Myeloid leukemia with transdifferentiation plasticity developing from T-cell progenitors

Pia Riemke^{1,†}, Melinda Czeh^{1,†}, Josephine Fischer^{1,†}, Carolin Walter², Saeed Ghani³, Matthias Zepper¹, Konstantin Agelopoulos⁴, Stephanie Lettermann⁵, Marie L Gebhardt⁶, Nancy Mah⁷, Andre Weilemann^{8,9}, Michael Grau^{8,9}, Verena Gröning¹, Torsten Haferlach¹⁰, Dido Lenze¹¹, Ruud Delwel¹², Marco Prinz^{13,14}, Miguel A Andrade-Navarro¹⁵, Georg Lenz^{8,9}, Martin Dugas², Carsten Müller-Tidow¹⁶ & Frank Rosenbauer^{1,*}

Abstract

Unfavorable patient survival coincides with lineage plasticity observed in human acute leukemias. These cases are assumed to arise from hematopoietic stem cells, which have stable multipotent differentiation potential. However, here we report that plasticity in leukemia can result from instable lineage identity states inherited from differentiating progenitor cells. Using mice with enhanced c-Myc expression, we show, at the single-cell level, that T-lymphoid progenitors retain broad malignant lineage potential with a high capacity to differentiate into myeloid leukemia. These T-cell-derived myeloid blasts retain expression of a defined set of T-cell transcription factors, creating a lymphoid epigenetic memory that confers growth and propagates myeloid/T-lymphoid plasticity. Based on these characteristics, we identified a correlating human leukemia cohort and revealed targeting of Jak2/Stat3 signaling as a therapeutic possibility. Collectively, our study suggests the thymus as a source for myeloid leukemia and proposes leukemic plasticity as a driving mechanism. Moreover, our results reveal a pathway-directed therapy option against thymus-derived myeloid leukemogenesis and propose a model in which dynamic progenitor differentiation states shape unique neoplastic identities and therapy responses.

Keywords leukemia; lineage plasticity; myeloid differentiation; T-cell progenitors

Subject Categories Cancer; Development & Differentiation; Immunology

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Introduction

Acute leukemia (AL) refers to a heterogeneous group of blood cell disorders in which differentiation-blocked malignant myeloid or lymphoid cells expand uncontrolled. The treatment for AL depends on how the disease is classified according to both cytogenetic and phenotypic markers (Bene *et al*, 1995; Dohner *et al*, 2010). However, up to 20% of ALs remain difficult to classify, as they express both lymphoid and myeloid antigens (Matutes *et al*, 1997; Golemovic *et al*, 2006). Clinically, these “ambiguous” AL cases are highly relevant because they result in particularly poor patient survival and are frequently resistant to standard therapies (Golemovic *et al*, 2006; Coustan-Smith *et al*, 2009; Dorantes-Acosta & Pelayo, 2012). In addition to lineage marker ambiguity,

1 Institute of Molecular Tumor Biology, University of Münster, Münster, Germany

2 Institute of Medical Informatics, University of Münster, Münster, Germany

3 Department of Hematology, Oncology, and Tumor Immunology, Robert-Rössle-Clinic, Berlin, Germany

4 Department of Dermatology, Competence Center Chronic Pruritus, University of Münster, Münster, Germany

5 Molecular Hematology and Oncology, Medical Clinics A, University of Münster, Münster, Germany

6 Department of Computational Biology and Data Mining, Max-Delbrück-Center for Molecular Medicine, Berlin, Germany

7 Berlin-Brandenburg Center for Regenerative Therapies, Charité - Universitätsmedizin Berlin, Berlin, Germany

8 Translational Oncology, Medical Clinics A, University of Münster, Münster, Germany

9 Cluster of Excellence EXC 1003, Cells in Motion, Münster, Germany

10 MLL Munich Leukemia Laboratory, Munich, Germany

11 Institute of Pathology, Charité - Universitätsmedizin Berlin, Berlin, Germany

12 Department of Hematology, Erasmus University Medical Center, Rotterdam, the Netherlands

13 Institute of Neuropathology, University of Freiburg, Freiburg, Germany

14 BIOSS Centre for Biological Signalling Studies, University of Freiburg, Freiburg, Germany

15 Department of Medical Informatics and Biomathematics, Institute of Molecular Biology, Johannes Gutenberg University of Mainz, Mainz, Germany

16 Department of Internal Medicine, Hematology and Oncology, University of Halle-Wittenberg, Halle, Germany

*Corresponding author. Tel: +49 251 83 55312; Fax: +49 251 83 55303; E-mail: frank.rosenbauer@ukmuenster.de

[†]These authors contributed equally to this work

ALs that switch lineages in treatment-associated relapse have been described (Stass *et al*, 1984; Risueno *et al*, 2011). These findings imply that hematopoietic malignancies can hold immanent potential to differentiate into alternative lineages. While earlier studies have assumed that leukemias with lineage infidelity and switching capacity can arise upon malignant transformation of a hematopoietic stem cell (HSC; Matutes *et al*, 1997; Neumann *et al*, 2012), the underlying cellular origin of such lineage plasticity remains poorly defined.

One type of AL, acute myeloid leukemia (AML), is believed to originate from transformed HSCs or myeloid-restricted progenitors (Walter *et al*, 2012). However, these bone marrow (BM)-resident precursors are not the only cells with myeloid potential: myeloid potential has also been found in thymic CD4/CD8 double negative 1 (DN1) and 2 (DN2) cells, which represent the earliest stages of T-cell development (Bell & Bhandoola, 2008; Wada *et al*, 2008). However, the contribution of DN1 and DN2 cells to physiological myelopoiesis *in vivo* has been questioned by lineage-tracing experiments (Schlenner *et al*, 2010). Nevertheless, the same authors found that approximately 15% of DN1 cells had robust myeloid differentiation capacity *in vitro*, confirming that thymic progenitors can produce myeloid progeny (Schlenner *et al*, 2010). In another study, myeloid potential was attributed to thymic progenitors *in vivo*, and early T cells were suggested as precursors of thymic granulocytes (De Obaldia *et al*, 2013). However, even if a contribution to myelopoiesis under homeostatic conditions is still unclear, these results demonstrated that the lymphoid fate of early T-cell progenitors is not yet fully stabilized and may compete with a residual option of myeloid differentiation. Therefore, we hypothesized that this myeloid potential may be exploited under pathological conditions so that T-cell precursors could transdifferentiate into leukemia with myeloid identity.

To test this hypothesis, we aimed to analyze the lineage potential of DN2 cells upon transformation. We reasoned that ectopic *c-Myc* (*Myc*) expression would serve our purpose best, as *MYC* has been demonstrated to transform cells, at least in part, by amplifying the expression of already transcribed genes without enforcing a lineage bias (Lin *et al*, 2012; Nie *et al*, 2012). Moreover, *MYC* is frequently overexpressed in both human acute myeloid and lymphoid leukemia (Majeti *et al*, 2009; Haferlach *et al*, 2010) (Appendix Fig S1A) and can rapidly induce hematopoietic neoplasias in mice (Luo *et al*, 2005). We therefore overexpressed *Myc*, together with *Bcl2* to prevent apoptosis, in murine thymic and BM-resident precursor cells and compared their lineage potential in leukemogenesis.

