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Prospective Multicenter Study of Single-Unit Cord Blood Transplantation with Myeloablative Conditioning for Adult Patients with High-Risk Hematologic Malignancies

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A B S T R A C T

Although the use of cord blood transplantation (CBT) is increasing, the optimal methods for conditioning and graft-versus-host disease (GVHD) prophylaxis remain to be established. Among previous reports, the Institute of Medical Science, University of Tokyo (IMSUT) has reported remarkably favorable results of CBT for hematologic malignancies as a single-institute experience. The aim of the present multicenter prospective study was to assess the safety and efficacy of CBT performed precisely according to IMSUT transplantation procedures. Thirty-three adult patients with hematologic malignancies, such as acute leukemia, chronic myelogenous leukemia, or myelodysplastic syndrome, either lacking an HLA-identical sibling/HLA-matched unrelated donor or requiring urgent transplantation were enrolled. Conditioning consisted of total body irradiation (12 Gy), cytarabine, and cyclophosphamide. Cyclosporine A and methotrexate were used for GVHD prophylaxis. Diagnoses were acute leukemia in 26 patients, chronic myelogenous leukemia in 4, and myelodysplastic syndrome in 3; 12 patients were in first complete remission, and the others were in advanced stages at the time of CBT. Thirty-one patients achieved engraftment, and the cumulative incidence of grade II-IV acute GVHD was 45% (95% confidence interval, 28%-62%). With a median follow-up of 46.2 months in 16 surviving patients, the 1-year cumulative incidence of nonrelapse mortality was 15% (95% confidence interval, 5%-30%). Causes of nonrelapse mortality were infection (n = 4) and graft failure (n = 1). The overall and disease-free survival rates were 51% (95% CI, 34%-68%) and 42% (95% CI, 26%-59%), respectively. These results suggest that the IMSUT CBT procedures can safely provide a high disease-free survival rate in patients with high-risk hematologic malignancies.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is the most promising curative treatment for hematologic malignancies. Several hematopoietic stem cell sources are now available, and the use of cord blood transplantation (CBT) has been increasing dramatically [1]. However, the outcomes of CBT are not necessarily satisfactory, because of the high nonrelapse mortality (NRM). Conditioning and prophylaxis against graft-versus-host disease (GVHD) used in CBT have varied significantly among previous studies, and the optimal approaches remain to be established [2-6]. Among those studies, the outcomes of CBT for hematologic diseases at the Institute of Medical Science, University of Tokyo (IMSUT) were notably favorable and in fact were superior to the outcomes of allogeneic bone marrow transplantation (BMT) or peripheral blood stem cell transplantation (PBSCT) from related and unrelated donors at the same institution [7,8].

The IMSUT transplantation procedures involve a myeloablative conditioning regimen using total body irradiation (TBI), cyclophosphamide (CY), and high-dose cytarabine. Cytarabine is combined with granulocyte colony-stimulating factor (G-CSF) for myeloid malignancies. In addition, cyclosporine A is given over 10 hours with short-term methotrexate (MTX) for GVHD prophylaxis. To date, however, no study has systematically assessed whether IMSUT's favorable results would be reproduced if CBT for hematologic malignancy were performed precisely according to IMSUT's transplantation procedures. Accordingly, we designed and performed a multi-institutional study to evaluate the safety

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and efficacy of single-unit CBT for hematologic malignancies by strictly following these procedures.

PATIENTS AND METHODS

This study is a multi-institutional prospective study of the Kanto Study Group for Cell Therapy. The protocol was approved by the Institutional Review Boards of the 9 participating institutions and registered at http://clinicaltrials.gov (NCT00270881). No patients were enrolled from IMSUT. Written informed consent was obtained from all patients in accordance with the Declaration of Helsinki.

Patient Eligibility and Cord Blood Unit Selection

Eligibility criteria for this study included (1) age 20-55 years; (2) diagnosis of acute myelogenous leukemia (AML), acute lymphoblastic leukemia (ALL), chronic myelogenous leukemia (CML), or myelodysplastic syndrome (MDS) suitable for allogeneic HSCT; (3) lack of an available 5/6 or fully HLA-matched related donor; (4) either lack of an available fully 6/6 HLA-matched unrelated donor or the need for immediate HSCT based on the features of the disease as judged by the treating physician; (5) availability of a 6/6, 5/6, or 4/6 serologically HLA-matched cord blood unit with a minimum of 2×10^7 total nucleated cells per kilogram of recipient body weight before cryopreservation in the Japan Cord Blood Bank Network; (6) Eastern Cooperative Oncology Group performance status of 0 or 1; (7) adequate function of main organs, including the liver, kidneys, lungs, and heart; and (8) lack of anti-HLA (class I and/or II) antibody. Patients with a previous history of HSCT, active infection, or active central nervous system disease or psychiatric disorders were excluded. This protocol was only for patients receiving single-unit CBT. HLA disparity was determined based on the antigen level of HLA-A, -B, and -DR loci specified by low- or high-resolution techniques.

Conditioning Regimen

All patients received the same myeloablative conditioning as described previously [7-9]. TBI 12 Gy was delivered in 4 or 6 fractions for 2 or 3 days (days -8, -7, and -6 or days -7 and -6). After completion of TBI, cytarabine at a dose of 2 or 3 g/m^2 was administered i.v. over 2 hours every 12 hours for 2 consecutive days (days -5 and -4). All patients received steroid eye drops for prophylaxis against keratoconjunctivitis due to cytarabine. For myeloid malignancies (AML, MDS, and CML), recombinant human granulocyte colony-stimulating factor (G-CSF; lenograstim) was given by continuous infusion at a daily dose of 5 μg/kg, starting 12 hours before the first dose of cytarabine and continuing until the last dose of cytarabine. Then CY 60 mg/kg was administered i.v. over 2 hours for 2 consecutive days (days -3 and -2). Cytarabine could be omitted in exceptional cases based on factors in the patient's background, such as a history of allergic reaction. No patient received antithymocyte globulin as part of the conditioning regimen.

Infusion of Cord Blood, GVHD Prophylaxis, and Supportive Care

Two days after the completion of CY administration (day 0), patients received CBT. The cord blood graft was thawed and immediately infused without washing. GVHD prophylaxis was provided with short-term methotrexate (MTX; 15 mg/m^2 on day 1, and 10 mg/m^2 on days 3 and 6) and cyclosporine A (CsA). Leucovorin was given i.v. to ameliorate its toxicity. CsA was given i.v. over 10 hours starting on day -1. The CsA dose was adjusted at the discretion of the physician only if the trough level of CsA was <100 ng/mL or adverse events associated with CsA developed.

Each patient was isolated in a laminar air-flow or high-efficiency particulate air-filtered room. The administration of lenograstim at a dose of 5 μg/kg was started 1 day after CBT and continued until neutrophil recovery was achieved. Prophylactic fluoroquinolone and fluconazole (200 mg/day) were given orally, starting 14 days before transplantation. For *Pneumocystis* pneumonia prophylaxis, cotrimoxazole was given for 14 consecutive days before transplantation and recommenced on a schedule of 2-3 days per week after sustained hematopoietic recovery was confirmed. Oral acyclovir at a dose of 1000 mg/day was given from day -7 to day 35. Cytomegalovirus (CMV) reactivation was routinely monitored by CMV antigenemia assay or PCR soon after neutrophil recovery, which triggered preemptive therapy with ganciclovir. Intravenous immunoglobulin was given in patients with a serum immunoglobulin G level <500 mg/dL.

Assessment of Chimerism, Engraftment, and GVHD

The chimerism study was performed on whole bone marrow cells at 1 month, 2 months, and 3 months after CBT. Analyses were performed by fluorescein in situ hybridization for X and Y chromosomes or by microsatellite PCR as appropriate. The day of myeloid engraftment was defined as the first day of 3 consecutive days when the absolute neutrophil count exceeded 0.5×10^9/L. The day of platelet engraftment was defined as the day when the absolute platelet count exceeded 20×10^9/L without platelet transfusion. Primary graft failure was defined as lack of myeloid engraftment until day 42; secondary graft failure, as a persistent loss of myeloid engraftment after having achieved engraftment. Both acute and chronic GVHD were diagnosed and graded based on published criteria [10,11].

Table 1
Patient and Transplant Characteristics (n = 33)

Characteristic	Value
Age, years, median (range)	37 (21-54)
Sex, males/females, n	21/12
Body weight, kg, median (range)	55.0 (39.1-97.0)
Diagnosis, n	
AML	20
ALL	6
CML	4
MDS	3
Disease status, n	
CR1	12
CR2, chronic phase 2	5
Not in CR, blast crisis	16
Conditioning, n	
TBI + cytarabine + G-CSF + CY	27
TBI + cytarabine + CY	5
TBI + CY	1
Cord blood units	
HLA disparity, n	
4-antigen match	29
5-antigen match	3
6-antigen match	1
Nucleated cells per kg body weight, median (range)	2.66 (2.00-4.58)
GVHD prophylaxis with CsA + short-term MTX, n	33

Statistical Analysis

The primary endpoint of this study was 1-year NRM, and secondary endpoints were engraftment, acute and chronic GVHD, infectious complications, day +100 NRM, relapse rate, disease-free survival (DFS), and overall survival (OS). Survival rates were calculated by the Kaplan-Meier method. Probability of acute GVHD, disease relapse, and NRM were estimated on the basis of cumulative incidence curves to accommodate the following competing events: death without GVHD and second transplantation for graft failure for acute GVHD, death for relapse, and relapse for NRM [12]. Comparisons were made using the log-rank test or Gray test as appropriate. Multivariate analyses were performed using the Cox proportional hazards model or the Fine and Gray proportional-hazards model as appropriate. $P < .05$ was considered to indicate statistical significance in all analyses.

