A Second Prophylactic MHC-Mismatched Bone Marrow Transplantation Protects Against Rat Acute Myeloid Leukemia (BNML) Without Lethal Graft-Versus-Host Disease

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Background. We have employed a rat model for human acute myeloid leukemia, a promyelocytic leukemia in the BN rat strain (BNML), to develop new protocols for immunotherapy in combination with allogeneic bone marrow transplantation (alloBMT). The status of mixed chimerism in allotransplanted rats provided an opportunity for immunotherapy using alloreactive donor cells. In addition to T or natural killer (NK) cells, we introduced a second infusion of bone marrow cells as prophylactic donor lymphocyte infusions (DLI) to test whether an effective graft-versus-leukemia (GVL) response could be obtained without clinical graft-versus-host disease (GVHD).
Methods. BN rats were sublethally irradiated and transplanted with T-cell depleted bone marrow cells from either fully major histocompatibility complex (MHC)-mismatched (PVG) donor rats or MHC-matched (PVG.1N) as controls. Seven days after transplantation, rats were given 500 leukemic cells to mimic minimal residual disease. Additional cellular therapy was given at day +7. The efficiency of DLI was monitored by chimerism analysis in peripheral blood.
Results. Rats receiving infusions of NK cells succumbed to leukemia. T-DLI induced complete donor T-cell chimerism and lethal GVHD. A second alloBMT protected against leukemia. This effect was dependent on an MHC incompatibility between the donor and host and also on the presence of alloreactive T cells in the second bone marrow inoculum, resulting in an increased, mixed donor T-cell chimerism.
Conclusion. A second prophylactic transplantation influenced the degree of T-cell chimerism to balance favorably between GVL and GVHD. If applicable to humans, repeated alloBMT may provide a novel approach to leukemia therapy.

Keywords: Acute myeloid leukemia, Graft versus leukemia, Donor lymphocyte infusions, Chimerism.

(*Transplantation* 2008;85: 102–111)

Clinical treatment of acute myeloid leukemia (AML) requires intensive induction therapy to attain remission followed by consolidation therapy. Relapse remains a major cause of treatment failure in AML, due to minimal residual disease (MRD) (*1*), such as persistence of residual leukemic blasts in the bone marrow surviving the induction therapy.

Allogeneic stem cells transplantation (alloSCT) represents a potentially curative option for advanced or high-risk AML (*2*). The therapeutic benefit of alloSCT relates to the graft-versus-leukemia (GVL) effect mediated by alloreactive donor lymphocytes (*3*).

Transplantation between human leukocyte antigen (HLA)-identical siblings is preferable, since a mismatch in HLA antigens involves a further risk of both graft rejection and graft-versus-host disease (GVHD) (*4–6*). Haploidentical stem cell transplantation has been developed for patients lacking an HLA-matched related donor or when HLA-matched unrelated donors are not eligible (*7*).

The continued development and implementation of reduced-intensity conditioning (RIC) protocols to diminish transplant-related mortality (TRM) has allowed a more widespread application of allogeneic transplantation (*8*). RIC was designed not to eradicate the malignancy completely, but rather to provide sufficient immunosuppression to achieve engraftment (*9*, *10*). Engraftment and the induction of initial mixed chimerism (MC) serve as an excellent basis for further adoptive cellular immunotherapy, such as donor lymphocyte infusions (DLI) to provide GVL reactivity.

The field of cellular immunotherapy has been dominated by transfer of peripheral donor-derived T cells. T-DLI has been used after alloBMT to treat relapsed or residual disease, to convert mixed to full donor chimerism, as an add-back to restore full immune function after T-cell depleted (TCD) transplantation and as prophylaxis (*11*). T-DLI has been used successfully for the treatment of chronic myeloid leukemia (*12*), but has been much less effective for patients with AML. The major complication after T-DLI is acute and chronic GVHD. A few pilot studies have been performed using T-DLI in a haploidentical transplantation setting (*13*, *14*).

This work was supported by The Research Council of Norway (Ph.D. grant to J.N.), The Norwegian Center for Stem Cell Research, The Research Council of Norway, Oddrun Mjåland's Fund for Cancer Research, and the Marie Curie Research and Training Network (EU 6FP "TRANS-NET").

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Received 24 July 2007. Revision requested 16 August 2007.
Accepted 22 September 2007.
Copyright © 2008 by Lippincott Williams & Wilkins
ISSN 0041-1337/08/8501-102
DOI: 10.1097/01.tp.0000296856.53493.1f

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After chemotherapy and alloSCT, natural killer (NK) cells are the first lymphoid cells to recover. NK cells express inhibitory receptors for major histocompatibility complex (MHC) class I molecules and activating receptors with diverse specificities. Inhibitory NK receptors discriminate between MHC class I positive and negative target cells (*15*). Faced with MHC-mismatched hematopoietic stem-cell transplants, donor NK cells may sense the absence of self–MHC class I alleles and mediate alloreactions (*16*). Some pilot studies have investigated the approach of NK-DLI in promoting engraftment and inducing GVL effects in patients after alloBMT from haploidentical donors (*17*, *18*).

Based on the Brown Norway promyelocytic leukemia (BNML) rat model, an experimental model for human AML, we have employed a rat transplant model where we reconstitute whole body irradiated rats with stem cell-enriched bone marrow cells (BMC) from MHC-matched or mismatched donors (*19*). We chose combinations of donor and recipient strains where we could expect an optimal effect of alloreactive NK and T cells derived from donor BMC against both residual MHC-mismatched BMC and leukemic cells. We attempted to simulate the minimal residual disease (MRD) phase of BNML by infusion of a low number of BNML cells (*20*). This status is comparable to the clinical situation after RIC: initial MC and a high incidence of MRD in most patients (*21*). We have evaluated the ability of DLI with T or NK cells to mediate beneficial GVL effects without exacerbating GVHD in this rat model. The efficiency of DLI was monitored by chimerism analysis. Even in a double MHC-mismatched combination, this therapy failed either because of recurrence of the disease (NK cells) or strong GVH reactions that obscured GVL (peripheral T cells). However, since the primary MHC-mismatched transplant used to reconstitute the rats itself had a weak GVL effect, we anticipated that the bone marrow (BM) contained leukemia preventing elements that could be exploited in further cellular therapy with BMC.

MATERIALS AND METHODS

Animals, Anesthesia, Injections, and Experimental Approval

The PVG, PVG.7B (differ only at one epitope of the leukocyte-common antigen [LCA]), and MHC congenic rat strains PVG.1U and PVG.1N were bred in our animal house. Brown, nonagouti BN/RijHsd rats were from Harlan U.K. Rowett athymic nude rats (*rnu/rnu*) were a kind gift from the Animal Facility at The Norwegian Radium Hospital. They closely resemble PVG rats as they have both the MHC and NK cell receptor complex derived from this strain (unpublished observations). Male BN rats, aged 8–10 weeks and weighing 200 to 250 g, were used as recipients and 2- to 4-month-old PVG or PVG congenic rats as donors. Rats were anesthetized with Hypnorm (fentanyl citrate and fluanisone) and terminated with CO_2.

