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The role of perforin-mediated cell apoptosis in murine models of infusion-induced bone marrow failure

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Abstract

Objective—To investigate the role of perforin-mediated cell apoptosis in murine models of immune-mediated bone marrow (BM) failure.

Methods—We compared C57BL/6J (B6) mice carrying a perforin gene deletion (*Prf*^{−/−}) with wild type (WT) controls for cellular composition in lymphohematopoietic tissues. Lymph node (LN) cells from *Prf*^{−/−} mice were co-incubated with BM cells from B10-H2^b/LilMcJ (C.B10) mice in an apoptosis assay *in vitro*. We then infused *Prf*^{−/−} and WT B6 LN cells into sublethally-irradiated C.B10 and CByB6F1 recipients with mismatches at the minor- and major-histocompatibility loci, respectively, in order to induce BM failure. Cellular composition was analyzed by flow cytometry.

Results—*Prf*^{−/−} mice showed normal lymphoid cell composition but *Prf*^{−/−} LN cells had reduced ability to induce C.B10 BM cell apoptosis *in vitro*. Infusion of 5–10 × 10⁶ *Prf*^{−/−} LN cells produced obvious BM failure in C.B10 and CByB6F1 recipients; pancytopenia and BM hypocellularity were only slightly less severe than those caused by infusion of 5 × 10⁶ WT B6 LN cells. Infused *Prf*^{−/−} LN cells showed less T cell expansion, normal T cell activation, and higher proportions of T cells expressing gamma-interferon, tissue necrosis factor alpha and Fas ligand CD178, in comparison to infused WT B6 LN cells. Fas expression was equally high in residual BM cells in recipient of both *Prf*^{−/−} and B6 LN cells.

Conclusion—Perforin deficiency alters T cell expansion but up-regulates T cell Fas ligand expression. Perforin-mediated cell death appears to play a minor role in mouse models of immune-mediated BM failure.

Destruction of primitive hematopoietic stem cells (HSCs) and immature progenitor cells is characteristic of human bone marrow (BM) failure diseases, including aplastic anemia (AA), myelodysplastic syndrome (MDS) and paroxysmal nocturnal hemoglobinuria (PNH) [1–2]. Engagement of Fas ligand (FasL) CD178 and Fas receptor (Fas) CD95 provides a major signal pathway leading to the elimination of patients' BM cells and the development of severe marrow hypoplasia and fatal pancytopenia [3–6]. Activation of self-reactive T cells leads to an up-regulation of FasL and the production of inflammatory cytokines gamma interferon (IFN-γ) and tissue necrosis factor alpha (TNF-α), which in turn stimulates the expression of Fas on patient BM cells, facilitating target cell destruction [3–8]. Involvement of other cell death pathways in immune-mediated BM destruction, such as the exocytotic perforin/granzyme

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pathway, is yet to be well characterized. It is well known that both FasL/Fas and perforin/granzyme pathways are involved in cytotoxic T cell-induced target cell apoptosis [9,10].

The role of perforin-mediated cell death has been well defined in certain human diseases and animal models of disease. Mutations in the perforin gene occur in familial hemophagocytic lymphohistiocytosis (HLH), a lethal inherited disorder in which marrow failure is prominent [11–13]. In chronic active Epstein-Barr virus infection, a life-threatening disease that shares clinical features with HLH, mutations in perforin genes were identified to cause accumulation of an uncleaved, immature form of perforin, resulting in reduced perforin-mediated cytotoxicity [14]. Germline mutations in the perforin gene cause childhood anaplastic large cell lymphoma [15], while variations in the perforin gene were detected in patients with type 1 diabetes [16]. In mouse models of T cell-mediated diabetes mellitus, destruction of pancreatic islet beta cells was largely mediated by the perforin/granzyme cell death pathway [17–19]. In a cell lysis assay *in vitro*, a CD4 cytotoxic T cell clone NT4.2 isolated from the BM of a patient with cyclosporine-dependent AA produced cytotoxic effects toward an autologous Epstein-Barr virus-transformed B-lymphoblast cell line, in which the effect was largely mediated by the perforin pathway [20]. We recently described polymorphisms in the perforin gene in circulating lymphocytes from patients with acute AA associated with significantly lower perforin mRNA and protein [21], suggesting a potential role of perforin in immune-mediated BM failure, although the nature and the extent of the role are currently unclear.

