A powerful new strategy for allogeneic bone marrow transplantation in mice

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Abstract
We have succeeded in treating intractable autoimmune diseases in chimeric resistant MRL/lpr mice by a new bone marrow transplantation (BMT) method consisting of fractionated irradiation (5.5 Gy x 2) followed by intra-bone marrow (IBM) injection of whole bone marrow cells (BMCs) from allogeneic normal C 57 BL/6 (B 6) mice [5.5 Gy x 2 + IBM]. In MRL/lpr mice treated with this method, the number of not only donor-derived cells but also donor-derived hemopoietic progenitor cells increased. Furthermore, donor-derived stromal cells were clearly detected in the cultured bone pieces from MRL/lpr mice treated with [5.5 Gy x 2 + IBM]. All the recipients thus treated survived more than one year (>60 weeks after birth) and remained free from autoimmune diseases. Autoantibodies decreased to almost normal levels, and abnormal T cells (Thy 1.2+/B 220k+/CD 4-/CD 8-) disappeared. These findings clearly indicate that this new strategy ("IBM-BMT") creates the appropriate hemopoietic environment for the early recovery of hemopoiesis and donor cell engraftment, resulting in the complete amelioration of intractable autoimmune diseases in chimeric resistant MRL/lpr mice without recourse to immunosuppressants. This strategy would therefore be applicable to human therapy.

Introduction
We have previously shown using various autoimmune-prone mice that conventional allo BMT can be used to prevent and treat a range of autoimmune diseases. 1-3 These findings have recently been confirmed even in humans. 4-6 However, in humans, the success rate of BMT across major histocompatibility complex (MHC) barriers is lowered by graft-versus-host disease (GvHD), graft rejection, and incomplete T-cell recovery. Therefore, autologous BMT (auto BMT) or auto peripheral blood stem cell transplantation (PBSCT) is the preferred treatment for autoimmune diseases. There have, however, been reports on the rapid recurrence or persistence of autoimmune diseases after auto BMT or auto PBSCT. 7 Therefore, it is important to establish a safe new method for allo BMT. 8

We have found that the MRL/lpr mouse, an animal model for autoimmune diseases, is a suitable model for establishing a safe new strategy for all BMT, since the MRL/lpr mouse itself is radio-sensitive (8.5 Gy), while the abnormal hemopoietic stem cells of the MRL/lpr mouse are radio-resistant (>8.5 Gy); conventional BMT (8.5 Gy plus allo BMT) has a transient effect on autoimmune diseases, which recur 3 months after the BMT. 8

To prevent the recurrence of autoimmune diseases in MRL/lpr mice, we carried out BMT plus bone grafts to replace not only the hemopoietic cells but also the stromal cells with donor cells. This is because there is a MHC restriction between pluripotent hemopoietic stem cells (P-HSCs) and stromal cells. 9,10 MRL/lpr mice that had been irradiated (8.5 Gy) and then reconstituted with C 57 BL/6 BMCs plus bone grafts survived more than 1 year.11,12
Although we found that allogeneic BMT plus bone grafts (to recruit donor stromal cells) could prevent the development of autoimmune diseases in MRL/lpr mice, this strategy (8.5 Gy/bone/BMT) was found not to be beneficial for the treatment of florid autoimmune diseases in MRL/lpr mice. MRL/lpr mice with small amounts of proteinuria (< +) can endure 8.5 Gy irradiation, whereas MRL/lpr mice with greater degrees of proteinuria (>2+) are more radiosensitive and are unable to withstand 8.5 Gy irradiation due to uremic enterocolitis. We therefore devised a new method, fractionated radiation (5 Gy X2), which reduces acute radiation injury and therefore reduces the side effects of radiation and prevents graft rejection.

