Allogenic mixed chimerism prevented autoimmune thrombocytopenia in BXSB lupus mice receiving donor BMT with nonlymphoablative conditioning of low-dose TBI and anti-CD40L mAb

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Objectives: We have demonstrated that allogeneic bone marrow transplant (BMT) with low-dose total body irradiation (TBI) and anti-CD40L mAb without lymphoablative treatment achieved durable mixed chimerism and permanent donor-specific tolerance. Also, allogeneic mixed chimerism induced by anti-CD40L-containing regimen overcomes both allo- and autoimmunity in NOD mice. BXSB mice with a lymphoproliferative disorder, splenomegaly, glomurulonephritis, and thrombocytopenia are adequate models of human systemic lupus erythematosus (SLE). Prolonged immunosuppressive therapy is required for treatment of autoimmune thrombocytopenia in human disease. We investigated whether or not allogenic mixed chimerism induced by nonlymphoablative regimen cured autoimmune thrombocytopenia in BXSB lupus mice.

Materials and Methods: Age-matched male BXSB (H-2b) mice were received 20 × 10^6 BALB/c (H-2d) BM cells. 4 Gy TBI on day -1 and anti-CD40L mAb (2 mg) on day 0 to BMT was given. Skin grafting (donor BALB/c and third party C3H/He) was performed one day after BMT to assess tolerance. Chimerism in peripheral blood was followed by flow cytometric analysis. Peripheral blood counting was performed by autoanalyzer. Antiplatelet antibody was detected by flow cytometric analysis.

Results: 4 Gy TBI on day -1 with anti-CD40L mAb and BMT on day 0 allowed induction of high levels of multilineage mixed chimerism. No clinical signs of graft versus host disease (GVHD) were observed. There was significant difference in number of platelets between chimeric BXSB mice and untreated BXSB mice. The antiplatelet antibodies could be detected in BXSB mice, but only scarcely detected in mixed chimeric BXSB mice.

Conclusions: Allogenic mixed chimerism achieved by BMT with less toxic, nonlymphoablative conditioning regimen, which induces donor specific tolerance without GVHD, improved the lymphoproliferative disorder and prevented thrombocytopenia in BXSB lupus mice without immunosuppressive therapy.

Key words: bone marrow mixed chimerism, immune thrombocytopenia, SLE

Introduction

BXSB mice spontaneously develop autoimmune disease with features similar to human systemic lupus erythematosus (SLE). The disease is associated with autoantibodies to self antigens (Ags) including dsDNA, ssDNA, antiplatelet Antibodies (Abs) and antienerythocyte Abs accompanying with splenomegaly and lymphadenopathy. Immune complex-mediated nephropathy is the hallmark of disease in BXSB. Histopathological changes are evident by 10 weeks of age, leading to end-stage renal disease and 70% mortality by 40 weeks of age. Disease in BXSB is known to be accelerated by a Y chromosome linked autoimmune accelerator gene Yaa, which manifests in a male mortality of 50% by the age of 24 weeks, compared with the age of 56 weeks for female mice. The Yaa gene by itself is unable to induce significant autoimmune responses in mice without an apparent SLE background, while it can induce and accelerate the development of SLE in combination with autosomal susceptibility alleles present in lupus-prone mice.

The etiologic and pathogenic bases of many autoimmune diseases ultimately reside in the primitive self-renewing hematopoietic stem cell population. The effect of bone marrow transplantation (BMT) as a
treatment and/or prevention of these autoimmune diseases in mice has been investigated extensively. BXS B was one of the first SLE mouse model to be treated by allogenic BMT. Fully allogenic BMT, after the marrow is purged of potentially damaging and destructive T cells, can prolong life spans, inhibit production of serum autoantibodies, and prevent the development of autoimmune associated histopathological lesions in autoimmune-prone strains of mice. However, when donors and recipients are fully mismatched in major histocompatibility complex (MHC), the resultant fully allogenic chimeras prepared experience immunodeficiencies after total body irradiation (TBI) followed by BMT. A second approach for treatment of disease in autoimmune-prone strains of mice included generation of a stable mixed chimerism. Because in mixed chimeric mice, immature T cells developed from both host and donor BM cells are positively selected by host stromal cells in thymus, the cognate interactions through T cell receptor (TCR) and at least host MHC are remained. Therefore, immunodeficiencies do not occur in mixed chimeric mice even if donor MHC is fully mismatched.