RESULTS

Patient Characteristics

Thirty-three patients were enrolled and underwent CBT. Patient and transplant characteristics are summarized in Table 1. At the time of CBT, 12 patients with AML/ALL were in the first complete remission (CR1) and were defined as standard-risk patients. The remaining 19 patients had AML/ALL in CR2 or CML in chronic phase 2 (n = 3) or AML/ALL not in remission, MDS with an excess of blasts, or CML in blastic crisis (n = 16), and were defined as high-risk patients. Three patients in CR1 had Philadelphia chromosome–positive ALL. All but 1 patient received TBI, cytarabine, and CY with or without G-CSF as conditioning.

Engraftment and Chimerism

Myeloid engraftment was obtained at a median of 26 days (range, 18-60 days) in 31 patients. Platelet engraftment was obtained at a median of 44 days (range, 25-140 days) in 26 patients. Two patients experienced primary graft failure, caused by graft rejection in 1 patient and early disease progression in 1 patient. One case of secondary graft failure occurred after hemophagocytic syndrome. In the 27 patients who underwent chimerism analysis, full donor chimerism

Table 2
Infectious Complications

Infection	Number
Bacterial (n = 14)	
Bacteremia	10
Enteritis	1
Meningitis	1
Pneumonia	1
Cholecystitis	1
Fungal (n = 5)	
Candidemia	1
Invasive aspergillosis	3
Pneumocystis pneumonia	1
Viral (n = 28)	
CMV infection	23
CMV disease	1
HHV-6 central nervous system disorder	1
Parainfluenza virus pneumonia	1
Encephalitis*	2

HHV-6 indicates human herpesvirus 6.
* Causative virus was not identified.

was obtained at 1 month posttransplantation in 24 patients, at 2 months in 2 patients, and at 3 months in 1 patient.

Acute and Chronic GVHD

Acute GVHD developed in 21 of 31 evaluable patients with myeloid engraftment (grade I in 6 patients, grade II in 10, grade III in 3, and grade IV in 2). The cumulative incidence of grade II-IV acute GVHD up to day +100 post-transplantation was 46% (95% CI, 27.8%-61.5%). Among the 10 patients with grade II acute GVHD, 6 did not require systemic glucocorticoid in addition to CSA for the treatment of acute GVHD. Among the 27 patients who survived more than 100 days after transplantation, 6 patients developed chronic GVHD (2 with extensive type and 4 with limited type).

Infectious Complications

All but 1 patient experienced at least 1 episode of infectious complications after CBT (Table 2). The most common infective pathogen was viruses, including CMV infection, followed by bacteria and fungus. Four cases of infectious complications were fatal (2 with bacteremia and 2 with encephalitis). All of these patients had grade II-IV acute GVHD (1 with grade II, 1 with grade III, and 2 with grade IV) and were receiving systemic glucocorticoid therapy when infectious complications developed.

NRM, Relapse, and Survival

At a median follow-up of 46.2 months (range, 31.0-65.8 months), 16 patients were alive. Causes of death in the other 17 patients included relapse and complications associated with treatment of relapse after transplantation (12 patients), infectious complications (4 patients), and graft failure (1 patient). The cumulative incidence of NRM was 9% (95% CI, 2%-22%) at 100 days post-HSCT and 15% (95% CI, 5%-30%) at 1 year post-HSCT (Figure 1A). The cumulative incidence of NRM at 3 years post-HSCT did not differ significantly between standard-risk and high-risk patients (25% versus 10%; $P = .254$). In 14 patients, disease relapse or progression occurred at a median of 9 months (range, 0.9-23.0 months) post-HSCT. The 3-year relapse rate was 42% (95% CI, 25%-59%) (Figure 1B). OS was 51% (95% CI, 34%-68%), and DFS was 42% (95% CI, 26%-59%) (Figure 1C). The 16 patients who were alive without disease remained in good condition, with an Eastern Cooperative Oncology Group performance status

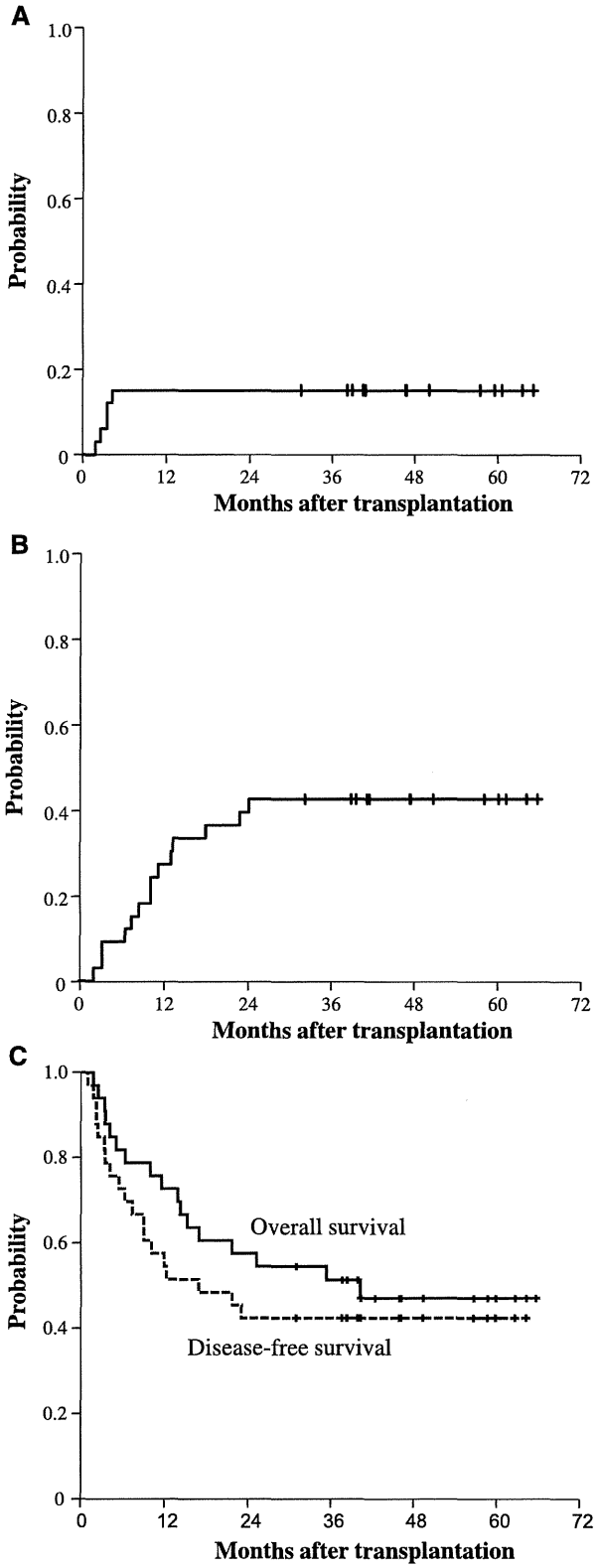

Figure 1. Cumulative incidences of NRM (A) and relapse rate (B), and Kaplan-Meier estimates of OS and DFS (C). "+" indicates a censored patient.

of 0 (n = 13) or 1 (n = 3). Two of the 3 patients who experienced primary or secondary graft failure and 4 of 14 patients who experienced disease relapse or progression subsequently underwent a second allogeneic HSCT. At the

time of this analysis, 2 patients were still alive without disease at 23 months and 62 months after the second HSCT.

As the possible factors affecting the rates of disease relapse, NRM, OS, and DFS, the following variables were analyzed: patient age (<40 years versus ≥40 years) and sex, risk categories based on disease status at transplantation (standard risk versus high risk), HLA disparity (4/6 versus 5/6 and full match), nucleated cell dose of cord blood graft ($<2.60 \times 10^7$/kg versus $\geq 2.60 \times 10^7$/kg), and development of acute GVHD (none or grade I versus grade II-IV). The development of grade II-IV acute GVHD had a negative impact on NRM (1-year NRM, 27% [95% CI, 8%-51%] versus 0%; $P < .05$); however, none of other variables, including patient age, had a significant impact on NRM, disease relapse, OS, and DFS.

DISCUSSION

The results of this prospective multicenter study demonstrate that single-unit CBT following myeloablative conditioning can provide favorable survival with low NRM in adult patients with high-risk hematologic malignancies. Our study is unique in that all patients received uniform conditioning, GVHD prophylaxis, and other supportive care. All of the CBT procedures applied in this study were identical to those reported by the IMSUT. In the IMSUT reports, the outcome of CBT was superior to that of allogeneic BMT or PBSCT from related and unrelated donors, which provided a notably favorable outcome compared with other studies [7,8]. Given the nature of the retrospective single-center study, we deemed it necessary to confirm the reproducibility of the results, and consequently planned and conducted a prospective multicenter study to evaluate the safety and efficacy of the IMSUT transplantation procedures. Of note, our study enrolled patients with high-risk hematologic malignancies regardless of disease status at transplantation, and indeed half of the patients enrolled were not in remission at the time of transplantation. Despite our use of such a high-risk cohort, the primary endpoint of 1-year NRM was 15%, which was comparable with the 9% reported by IMSUT and lower than those of other studies (30%~60%) [4-6,13-15]. The primary cause of NRM in the present study was infectious complications occurring after engraftment. All of those infectious complications occurred in patients who developed grade II-IV acute GVHD, and the sole factor significantly associated with the incidence of NRM was grade II-IV acute GVHD. Thus, to further reduce NRM, the optimal management of infectious complications should be explored, particularly in patients developing acute GVHD after CBT.