Recently, we have found that most donor HSCs are trapped and retained in the liver when they are injected either portal venously (PV) or even intravenously (IV), and that the HSCs induce anergy to host CD 8+ T cells. In addition, we have found a strategy (the PV plus the supplemental IV injections of whole BMCs) that induces persistent tolerance in the skin allograft system. On the basis of these findings, we have very recently established a new strategy for allo BMT: fractionated irradiation (5.5 Gy X2) and the PV plus IV injections of whole BMCs. However, this method has two demerits for patients: i) a laparoscope-guided injection of BMCs via the PV is necessary, and ii) an additional IV injection is essential for obtaining a 100% success rate. We have analyzed the mechanism underlying the tolerance induced by the PV injection of BMCs and noted the importance of donor-derived stromal cells trapped in the liver, which facilitate the proliferation and differentiation of donor HSCs. Based on these findings, we attempted to inject whole BMCs (including stromal cells) directly into the bone marrow (intra-bone marrow [IBM] injection). We here show that "IBM-BMT" is a powerful strategy for the treatment of intractable autoimmune diseases in chimeric-resistant MRL/lpr mice.

**Materials and methods**

**Mice**

Female MRL/Mp-lpr/lpr (MRL/lpr, H-2k), C 57 BL/6 (B6, H-2b), BALB/c (H-24) were purchased from SLC (Shizuoka, Japan) and maintained until use in our animal facilities under specific pathogen-free conditions.

**Preparation of donor BMCs and IBM injection of BMCs**

The BMCs were collected from the femurs and tibias of B6 mice. The IBM injection was carried out as a follow: The region from the inguen to the knee joint was shaved of hairs with a razor. The knee was flexed to 90 degrees and the proximal side of the tibia was shaved to the anterior. A 26-gauge needle was inserted into the joint surface of the tibia through the patellar tendon and then inserted into the bone marrow cavity. Using a microsyringe (50 μl, HAMILTON Co., Reno, NV) containing the donor BMCs (3X10^7/0.3 ml), the donor BMCs were injected from the said bone hole into the bone marrow cavity.

**PV injection of BMCs**

The BMCs were injected via the PV as described previously. In brief, a midline incision was made on the abdomen to expose the viscera. The donor BMCs (3X10^7/0.3 ml) were injected via the superior mesenteric vein using a 27-gauge needle.

**Experimental protocols**

The onset of autoimmune diseases in MRL/lpr mice was monitored by proteinuria (> 2.5 +) and lymphadenopathy. The mice (4 to 5 months of age) with autoimmune diseases were irradiated in fractionated doses (5.5 GyX2=11 Gy; 4-hour interval). One day after the irradiation, the mice were transplanted with the whole BMCs (3X10^7) by IBM injections [abbreviated as 5.5 GyX2+IBM]. Two groups were prepared as controls. The first group consisted of mice that were irradiated (5.5 Gy X2) and transplanted with the whole BMCs (3X10^7) via the PV. [abbreviated as 5.5 GyX2+PV]. The second group of mice were irradiated (5.5 GyX2) and transplanted with the whole BMCs (3X10^7) via the tail vein [abbreviated as 5.5 GyX2+IV].

**Analyses for surface marker antigens**

The spleen cells, HMNCs and BMCs were prepared from the recipient mice, and the cells were then stained with FITC-conjugated anti-H-2D^d and PE-conjugated anti-H-2K^d mAbs (PharMingen, San Diego, CA) to identify the donor-derived cells. The cell surface phenotypes were also analyzed by FITC-or PE-conjugated mAbs against Thy 1.2, B 220, CD 4, CD 8, CD 11 b, and Gr-1 (PharMingen). Furthermore, the cells were stained with
biotinylated mAbs against lineage (Lin) markers [anti-CD 4, anti-CD 8 (CALTAG, Burlingame, CA), anti-B 220, anti-Gr-1, and anti-CD 11 b (PharMingen)], followed by streptavidin-RED 670 (GIBCO BRL, Rockville, MD), then further stained with PE-anti-c-kit mAb and FITC-anti-H-2 D\(^b\) or anti-H-2 K\(^b\) (PharMingen). The cells with the immunophenotype of Lin\(^{-}\)/c-kit\(^{+}\)/H-2\(^{b+}\) were categorized as donor-derived hemopoietic progenitors. The stained cells were analyzed by a FACScan (Becton Dickinson & Co, Mountain View, CA).