To facilitate the investigation of pathophysiological mechanisms and to test potential new treatments, we have produced two murine models by infusing allogeneic lymph node (LN) cells into sublethally-irradiated recipients mismatched at major- (MHC) or minor-histocompatibility (minor-H) loci [22–26]. These animals developed severe pancytopenia and BM hypocellularity similar to the analogous human diseases [22,26]. Up-regulation of Fas expression on residual BM cells was a common feature in all affected animals, suggesting that FasL/Fas-mediated cell apoptosis contributed to the massive BM destruction [22,23]. We also tested the role of FasL/Fas-mediated cell death in the induction of BM failure, and found that abrogation of the FasL/Fas pathway causes a drastic decline in BM destruction (Omokaro et al., manuscript under review). In the current study, we specifically queried the role of perforin in the development of BM failure by using mice with germline deletion of the perforin gene (*Prf*^{-/-} mice). By testing effector T cell cytotoxicity *in vitro* and by infusing LN cells *in vivo* from *Prf*^{-/-} donors into sublethally-irradiated MHC or minor-H mismatched recipients, we found that *Prf*^{-/-} LN cells were capable of destroying host hematopoietic cells to cause pancytopenia and BM hypoplasia at an efficiency slightly lower than B6 LN cells, indicating that perforin-mediated cell death plays a minor role in immune-mediated BM failure. Together, our data provided a clear picture for immune-mediated BM failure: a small portion of marrow damage may be mediated by the perforin pathway while the most BM destruction requires the Fas/FasL signaling cascade.

Materials and methods

Mice and cell preparation

Inbred C57BL/6J (B6), congenic B6.SJL-PtprcaPepcb/BoyJ (B6-CD45.1), congenic C.B10-H2^b/LilMcdJ (C.B10), hybrid (B6 × BALB/cByJ)F1 (CByB6F1) and induced mutant C57BL/6-*Prf*^{tm1Sdz}/J (*Prf*^{-/-}) mice were all obtained from the Jackson Laboratory (Bar Harbor, ME), and were bred and maintained in the NIH animal facility under standard care and nutrition conditions [27]. *Prf*^{-/-} mice were on the B6 background and gene-deletion was confirmed by polymerase chain reaction using primers and conditions recommended by the Jackson Laboratory (www.jax.org). All mice were used at 2–6 months of age and were gender-matched between donors and recipients. Animal studies were approved by the National Heart, Lung, and Blood Institute Animal Care and Use Committee.

Inguinal, brachial, and axillary lymph nodes (LNs) were obtained from B6 and *Prf*^{-/-} mice and were homogenized and washed in IMDM. Peripheral blood (PB) was obtained through retro-orbital sinus bleeding. Cells were also obtained from spleen (SP) of B6 and *Prf*^{-/-} donor mice, homogenized and filtered through 90 μ M nylon mesh to obtain single cell suspension. BM cells were extracted from bilateral femurs and tibiae of donors and recipient animals. Cells were enumerated using a ViCell counter (Coulter Cooperation, Miami, FL).