Results

Single DN2 cells possess malignant myeloid and T-lymphoid potential *in vitro*

To evaluate the lineage potential of transformed T-cell precursors, we isolated DN2 cells from the thymus along with BM LSK cells (Lineage[−] Sca1⁺ c-kit⁺ as a population enriched for HSCs) and granulocyte-macrophage progenitors (GMPs) from C57BL/6 CD45.2 donor wild-type mice. Cells were transduced with a retrovirus co-expressing *Myc* and *Bcl2* from a bicistronic mRNA (*Myc/Bcl2*) (Appendix Fig S1B–D). From these populations, we sorted single cells into 96-well plates

containing 1:1 mixtures of OP9:OP9-DL1 as feeder cell layers and analyzed the resulting clones via flow cytometry for the expression of CD11b and Gr1 as myeloid markers, and CD25 as an early T-lineage marker which was in line with previous reports (Bell & Bhandoola, 2008; Wada *et al*, 2008). *Myc/Bcl2*-transduced DN2 cells generated immortalized clones with 8.6% plating efficiency, all of which had either mixed myeloid/T-cell or pure T-cell phenotypes (Fig 1A and B). From the mixed-lineage clones, flow-sorted myeloid cells or lymphoid cells demonstrated typical blast-like myeloid or lymphoid morphologies, respectively, and produced short-latency leukemias in transplanted mice, indicating their fully transformed states (Fig 1C). In contrast to DN2 cells, single *Myc/Bcl2*⁺ GMPs produced exclusively myeloid clones, and single *Myc/Bcl2*⁺ LSK cells produced predominantly pure myeloid clones but also mixed myeloid/T-cell and pure T-cell clones at lower frequencies (Fig 1B and D). These results show that at the single-cell level, transformed DN2 cells do not exclusively produce T-lymphoid leukemia, but can also develop into myeloid leukemia.

The myeloid potential of DN2 cells was stable across the leukemia transforming event, as transduction with the *MLL-AF9* [(9;11)] oncogenic fusion also generated mixed myeloid/T-cell clones at high frequency (Appendix Fig S2A and B). This suggests that the potential to produce myeloid blasts represents a feature that is endogenous to transformed DN2 cells.

DN2 cells with T-cell receptor rearrangement can generate myeloid leukemic progeny

The defining step in T-cell development is rearrangement of the T-cell receptor (TCR) locus. Thus, to underscore that T-cell progenitors can transform into myeloid blasts, we first identified rearrangement of the TCR beta locus in *Myc/Bcl2*⁺ DN2- and DN1-derived myeloid clones (Appendix Fig S2C) and subsequently transplanted one of each into mice. In both cases, the donor cells rapidly produced lethal myeloid leukemias, in which the blasts carried the same rearrangements as the initial clones (Fig 1E and F and data not shown). This experiment provides genetic evidence that myeloid leukemia can originate from T-cell progenitors.

Transformed DN2 cells give rise to leukemias with clonal myeloid and T-lymphoid populations *in vivo*

To directly assess the leukemic lineage potential *in vivo*, we first transduced DN2 cells with *Myc/Bcl2* and then immediately transplanted them intravenously into sublethally irradiated congenic mice. In line with the *in vitro* results, *Myc/Bcl2*⁺ DN2 cells produced leukemias consisting of myeloid, T-cell, and biphenotypic fractions in the same recipients (Fig 2A). We also did this for *Myc/Bcl2*⁺ LSK cells, which also produced mixed-lineage leukemia, but with reduced T-lymphoid and biphenotypic fractions. The *Myc/Bcl2*⁺ GMPs exclusively produced myeloid leukemia. We also generated *Myc/Bcl2*⁺ DN3 cells, representing a more differentiated T-cell progenitor, which produced exclusively T-lymphoid leukemia, in part with dim co-expression of myeloid markers, but never myeloid leukemia (Appendix Fig S3A).

The mean survival of *Myc/Bcl2*⁺ DN2, LSK and GMP recipients was comparably short (56 ± 5, 39 ± 5 and 49 ± 4 days, respectively) (Fig 2B). The animals showed massive infiltration of the BM

Figure 1. Single DN2 cells possess malignant myeloid and T-lymphoid potential.

- A** Representative FACS plot (left) showing a mixed-lineage clone with a T-lymphoid (CD25⁺/CD11b⁻Gr1⁻), myeloid (CD11b⁺Gr1⁺/CD25⁻), and biphenotypic (CD11b⁺Gr1⁺/CD25⁺) fraction derived from a single *Myc/Bcl2*-transformed DN2 cell grown on 1:1 OP9:OP9-D11 co-culture with lineage-promiscuous cytokines IL-2, IL-3, IL-6, IL-7, SCF, GM-CSF, Flt3. Morphology of the indicated flow-sorted cell fractions was analyzed by Giemsa staining (right).
- B** Phenotype frequencies of clones from *Myc/Bcl2*-transformed single cells (grown and phenotyped as mentioned in A). Analyzed were 31 clones from LSKs, 107 from GMPs and 28 from DN2 cells.
- C** Mice that received 5×10^4 to 5×10^5 T-lymphoid cells or myeloid cells flow-sorted from mixed *Myc/Bcl2*⁺ DN2-derived clones showed a cumulative survival of 31 ± 2 and 27 ± 6 days respectively. The summarized data are from three mice injected with the T-lymphoid and four mice injected with the myeloid fraction of two different clones.
- D** Representative FACS plots demonstrating predominant outgrowth of myeloid clones from single *Myc/Bcl2*-transformed LSK or GMP cells on OP9:OP9-D11 co-cultures.
- E** (top panels) Serial replating of *Myc-Bcl2*⁺ DN2 cells in methylcellulose generated myeloid clones with stable genomic reassembly of the Dβ1 TCR locus *in vitro* (gel: left two lanes). Upon transplantation, the same clones induced myeloid leukemia that retained the initial rearrangement *in vivo* (gel: right two lanes) as assessed by nested PCR. GL = germline. (bottom panels) Methylcellulose-based replating of *Myc-Bcl2*⁺ GMPs generated myeloid clones that exclusively displayed the TCRβ locus in germline configuration.
- F** Cumulative survival of mice that received 10⁶ cells of TCRβ-rearranged myeloid DN2-derived clones leading to death 33 ± 4 days after transplantation. Two DN2 clones were transplanted into five recipient mice.

Figure 2. *Myc/Bcl2*⁺ DN2 cells give rise to clonal myeloid and T-lymphoid leukemia *in vivo*.

