ARTICLE

Long-term serial xenotransplantation of juvenile myelomonocytic leukemia recapitulates human disease in Rag2$^{-/-}\gamma c^{-/-}$ mice

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ABSTRACT

Juvenile myelomonocytic leukemia is a clonal malignant disease affecting young children. Current cure rates, even with allogeneic hematopoietic stem cell transplantation, are no better than 50%-60%. Pre-clinical research on juvenile myelomonocytic leukemia is urgently needed for the identification of novel therapies but is hampered by the unavailability of culture systems. Here we report a xenotransplantation model that allows long-term *in vivo* propagation of primary juvenile myelomonocytic leukemia cells. Persistent engraftment of leukemic cells was achieved by intrahepatic injection of $1x10^6$ cells into newborn *Rag2*$^{-/-}\gamma c^{-/-}$ mice or intravenous injection of $5x10^6$ cells into 5-week old mice. Key characteristics of juvenile myelomonocytic leukemia were reproduced, including cachexia and clonal expansion of myelomonocytic progenitor cells that infiltrated bone marrow, spleen, liver and, notably, lung. Xenografted leukemia cells led to reduced survival of recipient mice. The stem cell character of juvenile myelomonocytic leukemia was confirmed by successful serial transplantation that resulted in leukemia cell propagation for more than one year. Independence of exogenous cytokines, low donor cell number and slowly progressing leukemia are advantages of the model, which will serve as an important tool to research the pathophysiology of juvenile myelomonocytic leukemia and test novel pharmaceutical strategies such as DNA methyltransferase inhibition.

Introduction

Juvenile myelomonocytic leukemia (JMML) is a malignant myeloproliferative disorder of infancy and early childhood with an aggressive clinical course. Clinical symptoms are caused by hematopoietic insufficiency and excessive proliferation of leukemic monocytes and granulocytes, leading to hepatosplenomegaly, lymphadenopathy, skin rash and respiratory failure.[1-3] JMML is caused by hyperactivation of the RAS signaling pathway due to acquired activating mutations in the *KRAS, NRAS* or *PTPN11* genes,[4-7] or due to acquired loss of heterozygosity of the constitutionally deficient *NF1* gene in patients with neurofibromatosis type 1 or of the *CBL* gene in the Noonan-like "CBL syndrome".[8-13] JMML is rapidly fatal unless allogeneic hematopoietic stem cell transplantation (HSCT) is performed, but even this approach is burdened with a significant risk of recurrence.[14,15]

A serious obstacle to research into JMML is the lack of suitable experimental models, impeding the development and pre-clinical evaluation of novel therapeutic

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approaches. Primary JMML leukemia cells cannot be maintained in culture as they differentiate and become apoptotic.[16] An immortalized cell line derived from JMML cells has not yet been successfully established.[17] The generation of induced pluripotent stem cell lines originating from JMML cells was reported, but conceptually such systems are limited by their artificial nature and the risk of further transformation during reprogramming.[18] Several of the "canonical" JMML mutations that deregulate the RAS signaling pathway were studied in genetically engineered mouse models, successfully inducing myeloproliferative disorders in the experimental animals.[19-28] Those were, however, still murine leukemias, and critical disease characteristics of JMML such as recurrent monosomy 7 or elevated fetal hemoglobin are not simulated in transgenic systems.

Xenotransplantation into murine hosts offers the unique possibility of basic and translational research into living primary JMML cells, while at the same time propagating and multiplying this precious clinical material. However, earlier attempts at JMML xenotransplantation were compromised by difficult leukemia cell engraftment (presumably owing to residual natural killer cell activity of the host strains) or rapid demise of engrafted animals within a few weeks, and not all reports documented the xenologous engraftment of long-term leukemia-initiating cells *via* successful serial transplantation.[29-31] In addition, the experiments depended on high input cell numbers (up to $5x10^7$ cells), a considerable practical obstacle concerning the limited availability of primary clinical JMML material, and on costly repeated application of human granulocyte-macrophage colony-stimulating factor (GM-CSF).

Here we report the suitability of the *Rag2*$^{-/-}\gamma c^{-/-}$ mouse strain for the reproduction of primary human JMML in recipient animals. The system is characterized by good phenotypic imitation of typical disease features, long duration of xenologous engraftment, quantitative expansion of leukemic cell material outside the human organism, and the possibility of retransplantation to further expand cell numbers and extend the duration of experiments without additional input of cryopreserved material. Not least, the data support the stem cell character of long-term leukemia-initiating cells in JMML.

Methods

Primary cells

Human cells were collected after obtaining informed consent from parents or legal guardians and approval from institutional review committees. Samples from JMML patients were collected in the context of the European Working Group of MDS in Childhood (EWOG-MDS). Clinical information is provided in *Online Supplementary Table S1*. Single cell suspensions obtained from mashed spleens were subjected to density gradient centrifugation (Ficoll) to separate and cryopreserve mononuclear cells (MNC). Where indicated, MNC were depleted from $CD3^+$ T cells (MACS immunobeads, Miltenyi; <0.15% remaining T cells). Cord blood was obtained from healthy newborns and $CD34^+$ cells were enriched by the MACS technique (Miltenyi; purity >90%).

Xenotransplantation

All experiments were approved by local authorities and followed the German "Tierversuchsgesetz". *Rag2*$^{-/-}\gamma c^{-/-}$ BALB/c mice[32] were maintained in a specific pathogen-free environment. Newborn mice were irradiated with 2.5 Gy within their first four days of life. Eight hours after irradiation, JMML MNC were thawed and $1x10^6$ viable cells were injected intrahepatically (30 µl). Alternatively, 5-week old mice were irradiated with 3 Gy and transplanted intravenously with $5x10^6$ viable MNC. Single cell suspensions were obtained from bone marrow (BM), spleen and blood. Liver, kidney and lung were digested with collagenase D and DNase (Roche) followed by density gradient centrifugation. For serial transplantation, 1-$4x10^6$ BM cells from engrafted mice (containing 60%-70% human cells) were injected into recipients.

