matched renal transplant recipients from sibling donors. Furthermore, van Rood *et al.* (2002) demonstrated that allogeneic stem cell transplantation (SCT) from NIMA-mismatched siblings showed a lower incidence of severe acute graft-versus-host disease (aGVHD) compared with that from the other family donors. Following these observations, we and others have demonstrated the feasibility of HLA-haploidentical SCT from NIMA-mismatched relatives without T-cell depletion (Ichinohe, Maruya & Saji, 2002; Shimazaki *et al.*, 2003). Stern *et al.* (2008) recently showed the advantage of maternal donor in T cell depleted HLA-haploidentical SCT. These clinical studies have been performed based on the presence of feto-maternal microchimerism as a result of feto-maternal immunological tolerance, nevertheless some cases developed severe aGVHD despite the existence of microchimeric cells (Ichinohe *et al.*, 2004).

Noninherited maternal HLA antigen allografts have been shown to accept better than NIPA allografts *in vivo*, and *in vitro* T-cell response to NIMA is significantly reduced in interleukin (IL)-2, IL-5 and interferon (IFN)-γ production (Andrassy *et al.*, 2003; Akiyama *et al.*, 2005). In human, Tsafrir *et al.* (2000) demonstrated such NIMA effect using umbilical cord blood mononuclear cells by a mixed lymphocyte reaction (MLC), but Hadley *et al.* (1990) could not detect it when using peripheral blood mononuclear cells from healthy individuals. Recently, van den Boogaardt *et al.*, (2005) also failed to detect any differences between NIMA and NIPA in T-cell reaction of MLC and ELISPOT assay *in vitro*.

In this study, we analyzed the T-cell reactivity by devising an IFN-γ ELISPOT assay using sorted responder T cells and stimulator B cells in three cases of HLA fully matched related SCT, and healthy individuals from one family to compare immune responses of T cells between NIMA and other combinations.

MATERIALS AND METHODS

Subjects

Three cases of HLA fully matched related SCT, four healthy volunteers including a family member and a one HLA-haploidentical unrelated donor were tested. All individuals were typed for major histocompatibility complex class I and class II at a high-resolution level by DNA-based WAKFlow HLA typing system (Wakunaga Co., Hiroshima, Japan). All the donors provided written informed consents which were approved by the

	Donor	Engraftment	aGVHD	cGVHD	Outcome
Case 1	Brother	Day 14	Grade 1 (skin 1)	none	Alive in day 187
Case 2	Brother	Day 12	Grade 0	none	Alive in day 155
Case 3	Brother	Day 23	Grade 1 (skin 1)	none	Alive in day 153

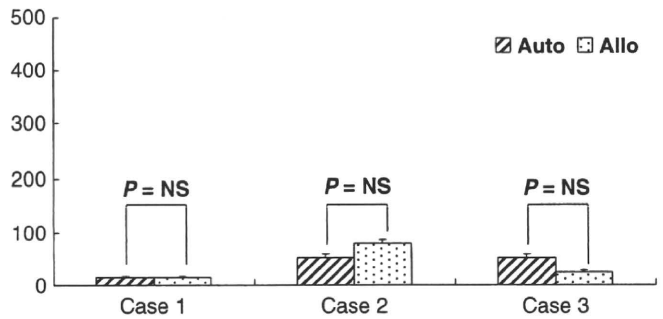

Figure 1. Clinical outcomes in cases of HLA fully matched related SCT and results of a modified IFN-γ ELISPOT assay. The relationship between donor and recipient, the engraftment, the grade of graft-versus-host disease (GVHD), and outcomes in each case are shown in upper table. There is no significant difference in the reaction of T cells from donor against B cells from donor (autologous) and recipient (allogeneic). Acute GVHD was graded by according to standard criteria.

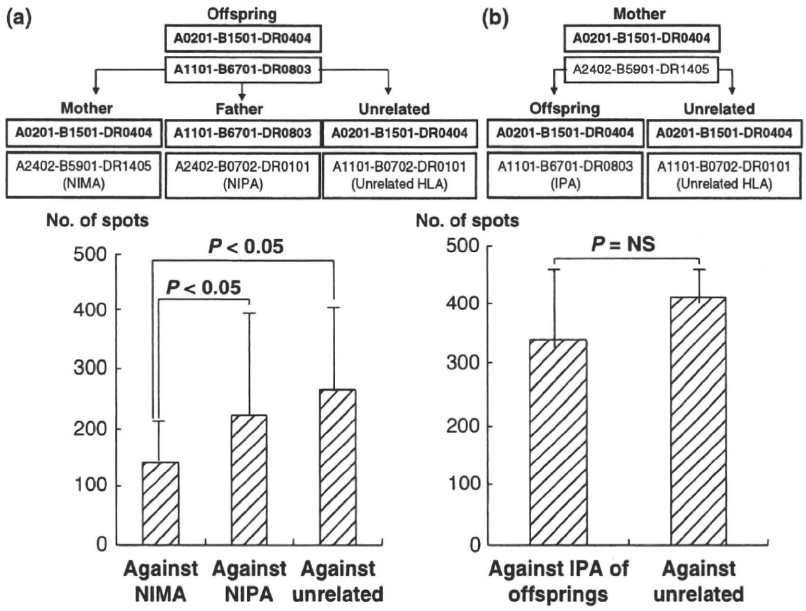

Figure 2. The relationship between responder and stimulator and T-cell responsiveness from the offspring or mother against stimulator B cells using a modified IFN-γ ELISPOT assay. The HLA haplotypes of each individual are shown in upper panel. The offspring, his mother, his father and unrelated donor shared one HLA haplotype (bold characters), and the number of mismatch HLA was same in each combination. T cells from the offspring were activated less remarkably by B cells from his mother (i.e. against NIMA) than those from his father (i.e. against NIPA) and from an unrelated individual (a). T-cells from the mother did not show any specific hyporesponsiveness to stimulator B cells from her offspring (i.e. against IPA) (b). NIMA: noninherited maternal HLA antigen; NIPA: noninherited paternal HLA antigen; IPA: inherited paternal HLA antigen.

institutional review board. The relationship between donor and recipient in HLA fully matched related SCT showed in Figure 1. The HLA-phenotypes of healthy individuals and the relationships in tests are shown in Figure 2. To exclude the effects of mismatched HLA, one haplotype was matched among mother, father, offspring and unrelated control, and the number of mismatches toward NIMA is equal to those in the unrelated control.