- A** Representative FACS plots of spleens from leukemic recipient mice that had received LSK, GMP, or DN2 cell grafts immediately after retroviral *Myc/Bcl2* transduction. Numbers indicate frequencies (%) of CD45.2 donor cells within the indicated gates summarizing the mean ± SEM of at least eight recipients per group from a total of 10 independent experiments.
- B** Cumulative survival of mice that received 2×10^4 to 2×10^5 freshly *Myc/Bcl2*-transduced LSK, GMP, or DN2 cells. Graph shows $n = 11$ *Myc-Bcl2*⁺ LSK-, $n = 14$ DN2-, and $n = 8$ GMP-transplanted animals.
- C** Histological spleen sections of *Myc-Bcl2*⁺ LSK-, GMP-, or DN2-transplanted mice displaying myeloperoxidase staining. Leukemic cell engraftment in spleens ranged from 75.1% to 95.5%.
- D** Multiplex quantitative RT-PCR demonstrating strong expression of PU.1 in the myeloid (M3L: CD11b⁺Gr1⁺/CD3⁺CD4⁺CD8⁺) and biphenotypic T/myeloid (BL: CD11b⁺Gr1⁺/CD3⁺CD4⁺CD8⁺) fractions but not in the T-lymphoid (TL: CD11b⁺Gr1⁺/CD3⁺CD4⁺CD8⁺) fraction that were flow-sorted from diseased *Myc-Bcl2*⁺ DN2-transplanted mice. Myeloid progeny of *Myc/Bcl2*⁺ GMP-transplanted or T-lymphoid progeny of *Myc/Bcl2*⁺ DN3-transplanted animals as well as healthy thymocytes (Thy) and BM cells from WT mice served as controls. Results are shown as fold over actin transcripts. Error bars define standard deviation (SD), number of replicates $n = 2$.
- E** D-J rearrangement PCR of flow-sorted T-lymphoid (TL), biphenotypic (BL) and myeloid (ML) fractions of donor splenocytes of a diseased *Myc/Bcl2*⁺ DN2-transplanted mouse. All three fractions revealed a stable genomic reassembly of the Dβ1 TCR locus (Dβ1Jβ1.5) demonstrating clonality in DN2 leukemia. thy = thymus.
- F** FACS plot of diseased intrathymic grafts (CD45.2-gated), demonstrating that *Myc/Bcl2*⁺ DN2 cells could produce multi-lineage leukemia within a T-cell-supporting environment; *Myc/Bcl2*⁺ GMPs remained myeloid-restricted under these conditions. Numbers indicate cell frequencies within the gates (mean ± SEM) representing six independent experiments.
- G** Intrathymic transplantation of mock-infected non-leukemic DN2 cells produced predominantly T-lymphoid but also myeloid progeny 23 days after injection. This differentiation outcome resembles that of freshly isolated DN2 cells (Bell & Bhandoola, 2008; Richie Ehrlich et al, 2011) and indicates that the short-term culture necessary for retroviral transduction did not interfere with the physiological lineage potential of DN2 cells.

with myeloid blasts, leukocytosis, infiltration of lymphatic tissues (splenomegaly, lymphadenopathy), and indications of extralymphatic infiltration (hind limb paralysis). In many mice, we observed elevated white blood cell counts ($> 50 \times 10^3/\mu\text{l}$ as compared to $9.2 \pm 2.4 \times 10^3/\mu\text{l}$ in healthy animals; data not shown). A detailed flow-cytometric characterization of *Myc/Bcl2*⁺ DN2-leukemia is given in Appendix Fig S3B and C. This revealed heterogeneous marker expression in the biphenotypic population. The myeloid population expressed both Gr1 and/or CD11b, as well as myeloperoxidase and PU.1, a key myeloid identity transcription factor that is not expressed in T cells (Rothenberg *et al*, 2008; Fig 2C and D, and Appendix Fig S3B–F). Notably, TCR rearrangement analyses demonstrated clonal relationships between the T-lymphoid, biphenotypic, and myeloid fractions in leukemic *Myc/Bcl2*⁺ DN2-recipient mice (Fig 2E), suggesting that single DN2 cells have multi-lineage potential *in vivo*.

To exclude the possibility that the short-term cultivation required for retroviral transduction imposed a myeloid lineage bias on DN2 cells, we showed that the *in vivo* differentiation capacity of mock virus-transduced cells was similar to that of DN2 cells freshly isolated from the thymus (Fig 2G; Richie Ehrlich *et al*, 2011). Moreover, to more closely model the physiological transformation process *in vivo*, we also transplanted *Myc/Bcl2*⁺ DN2 cells directly into the thymus, and confirmed their myeloid leukemic potential (Fig 2F). Intrathymic *Myc/Bcl2*⁺ DN2- or GMP-grafts developed full-blown leukemia after 36 ± 2 and 46 ± 5 days, respectively (Appendix Fig S3G).

When comparing cell engraftment in BM, spleen, and thymus, we found that DN2- and GMP-derived blasts disseminated differently. *Myc/Bcl2*⁺ cells from GMPs preferentially infiltrated the organ(s) associated with their site of transplantation. Intravenously transplanted GMP-derived blasts primarily infiltrated the BM and spleen rather than the thymus (Appendix Fig S3H), while intrathymically transplanted GMP-derived blasts mainly stayed within the thymus. In contrast, *Myc/Bcl2*⁺ DN2-derived blasts infiltrated equally into all organs, suggesting an enhanced dissemination capacity.

DN2-myeloid blasts have DC-like identity and retain a cell-of-origin memory

To determine the molecular mechanisms that drive development of myeloid blasts from DN2 cells, we performed gene expression profiling of flow-sorted myeloid fractions of *Myc/Bcl2*⁺ DN2-, LSK- and GMP-derived leukemias from sick mice, along with normal DN2 and GMP cells from healthy wild-type (WT) mice (Dataset EV1). To better disclose the lineage identity of the diseased cells, we used gene set enrichment analysis (GSEA) to compare transcriptomes of the flow-sorted myeloid fractions to gene signatures defining

different myeloid cell populations (Schonheit *et al*, 2013). Whereas the GMP- and LSK-derived myeloid blasts mainly demonstrated a neutrophil identity, DN2-derived myeloid blasts exhibited a pan-dendritic cell (DC) signature (Fig 3A), a finding confirmed with another DC signature (Chan *et al*, 2006; data not shown). This was in line with residual DC potential that has been assigned to early T-cell progenitors (Moore *et al*, 2012).

Through a heat map comparison of the top 100 genes most differentially expressed ($P \leq 0.05$) in each of the analyzed cell populations, we revealed that DN2- and GMP-derived myeloid blasts clearly differed from each other, but LSK-derived blasts had an intermediate phenotype (Fig 3B and Dataset EV2). Yet, unsupervised hierarchical clustering across the entire transcriptomes revealed that LSK- and GMP-derived cells are more closely related to each other than they are to DN2-derived cells (Fig 3C). Moreover, GSEA demonstrated that a signature of genes highly expressed in WT DN2 cells was significantly enriched in the DN2- but not in GMP- or LSK-derived myeloid blasts (Fig 3D and Dataset EV3). Collectively, gene expression profiling revealed that DN2-leukemia adopts a unique myeloid lineage identity, and still retains expression of a memory of its pre-malignant T-cell origin.

The T-cell memory is required for DN2-leukemia growth

We next assessed if the T-cell memory was involved in driving *Myc/Bcl2*⁺ DN2-leukemia. Because cell-specific gene programs are mainly regulated by transcription factors (Rosenbauer & Tenen, 2007; Rothenberg *et al*, 2008), we searched for genes in the thymic memory that encode transcription factors and identified *Bcl11b* and *Gata3* as two promising candidates (Sambandam *et al*, 2005; Rothenberg, 2012). We confirmed expression of both proteins in DN2-derived leukemia, including the myeloid and biphenotypic fractions (Fig 4A and Appendix Fig S4A and B), and generated a series of lentiviral constructs expressing different shRNAs to silence either factor (Appendix Fig S4C and D). Indeed, knocking down *Bcl11b* or *Gata3* impaired growth of DN2- but not GMP-derived leukemia in culture, and completely abolished DN2-leukemic development in transplanted animals (Fig 4B–D). Because both factors are important for normal T cells but not myeloid cells, we flow-sorted the DN2-derived biphenotypic and myeloid fractions and individually transduced them with the lentiviral shRNA constructs, revealing that both fractions required *Bcl11b* and *Gata3* for growth (Appendix Fig S4E). These experiments suggest that DN2-leukemogenesis requires the T-cell memory.

