABL1, or with ^{32}P-α-CTP labeled SRY probe to detect male sexuality. ^{32}P-α-CTP was purchased from PerkinElmer; all probes were generated using the Random Primers DNA labeling System (Invitrogen).

Male-specific *SRY* PCR assay

Genomic DNA samples were collected from the secondary recipients at time of death or when reaching moribund state. 0.5μg of phenol-chloroform purified samples were added to each amplification reaction of one cycle of 94°C for 5 mins and 30 cycles of (94°C, 5 seconds→67°C, 40 seconds), followed by one cycle of 72°C for 1 min (174). The 441 bp SRY product was amplified using the forward primer: TCA TGA GAC TGC CAA CCA CAG, and reverse primer: CAT GAC CAC CAC CAC CAC CAA; the 245 bp myogenin internal control was amplified in the same reaction using the forward primer: TTA CGT CCA TCG TGG ACA GC, and reverse primer: TGG GCT GGG TGT TAG TCT TA. For each amplification reaction, all primers were used at 40 ng per test.

Generation of the Rag2-deficient B10.D2 mice colony (B10.D2-Rag)

The B10.D2-Rag line was generated by crossing the TCR-DO11.10/RAG2 [KO] line (Taconic Farm) with wild type B10.D2 mice. The TCR-DO11.10/RAG2 [KO] line is homozygous for a transgene that encodes the T cell receptor specific for a chicken ovalbumin (OVA) peptide presented by the class II MHC molecule I-A^d. Starting form the commercially available line, the B10.D2-Rag line was created after two rounds of crossing. Presence of DO11.10 transgene can be phenotyped by mAb staining against DO11.10 TCR (BD pharmingen). Rag2-deficiency can be detected by genotyping using Rag A primer (final concentration at 0.25 μM): GGG AGG ACA CTC ACT TGC CAG, Rag B primer (1 μM): AGT CAG GAG TCT CCA TCT CAC, and Neo primer (0.5 μM): CGG CGG GAG AAC CTG CGT GCA A, with the Thermal cycling condition of one cycle of 95°C for 15 mins, 35 cycles of (94°C, 45 seconds→55°C, 1 min→72°C, 1 mins), followed by one cycle of 72°C for 5 mins. All primers were synthesized at Eurofins

MWG/Operon. According to the protocol, the amplicons of wild type and Rag2^{tm1} alleles are 263-bps and 350-bps, respectively. Rag2-deficient phenotype can further be confirmed by mAb staining against mouse CD3 (BD Biosciences).

Blood smear

Blood smear slides were generated by sandwiching one drop (about ~ 2 μL) of fresh blood sample between cover slides and quickly removed the top piece. The smears were then subjected to Wright-Giemsa staining (Sigma-Aldrich) for morphological examination.

Histopathological examination

Organs of individual recipient were collected at time of death or when reaching a moribund state; collected organs were fixed in 10% buffered formalin solution for at least 24 hours at room temperature. Fixed organs were processed by the standard dehydration protocol and embedded in paraffin blocks prior sectioning and H&E staining by Tufts New England Medical Center Histology Laboratory.

Imatinib preparation and dosing

Imatinib tablets (100 mg tabs, Novartis) were sequentially dissolved in water and centrifuged at 2500 rpm for 10 mins. The reconstitution process was repeated twice to ensure maximal solubility. All supernatants were carefully pooled through 0.45 μM filters (Millipore) and were adjusted to final concentration of 20 mg/mL. The stock solution was stored at -20°C in 2-mL aliquots; each vial was dissolved readily upon use. For a 20g mouse, 100 μL of the stock solution (100 mg/kg) was orally administered via

disposable feeding needle (Fisher) on a once daily basis until time of treatment withdrawal.

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