Rheumatoid arthritis (RA) is an autoimmune disease affecting approximately 0.5–1.0% of the adult population worldwide.\textsuperscript{1,2} Despite its importance, the etiological genetic and environmental factors of RA are poorly understood.\textsuperscript{1,2} Although there are well-known associations between certain genetic polymorphisms and the development of RA, the concordance rate in monozygotic twins is no more than 15–50%.\textsuperscript{1,3,4} Moreover, RA is thought to be caused by the interactions of many factors, such as susceptibility genes, stem cell disorders, viral infection, and environment elements.\textsuperscript{1,3,4–9} Therefore, additional studies are necessary to elucidate the causes of RA.

Stem cells in the bone marrow consist of hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). HSCs differentiate into hematopoietic cells, which include the immune cells. They may therefore represent the source of the hypothetically abnormal hematopoietic cells in RA that produce inflammatory cytokines and attack different organs.\textsuperscript{10,11} In contrast, MSCs differentiate into synovial cells and osteoblasts. Potentially abnormal synovial cells can cause synovitis and produce several inflammatory cytokines.\textsuperscript{12} It has been hypothesized that if RA is a stem cell problem rather than a systemic problem, bone marrow transplantation (BMT) to replace both HSCs and MSCs may be curative. In humans, several autoimmune diseases, including RA, systemic lupus erythematosus (SLE), multiple sclerosis, and Crohn’s disease, have been reported to be cured after allogeneic BMT.\textsuperscript{13–17} Reciprocally, the adoptive transfer of autoimmune diseases, such as myasthenia gravis, insulin-dependent diabetes mellitus (IDDM), and Graves’ disease, by allogeneic BMT from unaffected recipients has also been reported.\textsuperscript{18–20} In mice, we have previously shown that allogeneic BMT from normal mice to autoimmune disease-prone mice can be used to prevent and treat autoimmune diseases, such as SLE, RA, immune thrombocytic purpura, IDDM, chronic glomerulonephritis, and non-insulin-dependent diabetes mellitus.\textsuperscript{21–23} We have previously shown that allogeneic BMT from normal mice to estrogen-deficient mice can be used to prevent osteoporosis and hypogonadism.\textsuperscript{24} In contrast, we have found that the transplantation of T cell-depleted bone marrow cells (BMCs) or partially purified HSCs from autoimmune disease-prone mice to normal mice leads to the induction of autoimmune disease in the recipients.\textsuperscript{25} These data are all consistent with the notion that susceptibility to autoimmune disease resides in some abnormality of BMCs, most likely the stem cells.\textsuperscript{25}

SKG/Jcl (SKG) strain mice have a point mutation in the gene encoding the Src homology 2 domain of the \( \zeta \)-associated protein of 70kDa (Zap-70), a key signal transduction molecule in T cells, resulting in spontaneous development of T cell-mediated chronic autoimmune arthritis.\textsuperscript{26} These mice produce antibodies, such as rheumatoid factor, and manifest clinically relevant joint swelling and hyperemia. Hence, they have been proposed as an animal model for human RA. Recent reports have shown that immune-deficient mice transplanted with BMCs or thymocytes from SKG mice develop arthritis.\textsuperscript{26} This may have occurred due to the secretion of inflammatory cytokines from the transplanted cells; however, the cause and...
duration of inflammation and mechanisms responsible for causing arthritis are not clear.

Recently, we reported that allogeneic BMCs from B6 mice transferred into irradiated SKG mice did indeed prevent the development of arthritis in the latter model.25 However, irradiation itself may have inhibited the abnormal SKG lymphocytes, and age-associated “burnout” may also have affected the degree of inflammation, as in human RA.28 In the present study, we attempted to transfer systemic arthritis by injecting lineage-negative (lin−) immature cells isolated from arthritis-prone SKG mice into normal recipients to confirm that arthritis occurred due to a “stem cell disorder” and that BMCs (both HSCs and MSCs) were the key factors mediating inflammation and joint destruction.

MATERIALS AND METHODS

Mice Female SKG (H-2b) were purchased from Clea Japan (Osaka, Japan) and maintained in our animal facility under specific pathogen-free conditions. We chose female B6 (H-2b) mice to confirm successful allograft transplantation and the induction of autoimmune arthritis by using 2 strains with complete mis-match for H-2 antigen type. All mice (10 weeks of age) were given a single intraperitoneal injection of 30 mg laminarin (Sigma Chemical Co., St. Louis, MO, U.S.A.), which triggers severe chronic arthritis in SKG mice.29 All mice were kept in single cages and fed a standard diet. The study protocol was approved by the Animal Experimentation Committee, Kansai Medical University (Osaka, Japan).