Preparation of blood samples and cell separation

Lymphocytes were separated from peripheral blood using a Ficoll-Hypaque method. $CD3^+$ T cells and $CD19^+$ B cells were separated using MACS cell separation system (Miltenyi Biotec GmbH, Gladbach, Germany). The purity of isolated cell fractions were checked by a FACSCalibur (BD Biosciences, Bedford, MA, USA) and those showing more than 90% purity were used.

Modified IFN-γ ELISPOT assay

Each purified sample was cultured in RPMI 1640 medium supplemented with 10% pooled human serum and 40 U/ml human recombinant IL-2 (Sigma-Aldrich, St. Louis, MO, USA) overnight at 37 °C in 5% CO_2. After preincubation, responder (4.0×10^6 cells/ml) and stimulator cells (6.0×10^5 cells/ml) were mixed and cultured in 96-well plates (Coaster, Cambridge, MA, USA) for 72 h. ELISPOT plates (Millipore, Billerica, MA, USA) were coated with anti-IFN-γ antibody (10 μg/ml, Sigma) at 4 °C overnight. Stimulated $CD3^+$ T cells were added to the plates and incubated at 37 °C in 5% CO_2 for overnight. Then plates were washed, and biotinylated anti-IFN-γ mAb (1 μg/ml; Endogen, Rockford, IL, USA) were added and incubated at room temperature for 2 h. Plates were washed, and SA-AP conjugate horse radish peroxidase (diluted 1/1000; Promega, Madison, WI, USA) was added for 1 h. After washing, AP conjugate substrate

kit (Bio-Rad Laboratories, Hercules, CA, USA) was added. Visualized spot was counted by ELISPOT Reader (Carl Zeiss, Tokyo, Japan).

Statistical analysis

The Cochran and Cox, and Welch test were used to detect the differences of T cell responses. *P*-values < 0.05 with a 95% confidence interval were considered significant.

RESULTS AND DISCUSSION

In three cases of HLA fully matched related SCT, the reactions of T cells from HLA fully matched related donors to B cells from patients were almost same as those to B cells from donors themselves (Figure 1). Immunological reactions of T cells such as aGVHD in these cases were easily controlled after allogeneic SCT. According to these results, we tested whether modified IFN-γ ELISPOT assay could detect the existence of feto-maternal immunological tolerance in healthy volunteer family members. Our experiments demonstrated that T cells from the offspring were activated less remarkably by B cells from his mother (i.e. against NIMA) than those from his father (i.e. against NIPA) and from an unrelated individual (Figure 2a). The number of mismatched HLA against his mother for the offspring T cells was equal to that against his father and against the unrelated control, implying that T cells from the offspring were hyporesponsive to NIMA compared with NIPA and unrelated antigens.

We have modified the ordinary ELISPOT assay in order to maximally detect the subtle differences in IFN-γ production in response to NIMA or others. First, the significant difference was only observed when purified $CD3^+$ T cells and $CD19^+$ B cells were used as responders and stimulators, respectively. Moreover, we could not detect any differences in T-cell reaction between against NIMA and NIPA with $CD8^+$ T cells only (data not shown), and these observations introduce us that $CD4^+$ T cell might have important ability in the allogeneic recognition of feto-maternal immunological tolerance. Second, responder T cells were stimulated with B cells for 72 h which was longer than usual ELISPOT assay, because this stimulation time demonstrated the most distinguishable results between NIMA and other stimulators (data not shown). Third, use of nonirradiated stimulator B cells seemed to be critical for best readout, because irradiation might damage stimulator cells and blunt their antigen presentation capacity. In comparison our method with previous ones, our method was more 'selective' and 'natural' in immunological reaction of T-cell immunity, and these differences might indicate us to be able to detect the significant difference in T-cell reaction of offspring between to NIMA and to NIPA in our experiments.

In contrast, T cells from the mother did not show any specific hyporesponsiveness to B cells from her offspring when compared with those from unrelated control (Figure 2b). A previous clinical study showed that six of 14 patients receiving SCT from maternal donors developed severe aGVHD, while only two of 20 patients receiving SCT from their offsprings or NIMA-mismatched siblings showed severe aGVHD (Ichinohe *et al.*, 2004). This suggests that the tolerance between mothers to their offspring and vice versa is of different quality or nature, and the tolerance of mother's T cells to the offspring might be more unstable than that of offspring. The persistence of feto-maternal immunological tolerance in healthy individuals varies among cases. Therefore, prediction of the immunological tolerance in HLA-haploidentical SCT is crucial to select an appropriate donor from the family members. The mechanisms involved in the unstable tolerance to her offspring in the mothers are explained by two hypotheses. One is that the mother's T cells become tolerant to the inherited paternal antigens expressed on the fetus during pregnancy, while the fetus' T cells become anergic to NIMA during immunological development before birth (van Rood & Claas, 2000). And this T-cell tolerance of offspring to NIMA is reported to be maintained by mother's breast-feeding after birth (Matsuoka *et al.*, 2006). The other is that T-cells from the mother might have been 'pre-sensitized'minor histocompatibility antigens (mHAs), because mHAs are less immuno-dominant than HLA and it might be difficult for mother's T-cell immunity to get tolerance against offspring's mHAs during pregnancy. Certainly, Verdijk *et al.* (2004) showed the existence of mHA specific cytotoxic T cells in healthy postpartum women, and these observations suggest that the difference of immuno-dominancy between HLA and mHAs. Therefore, we need to take into account the

two histocompatibilities including HLA and mHA to predict the immunological reactivity in HLA-haploidentical SCT between family members. In addition, there are many cytokines influencing to the result of allogeneic SCT, and further studies are required to examine the effect of the genetic polymorphisms of cytokines on the outcome of allogeneic SCT (Dickinson *et al.*, 2004). From this standpoint, our modified ELISPOT assay might be useful to detect the total immunological reaction of donor T cells to recipient including HLA, mHA and genetic polymorphisms of cytokines.