Disease relapse greatly interferes with the success of allogeneic HSCT, especially in patients with chemorefractory hematologic malignancies, and the intensity of conditioning plays a crucial role. Although TBI plus CY (TBI-CY) remains the most common myeloablative conditioning regimen for allogeneic HSCT, further intensification of conditioning by administering additional antileukemic agents has been attempted in an effort to reduce disease relapse. Although this further intensification can lead to more effective disease control, the benefit is generally offset by the higher rates of NRM, and thus the effect of this approach on survival remains controversial [16]. Cytarabine has been extensively investigated as an additional agent in this setting, but this drug is also associated with significant toxicity [17-22]. However, in a recent study we found favorable outcomes with low NRM with the use of TBI-CY plus cytarabine as conditioning in patients with ALL [9]. Based on these findings and the fact that the IMSUT protocol uses mainly TBI-CY plus cytarabine as conditioning for CBT [7,8], we followed the same regimen. In addition, we combined cytarabine with G-CSF infusion in patients with myeloid malignancies, based on the hypothesis that G-CSF increases the susceptibility of myeloid leukemic cells to cytarabine, thereby contributing to decreased relapse rate [23-30]. In the setting of allogeneic BMT and PBSCT, favorable outcomes of the conditioning consisting of TBI and G-CSF with cytarabine in patients with AML and advanced MDS have been reported [31-34]. In the present study, despite the high-risk features of the disease, approximately half of the patients achieved long-term DFS with this unique intensified myeloablative conditioning because of the low NRM and disease relapse, as expected, which seemed more favorable than the results of previous studies [5,14]. These results suggest that the myeloablative conditioning regimen used in the present study is safe and highly effective in eradicating leukemic cells even in patients with high-risk leukemia. However, the outcomes of our study patients seem inferior to those reported by IMSUT in terms of disease relapse and survival. The major difference is in the incidence of disease relapse, which was higher in the present study (40% versus 16%~17%) and consequently had an effect on DFS (42% versus 70%~74%). The most plausible explanation for this difference is the differing study designs (prospective multicenter versus retrospective single-center). Another possible explanation may be related to demographic differences in the patient cohorts. The standard variables, such as age and disease status at transplantation, seemed similar in the 2 studies. However, BMT from unrelated donors takes priority over CBT in clinical practice in all of the institutions participating in the present study. Thus, it is possible that high-risk patients, particularly those with diseases in CR, might be selectively enrolled into the present study based on the inclusion criteria defining the requirement of immediate HSCT based on disease features at the discretion of each treating physician. Disease status at transplantation cannot precisely reflect the risk of such features of the disease and could have significantly affected the outcome.

Along with conditioning, GVHD prophylaxis plays an important role in the outcomes of allogeneic HSCT. However, a standard GVHD prophylaxis regimen for CBT has yet to be established, and GVHD prophylaxis varies widely among previous studies, variously consisting of CsA or tacrolimus alone or in combination with MTX, glucocorticoids, mycofenolate mofetil (MMF), and/or antithymocyte globulin [2-6,13-15,35]. To avoid the toxicity and negative effects of MTX on hematopoietic reconstitution, several studies have applied non–MTX-containing GVHD prophylaxis. In contrast, CsA in combination with short-term MTX, which remains a common GVHD prophylaxis regimen in allogeneic HSCTs other than CBT, was used in the present study precisely according to the IMSUT protocol. Leucovorin was routinely given to ameliorate the toxicity of MTX. In addition, CsA was given over 10 hours at a dose of 3 mg/kg/day, with the dose adjusted only when the CsA trough level was <100 ng/mL or adverse events associated with CsA developed. With this relatively unique regimen, neutrophil engraftment was obtained at a median of 26 days after CBT, which is comparable with those in previous studies. The incidence of grade II-IV acute GVHD of 48% is identical to that reported by IMSUT (44%~52%) [7,8]. Of note, however, acute GVHD was manageable without systemic glucocorticoid administration in approximately 60% of patients with grade II acute GVHD, suggesting that the incidence of clinically

significant acute GVHD requiring systemic glucocorticoid therapy was actually lower with this prophylactic regimen. In addition, the development of acute GVHD requiring systemic glucocorticoid therapy was associated with NRM due to fatal infectious complications. Therefore, further investigations should focus on the more effective GVHD prophylaxis, although the balance between the immunosuppressive effect and graft-versus-tumor effect is also important. In this setting, less-toxic GVHD prophylaxis using MMF instead of MTX could be a good option, because it has a potential to allow faster engraftment and could decrease the risk of infectious complications.

Secondary graft failure due to graft rejection was observed in 1 patient, who was rescued by the second CBT and alive at 26 months after the second CBT. Other complications interfering with survival and quality of life, such as severe chronic GVHD and secondary malignancies, were seen in no patients during the follow-up period. All surviving patients were in good clinical condition without disease recurrence. These results demonstrate the long-term safety and tolerability of these CBT procedures despite the highly intense conditioning.

Two major limitations of the present study are the small number of patients evaluated and the heterogeneous disease background in these patients, including the types of disease, high-risk features, and disease status at transplantation. However, the study's primary endpoint was to evaluate 1-year NRM with respect to safety of the transplantation procedures. Thus, we believe that safety of the procedures can be confirmed even with our relatively small, heterogeneous cohort.

We conclude that the transplantation methods used in the present study, including intensified myeloablative conditioning, CsA with MTX as GVHD prophylaxis, and other supportive care measures, can provide low NRM and high survival rates in patients undergoing single-unit CBT for hematologic malignancies and could possibly become a standard treatment. However, because of the limited number of patients and high incidence of life-threatening infectious complications associated with GVHD, a larger-scale study with some modifications to GVHD prophylaxis is needed to establish the optimal CBT techniques.

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ORIGINAL ARTICLE

B-cell function after unrelated umbilical cord blood transplantation using a minimal-intensity conditioning regimen in patients with X-SCID

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Abstract Patients with X-linked severe combined immunodeficiency (X-SCID) suffer from severe and persistent infections, and usually die early in life unless treated by hematopoietic stem cell transplantation. If a patient has an HLA-identical sibling donor, preparative conditioning is not necessary for T-cell engraftment and B-cell function. However, in the absence of such a donor, long-term reconstitution of full B-cell function is often problematic, leading in many cases to a lifetime requirement for immunoglobulin replacement therapy. Preparative myeloablative conditioning has been shown to improve long-term B-cell function, but may aggravate pre-existing infection and transplant-related toxicity. It is thus important to determine the minimum intensity of conditioning that assures immunoglobulin production. In the present study, we performed reduced-intensity conditioning (RIC), consisting of fludarabine 125 mg/m^2 and melphalan 80 mg/m^2, prior to unrelated umbilical cord blood transplantation (UCBT) for five patients with X-SCID, none of them had an HLA-identical donor. Four patients survived more than 4 years without sequelae, and none required long-term immunoglobulin replacement therapy. One patient succumbed to sepsis in conjunction with severe GVHD. Our result demonstrates that the RIC regimen described above in combination with UCBT is an effective and less toxic conditioning to correct B-cell function in patients with X-SCID.

Keywords X-SCID · Reduced-intensity conditioning · Umbilical cord blood transplantation · Fludarabine/melphalan

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Introduction

X-linked severe combined immunodeficiency (X-SCID), which accounts for approximately half the cases of SCID, is caused by mutations of the γc chain. Immunological characteristics of this disease include profound impairment of both cellular and humoral immunity due to the absence or diminished numbers of T cells and natural killer (NK) cells, and abnormal B-cell function in spite of normal or elevated numbers of B cells. Therefore, patients with X-SCID suffer from severe and persistent infections, including opportunistic pathogens, and usually die early in life unless treated by hematopoietic stem cell transplantation (HSCT) or gene therapy [1]. Previous reports demonstrated excellent results of HLA-identical BMT with

survival rate over 90 %, and full restoration of T- and B-cell function [2, 3]. Since most patients do not have an HLA-identical sibling donor, HLA-haploidentical bone marrow transplantation (BMT) was developed in the early 1980s, when better T-cell depletion methods became available. However, the survival rate was lower, at around 60–80 %, and about half of the patients required life-long immunoglobulin replacement therapy despite normal T-cell immunity with or without pre-transplant conditioning [2–4]. These results suggested that T-cell-depleted, HLA-haploidentical bone marrow cells might not be a suitable source of HSCT for correcting B-cell function. Another strategy to treat this condition is to use unrelated donor HSCT with conventional myeloablative conditioning regimens, which leads to stable reconstitution of T- and B-cell function [5]. However, this approach has been associated with significant treatment-related toxicities and aggravation of pre-existing infections. To avoid these problems, reduced-intensity conditioning (RIC) regimens have been developed. Recently, Rao et al. [6] reported that a RIC regimen using a total dose of 150 mg/m^2 of fludarabine, 140 mg/m^2 of melphalan, and Campath 1H or ATG resulted in an improved survival and reduced transplantation-related mortality, compared with myeloablative conditioning, in children with primary immunodeficiency (PID) undergoing unrelated BMT. They used the same regimen for patients with X-SCID who would be expected to need less intensive conditioning because their immune system is already profoundly impaired. Based on their report, we performed unrelated umbilical cord blood transplantation (UCBT) with pre-transplant conditioning using a further reduced dosage of fludarabine and melphalan, in the absence of Campath 1H or ATG, to investigate whether a minimally intensive conditioning regimen could assure correction of B-cell function in X-SCID patients.

Patients and methods

Patients

Five patients with typical X-SCID received unrelated UCBT because they had no HLA-matched sibling donor. As shown in Table 1, mutations in the γc chain gene were detected in all patients. Patient 3 suffered from pneumonia caused by *Pneumocystis jiroveci* at the time of diagnosis of X-SCID. All patients except for patient 3 were diagnosed with X-SCID at birth because their brothers had the same disease. Immunoglobulin replacement therapy was initiated once hypogammaglobulinemia was confirmed, and IgG trough levels were maintained over 500 mg/dL. This study was performed with the approval of Institutional Review Board at each university and with the written informed consents of the parents.

