Bloodstream Infection after Stem Cell Transplantation in Children with Idiopathic Aplastic Anemia

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A B S T R A C T

Bloodstream infection (BSI) is the most common infectious complication of hematopoietic stem cell transplantation (HSCT) and can cause substantial morbidity and mortality. Identification of risk factors for BSI might be helpful in efforts to reduce transplantation-related death. This study analyzed the incidence of BSI and risk factors for BSI after HSCT in pediatric patients with aplastic anemia (AA). BSI occurred in 39 of the 351 patients with AA (11.1%). Onset of BSI occurred at a median of 8 days after HSCT (range, 0 to 92 days). The 5-year overall survival rate was lower in patients with BSI than in patients without BSI (63.32% ± 7.90% versus 93.35% ± 1.44%; $P < .0001$). Univariate analysis identified the following variables as associated with BSI: history of immunosuppressive therapy with antithymocyte globulin (ATG), transplantation from an unrelated donor, frequent blood transfusion before transplantation, major or major plus minor ABO type mismatch, graft-versus-host disease prophylaxis with tacrolimus and without cyclosporine, and long interval from diagnosis to transplantation. Among these factors, long interval from diagnosis to transplantation was the sole statistically significant risk factor for BSI on multivariate analysis. In patients who underwent HSCT from a related donor, age ≥14 years at transplantation was risk factor for BSI. In contrast, history of immunosuppressive therapy with ATG, frequent blood transfusion before HSCT, graft failure, and major or major plus minor ABO type mismatch were risk factors for BSI in patients who underwent HSCT from an unrelated donor. Because the overall 5-year survival rate without BSI was >90%, even in patients who were received a transplant from an unrelated donor, control of BSI is very important for successful HSCT in pediatric patients with AA.

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) is first-line therapy for severe aplastic anemia (AA). HSCT from an HLA-matched sibling donor is an established standard therapy for children with severe AA and is associated with high survival rates [1]. Outcomes of HSCT from an unrelated donor have gradually improved [2,3].

Bloodstream infection (BSI) is the most common infectious complication of HSCT and causes substantial morbidity and mortality [4,5]. Identification of risk factors for BSI may aid efforts to reduce transplantation-related deaths. We previously identified AA as a common risk factor for BSI in a retrospective multicenter study [6]. In the present study, we analyzed the incidence of BSI and risk factors for BSI after

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HSCT in pediatric patients with AA using the Transplant Registry Unified Management Program (TRUMP) system of the Japanese Society of Stem Cell Transplantation.

PATIENTS AND METHODS

Between 1980 and 2011, 1098 patients age ≤19 years who underwent HSCT for AA (excluding hereditary bone marrow failure, paroxysmal nocturnal hemoglobinemia, and secondary AA) were registered with the TRUMP system of the Japanese Society of Stem Cell Transplantation. Of these 1098 patients, 516 who underwent HSCT before 2000 were excluded from this analysis, owing to the drastic changes in infection control practices promulgated by the Japanese Society of Stem Cell Transplantation in 2000, including antibiotics and antifungal drugs and guidelines for infection management in the early post-transplantation period. Of the remaining 582 patients, 231 were excluded due to insufficient data; thus, our study group comprised 351 pediatric patients with AA who underwent HSCT, including 193 males and 158 females, with a median age of 11 years (range, 0 to 19 years).

Diagnosis and assessment of severity of disease were established according to published criteria [7]. Severity of AA at initial diagnosis was as follows: very severe, n = 84; severe, n = 137; nonsevere, n = 130. Severity of AA at HSCT was as follows: very severe, n = 122; severe, n = 166; nonsevere, n = 63. The median interval from diagnosis to transplantation was 337 days (range, 9 to 5261 days). Two hundred and seventy-eight patients had received some specific treatment for AA before transplantation, including steroids (n = 171), antithymocyte globulin (ATG; n = 210), cyclosporine (CsA; n = 244), and granulocyte colony-stimulating factor (n = 141). Stem cell source was bone marrow in 315 patients, peripheral blood in 12 patients, bone marrow plus peripheral blood in 1 patient, and cord blood in 23 patients. One hundred seventy-three patients had a related donor, 1 patient had a syngeneic donor, and 177 patients had an unrelated donor.