In conclusion, we developed a modified IFN-γ ELISPOT assay to predict the presence of T cell nonresponsiveness to NIMA in comparison with NIPA or unrelated HLAs, which may be useful to select an appropriate donor in HLA-haploidentical allogeneic SCT from family donors. Further studies testing (i) the reproducibility of this assay in other families and (ii) the clinical applicability by comparing the clinical outcome of allogeneic SCT with the *in vitro* results by this ELISPOT assay will be definitely warranted to validate our observations.

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Title	ABO-incompatible living-donor lobar lung transplantation.
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ABO-incompatible living-donor lobar lung transplantation

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Abstract

ABO-incompatible living-donor lobar lung transplantation was performed in a 10-year-old boy with bronchiolitis obliterans (BO) after bone marrow transplantation (BMT) for recurrent acute myeloid leukemia (AML). His blood type had changed from AB to O since he received BMT and he had no anti-A/B antibody, and received type B and AB donor lobar lungs. To our knowledge, this case represents the first successful living-donor lobar lung transplantation from ABO-incompatible donors.

Introduction

ABO-incompatible organ transplantation, especially kidney and liver transplantation have been performed to overcome donor organ shortage. However, very few cases have been reported involving ABO-incompatible lung transplantation, and furthermore, an intentional lung transplant has been reported only one case. Herein, we report ABO-incompatible lung transplantation in a 10-year-old boy with bronchiolitis obliterans (BO) after bone marrow transplantation (BMT).

Case Report

A 6-year-old boy was diagnosed with AML in 2005 and was treated with chemotherapy. In May 2008, at the age of 9, he underwent BMT from an unrelated, HLA-identical and ABO-mismatched donor for recurrent AML. His blood type was originally AB (+) and after receiving BMT from blood type O (+) donor, his blood type changed to O (+). In early 2009, at the age of 9, he began complaining of dyspnea and was diagnosed as having bronchiolitis obliterans, with the presumption that the cause was pulmonary GVHD. Respiratory distress continued to deteriorate with respiratory *Pseudomonous Aeruginosa* infection despite home oxygen therapy.

In January 2010, at the age of 10, the patient was transferred to Kyoto University Hospital. On admission, his vital capacity was 0.72 L (39.6% predicted), FEV1 was 0.27L (16.3% predicted), and arterial blood gas demonstrated a pH of 7.40, PaO_2 of 87.0 mmHg, and $PaCO_2$ of 55.8 mmHg with 2L/min oxygen administered via a nasal cannula.

Cadaveric lung transplantation was not a realistic option because brain death is accepted only for persons over than 15 years old in Japan. His parents, mother, 43 years old, ABO type AB (+) and father, 44 years old, ABO type B (+) each offered to be lung donors. The patient's ABO type had changed to type O according to ABO testing of red cells, but ABO serum test did not detect any anti-A/B antibody in his serum and tolerance to A and B antigens had been established. After carefully discussion, we thought that the risk of ABO-incompatible lung transplant in this particular case would be equivalent to ABO-

compatible transplant since the production of anti-A and anti B antibody would be unlikely even if new A and B antigen was presented from donor after lung transplantation.

In February 2010, he underwent living-donor lobar lung transplantation with a left lower lobe from his mother and a right lower lobe from his father. The surgical aspects of the donor lobectomy, donor back table preservation technique, and recipient bilateral pneumonectomy and lobar implantation have been previously described by Starnes' group [1]. For perioperative transfusion, type O red blood cells and type AB fresh frozen plasma and platelets were used for the recipients. Postoperative immunosuppression included cyclosporine, mycophenolate mofetil, and prednisone.

The postoperative course was relatively uneventful. The patient was completely weaned from the ventilator on postoperative day (POD) 3. There was transient very weak detection of anti-A antibody (Table 1). However, there was no apparent acute cellular rejection (ACR) or antibody-mediated rejection (AMR) postoperatively. Since there is no clinical finding suggesting rejection, No lung biopsy was performed postoperatively.

He was discharged from the hospital on POD 75. At that time, arterial blood gas in room air demonstrated a pH of 7.43, PaO_2 of 92.6 mmHg, and $PaCO_2$ of 37.6 mmHg. FVC was 1.53 L (83.2% predicted) and FEV1 was 1.12 L (67.9% predicted) (Table 2). Five months postoperatively, he returned to a normal life without oxygen inhalation and is able to perform daily activities.

Discussion

After bone marrow transplantation, if the patient has received marrow from a compatible but dissimilar ABO type, serum antibodies will not agree with red cell antigens. The present pediatric patient, who was originally type AB received type O marrow, had circulating type O red cells but produced no anti-A/B antibody in the serum at the time of lung transplantation. According to ABO testing of red cells, recipient (type O)-donors (B and AB) ABO type matching was incompatible, however, since the recipient had no anti-A/B antibody in serum, we could perform this surgical procedure with ABO incompatible donors. Other possible hematological change that may occur in the recipient after lung transplantation was

carefully discussed. Theoretically, the lymphocytes derived from type B lung donor might produce anti-A antibodies in the recipient, and not only attack the recipient's other organs which were originally type AB, but also attack contralateral type AB donor lung. However, there was only transient weak detection of anti-A antibodies and no AMR occurred postoperatively.