Lineage plasticity of DN2-leukemia

While leukemias with enhanced lineage plasticity have not yet been linked to a distinct cell-of-origin, a good candidate is stem cells

Figure 3. DN2-myeloid leukemia retains a T-cell memory.

- GSEA demonstrates that *Myc/Bcl2*⁺ DN2-derived myeloid blasts were significantly ($\text{FDR} \geq 0.05$) enriched for expression of a pan-dendritic cell gene set. Gene sets were published in Schonheit *et al* (2013).
- Heat map of the top 100 differentially expressed genes (adjusted P -value of ≤ 0.05) in each myeloid fraction (ML: CD11b⁺Gr1⁺/CD3[−]CD4[−]CD8[−]) from GMP-, LSK-, and DN2-derived leukemias.
- Dendrogram revealing transcriptional relationships between *Myc/Bcl2*⁺ DN2-, LSK-, and GMP-derived myeloid blasts based on unsupervised hierarchical clustering of all probes on the microarray.
- GSEA demonstrates significant enrichment of a normal DN2-specific gene set (see Materials and Methods section) in the *Myc/Bcl2*⁺ DN2-derived myeloid blasts.

Figure 3.

Figure 4. *Bcl11b* and *Gata3* are required for DN2-leukemia.

- A** Western blot illustrating abundant Bcl11b and Gata3 protein expression in *Myc/Bcl2*⁺ DN2- but not LSK- or GMP-derived blasts. Thymus (Thy) and bone marrow (BM) extracts from healthy WT mice served as controls. Detection of α -tubulin or valosin-containing protein (VCP) ensured comparable loading.
- B, C** Proliferation assays illustrating growth of *Myc/Bcl2*⁺ DN2- (**B**) or GMP-derived (**C**) blasts after lentiviral transduction with the indicated shRNAs. The shRNA-transduced cells were flow-sorted based on co-expression of the RFP reporter and seeded on OP9/OP9-D11 1:1 layers. Growing cells were RFP sorted for three rounds within 21 days of culture and percent growth was calculated over the initially seeded cell number in relation to the Luc control. Shown are results of four independent experiments with cells from four different DN2- and three different GMP-leukemic animals. Luc = control shRNA targeting luciferase (Suzuki *et al*, 2003). At least two different shRNAs were used against *Bcl11b* or *Gata3* as indicated. * $P \leq 0.05$, Student's *t*-test. Error bars define SEM.
- D** Representative FACS plots showing CD45.2-donor splenocytes of a secondary (CD45.1) recipient 23 weeks after transplantation with 4×10^3 RFP⁺ *Myc-Bcl2*⁺ DN2-leukemia blasts carrying the indicated shRNAs. Luc: luciferase control shRNA. The data are representative of 2 *Gata3*, 4 *Bcl11b*, and 2 luciferase shRNA-transplanted animals.

(Cheng *et al*, 1986; Dorantes-Acosta & Pelayo, 2012). However, because we identified a T-cell memory in the DN2-derived myeloid blasts, we hypothesized that they may harbor myeloid/T-lymphoid

infidelity. To test this, we flow-sorted the myeloid fractions of *Myc/Bcl2*⁺ LSK-, DN2-, or GMP-derived leukemias established in primary mice and performed transplantation assays into secondary

Figure 5. Enhanced plasticity of DN2-derived myeloid leukemia depends on dynamic PU.1 expression.

- A** Representative FACS plots of splenocytes from diseased secondary recipients that were transplanted with 10^4 to 10^5 flow-sorted myeloid leukemia blasts (ML: $CD11b^+Gr1^+/CD3^-CD4^-CD8^-$) from sick primary *Myc/Bcl2*⁺ LSK-, GMP-, or DN2-transplanted mice. Cell percentages in indicated gates are shown.
- B** Table summarizing the phenotype frequencies within the spleens of secondary recipients that were retransplanted with *Myc/Bcl2*⁺ LSK-, GMP-, or DN2-derived myeloid blasts as described in (A). T-lymphoid leukemia: (TL: $CD11b^-Gr1^-/CD3^+CD4^+CD8^+$), biphenotypic leukemia: BL ($CD11b^+Gr1^+/CD3^+CD4^+CD8^+$), myeloid leukemia: ML ($CD11b^+Gr1^+/CD3^-CD4^-CD8^-$). Numbers before the dash represent mice holding the indicated leukemia fraction; numbers after the dash indicate the total mouse count analyzed.
- C** Single myeloid blasts from four different *Myc/Bcl2*⁺ DN2-derived leukemias were flow-sorted from transplanted mice and grown on irradiated OP9:OP9-DL1 stroma. The clones were analyzed at two different time points. FACS plots of two representative clones, one with strong (top), and one with weak (bottom) lineage switching potential are shown.
- D** Table summarizing the lineage switching outcome of 20 independent clones that were grown from single DN2-derived myeloid blasts of four different leukemias. Percentages at the right indicate the relative amounts of cells with T-lymphoid (TL), biphenotypic (BL), or myeloid (ML) phenotype as analyzed by FACS at the end of the experiment.
- E** FACS plot of DN2-derived primary leukemic cells transduced with a retrovirus carrying GFP alone or GFP together with PU.1 as a bicistronic RNA. The transduced cells were grown on OP9/OP9-DL1 1:1 layer for 8–13 days and analyzed using a GFP gate. The data are representative of 3 different experiments with similar outcome.

recipients. Whereas the GMP-derived blasts remained exclusively myeloid, indicating a complete lack of plasticity, those originating from LSKs converted into a biphenotypic population which in some

mice was accompanied by a myeloid fraction (Fig 5A and B). However, the LSK-derived myeloid blasts did not adopt a pure T-lymphoid phenotype, indicating incomplete plasticity. In contrast,

only DN2-derived blasts were able to completely convert from the myeloid into a T-lymphoid phenotype *in vivo*. We next sorted single myeloid blasts from DN2-leukemic mice onto OP9:OP9-DL1 stromal cells, and confirmed their potential to switch phenotype at the clonal level (Fig 5C). Intriguingly, not all clones possessed plasticity to the same extent (Fig 5D), suggesting that the cells in the myeloid fraction had differential lineage potential.

We also assessed plasticity of the T-lymphoid and biphenotypic populations from *Myc/Bcl2*⁺ LSK- and DN2-derived leukemias upon transplantation into secondary recipients; we found comparable abilities to switch between T-lymphoid and biphenotypic states, but low abilities to adopt the myeloid phenotype (Appendix Fig S5A). Taken together, these experiments demonstrate significant plasticity in both LSK- and DN2-derived leukemias and reveal a unique lineage switching capacity especially in the DN2-derived myeloid leukemia population.

PU.1 expression can determine plasticity in DN2-leukemia

PU.1 strongly regulates myeloid gene expression (Rosenbauer & Tenen, 2007) and is expressed in the myeloid and biphenotypic but not T-lymphoid fraction of DN2-leukemia (Fig 2D). To test whether dynamic PU.1 expression determines lineage conversion of *Myc/Bcl2*⁺ DN2 cells, we transduced DN2-leukemia bulks from sick mice with a retrovirus coexpressing PU.1 and GFP and analyzed lineage distribution in culture. While control virus-transduced cells predominantly gave rise to progeny with T-lymphoid markers, PU.1-expressing cells produced mainly myeloid or biphenotypic progeny (Fig 5E). Next, we transduced flow-sorted T-lymphoid cells from DN2-derived leukemias with the PU.1-expressing retroviruses. After just 4 days, forced PU.1 expression reprogrammed most of them into biphenotypic or, to a lesser extent, myeloid blasts (Appendix Fig S5B), suggesting that dynamic PU.1 expression may dictate lineage outcome in DN2-derived leukemia.