All animal experiments were approved by the Experimental Animal Board under the Ministry of Agriculture of Norway and conducted in conformity with the Norwegian Regulations on Animal Experimentation and the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes. The laboratory animal facilities are subject to a routine health monitoring program and were tested for infectious organisms according to a modification of the FELASA (Federation of European Laboratory Animal Science Associations) recommendation (*22*).

Irradiation and BMC Isolation

Rats were irradiated at 8.5 Gy from a ^{137}Cs source (Gammacell 3000 Elan, MDS Nordion) being close to LD_{50} in this rat strain, since about 50% of the rats survived this dose without stem cell treatment. They were then injected i.v. with 30×10^6 BMC 1–2 h later. BMC were obtained by flushing the tibias and femurs with 20 ml Roswell Park Memorial Institute 1640 containing 2% fetal calf serum (FCS). Cells were passed through a nylon cell-strainer, and mononuclear cells were purified by density gradient centrifugation on Nycoprep 1.077A (Medinor ASA, Norway). BMC cells were TCD with a combination of Ox19 (anti-CD5) and R73 (anti-TcR α/β) antibodies (supernatants or ascites fluid) and antimouse immunoglobulin G (IgG)–coated Dynabeads (Dynal Biotech ASA, Oslo, Norway); the number of $CD3^+$ T cells ($G4.18^+$ cells assessed by flow cytometry) was reduced more than 10-fold, from 3.0% to 0.2% among the BMC.

The BNML Model

The BNML rat model is an experimental model for human AML and has been essential for understanding the interaction between hematopoietic and leukemic cells and for the characterization of MRD (*23*, *24*). The leukemia was passaged by i.v. injection of BNML cells. BN rats injected i.v. with 10×10^6 BNML cells developed signs of leukemia and died 17–18 days after injection.

Characterization of BM Chimeras and BNML by Flow Cytometric Analysis

Heparinized venous peripheral blood samples were taken from rats at various time points after transplantation (days +14, +21, +28, +35). Then 0.3 ml of blood from the lateral tail vein was collected in 75-mm heparinized tubes (Drummond, Hemato-Clad, Heparinized, Sigma-Aldrich) and lysed in 0.8% ammonium chloride. Fluorescein isothiocyanate (FITC)–conjugated His41 (anti-CD45.2 monoclonal antibodies [mAb]) (*25*) was used to determine the proportion of PVG.7B donor cells after MHC-mismatched transplantation. Cells were double-labeled with His41-FITC and a marker for T cells, G4.18-PE (anti-rat CD3, BD Biosciences-Pharmingen, San Diego, CA) or a marker for NK cells, phycoerythrin (PE)-conjugated anti-rat NKR-P1A (BD Biosciences-Pharmingen). In the MHC-matched setting (PVG.1N→BN), there is no rat antibody-based detection system available to separate donor and host cells.

For tracing of BNML in blood, we used mAb RM124 (*20*), which is mainly specific for BNML with IgM-biotin as a secondary step and then PE-conjugated streptavidin (Jackson ImmunoResearch Europa Ltd, UK).

Cell surface phenotype of BNML was determined with a panel of mouse mAbs against rat leukocyte antigens. BNML were stained with the mAbs and GaM IgG-FITC (Sigma-Aldrich Norway A/S, Oslo, Norway), as secondary antibody, and the rat mAbs AAS5 and AAS6 (anti-MHC-I against $RT1^c$ and crossreacting with $RT1^n$) (*26*) with GaR IgG-FITC (Jackson ImmunoResearch) as secondary antibody. Labeled cells

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were analyzed on a FACScalibur (BD Biosciences, San Jose, CA) equipped with the CellQuest software (BD Biosciences).

Assessment of BNML Development and GVHD

GVHD was assessed by weight loss, skin lesions (alopecia, dermatitis), diarrhea, and reduced spleen weight.

The leukemic cells were identified both morphologically in cytospins and with the RM124 antibody and FACS (*20*). When approaching 70–80% of peripheral blood lymphocytes (PBL), defined as the survival end point, the rats were killed and the increase in spleen weights confirmed the BNML diagnosis.

Statistics

The data were analyzed with SPSS (Version 13.0, Chicago, Illinois). Survival was estimated with the Kaplan-Meier method and compared with the log-rank test for all groups. The significance level was set to 0.05.

RESULTS

Cell Surface Phenotype of BNML

A panel of mAbs against rat cell surface antigens showed that BNML cells expressed a number of myeloid cell markers, including CD11b/c, CD43, CD45, CD47, CD48, CD93, and CD147, but not T, B, and NK cell markers such as CD2, CD3, CD4, CD5, CD8, CD134, Ig-kappa (OX12), or CD161 (NKR-P1; Fig. 1). They expressed MHC class I (OX27) but not class II molecules (OX6). Further staining with *RT1^n* haplotype specific mAbs (AAS5 and AAS6) showed that BNML expressed both classical (Ia) and nonclassical (Ib) MHC-I molecules. AAS6 reacts with both Ia (RT1.A) and Ib (RT1.C/E) molecules, while AAS5 only reacts with Ib in this haplotype (*26*). The staining with AAS5, although weak, was significantly higher than background staining with isotype-matched control mAb, as shown in several independent experiments. This is consistent with a much lower level of expression of Ib than Ia molecules on the cell surface in general. Since BNML expressed both classical and nonclassical MHC-I molecules, they may be targets for both NK and T cells in BM allotransplanted rats.

Design of the In Vivo Experiments

Recipient BN rats were sublethally irradiated and transplanted with TCD BMC from either MHC-matched or mismatched donor rats (day −7). They received an infusion of 500 BNML cells seven days later (day 0). To allow the leukemic cells to establish before exposure to the transplanted immune cells, additional cellular therapy was given on day +7 (Table 1). The rats lost approximately 20% of their body weight after transplantation, but were restored according to weight 14 days later. During the experiments, a weight loss of 5 g served as an early indicator of disease development (leukemia or GVHD).

Transplantation and Immune Recovery

Several studies have reported that high numbers of BMC are required for the transplantation over MHC barriers (*27–29*). We used a standard dose of 30×10^6 TCD BMC for transplantation, since lower doses often failed to reconstitute the animals. BMC was depleted of T cells, since an undepleted BMC transplant (30×10^6 BMC containing approximately 3.0% CD3$^+$ T cells) developed GVHD in recipient rats and they died around day 30 posttransplant.