Preparation and Inoculation of Donor BMCs BMCs were collected from the femurs and tibias of donor SKG mice at 8 weeks of age. To deplete lineage-positive cells, HSCs were prepared by treating the whole population of BMCs with a cocktail of monoclonal antibodies (mAbs) against T cells (CD5), B cells (B220), macrophages (CD11b), and granulocytes (Gr-1) and mixed with magnetic beads coupled to a monoclonal anti-biotin antibody (Lineage Cell Depletion Kit; Miltenyi Biotec, Auburn, CA, U.S.A.). Under intraperitoneal anesthesia with 40 mg/kg of pentobarbital sodium, whole BMCs or lineage-depleted BMCs (lin− BMCs) were directly injected into the bone cavities of recipient B6 mice according to the method described previously to facilitate the early recovery of donor-derived hematopoiesis (intra-bone marrow BMT [IBM-BMT]).30 In brief, 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. After removal of the guide, donor BMCs were injected through the hole in the bone into the bone cavity using a micro syringe (50 µL) or lin− BMCs (1×107 cells/10 µL) from SKG mice (8 weeks of age) via IBM-BMT as described above. The group transplanted with whole BMCs is referred to as the whole [SKG→B6] group, and those receiving lin− BMCs is referred to as the lin− [SKG→B6] group. Furthermore, we used untreated SKG mice (8 weeks of age) as the positive control and untreated B6 mice (8 weeks of age) as the negative control. We performed all experiments using 6 mice in each group, and all mice were sacrificed by cervical dislocation.

Arthritis Scores As previously described,29 joint swelling was monitored by inspection by an unbiased observer and was scored as follows: 0, no joint swelling; 0.1, swelling of one finger joint; 0.5, mild swelling of wrist or ankle; 1.0, severe swelling of wrist or ankle. Scores for all digits of forepaws and hind paws as well as the wrists and ankles were summed to give a single value for each individual mouse.

Surface Marker Analyses Spleen cells, BMCs, and thymocytes were prepared from untreated B6 mice (28 weeks old), untreated SKG mice (28 weeks old), whole [SKG→B6] mice (28 weeks old; 20 weeks after BMT), and lin− [SKG→B6] mice (28 weeks old; 20 weeks after BMT). To distinguish between cells of donor or recipient origin, samples were stained with fluorescein isothiocyanate (FITC)-conjugated anti-H-2Kb and phycoerythrin (PE)-conjugated anti-H-2Kd mAbs (BD PharMingen, San Diego, CA, U.S.A.). FITC-conjugated anti-H-2Kb or anti-H-2Kd mAbs and PE-conjugated anti-CD4, CD8, and B220 mAbs (BD PharMingen) were used to analyze cells with mature lineage markers. To detect regulatory T cells (Tregs), samples were stained with FITC-conjugated anti-CD4 and PE-conjugated anti-Foxp3 mAbs (eBioscience Inc., San Diego, CA, U.S.A.). Before staining with anti-Foxp3 mAbs, the cells were preloaded with Foxp3 Fixation/Permeabilization Concentrate and Diluent (eBioscience Inc.).27 Further, to detect receptor activator of nuclear factor kappa-B ligand (RANKL)+ cells among CD4+ T cells in the spleen, the cells were stained with FITC-conjugated anti-H-2Kb or anti-H-2Kd, peridinin-chlorophyll-conjugated anti-CD4 mAbs (BD PharMingen), and PE-conjugated anti-RANKL (CD254) mAbs (BioLegend, San Diego, CA, U.S.A.). The stained cells were analyzed by FACSscan (Block Scientific Inc., Bohemia, NY, U.S.A.).