Conditioning regimen and GVHD prophylaxis

Pre-transplant conditioning for all patients consisted of fludarabine (25 mg/m^2 per day) from day −7 to day −3

Table 1 Patient characteristics

Patient	1	2	3	4	5
Age at diagnosis (months)	0	0	4	0	0
Age at UCBT (months)	3	3	10	3	3
Mutations in the γc chain	868 G > A	691 G > A	c.735 741*	IVS4 + 2 T > A	568A > G
HLA identity	6/6	5/6	6/6	5/6	5/6
Nucleated cell dose (×10^7/kg)	7.1	10.0	5.0	9.5	11.2
CD34+ cell dose (×10^5/kg)	1.09	3.65	ND	3.50	1.68
Hematological recovery					
Nt > 500/μL	30	20	36	20	12
Plt > 5 × 10^4/μL	10	16	95	17	16
Ret > 1 %	18	16	38	20	15
Complications at UCBT	None	None	Pneumonia	None	None
Additional infections during UCBT	None	None	Sepsis	CMV	Sepsis
GVHD					
Prophylaxis	CyA	FK + sMTX	FK	FK + sMTX	CyA + PSL
Acute (grade)	0	0	II	II	III
Chronic	–	–	Extensive	–	Extensive
Therapy	–	–	FK + mPSL	FK + PSL	FK + MMF + PSL

*c.735 741** c.735_741delAGCCACC→insGGGAGCAATACTT, *ND* not determined, *Nt* neutrophils, *Plt* platelets, *Ret* reticulocytes, *sMTX* short-term methotrexate

(total dose 125 mg/m^2) and melphalan (40 mg/m^2 per day) from day −4 to day −3 (total dose 80 mg/m^2). Neither ATG nor Campath 1H was included in the conditioning regimen.

Prophylaxis for acute GVHD was performed with either cyclosporine A (CyA) with/without prednisolone or FK506 with/without short-term methotrexate as shown in Table 1.

Graft characteristics

As shown in Table 1, UCB units were either serologically full-matched or one locus mismatched at 6/6 (A, B, DR) HLA loci. Infused nucleated cell doses were 5.0 × 10^7/kg–11.2 × 10^7/kg (mean 8.6 × 10^7/kg), which contained CD34+ stem cells, ranging from 1.09 × 10^5/kg to 3.65 x 10^5/kg (mean 2.48 × 10^5/kg) except for patient 3, whose information on CD34+ cells was not available.

Chimerism studies

Hematological recovery was defined as achievement of absolute neutrophil count (ANC) >500/μL for 3 consecutive days and a platelet count >5.0 × 10^4/μL for 7 consecutive days without need for further transfusion. Chimerism was analyzed at Human Leukocyte Antigen Laboratory (Kyoto, Japan) as described previously [7]. Briefly, T cells, B cells and NK cells were separated by anti-CD3, anti-CD19 and anti-CD56 microbeads (Invitrogen Dyanl AS, Oslo, Norway), respectively. Donor- and recipient-specific polymorphic short tandem repeats (STR) were amplified by PCR, and subsequently analyzed by SDS-PAGE.

Immunological reconstitution studies

Immunological reconstitution status after transplantation was monitored by serum immunoglobulin levels (IgG, IgA, IgM and IgE), isohemagglutinin, and specific antibodies, and by flow cytometry analyses of peripheral mononuclear cells for CD3, CD4, CD8, CD19, CD16 and CD56.

Results

The age at transplantation was 3 months in four patients and 10 months in one patient (Table 1). All patients received UCBT using fludarabine (125 mg/m^2) and melphalan (80 mg/m^2) as a pre-transplant conditioning. They all achieved engraftment of ANC > 500 μL and platelets > 5.0 × 10^4/μL at a mean of 23.6 days (range 12–36 days) and 30.8 days (range 10–95 days), respectively. All but one survived more than 4 years without complication. One patient, patient 5, succumbed to sepsis in conjunction with severe GVHD.

Infections

Patient 3 suffered pneumonia due to *P. jiroveci* infection prior to admission and intravenous trimethoprim/sulfamethoxazole therapy was initiated. The pneumonia resolved with the engraftment of donor cells. He also experienced an episode of sepsis due to enterococci after UCBT, which was cured by appropriate antibiotics. Patients 1, 2, 4, and 5 were diagnosed with X-SCID at birth by sequencing of the γc chain because their brothers had the same disease. They had been protected in a clean environment soon after birth and they did not experience any infection until UCBT. Patient 5 developed sepsis due to a catheter infection, which was the cause of death at day 491 after UCBT.

Regimen-related toxicity and GVHD

Mild mucositis and myelosuppression were observed with this reduced-intensity conditioning, and no other regimen-related toxicity was noted.

Patient 3 developed acute GVHD grade II (skin stage 3) and extensive chronic GVHD, while patient 4 developed acute GVHD grade II (skin stage 3, liver stage 1 and gut stage 1). Symptoms in both cases resolved on prednisolone and FK506. Patient 5 developed acute GVHD grade III (skin grade 1, liver grade 3 and gut stage 3), followed by extensive chronic GVHD. He succumbed to sepsis in conjunction with uncontrolled GVHD, although he was treated with prednisolone, FK506 and mycophenolate mofetil (MMF).

Chimerism

Median follow-up was 68 months (range 48–73 months). As shown in Table 2, all survivors had complete donor T-cell chimerism. One survivor, patient 3, also had complete lymphocyte and granulocyte chimerism, which was confirmed by day 52. The others demonstrated mixed chimerism in these cell lineages. The percentage of the donor cells in each cell lineage had been stable since day 168 after UCBT in patient 1. In patients 2 and 4, detailed chimerism using fractionated cells was analyzed only the date indicated in Table 2. Donor cells of patient 5 constituted only 5 % of his peripheral blood nucleated cells at day 420 after UCBT, although T cells were 100 % of donor origin.

Immune reconstitution

Table 3 shows the results of immunologic evaluation at the most recent follow-up after UBCT in all survivors. Absolute numbers of lymphocytes were normal after

Table 2 Leukocyte chimerism

Patient	1		2		3		4		5
Days after UCBT[a]	168	1620	60	1098	52	2021	90	2078	420
T cell (donor %)	100	>95	90	94		100	100	92	100
B cell (donor %)	24	20	20	8		100	70	50	ND
NK cell (donor %)	55	69	15	33		100	90	84	ND
Granulocyte (donor %)	65	59	18	48	>95	100	20	13	ND
Lymphocyte (donor %)					>95				

ND not determined

[a] Days after UCBT when chimerism was determined

Table 3 Immune reconstitution

Patient	1	2	3	4	5 (at day 470)
WBC (/μL)	7700	7400	4710	9200	1000
Lymphocyte (/μL)	3700	4370	4120	4100	400
CD3 (%)	82.2	60.4	63.7	81.3	38.6
CD4 (%)	48.4	24.4	35.3	42.0	33.0
CD8 (%)	27.8	28.7	25.7	32.2	11.5
CD19 (%)	13.4	37.9	32.0	15.1	0.0
CD16/56 (%)	1.8	0.6	4.0	2.7	13.0
B-cell function					
IgG (mg/dL)	937	531	692	1157	660 (under i.v.Ig)
IgA (mg/dL)	58	32	55	101	89
IgM (mg/dL)	117	77	115	231	112
IgE (IU/mL)	37	<3	4.2	1	ND
Isohemagglutinin	+	+	−	+	−
Specific antibody	−	+	+	+	ND
T-cell function					
PHA stimulation (SI)	164.4	243.6	220.9	1213.4	1.1
ConA stimulation (SI)	897.7	322.1	225.5	713.1	1.1
NK activity (%)	15	4	10	19	ND

Normal values; PHA stimulation (SI) >100, ConA stimulation (SI) >75, NK activity 18–40 %

ND not determined, *SI* stimulation index

transplantation (Table 3). Numbers of CD3+, CD4+, CD8+ T cells and CD19+ B cells were within normal ranges, and T-cell function was normal by assessment with PHA and ConA stimulation. Immunoglobulin serum levels were within normal ranges of age-matched controls in all four patients, and none requires IgG substitution (Tables 3, 4). Also each patient had a positive antibody response. NK activity was lower than normal in all but patient 4.

Growth and psychomotor development

As shown in Table 4, all survivors have shown normal height, body mass index (BMI), psychomotor development and performance status to date.

Discussion

We report the outcome of unrelated UCBT in five patients with X-SCID using a RIC regimen. The most important result of this study is all four survivors are free from immunoglobulin replacement therapy.

Previous studies showed that about two-thirds of SCID patients required immunoglobulin replacement therapy after T-cell-depleted, HLA-haploidentical BMT from related donors without pre-transplant conditioning [2, 8]. In Europe, about half of SCID patients who received HLA-haploidentical related marrow cells were conditioned mostly with busulfan (8 mg/kg) and cyclophosphamide (200 mg/kg) [3]. However, the mortality rate for this type of conditioning was higher than that of patients without conditioning. Further, pre-transplant conditioning in combination with HLA-haploidentical related marrow cells did not always result in correction of B-cell function, and about one-third of the SCID patients continue to require immunoglobulin replacement therapy. In contrast, all surviving SCID patients, who had received bone marrow cells from unrelated donors after conventional conditioning with busulfan (16 mg/kg) and cyclophosphamide (200 mg/kg), did not require immunoglobulin replacement therapy [5, 6]. This conventional conditioning regimen, however, has been associated with a significant mortality rate due to treatment-related toxicities such as profound pancytopenia, severe organ toxicity, and exacerbation of pre-existing infections. In addition, children treated with myeloablative regimens often suffer from delayed effects such as infertility, hormonal dysfunction, growth failure and secondary malignancies [9]. Recently, Rao et al. [6] reported the outcome of 33 patients with primary immunodeficiency (PID) [SCID ($n = 6$) and non-SCID ($n = 27$)] who received unmodified unrelated donor marrow grafts following reduced-intensity conditioning consisting of fludarabine (150 mg/m^2), melphalan (140 mg/m^2), and alemtuzumab (Campath 1H) or anti-thymocyte globulin (ATG). All patients had primary engraftment, and most patients achieved normal immunoglobulin production and B-cell function, although it is not clear whether patients with SCID were on immunoglobulin replacement therapy or not. From these