Delayed immune reconstitution (IR) limits the success of transplantation and increases the risk for infectious complications and relapse (*30*). In order to test the effect of an MHC-mismatch on the recovery after transplantation, we performed an experiment where PVG rats were transplanted with allotype-marked PVG.7B or MHC-mismatched PVG.1N. NK cells rapidly recovered in both groups and returned to normal levels within the first month. Reconstitution of T cells was more delayed, but was faster for MHC-mismatched and reached higher percentages than for matched transplants. Mean±SD of donor T and NK cells were calculated from dot plots (Fig. 2). In MHC-matched transplantation, there was a MC both in the NK cell (91.7±3.1% donor cells) and T cell (34.3±6.0%) fraction at day 35 (Table 1, experiment 1; Fig. 2B). For MHC-mismatched transplanted rats, we found a higher level of donor T-cell chimerism (77.7±7.8%) and the NK cells were 100% donor type (Table 1, experiment 2; Fig. 2A).

Minimal Residual Disease

In order to mimic MRD, we determined the minimal number of BNML cells needed to consistently induce leukemia. BNML cells in 5- to 10-fold dilutions ranging from 10×10^6 to 100 cells were injected i.v. into groups of BN rats. To follow the kinetics of leukemia development, we determined the number of leukemic cells both morphologically and with the mAb RM124 against BNML in blood at timed intervals after BNML injection. The lowest dosage of BNML that reproducibly induced leukemia was 500 cells. The rats exhibited a rapid and consistent rise in percentage of BNML cells in blood after day 30 and died or were terminated between day 34 and 39 with spleen weights ranging from 2.9 to 4.0 g and 70–80% BNML cells in blood.

One BMC Allotransplantation Is Not Protective Against BNML

The average time for nontransplanted rats in the control group to develop leukemia was 35 days. The outcomes of allotransplanted rats are shown in Table 1 and Figure 3B.

Nine rats transplanted with MHC-matched BMC and injected with 500 BNML cells, succumbed to leukemia at the same time as control rats. Twelve rats transplanted with TCD MHC-mismatched BMC also died at the same time, with the exception of one long-term survivor. However, there was a slightly delayed rise in proportion of BNML in blood in these rats (Fig. 3A). The percentages of donor-derived T cells in the blood at day 35 after BNML injection are shown in Table 1. Means±SD were calculated from dot blots (Figs. 2 and 4), where numbers in upper right and upper left quadrant correspond to host and donor T cells, respectively. After MHC-mismatched transplantation, NK cells were 100% donor derived at any point tested. MC in the CD3$^+$ T-cell population was estimated in six leukemic rats, at a level of 53.4±8.3% donor T cells (Table 1, experiment 5; Fig. 4A).

Failing Adoptive Immunotherapy With NK Cells

In the allotransplanted rats, we measured the effect of infusion of interleukin (IL)-2 activated NK cells (LAK cells) on BNML development. The NK cells were generated according to a standard protocol (*31*) and were shown to kill both Con A-

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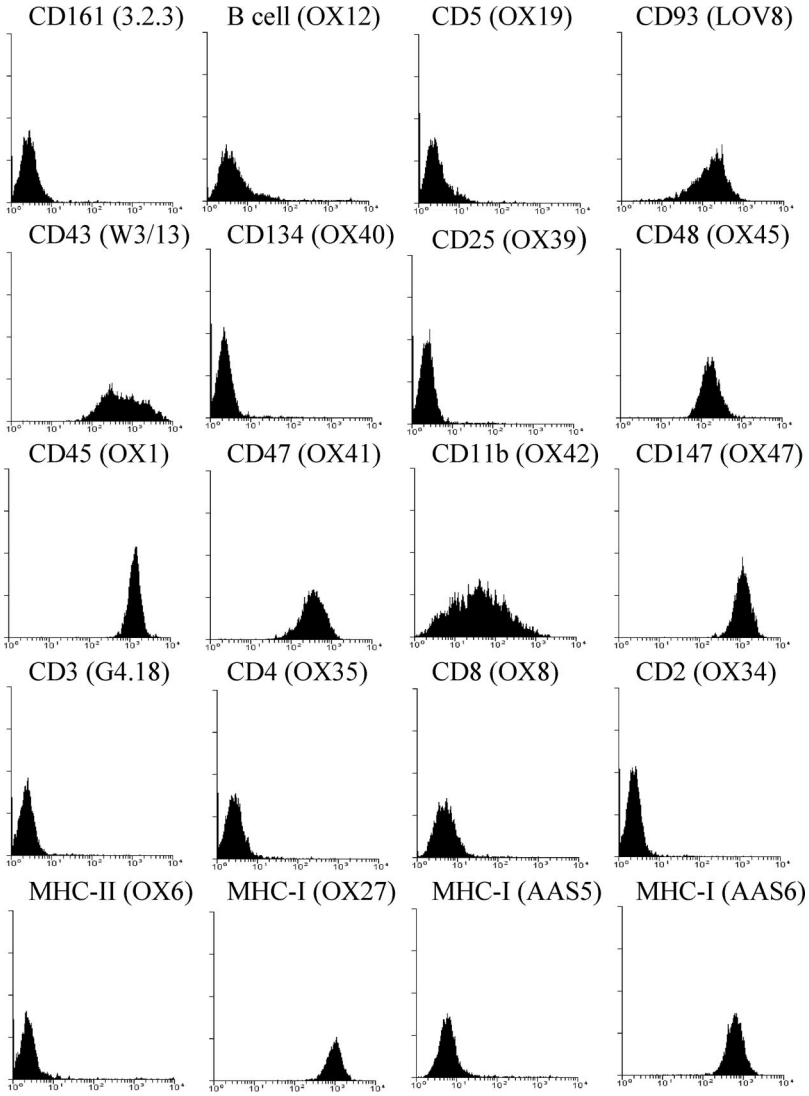

FIGURE 1. Phenotype of BNML as determined by FACS-analysis with a panel of mAbs against rat leukocyt differentiation markers. Note the expression of a number of myeloid cell markers and also classical and nonclassical MHC-I (OX27, AAS6, AAS5) but not MHC-II (OX-6) or T, B, and NK cell markers on the cell surface. (AAS5 only labels nonclassical RT1.C/E MHC-I in BN.)

stimulated BN lymphocytes and BNML targets in vitro (data not shown). Various regimens were tested, including one single injection of 70×10^6. LAK cells on the day of BNML administration, as well as regimens with repeated injections of LAK cells at dosages of 50×10^6 cells i.v. on days 0, 7, and 14. No effect on BNML development was observed, whether the LAK cells were MHC-matched (PVG.1N) or mismatched (PVG.7B) with the recipient (data not shown). This indicated that IL-2 activated alloreactive NK cells from PVG.7B, although clearly reactive against BNML in vitro, did not by themselves protect the rats from BNML with this therapy protocol.