Takeuchi et al. showed that administration of MHC mismatched donor bone marrow to mice receiving non-lethal dose TBI on day -1, and a single injection of anti-CD40L mAb intraperitoneally (I.P.) on day 0 permitted the induction of permanent mixed chimerism and tolerance without T cell depletion. Recently, we showed that the induction of mixed chimerism using this simple, less toxic regimen prevented the development of glomerulonephritis in BXSB mice. Here we demonstrate that highly frequent induction of MHC mismatched mixed chimerism with low-dose TBI improved not only proliferative glomurulonephritis but also thrombocytopenia because of significant decrease of antiplatelet Ab without any abnormalities of other blood cell counts, and extended the lifespan of mice with a BXSB genetic background.

Materials and Methods

Mice
Male BXSB (H-2b) mice purchased from Japan SLC (Shizuoka, Japan) were the recipient mice. The donor mice were BALB/c (H-2d) bred and C3H/HeN (H-2k) purchased from CLEA Japan (Tokyo, Japan).

BMT
Seven-week-old recipient mice were treated with a non-myeloablative dose TBI (4 Gy) on 1 day before BMT (day -1). Twenty-million MHC-mismatched BALB/c bone marrow cells were injected intravenously with administration of hamster anti-mouse CD40L mAb (MR1, hybridoma kindly provided by Dr. Sykes, TBRC, MA) 2 mg I.P. BXSB mice were divided into 4 groups: I; BMT with TBI and anti-40L, II; TBI, III; TBI and anti-CD40L, IV; no treatment.

Skin graft
Full-thickness tail skin (~1.0 cm²) from BALB/c (donor specific) or fully MHC mismatched C3H/HeN (third party) mice were grafted on the dorsal thoracic wall, bandaged and followed by daily visual inspection. Grafts were defined as rejected when <10% of the graft remained viable.

Flow cytometric analysis
Single-cell suspension of peripheral blood cells from BXSB, BALB/c, or transplanted mice were stained with fluorochrome-conjugated mAbs (BD Biosciences, San Diego, CA, USA) for surface markers: B cells (PE conjugated B220), T cells (PE conjugated anti-CD4, anti-CD8) and monocytes/macrophages (PE conjugated anti-CD11b). Donor-derived cells were identified by fluorescein isothiocyanate (FITC) conjugated anti-H-2Dd mAb. Cell suspensions were analyzed with a 3-color FACSCalibur flow cytometer. The percentage of chimerism was calculated from the ratio of FITC positive cells of all applicable PE positive cells.

Complete blood counts
Complete blood counts in the peripheral blood were made in heparinized blood samples from the BXSB mice with or without treatment or age-matched C57BL/6 and BALB/C mice on a hemocytometer.

Detection for antiplatelet Abs
The detection method for antiplatelet Ab was described previously. In summary, platelet-rich plasma was suspended in 1% paraformaldehyde solution for 1 minute. After washing, the platelets were resuspended in EDTA-PBS. To detect the circulating antiplatelet Ab, platelets were incubated with serum from BXSB with or without treatment for 30 minutes at room temperature. After washing, they were incubated with FITC conjugated goat anti-mouse IgG or mouse IgM. Samples were analyzed on a FACS analyzer with gating to exclude debris.

Statistical analysis
Results are expressed as means ± standard deviations (SD). Statistical analysis was performed with Student’s
$t$-test for two samples. Non-parametric samples were analyzed with the Mann-Whitney U test; results are expressed as means. Values of $P < 0.05$ were considered to be statistically significant.

**Results**

**Chimerism**

Recently, Takeuchi et al. showed that 100% of mice receiving 4 Gy TBI on day -1 with MR1 and BMT on day 0 achieved lasting high levels of donor chimerism without GVHD.\(^{10}\) We investigated whether or not this regimen allowed the induction of long-term mixed chimerism to BXSB mice. \(2.0 \times 10^7\) normal BALB/c bone marrow cells were injected with 2.0 mg anti-CD40L (I.P.) to 7-week-old BXSB mice irradiated 4 Gy non-lethal dose TBI on day -1. As shown in Figure 1, this regimen allowed induction of multilineage mixed chimerism in 9 of 10 mice: donor CD4$^+$ T cell; 80.3 $\pm$ 9.7%, CD8$^+$ T cell; 57.1 $\pm$ 17.8%, B cell; 58.6 $\pm$ 14.7%, granulocyte; 91.2 $\pm$ 5.1% in 32 weeks after BMT. No clinical signs of GVHD were shown throughout the whole observation period until 40 weeks after BMT. The mice in groups I (TBI + MR1 + BMT), III (TBI + MR1) and IV (untreated) received skin grafts from donor (BALB/c, H-2$^d$) and third party (C3H/HeN, H-2$^k$) 1 day after BMT. The result of the skin grafts showed that all chimeric BXSB mice accepted donor skin specifically, but rejected both donor and third-party skin grafts by 20 days (Figure 2). This indicated that chimeric BXSB mice acquired donor-specific tolerance without immunodeficiencies.