Flow cytometry

Cell suspensions were subjected to red blood cell lysis and stained with antibodies listed in *Online Supplementary Table S2*. Cytometric Bead Array kits (human inflammatory and Th1/Th2 cytokines; BD) were used according to the manufacturer's instructions. A FACSCalibur (BD) was used; analyses were performed using FlowJo (FlowJo) and Cyflogic (Cyflo). The gating strategy is shown in *Online Supplementary Figure S1*.

Immunohistochemistry

Organs were fixed in 4% buffered formalin, and sternums were decalcified. After paraffin-embedding, sections were deparaffinized in xylene and graded alcohols. H&E and chloracetate esterase staining followed standard protocols. Immunohistochemical staining was performed after specific antigen retrieval in "low pH target retrieval solution" (Dako) for 30 min. Primary and secondary antibodies are listed in *Online Supplementary Table S2*. The EnVision FLEX System or the AP-K5005 system were used for visualization (Dako). Sections were counterstained with hematoxylin (Dako) and mounted.

Genetic analysis

Human-specific PCR for *PTPN11* was performed on hematopoietic cells isolated from murine organs (forward primer ATCCGACGTGGAAGATGAGA, reverse primer TCAGAGGTAGGATCTGCACAGT). Human HL60 cells and hematopoietic cells from non-transplanted mice were used as positive and negative controls. PCR products were sequenced bidirectionally (BigDye Terminator kit, Life Technologies; ABI 3730xl or 3130xl capillary sequencers).

Pyrosequencing

Human-specific *PTPN11* PCR products were generated as above using a biotinylated reverse primer and pyrosequenced on a Pyromark Q24 (Qiagen) using sequencing primer ACATCAAGATTCAGAACACT. The wild-type/mutant allelic ratio of *PTPN11* point mutations was calculated using PyroMark Q24 software v.2.0 (Qiagen).

Statistical analysis

Charts show mean values and standard errors of the mean (SEM). Mann-Whitney test, Kaplan-Meier analysis and Mantel-Cox log rank test were used (Statview 4.1 software). $P<0.05$ was considered statistically significant.

Results

Xenotransplantation of human JMML cells into *Rag2*$^{-/-}\gamma c^{-/-}$ mice results in leukemic engraftment

We chose Rag2 and interleukin-2 receptor gamma chain double-deficient mice (*Rag2*$^{-/-}\gamma c^{-/-}$) as recipients for the JMML xenografts. The genetic defect of this strain leads to near-complete abolishment of residual T cell, B cell, and

natural killer cell activity,[32] a prerequisite for successful JMML xenotransplantation.[31] The mice were transplanted with MNC isolated from splenectomy preparations of 5 children with JMML. Flow cytometry showed that the pre-transplantation MNC samples consisted of a median of 21% CD34$^+$ stem/progenitor cells (range 1%-65%), 44% CD33$^+$ myeloid cells (range 18%-89%), 13% CD3$^+$ T cells (range 3%-23%) and 27% CD19$^+$ B cells (range 9%-31%). The cellular viability upon thawing and the cell composition of xenotransplanted material from individual patients is shown in *Online Supplementary Figure S2*. Based on our own previous experience with a xenotransplantation system for healthy human hematopoiesis using the same host strain,[33] we started by using intrahepatic injection of graft cells into newborn mice (1x10^6 viable JMML MNC per mouse) as route of transplantation. To see if the procedure could be simplified and make the model less dependent on the timely birth of pups, we also transplanted 5-week old mice *via* conventional intravenous injection; these mice received 5x10^6 JMML MNC to compensate for the higher body weight at that age. The xenografted cells were monitored in all mice by biweekly collection of blood and flow cytometry of human CD45$^+$ cells. Whereas most animals were sacrificed at elected time points (ranging from 10 to 20 weeks after transplantation) for phenotype analysis and harvest of JMML cells, a subset of mice was euthanized only when in poor condition so as to learn about the natural disease course.

We defined the level of human engraftment in a given murine organ as the proportion of human CD45$^+$ cells within the total population of murine and human CD45$^+$ cells. Following an accepted convention in xenotransplantation models,[30,34] the occurrence of 0.5% or more human CD45$^+$ cells in the murine BM was scored as successful engraftment. Using these definitions, JMML MNC from 4 children (Patients #1, #2, #3 and #5) engrafted into recipient mice with an overall leukemic engraftment rate of 58/82 mice (Figure 1), not counting 16 animals with non-leukemic T-cell engraftment (see below). Transplantation from one patient was unsuccessful (Patient #4, n=9 recipient mice) (Figure 1). In total, 64% (58/91) mice engrafted. Levels of human engraftment were variable and there was no correlation between percentage of human CD45$^+$ cells and time from transplantation or condition of the mice. However, we cannot exclude the possibility that leukemic engraftment in mice might have reached higher levels in some mice if sacrificed later.

To confirm the presence of JMML cells and rule out the possibility that co-transplanted healthy hematopoietic stem cells were engrafted into recipient mice, human