Cultured Osteoblast-Like Cells Cultured stromal cells were obtained as previously described.30,31 BMCs were extensively washed out from the femurs, tibias, and humeri of untreated B6 mice (28 weeks old), untreated SKG mice (28 weeks old), whole [SKG→B6] mice (28 weeks old; 20 weeks after BMT), and lin− [SKG→B6] mice (28 weeks old; 20 weeks after BMT). The bone pieces were then cut into pieces and cultured in a flask. Minimum Essential Medium (α-MEM; Invitrogen, Life Technologies Corp., Carlsbad, CA, U.S.A.) containing 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, U.S.A.) and 10% fetal bovine serum was replaced weekly with the same volume of fresh culture medium. Two weeks later, nonadherent cells were removed, and adherent cells were then freed from the surface using Cell Dissociation Solution (Sigma-Aldrich). These collected cells were stained with FITC-conjugated anti-H-2Kb mAbs, FITC-conjugated anti-H-2Kd, or FITC-conjugated IgG2a-K-isotype mAbs (BD PharMingen).

Measurement of Cytokines in Sera Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) levels in the sera of untreated B6 mice (28 weeks old), untreated SKG mice (28 weeks old), whole [SKG→B6] mice (28 weeks old; 20 weeks after BMT), and lin− [SKG→B6] mice (28 weeks old; 20 weeks after BMT) were quantified using an enzyme-linked immunosorbent assay (ELISA) kit (BioSource International Inc., Camarillo, CA, U.S.A.).

Histological Findings The ankle joints of untreated B6
mice (28 weeks old), untreated SKG mice (28 weeks old),
whole [SKG→B6] mice (28 weeks old; 20 weeks after BMT),
and lin−[SKG→B6] mice (28 weeks old; 20 weeks after
BMT) were removed, fixed in 10% formalin, and decalcified
with ethylenediaminetetraacetic acid (EDTA). Thereafter, the
specimens were embedded in paraffin, cut into 4-
µm sections,
and stained with hematoxylin and eosin (H–E). Osteoclasts
were identified by tartrate-resistant acid phosphatase (TRAP)
staining using TRAP/ALP Stain kits (Wako Pure Chemical
Industries, Ltd., Osaka, Japan). The stained areas were quanti-
fied using NIH ImageJ software.

**Statistical Analysis**
Statistical analysis was performed
using Mann–Whitney tests. Differences with p-values less
than 0.05 were considered significant.

**RESULTS**

**Arthritis Scores** In untreated SKG mice, ankle joint
swelling with hyperemia was observed at about 12 weeks of
age, after which the finger, wrist, and ankle joints developed
severe swelling. In whole [SKG→B6] and lin−[SKG→B6]
mice, joint swelling also started from 8 weeks after BMT and
gradually developed into severe joint swelling. In contrast, in
untreated B6 mice, joint swelling or hyperemia did not occur,
even after 36 weeks. The arthritis scores were significantly
higher (p<0.05) in whole [SKG→B6] and lin−[SKG→B6]
mice compared to untreated B6 mice, but lower than those of
untreated SKG mice (Fig. 1).

**Macroscopic and Histological Findings** Untreated SKG
mice showed macroscopic severe joint swelling with hyper-
emia (Fig. 2A) and hypertrophic synovitis with lymphocytes
accumulated around the ankle joints (as seen by histology in
Fig. 2B). Whole [SKG→B6] and lin−[SKG→B6] mice also
showed symptoms of arthritis in the ankle joints similar to un-
treated SKG mice (Figs. 2C, D). On the other hand, untreated
B6 mice showed no joint swelling or hyperemia (Figs. 2E, F).

**Analyses of Donor-Derived Hematolymphoid Cells** Inv-
estigation of the nature of T cells in the thymus of untreated
SKG mice showed that more than 90% were CD4+CD8−
double positive (DP), with CD4+CD8− and CD4+CD8−
single positive (SP) T cells present at less than 5% each. In contrast,
the percentage of DP cells in B6 mice was significantly lower,
at around 60%. In their spleens and bone marrow, the percentages of CD4$^+$ and CD8$^+$ T cells were lower than in normal B6 mice ($p<0.05$). In whole [SKG$^\text{ergy}$ B6] and lin$^-$ [SKG$^\text{ergy}$ B6], and untreated B6 mice, the percentages of H-2d (donor type) cells in the spleen, bone marrow, and thymus were essentially 100%, indicating virtually complete replacement by donor cells. Importantly, the percentages of CD4$^+$CD8$^+$ DP T cells in the thymus and the percentages of CD4$^+$ and CD8$^+$ T cells in spleen and bone marrow now appeared very similar to those of untreated SKG mice (Fig. 3).

Cultured Osteoblast-Like Cells

Osteoblast-like cells collected from each group of mice undergoing IBM-BMT and were analyzed for expression of H-2$^d$ (indicating donor origin).