DN2-leukemia is distinct from human ETP-ALL

We next set out to identify a human disease that may correlate with murine DN2-leukemia. Comparison of orthologous gene expression has recently been used to define a novel human T-cell acute lymphoblastic leukemia (T-ALL) that shows co-expression of myeloid and stem cell markers (Coustan-Smith *et al*, 2009). Based on gene expression similarity with murine early T-cell precursors (ETP), representing a DN1 subpopulation, this disease has been speculated to originate from ETPs and was congruently termed ETP-ALL (Neumann *et al*, 2012). Thus, using GSEA, we tested whether murine DN2-leukemia corresponds to human ETP-ALL; however, we detected a negative correlation between both leukemia types

using two independently published ETP-ALL gene expression signatures (Appendix Fig S6A and B).

A cohort of immature human AML cases resembles mouse DN2-leukemia

To determine a possible human counterpart of mouse DN2-leukemia, we conducted an exploratory search concentrating on myeloid entities (AMLs). We used the set of the top 100 up- and downregulated genes ($P \leq 0.05$) in the analyzed myeloid blasts from our mouse model, and compared it to the transcriptomes of 542 human AML samples (Haferlach *et al*, 2010). Indeed, the DN2-derived myeloid leukemia gene signature was similar to 26 samples (frequency: 4.8%) as assessed via a Spearman's rank correlation coefficient higher than 0.5 (Fig 6A and Dataset EV4). A bootstrapping approach (see Materials and Methods) yielded a highly significant enrichment value ($P = 0.006$) of the mouse DN2-leukemia signature in the human samples, thus confirming a non-random correlation. All DN2-like human AML samples of which metadata were available (8 of the 26 patients) had an immature phenotype, as evident from their French-American-British (FAB) classification as M0 (2 patients), M1 (1 patient), or M2 subtypes (5 patients), whereas the whole cohort consisted of M0-M6 patients (Dataset EV5).

We next validated this finding using a second human AML dataset (Verhaak *et al*, 2009) as it contained metadata for all patients, and focused on the FAB M2 subtype to achieve higher comparability within the samples. We identified nine M2 AMLs resembling the murine DN2-leukemia, which clustered together and were significantly enriched for the mouse DN2-myeloid leukemia signature (Fig 6B and C). Their mutational spectrum resembled that of other AMLs (Appendix Fig S7A and Dataset EV4). Moreover, the DN2-like M2 AML patients expressed significantly more *GATA3* and *BCL11B* as well as T-cell receptor alpha (*TRAC*) transcripts, but had unchanged *MYC* levels (Fig 6D and Appendix Fig S7B and C). They also had DC-like transcriptional identity (Fig 6E). Collectively, transcriptome comparisons identified a potentially novel cohort of immature human AMLs that resembles mouse DN2-derived leukemia.

DN2-leukemia is refractory to standard AML therapeutic agents

Because lineage plasticity represents a possible non-mutational mechanism for cancer cells to escape therapy (Glickman & Sawyers, 2012), we used our mouse model to test if *Myc/Bcl2*⁺ DN2-derived blasts would be refractory to standard AML drugs (Estey, 2009). Indeed, compared to their GMP-derived counterparts, DN2-derived cells were significantly less sensitive to the anthracyclines daunorubicin and doxorubicin *in vitro* (Fig 7A).

Figure 6. A cohort of immature human AML patients resembles mouse DN2-leukemia.

- A Heat map depicting all human AML samples from the Haferlach study (Haferlach *et al*, 2010) whose transcriptomes resembled DN2-leukemia by a Spearman's rank correlation coefficient ≥ 0.5 . The top 100 up- and downregulated gene signature of *Myc/Bcl2*⁺ DN2-derived myeloid blasts was applied (see also Fig 3B).
- B Hierarchical clustering of all FAB M2 AML samples from the Verhaak study (Verhaak *et al*, 2009) based of the *Myc/Bcl2*⁺ DN2-derived myeloid blast top 100 upregulated (UP) gene signature (see Fig 3B).
- C–E GSEA confirming enrichment of the *Myc/Bcl2*⁺ DN2-derived myeloid blast top 100 UP (C) and pan-DC (Schonheit *et al*, 2013) (E) gene signatures in the human FAB M2 DN2-like AML samples as compared to all residual M2 AMLs from the Verhaak study (Verhaak *et al*, 2009). (D) Relative transcript levels of the T-cell receptor alpha locus (*TRAC*) in human DN2-like M2 AML compared to residual M2 AML patient samples from the Verhaak study (Wilcoxon rank-sum test, $**P \leq 0.01$). Boxes represent 25–75% quantiles, whiskers represent minimum and maximum values.

Figure 6.

An *in vivo* shRNA screen reveals sensitivity to Jak2/Stat3-pathway inhibition

We next sought to explore other therapeutic options to target DN2-leukemia. To this end, we conducted an *in vivo* genetic loss-of-function screen using an in-house generated retroviral library expressing shRNAs against 142 genes selected for their general roles in leukemogenesis and/or HSC function (Dataset EV6). We isolated primary *Myc/Bcl2*⁺ DN2-leukemic cells from diseased mice and transduced them with the pooled shRNAs (Fig 7B), after which the transduced cells were sorted and either processed as reference samples or intravenously injected into sublethally irradiated secondary hosts. Upon harvesting the BM, spleen and thymi of the diseased hosts, we determined the relative frequencies of each integrated shRNA (compared to reference samples) by deep sequencing. The shRNA frequency changes correlated in the three analyzed organs (Fig 7C and Appendix Fig S8A). Depletion of a particular shRNA during the disease course indicated a candidate target gene promoting *Myc/Bcl2*⁺ DN2-leukemogenesis. Strikingly, among the five top candidate genes, two were different components of the Jak/Stat pathway, *Jak2* and *Stat3* (Fig 7D).

To corroborate a functional role of Jak/Stat signaling, we retrovirally transduced five *Myc/Bcl2*⁺ cell lines derived from independent primary DN2-leukemic mice with two different shRNAs targeting *Jak2*. After demonstrating *Jak2* knockdown (Appendix Fig S8B), we performed proliferation assays in culture (Fig 7E). Indeed, with the exception of one outlier, *Jak2* knockdown reproducibly impaired proliferation of the DN2-leukemic lines.

Jak2 (and *Jak1*) can be selectively inhibited by ruxolitinib, which is approved for the treatment of myelofibrosis (Treanor *et al*, 2014). To determine whether ruxolitinib would have an anti-proliferative effect, we cultured murine *Myc/Bcl2*⁺ DN2-leukemic lines with the drug and measured proliferation. To test specificity, we also treated cell lines derived from sick *Myc/Bcl2*⁺ GMP- and LSK-transplanted mice. All of the DN2- but none of the GMP- or LSK-derived lines showed a significant retardation in cell

growth (Fig 7F and Appendix Fig S8C). We confirmed activated STAT3 and its inhibition by ruxolitinib in the DN2-leukemic lines (Fig 7G). Finally, significant enrichment of JAK/STAT signaling signature genes suggested that this pathway is also activated in human DN2-like M2 AML cells (Fig 7H). Collectively, these results propose Jak2/Stat3 signaling as a potentially druggable target in DN2-leukemogenesis.