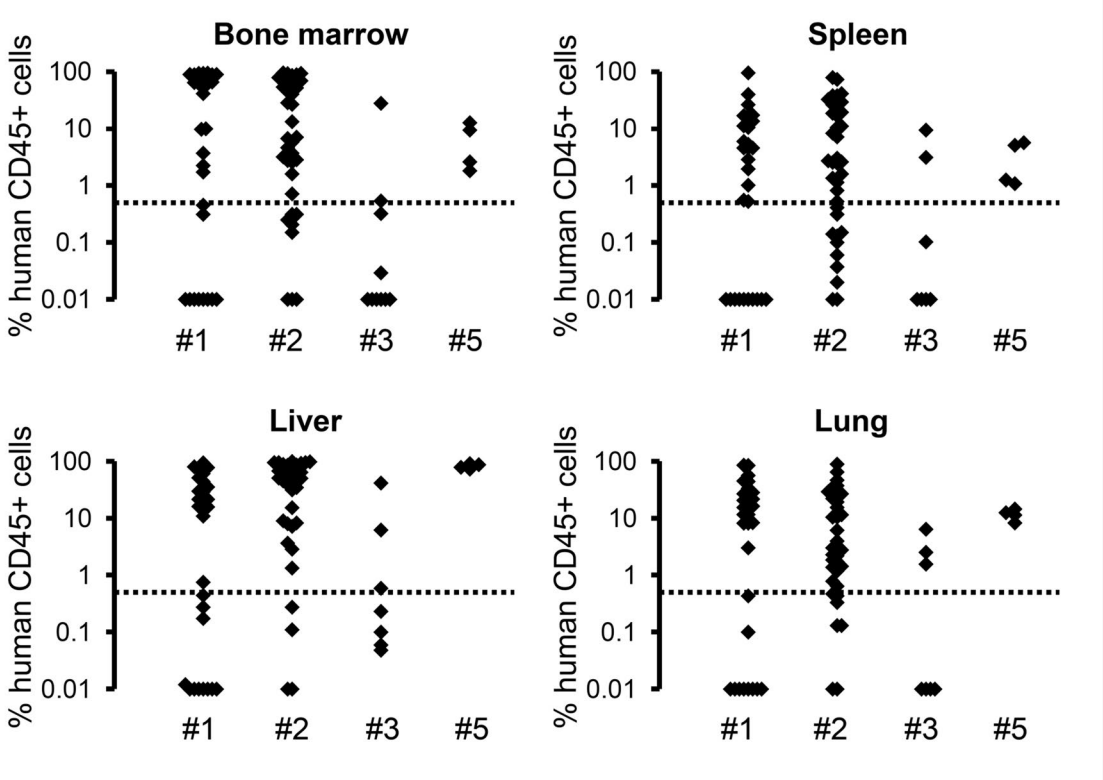

Figure 1. Sustained engraftment of xenotransplanted juvenile myelomonocytic leukemia (JMML) cells in *Rag2*$^{-/-}\gamma c^{-/-}$ mice. Spleen mononuclear cells (MNC) from JMML Patients #1 to #5 were transplanted into sublethally irradiated mice (1x10^6 cells per mouse *via* intrahepatic injection or 5x10^6 cells per mouse *via* intravenous injection). Hematopoietic cells were obtained from indicated organs at 7-37 weeks post transplant. Human cell engraftment as assessed by flow cytometry of CD45$^+$ cells is shown for animals transplanted from Patient #1 (n=31 mice), Patient #2 (n=37 mice), Patient #3 (n=10 mice) and Patient #5 (n=4 mice). The level of human engraftment was defined as proportion of human CD45$^+$ cells within the total population of murine and human CD45$^+$ cells. Cells from Patient #4 consistently failed to engraft (n=9 mice). The dotted line represents the definition of successful engraftment (≥0.5% human CD45$^+$ cells).

CD45+ cells were isolated from BM of 43 recipient mice after transplantation from Patients #1 and #2 and used for sequence analysis of the *PTPN11* gene. In all mice, the JMML-related mutation (*PTPN11* c. G181T and c. C215T, respectively) was detected in infiltrating cells.

Sustained leukemic engraftment in Rag2$^{-/-}\gamma c^{-/-}$ xenotransplanted mice recapitulates characteristic features of human JMML

To analyze the leukemic phenotype of xenografted animals, BM, spleen, liver, lung, and kidney of all mice were evaluated for human cell infiltration by flow cytometry, histopathology and immunohistochemistry. We observed consistent involvement of BM, spleen, liver and, importantly, lung. The kidney was unaffected in all animals. Flow cytometry revealed a strong predominance of human myeloid CD33+ cells in infiltrated murine organs (Figure 2A). The number of immature CD34+ cells was highest in BM and liver, reflecting the sites of perinatal hematopoiesis. Mature myeloid CD13+ cells were most abundant in lung. Only a minor proportion of human cells were B or T lymphocytes. On gross examination, significant splenomegaly was observed in mice with successful xenologous leukemic engraftment as opposed to non-engrafted animals (Figure 2B). Together, these features closely resemble JMML in children.

A total of 19 mice was informative for survival analysis. These mice were euthanized only at terminal disease and fulfilled the criteria of leukemic engraftment outlined above (Figure 3). Leukemia established in the recipient mice by JMML-initiating cells led to death of host animals at 51-224 days post transplantation whereas the survival of non-engrafted or non-transplanted mice was not reduced.

The histopathology of JMML-xenotransplanted mice showed that the BM, spleen and liver were infiltrated by a