**Longevity**

Longevity was compared between each group (Figure 3). As expected, 80% of untreated BXSB mice died by 45 weeks of age. In contrast, no chimeric BXSB mice died up to this time point. The BXSB mice received TBI + MR1 or TBI only showed higher longevity than the untreated

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**Figure 1.** Long-term multilineage chimerism in peripheral blood of mice receiving 4 Gy TBI on day -1 with MR1 on day 0. The mean percentage of donor cell (detected as H-2D$^d$ positive cells) among peripheral cells of each lineage as determined by 2-color FACS at various times after bone marrow transplantation. Results are shown as means $\pm$ SDs for each group.

**Figure 2.** Donor-specific skin graft tolerance in recipient received BALB/c BMT and MR1 with 4 Gy TBI. Donor (BALB/c: H-2$^d$) and third-party (C3H: H-2$^k$) skin was grafted 1 day after BMT. The chimeric mice (◆ $n = 9$) accepted donor skin grafts permanently, while third-party skin graft was rejected by 20 days postgrafting. Untreated mice (● $n = 5$) and mice treated with TBI and MR1 (△ $n = 5$) rejected both donor and third party skin grafts until 20 to 40 days. This indicated that chimeric BXSB mice acquired donor-specific tolerance.
BXSB mice, but the survival rates of these two groups were significantly lower than those in the chimeric mice in a long-term observation. Moreover, the mice that received TBI or TBI + MR1 developed apparent lymphadenopathy, which was not observed in the chimeric mice. This indicated that TBI might be effective to postpone the onset of the disease, but this treatment is not sufficiently effective to suppress lupus disease. Induction of BM chimerism was needed to suppress progression of autoimmune disease in BXSB mice.

**Complete blood count**

It is known that BXSB mice develop thrombocytopenia associated with autoantibody. To investigate the suppressive effect of induction of mixed chimerism, platelet counts in the peripheral blood were individually made on a hemocytometer. As shown in Figure 4, there was a significant difference in the number of platelets between the chimeric BXSB mice (50.4 ± 12.9 × 10^4/μl) and the untreated BXSB mice (26.0 ± 18.5 × 10^4/μl) (P < 0.01). Therefore, the treatment to induce mixed chimerism significantly suppressed the development of thrombocytopenia. The red blood cell (RBC) and white blood cell (WBC) counts and the concentration of hemoglobin (Hb) were not different between the experimental groups and the controls (Figure 5).

![Figure 3](image1.png)

**Figure 3.** Survival curves of male BXSB in each group. While All BXSB lupus mice with donor chimerism (● n = 10) survived, untreated BXSB mice (◆ n = 10) showed high mortality. The mice treated with TBI (▲ n = 5) or TBI and MR1 (△ n = 5) showed the middle level of mortality between chimeric mice and untreated mice.

![Figure 4](image2.png)

**Figure 4.** Thrombocyte counts in peripheral blood 26 weeks after BMT. The induction mixed chimerism significantly suppressed the development of thrombocytopenia compared with untreated mice (P < 0.01).

![Figure 5](image3.png)

**Figure 5.** Red blood cell (RBC) and white blood cell (WBC) counts and the concentration of hemoglobin (Hb). The RBC and WBC counts and concentration of Hb were not different between the experimental groups and the controls. The peripheral blood cell counts of age-matched normal C57BL/6 (B6) were shown as normal controls.
Antiplatelet Abs
To assess whether the improvement of thrombopenia was caused by disappearance of antiplatelet Abs, in 26 weeks after BMT serum, anti-platelet IgG and IgM were detected by FACS (Figure 6). As shown in Table 1, antiplatelet IgM could be detected in 25% (4/16) of the untreated mice, and antiplatelet IgG was detected in 50% (8/16) of the untreated mice. In contrast, only 1 of 10 mice that received BMT showed antiplatelet Abs. The mice that received TBI only or TBI + MR1 also showed fewer positive of antiplatelet Abs because TBI effectively decreased B cells. However, this effect was only temporal, an even higher rate of antiplatelet Abs positive

Table 1. The number of antiplatelet positives/total. Certain number of untreated mice and mice with TBI, or TBI and MR1 showed either antiplatelet IgG or IgM. Otherwise, antiplatelet Abs hardly detected in chimeric mice. The number indicated antiplatelet positives/total (percentage of antiplatelet positives).