Recently, many cases of ABO-incompatible organ transplantation, especially kidney and liver transplantation have been performed to overcome donor organ shortage. Japanese groups reported excellent patient and graft survival of ABO-incompatible kidney transplantation using regimens including plasmapheresis, immunosuppression, immunoabsorption and splenectomy, which showed a similar outcomes to those of ABO-compatible donor transplants[2, 3]. However, intentional ABO-incompatible lung transplant was reported in only one case. [4] Pierson et al. reported 42 cases (0.4%) of accidental ABO-incompatible lung transplants among 9,804 primary lung transplants according to the database of the Organ Procurement and Transplant Network in the United States [5] and the outcome was acceptable compared with those of ABO-compatible lung transplants when the intensive therapy described above has been used.

Although the present case showed a unique blood type background because of prior bone marrow transplantation, to our knowledge, this case represents the first successful living-donor lobar lung transplantation from ABO-incompatible donors. Although the short-term outcome was satisfactory, long-term follow-up is needed to assess whether this procedure is ultimately justified.

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Table 1. The serological analysis of anti-A and anti-B antibody in the recipient

POD after transplant	pre	7	12	19	26	54	82
Aggregation to type A RBC*	0	w+**	0	w+	w+	0	0
Aggregation to type B RBC	0	0	0	0	0	0	0

* Red blood cells, ** very weak aggregation

Table 2. The time trend of pulmonary function test in the recipient

POD after transplant	pre	82	188
Height (cm)	127.0	127.4	128.0
Body Weight (kg)	24.0	25.0	28.0
VC (L)	0.72	1.62	1.61
FVC (L)	0.72	1.53	1.60
FEV1.0 (L)	0.27	1.12	1.09

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PLoS one

Noninvasive Tracking of Donor Cell Homing by Near-Infrared Fluorescence Imaging Shortly after Bone Marrow Transplantation

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Abstract

Background: Many diseases associated with bone marrow transplantation (BMT) are caused by transplanted hematopoietic cells, and the onset of these diseases occurs after homing of donor cells in the initial phase after BMT. Noninvasive observation of donor cell homing shortly after transplantation is potentially valuable for improving therapeutic outcomes of BMT by diagnosing the early stages of these diseases.

Methodology/Principal Findings: Freshly harvested near-infrared fluorescence-labeled cells were noninvasively observed for 24 h after BMT using a photon counting device to track their homing process. In a congenic BMT model, the homing of Alexa Fluor 750-labeled donor cells in the tibia was detected less than 1 h after BMT. In addition, subsequent cell distribution in an intraBM BMT model was successfully monitored for the first time using this method. In the allogeneic BMT model, T-cell depletion decreased the near-infrared fluorescence (NIRF) signals of the reticuloendothelial system.

Conclusions/Significance: This approach in several murine BMT models revealed that the transplanted cells homed within 24 h after transplantation. NIRF labeling is useful for tracking transplanted cells in the initial phase after BMT, and this approach can contribute to in vivo studies aimed at improving the therapeutic outcomes of BMT.

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Introduction

Bone marrow transplantation (BMT) is an important procedure for curing hematological malignancies, although engraftment failure [1] and graft-versus-host disease (GVHD) [2] remain serious complications following allogeneic BMT. To examine these complications, it is important to monitor the transplanted donor cells in the initial phase after BMT because donor cell homing is a rapid process. Homing is defined as the anchoring of hematopoietic cells in their niche before cell proliferation [3]. Results of a colony-forming assay revealed that donor cell homing occurs within minutes or a few hours, rather than days, after BMT [4]. BMT-associated complications are suspected to begin just after homing of the donor cells; for example, in studies of engraftment failure, homing of hematopoietic stem cells (HSCs) is crucial and is the first step in hematopoietic reconstitution [5]. In acute GVHD (aGVHD), T-cell activation by host antigen-presenting cells [6,7] begins after homing in the reticuloendothelial system, and this process starts within a few days after BMT in murine GVHD models [8].

Recent molecular imaging techniques have facilitated significant advances in noninvasive optical imaging [9], which enable tracking of transplanted hematopoietic cells with greater accuracy in vivo. Bioluminescence imaging can precisely analyze the processes during BMT [10]. However, until now, bioluminescence imaging has provided sparse spatiotemporal information for donor cell homing in the initial phase after BMT.

In this study, we directly labeled donor BM cells using a near-infrared fluorescence (NIRF) dye with high tissue permeability and monitored the homing of transplanted cells shortly after BMT using a noninvasive whole-body imaging device IVIS-Spectrum. The overall results provide information regarding the homing of donor cells after BMT and intraBM-BMT (IBM-BMT), and the onset of GVHD. Noninvasive tracking of transplanted donor cells in the initial phase after BMT enables acquisition of spatiotemporal information regarding HSC homing, which can help identify

factors supporting HSC homing and mechanisms of hematopoietic reconstitution.

Materials and Methods

Ethics statement

All animal experiments in this study were performed with the approval of the Animal Experiment Committees of Kyoto University, Graduate School of Medicine. The approval number of the experiment is Med Kyo 09247. Approved experiments included use of transgenic mice and primary cells, cell transplantation, in vivo and ex vivo optical imaging, and UV irradiation.

Mice

Balb/c nu/nu (H-2^d, Thy 1.2), Balb/c (H-2^d, Thy 1.2), C57BL/6 (H-2^b, Thy 1.2), and FVB/N (H-2^q, Thy 1.1) mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). Enhanced green fluorescent protein (EGFP) transgenic mice were generated by injecting the CAAG-EGFP expression vector into a one-cell embryo of an ICR closed colony (ICR/EGFP) [11], followed by breeding in an SPF animal facility. All mice were 7–9 weeks of age and were fasted according to our original fasting protocol from the day before BMT until 24 h after BMT (Supplemental Fig. S1) for suppression of autofluorescence from food particles in the gastrointestinal (GI) tract.