Lymphocyte cytotoxicity in vitro

BM cells from normal C.B10 mice were extracted and used as targets to test LN cell cytotoxicity from B6 or *Prf*^{-/-} donors using a CyToxilux assay as described previously [26,28]. Target C.B10 BM cells were pre-loaded with a blue fluorescent dye (OncoImmunin, Inc., Gaithersburg, MD) at 37°C for 30 minutes according to the manufacturer's instructions, dispensed into 96-well plates at 2×10^4 cells per well, and then mixed with 20×10^4 , 40×10^4 and 60×10^4 B6 or *Prf*^{-/-} LN cell effectors to produce effector:target ratios (E:T ratios) of 10:1, 20:1 and 30:1, respectively. Effector and target mixtures were incubated at 37°C for 60 minutes, washed and stained with annexin V (BD Biosciences, San Diego, CA) for 30 minutes on ice and then propidium iodide (PI, 10 μ L of a 5 μ g/ μ L) was added immediately before acquisition. Stained cells were analyzed by a LSR-II flow cytometer (Becton Dickinson, San Jose, CA) in which target cells without effectors were stained with annexin V and PI as negative controls.

Induction of BM failure

LN cells from B6 and *Prf*^{-/-} mice were infused into C.B10 or CByB6F1 mice at $5\text{--}10 \times 10^6$ cells per recipient to induce BM failure. All recipient mice received a sub-lethal dose of 5 Gy total body irradiation (TBI) from a Shepherd Mark 1 ¹³⁷cesium gamma source (J. L. Shepherd, Glendale, CA) four to six hours before cell infusion. In each experiment, a group of mice that received 5 Gy TBI only were used as controls. Recipient mice were bled at two and three weeks after cell infusion. Complete blood counts (CBC) were performed using a Hemavet 950 analyzer (Drew Scientific, Oxford, CT). Mice were euthanized two or three weeks after the infusions and cells were extracted for analyses as specified in each experiment.

Flow cytometry

Monoclonal antibodies for mouse CD3 (clone 145-2C11), CD4 (clone GK 1.5), CD8 (clone 53-6.72), CD11a (clone 2D7), CD11b (clone M1/70), CD25 (clone 3C7), CD45R (B220, clone RA3-6B2), CD95 (Fas, clone Jo2), CD178 (FasL, clone Kay-10), IFN- γ (clone XMG1.2) and TNF- α (clone MP6-XT22) were all from BD Biosciences (San Diego, CA). Mouse Fox-P3 (clone FJK-16s) antibody was obtained from eBioscience (San Diego, CA). All antibodies were conjugated to either fluorescein isothiocyanate (FITC), phycoerythrin (PE), CyChrome, PE-Cyanin 5, or allophycocyanin (APC).

Cells were first incubated with Gey's solution (130.68 mM NH₄Cl, 4.96 mM KCl, 0.82 mM Na₂HPO₄, 0.16 mM KH₂PO₄, 5.55 mM Dextrose, 1.03 mM MgCl₂, 0.28 mM MgSO₄, 1.53 mM CaCl₂ and 13.39 mM NaHCO₃) for ten minutes on ice to lyse red blood cells, and then washed and stained with various antibody mixtures in FACS buffer (2.68 mM KCl, 1.62 mM Na₂HPO₄, 1.47 mM KH₂PO₄, 137 mM NaCl, 7.69 mM NaN₃, and 1% BSA) for 30 minutes on ice. For the measurement of intracellular cytokines, samples stained with cell surface markers were fixed, permeabilized, and incubated with specific cytokine antibodies for intracellular staining. Stained cells were all analyzed on a BD LSR II flow cytometer.

Data analyses

Data were analyzed using the JMP Statistical Discovery Software (SAS Institute, City NC) on one-way or two-way variance analysis platforms [29]. Results are shown as means and standard errors. Statistical significance was identified at $p < .05$ and $p < .01$ levels.