Adoptive Immunotherapy With T Cells Leads to GVHD

Six allotransplanted rats received a T-DLI. Lymph node cells from PVG.7B rats, given i.v. in doses of 1×10^6 (0.6×10^6 $CD3^+$ cells), and 30×10^6 cells induced acute, brisk GVH reactions with dermatitis and skin lesions. The rats had to be sacrificed on days 31 and 22 after T-cell injection, respectively, with no signs of BNML development. Administration of DLI led to conversion from mixed to full donor chimerism (CC) in the T-cell population (Table 1, experiments 11 and 12; Fig. 4C).

A Second BMC Transplant Protects Against BNML

In contrast to NK or T cells, a clearcut effect on survival was observed when a second round with MHC-mismatched BMC was given as cellular therapy after alloBMT and BNML administration. A fully MHC-mismatched non-TCD alloBMT 7 days after BNML injection had a life-saving effect on the rats (Fig. 3B). Of the nine rats in this group, only one rat developed leukemia after 52 days, with a mixed T-cell chimerism of 54.4%. The other eight rats survived beyond 100 days with a mixed T-cell chimerism of $83.3\pm3.8\%$ (estimated for six rats; Table 1, experiment 6; Fig. 4B1). These rats had no signs of leukemia or

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TABLE 1. Summary of all experiments involving irradiated and allotransplanted rats

Experiment	Number of recipients	Protocol: Irradiation + 30×10^6 BMC donor type day −7	Protocol: 500 BNML day 0	Protocol: Cellular immunotherapy donor type day +7	Percentage donor T-cell chimerism day +35 (mean±SD)	Outcome
1	3 (PVG)	Match TCD (PVG.7B)	No		43.3±6.0	Terminated
2	3 (PVG.1N)	Mismatch TCD (PVG.7B)	No		77.7±7.8	Terminated
3	9 (BN)	Match (PVG.1N)	Yes			9→leukemia
4	10 (BN)	Match (PVG.1N)	Yes	Match (PVG.1N) $15–20\times10^6$ BMC		10→leukemia
5	12 (BN)	Mismatch TCD (PVG.7B)	Yes		53.4±8.3	11→leukemia 1→alive
6	9 (BN)	Mismatch TCD (PVG.7B)	Yes	Mismatch (PVG.7B) $15–20\times10^6$ BMC	83.3±3.8 54.4	8→alive 1→leukemia
7	6 (BN)	Mismatch TCD (PVG)	Yes	Mismatch (PVG.7B) $15–20\times10^6$ BMC	3.3±11.1	Terminated
8	2 (BN)	Mismatch TCD (PVG.7B)	Yes	Mismatch TCD (PVG.7B) $15–20\times10^6$ BMC	56.4±18.6	2→leukemia
9	3 (BN)	Mismatch TCD (PVG.7B)	Yes	Mismatch (nude)[a] 30×10^6 BMC	54.4±7.1	3→leukemia
10	6 (BN)	Mismatch (nude)[a]	Yes	Mismatch (nude)[a] 30×10^6 BMC		5→leukemia 1→alive
11	3 (BN)	Mismatch TCD (PVG.7B)	Yes	1×10^6 lymph node cells/T cells		3→GVHD
12	3 (BN)	Mismatch TCD (PVG.7B)		$\sim30\times10^6$ lymph node cells/T cells	100, 100	3→GVHD
13	5 long-term survivors		Yes			3→leukemia 2→dead

1×10^6 lymph node cells $\approx0.6\times10^6$ $CD3^+$ T cells.
[a] Nude BM lack functional T cells.

clinical GVHD and no detectable leukemic cells in blood, lymphoid organs or the bone marrow (data not shown). Some of these rats were terminated due to old age or used for further experiments, but the ultimate survival has been close to one year. The striking effect was dependent on an MHC disparity between the donor and recipient, since ten BN rats receiving an allogeneic MHC-matched BMC transplant twice from the PVG.1N strain succumbed to leukemia (Table 1, experiment 4; Fig. 3A).

Success of AlloBM Retransplantation Depends on T Cells in the Second Graft

T cells in the second transplant seemed to be essential for the protective effect, as of six BN rats transplanted twice with MHC-mismatched BMC from athymic nude rats (PVG-like, lacking functional T cells), only one rat was protected from BNML (Table 1, experiment 10). This was further confirmed by other experiments where three rats received a second mismatched transplant from athymic nude rats and two rats were transplanted twice with TCD-mismatched BMC (Table 1, experiments 8 and 9). These rats developed leukemia and showed a level of T-cell chimerism (54.4±7.1% and 56.4±18.6% respectively) close to rats transplanted once with TCD BMC (53.4±8.3%; Table 1, experiment 5). The antileukemic effect was furthermore dependent on the dosage of BMC cells given in the second transplant; all the rats depicted in Table 1, Figure 3A and B, and Figure 4B1 were given $15–20\times10^6$ BMC cells (containing 0.6×10^6 $CD3^+$ cells), titrated to the dose interval of cells that gave long-lasting protection. Of these, $5–10\times10^6$ cells did not protect and higher doses (30×10^6 cells or more) often induced symptoms of GVHD.

To estimate the contribution to T-cell chimerism by the second transplant, six rats were transplanted first with a mismatched-TCD transplant (PVG) and 14 days later with a second allotype-marked mismatched undepleted transplant (PVG.7B).

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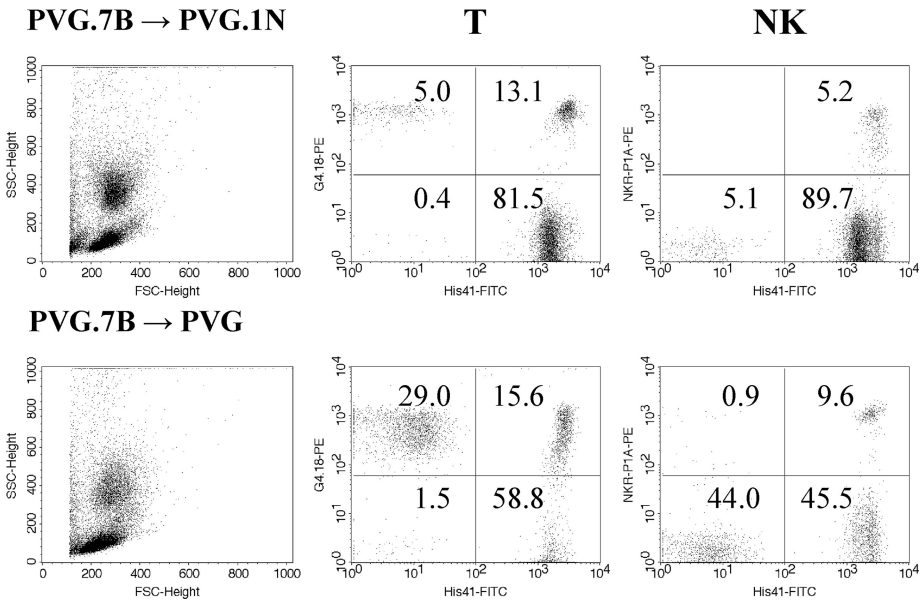