**Analyses for donor-derived stromal cells**

To prepare the bone marrow-derived stromal cells, the humeri from which the BMCs had been extensively washed out were cut into pieces, and the bone pieces were then cultured in a flask containing PRMI with 10% FBS at 37°C in 5% CO\(_2\) in air. Three weeks later, the adherent cells were then collected from the surface of the flask using CELL DISSOCIATION SOLUTION (SIGMA). To detect donor-derived stromal cells, the cultured cells were stained with anti-PA 6 mAbs, which were reactive to the stromal cells,\(^\text{17}\) followed by PE-anti-Rat IgG (GIBCO BRL). After blocking with normal rat IgG (PharMingen), the cells were further stained with FITC-anti-H-2 D\(^b\) or anti-H-2 K\(^b\), and analyzed by a FACScan\(^\text{®}\). The cultured cells stained with isotype-matched lgs served as a negative control.

**Measurement of autoantibodies**

RFs (IgG and IgM) and anti-ssDNA Abs (IgG and IgM) in the sera of the recipient mice were determined by a standard enzyme-linked immunosorbent assay (ELISA) as described previously.\(^\text{16}\)

**Analyses for immunological functions**

Antibody production against sheep red blood cells (SRBCs) and mixed leukocyte reaction (MLR) were performed as immunological functions of the treated mice. Anti-SRBC antibody response was evaluated as described previously.\(^\text{16}\)

**Statistical analyses**

Statistical analyses of the survival rate of the recipient mice were performed using a logrank test.

**Results**

**Survival rates after BMT**

MRL/lpr mice (4-5 months of age) that had developed the symptoms of autoimmune diseases such as massive lymphadenopathy and proteinuria (more than 2.5+) were first treated with (5.5 Gy\(\times\)2+PV). As shown in Figure 1, more than 70% of the mice thus treated survived more than 1 year, indicating that this treatment has some effect on the treatment of autoimmune diseases. We next treated autoimmune diseases in MRL/lpr mice with [5.5 Gy\(\times\)2+PV+IV]. Thus-treated MRL/lpr mice showed a 100% survival rate 1 year after the treatment, indicating that the supplemental IV injection of BMCs is helpful for successful engraftment.\(^\text{16}\) In contrast, all the recipients treated with (8.5 Gy\(\times\)2) died within 4 weeks because of the side effects of radiation, as previously described.\(^\text{12}\)

We analyzed the mechanism underlying the tolerance induced by the PV injection of BMCs and noted the importance of donor-derived stromal cells trapped in the liver, these cells facilitating the proliferation and differentiation of donor HSCs.\(^\text{18}\) Based on these findings, we attempted to inject whole BMCs (including stromal cells) directly into the bone marrow cavity (intra-bone marrow [IBM] injection).

We reduced the radiation dose from 5.5 Gy\(\times\)2 to 5 Gy\(\times\)2 in order to compare the effect of IBM-BMT with that of PV-BMT. MRL/lpr mice treated with either (5 Gy\(\times\)2+PV) or (5 Gy\(\times\)2+PV+IV) showed survival rates of 30% and 50%, respectively (Figure 1). These findings indicate that the 5 Gy\(\times\)2 irradiation followed by the treatment with PV+IV is insufficient to prevent graft rejection. In contrast, all the recipients that had received "IBM-BMT" survived 48 weeks after the treatment without showing any signs of graft rejection or recurrence of autoimmune diseases even when treated with 5 Gy\(\times\)2+IBM (Figure 1). Furthermore, more than 85% of the MRL/lpr mice survived 30 weeks after the treatment, even when treated with 4.5 Gy\(\times\)2+IBM.

**Analyses of donor-derived hemopoietic cells**

The percentages of the donor-derived hemopoietic cells
in the bone marrow, spleen, and liver were kinetically examined on days 3, 7, 10, and 14 after the treatment with [5.5 Gy×2+IBM], and compared with those from the recipients that received [5.5 Gy×2+IV] or [5.5 Gy×2+PV]. As shown in Fig. 2 B, when treated with [5.5 Gy×2+PV], the percentages of donor-derived cells in the hemopoietic organs had increased by 10 days after the treatment, but then gradually decreased. When treated with [5.5 Gy×2+PV], the percentages of donor-derived cells had rapidly increased by 10 days and reached almost 100% on day 14. When treated with [5.5 Gy×2+IBM], the percentages of donor-derived cells remarkably increased to approximately 70% both in the bone marrow from the tibia (directly injected with BMCs) and the femur (not directly injected with BMCs), and 40% in the spleen on day 3 after the treatment, and reached to
Figure 2. Percentages of donor-derived cells in MRL/lpr mice treated with \([5.5 \text{ Gy} \times 2 + \text{IBM}], [5.5 \text{ Gy} \times 2 + \text{PV}], \) or \([5.5 \text{ Gy} \times 2 + \text{IV}].\)