The conditioning regimen included ATG for 240 patients, cyclophosphamide for 317, fludarabine for 244, melphalan for 39, total body irradiation for 145, thoracoabdominal irradiation for 49, and total lymphoid irradiation for 70 patients. Graft-versus-host disease (GVHD) prophylaxis, defined as planed administration of immunosuppressive drugs before evidence of acute GVHD, included steroids in 17 patients, CsA in 160, tacrolimus in 191, and methotrexate in 319.

Twenty-four patients underwent a second HSCT, 3 patients underwent a third HSCT, and 1 patient underwent a fourth HSCT. Twenty-one patients had a bacterial or fungal infection at the time of transplantation. In patients with multiple HSCTs, each transplantation was analyzed separately.

BSI was defined as isolation of 1 or more recognized bacterial or fungal pathogens from 1 or more blood cultures and at least 1 of the following signs and symptoms within 24 hours of collection of a positive blood culture: fever (>38°C), chills or rigors, or hypotension. We classified ABO compatibility as minor (eg, from an type O donor to a type A, B, or AB recipient), major (eg, from a type A, AB, or B donor to an type O recipient), and major and minor (eg, type A donor to type B recipient). We defined an HLA match donor as a 6/6 HLA-A, -B, and -DR antigen match between recipient and donor, using low-resolution typing. The median duration of follow-up was 39 months. Data collected as of October 2012 were analyzed.

In univariate analysis, the chi square test and Fisher's exact test were used to assess risk factors for BSI. Multivariate stepwise regression was performed to explore the independent effects of variables that demonstrated a significant influence in univariate analysis ($P < .10$). Overall survival was analyzed using the Kaplan-Meier method, with differences compared using the log-rank test. Statistical analyses were performed using SPSS 11.0 for Windows release 11.0.1J (SPSS Japan, Tokyo, Japan).

RESULTS

Assessment of BSI in All 351 Patients Who Underwent HSCT

BSI occurred in 39 of the 351 patients with AA (11.1%). Onset of BSI occurred at a median of 8 days after transplantation (range, 0 to 92 days). The bacteria that were isolated are summarized in Table 1. *Staphylococcus* spp were detected in 11 patients, and *Streptococcus* spp were detected in 7 patients. Gram-positive cocci were detected in 20 patients (51.3%); gram-positive bacilli, in 5 patients (12.8%); gram-negative bacilli, in 11 patients (28.2%); and *Candida* spp, in 3 patients (7.7%). The 5-year overall survival rate was lower in patients with BSI compared with patients without BSI (65.32% ± 7.90% versus 93.35% ± 1.44%; $P < .0001$) (Figure 1). The cause of death was directly associated with BSI in 5 of the 13 patients with BSI who died.

Table 1
Organisms Isolated from Blood Cultures of Patients with AA Who Underwent HSCT

Organism	n
Staphylococcus	11
Staphylococcus epidermidis	8
Staphylococcus haemoliticus	1
Coagulase-negative staphylococci	1
Staphylococcus sp	1
Streptococcus	7
Streptococcus mitis	4
Streptococcus viridans	1
α-streptococci	1
Streptococcus sp	1
Micrococcus	1
Enterococcus	1
Bacillus	4
Gram-positive rods	1
Escherichia coli	1
Enterobacter cloacae	2
Acinetobacter	1
Pseudomonas aeruginosa	4
Stenotrophomonas maltophilia	3
Candida	3

We performed univariate and multivariate analyses to identify risk factors for BSI in the patients with AA (Table 2). Variables associated with BSI on univariate analysis included (1) history of immunosuppressive therapy with ATG, (2) transplantation from an unrelated donor, (3) frequent blood transfusions before HSCT, (4) major or major plus minor ABO mismatch, (5) tacrolimus as acute GVHD prophylaxis with use of CsA, and (6) extended interval from diagnosis of AA to HSCT. Infectious complications at the time of HSCT were not associated with BSI after transplantation. Multivariate analysis identified extended interval from diagnosis to HSCT (>300 days) as the sole statistically significant risk factor for BSI (Table 3).