FIGURE 2. Representative dot-blot diagrams of blood leukocytes from experiments 1 and 2 in Table 1. The data shown the frequencies of donor and host-derived leukocytes in irradiated PVG.1N and PVG rats transplanted once with PVG.7B BMC on day 35 after transplantation. Donor cells were identified with mAb His-41 that binds to CD45.2 present on PVG.7B leukocytes, T cells with the mAb G4.18 against CD3, and NK cells with the mAb 3.2.3 against CD161. The percentage of cells in each quadrant is indicated. Percentage of donor-derived T or NK cells is calculated from the numbers given in the upper right quadrant (donor) divided by the sum of the numbers in the upper right+upper left quadrant (donor+host). Mean±SD of all the experiments in each group are given in Table 1 and in the text. Note the increased degree of donor chimerism in the MHC-mismatched chimera (Fig. 2A).

The number of T cells derived from the second injection was only 3.3±1.1% (Table 1, experiment 7; Fig. 4B2), but this small proportion of cells enhanced the degree of donor T-cell chimerism from 53.4±8.3% to 83.3±3.8% (Table 1, experiment 6). The contribution of NK cells from the second infusion was 6.6±1.7%.

T-cell chimerism was determined for four long-term surviving rats to a level of 87.6±5.2%. This chimerism status remained stable for up to 1 year after transplantation.

No Long-Term Protection Against a New Challenge With BNML

Finally, we tested the resistance of long-term survivors against rechallenge with BNML. Five BN rats that had received MHC-mismatched BMT and BNML 4–10 months previously were again given 500 BNML cells (Table 1, experiment 13). One rat died with bone marrow aplasia on day 21 without leukemic cells in blood, three rats were terminated on days 29–54 with splenomegaly and BNML in blood, while one rat was terminated on day 62 due to weight loss but with no signs of leukemia. The doubly MHC-mismatched alloBMT rats surviving the first encounter with BNML therefore had not developed long-lasting protective memory to BNML.

DISCUSSION

Here we have applied a rat alloBMT model for studying the effect of allogeneic BMT, with or without additional therapy with DLI from various sources, on the resistance against a promyelocytic leukemia (BNML). We employed a transplantation protocol that enabled a state of mixed chimerism to develop among the peripheral T cells. We chose BM donor strains that were either fully MHC-matched (PVG.1N) or mismatched (PVG) with the recipient BN rats. As expected, an MHC mismatch was required for investigating further antileukemia effects of alloBMT. BN rats transplanted twice with BMC from the MHC-matched, but otherwise allogeneic, PVG.1N strain, were not protected from BNML. Furthermore, to avoid serious GVH reactions resulting from an MHC-mismatched transplant, the initial BMC had to be depleted of T cells; such transplanted rats were minimally protected against leukemia.

The mixed chimerism in rats transplanted with TCD MHC-mismatched BMC served as a platform to study effects of further cellular therapy on leukemia development. We assessed peripheral T cells, NK cells, or BMC as a source of DLI. Because administration of peripheral T cells led to brisk lethal GVH reactions, even in numbers that were equivalent to T-cell numbers in the second BMT, they were not feasible therapeutic cells in our fully MHC-mismatched model.

Clinically, a second BMT is offered as salvage therapy for patients who relapse postBMT. Rationale for using prophylactic DLI in this study was to treat BNML disease while the leukemic cell load was still low. A non-T-cell-depleted BMC as DLI protected against leukemia without provoking a GVH response, indicating that T cells of the BM were prime contributors to the protection against leukemia. This was verified in experiments where rats had a second MHC-mismatched BMT from athymic nude rats, lacking functional T cells, or transplanted twice with BMC from TCD MHC-mismatched rats; all rats in both groups developed leukemia. The status of chimerism after MHC-mismatched transplantation was a particular relevant marker for outcome. After the first transplantation, the proportion of donor T-cells was approximately 50%. A level of 50% was associated with development of leukemia. The second prophylactic infusion of T cell containing BMC had a curative antileukemic effect, which coincided with increased level of donor

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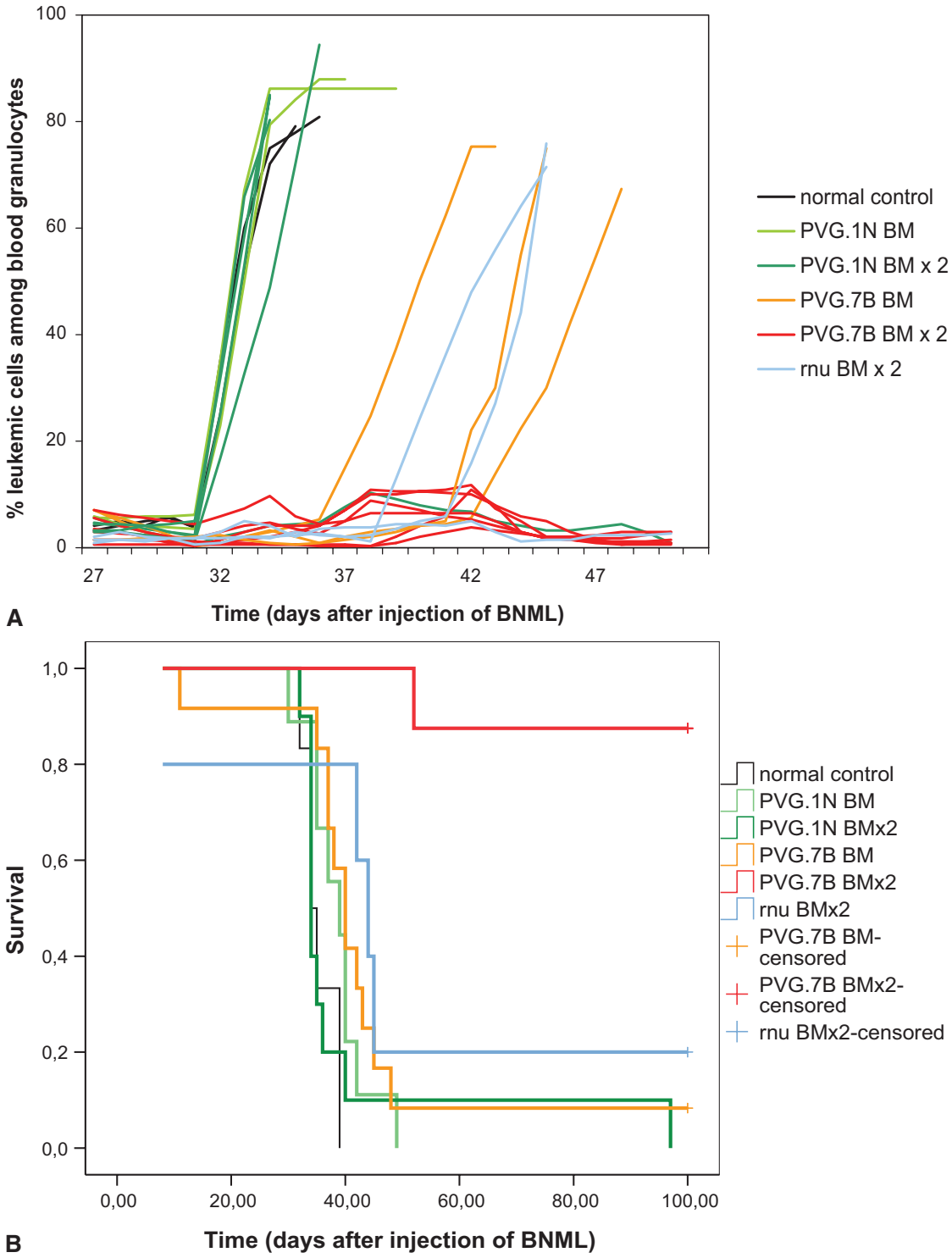