(A) Representative dot-plot profiles of BMCs, spleen cells and HMNCs obtained from MRL/lpr mice 3 or 10 days after the treatment with \([5.5 \text{ Gy} \times 2 + \text{IBM}], [5.5 \text{ Gy} \times 2 + \text{PV}], \) or \([5.5 \text{ Gy} \times 2 + \text{IV}].\) The cells in recipients were collected and stained with donor (FITC-anti-H-2\(b\)) and recipient (PE-anti-H-2\(k\))-specific mAbs. (B) Kinetic analysis of donor-derived cells. Donor-derived cells in the bone marrow, spleen, and liver were analyzed at the days indicated on the X-axis after staining with FITC-anti-H-2\(b\) and PE-anti-H-2\(k\) mAbs. The results are expressed as the mean ± SD of 6 mice. Symbols representing the sites of the injection of BMCs are shown as follows: Closed circles represent donor-derived BMCs collected from the tibia where the donor BMCs were directly injected, and closed diamonds show donor-derived BMCs obtained from the femur. Closed triangles indicated PV and closed squares indicated IV.

Almost 100% in both bone marrow (tibia and the femur) and spleen on day 10 (Fig. 2 B).

These were histologically confirmed (Fig. 3); both the tibia (Fig. 3 A) where directly injected with the BMCs, and femur (Fig. 3 B), not directory injected with BMCs, showed a remarkable proliferation of hematopoietic cells in comparison with the tibia (Fig. 3 C) and femur (Fig. 3 D) of the mice treated with \([5.5 \text{ Gy} \times 2 + \text{IV}].\) when examined 14 days after the treatment. The tibia and femur of the mice treated with \([5.5 \text{ Gy} \times 2 + \text{IV}].\) showed hypoplastic bone marrow. These findings clearly indicate that “IBM-BMT” facilitates engraftment.

Furthermore, Donor-derived cells with mature lineage markers (B 220\(^+\), CD 4\(^+\), CD 8\(^+\), Mac-1\(^+\), and Gr-1\(^+\)) had also been generated in the bone marrow, spleen, and liver by 14 days after the treatment with \([5.5 \text{ Gy} \times 2+\)
Figure 3. Histological findings in the tibia and femur of a MRL/lpr mouse 14 days after the treatment with [5.5 Gy×2+IBM] or [5.5 Gy×2+IV]. The tibia (directly injected with the BMCs: A) and femur (not directly injected: B) of the MRL/lpr mouse treated with [5.5 Gy×2+IBM] showed hyperplastic bone marrow 14 days after the treatment. On the other hand, the tibia (C) and femur (D) of the mouse treated with [5.5 Gy×2+IV] showed the hypoplastic bone marrow 14 days after the treatment. Immunohistochemical analysis of bone marrow treated with [5.5 Gy×2+IBM] (E). Note that cells stained both with anti-PA 6 mAb and anti-H-2 D^b mAb (yellow-colored) were scatteringly observed. Donor-derived BMCs were stained by green.

IBM], and remained at normal levels 1 year after the treatment (data not shown). In contrast, 14 days after the treatment with [5.5 Gy×2+IV], hardly any donor-derived cells bearing lineage markers could be detected in the hemopoietic organs. These findings indicate that "IBM-BMT" significantly facilitates the early engraftment and continuous proliferation and differentiation of donor cells.

Analyses of donor-derived progenitor cells

The engraftments of hemopoietic stem cells and progenitor cells are crucially important to maintain hemopoiesis. Since many mice (>10 mice in each group) are necessary to analyze the pluripotent hemopoietic stem cells (P-HSCs) (c-kit^{low}/Lin^−),^1^ we examined the frequency only of donor-derived progenitor cells (c-kit^{+/Lin^-}cells) in the hemopoietic organs. As
Figure 4. Percentages of donor-derived c-kit+/Lin−-cells in a MRL/lpr mice treated with [5.5 Gy×2+IBM], [5.5 Gy×2+PV] or [5.5 Gy×2+IV].