Results

Normal cellular composition but reduced lymphocyte cytotoxicity in *Prf*^{-/-} mice

We first examined cellular composition comparing *Prf*^{-/-} mice with normal B6 controls. Similar to the results reported previously [30], *Prf*^{-/-} mice had normal proportions of CD4, CD8, CD11b, and CD45R cells similar to WT B6 controls in the BM, LN, PB and SP. There was also no significant difference in the proportions of CD4⁺CD25⁺FoxP3⁺ regulatory T cells in the four lymphoid tissues between *Prf*^{-/-} (0.13 ± 0.03 , 3.10 ± 0.34 , 0.57 ± 0.11 , 0.78 ± 0.17) and WT B6 (0.05 ± 0.05 , 3.15 ± 0.59 , 0.50 ± 0.13 , 0.31 ± 0.22) mice.

To elucidate the role of perforin in lymphocyte-mediated cytotoxicity, we incubated effector LN cells from B6 and *Prf*^{-/-} mice with target BM cells from C.B10 mice in a CyToxilux assay *in vitro* [26,28]. The B6 and C.B10 mice differ at multiple minor-H loci, permitting LN cells from B6 donors to recognize C.B10 BM cells as foreign targets. Incubation of effectors and targets for 60 minutes at 37°C resulted in marked increases in target cell apoptosis, detectable by the staining of annexin V, with or without the uptake of PI (Figure 1A). In one experiment using an Effector:Target ratio of 20:1, we found that the proportions of annexin V⁺PI⁻ and annexin V⁺PI⁺ target cells were $14.1 \pm 1.8\%$ and $25.5 \pm 2.0\%$ for B6 effectors, and $8.2 \pm 1.8\%$ and $11.7 \pm 2.0\%$ for *Prf*^{-/-} effectors respectively, showing significant decline ($P < 0.05$ and $P < 0.01$) in *Prf*^{-/-} effector-induced apoptosis (Figure 1B). Similar results were also obtained in another experiment when different E:T cell ratios were used (data not shown).

Induction of BM failure by *Prf*^{-/-} LN cells

We evaluated the role of perforin deficiency on lymphocyte function *in vivo* by infusing LN cells from B6 and *Prf*^{-/-} donors into sublethally-irradiated CByB6F1 and C.B10 recipients. In four separate experiments, infusion of *Prf*^{-/-} LN cells induced BM failure in both types of recipients (Table 1). In the MHC-mismatched CByB6F1 recipients, infusion of $5\text{--}10 \times 10^6$ *Prf*^{-/-} LN cells caused obvious pancytopenia with significant ($P < 0.01$) declines in WBCs, neutrophils, RBCs and platelets, and severe marrow hypoplasia with a significant ($P < 0.01$) reduction in residual BM cells, in comparison to TBI controls. The severities of pancytopenia and BM hypoplasia were relatively similar for recipients of *Prf*^{-/-} and B6 LN cells (data not shown, similar to those in Figure 2). Notably, three recipients of 5×10^6 B6 LN cells died between 2–3 weeks while only one recipient of 10×10^6 *Prf*^{-/-} LN cells died during the same time period, indicating that *Prf*^{-/-} LN cells maybe less effective than are WT B6 LN cells in causing fatality in CByB6F1 mice. Although we did not study pathological changes in these specific animals, their death was likely secondary to BM failure. We have reported previously that C.B10 mice treated with 5Gy irradiation and 5×10^6 B6 LN cell infusion developed severe pancytopenia and fatal marrow hypoplasia showing an empty BM with only mild to no GVHD responses in other tissues [26].

We also infused *Prf*^{-/-} and B6 LN cells into minor-H-mismatched C.B10 mice and found that infusion of $5\text{--}10 \times 10^6$ *Prf*^{-/-} LN cells caused significant declines in blood ($P < 0.05$) and BM cell counts similar to the infusion of 5×10^6 ($P < 0.01$, Figure 2A). Total residual BM cells were also reduced in recipients of *Prf*^{-/-} and B6 LN cells when compared to TBI only controls ($P < 0.01$, Figure 2B). The declines in WBCs, RBCs, platelets and BM cells in *Prf*^{-/-} LN cell-infused animals were less severe than those in B6 LN cell-infused animals although the differences were not statistically significant (Figure 2, A & B). There was a significant

expansion of CD4 ($P<0.05$) and CD8 ($P<0.01$) T cell in the BM of recipients that received LN cell infusion than in the BM of TBI only control mice ($P<0.01$, Figure 2B); the level of T cell expansion, however, was significantly lower in recipients of *Prf*^{-/-} than in recipients of B6 LN cells (Figure 2, B & C).