Table 4 Current status

Patient	1	2	3	4	5
Clinical status	Alive	Alive	Alive	Alive	Dead (at 17 months)
Follow-up (months)	68	48	73	69	17 months
Last i.v.Ig (months)	44	32	8	3	17 months
i.v.Ig at present	Off	Off	Off	Off	NA
Height	−1.0 SD	+1.92 SD	−1.0 SD	−0.2 SD	Short stature
Body mass index	15.9	14.5	14.5	15.2	BW 6 kg
Mental status	Normal	Normal	Normal	Normal	Normal
Karnofsky performance status	100 %	100 %	100 %	100 %	30 %

i.v.Ig intravenous immunoglobulin, *NA* not applicable, *SD* standard deviation, *BW* body weight

results, we speculated that T-cell depletion might interfere with B-cell engraftment and function. In this context, it is interesting to note that patients in our study who had acute GVHD complications showed higher B-cell chimerism and early immunoglobulin production after UCBT. However, one of our patients succumbed to sepsis in conjunction with severe GVHD. Unlike patients with hematologic malignancies, who benefit from the graft-vs-leukemia effect of donor cells, there is no such benefit from GVHD in patients with PID [10]. Thus, it is inevitable to use immunosuppressive drugs to prevent GVHD, and modifications such as the addition of ATG to our protocol to reduce the risk of GVHD will need to be evaluated in a future study [11]. Of note, two of our patients who did not develop acute GVHD gradually corrected their B-cell function, and immunoglobulin replacement therapy could be discontinued 32 and 44 months after UCBT. These results suggest that the RIC regimen described here may provide a minimal-intensity conditioning regimen in combination with UCB, which can assure sufficient production of immunoglobulin.

Some reports have raised concern about cardiac toxicity associated with high-dose melphalan and fludarabine used in combination [12, 13]. However, patients with this adverse event had been suffering from advanced hematologic malignancies and had been heavily treated with cytotoxic drugs including anthracyclines prior to pre-transplantation conditioning, and the total dosage of fludarabine (150 mg/m^2) and melphalan (140 mg/m^2) used for conditioning was much higher than the present study. In addition, reduction of melphalan from 140 to 80 mg/m^2 is expected to result in a lower frequency of cardiac toxicity. We only observed mild myelosuppression and mucositis as adverse events of the RIC regimen. Engraftment of unrelated cord blood cells, which might not be achieved with lower concentration of melphalan, was observed in all patients in our study. To date, none of our patients has shown any delay in growth or mental development. Long-term follow-up is necessary to validate the efficacy and safety of this RIC regimen.

Regarding B-cell engraftment and function, T-cell depletion from related donor bone marrow cells may not be a suitable source of HSCT for PID patients who do not have an HLA-identical sibling donor as described above. Recently, it was reported that UCB from unrelated donors could be used successfully for patients with PID [14, 15]. As UCB contains T cells, faster emergence of donor T cells is expected even though the infused T cells are functionally naïve. UCB recipients were able to discontinue immunoglobulin replacement therapy sooner and more frequently compared with T-cell-depleted bone marrow recipients although the estimated 5-year over all survival rates were comparable when UCB recipients received a myeloablative conditioning regimen [15]. In addition, UCBT is more tolerant of HLA disparity because the incidence and severity of GVHD is lower than for unrelated BMT. These results together with ours support the application of UCBT for patients with X-SCID who do not have an HLA-identical sibling donor.

Another risk factor for a poor outcome using HSCT for SCID is a pre-existing infection [8]. In our patients, all but one were diagnosed with X-SCID at birth from their family histories, and they had been kept in a protective environment for 3 months until they received UCBT. There are two reasons why we performed UCBT at the age of 3 months. One is to minimize regimen-related toxicities because infants are more susceptible to cytotoxic drugs, and the other is to expect higher survival rate after transplantation in the first 3.5 months of life as described previously [2, 16]. Early diagnosis before any infectious episodes is necessary for safe HSCT in the patients with SCID. Recently, screening of newborns for SCID has been recommended [17], and the RIC regimen described above in combination with UCBT is an alternative to HLA-haploidentical BMT for such patients.

In conclusion, our regimen in combination with UCBT is well tolerated and resulted in normal immunoglobulin production and B-cell function in our patients. Future studies with a modification of GVHD prophylaxis for patients with X-SCID who do not have an HLA-matched

sibling donor will be needed to further improve the outcome.

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Conflict of interest The authors declare no conflict of interest.

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【第54回日本小児血液・がん学会学術集会】シンポジウム5：細胞治療（腫瘍免疫・感染免疫・移植免疫）

移植後ウイルス感染に対する多ウイルス特異的CTL療法

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要　旨

造血細胞移植や臓器移植の成功率は免疫抑制薬の進歩などにより次第に向上しているが，一方移植後の日和見感染症は大きな問題となっている．ウイルス感染症は生存率，生着不全に関与するのみならず，有効薬剤の欠如，薬剤耐性誘導，長期使用による費用負担の増大などの問題も内包している．薬剤に頼らず，拒絶やGVHDを誘導しない特異的細胞治療の導入は喫緊の課題である．古典的なウイルス特異的T細胞治療は有効性が検証されているが，臨床応用にあたりいくつかの解決すべき問題点がある．1つは抗原提示にあたりウイルスそのもの，あるいはプラスミドを用いる点である．安全性の点から可能であれば使用を控えたい．2点目は作成作業の煩雑さである．高度な技術を必要とし，手順も多い．3つは作成に要する時間である．EBV特異的T細胞の作成には最低6–8週以上必要とされている．さらに現時点では特定のHLAに拘束されたT細胞を作成する方法が主流であり，すべてのHLAはカバーできない．また特異的CD4T細胞の作成は難しいという問題もある．これらを克服するために，ウイルス抗原の主要エピトープをカバーする15merの（11アミノ酸ずつ重なる）ペプチドをパルスし，最適化したサイトカインと共に培養し，2週間で特異的T細胞を作成する手法が開発された．この方法を用いれば多ウイルス多抗原特異的T細胞を短期間に効率的に作成することが可能である．すでに臨床応用され，第三者からの特異的T細胞輸注の形で検証が始まっている．

キーワード：ウイルス特異的T細胞，オーバーラッピングペプチド，造血幹細胞移植，アロ反応性
Key words: virus-specific T cells, overlapping peptides, hematopoietic stem cell transplantation, allo-reactivity

I　はじめに

造血細胞移植（hematopoietic stem cell transplantation: HSCT）後のウイルス感染症は未だに大きな問題であり，移植後の様々な時期に，EBV関連リンパ増殖症候群，CMV感染症，出血性膀胱炎（BKウイルス（BKV）/アデノウイルス（AdV）），HHV6脳炎，内臓播種性VZV感染症，RSV感染症，Parainfluenza感染症など種々な感染症が発症する．

ウイルス感染症への対応に当たっては，モニタリング，適切な抗ウイルス薬の使用に加えて，（特異的・非特異的）免疫学的再構築の促進が重要である．

II　移植後ウイルス特異的T細胞治療の歴史

1. 非特異的T細胞治療

移植後ウイルス感染症の中で，早くから注目を集めていたものとしてEBV関連リンパ増殖症候群（EBV-associated lymphoproliferative disorder: EBV-LPD）が挙げられる．EBV-LPDでは免疫抑制薬の中止あるいはドナーリンパ球輸注が選択され，免疫能の増強によりウイルス感染細胞を排除できることが明らかとなっていた[1]．CMV感染症，AdV感染症，RSウイルス感染症などのウイルス感染症も，ドナーリンパ球輸注によって改善する可能性がある．これはおそらく，ドナーリンパ球中の特異的T細胞によるものである以上に，免疫系の全般的な賦活化による間接的な作用が重要と思われる．

2. ウイルス特異的CD8T細胞治療（*in vitro* expansion）

1）単ウイルス特異的T細胞調製

ウイルス特異的細胞傷害性T細胞（CTL）療法の歴史は比較的古い．1992年にRiddellらはCMV感染症に対して特異的T細胞を用い，効果を認めたと報告した[2]．彼らは骨髄移植ドナーの線維芽細胞をCMVで感染させ，末梢血単核球とIL-2存在下で共培養し，さらに反復刺激を行うことで特異的CTLを樹立している．さらに1995年にRooneyらはEBV特異的T細胞をHSCT前に予め作成しておき，EBV再活性化を認めた時点で投与したところ効果を認めたと報告している[3]．ここではドナーから先にEBVを感染させたリンパ芽球様リンパ球を作成しておき，ドナー単核球とIL-2存在下で共培養することによって特異的CD4/CD8

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細胞を得ている．これらの単純な共刺激系を用いたウイルス特異的T細胞では，特異的細胞傷害活性はE:T比20:1で最大70%程度であったが，10^7程度の細胞でも効果を示し，また明らかなGVHDなどの有害事象を認めなかった．

その後開発された単ウイルス特異的T細胞療法では，さらに特異性を高くし，より増殖高率を高める工夫が加えられている．例えばEBV特異的T細胞調製では，まずEBV-LCLにLMP2発現ベクターを導入し，LMP高発現EBV-LCLを作成しておく．さらに樹状細胞を分化・調製して同様にベクターを導入してLMP2を発現させ，それを末梢血単核球と共培養する．さらに予め用意したLMP2発現EBV-LCLで刺激を加え，IL-2存在下で培養するなどの方法がそれに当たる[4]．明らかに手順が多く，最低12週以上の時間を必要とするが，十分量の特異性の高いT細胞が得られる点が最大の特徴である．有効性と安全性も検証されている．

2）多ウイルス特異的T細胞調製

造血細胞移植後には様々なウイルス感染症に罹患する危険性がある．感染症の種類が明らかになってから特異的T細胞調製に当たる場合には，調製までの時間が死活的に重要となる．10週前後の時間は許容されないことも多い．さらに予め特異的T細胞を作成していても，異なるウイルスによる感染に罹患することも想定される．従って適切な時期に有事対応するためには，予めいくつかのウイルスを標的にしたT細胞を作成しておく方策が妥当と考えられる．