Cell preparation for transplantation and in vitro assays

BM cells were prepared from the medullary cavities of the humerus, femur, and tibia. Splenocytes were prepared by homogenization of the spleen. BM mononuclear cells (BM-MNCs) and spleen mononuclear cells (Sp-MNCs) were obtained by density gradient centrifugation using Lympholyte-M solution (Cedarlane Labs, Hornby, ON, Canada). T-cell-depleted (TCD) BM-MNCs were obtained by negative selection using Thy1.2^+ microbeads (Miltenyi Biotech, Auburn, CA, USA).

NIRF and 5-(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling

Cy5.5 monofunctional dye (peak excitation: 675 nm, peak emission: 694 nm; GE Healthcare UK Ltd, Buckinghamshire, UK) and Alexa Fluor 750 carboxylic acid, succinimidyl ester (AF750) (peak excitation: 749 nm, peak emission: 775 nm; Invitrogen, Eugene, OR, USA) were dissolved in N, N-dimethylformamide. To prepare Cy5.5- or AF750-labeled cells, BM-MNCs and Sp-MNCs were incubated with Cy5.5 (0.4 mg/mL) or AF750 (0.1 mg/mL) for 15 min at 37°C under 5% CO_2. The cells were washed twice with phosphate-buffered saline (PBS) and then once with 10 mM Tris in PBS to disturb the active groups. For CFSE (Invitrogen) labeling, the cells were incubated in 5 μM CFSE in PBS for 10 min at 37°C, followed by washing with excess ice-cold PBS on ice for 5 min to quench staining. The cells were then washed 3 times with PBS. To prepare CFSE and NIRF double-positive cells, CFSE staining was performed before NIRF labeling.

Cell proliferation assays

NIRF-labeled Sp-MNCs (1×10^5) were seeded in a 96-well plate with 100 μL of phenol red-free RPMI 1640 (Wako Pure Chemical Industries Ltd., Osaka, Japan) supplemented with 1 mM sodium pyruvate, 10 mM HEPES, 100 units/mL penicillin, 100 μg/mL streptomycin, 50 μM mercaptoethanol, and 10% FCS. To stimulate splenocyte proliferation, recombinant mouse IL-2 (mIL-2) (20 ng/mL; R&D Systems, Minneapolis, MN, USA) and anti-CD3/CD28 antibody-coated beads (Dynabeads mouse CD3/CD28 T-cell expander; Invitrogen Dynal AS, Oslo, Norway) were added to the medium. The CD3/CD28 T-cell expander was used at a 1:1 bead-to-cell ratio according to the manufacturer's instructions. The viable cell number was analyzed as previously described [12] with slight modifications. The absorbance (450 nm) was measured 3 h after adding the cell-counting reagent.

Analysis of clonogenic myeloid and erythroid progenitors

BM cells (2×10^4) were harvested from 6- to 8-week-old Balb/c nu/nu mice and cultured in Methocult GF M3434 (Stem Cell Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions after NIRF labeling. Granulocyte-macrophage colony-forming units (GM-CFUs); erythroid burst-forming units (BFU-Es); and granulocyte, erythrocyte, macrophage, and megakaryocyte colony-forming units (GEMM-CFUs) were scored after 7 days in culture.

Fluorescence-activated cell sorter (FACS) analyses

FACS data were obtained using a Canto II flow cytometer (Becton Dickinson, Mountain View, CA, USA) and analyzed using FACSDiva (Becton Dickinson) or FlowJo software (TreeStar, Ashland, OR, USA). A red helium-neon (633 nm) laser was used for excitation of Cy5.5 (analyzed using an APC filter) and AF750 (analyzed using an APC-Cy7 filter). NIRF-labeled ICR/EGFP BM-MNCs were used for analysis of chimerism of transplanted cells. Antibodies against FITC-conjugated Thy1.1 (CD90.1) and PE-conjugated Thy1.2 (CD90.2) were purchased from BD Pharmingen, San Diego, CA, USA. AF750-labeled Balb/c Thy1.2^+ cells (1×10^6) were mixed with unlabeled FVB/N Thy1.1^+ cells (1×10^6) in a 1:1 ratio and incubated together in PBS at 37°C for 3 h, and the AF750 intensity of Thy1.1^+ and Thy1.2^+ cells was determined. We estimated the percentages of EGFP$^+$ cells in the tibiae and peripheral blood of the recipients at 24, 48, and 72 h after BMT. Erythrocytes were removed using ACK lysis buffer (Invitrogen). DNA content was analyzed as previously described [13].

Pathological examinations

For ex vivo analyses of the chimerism of transplanted EGFP$^+$ cells in the tibiae, the recipient mice were sacrificed at 24, 48, or 72 h after BMT. BM cells were flushed from the tibiae using PBS and dyed with 100 μg/mL DAPI (Sigma-Aldrich, St. Louis, MO, USA). These cells were then observed with an inverted fluorescent microscope. To detect EGFP$^+$ cells in the organs after BMT, the Balb/c nu/nu mice receiving ICR/EGFP BM-MNCs were sacrificed at 24, 48, or 72 h after BMT. Organs from the mice were then removed, fixed in 10% formalin, and embedded in paraffin. Furthermore, 3-μm-thick sections were deparaffinized and incubated with blocking solution (1% Block Ace; Dainippon Pharmaceutical Co. Ltd., Osaka, Japan) for 15 min at room temperature. Sections were incubated with polyclonal rabbit anti-EGFP antibody (1:1000 dilution in PBS; Abcam, Cambridge, MA, USA) at 4°C overnight and then with FITC-conjugated swine anti-rabbit immunoglobulin (1:30 dilution in PBS; Dako, Glostrup, Denmark) for 1 h at room temperature. They were then washed and mounted in VECTASHIELD with DAPI (Vector Laboratories, Burlingame, CA, USA). All photos were taken using a BZ-9000 microscope (KEYENCE, Osaka, Japan).