FIGURE 3. Prolonged survival of BNML-exposed BN rats transplanted twice with BMC from MHC-mismatched (PVG.7B) donors. Rats were irradiated and given the first alloBMT on day −7, given 500 BNML on day 0 and additional BMC therapy on day +7. Details of the transplantation protocol are given in Table 1. (A) Leukemia development in alloBMT BN rats from the different strain combinations. Results from representative rats in each group are presented. Percentage of BNML among blood granulocytes was determined with mAb RM124 that reacts strongly with BNML. Note the lack of BNML development of selected rats transplanted twice with PVG.7B BMC but also the slightly delayed leukemia development in rats transplanted once with MHC-mismatched PVG.7B or twice with PVG rnu/rnu BMC compared to normal control rats. (B) Only a double MHC-mismatched transplantation had a significant effect on survival. Note the failing effect on survival of all other therapies (rats transplanted once or twice with MHC-matched PVG.1N, rats transplanted twice with PVG rnu BMC, or rats given single MHC-mismatched BMT from PVG.7B) compared to rats transplanted twice with MHC-mismatched BMC. The differences between the survival of rats transplanted once (12 rats) and twice (9 rats) with MHC-mismatched BMC was highly significant ($P<0.001$).

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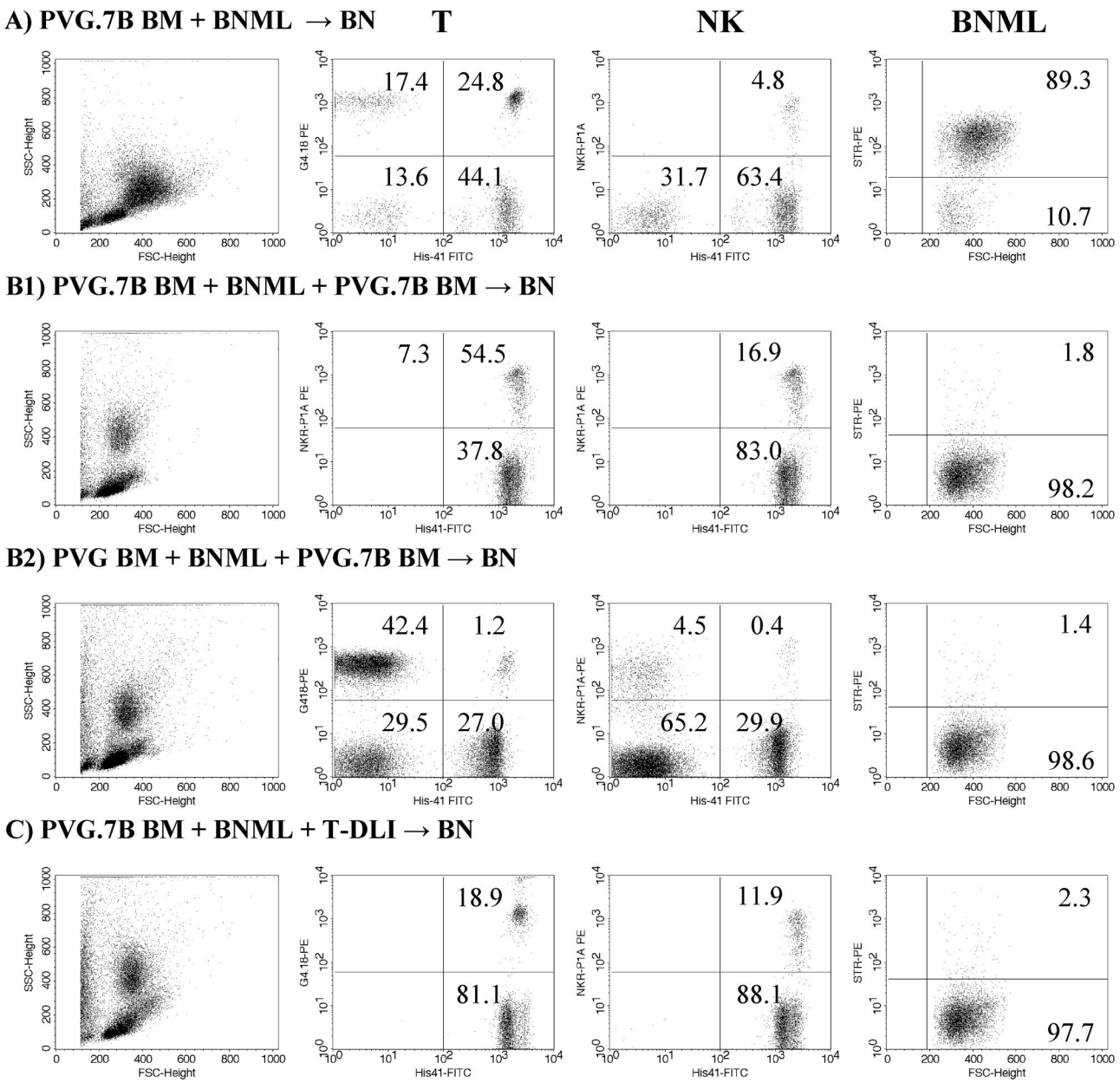

FIGURE 4. Representative dot-blot diagrams of blood leukocytes from experiments 5, 6, 7, and 11 in Table 1. Degree of donor T and NK cell chimerism of BN rats (A) transplanted once with BMC from PVG.7B (experiment 5). Rats receiving further cellular therapy with either (B1) a second BMT from PVG.7B (experiment 6) or (C) peripheral T cells from PVG.7B (experiment 11). Note the switch to 100% donor T-cell chimerism in the latter group. (B2) Contribution of the second BMT to the donor T and NK cell chimerism observed in BMC animals transplanted twice, the first time with PVG and the second with PVG.7B BMC (experiment 7).