***Prf*^{-/-} T cell activation in vivo**

To further study the role of *Prf*^{-/-} LN cells in immune-mediated BM failure, we next infused sublethally-irradiated CB6B6F1 and C.B10 recipients with LN cells from the same B6-CD45.1 and *Prf*^{-/-} donors (Figure 3A). B6-CD45.1 LN cells induced pancytopenia with significant declines ($P<0.05$) in residual BM cells in both CByB6F1 and C.B10 recipients, as expected (Figure 3B). About 75–90% of residual BM CD8 T cells from recipients of B6-CD45.1 LN cells were of the CD45.1 genotype, indicating that the expanded T cells in recipient BM originated from infused donor LN cells (Figure 3C). *Prf*^{-/-} LN cells, on the other hand, caused moderate pancytopenia and marrow hypoplasia in C.B10 recipients but only mild cytopenia in CByB6F1 recipients (Figure 3B). There was significantly less ($P<0.05$) CD4 and CD8 T cell expansion in recipients of *Prf*^{-/-} LN cells than in recipients of B6-CD45.1 LN cells (Figure 3C), however expanded T cells from both B6-CD45.1 and *Prf*^{-/-} donors had significantly higher ($P<0.01$) proportions of CD4 and CD8 T cells that are CD11a⁺ than T cells from TBI controls (Figure 4A). By intracellular staining, CD8 T cells from recipients of *Prf*^{-/-} LN cells had significantly higher ($P<0.05$) percentage of IFN- γ ⁺ cells than did those from recipients of B6-CD45.1 LN cells (Figure 4B). Among *Prf*^{-/-} LN cell-infused mice, C.B10 recipients had a much higher ($P<0.05$) proportion ($46.9 \pm 8\%$) of IFN- γ ⁺ CD8 T cells than CByB6F1 ($26.0 \pm 6.2\%$) recipients (Figure 4B). Recipients of *Prf*^{-/-} LN cells had higher proportion of TNF- α ⁺ CD8 T cells than recipients of B6 LN cells (Figure 4C).

We further examined the expression of FasL on BM CD8 T cells and Fas on residual BM cell in all animals. In recipients of *Prf*^{-/-} LN cells, the proportion of FasL-expressing CD8 T cell percentage ($36.0 \pm 2.0\%$) was significantly higher ($P<0.01$) than that in recipients of B6 LN cells ($23.6 \pm 2.4\%$) (Figure 4D). Conversely, the proportion of Fas positive BM cells was higher in B6 and *Prf*^{-/-} LN cell-infused recipients than in TBI controls (Figure 4D), indicating that infusion of B6 or *Prf*^{-/-} LN cells augmented Fas expression on recipient BM cells to facilitate marrow destruction.

Discussion

The *Prf*^{-/-} mice had normal cellular composition in lymphoid tissues which is consistent with earlier reports showing that lack of perforin does not affect differentiation and maturation of T cells, B cells and NK cells [17,30]. Upon infusion into C.B10 or CByB6F1 recipients, LN cells from both *Prf*^{-/-} and B6 donors showed similar levels of T-cell activation which confirms an earlier report indicating that T cell activation was not compromised in *Prf*^{-/-} mice [17]. Over the years, the role of the perforin/granzyme pathway in cell cytotoxicity has been well studied in the clearance of viral, parasite and fungal infections [17,31–35], in the induction of autoimmune disorders [18,19,36–39], in the eradication of progressive cancerous cells through anti-tumor immunotherapy [40–43], and in the control of immune response by regulatory T cells [44]. In a rat model of crescentic glomerulonephritis, CD8 T cells play a role in glomerular injury as effectors in part through a perforin/granzyme-mediated pathway [45]. In a cell cytotoxicity assay *in vitro*, perforin/granzyme B-mediated cell cytotoxicity was responsible for cell death in mouse pancreatic islet cells but not in mouse hematopoietic cells, showing tissue/cell type-specific regulation of apoptosis [19]. Our observation that perforin-deficient LN cells had only a slightly reduced ability to induce BM failure is in line with the notion that hematopoietic cells are less sensitive to perforin-mediated cell death.