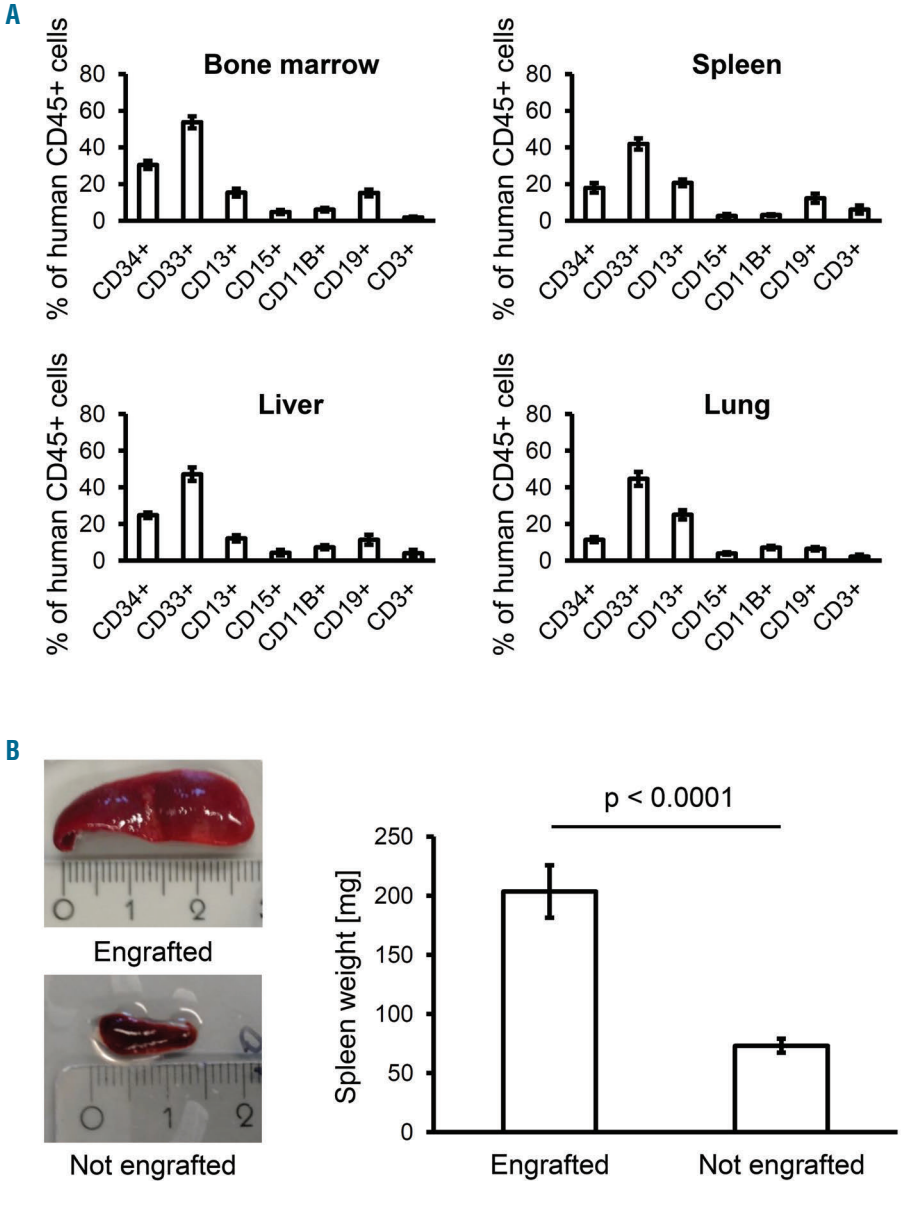

Figure 2. Organ infiltration and human cell subpopulations after xenotransplantation of juvenile myelomonocytic leukemia (JMML) cells in *Rag2$^{-/-}\gamma c^{-/-}$* mice. (A) Hematopoietic cells were obtained from indicated organs at 7-37 weeks after transplantation from 4 patients with JMML (n=58 mice). Cell subpopulations were assessed by flow cytometry with antibodies to human CD45, CD34, CD33, CD13, CD15, CD11B, CD19 and CD3. Bars indicate mean value and standard error. (B) Representative example of splenomegaly in mice with JMML cell engraftment (top) and normal spleen size in non-engrafted mice (bottom). The spleen weight of 49 engrafted and 17 non-engrafted mice was measured (right panel).

predominant population of differentiating myelomonocytic cells (Figure 4A). Immature forms with blast-like appearance were detected to a lesser degree. Leukemic infiltrates were focal and displaced the normal murine hematopoiesis in BM and spleen or the murine hepatocytes in liver. Some parts of the organs were completely destroyed by the leukemic foci, while other parts remained unaffected (Figure 4B). Consistent with flow cytometry and the observation that many animals developed respiratory distress after xenologous engraftment, histopathology demonstrated human cell infiltration also in the lung (Figure 4A). BM immunohistochemistry showed that human CD34$^+$ immature cells resided closer to the endosteal niches whereas more mature cells (lysozyme-positive or CD68$^+$) were located towards the medulla. Not all cells with leukemic morphology stained positive for human CD45. However, mouse-specific antibodies excluded the murine origin of these cells (Figure 4A). We assume that such cells represent CD45-negative JMML progeny, for example early erythroid progenitors. We noted that several mice transplanted with JMML cells from Patient #2 carried a predominant blast cell population, while the majority of mice receiving cells from this donor showed the usual infiltration with differentiating myelomonocytic cells. This suggests the outgrowth of an acute myeloid leukemia (AML)-like subclone in single animals.

To determine if the two transplantation techniques resulted in different disease phenotypes, we prospectively compared 6 mice after neonatal intrahepatic transplantation (*Online Supplementary Figure S3*) with 5 mice transplanted intravenously at 5-weeks of age (*Online Supplementary Figure S4*). For the purpose of this experiment, the mice were killed only at terminal disease. We found no difference in engraftment levels in BM, spleen, liver, or lung (*Online Supplementary Figures S3A and S4A*). The length of survival was identical between the intrahepatic (*Online Supplementary Figure S3B*) and the intravenous (*Online Supplementary Figure S4B*) group. Likewise, the differentiation profile of infiltrating cells was the same in both groups (*Online Supplementary Figures S3C and S4C*).

To compare JMML engraftment with non-leukemic xenologous hematopoiesis, we transplanted human CD34$^+$ cells derived from umbilical cord blood of a healthy newborn into 7 *Rag2*$^{-/-}\gamma c^{-/-}$ mice (*Online Supplementary Figure S5*). Contrary to mice transplanted with JMML cells, and in line with previous observations,[33] the human cells found in these mice were predominantly B cells and myeloid differentiation was barely detectable. The mice did not develop organomegaly and their survival after transplantation was no shorter.

The *Rag2*$^{-/-}\gamma c^{-/-}$ xenotransplantation model is independent of exogenous stimulation with GM-CSF

A hallmark feature of JMML progenitor cells is their hypersensitivity to GM-CSF,[17,35-37] and previous JMML xenograft models depended on continuous administration of human GM-CSF.[30] To analyze the effect of exogenous GM-CSF in our model, we xenotransplanted 9 newborn mice, 4 of which received weekly injections of 5 μg human GM-CSF beginning eight weeks after transplantation (Figure 5A). We observed no difference to 5 unstimulated mice regarding human CD45$^+$ or CD34$^+$ cell content in bone marrow or spleen even when GM-CSF treatment was continued for as long as 20 weeks after transplant

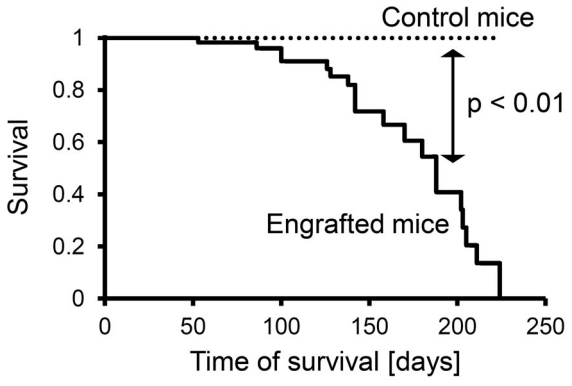

Figure 3. Xenologous engraftment of juvenile myelomonocytic leukemia (JMML) cells in *Rag2*$^{-/-}\gamma c^{-/-}$ mice results in decreased survival. Of 58 mice with xenologous JMML cell engraftment after transplantation, 39 were sacrificed at elected time points (not included in this Figure) and 19 were informative for survival analysis (solid line). Survival of non-transplanted control mice was unaffected (dotted line, n=5) (*P*=0.0004, Mantel-Cox log rank test).