(A) A representative dot-plot profile of BMCs (the femur) obtained from MRL/lpr mice 14 days after the treatment with [5.5 Gy×2+IBM] is shown. The cells in recipients were collected and stained with FITC-anti-H-2b mAb or FITC-anti-H-2k mAb to detect the donor- or host-derived cells (gated as R1). Cells were then stained with PE-anti-c-kit and biotinated mAbs (anti-CD 4, anti-CD 8, anti-CD 45 R, anti-CD 11 b, and anti-Gr-1) plus avidin-RED 670. The donor- or host-derived hemopoietic progenitor cells (c-kit+/Lin−-cells) were observed (gated as R2), and the percentages of these cells to total cells were calculated, respectively. The FACS profile of negative control where cells stained with isotype-matched biotinated-IgG 2α/IgG 2β plus RED 670 and PE-IgG 2α (instead of PE-anti-c-kit mAb), is also represented.

(B) Kinetical analysis of donor- or host-derived progenitor cells. The percentages of donor- (closed symbols and straight lines) or host- (open symbols and dashed lines) of c-kit+/Lin− progenitor cells in the bone marrow, spleen and liver (hepatic mononuclear cells) in MRL/lpr mice treated with [5.5 Gy×2+IBM] (circles; tibia. diamonds; femur), [5.5 Gy×2+PV] (triangles), or [5.5 Gy×2+IV] (squares) are shown. The results are expressed as the mean ± SD of 6 mice. Symbols representing the sites of the injection of BMCs are shown in the next the right figure figure.

shown in Fig 4 B, the percentages of donor-derived progenitor cells (H-2b+/c-kit+/Lin−-cells) in the bone marrow and spleen significantly increased when treated with [5.5 Gy×2+IBM] in comparison with [5.5 Gy×2+PV] or [5.5 Gy×2+IV] till 14 days after the treatment, and the progenitors cells of the host-origin (H-2b+/c-kit+/Lin−-cells) could be hardly detected (Fig. 4 B, dashed line; it is noted that the residual host cells gradually decreased to ~0% when treated with [5.5 Gy×2+IBM], as shown in Fig. 2 B). As shown in Fig. 4 A, 14 days after the treatment with [5.5 Gy×2+IBM], almost all the progenitor cells were of donor-origin. It should be noted that the percentages of donor-derived progenitor cells had increased even in the spleen of recipients treated with [5.5 Gy×2+IBM], although the percentages in the spleens in the other groups had decreased. The
Figure 5. Generation of donor-derived stromal cell. The bone pieces without BMCs from MRL/lpr mice 10 days after the treatment with [5.5 Gy×2+ IBM] were cultured for 3 weeks and then the adherent cells were collected. The adherent cells were stained with anti-PA 6 mAb followed by PE-anti-Rat IgG, then blocked with normal rat IgG. They were further stained with FITC-anti-H-2 D^b mAb. Quadrants in the figures were set by the staining profile of the cells treated with isotype-matched Ig controls.

percentages of donor-derived progenitor cells in the bone marrow remained >3% up to 1 year after the treatment (data not shown). These findings indicate that "IBM-BMT" accelerates and maintains the proliferation of donor-derived progenitor cells, possibly P-HSCs.

Analyses of donor-derived stromal cells

We have recently found that donor-derived stromal cells are necessary for successful allogeneic BMT, since there is an MHC restriction between P-HSCs and stromal cells. To examine whether donor-derived stromal cells are actually present in the recipient bone marrow after the treatment, bone pieces without BMCs from the treated MRL/lpr mice were cultured for 3 weeks, and the adherent cells were then collected. The number of adherent cells from the recipient bone treated with [5.5 Gy×2+] was 1.5 ± 1.5×10^4/culture (humerus), and that of [5.5 Gy×2+PV] was 4.2 ± 1.2×10^5, whereas that of [5.5 Gy×2+IBM] was 5.9 ± 0.7×10^5 (the mean ± SD of 5 mice). Furthermore, as shown in Figure 5, these adherent cells from the recipients treated with [5.5 Gy×2+IBM] were positive for H-2 D^b and stained by stromal cell-reactive anti-PA 6 mAb, indicating the replacement of stromal cells by donor-derived stromal cells. This was also confirmed by the immunohistochemical analysis where the stromal cells that stained by anti-PA 6 mAb were also detected by anti-H-2 D^b mAb (Fig. 3 E, cells coloured by yellow), but not by anti-H-2 K^k mAb, again indicating the donor-origin of the stromal cells. These findings indicate that the donor-derived stromal cells can efficiently proliferate in the recipient bone marrow when "IBM-BMT" is carried out. In addition, abnormal T cells (Thy 1.2+/B 220+) of MRL/lpr mice disappeared, and all the cells in the spleen and
lymph nodes had been replaced by donor-derived cells by 40 weeks after the treatment of [5.5 Gy×2+IBM] (at the age of 60 weeks), indicating that the hematolymphoid cells are completely reconstituted with donor-derived cells (data not shown).