Discussion

In the present study, we demonstrated that oncogene-transformed murine DN2 T-cell progenitors harbor malignant multi-lineage capacity and a high intrinsic potential to transdifferentiate into myeloid and biphenotypic leukemia. Focusing particularly on the myeloid disease, we showed that DN2-derived leukemia is characterized by several unique features: It has (i) transcriptional similarity with DCs, (ii) functional thymic origin memory, (iii) persisting myeloid/T-lymphoid plasticity, (iv) resistance to standard chemotherapy, and (v) dependency on Jak2/Stat3 signaling. Importantly, we identified a possibly correlating human AML cohort (comprising ~5% of all cases) of potential thymic origin and pathogenesis similar to the murine disease. Congruently, the disease of the human cohort may represent a new subgroup of thymus-derived AML. Moreover, our results propose a general model in which not-yet-fully stabilized competing differentiation states of developing progenitors shape plastic lineage identities in cancer.

Leukemic lineage outcomes of T-cell progenitors have not yet been systematically investigated. To dissect possible lineage fate options of transformed DN2 cells, we delivered ectopic expression of *Myc* together with *Bcl2* and compared these cells with equally treated LSK and GMP cells. In humans, MYC expression is increased (e.g., through activating *NOTCH1* mutations) in more than 50% of T-ALLs (Palomero *et al*, 2006; Weng *et al*, 2006) and more than 40% of AMLs (Larramendy *et al*, 2002; Court *et al*, 2004). Overall, this highlights that MYC is among the most

Figure 7. A targeted shRNA *in vivo* screen reveals requirements of Jak2/Stat3 signaling in DN2-leukemia.

- Survival assay of *Myc/Bcl2*⁺ GMP- and DN2-derived leukemia cells that were isolated from diseased mice and treated for 24 h with 0.15 μ M daunorubicin or 1 μ M doxorubicin in liquid culture. Cell death was measured by FACS of 7-AAD⁺ cells and was normalized to the solvent controls. The graphs show the mean+SEM of three different DN2- and five GMP-leukemias. ** $P \leq 0.01$, two-tailed Student's *t*-test.
- Experimental procedure of the shRNA library *in vivo* screen. Blasts from leukemic mice that were transplanted with *Myc/Bcl2*⁺ DN2-cells were infected with a retroviral shRNA library consisting of ~300 GFP- or YFP-tagged shRNAs targeting a total of 142 different genes. Cells were harvested 72 h postinfection, the GFP⁺ and YFP⁺ fractions were flow-sorted, and 6×10^4 cells were transplanted into secondary recipients. The residual cells were expanded for five days on OP9:OP9-Dll1 1:1 layers for amplification (T0 reference samples). Secondary recipients were sacrificed after development of leukemia, and T1 samples were generated from BMs, spleens, and thymi, which confirmed BM infiltration > 20%. T1 and T0 samples were deep sequenced, and changes in individual shRNAs were calculated.
- Scatterplot depicting the averaged quantitative changes (log10) of each shRNA within the library in the T1 spleen and BM samples in reference to T0. The changes in both organs were concordant ($R^2 = 0.72$). T1 samples from 11 mice were compared to 3 T0 reference samples. Blue dots represent shRNAs with a significant depletion by ≥ 10 -fold.
- Top five genes with ≥ 10 -fold shRNA depletion in BM, spleen, and thymus in comparison with the reference.
- Percent cell growth of five different DN2-derived leukemic cell lines transduced with two different shRNA constructs targeting *Jak2*. The cells were sorted for the GFP or YFP reporter and normalized to the control shRNA targeting the lacZ gene (lacZ). Four of five DN2-derived leukemias show reduced growth with both shRNAs after the second round of sorting within eight days in culture. The lacZ control was set to 100%.
- Proliferation curves of 3 *Myc/Bcl2*⁺ DN2- and 3 GMP-derived leukemia lines during treatment with 0.5 μ M ruxolitinib. 1.5×10^5 cells were seeded in culture and counted at the indicated time points. Results are shown as fold growth relative to the starting cell number which was set to 1. * $P \leq 0.05$, ** $P \leq 0.01$, Student's *t*-test. Error bars define standard deviation (SD), number of replicates $n = 3$.
- Western blot indicating active Jak/Stat signaling in DN2-derived leukemia with Stat3 as well as pStat3 protein expression that diminishes after treatment with the Jak-inhibitor ruxolitinib.
- GSEA indicating significant enrichment of genes of the JAK/STAT signaling pathway of the KEGG pathway database in the DN2-like M2 AML samples as compared to the residual M2 AML samples using the Verhaak AML dataset (Verhaak *et al*, 2009).

Figure 7.

frequently upregulated proto-oncogenes in human acute leukemias and thus represents a useful model in our study. Although there are already published experimental examples of lymphoid/myeloid infidelity in leukemia, it has remained unclear if the transforming oncogenic events inadvertently activate this plasticity, thus overshadowing the possibility that plasticity is an endogenous characteristic of the transformed cells. MYC has been suggested to function as a general amplifier of gene expression rather than activating specific target gene sets (Lin *et al*, 2012; Nie *et al*, 2012). Although this view has recently been challenged (Sabo *et al*, 2014; Koh *et al*, 2015), our single cell-based experiments suggest at the functional level that ectopic *Myc* expression “freezes” endogenous lineage fate options because the lineage outcomes of *Myc*-transformed progenitors were similar to those of their healthy counterparts (Bell & Bhandoola, 2008; Wada *et al*, 2008; Richie Ehrlich *et al*, 2011). Thus, ectopic *Myc* expression appeared instrumental to dissect endogenous lineage options of transformed hematopoietic cells. Moreover, transformation of DN2 cells by MLL-AF9 also produced multi-lineage leukemias, providing further evidence for an intrinsic myeloid/T-lymphoid potential. Finally, this conclusion also agrees with the myeloid-based model of hematopoiesis, which explains the retained myeloid potential in other lineages (Kawamoto *et al*, 2010).

We focused our study primarily on the myeloid fraction of *Myc/Bcl2*⁺ DN2-derived leukemia and found several unique properties, one of which was expression of DC genes. Previous reports have shown DC potential of healthy thymic progenitors (Li *et al*, 2009; Moore *et al*, 2012), thus explaining our observation that DC-like blasts arise from transformed DN2 cells. Another unique feature of DN2-derived myeloid blasts was that they expressed a gene set shared by normal DN2 T-cell progenitors. This set likely represents an inherited transcriptional ancestor memory and includes genes encoding important T-cell transcription factors such as *Bcl11b* and *Gata3*, which, as our knockdown data show, are required for DN2-leukemia growth. Congruently, genes of the T-cell origin memory are of functional importance.