実際にこの手法は既に臨床応用されている．例えばアデノウイルスベクターでCMV抗原を発現させたEBV-LCLを用いて刺激を行うことにより，AdV，CMV，EBVの3者に特異的なT細胞を作成することが可能である．当初は移植後ウイルス感染症に対して用いられていたが，近年では感染症予防にも用いられるようになり，効果を上げている[5,6]．

予防的投与に際して重要なことは，effector memory T細胞のみならずcentral memory T細胞を用意しておくこと，およびできれば特異的CD4T細胞を調製しておくことである．Central memory T細胞の輸注により長期生存可能なT細胞が供給され，また特異的CD4T細胞は，*in vivo*でウイルスのチャレンジを受けた際に抗原提示細胞から指令を受け，特異的CD8T細胞の分化や増殖に関与する．

3）古典的ウイルス特異的T細胞調製の問題点

上記の方法で調製されたT細胞の主体はCD8 T細胞であるが，CD4 T細胞も様々な割合で混在する．この方法の臨床応用上の（広く臨床に展開する上での）問題点は抗原を提示するためにウイルスあるいは発現ベクターを用いることであろう．品質保証や安全管理上，これらの非自己産物は安定した特性を示す必要があり，また一方最終産物でのウイルスや発現ベクターの混在は否定される必要がある．また調製に時間を要すること，専門性の高い調製技能が必要であること，などの問題も存在する．

3. ウイルス特異的T細胞治療（ドナー由来特異的T細胞直接採取）[7,8]

上に挙げたような*in vitro*分化・増幅古典的ウイルス特異的T細胞の欠点を補うために，ドナーに内在する特異的T細胞を採取して，投与する試みがなされている．抗原提示にあたり取り込まれた抗原はproteaseで分解を受け，MHC class IIには10～30アミノ酸長（多くは15前後）の，MHC class Iには8～12アミノ酸長（多くは9）のペプチドとなり，それぞれのMHC分子の溝にはまって提示される．Class I MHCはCD8 T細胞に，Class II MHCはCD4 T細胞に抗原提示することは周知の通りである．この知見を応用して，実際のウイルスで抗原となる部位の全ての領域を11アミノ酸ずつoverlapする15-merのペプチドとして用意し，それを末梢血単核球（リンパ球，単球，樹状細胞などを含む）にパルスして，IFN-γを産生するものを磁気ビーズで回収する手法がある．Class I，class IIが提示するdominantなペプチドはMHCにより異なる（例えばA24とA11では異なったペプチドを主としてはめ込んでいる）が，この「オーバーラップペプチド」を用いれば，ドナーのHLAタイプに関わらず特異的T細胞を集めてくることが可能である．さらにCD4，CD8 T細胞を共に採取できることが利点である．実際にHSCT後の感染症の場で用いられており，10^5レベルの細胞数でも有効である．現時点ではEBV（LMP1，LMP2，EBNA1，BZLF1），CMV（IE1，pp65），AdV（hexon，penton）などが臨床応用され，さらにBKV，JCV，Influenza B，Papilloma virusなどに対するペプチド混合物も用意されている．

本治療法の最大の利点は増幅が不要，従って調製当日に投与が可能，という点であり十分な有効性が検証されている．投与数も少なく有害事象も少ない．問題点としては特異的T細胞の%が低いものが多く（特にAdV），多くの場合は有効な細胞数を確保するためにapheresisを必要とすることである．例えばIE-1特異的T細胞が全リンパ球中0.5%あったとした場合，2,000/μLのリンパ球があるドナーでは1×10^4/mLの特異的T細胞が存在することになり，5×10^5個投与を希望する場合50%回収できたとしても100 mLの血液を必要とする．さらに1抗原ごとに費用が発生するため大変高価な治療手段となってしまう．

4. ペプチド＋サイトカインによる多ウイルス特異的T細胞治療

以上のような経緯を経て，古典的ウイルス特異的T細胞調製，ドナー由来特異的T細胞直接採取の両者の利点を生かし，問題点を克服するような細胞調製法がAnn Leenら

表1　特異的T細胞治療の対象となるウイルスとT細胞の標的となる抗原

Virus	Antigen
CMV	IE-1, pp65
EBV	EBNA1, LMP2, BZLF1
Adenovirus	Hexon, Penton
BK virus	LT, VP-1
Influenza	MP1, NP1
RSV	N, F
HHV6	U14, U90

Abbreviations: CMV, cytomegalovirus; EBV, Epstein-Barr virus; RSV, respiratory syncytial virus

により開発された[9]．私たちの施設でも2012年より同手法を用いて基礎的検討に入っている．

1）調製方法と調製細胞の特性

調製方法は極めて簡便である．末梢血から単核球を分離し，表1に記載するペプチドにより100 ng/peptide/15×10^6 PBMCsで30–60分刺激し，さらにIL-4，IL-7の存在下で培養することにより，特異的T細胞を増幅する（図1）．特異的T細胞は12日間にて約10–200倍に増幅することが可能である．調製した細胞は約95%以上がCD3陽性で，CD4/CD8はほぼ開始時の比率を維持し，CD45RO陽性比率には変化なく，またCD62L陽性のcentral memory細胞も豊富に含まれている．一方B，NK細胞はほぼ混在しない状態になっている．単刺激でも複数刺激でも同程度の増幅が可能なことは重要である．現時点ではEBV: BZLF1，EBNA1，LMP2，CMV: IE1，pp65，AdV: Penton，Hexonの3ウイルス，7抗原を用いて最終的に調製された細胞における特異的T細胞は35%を超えるものとなっている．各特異的T細胞の増幅はペプチド刺激後の細胞内IFN-γ陽性細胞比率に加えて，ELISPOTアッセイにても確認されている（図2，3）．一方Ann Leenらの報告では7ウイルス，15抗原刺激では最終産物の特異的T細胞は98%以上である．

これらの細胞はさらに，ペプチドパルスした自己由来単核球あるいはPHA刺激単核球＋サイトカイン（IL-4，IL-7，IL-15など）で増幅可能である．調製した特異的T細胞を用いて自己EBV-LCLに対する細胞傷害活性を検討したところ，E:T比2:1で53%という高値を示した．現在それぞれのHLAに合致した既知のペプチドに対して反応を示すかどうかを解析しているところであるが，A2402を有するドナーでは特定のペプチド断片に反応するA2402拘束性T細胞が検出される．

用いるサイトカインはIL-4+IL-7であり，多くの方は違和感をもたれると思われる．Ann Leenらにより様々な組み合わせとdoseが検証された結果の最適化されたものである．IL-2は増幅には効果的であるがNK細胞や非特異的な細胞の増幅などが問題になる．初期の段階での特異的T細胞の誘導においてIL-15はあまり効果的ではない（一方濃縮後の二次増殖では効果を発揮する）．

2）臨床応用に向けての検証と課題

さらに私たちは本調製方法の臨床応用に向けて，無血清化と（EBV以外の）ウイルス特異的T細胞による細胞傷害活性検証系開発を試みている．M社などの無血清培地は増幅を指標とした場合，現行のAIM-5+ヒト血清と遜色はない．今後は難治疾患研究所ウイルス治療学分野の清水則夫博士などが開発する新規無血清培地を含め，細胞亜群や細胞傷害活性の差異などについても検証が進められる．小児においては最小限の侵襲という意味で自己血清を回避する手段が必要である．反復投与の可能性がある免疫細胞治療においては，異種血清，同種血清ともに回避できる方法が好ましい．

細胞傷害活性測定もEBV-LCLを用いることのできるEBV特異的キラー活性測定以外は比較的難しい．現在はペプチドをパルスした自己の単核球を^{51}Crラベルし，通常%^{51}Cr releaseの形で活性を測定する場合が多い．CMVに関しては間葉系幹細胞に感染させることが可能であるが，真の標準標的細胞作成にはまだ工夫を必要とする．さらに非アイソトープ化も現時的な問題である．私達は標的細胞をCell

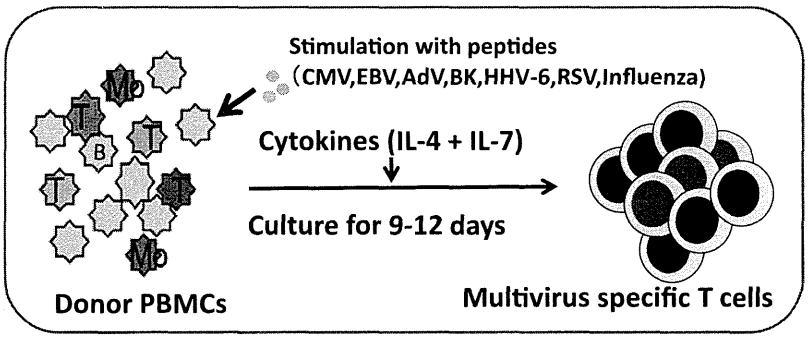

図1　多ウイルス特異的T細胞の作成法の模式図．ドナー末梢血単核球（PBMCs）を各ウイルス抗原（ペプチドミクスチャー）で刺激し，さらにIL-4，IL-7と共に培養する．