Transplantation of donor cells

Balb/c nu/nu mice (7–9 weeks old) or Balb/c mice (7–9 weeks old) were lethally irradiated (8 Gy) in a single fraction by a ^{137}Cs γ-ray using a Gammacell 40 Exactor (MDS Nordion International

Inc., Ontario, Canada). BM-MNCs and Sp-MNCs were injected into the recipient mice through the tail vein at 6–8 h after irradiation. For ex vivo analyses of the chimerism of transplanted EGFP$^+$ cells in the tibiae or peripheral blood, 1×10^7 Cy5.5- or AF750-labeled ICR/EGFP BM-MNCs were transplanted into the Balb/c nu/nu mice. The recipient mice were sacrificed at 24, 48, or 72 h. For in vivo imaging with congenic BMT, 1×10^7 AF750-labeled Balb/c nu/nu BM-MNCs were injected into the Balb/c nu/nu mice. For allogeneic BMT, 1×10^7 AF750-labeled C57BL/6 BM-MNCs, 1×10^7 AF750-labeled TCD C57BL/6 BM-MNCs, or 5×10^6 AF750-labeled C57BL/6 TCD BM-MNCs plus 5×10^6 AF750-labeled C57BL/6 Sp-MNCs were injected into 7- to 9-week-old Balb/c nu/nu mice.

IBM-BMT model

IBM-BMT was performed as described previously [14]. In brief, 7- to 9-week-old Balb/c nu/nu mice were lethally irradiated (8 Gy) in a single fraction by a ^{137}Cs γ-ray 24 h before BMT. For IBM-BMT, the mice were then anesthetized with isoflurane, and the left tibia was gently drilled using a 26-G microsyringe (50 μL; Hamilton, Reno, NV, USA) through the patellar tendon. The AF750-labeled BM-MNCs (1×10^7 in 30 μL PBS) were directly injected into the BM cavity thereafter.

In vivo and ex vivo imaging of transplanted mice

In vivo imaging was performed using an IVIS Spectrum system (Xenogen, Alameda, CA, USA) at 5, 15, or 30 min and 1, 3, 6, 12, 18, or 24 h after BMT. During imaging, the mice were kept on the imaging stage under anesthesia with 2.5% isoflurane gas in oxygen flow (2 L/min). Transplanted Cy5.5- or AF750-labeled cells were detected using emission and excitation filters (excitation/emission: 640/700 for Cy5.5-labeled cells, 710/780 for AF750-labeled cells). The conditions were as follows: exposure time = 5 s, lamp level = high, binning = medium, field of view = 12.9×12.9 cm, and f/stop = 1. Ex vivo imaging was performed using the IVIS Spectrum system 24 h after BMT, under the same conditions as for in vivo imaging. For free NIRF injection, free Cy5.5 (625 ng) or AF750 (40 ng) in 100 μL Tris in PBS (10 mM), with the same fluorescence intensity as 1×10^7 NIRF BM-MNCs, was injected through the tail vein.

Statistical analysis

Student's t tests were used to determine the statistical significance. $P<0.05$ was considered significant.

Results

Direct NIRF labeling technique shows low cytotoxicity

Since most diseases associated with BMT are caused by transplanted hematopoietic cells, it is very important to label all the donor cells. Freshly harvested BM-MNCs from Balb/c nu/nu mice were labeled with 0.4 mg/mL Cy5.5 and 0.1 mg/mL AF750, which did not show obvious cytotoxicity or growth inhibition (Supplemental Figs. S2 and S3). Furthermore, the NIRF dyes did not affect the colony-forming efficiency at these concentrations (Fig. 1A). BM-MNCs were efficiently labeled regardless of cell size and diversity (Fig. 1B). Using the NIRF dyes, homogeneous labeling on the cell surfaces was achieved despite using a variety of cell populations (Fig. 1C).

Fluorescence on the cell surface is retained and does not influence surface antigen recognition

Balb/c Sp-MNCs were double-stained with CFSE and NIRF dyes, and their mean fluorescence intensity (MFI) was measured every 24 h until 72 h using FACS (Fig. 1D). To activate and expand Sp-MNCs, we cultured them with CD3/28 beads and mIL-2. MFI of CFSE decreased by 1 log during the first 24 h, while MFI of NIRF hardly changed for 24 h; it was halved following cell division after 48 h. Because Sp-MNCs were labeled using the same method, the difference in MFI between CFSE and NIRF can be attributed to the faster fluorescence decay of CFSE, as its excitation wavelength is in the visible range, rather than a difference in dye retention time on the cell surface. These results indicate that NIRF retains more stable MFI than CFSE.

During in vivo fluorescence imaging, recipient mice received approximately 20 excitations over a 24-h period. We confirmed that repeated excitations did not influence cell viability (Supplemental Fig. S4) or the NIRF MFI on the cell surface (Supplemental Fig. S5). It was also considered that precise evaluation of the obtained fluorescence images would be influenced by transfer of the NIRF dye from the donor cells to neighboring recipient cells after BMT and that the NIRF dyes on the cell surface would hinder recognition of cell surface markers. To examine these possibilities, we co-cultured Balb/c-derived Thy1.2$^+$ Sp-MNCs (NIRF labeled) and FVB/N-derived Thy1.1$^+$ cells for 3 h and examined their NIRF-labeling status and recognition by corresponding antibodies using FACS. The cells labeled with NIRF were recognized as Thy1.2$^+$. Co-cultured Thy1.1$^+$ cells showed no increase in NIRF (Fig. 1E). These results indicate that NIRF dyes were universally retained on the cell surface and did not influence the recognition of cell surface antigens, suggesting that in vivo fluorescence imaging with NIRF-labeled cells would contribute to acquisition of spatiotemporal information regarding donor cells shortly after BMT.