Another important question we addressed in this study was if mouse model DN2-leukemia has a human disease counterpart. We approached this by comparing orthologous gene expression. The most obvious correlating human disease seemed to be the recently described ETP-ALL, which is believed to arise from the earliest thymic precursors and expresses lymphoid together with myeloid and stem cell antigens on the diseased cells (Coustan-Smith *et al*, 2009; Zhang *et al*, 2012; Treanor *et al*, 2014). However, GSEA with two independently published ETP-ALL gene signatures clearly showed that DN2-leukemia is a different disease. Supporting this, ETP-ALL has shown stronger correlation in gene expression with human HSCs and GMPs than with ETPs (Zhang *et al*, 2012). Moreover, ETP-ALL lacks *GATA3* and *BCL11B* expression (Zhang *et al*, 2012), while DN2-leukemia requires both factors. In contrast with ETP-ALL, comparing the DN2-leukemia gene signature with published AML transcriptome data (Verhaak *et al*, 2009; Haeflrich *et al*, 2010) allowed us to identify a novel patient cluster of immature AMLs that significantly resembles mouse DN2-leukemia. Importantly, these patients expressed significantly more TCR alpha mRNA than the other AML patients. Furthermore, our first preliminary analysis of two DN2-like AML patients from which genomic DNA was available, suggested TCR rearrangement in at least one of

them (data not shown), supporting the view that a transformed T cell served as the ancestor. The DN2-like human AMLs harbored mutations in *DNMT3a*, *FLT3*, *IDH1*, *IDH2*, and *NPM1*, all of which are frequent in AML (Falini *et al*, 2011; Patel *et al*, 2011; How *et al*, 2013; Mayle *et al*, 2015). Interestingly, with the exception of *NPM1*, all of these mutations have also been found in T-ALL (Van Vlierberghe *et al*, 2005, 2013; Grossmann *et al*, 2013; Neumann *et al*, 2013), and their occurrence in both lineages has been explained by the assumption that they were acquired in a stem cell (Matutes *et al*, 1997; Dorantes-Acosta & Pelayo, 2012). Supporting this notion, we detected significant plasticity in leukemias derived from LSK cells. In this context, it was interesting that in addition to the polyclonal leukemic T-lymphoid fractions that developed in *Myc/Bcl2*⁺ LSK-transplanted mice, some of the animals also had monoclonal biphenotypic fractions (Appendix Fig S9). This finding suggests that individual T-lymphoid leukemia clones had the plasticity to switch their phenotype *in vivo*. Moreover, because we found that DN2 cells had leukemic multi-lineage potential, an important conclusion from our study could be that the same mutations in T-ALL and AML may also originate from a transformed T-cell progenitor instead of a stem cell. In line, the view of a T-cell ancestor for certain AMLs is supported by studies that have observed frequent TCR rearrangement in MLL-translocated adult AML and the hypogranular variant subtype of acute promyelocytic leukemia (APL) (Dupret *et al*, 2005; Chapiro *et al*, 2006).

Our data suggest that biphenotypic leukemia—now termed mixed-lineage acute leukemia (MPAL) by the WHO (Borowitz *et al*, 2008)—may develop from T-cell progenitors as well. Many of these cases carry rearrangement or deletions of the TCR or immunoglobulin heavy chain genes (Buccheri *et al*, 1993), underlining the idea that a transformed lymphoid progenitor with residual myeloid potential could be their origin. Importantly, patients with biphenotypic leukemia have a worse clinical outcome than matched AML or ALL patients, and there is no set therapy for this disease (reviewed in Weinberg & Arber, 2010). Hence, pathogenic mechanisms leading to biphenotypic leukemia are of high clinical interest.

A key feature of DN2-leukemia in mice was its myeloid/T-lymphoid plasticity, which we linked to dynamic PU.1 expression. PU.1 is an important transcription factor for myeloid differentiation, and forcing its expression can lead to reprogramming of normal lymphoid into myeloid cells (Laiosa *et al*, 2006a,b). During normal T-cell development, PU.1 expression is switched off between the DN2 and DN3 stages, representing the point in time when myeloid potential is lost (Rothenberg *et al*, 2008). From the results presented herein, it appears that the DN2-derived leukemia blasts inherited a unique epigenetic flexibility to dynamically control PU.1 expression from their healthy ancestors. Importantly, because our single-cell assays revealed that not all DN2-derived blasts had the same potential to switch phenotype, it is likely that this epigenetic flexibility is confined to special plastic cells within the leukemia population. On the other hand, lineage plasticity in leukemia can also result from particular properties of the transforming oncogene. For example, the CALM-AF10 t(10:11) fusion product can drive trans-differentiation of B-cell progenitors into leukemia with a myeloid phenotype (Deshpande *et al*, 2006). Thus, lineage infidelity in leukemia likely involves both intrinsic programs inherited from the healthy ancestor cells and epigenetic properties as a result of the transforming events.

Why might lineage infidelity be advantageous for a leukemia cell? The answer might relate to the fact that lineage switching can be observed after treatment-associated leukemia relapse; this switching includes the conversion of myeloid to lymphoid leukemia or vice versa (Stass *et al*, 1984; Gagnon *et al*, 1989; Liso *et al*, 1998). Indeed, lineage plasticity in human leukemia is associated with particularly poor survival (Matutes *et al*, 1997; Killick *et al*, 1999; Golemovic *et al*, 2006). In this context, we also investigated a drug response scenario and found that treating DN2-leukemic cells with daunorubicin or doxorubicin—both frequently used drugs for human AML therapy (Estey & Dohner, 2006; Estey, 2009)—negligibly affected the survival of *Myc/Bcl2*⁺ DN2-leukemic cells. Therefore, we were eager to uncover pathways in DN2-leukemia that might serve as druggable targets. Because shRNA libraries are instrumental for identifying such targets (Miller *et al*, 2013; Sroczynska *et al*, 2014), we developed a retroviral shRNA library and conducted an *in vivo* genetic loss-of-function screen. Importantly, Jak2 and Stat3 arose as top candidates. *JAK2* mutations are frequent in polycythemia vera (Scott *et al*, 2007), thrombocythemia (Campbell *et al*, 2005), and myelofibrosis (Campbell *et al*, 2006), and specific *JAK2* inhibitors such as ruxolitinib are now in clinical use (Egthedar *et al*, 2012; Harrison *et al*, 2012). Indeed, ruxolitinib treatment led to impaired growth of DN2- but neither GMP- nor LSK-derived leukemia, indicating that *JAK2* inhibition may be clinically beneficial. This view is supported by our finding that the human DN2-like AML samples also had enriched expression of the KEGG database *JAK/STAT* pathway signature, indicative of activated *JAK/STAT* signaling.

Taken together, this study presents evidence that T-cell progenitors may represent the cellular origin of a unique type of lineage-unstable leukemia. More generally, our results propose that in cancer, the cells' associated plasticity, growth, and therapy responses can be the result of genetic programs inherited from healthy ancestor cells. Thus, determining the ancestry of human cancers may aid the development of better treatment protocols.

Materials and Methods

Mice and cell lines

Thymus and BM were extracted from 6- to 12-week-old WT BL6 (C57BL/6) donor mice, purchased from Charles River Laboratories. *Myc/Bcl2*⁺ LSKs, GMPs, or DN2 cells were intravenously or intrathymically injected into B6.SJL (B6.SJL-Ptprca/BoyAiTac) congenic recipient mice. BL6 mice express CD45.2 (Ly5.2; Ptprc; protein tyrosine phosphatase, receptor type, C) and B6.SJL CD45.1 (Ly5.1; Ptprc; protein tyrosine phosphatase, receptor type, C), allowing for distinction of leukemic donor and healthy recipient cells. All mouse experiments were approved by the local authorities according to the German Federal Animal Protection Act.