<table>
<thead>
<tr>
<th></th>
<th>Anti-plt IgG</th>
<th>Anti-plt IgM</th>
</tr>
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<tbody>
<tr>
<td>No treatment</td>
<td>8/16 (50%)</td>
<td>4/16 (25%)</td>
</tr>
<tr>
<td>TBI + MR1 + BMT</td>
<td>1/10 (10%)</td>
<td>0/10 (0%)</td>
</tr>
<tr>
<td>TBI only</td>
<td>2/5 (40%)</td>
<td>1/5 (20%)</td>
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<tr>
<td>TBI + MR1 only</td>
<td>2/5 (20%)</td>
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Figure 6. Antiplatelet IgG and IgM were detected by FACS. Antiplatelet positive (untreated) and negative (BMT with TBI + MR1) histograms are shown. Upper panel, antiplatelet IgG; Lower panel, antiplatelet IgM.
mice was observed in these groups than in the chimeric mice group. At 40 weeks after BMT, still only 1 of 10 chimeric mice showed antiplatelet Ab positive (data not shown). This result indicated that the induction of mixed chimerism might have cured antiplatelet Abs positive thrombocytopenia.

Discussion

It was shown that the induction of a stable mixed bone-marrow chimerism is possible to treat or prevent many autoimmune diseases in mice. In the present study, we investigated whether such a stable mixed chimerism was established by the regimen with low-dose TBI and injection of anti-CD40L. It is known that this regimen allowed to induce mixed chimerism to normal C57BL/6 mouse with MHC fully mismatched donor BALB/c bone marrow, though this is the first trial with the induction mixed chimerism to lupus mice using the same regimen. Until now, several groups reported that mixed hematopoietic chimerism can prevent the development of autoimmune disease in BXSB mice. However, their regimen respectively required particular condition of donor bone marrow cell such as fully matched donor, T cell depleted marrow, and/or a lethal dose of TBI so that clinical application is prevented. This study is a clinically significant trial with the regimen that does not require host T cell depletion, donor myeloablation, MHC fully matched donor bone marrow, or lethal dose of TBI. As a result, this regimen made it possible to induce fully MHC mismatched allogenic mixed chimerism to BXSB by a high frequency, contrary to its lower toxicity.

Cellular engineering by BMT using anti-CD40L and TBI is thought to partly replace the primitive self-renewing hematopoietic stem cells of the recipient with those of the donor or balancing between recipient cells and donor cells. Anti-CD40L is known to block the co-signal between CD4+ T cells and dendritic cells so that T cell tolerance to allogenic antigen might be induced by this regimen. It is speculated that re-arrangement of T cells by antigen presenting cells with host-MHC may replace host autoreactive T cells in thymus or eliminate autoreactive cells in autoimmune disease mice (data in preparation).

In the present study, complete blood counts at the age of 26 weeks were compared among the groups. By this time point, of 26 weeks, half of the untreated BXSB mice were dead due to renal failure. Therefore, we could not compare the number of platelets between the chimeric mice and the untreated mice at later time points; however, antiplatelet Abs were only scarcely detected in the chimeric mice at 40 weeks after BMT. Moreover, almost all of the untreated BXSB mice showed obvious cervical lymphadenopathy, splenomegaly, and glomerulonephritis. In contrast, the mice that achieved mixed chimerism did not present with these symptoms. The results of complete blood counts showed the effect upon inhibition of the thrombocytopenia caused by production of autoreactive antibody in the BXSB mice. It is likely that some kinds of populations of autoreactive B cells could be down-regulated by re-arranged T cells.

Immune thrombocytopenia is a common manifestation of human SLE, and severe thrombocytopenia occurs in about 5% to 10% of patients, usually in the context of active disease. High-dose glucocorticoids are considered first-line therapy. Although most SLE patients with immune thrombocytopenia respond initially to this therapy, long-term response is sustained in only 20% of patients. Intravenous immunoglobulins and high-dose methylprednisolone pulses have also been reported as effective but these effects are only transient, even in patients receiving repeated infusions. Radical treatment, not remission, will be needed especially for steroid-resistant patients. The induction of mixed chimerism re-arranges the host autoreactive immune system. Therefore, this strategy will likely be a powerful treatment option for autoimmune diseases. Further studies are warranted to elucidate the mechanism of the regulation of autoreactive cells.

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