In vivo tracking of Cy5.5-labeled hematopoietic cells during the first 24 h after BMT

Cy5.5-labeled Balb/c nu/nu BM-MNCs were transplanted to the Balb/c nu/nu recipient mice. Since abdominal autofluorescence caused by food decreases the precision of in vivo imaging, all mice were fasted before in vivo imaging to suppress autofluorescence from food particles in the GI tract, (Supplemental Figs. S1 and S6). The Cy5.5 signal was observed from both sides of the tibiae and spines at 5 min after BMT through the tail vein (Supplemental Fig. S7). We further examined the status of transplanted BM-MNCs with Cy5.5 labeling (Fig. 2) and found that BM-MNCs from ICR/EGFP were homogeneously labeled with Cy5.5 on the cell surface (Fig. 2A). FACS analysis revealed that the EGFP$^+$ cells included all leukocyte fractions (Fig. 2B).

To confirm whether the in vivo Cy5.5 signals from the tibia (Supplemental Fig. S7) were derived from Cy5.5-labeled transplanted BM-MNCs, we transplanted Cy5.5-labeled ICR/EGFP BM-MNCs to the Balb/c nu/nu mice. Cy5.5 signals were observed in the tibia 24 h after BMT; these signals decreased thereafter (Fig. 2C). BM cells in the tibia were flushed and then observed by fluorescence microscopy on the same schedule as for in vivo imaging. We confirmed that the Cy5.5 signals detected in the tibia 24 h after BMT were from the transplanted BM-MNCs and belonged to the Cy5.5$^+$ and EGFP$^+$ cell populations (Fig. 2C, upper row). The cell surface Cy5.5 signal decreased throughout the experiment, probably due to cell division (Fig. 2C), while the chimerism of the EGFP$^+$ cells increased rapidly (Fig. 2D). The NIRF signals emanating from the transplanted donor cells decreased with inverse proportion to cell division.