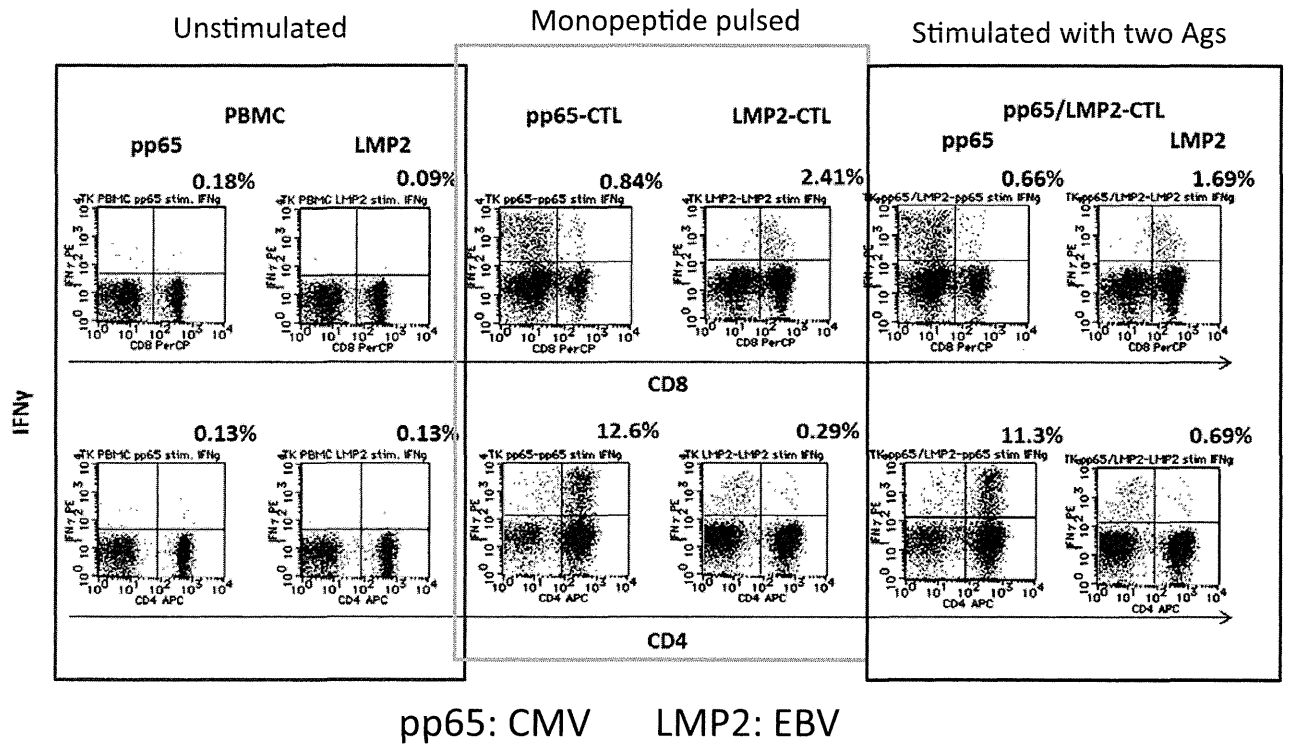

図2　抗原特異的T細胞比率の測定（フローサイトメトリー）．末梢血単核球（PBMC），単独抗原（ペプチドミクスチャー）刺激を加えサイトカインで増幅したもの，2抗原で刺激し増幅したもので，CD4，CD8細胞群におけるIFN-γ産生細胞比率を測定した．

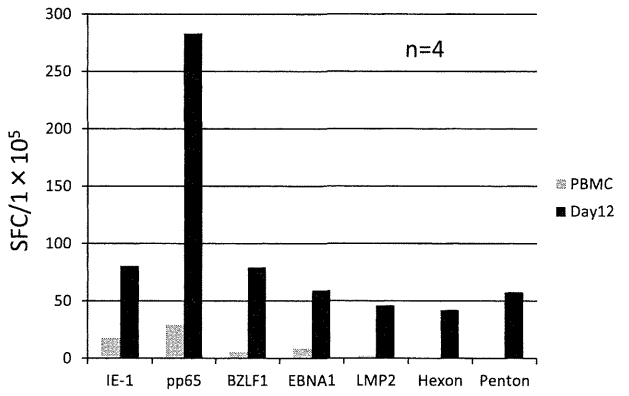

図3　抗原特異的T細胞比率の測定（ELISPOTアッセイ）．末梢血単核球（PBMC）を3ウイルス7抗原（ペプチドミクスチャー）で刺激を加えサイトカインで増幅した．刺激前と刺激後の細胞を用いてIFN-γ産生ELISPOTアッセイで測定した．なおここでは細胞は無血清培地を用いて培養した．

tracker orange/violet，CFSE，Protein transduction domain tagged EGFPなどでラベルし，調製したT細胞と共培養し，細胞外に放出された上記色素をFluorometerで測定する方法を試みたが比較的感度が低かった．他の方法として，標的細胞をCell tracker orange/violetなどでラベルし，アポトーシスの際に発動するCaspase 3で切断されると傾向を発する物質（PhiPhiLuxなど）を細胞内にパルスし，FACSにて切断物質を含む細胞の%を測定するという方法も報告されている．私達は細胞の標識は同様として，Caspase 3 cleavageをcleaved Caspase 3抗体で検出することにより，また同時にAnnexinV/7AAD測定することにより，傷害細胞の%を算出する方法を用いている．

さらに重要な課題はアロ反応性の検証であろう[10–12]．2つの課題がある．1つは特異性を高める（非特異的クローンを最小限にする）点であり，もう1つは特異的T細胞のアロ反応性である．前者に関しては調製および濃縮手法により克服可能である．後者に関しては，特異的T細胞でも*in vitro*ではアロ反応性を示すというデータが蓄積している．一方100例前後に達している臨床データからは，特異的T細胞治療によるGVHD（あるいはその明らかな増悪）は認められていない．アロ反応性については同一ドナーからの投与において議論されているが，現時点ではアロ反応性の問題は極めて低いという認識である．一方，今後次項に述べるようなthird party，off-the-shelf細胞治療が開発された際には，さらに掘り下げた科学的検証が必要となるであろう．

この細胞調製において最も費用が必要な部分はサイトカインであるが，試算でも現行の一般的な活性化T細胞調製に比べ，より安価に調製できる可能性が高い．無血清化した場合は原材料としての血清の品質評価が必要となくなり，さらにコストダウンをはかることができる．

	DLI	Expanded T	Specific T (in donor)	Specific CTL (plasmid/virus)	Specific CTL (peptide)
Memory T	△	△	○	△	○
Specific CD4	X	X	○	X	○
From 10mL?	X	○	X	X	△
Simple	○	△	△	X	△
Rapid	◎	X	◎	X	○
Side effect	X	△	○	○*	○*
Effect	△	△	○	◎	◎
Multi-viruses	○	○	△	△	◎
Low cost	○	△	X	X	△
From CB	X	○	X	○	X

図4　移植後のウイルス対応T細胞治療法の比較．Expanded T：例えばanti-CD3/IL-2やanti-CD3/CD28で増幅したT細胞あるいはCD4T細胞，Specific T（in donor）：ドナー血液中の特異的T細胞をそのまま収集する方法

III　ペプチド＋サイトカインによる多ウイルス特異的T細胞治療成績

米国Bayler大学では既にphase I/II多施設共同研究が行われている．対象は薬剤治療に反応しないEBV，CMV，AdV感染症であり，最もHLAが合致したドナーの特異的T細胞を2×10^7 cells/m^{2}の量で一度投与した．反応を認めない場合は2週おきに4回までの輸注を追加可能とされている．登録された患者は45名で（CMV感染症21，EBV感染症10，AdV感染症13，CMV+AdV 1），40%が末梢血肝細胞を24%が骨髄を，24%が臍帯血（ダブルユニット）を，12%が臍帯血（シングルユニット）を移植されていた．HLA合致度は1/6が26%，2/6が45%，3/6が26%，4/6が3%で半合致であれば良い程度であった．45名の内33名は一度の輸注で終了し，7名は2回投与を受けている．部分的反応あるいは完全寛解はCMVで88.2%，EBVで78.2%，AdVで78.6%である．GVHDは0が33症例，皮膚のGVHDが5例，肝臓GVHDが1例，慢性GVHDが1例で，TMAが2症例で認められた．

以上より，今後ドナー由来特異的T細胞療法から，第三者からのHLAが最も合致した特異的T細胞を輸注する方向性への転換を考慮できることを強く示した結果と言える．

IV　ウイルス特異的T細胞治療の展望

今までのウイルス特異的T細胞治療は，HLAに適合したペプチドと簡易抗原提示細胞系（名古屋大学ではT細胞）を用いて迅速T細胞誘導系を除き，安全性，迅速性，簡便性，経済性の面での問題を内包していた．今回紹介したウイルス特異的T細胞調製では，さらにHLAに関わらず細胞を調製できること，多ウイルス特異的なT細胞を用意できること，また第三者からの特異的T細胞も用いることができることなどを示唆しており，より汎用性のある治療法へと向かおうとしている．図4に現時点で実施されている主要な移植後ウイルス感染症に対する細胞治療の利点と欠点についてのまとめを掲載する．

再生医療新法案（再生医療等の安全性の確保等に関する法律案）の中でもこのペプチド+サイトカインによる調製は第3種再生医療として，医療機関としては届け出のみで良い部類に入る治療となると思われる（一方プラスミドやウイルスを用いた調製は第1種などに分類される）．一方本治療の普及のためには，いずれアカデミアから細胞調製機関への細胞培養加工委託や，あるいは再生医療医薬として改正薬事法の中で薬として展開するような方向性が模索されることも重要と思われる．さらに，その中で（もし本治療が標準となるのであれば）培養の全自動化も重要な課題と言える．

特異的T細胞のバンク化については現在，適応拡大に向けた臍帯血移植の先進化による成績向上と普及に関する研究」（H24-難治等（免）-一般008）（研究代表者：東京大学医科学研究所分子療法分野・高橋聡先生）の元で体制作りについて模索されており，今後のすりあわせが期待される．またこの多ウイルス特異的T細胞調製の最適化と妥当性の検証は，臓器移植・造血細胞移植後日和見感染症に対する有効かつ安全な多ウイルス特異的T細胞療法の開発と実用化に関する研究（H24-難治等（免）-一般105）（研究代表者：森尾友宏）の中で検証されようとしており，様々な場面で進捗状況を報告させていただきたいと願っている．