Perforin/granzyme-mediated cell death has also been studied in graft tolerance/rejection and graft versus host diseases (GVHD). By transplanting syngeneic BM cells into lethally-irradiated recipients, Graubert et al found that perforin/granzyme-mediated cytotoxicity is essential for class I-restricted, but not class-II restricted, GVHD responses as measured by animal mortality [30]. In a skin graft model, Bose et al found that perforin is involved in the long-term survival of either MHC-I or MHC-II mismatched grafts[46]. Maeda et al reported more recently that both perforin and FasL are required for the regulation of alloreactive CD8 T cells during acute GVHD[47]. In our study, both MHC and minor-H mismatch models involve multiple antigens without separate class I and II immune responses. While we have no definitive explanation for the published diverse results, we can speculate that the complexity of cell death process mediated by multiple signaling pathways that function in a competitive, interactive, or complementary fashion likely account for varied observations in different experimental settings.

Both FasL/Fas and perforin/granzyme share certain functional elements, such as utilization of caspase-8[48], and may function to mutually compensate when one pathway is down regulated. Our results are in good agreement with this theory as we observed FasL up-regulation on activated T cells from *Prf*^{-/-} donors. This observation is also consistent with an earlier report showing perforin-dependent cell death as compensatory for Fas deficiency in activation-induced lymphocyte apoptosis from patients with autoimmune lymphoproliferative syndrome [49]. Results from current study also concur with results from another study we performed recently in which infusion of LN cells from Fas- and FasL-deficient donors produced only mild to no BM failure in minor-H mismatched C.B10 recipients (Omokaro et al., manuscript under review), suggesting that Fas/FasL pathway plays a major role in marrow destruction.

Our current study specifically explored the role of the perforin/granzyme pathway in immune-mediated BM failure. LN cells from *Prf*^{-/-} donors showed reduced cytotoxicity *in vitro* but caused moderate to high level BM failure when infused into MHC- and minor-H-mismatched recipients *in vivo*. The efficacy of *Prf*^{-/-} LN cells for BM destruction was slightly lower than that of normal B6 LN cells, indicating that perforin-mediated cell death plays a minor role in immune-mediated BM failure.

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Apoptosis and Cell Death

Figure 1. Reduced BM cell apoptosis mediated by *Prf*^{-/-} LN cells

LN cells from B6 and *Prf*^{-/-} donors (effectors) were co-incubated with C.B10 BM cells (targets) at 10:1 to 30:1 ratios respectively for 60 minutes at 37°C. Proportions of target cells staining positive for Annexin V, with or without uptake of Propidium Iodide, were shown as representative dot plots (**A**) as well as means with standard errors from 8 culture wells per cell type in one experiment as representatives of four independent experiments (**B**).

Figure 2. *Prf*^{-/-} LN cell-induced BM failure in MHC or minor-H mismatched cell infusion models
 In the four experiments described in Table 1, we infused LN cells from B6 and *Prf*^{-/-} donors into sublethally-irradiated, minor-H mismatched C.B10 or MHC-mismatched CByB6 F1, recipients respectively. Recipients of *Prf*^{-/-} LN cells developed neutropenia, thrombocytopenia, and anemia (A) with obvious signs of BM hypoplasia (B) similar to recipients of B6 LN cells. However, recipients of *Prf*^{-/-} LN cells had lower level of T cell expansion in the residual BM at three weeks after cell infusion shown as means with standard errors (B) as well as representative histograms (C). Data shown are from experiments III and IV as described in Table 1 with five to six animals in each treatment group. Similar changes were also observed in experiments I and II.