**Immunological and immunopathological findings in MRL/lpr treated with [5.5 Gy×2+IBM]**

Non-treated MRL/lpr mice at the age of 20 weeks showed increased anti-ssDNA Abs (IgG and IgM) and RFs (IgG and IgM), whereas MRL/lpr mice treated with [5.5 Gy×2+IBM] showed almost normal values in all these parameters 40 weeks after the treatment (Fig. 6). In addition, the immunological functions were completely restored after the treatment (40 weeks) when assessed by in vitro anti-SRBC antibody response (number of PFC /culture: 198.3 ± 16.1 in the recipients treated with [5.5 Gy×2+IBM] and 257.7 ± 13.2 in normal B 6 mice). Furthermore, newly developed T cells showed tolerance to both host (MRL/lpr)-type and donor (B 6)-type MHC determinants, whereas they showed normal responsiveness to third party (BALB/c) cells when examined in MLR (data not shown). These findings indicate that successful cooperation can be achieved among newly developed T cells, B cells, and antigen-presenting cells in MRL/lpr mice treated with [5.5 Gy×2+IBM].

The glomeruli in the non-treated MRL/lpr mice showed the proliferation of mesangial cells and marked IgG deposits (Fig. 7 A and 7 B), whereas the glomeruli in the MRL/lpr mice treated with [5.5 Gy×2+IBM] showed normal appearance without IgG deposits (Fig. 7 C and 7 D).

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**Figure 6. Serum autoantibody levels in MRL/lpr mice treated with [5.5 Gy × 2 + IBM].**

The levels of anti-ssDNA Abs and RFs were measured 40 weeks after the treatment (striped bars). Sera from B 6 (black bars) and non-treated MRL/lpr mice (white bars) at 20 weeks of age, served as positive and negative controls, respectively. Results are expressed as the mean ± SD of 6 mice. Asterisks represent P values of treated versus non-treated MRL/lpr mice; * p < 0.01 and **p < 0.001.
Discussion

Allo BMT has become an established technique and is generally performed as the treatment of preference for leukemia and aplastic anemia. However, problems such as graft failure and GvHR have been reported; only 60% of patients with leukemia or 55% of patients with aplastic anemia survive disease-free.\textsuperscript{19-21} We have previously found that allo BMT via the IV or the PV with or without bone grafts (to recruit donor stromal cells) has curative effects on autoimmune diseases in various autoimmune-prone mice except MRL/lpr mice.\textsuperscript{22,23} In humans, allo BMT has been performed on patients with autoimmune diseases such as RA, SLE, multiple sclerosis, psoriasis and Crohn's disease, and has been reported to be effective in treating these autoimmune diseases.\textsuperscript{24-28} Moreover, these diseases do not recur after allo BMT during long-term observation (range 7-20 years).\textsuperscript{29-30} In general, patients with autoimmune diseases will have undergone long-term administration of anti-inflammatory drugs, immunosuppressants and cytotoxic reagents, all of which produce various side effects. Therefore, a safe new strategy for allo BMT has been long awaited.