V　おわりに

移植後ウイルス感染に対する多ウイルス特異的CTL療法について概説した．日本はこの領域で諸外国に著しく遅れをとっているが，明らかな非自己抗原を表出する標的を根絶できる特異的T細胞治療の原型として，本治療がさらに安全に簡便に用いることができる日が近いことを願っている．またこの手法は抗原部位さえ明らかになれば様々な微生物に応用可能であり，さらなる応用や展開が期待される．

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Microenvironment and Immunology

Cancer Research

Bone Marrow–Derived CD11b$^+$Jagged2$^+$ Cells Promote Epithelial-to-Mesenchymal Transition and Metastasization in Colorectal Cancer

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Abstract

Timely detection of colorectal cancer metastases may permit improvements in their clinical management. Here, we investigated a putative role for bone marrow–derived cells in the induction of epithelial-to-mesenchymal transition (EMT) as a marker for onset of metastasis. In ectopic and orthotopic mouse models of colorectal cancer, bone marrow–derived CD11b(Itgam)$^+$Jagged2 (Jag2)$^+$ cells infiltrated primary tumors and surrounded tumor cells that exhibited diminished expression of E-cadherin and increased expression of vimentin, 2 hallmarks of EMT. *In vitro* coculture experiments showed that the bone marrow–derived CD11b$^+$Jag2$^+$ cells induced EMT through a Notch-dependent pathway. Using neutralizing antibodies, we imposed a blockade on CD11b$^+$ cells' recruitment to tumors, which decreased the tumor-infiltrating CD11b$^+$Jag2$^+$ cell population of interest, decreasing tumor growth, restoring E-cadherin expression, and delaying EMT. In support of these results, we found that peripheral blood levels of CD11b$^+$Jag2$^+$ cells in mouse models of colorectal cancer and in a cohort of untreated patients with colorectal cancer were indicative of metastatic disease. In patients with colorectal cancer, the presence of circulating CD11b$^+$Jag2$^+$ cells was accompanied by loss of E-cadherin in the corresponding patient tumors. Taken together, our results show that bone marrow–derived CD11b$^+$Jag2$^+$ cells, which infiltrate primary colorectal tumors, are sufficient to induce EMT in tumor cells, thereby triggering onset of metastasis. Furthermore, they argue that quantifying circulating CD11b$^+$Jag2$^+$ cells in patients may offer an indicator of colorectal cancer progression to metastatic levels of the disease. *Cancer Res; 73(14); 4233–46. ©2013 AACR.*

Introduction

Metastatic disease is a major cause of cancer-associated mortality. Despite significant advances in the treatment of primary tumors, metastases remain a significant clinical problem, likely reflecting our limited knowledge of the mechanisms governing this complex process (1). It is accepted that metastasization follows a series of interrelated steps, each of which can be rate-limiting. These steps include: local invasion by tumor cells, entry into systemic circulation (intravasation), invasion of the target organ (extravasation), and finally proliferation and growth of the secondary tumor (2). One of the major processes regulating local invasion in epithelial tumors is termed epithelial-to-mesenchymal transition (EMT; refs. 3, 4). EMT is a transcriptional regulated transdifferentiation process characterized at the tumor cell level, by a decrease in epithelial markers such as E-cadherin, loss of cell–cell adhesion, apical–basal polarity, and acquisition of mesenchymal markers such as vimentin associated with an increase in cell motility and invasion capacity (5–8). EMT has been positively correlated with increase breast and colon cancer metastasis and decreased patient survival (3, 9, 10).

In the last decade there has been increasing evidence suggesting that tumor metastasis is also regulated by nonmalignant cells of the tumor microenvironment, namely by bone marrow–derived cell populations (11). In fact distinct bone marrow–derived populations such as tumor-associated macrophages (12, 13), premetastatic niche cells (14), and endothelial progenitor cells (15) have been shown to enhance metastasization via multiple processes. Nevertheless, a direct role of bone marrow–derived cells in promoting EMT at the

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primary tumor has not been described, and was the focus of the present study.

Using ectopic and orthotopic colorectal cancer models in mice, we show that a population of bone marrow–derived myeloid ($CD11b^+F4/80^+$) expressing Jagged2 (Jag2) is actively recruited into colon tumors and accumulates in tumor areas undergoing EMT. Detailed analysis of this tumor: bone marrow–derived cell interaction shows the latter induce EMT via Notch activation on the tumor cells. Importantly, *in vivo* depletion of $CD11b^+$ cells in ectopic colorectal cancer models reduced the recruitment of $CD11b^+Jag2^+$ cells into the tumors and significantly decreased EMT. Quantification of circulating (peripheral blood) and tumor-derived $CD11b^+Jag2^+$ cells in patients with colorectal cancer was significantly correlated with the presence of metastatic disease. Together, the data presented here reveal a novel undisclosed role for bone marrow–derived cells in inducing EMT in primary colorectal cancer and identifies a bone marrow–derived cell population that may be targeted and studied as a biomarker for colorectal cancer metastases formation.

Materials and Methods

Human peripheral blood samples collection and processing

Peripheral blood samples of patients with sequential colorectal cancer evaluated at diagnosis by the Multidisciplinary Colorectal Cancer Team were collected at the Gastroenterology Department at Instituto Português de Oncologia (IPO, Lisbon, Portugal) after informed consent and Institutional Review Board approval (IPO), in accordance with the Declaration of Helsinki. Patients were included if they had a pathology exam showing colorectal adenocarcinoma. All patients previously submitted to endoscopic, surgical, or medical treatment for colorectal cancer were excluded. Peripheral blood samples were collected in 4 EDTA-coated tubes to a total volume of 12 mL. Samples were centrifuged at 4°C for 8 minutes at 1,500 rpm. Plasma was collected and stored at −80°C. The remaining fraction was lysed using 50 mL of red cell lysis buffer for 20 minutes at room temperature. The resulting mononuclear cell fraction was washed in PBS EDTA 2 mmol/L + 0.5% bovine serum albumin (BSA) and used for further analysis. Staging of patients with colorectal cancer was done according to the American Joint Committee on Cancer (AJCC) Staging System.

Mouse strains, bone marrow transplants, ectopic, and orthotopic colon carcinoma model

Animal experiments were carried out with the approval of the Animal Care Committee and Review Board at the Instituto Gulbenkian de Ciência (Oeiras, Portugal). *In vivo* experiments were carried out on 4- to 8-week-old female nude mice (C57/BL6 background). For bone marrow transplants, nude mice received a whole body lethal irradiation (800–950 rads) and 24 hours later received an intravenous injection of $2–3 \times 10^6$ bone marrow mononuclear cells collected from Actin-GFP male mice (C57/BL6 background). Mice were allowed to recover for 2 to 4 weeks. After this period peripheral blood samples were collected from the facial vein in EDTA-coated tubes (Multivette 600, Sarstedt) and analyzed by flow cytometry for GFP^+ cells. Mice were considered suitable for further experiments when the percentage of GFP^+ cells in the peripheral blood was more than 80% of total cells. Xenografted ectopic colon carcinoma tumors were induced by inoculation of 5×10^6 HCT15, HCT116, DLD-1, or HT-29 cells (human colorectal carcinoma cell lines; these were obtained from American Type Culture Collection, in 2012, and were not passaged for more than 6 months in our Laboratory) subcutaneously in nude mice. Tumors were allowed to grow and at specific time points (1–3 weeks) mice were sacrificed and tumors were collected. Tumors were fixed (10% formalin or paraformaldehyde) and included (paraffin or gelatin, respectively), frozen at −80°C for further RNA isolation, or digested for fluorescence-activated cell sorting (FACS) analysis. Xenografted orthotopic colorectal carcinoma tumors were induced by inoculation of 1×10^6 HCT15 or HCT116 cells, into the visceral cecal wall of nude mice. Peripheral blood samples were collected from the facial vein at different time points and further processed for flow cytometric analysis. CD11b-neutralizing antibody *in vivo* administration was conducted as follows: briefly, 500 μg anti-CD11b (clone 5C6) neutralizing monoclonal antibodies against CD11b were administered intraperitoneally every 3 days into tumor-bearing mice, starting on day 5 postinoculation.

Isolation of bone marrow–derived cells from the tumors

Tumor samples were mechanically fragmented into 2×2 mm^2 pieces and then digested with collagenase (Sigma-Aldrich, 2 mg/mL in serum-free Dulbecco's Modified Eagle Medium (DMEM) for 1 hour at 37°C and 5% CO_2. After digestion, tumor cell suspensions were passed through a mesh and washed in sterile PBS. Further isolation of tumor cell population was conducted by cell sorting using FACSaria (BD Biosciences). Isolation of tumor-derived GFP^+ population was done without the use of any antibody staining, whereas isolation of $Jag2^+$ and $CD11b^+$ population required previous staining with fluorochrome-conjugated antibodies (PE anti-mouse Jag2, 131007, BioLegend and FITC anti-mouse CD11b, 101205 BioLegend). Antibodies used were diluted 1:100 in PBS + BSA 0.5% and incubated in the dark with rotation at 4°C for 45 minutes.

In vitro co-culture assays

HCT15 cells were cultured at $1 \times 10^4/cm^2$ cell density in DMEM-supplemented medium (GIBCO) with 10% FBS (Sigma-Aldrich). After 24 hours, medium was changed to DMEM-supplemented medium with 2% FBS and 1×10^5 tumor-associated bone marrow–derived cells were added (GFP^+, GFP^+Jag2^+, GFP^+Jag2^-, or $Jag2^+$ $CD11b^+$ cells). Coculture was maintained for 48 hours. After this period, tumor-associated bone marrow–derived cells were gently washed from the culture and HCT15 cells collected for mRNA extraction or fixed with 2% paraformaldehyde for 15 minutes for further immunocytochemistry staining. γ-Secretase inhibitor (GSI; DAPT, Sigma-Aldrich) was added to cocultures at a final concentration of 10 μmol/L and respective controls received DMSO (Sigma-Aldrich).