Assessment of BSI in 158 Patients Who Underwent First HSCT from a Related Donor

BSI occurred in 11 of 158 patients with AA who underwent first HSCT from a related donor (7.0%). The 5-year overall survival rate was lower in patients with complicated BSI compared with patients without BSI (81.82% ±

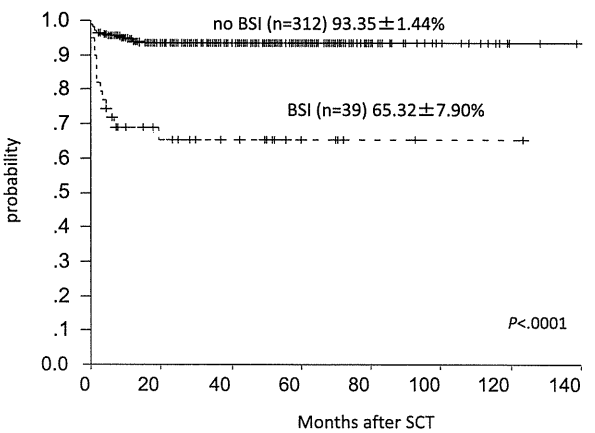

Figure 1. Kaplan-Meier estimate of overall survival for patients with BSI (n = 40; 63.68% ± 7.87%) and patients without BSI (n = 311; 93.65% ± 1.41%); $P < .0001$.

Table 2
Clinical Characteristics of 351 Patients Who Underwent HSCT

	BSI (n = 39)	No BSI (n = 312)	*P*
Sex, male/female, n	21/18	172/140	1.000
Age, yr, median	11	11	.568
Previous treatment, n (%)			
ATG	33 (85)	177 (57)	**.001**
CsA	34 (87)	210 (67)	**.010**
Both ATG and CsA	33 (85)	171 (55)	**<.001**
Bacterial/fungal infection at HSCT, n (%)	3 (8)	18 (6)	.716
First HSCT, n (%)	37 (95)	286 (92)	.754
Stem cell source, n (%)			.959
BM	35 (90)	280 (90)	
PB	1 (3)	11 (4)	
BM + PB	0 (0)	1 (0)	
CB	3 (8)	20 (6)	
Donor, n (%)			**.044**
Syngeneic	0 (0)	1 (0)	
Related	12 (31)	161 (52)	
Unrelated	27 (69)	150 (48)	
Receipt of ≥20 transfusions before HSCT, n (%)			
RBCs	20 (51)	84 (27)	**.003**
Platelets	21 (54)	102 (33)	**.012**
ABO type match, n (%)			**.014**
Match	16 (41)	172 (55)	
Major mismatch	14 (36)	47 (15)	
Minor mismatch	6 (15)	54 (17)	
Major plus minor mismatch	3 (8)	39 (13)	
HLA compatibility (low-resolution typing), n (%)			.846
Matched	30 (77)	231 (74)	
Mismatched	9 (23)	81 (26)	
Conditioning regimen, n (%)			
ATG	30 (77)	210 (67)	.275
Fludarabine	27 (69)	217 (70)	1.000
Cyclophosphamide	36 (92)	281 (90)	1.000
Irradiation	33 (85)	231 (74)	.173
GVHD prophylaxis, n (%)			
Steroid	0 (0)	17 (5)	.235
CsA	11 (28)	149 (48)	**.040**
Tacrolimus	28 (72)	163 (52)	**.026**
Methotrexate	37 (95)	282 (90)	.555
Acute GVHD grade II-IV, n (%)	5 (13)	55 (18)	.651
Graft failure, n (%)	5 (13)	16 (5)	.070
Time from diagnosis to HSCT, d, median	447	305.5	**.020**
Time from diagnosis to HSCT >300 d, n (%)	30 (77)	157 (50)	**.002**
Severity at diagnosis, n (%)			.641
Very severe	7 (18)	77 (25)	
Severe	16 (41)	121 (39)	
Nonsevere	16 (41)	114 (37)	
Severity at HSCT, n (%)			.426
Very severe	17 (44)	105 (34)	
Severe	15 (38)	151 (48)	
Nonsevere	7 (18)	56 (18)	

BM indicates bone marrow; PB, peripheral blood; CB, cord blood.
*Irradiation included total body irradiation, thoracoabdominal irradiation, and total lymphoid irradiation.

11.63% versus 95.84% ± 1.66%; $P = .0379$). Univariate analysis of variables associated with BSI identified age ≥14 years at HSCT as the sole risk factor (Table 4).