Figure 3. Differential responses of *Prf*^{-/-} LN cells in MHC and minor-H mismatched recipients
 In a separate experiment, we infused 5×10^6 LN cells from the same B6-CD45a and *Prf*^{-/-} donors into sublethally-irradiated CByB6F1 and C.B10 recipients respectively (**A**). *Prf*^{-/-} LN cells caused similar levels of cytopenia in blood and BM as B6 LN cells in C.B10 recipients but less severe thrombocytopenia and marrow hypoplasia in CByB6F1 recipients (**B**). In both recipient types, *Prf*^{-/-} LN cells, in comparison to B6 LN cells, showed significantly less T cell expansion in recipient BM. The expanded T cells were originated from the infused donor LN cells (**C**). The *Prf*^{-/-} to CByB6F1 LN cell infusion group used five recipients while all other treatment groups each used three recipients.

BM T Cell Activation

Figure 4. T cell activation, cytokine production, and Fas/FasL expression

Animals from the LN cell infusion experiment described in Figure 3 were euthanized at day 14. Residual BM cells were analyzed for the expression of the T cell activation marker CD11a (A). BM CD8 T cells were also analyzed for the production of inflammatory cytokines IFN- γ (B) and TNF- α (C). Expression of FasL was measured on BM CD8 T cells while expression of Fas was measured on residual BM cells (D). Infusion of B6 and *Prf*^{-/-} LN cells caused

significant T cell activation with up-regulation in the expression of TNF- α , FasL and Fas in the BM.

Table 1
Perforin deficiency on immune-mediated BM failure

LN Donor	Cell Dose	Recipients	N	Results
Experiment I				
None	0	CByB6F1	2	Self-recovery
B6	5 × 10 ⁶	CByB6F1	3	Pancytopenia, BM hypoplasia
<i>Prf</i> ^{-/-}	5 × 10 ⁶	CByB6F1	3	Pancytopenia, BM hypoplasia
Experiment II				
None	0	CByB6F1	3	Self-recovery
B6	5 × 10 ⁶	CByB6F1	3	All died at three weeks
<i>Prf</i> ^{-/-}	5 × 10 ⁶	CByB6F1	3	Pancytopenia, BM hypoplasia
<i>Prf</i> ^{-/-}	10 × 10 ⁶	CByB6F1	3	One died at three weeks
Experiment III				
None	0	C.B10	2	Self-recovery
B6	5 × 10 ⁶	C.B10	2	Pancytopenia, BM hypoplasia
<i>Prf</i> ^{-/-}	5 × 10 ⁶	C.B10	3	Pancytopenia, BM hypoplasia
<i>Prf</i> ^{-/-}	10 × 10 ⁶	C.B10	3	Pancytopenia, BM hypoplasia
Experiment IV				
None	0	C.B10	3	Self-recovery
B6	5 × 10 ⁶	C.B10	3	Pancytopenia, BM hypoplasia
<i>Prf</i> ^{-/-}	5 × 10 ⁶	C.B10	3	Pancytopenia, BM hypoplasia
<i>Prf</i> ^{-/-}	10 × 10 ⁶	C.B10	3	Pancytopenia, BM hypoplasia

All recipient mice received 5 Gy TBI 4–6 hours before LN cell infusion. Recipient mice were euthanized at three weeks after cell infusion for BM cellular composition analysis. Criteria for pancytopenia and marrow hypoplasia used in the current study: WBCs <0.4 × 10⁶/mL, neutrophils <0.1 × 10⁶/mL, RBCs <6 × 10⁹/mL; platelets <150 × 10⁶/mL; and total BM cells <100 × 10⁶/mouse.