(Figure 5B). Histopathology did not reveal any noteworthy differences either. As expected, higher proportions of human CD33$^+$ myeloid cells were noted in mice treated with GM-CSF (Figure 5B). It appears that paracrine secretion of human cytokines by differentiating monocytes is sufficient to sustain the JMML-initiating cells in the *Rag2*$^{-/-}\gamma c^{-/-}$ microenvironment. Accordingly, we detected the human cytokines interleukin-8, tumor necrosis factor-alpha and interferon-gamma in the serum of engrafted mice (*Online Supplementary Figure S6*).

Graft-versus-host disease originating from T lymphocytes may overwhelm the leukemic engraftment of individual JMML samples

Whereas the xenotransplantation of unfractionated spleen MNC from Patients #1, #2 and #5 invariably led to myeloid leukemic engraftment in recipient mice, the spleen MNC from Patient #3 caused massive T lymphocyte infiltration of all organs and rapid death within 22-36 days after transplantation (*Online Supplementary Figure S7*). Flow cytometry with human-specific antibodies confirmed the human origin of these cells. Upon genetic analysis, the JMML-specific *PTPN11* mutation was undetectable, indicating that co-transplanted non-leukemic T cells had expanded in the animals. When CD3$^+$ lymphocytes were depleted from spleen MNC prior to transplantation using immunomagnetic beads, regular JMML cell engraftment but no T-cell expansion or graft-*versus*-host disease occurred in the recipient animals (see Figure 1, Patient #3). Hence, a T-cell depletion step may be required for successful xenotransplantation depending on the individual cell composition of the clinical material.

JMML-initiating cells are serially retransplantable and re-establish disease in mice

To assess the self-renewal capacity of long-term JMML-initiating cells, serial transplantations were performed. Seventeen weeks after xenotransplantation with JMML cells from Patient #1, BM was obtained from 2 mice and injected into 9 mice as second-generation xenograft. Successful leukemic engraftment was observed in all 9

mice (Figure 6A). The survival of recipient mice was similar to that of primary recipients (Figure 6B). The secondary recipients developed splenomegaly and showed predominant myeloid infiltration (Figure 6C). The overall level of organ infiltration with human cells and the lineage distribution of human cell progeny were comparable to first-generation xenograft mice.

At ten weeks after xenotransplantation with JMML MNCs from Patient #2, 8 secondary recipients were transplanted with BM harvested from 4 mice. Four of the sec-