Assessment of BSI in 165 Patients Who Underwent First HSCT from an Unrelated Donor

BSI occurred in 26 of 165 patients with AA who underwent a first HSCT from an unrelated donor (15.8%). The 5-year overall survival rate was lower in patients with complicated BSI compared with those without BSI (55.75% ± 10.14% versus 90.77% ± 2.25%; $P < .0001$). In univariate analysis, variables associated with BSI included a history of immunosuppressive therapy with ATG, frequent blood transfusion before transplantation, graft failure, and major or major plus minor ABO type mismatch (Table 4).

Table 3
Multivariate Analysis of 351 Patients with AA

	Hazard Ratio	*P*	95% Confidence Interval
Interval from diagnosis to HSCT >300 d	2.430	**.041**	1.036-5.702
≥20 RBC or platelet transfusions before HSCT	1.843	.109	0.873-3.891
Major or major plus minor ABO type mismatch	1.595	.199	0.783-3.250
Unrelated donor	1.233	.673	0.466-3.262
Use of tacrolimus	1.167	.755	0.442-3082
Previous treatment with ATG or CsA	1.115	.839	0.392-3.170

DISCUSSION

In the literature, the incidence of BSI after the early phase of HSCT in children has ranged from 25% to 30% [4-8]. In our previous study, the incidence of BSI after HSCT (including patients with malignant and nonmalignant diseases) was 8.7% [6]. In the study of Sarashina et al. [6], nonmalignant disease, especially AA and Wiskott-Aldrich syndrome, were identified as risk factors for BSI after HSCT (17.2%).

The present study is the first to analyze BSI after HSCT in pediatric patients with AA. Our data show a lower incidence of BSI in these patients (11.1%) compared with our previous study, but a higher incidence than that seen in patients with other diseases in that study. In our previous study, BSI was not associated with survival, and the survival rate was nearly identical in patients with BSI and those without BSI; however, in the present study, the survival rate was lower in patients with BSI. Patients with malignant diseases were included in the previous study, whereas only patients with AA were analyzed in the present study. In patients with malignant disease, the relapse rate of the original disease was lower in patients with BSI; this difference might account for the discrepant results between the previous and present studies.

In the present study, univariate analysis identified a history of immunosuppressive therapy with ATG, receipt of a transplant from an unrelated donor, frequent blood transfusions before HSCT, major or major plus minor ABO type mismatch, GVHD prophylaxis with tacrolimus but without CsA, and extended interval from diagnosis to HSCT as risk factors for BSI. Poutsiaka et al. [9] previously reported an association between BSI after HSCT and acute GVHD; however, our data do not corroborate this finding. Interestingly, the risk factors for BSI identified in the present study are associated with one another. Generally, patients without a related HLA-matched donor are treated with immunosuppressive therapy. If this therapy is not effective, then HSCT with an unrelated donor is performed. These patients often receive numerous blood transfusions and have a extended interval between diagnosis and transplantation. Furthermore, tacrolimus (rather than CsA) may be selected for GVHD prophylaxis. Among these factors, an extended interval between diagnosis and HSCT was the sole statistically significant risk factor for BSI identified by multivariate analysis.

Regarding numerous blood transfusions before HSCT, some recent studies have investigated the relationship

Table 4
Clinical characteristics of 158 patients who underwent HSCT from related donor as first HSCT and 165 patients who underwent HSCT from unrelated donor as first HSCT