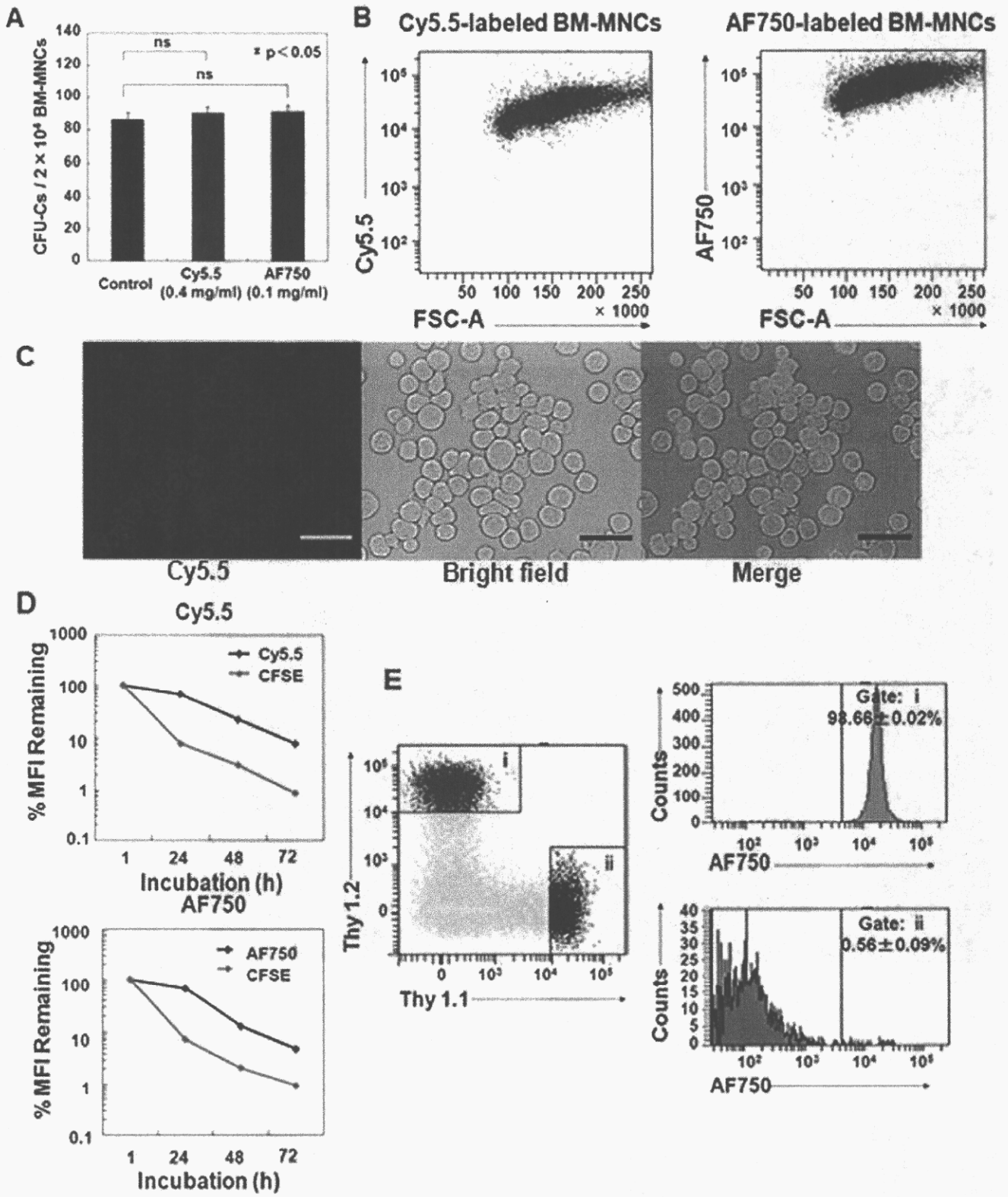

Figure 1. Evaluation of cytotoxicity and in vitro fluorescence intensity of NIRF labeling. (A) GM-CFUs, BFU-Es, and CFU-GEMMs were counted (n = 5); mean CFUs per 2×10^4 BM-MNCs are shown. (B) FACS analysis of Balb/c nu/nu BM-MNCs labeled with Cy5.5 (0.4 mg/mL) and AF750 (0.1 mg/mL). (C) Representative fluorescence image of Cy5.5-labeled Balb/c nu/nu BM-MNCs. Bar = 100 μm. (D) In vitro retention of NIRF and CFSE in Balb/c Sp-MNCs for the indicated times after labeling (n = 8). Sp-MNCs (1×10^5) were seeded in a 96-well plate with phenol red-free RPMI 1640 medium containing CD3/28 beads and mIL2. Values are %MFI ± SEM. Error bars are less than 1.1%. (E) AF750-labeled Balb/c Thy1.2$^+$ Sp-MNCs (1×10^6) (i) were mixed in a 1:1 ratio with unlabeled FVB/N Thy1.1$^+$ Sp-MNCs (1×10^6) (ii) at 37°C for 3 h, and the AF750 intensity of Thy1.1$^+$ and Thy1.2$^+$ cells was analyzed by FACS.

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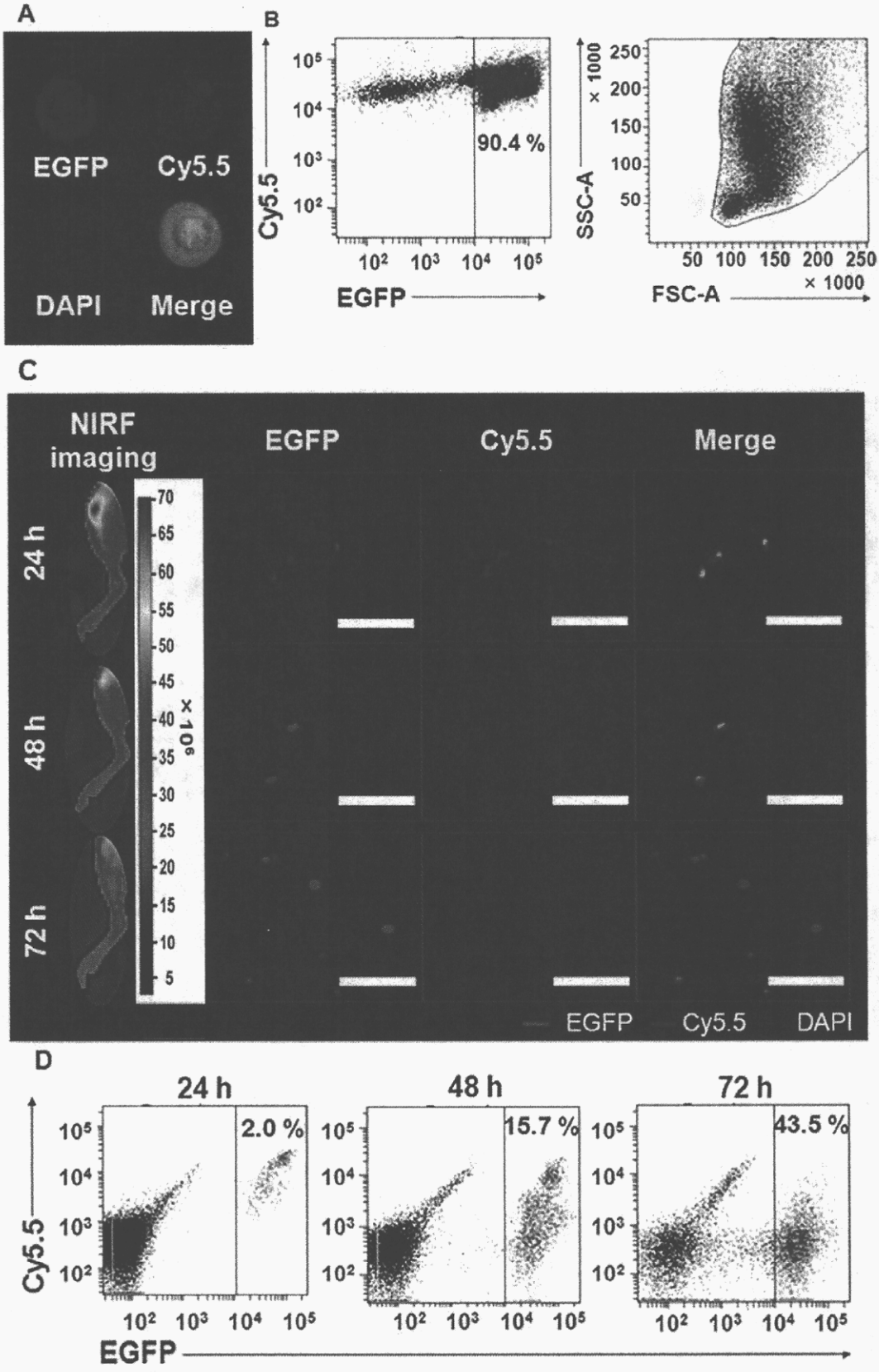

Figure 2. EGFP and Cy5.5 double-positive cells were detected in BM at 24 h after BMT. (A) Representative fluorescence image of Cy5.5-labeled ICR/EGFP BM-MNCs. Green, EGFP; blue, DAPI; red, Cy5.5. (B) FACS analysis of Cy5.5-labeled ICR/EGFP BM-MNCs at 1 h after labeling. The $EGFP^+$ cells included all leukocyte fractions. (C) Analysis of Cy5.5 and EGFP double-positive cells in the tibiae at the indicated times. Recipient Balb/c nu/nu mice ($H\text{-}2^d$) were transplanted with 1×10^7 Cy5.5-labeled ICR/EGFP BM-MNCs (closed colony). In vivo fluorescence imaging of the right legs of mice