We have previously found that MHC-matched stromal cells are required for the proliferation and differentiation of P-HSCs, and that the donor-derived stromal cells play a crucial role in successful allo BMT.\textsuperscript{31-33} In addition, we have recently found that allo BMT via PV plus supplemental IV injections completely cures autoimmune diseases in chimeric-resistant MRL/lpr mice even after the onset of autoimmune diseases, and that all the recipients thus treated survived more than 1 year without any recurrence of the diseases.\textsuperscript{34} These findings suggest
that the PV injection of donor whole BMCs (including donor stromal cells) creates a suitable microenvironment in the liver, and that this facilitates the proliferation of donor HSCs in collaboration with MHC-matched donor stromal cells. Indeed, we have very recently found that donor-derived cells trapped in the liver play a crucial role in the success of BMT;33 when adherent cell-depleted BMCs were injected, 75% of recipients died within 90 days, whereas all the recipients injected with adherent cell-depleted BMCs with cultured stromal cells survived more than 80 days.34 Although this method is effective, there are risks associated with the abdominal section, and supplemental IV injection is required. In addition, the radiation dose could not be reduced to 5 GyX2.

It has been reported that the transplantation of a large number of HSCs overcomes MHC barriers.36 We have very recently found that a large number of HSCs can proliferate in collaboration with MHC-mismatched stromal cells,9 although there is an MHC restriction between HSCs and stromal cells.37 However, in humans, this strategy suffers from the difficulty of obtaining a large number of HSCs from one donor. In the present study, we attempted to inject the allogeneic whole BMCs (including a small number (<3%) of T cells, HSCs, and stromal cells) directly into the recipient bone marrow cavity (“IBM-BMT”) so that donor-derived hemopoietic cells including stromal cells can effectively accumulate in the bone marrow. To our knowledge, this is the first report on the effect of “IBM-BMT” on autoimmune diseases in MRL/lpr mice, although there are a few reports in which bone marrow cells aspirated from the bone marrow cavity were used for serial BMT38 and gene therapy.39 All the MRL/lpr mice survived more than one year (>60 weeks after birth) without the recurrence of autoimmune diseases, and immunological functions were completely restored even when the radiation dose was reduced to 5 GyX2 ([5 GyX2+IBM]). It should be noted that >85% of MRL/lpr mice treated only with [4.5 GyX2+IBM] also survived >30 weeks after the treatment. These findings suggest that “IBM-BMT” can be used to treat intractable autoimmune diseases under reduced radiation doses without using any immunosuppressants. This seems to be attributable to the enhanced engraftment of donor-derived cells in the early stage after this treatment; the percentage of donor-derived cells reached almost 100%, and donor-derived cells bearing mature lineage markers were generated in the bone marrow, spleen, and liver by 14 days after the treatment with “IBM-BMT” (Figure 2 and Table 1). Furthermore, the percentages of donor-derived progenitor cells in the bone marrow of both the tibia (BMCs were directly injected) and the femur (BMCs were not injected) significantly increased by 14 days after the treatment (Figure 4). Therefore, the IBM injection rapidly accelerates the proliferation of donor-derived progenitor cells and simultaneously maintains hemopoietic progenitor cells, resulting in the recovery of hemopoiesis.

In the present study, we have shown that “IBM-BMT” is, so far, the best strategy for allo BMT: i) no GvHD develops even if T cells are not depleted from the bone marrow, ii) no graft failure occurs even if the dose of radiation as the conditioning for BMT is reduced to 5 GyX2, iii) hemopoietic recovery is rapid, and iv) the restoration of T cell functions is complete even in donor-recipient combinations across the MHC barriers. We believe that this “IBM-BMT” is applicable to humans, since intraosseous (IO) infusion (IBM injection) is now an established method for administering fluids, drugs, and blood to critically ill patients, particularly infants.40-43 Indeed, Hagglund et al. have recently compared the effectiveness of IO infusion with that of IV infusion in human allo BMT;44 they have concluded that allo BMT can be safely performed by IO infusion, but the incidences of acute and chronic GvHD, transplantation-related mortality, and survival rates are similar. However, they aspirated the donor BMCs from the iliac bones and infused these BMCs into the iliac bones of the recipients. Using cynomolgus monkeys, we have just established a new method (“Perfusion Method”) for collecting BMCs from the long bones (femur, humerus etc.) without peripheral blood contamination.45-46 This method has various merits: i) no GvHD develops even in cynomolgus monkeys, since the percentage of T cells in the BMCs thus collected is less than 3%, and ii) a large number of BMCs can be collected quickly and safely. We therefore believe that this method (“IBM-BMT”) will become a powerful new strategy for not only allo BMT but also organ transplantation in conjunction with BMT.

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