The percentages of Tregs (Foxp3$^+$ of CD4$^+$) in the spleen of untreated B6, untreated SKG, whole [SKG$\rightarrow$B6], and lin$^-$ [SKG$\rightarrow$B6] mice were 29.3±3.2%, 19.9±5.1%, 19.2±2.8%, and 20.2±2.9%, respectively. The percentages of Tregs in untreated SKG, whole [SKG$\rightarrow$B6], and lin$^-$ [SKG$\rightarrow$B6] mice were significantly lower than that in untreated B6 mice (unpublished data).

Cultured Osteoblast-Like Cells

Osteoblast-like cells collected from whole [SKG$\rightarrow$B6] and lin$^-$ [SKG$\rightarrow$B6] mice were confirmed also to be of donor origin (H-2$^d$; Fig. 4). RANKL has been reported to stimulate bone resorption and joint destruction through the activation of osteoclasts. Therefore, we analyzed the percentages of RANKL$^+$ cells among CD4$^+$ T cells (RANKL$^+$/CD4$^+$ T cells) in the spleen and cultured osteoblast-like cells that may contribute to the development of joint destruction. The percentages of RANKL$^+$ cells among cultured osteoblast-like cells were significantly greater ($p<0.05$) in untreated SKG, whole [SKG$\rightarrow$B6], and lin$^-$ [SKG$\rightarrow$B6] mice than they were in untreated B6 mice (Fig. 5A). Furthermore, the percentages of RANKL$^+$ cells among cultured osteoblast-like cells...
(RANKL\+/osteoblast-like cells) in untreated SKG, whole [SKG→B6], and lin− [SKG→B6] mice were significantly higher \((p<0.05)\) than in untreated B6 mice (Fig. 5B).

**TRAP Staining** To confirm joint destruction due to the activation of osteoclastogenesis, we investigated the activity of osteoclasts in tarsal bone by TRAP staining. In whole [SKG→B6], lin− [SKG→B6], and untreated SKG mice, areas of osteoclasts (TRAP\(^+\) cells) in the metatarsal bone were significantly larger \((p<0.05)\) than those in untreated B6 mice (Fig. 6).

**Analysis of Cytokines** TNF-\(\alpha\) and IL-6 play essential roles in the activation of osteoclasts. Therefore, we next investigated the relative amounts of these cytokines in the sera. We found that TNF-\(\alpha\) and IL-6 levels in untreated SKG, whole [SKG→B6], and lin− [SKG→B6] mice were significantly higher \((p<0.05)\) than those in untreated B6 mice (Fig. 7).

**DISCUSSION**

SKG mice present with subsynovial infiltration of inflammatory cells and plasma cells and with synovial cell proliferation with pannus formation and neovascularization. Sakaguchi et al. recently reported that immunodeficient mice (nude mice and severe combined immunodeficiency [SCID] mice) receiving BMCs, peripheral T cells, or thymocytes from SKG mice subsequently develop arthritis.26) A few reports have shown that arthritis in SKG mice can be suppressed by the administration of nondepleting anti-CD4 mAbs or methotrexate, trichostatin A, and N-acetyl-d-glucosamine.33–36) Charles et al. have reported that adoptive transfer of CD11b\(^{−/−}\)Ly6C\(^{hi}\) osteoclast precursors (OCP) from syngenic BALB/c mice can suppress joint arthritis in SKG mice.37) However, this treatment did not restore cytokine levels and improve CD4\(^{+}/\)CD8\(^{−}\)
T cell population in the thymus. These outcomes are crucial in the general clinical management of autoimmune arthritis patients. OCP-positive CD11b<sup>−</sup>/Ly6<sup>Ch</sup> BMCs contribute to a very small amount of BMCs, and it is unclear whether the major OCP-negative sub-population of CD11b<sup>−</sup>/Ly6<sup>Ch</sup> is the fundamental cause of autoimmune arthritis.