T-cell chimerism, to approximately 80%. One possible interpretation of this finding was that the second MHC-mismatched transplant had induced a donor antihost response that led to a more complete eradication of host hematopoietic cells.

We found a correlation between persistence of recipient T cells, such as MC and the absence of clinical signs of GVHD. The number of $CD3^+$T cells (0.6×10^6) was approximately the same in the second BM inoculum as in the lowest dose of peripheral T cells in the T-DLI. Administration of T-DLI led to conversion of MC in the T-cell population to full donor chimerism and involved acute GVHD in all rats. Our data correspond to previous findings reporting that full donor T-cell chimerism after transplantation is strongly associated with GVHD (*32*). Based on these results, we hypothesized that BMC contain unique T-cell subsets that are responsible for the GVL effect without inducing GVHD.

Among candidates for cells mediating immunomodulatory effects, studies of T-regulatory cells (Tregs) have identified several populations of cells with different actions. It has been demonstrated that $CD4^+CD25^+$ Tregs inhibit GVHD in animal models (*33*), but in humans, data on the suppressive capacity of Tregs on GVHD are conflicting (*34*, *35*).

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Other Tregs, such as CD8 Tregs (*36*) and NKTregs (*37*, *38*), have been reported to be involved in various models of transplantation tolerance.

Alloreactive NK cells may play a central role in protection against leukemia in allotransplantation, indicated by studies in humans (*39*). Original studies of F1 hybrid resistance patterns in rats to parental BMT and BNML gave rise to this idea (*40*). Although we could not dismiss a role of NK-DLI in protection against BNML, the experiments did not support a primary role of NK cells either: 1) therapy of the PVG→BN chimeras with repeated injections of IL-2 activated alloreactive NK cells from PVG rats had no ameliorating effect on BNML development; or 2) BN rats that had been reconstituted twice with BMC from athymic nude rats (PVG-like) rather than normal PVG rats, were minimally protected from BNML. Athymic nude rats lack alloreactive T cells, but do have NK cells that are strongly alloreactive against MHC-mismatched target cells (*41*, *42*). Therefore, if NK cells contributed to the leukemia resistance observed here, this was dependent on other mechanisms.

Long-term surviving rats were not protected from a new exposure to BNML, but showed a delayed development of the disease. This was probably due to induction of tolerance, as evidenced by the stable MC in these rats (*43*).

Great effort has been undertaken in separating GVHD and GVL reactions by further defining those cells that are responsible for GVHD and GVL, respectively. In our study, we have used an additional injection of bone marrow cells to improve the development of T-cell chimerism to prevent GVHD without affecting GVL. This was obtained by TCD of a high dose of BMC in the first transplantation followed by a second T cell containing BMC injection, resulting in an increased but not complete donor cell chimerism. The phenotype of host and donor T cells that contribute to this balance between GVL and GVHD and the mechanism behind our findings is an issue for further investigations. If applicable to humans, repeated alloBMT from donors other than HLA-identical siblings, with a careful control of the T cell content of the first and second transplant, may provide a novel approach to leukemia therapy.

ACKNOWLEDGMENTS

This work is supported by the Norwegian Cancer Society and the Research Council of Norway and Oddrun Mjåland's Fund for Cancer Research and a Marie Curie research and training network under EU 6FP "TRANS-NET." We thank Dr. A. Neil Barcly, Oxford, England, for the kind gift of all the OX hybridomas and Dr. J. Kampinga, Groningen, the Netherlands, for the His-41 hybridoma.