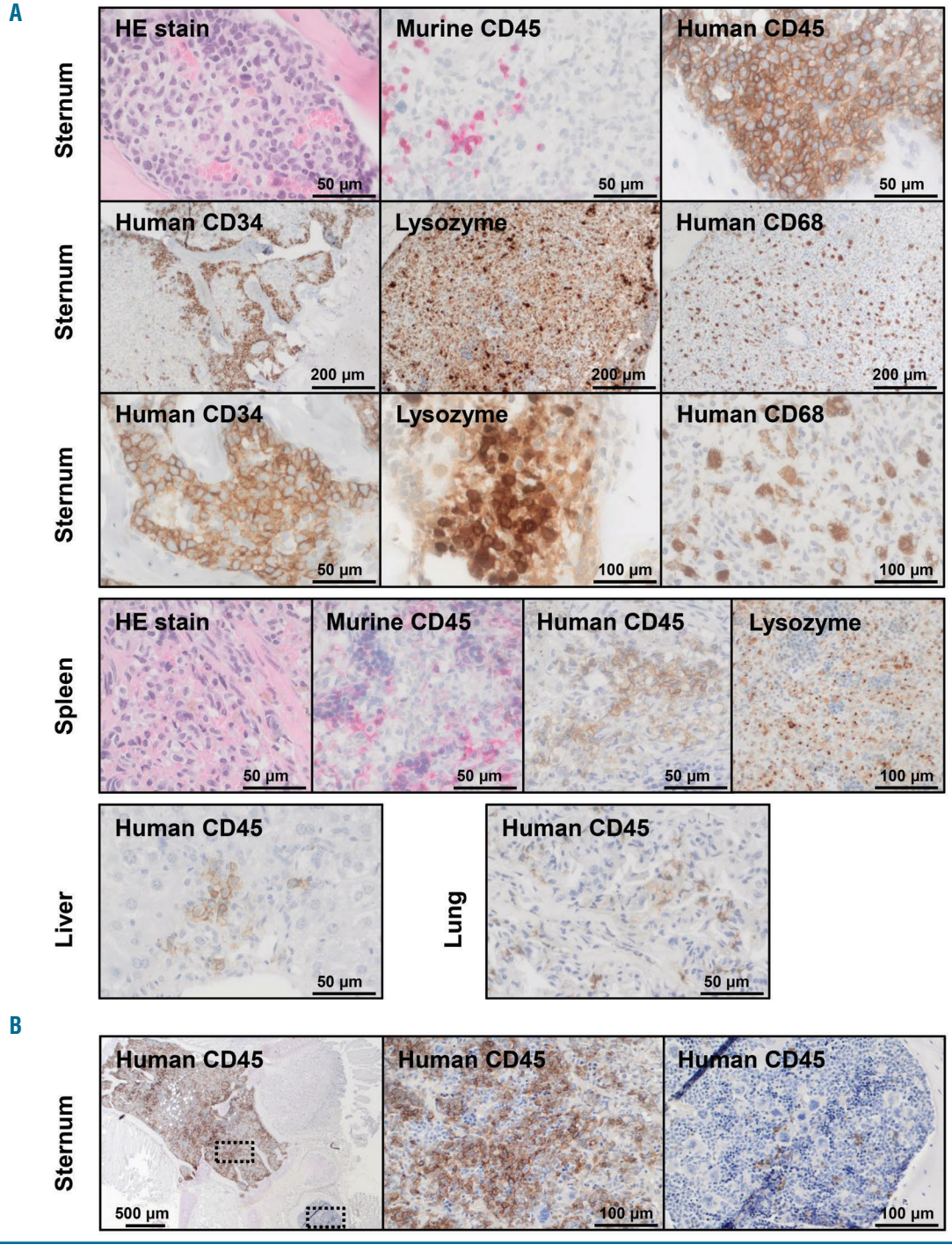

Figure 4. Histopathology demonstrates human myelomonocytic infiltration in murine tissues. (A) Histology and immunohistochemistry of bone marrow, spleen, liver and lung at time of terminal illness revealed leukemic infiltration with differentiating myelomonocytic cells that replaced regular murine tissue. Immunostaining with antibodies to murine CD45 and human CD45 confirmed the human origin of the leukemic cells (top panel, first row). Immature CD34-positive cells were located in the peritrabecular region and had a blast-like appearance. By contrast, lysozyme- and CD68-positive, more differentiated myelomonocytic cells were found in the center of the medullary cavity (top panel, second and third row). The murine spleen was infiltrated by myelomonocytic cells positive for human CD45 and lysozyme but negative for murine CD45 (middle panel). Human myelomonocytic cells were also found in murine liver and lung (bottom panel). (B) Focal displacement of murine hematopoiesis by human CD45-positive myelomonocytic cells. Dotted frames indicate areas with higher magnification shown on the right.

ondary recipients showed successful engraftment. BM cells from these mice were then used for tertiary transplantation and led to engraftment in 8 of 8 mice (*Online Supplementary Figure S8*). Again, the level of leukemic organ infiltration and length of survival were comparable to first-generation xenograft mice. Mutation analysis demonstrated that the leukemia-specific *PTPN11* mutation was invariably present when human cells were retrieved from serially engrafted mice, regardless of infiltrated organ or graft generation (*data not shown*). This ruled out the possibility that the leukemic clone might have been lost after repeated xenotransplantation and that long-lived non-leukemic progenitors might have prevailed. In addition, quantitative pyrosequencing was employed to compare the mutant allele fraction between source material (spleen MNC) and purified human CD45$^+$ cells retrieved from BM cells of serially xenografted mice (Figure 7). We observed that close to 100% of human cells were of leukemic origin in primary, secondary, or tertiary recipients.

Discussion

The need for a pre-clinical model of JMML that can be used for basic research, biomarker identification and drug testing prompted us to establish a xenotransplantation system for this leukemia in immunodeficient mice. Other investigators have previously xenografted JMML cells but reported various difficulties.[29-31] Lapidot *et al.*, using SCID mice as host strain, observed a rapid decline in well-being and cachexia as soon as 2-4 weeks after xenotransplanta-