Allogenic BMT potentially induces GvHD. We have developed IBM-BMT, which is a technique involving injection of whole BMCs (including stromal cells) directly into the bone marrow. IBM-BMT is the optimal strategy for allogeneic BMT, because it (1) reduces GvHD and graft failure, (2) reduces the radiation dose required for BMT conditioning, and (3) facilitates early hematopoietic recovery and restoration of T-cell functions. Furthermore, because IBM-BMT can suppress GvHD, IBM-BMT with donor lymphocyte infusion and IBM-BMT with thymus transplantation have been reported. Recently, Kushida et al. reported that SKG mice transplanted with BMCs from normal B6 mice by IBM-BMT (B6→SKG) did not develop arthritis up to 12 months after transplantation. Furthermore, the percentage of RANKL<sup>+</sup> CD4 cells and the serum levels of TNF-α and IL-6 were normalized in the [B6→SKG] mice. In the present study, we investigated whether the putatively abnormal BMCs from SKG mice could permanently engraft, differentiate, and proliferate in normal allogeneic B6 mice, and if so, whether this would lead to autoimmune arthritis as in donor SKG mice themselves. The application of this procedure was indeed found to result in joint swelling with hyperemia and hypertrophic synovitis with lymphocyte accumulation around the joints. This was seen in whole [SKG→B6] and lin<sup>−</sup> [SKG→B6] mice, with the same symptoms as in untreated SKG mice. The mechanism responsible for the development of autoimmune arthritis in whole [SKG→B6] and lin<sup>−</sup> [SKG→B6] mice was similar in that donor-derived hematolymphoid cells (hematopoietic cells) and osteoblast-like cells (mesenchymal cells) had completely replaced autochthonous cells in the allogeneic B6 recipients. Therefore, these data indeed supported that injection of cells from an autoimmune donor could induce autoimmune disease in normal individuals.

Generally, inflammatory cytokines such as TNF-α and IL-6 are thought to have important roles in the development of autoimmune arthritis in RA patients. TNF-α can directly activate osteoclasts, and TNF-α and IL-6 both upregulate RANKL in different cells, which can then activate osteoclasts, with consequent development of osteoporosis and joint destruction. In SKG mice, high levels of TNF-α and IL-6 have been reported in the sera and joint fluid. In the present study, to analyze the function of donor-SKG HSCs in displacing the original-B6 HSCs, we investigated serum cytokines and blood cells. Not only untreated SKG mice but also whole [SKG→B6] and lin<sup>−</sup> [SKG→B6] mice had much more TNF-α and IL-6 in their sera than normal B6 mice. Therefore, we suggest that hematolymphoid cells, which differentiate from abnormal donor HSCs, secrete large quantities of these cytokines, causing arthritis. Furthermore, to analyze the function of donor MSCs, we investigated cultured osteoblast-like cells using flow cytometry. We found that whole [SKG→B6] and lin<sup>−</sup> [SKG→B6] mice had higher levels of RANKL on osteoblast-like cells than normal B6 mice, resulting in greater numbers of osteoclasts (TRAP<sup>+</sup> cells) in whole [SKG→B6] and lin<sup>−</sup> [SKG→B6] mice than in normal B6 mice. These findings suggest that donor-derived hematolymphoid (hematopoietic) cells and osteoblast-like (mesenchymal) cells persisted in the recipients and caused autoimmune arthritis and increased osteoclastogenesis.

Peripheral lymphocytes, especially CD<sup>+</sup> T cells, cause autoimmune arthritis in RA patients. Spleen cells or thymocytes from SKG mice were able to cause autoimmune arthritis in immunodeficient mice (nude mice and SCID mice). Furthermore, several stimulator cell types in BMCs have also been reported to cause autoimmune arthritis in SKG mice. In our study, injection of the whole population of BMCs caused autoimmune arthritis in normal B6 mice. Moreover, to confirm that the autoimmune disease in SKG mice was due to a “stem cell disorder,” we transplanted additional immature cells that were depleted of lineage-positive cells into normal B6 mice. Subsequently, abnormal lymphocytes and osteoblast-like cells were found in normal B6 mice, resulting in autoimmune arthritis characterized by accumulated lymphocytes, synovitis, and increased osteoclasts in the articular bone. These findings
suggest that autoimmune disease in SKG mice occurred due to a “stem cell disorder” and that normalization thereof would be a novel method to cure autoimmune diseases permanently. Currently, few RA patients remain in long-term remission using disease-modifying antirheumatic drugs, and many patients require outpatient treatment every month. Furthermore, some RA patients do not respond sufficiently and still suffer treatment side effects. Our data suggests that the etiology of autoimmune arthritis is probably a stem cell disorder of lin−immature cells isolated from abnormal BMCs. Therefore, we think radical therapy with bone marrow from normal donors should replace lin−immature cells of recipients suffering from autoimmune diseases. Moreover, we believe transplanting normal BMCs using direct injection into the bone cavity could be a secure option for treatment of autoimmune diseases.

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