REFERENCES

1. Venditti A, Buccisano F, Del Poeta G, et al. Level of minimal residual disease after consolidation therapy predicts outcome in acute myeloid leukemia. *Blood* 2000; 96: 3948.
2. Appelbaum FR. Haematopoietic cell transplantation as immunotherapy. *Nature* 2001; 411: 385.
3. Horowitz MM, Gale RP, Sondel PM, et al. Graft-versus-leukemia reactions after bone marrow transplantation. *Blood* 1990; 75: 555.
4. Thomas ED, Storb R, Clift RA, et al. Bone-marrow transplantation (second of two parts). *N Engl J Med* 1975; 292: 895.
5. Ferrara JL, Deeg HJ. Graft-versus-host disease. *N Engl J Med* 1991; 324: 667.
6. Anasetti C, Beatty PG, Storb R, et al. Effect of HLA incompatibility on graft-versus-host disease, relapse, and survival after marrow transplantation for patients with leukemia or lymphoma. *Hum Immunol* 1990; 29: 79.
7. Aversa F, Terenzi A, Tabilio A, et al. Full haplotype-mismatched hematopoietic stem-cell transplantation: A phase ii study in patients with acute leukemia at high risk of relapse. *J Clin Oncol* 2005; 23: 3447.
8. Barrett AJ, Savani BN. Stem cell transplantation with reduced-intensity conditioning regimens: A review of ten years experience with new transplant concepts and new therapeutic agents. *Leukemia* 2006; 20: 1661.
9. Slavin S, Morecki S, Weiss L, et al. Nonmyeloablative stem cell transplantation: Reduced-intensity conditioning for cancer immunotherapy–from bench to patient bedside. *Semin Oncol* 2004; 31: 4.
10. Champlin R, Khouri I, Shimoni A, et al. Harnessing graft-versus-malignancy: Non-myeloablative preparative regimens for allogeneic haematopoietic transplantation, an evolving strategy for adoptive immunotherapy. *Br J Haematol* 2000; 111: 18.
11. Dey BR, McAfee S, Colby C, et al. Impact of prophylactic donor leukocyte infusions on mixed chimerism, graft-versus-host disease, and antitumor response in patients with advanced hematologic malignancies treated with nonmyeloablative conditioning and allogeneic bone marrow transplantation. *Biol Blood Marrow Transplant* 2003; 9: 320.
12. Kolb HJ, Schattenberg A, Goldman JM, et al. Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. *Blood* 1995; 86: 2041.
13. Lewalle P, Triffet A, Delforge A, et al. Donor lymphocyte infusions in adult haploidentical transplant: a dose finding study. *Bone Marrow Transplant* 2003; 31: 39.
14. Huang XJ, Liu DH, Liu KY, et al. Donor lymphocyte infusion for the treatment of leukemia relapse after HLA-mismatched/haploidentical T-cell-replete hematopoietic stem cell transplantation. *Haematologica* 2007; 92: 414.
15. Rolstad B, Naper C, Lovik G, et al. Rat natural killer cell receptor systems and recognition of MHC class I molecules. *Immunol Rev* 2001; 181: 149.
16. Ljunggren HG, Karre K. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol Today* 1990; 11: 237.
17. Passweg JR, Tichelli A, Meyer-Monard S, et al. Purified donor NK-lymphocyte infusion to consolidate engraftment after haploidentical stem cell transplantation. *Leukemia* 2004; 18: 1835.
18. Koehl U, Sorensen J, Esser R, et al. IL-2 activated NK cell immunotherapy of three children after haploidentical stem cell transplantation. *Blood Cells Mol Dis* 2004; 33: 261.
19. Engh E, Strom-Gundersen I, Benestad HB, Rolstad B. Long-term donor chimerism after MHC (RT1) mismatched bone marrow transplantation in the rat: The role of host alloreactive NK cells. *Scand J Immunol* 2001; 54: 198.
20. Martens AC, Johnson RJ, Kaizer H, Hagenbeek A. Characteristics of a monoclonal antibody (RM124) against acute myelocytic leukemia cells. *Exp Hematol* 1984; 12: 667.
21. Perez-Simon JA, Caballero D, Ez-Campelo M et al. Chimerism and minimal residual disease monitoring after reduced intensity conditioning (RIC) allogeneic transplantation. *Leukemia* 2002; 16: 1423.
22. Recommendations for the health monitoring of mouse, rat, hamster, guineapig and rabbit breeding colonies. Report of the Federation of European Laboratory Animal Science Associations (FELASA) Working Group on Animal Health accepted by the FELASA Board of Management November 1992. *Lab Anim* 1994; 28: 1.
23. McCormack E, Bruserud O, Gjertsen BT. Animal models of acute myelogenous leukaemia: Development, application and future perspectives. *Leukemia* 2005; 19: 687.
24. Martens AC, van Bekkum DW, Hagenbeek A. The BN acute myelocytic leukemia (BNML) (a rat model for studying human acute myelocytic leukemia (AML)). *Leukemia* 1990; 4: 241.
25. Kampinga J, Kroese FG, Pol GH, et al. RT7-defined alloantigens in rats are part of the leucocyte common antigen family. *Scand J Immunol* 1990; 31: 699.
26. Leong LYW, Le Rolle AF, Deverson EV, et al. RT1-U: Identification of a novel, active, class Ib alloantigen of the rat MHC. *J Immunol* 1999; 162: 743.
27. Reisner Y, Gur H, Reich-Zeliger S, et al. Hematopoietic stem cell transplantation across major genetic barriers: Tolerance induction by megadose CD34 cells and other veto cells. *Ann NY Acad Sci* 2005; 1044: 70.
28. Bachar-Lustig E, Rachamim N, Li HW, et al. Megadose of T cell-

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depleted bone marrow overcomes MHC barriers in sublethally irradiated mice. *Nat Med* 1995; 1: 1268.
29. Aversa F, Tabilio A, Velardi A, et al. Treatment of high-risk acute leukemia with T-cell-depleted stem cells from related donors with one fully mismatched HLA haplotype. *N Engl J Med* 1998; 339: 1186.
30. Schulenburg A, Fischer M, Kalhs P, et al. Immune recovery after conventional and non-myeloablative allogeneic stem cell transplantation. *Leukemia Lymphoma* 2005; 46: 1755.
31. Naper C, Vaage JT, Lambracht D, et al. Alloreactive natural killer cells in the rat: complex genetics of MHC control. *Eur J Immuol* 1995; 25: 1249.
32. Hanash AM, Levy RB. Donor CD4+CD25+ T cells promote engraftment and tolerance following MHC-mismatched hematopoietic cell transplantation. *Blood* 2005; 105: 1828.
33. Childs R, Clave E, Contentin N, et al. Engraftment kinetics after nonmyeloablative allogeneic peripheral blood stem cell transplantation: Full donor T-cell chimerism precedes alloimmune responses. *Blood* 1999; 94: 3234.
34. Meignin V, de Latour RP, Zuber J, et al. Numbers of Foxp3-expressing CD4+CD25high T cells do not correlate with the establishment of long-term tolerance after allogeneic stem cell transplantation. *Exp Hematol* 2005; 33: 894.
35. Zorn E, Kim HT, Lee SJ, et al. Reduced frequency of FOXP3+ CD4+CD25+ regulatory T cells in patients with chronic graft-versus-host disease. *Blood* 2005; 106: 2903.
36. Gilliet M, Liu YJ. Generation of human CD8 T regulatory cells by CD40 ligand-activated plasmacytoid dendritic cells. *J Exp Med* 2002; 195: 695.
37. Pillai AB, George TI, Dutt S, et al. Host NKT cells can prevent graft-versus-host disease and permit graft antitumor activity after bone marrow transplantation. *J Immunol* 2007; 178: 6242.
38. Zeng D, Lewis D, Jbakhsh-Jones S, et al. Bone marrow NK1.1- and NK1.1+ T cells reciprocally regulate acute graft versus host disease. *J Exp Med* 1999; 189: 1073.
39. Farag SS, Fehniger TA, Ruggeri L, et al. Natural killer cell receptors: New biology and insights into the graft-versus-leukemia effect. *Blood* 2002; 100: 1935.
40. Williams RM, Singer DE, Rodday P, Bennett M. F1 Hybrid resistance to bn rat myelogenous leukemia parallels resistance to transplantation of normal bn bone marrow. *Leukemia Res* 1980; 4: 261.
41. Løvik G, Vaage JT, Naper C, et al. Recruitment of alloreactive natural killer cells to the rat peritoneum by a transfected cell line secreting rat recombinant interleukin-2. *J Immunol Meth* 1995; 179: 59.
42. Rolstad B, Benestad HB. Spontaneous alloreactivity of natural killer (NK) and lymphokine- activated killer (LAK) cells from athymic rats against normal haemic cells. NK cells stimulate syngeneic but inhibit allogeneic haemopoiesis. *Immunology* 1991; 74: 86.
43. Sykes M. Mixed chimerism and transplant tolerance. *Immunity* 2001; 14: 417.

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