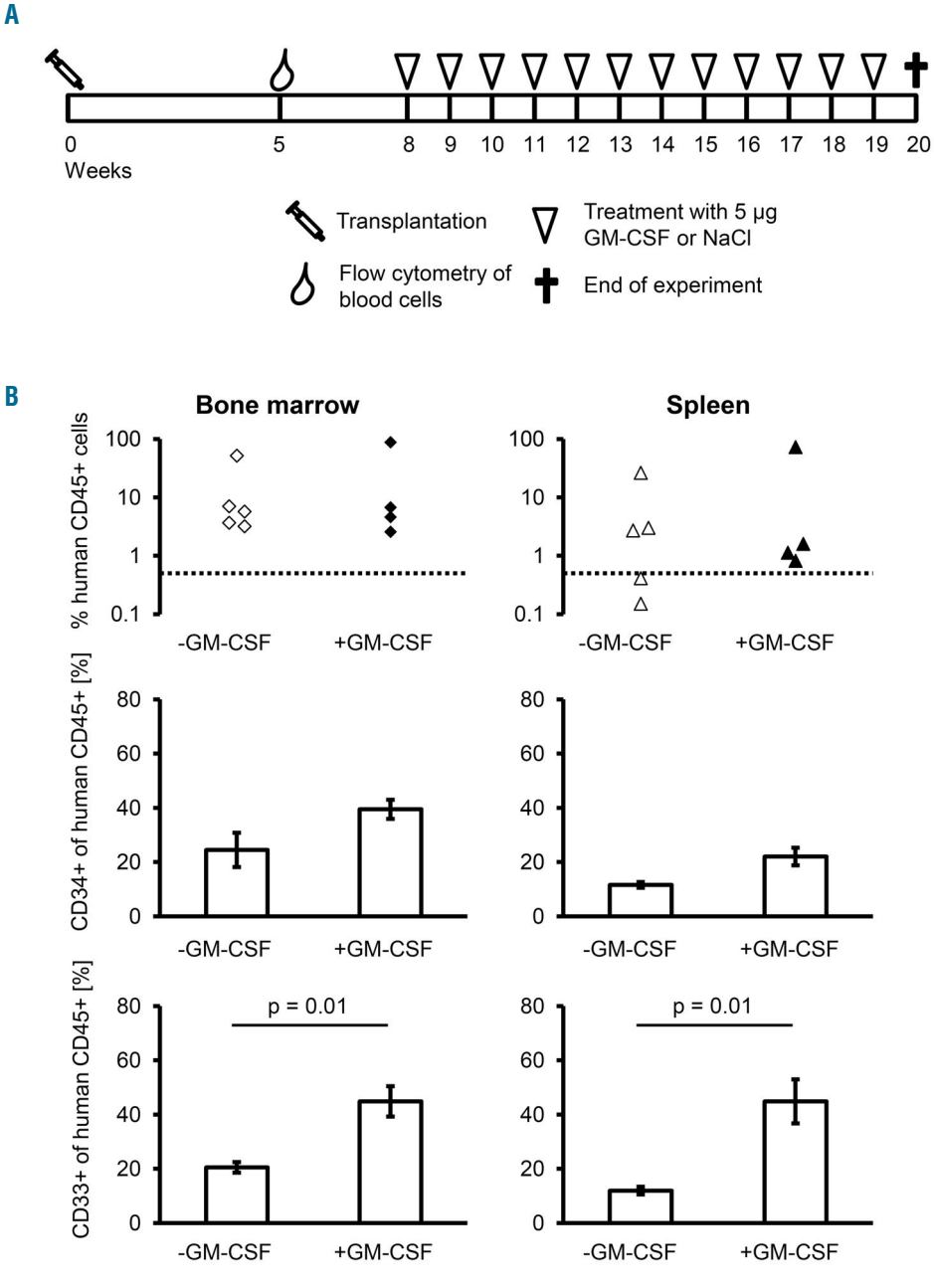

Figure 5. Application of exogenous granulocyte-macrophage colony-stimulating factor (GM-CSF) increases myeloid differentiation without affecting overall engraftment. (A) Schematic diagram of the experimental set up. Nine mice were transplanted intrahepatically with 1x10^6 juvenile myelomonocytic leukemia (JMML) mononuclear cells of Patient #2. Five weeks later, human cell engraftment was confirmed by flow cytometry of CD45$^+$ peripheral blood cells. Mice were divided into two experimental groups matched for level of engraftment in peripheral blood. Four mice received weekly injections of 5 µg recombinant human GM-CSF, while saline was administered in 5 mice. Applications were started eight weeks after transplantation. The animals were analyzed 12 weeks later. (B) The human CD45$^+$ cell engraftment, the proportion of CD34$^+$ progenitor cells and the proportion of CD33$^+$ myeloid cells were determined in bone marrow (left) and spleen (right). Levels of human CD45$^+$ and CD34$^+$ cells were comparable between untreated (open symbols) and treated (filled symbols) animals. The proportion of CD33$^+$ cells was significantly higher in bone marrow and spleen of treated animals (P=0.01, Mann-Whitney test).

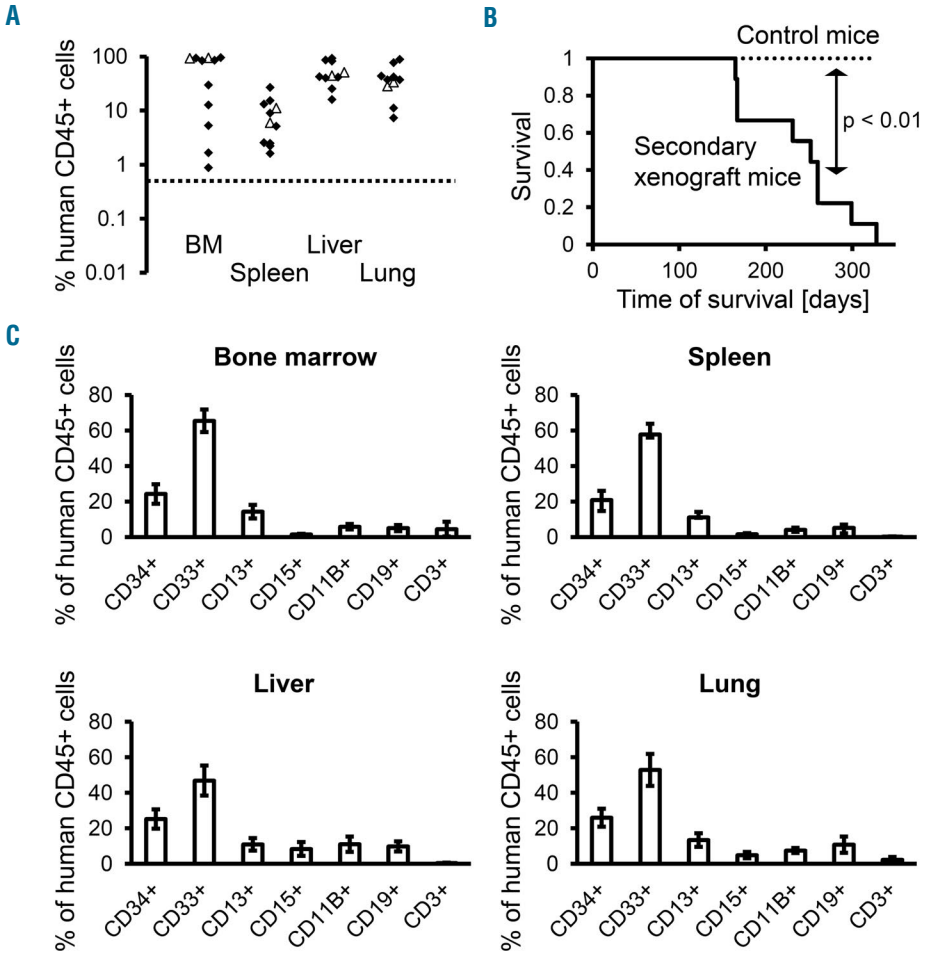

Figure 6. Analysis of secondary recipient mice after serial xenotransplantation. (A) Seventeen weeks after xenotransplantation with juvenile myelomonocytic leukemia (JMML) cells from Patient #1, bone marrow (BM) cells from 2 mice were re-transplanted into 9 second-generation mice ($1x10^6$ cells per animal). The secondary recipient animals were sacrificed for analysis when terminally sick (165-328 days after transplantation). The level of human engraftment was defined as proportion of human $CD45^+$ cells within the total population of murine and human $CD45^+$ cells. Open triangles indicate first-generation (donor) mice; closed diamonds, second-generation recipients. The dotted line represents the definition of successful engraftment (≥0.5% human $CD45^+$ cells). (B) Secondary recipient mice (solid line) had significantly reduced survival compared to untransplanted control mice (dotted line, n=5) ($P<0.01$, Mantel-Cox log rank test). (C) Hematopoietic cells were obtained from indicated organs and cell subpopulations were assessed by flow cytometry with antibodies to human CD45, CD34, CD33, CD13, CD15, CD11B, CD19 and CD3. Bars indicate mean value and standard error.

tion of JMML cells.[30] Their experiments involved continuous treatment of host mice with human GM-CSF. In the absence of exogenous human GM-CSF the leukemic cells did not engraft at all. Nakamura *et al.* reported highly variable levels of myeloid engraftment in NOD/SCID/$\gamma c^{-/-}$ mice (average 18.7% $CD33^+$ of human $CD45^+$ cells in the bone marrow, range 9.4%-37.7%), although the number of cells transplanted was fairly high (10^7 cells per mouse).[31] In addition, those previous reports on JMML xenotransplantation lacked a detailed analysis of the natural disease course of recipient mice as all animals were sacrificed no later than 12 weeks after transplantation.[29-31] However, we felt that a system with a more chronic disease course would be desirable if JMML were to be modeled in experimental animals for pre-clinical research.