Transplantation experiments

For cell transfer, we used 4- to 8-week-old recipient mice that received 4.5–6 Gy total body irradiation with the 18 MeV photon beam of a linear electron accelerator using a dose rate of

6.79 Gy/min or the Faxitron CP160 X-ray irradiation system with 0.55 Cu filter and a dose of 0.603 Gy/min. 2×10^4 – 1×10^6 malignant cells were intravenously injected. Leukocytosis with blast counts > 10% in peripheral blood derived from donor cells indicated acute leukemia (20% bone marrow infiltration). If recipient animals showed physical signs of full-blown leukemia (hind limb paralysis, loss of body weight, palpable spleen, or submandibular lymph nodes), they were euthanized. Intrathymic injections were performed as previously described (Goldschneider *et al*, 1986). Recipient mice were narcotized with a mixture of ketamine–xylazine, fixated with the ventral side pointing upwards the sterilized thorax area, and opened with a medial longitudinal skin section to expose the thymus. 3×10^4 donor cells were injected with a 27G canula into the two lobes of the thymus and the incision closed with wound clips.

Histology

Maximally 1×10^4 of cells were cytospun (Tharmac) at 33.5 g for 10 min onto object slides. Air-dried object slides were fixed in methanol for 10 min and incubated 10 min in May-Gruenwald staining solution (Sigma-Aldrich). Slides were washed with water and stained with Giemsa staining solution (Sigma-Aldrich) for 40 min. After the final washing step with water, slides were air-dried and closed with a drop of Roti[®]-Histokitt II and cover slips. For histology, parts of the spleen, sternum, or liver were transferred into a Histofix solution (Roth) and cross sections were prepared as described previously (Erny *et al*, 2015). May-Gruenwald Giemsa (MGG) and Myeloperoxidase (Ab-1, RB 373-A0 Thermo Scientific, 1:200) staining was conducted, and the slides were photographed with an Axioplan2 microscope/camera system (Zeiss).

Flow cytometry and cell sorting

Twenty 4- to 5-week-old donor mice were sacrificed per experiment to obtain approximately 5×10^4 DN2 cells from thymi and 6×10^4 LSK and 1×10^5 GMP from BM washout. Single-cell suspensions from thymi and BM were stained with the following antibodies conjugated with V450, PE, APC, or FITC: CD3 (500A2), CD4 (RM4-5), CD8a (53-6.7), CD19 (1D3), Gr1 (RB6-8C5), CD11b (M1/70), CD45R/B220 (RA3-6B2), CD25 (PC61), FcγRII/III (2.4G2), CD34 (RAM34), Sca-1 (D7), and c-Kit (2B8). Thymic and BM suspensions were incubated with CD117 (c-Kit) mouse microbeads (Miltenyi) according to the instructions of the supplier and enriched for c-Kit via magnetic cell separation in MACS LS columns (Miltenyi). Cell sorting was performed with a high-speed multi-color cell sorter (Aria II or III). Thymic precursors were defined via surface markers as described previously (Wada *et al*, 2008). All cells were sorted by exclusion of 7-AAD⁺ dead cells as well as doublets. After sorting, an aliquot of the sorted fractions was used for re-analysis and confirmation of at least 95% sort purity.

Primary leukemias were analyzed by FACS with antibodies against CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD11b (M1/70.15), Gr1 (RB6-8C5), CD45.1 (A20) and CD45.2 (104), TCR beta (H57-597), or TCR gamma/delta (GL3) conjugated with PE, APC, Pacific and Alexa Fluor 700. Intracellular CD3 staining was performed by Cell Fixation/Permeabilization Kits (BD).

Plasmids

MSCV-c-Myc-IRES-Bcl2 was described in Luo *et al* (2005). The shRNA library was subcloned into the PINCO-RFB-GFP-IRES-RE9 (Grignani *et al*, 1998) following the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit (Invitrogen) together with the Gateway® technology (Invitrogen). shRNAs against Bcl11b, Gata3, and luciferase were subcloned into the pRSI9-U6-(sh)-UbiC-TagRFP-2A-Puro (Addgene) following the Cellecta protocol. All shRNA sequences are available on request. The plasmids pLP1, pLP2 and VSVG, and pcDNA™ 6.2-GW/± EmGFP-miR-lacZ were from Invitrogen. MSCV-IRES-GFP-IRES-PU.1 was described in Rosenbauer *et al* (2004) and MSCV-IRES-GFP in Huntly *et al* (2004).

In vitro colony forming assays and single-cell sorts

Semi-solid culture of transformed precursors in methylcellulose was used to generate immobilized colonies; 24-h postinfection, ~1,000 *Myc/Bcl2*-transduced cells were plated in methylcellulose (M3234, Stem Cell Technologies) supplemented with 10 ng/ml IL-2, IL-3, IL-6, IL-7, SCF, GM-CSF, FLT3, and 20 µg/ml gentamicin; 500 ng/ml Fungizole; and 20 µg/ml ciprofloxacin. Methylcellulose suspensions were plated onto 35-mm cell culture dishes for 5–7 days before leukemic colonies were picked and separately expanded in a 96-well plate with fresh methylcellulose. Clones were expanded for extraction of genomic DNA.

Western blot analysis

Protein samples were prepared as described in Rosenbauer *et al* (2006). Proteins were detected with the antibodies anti-Ctip2/Bcl11b (25B6) (Abcam), anti-GATA-3 (510-20) (Abcam), anti-β-actin (AC-15 or A2066) (Sigma-Aldrich), anti-α-tubulin (B-5-1-2) (Santa Cruz), anti-VCP (5) (Abcam), anti-PU.1 (T-21X) (Santa Cruz), anti-pStat3 (Tyr705) (D3A7) (Cell Signaling Technology), and anti-Stat3 (124H6) (Cell Signaling Technology). For the detection of horseradish peroxidase coupled secondary antibodies, the ECL kit (Invitrogen) was used.

Gene expression profiling

The Affymetrix GeneChip® (*Mus musculus*) MG 430 PM was applied to investigate the expression of approximately 45,000 features in flow-sorted LSK-, GMP-, and DN2-derived myeloid leukemia cells. Sample processing and microarray hybridization were carried out at the Center of Excellence for Fluorescent Bioanalytics (KFB, University of Regensburg, Germany). The raw gene expression data were imported into R (R Core Team, 2015) and RMA normalized with the R package “affy” (Gautier *et al*, 2004). Murine gene signature generation, gene set enrichment analysis, and identification of human AML samples correlating to mouse DN2-leukemia are described in detail in the Appendix Supplementary Methods.

Statistical analysis

No statistical methods were used to predetermine sample sizes, and exact group numbers were determined by animal availability. However, we did ensure that our sample sizes were similar to those

generally employed in the field. To obtain unbiased data, experimental mice were not randomized or blinded but processed by scientists together with technicians and cell quantifications were performed by two scientists independently and separately. Microarray analysis was blinded, and sample preparation, processing, and analysis were performed by different scientists.

Two-tailed Student's *t*-test was carried out to determine the statistical significance of experimental results for normally distributed values. **P* ≤ 0.05 and ***P* ≤ 0.01. Alternatively, Wilcoxon rank-sum tests were used to determine statistical significance. ****P* ≤ 0.01.

Accession codes

GEO: array data, GSE80739.

Expanded View for this article is available online.

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Author contributions

PR, MC, JF, SG, VG, DL, and AW conducted experiments. CW, MZ, MLG, NM, and MG analyzed data; KA and SL generated the shRNA library; TH and RD provided patient data; MP conducted pathological analyses; MAA-N, GL, MD, and CM-T provided experimental support and supervised parts of this study. PR and FR designed the study and wrote the manuscript. FR supervised the entire project and provided financial support.

Conflict of interest

The authors declare that they have no conflict of interest.

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