In an attempt to overcome the obstacles discussed above we chose *Rag2*$^{-/-}\gamma c^{-/-}$ mice as hosts and intrahepatic injection into newborn recipients as mode of transplantation. We favored this technique because of an earlier description by Traggiai and the documented suitability for transplantation of healthy cord blood-derived $CD34^+$ cells.[33,38] Adopting an intrahepatic strategy seemed especially appropriate for JMML since it is a disease of early childhood, frequently affects the liver and most probably originates from fetal hematopoietic cells, which appear to be supported better by neonatal than adult tissues.[39,40] In addition, intravenous injection would be technically challenging in newborn mice. When we later compared the phenotype of mice transplanted intrahepatically with that of animals xenografted at older age *via* the intravenous route we found no significant differences in level of organ infiltration, length of survival after transplantation, or other aspects of the ensuing leukemia. Although we did not perform systematic titrations of input cell numbers, we believe that the intrahepatic technique might be the better choice if clinical samples with limited cell number were to be transplanted.

Juvenile myelomonocytic leukemia cells from 4 patients readily engrafted in the mice whereas transplantation from one child was unsuccessful. We can only speculate whether this failure relates to low amounts of JMML-initiating cells in the spleen MNC preparations (*Online Supplementary Table S1*) or to poor material quality (i.e. latency between splenectomy and cryopreservation). After successful engraftment, the xenotransplanted mice displayed symptoms similar to those observed in children with JMML, including hepatosplenomegaly, cachexia and pulmonary infiltration with respiratory distress. Detailed analysis of murine hematopoietic organs revealed focal infiltration by human myelomonocytic $CD33^+$ cells. CD13 expression indicated the presence of cells at more mature stages of differentiation and was stronger in spleen and lung than BM and liver, consistent with the physiological route of myeloid differentiation. Immature $CD34^+$ leukemic cells were located in the endosteal regions of bone indicating that they shared hematopoietic niches

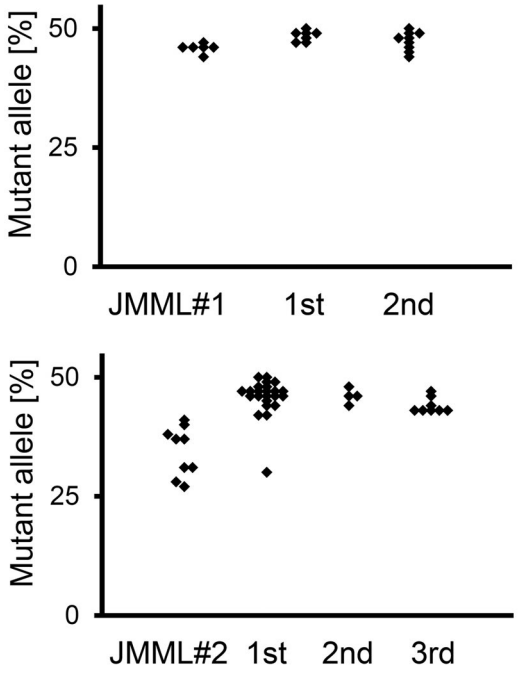

Figure 7 (right). Leukemic allele frequency was maintained during serial transplantations. Pyrosequencing was performed on human CD45^{+} cells obtained from murine bone marrow to determine the mutant allele frequency of *PTPN11* c.C215T (Patient #1, upper panel) and *PTPN11* c.G181T (Patient #2, lower panel) in serially transplanted animals. Since the leukemic mutations were heterozygous, a 50% allele frequency corresponds to 100% leukemic cells.

with their normal counterparts, similar to a recent observation in AML.[41] The lineage composition of engrafted JMML cell progeny clearly differed from that of xenotransplanted cord blood-derived healthy CD34^{+} cells, where B cells predominated and only minor myeloid populations were observed. Importantly, xenotransplanted mice showed a chronic disease course with a median survival of more than 20 weeks. This makes it possible to evaluate pharmaceuticals with delayed activity, in particular the DNA-hypomethylating agent azacitidine which has recently gained clinical interest for use in JMML.[42,43] In contrast to previous JMML xenotransplantation models, *Rag2*$^{-/-}\gamma c^{-/-}$ mice efficiently sustained engrafting JMML cells in the absence of exogenous human GM-CSF. Weekly application of human GM-CSF enhanced myeloid differentiation but did not influence the total level of engraftment or time to leukemia.

Serial transplantation of JMML cells confirmed the presence of long-term JMML-initiating cells with self-renewal capacity. Phenotype and disease kinetics were similar in primary, secondary and tertiary recipients. Importantly, typical morphology with differentiating myelomonocytic cells was preserved over time and no disease acceleration was observed. Serial transplantability is not only important to the scientific concept of leukemia-initiating cells, but is also a valuable tool for the expansion of primary JMML cells from a practical perspective. Using the *Rag2*$^{-/-}\gamma c^{-/-}$ JMML system and serial retransplantation we have maintained JMML cells *in vivo* for 1.5 years in total. In the process, unmanipulated clinical JMML material was expanded rather than consumed. This has not so far been feasible by *in vitro* culture.

In summary, we present a novel xenotransplantation model of JMML that closely mimics human disease. We are confident that the model will be useful to further characterize the JMML-initiating cell, amplify scarce and valuable clinical material, and complement the recently evolving early-phase clinical trials for novel pharmaceutical strategies such as epigenetic therapy.

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