	Related Donor (n = 158)			Unrelated Donor (n = 165)		
	BSI (n = 11)	No BSI (n = 147)	*P*	BSI (n = 26)	No BSI (n = 139)	*P*
Sex, male/female, n	6/5	84/63	1.000	10/16	77/62	.136
Age, yr, median	15	11	**.050**	11	11	.675
Age ≥14 yr, n (%)	8 (73)	45 (31)	**.007**			
Previous treatment, n (%)						
ATG	5 (45)	49 (33)	.512	25 (96)	110 (79)	**.050**
CsA	6 (56)	68 (46)	.756	25 (96)	124 (89)	.472
Both ATG and CsA	5 (45)	48 (33)	.509	25 (96)	107 (77)	.030
Bacterial/fungal infection at HSCT, n (%)	2 (18)	10 (7)	.198	1 (4)	7 (5)	1.000
Stem cell source, n (%)			.863			.734
BM	10 (91)	139 (95)		23 (88)	125 (90)	
PB	1 (9)	6 (4)		0 (0)	0 (0)	
BM + PB	0 (0)	1 (1)		0 (0)	0 (0)	
CB	0 (0)	1 (1)		3 (12)	14 (10)	
Donor, n (%)			1.000			
Syngeneic	0 (0)	1 (1)		-	-	
Related	11 (100)	146 (99)		-	-	
Receipt of ≥20 transfusions before HSCT, n (%)						
RBCs	2 (18)	10 (7)	.198	17 (65)	57 (41)	**.031**
Platelets	3 (27)	26 (18)	.425	17 (65)	58 (42)	**.032**
ABO type match, n (%)			.718			**.024**
Match	7 (64)	97 (66)		8 (31)	62 (45)	
Major mismatch	2 (18)	18 (12)		12 (46)	26 (19)	
Minor mismatch	2 (18)	20 (14)		3 (12)	29 (21)	
Major and minor mismatch	0 (0)	12 (8)		3 (12)	22 (16)	
HLA compatibility (low-resolution typing), n (%)			1.000			.248
Matched	9 (82)	122 (83)		21 (78)	95 (68)	
Mismatched	2 (18)	25 (17)		5 (22)	44 (32)	
Conditioning regimen, n (%)						
ATG	7 (64)	91 (62)	1.000	22 (81)	108 (78)	.602
Fludarabine	5 (45)	87 (59)	.528	20 (77)	109 (78)	.333
Cyclosphosphamide	10 (91)	138 (94)	.525	24 (92)	130(94)	.685
Irradiation	6 (56)	85 (58)	1.000	25 (96)	128 (92)	.693
GVHD prophylaxis, n (%)						
Steroid	0 (0)	4 (3)	1.000	0 (0)	10 (7)	.365
CsA	10 (91)	119 (81)	.690	1 (4)	20 (14)	.203
Tacrolimus	1 (9)	28 (19)	.690	25 (96)	119 (86)	.203
Methotrexate	10 (91)	131 (89)	1.000	25 (96)	132 (95)	1.000
Acute GVHD grade II-IV, n (%)	0 (0)	13 (9)	.601	5 (19)	37 (27)	.624
Graft failure, n (%)	0 (0)	5 (3)	1.000	5 (19)	7 (5)	**.024**
Mean time from diagnosis to HSCT, d	91	80	.426	474.5	455	.183
Severity at diagnosis, n (%)			.735			.664
Very severe	2 (18)	41 (28)		5 (19)	35 (25)	
Severe	5 (45)	65 (44)		10 (38)	42 (30)	
Nonsevere	4 (36)	41 (28)		11 (42)	62 (45)	
Severity at HSCT, n (%)			.490			.094
Very severe	5 (45)	59 (40)		12 (46)	35 (25)	
Severe	3 (27)	64 (44)		10 (38)	75 (54)	
Nonsevere	3 (27)	24 (16)		4 (15)	29 (21)	

*Irradiation included total body irradiation, thoracoabdominal irradiation, and total lymphoid irradiation.

between pretransplantation hyperferritinemia and post-transplantation outcomes [10,11]. In many of these reports, hyperferritinemia was associated with adverse outcomes after allogeneic HSCT. Moreover, iron overload is associated with proliferation of bacteria and fungus [12]. These observations suggest that iron chelating agents should be administered before HSCT in patients who have received frequent blood transfusions.

We analyzed BSI after HSCT in patients undergoing first transplantation from related and unrelated donors to clarify the risk factors for BSI. In both groups, survival rates were significantly lower in patients with BSI than in those without BSI. Surprisingly, the survival rate of patients undergoing HSCT from an unrelated donor without BSI exceeded 90%, not significantly different from that seen in patients undergoing HSCT from a related donor. This finding suggests that prevention of BSI is important to improving outcomes after HSCT. In patients who underwent HSCT from a related donor, age ≥14 years at transplantation was identified as a risk factor for BSI, although the incidence of BSI was evidently lower than that in patients undergoing HSCT from an unrelated donor. Older patients tend to have more severe oral mucositis. Furthermore, we previously identified age >10 years as a risk factor for fungal infection in patients with hematologic malignancies [13]. In contrast, in patients who underwent HSCT from an unrelated donor, variables associated with BSI included a history of immunosuppressive therapy with ATG, frequent transfusions before transplantation, graft failure, and major or major plus minor ABO type mismatch.

The impact of ABO incompatibility on clinical outcomes remains controversial [14,15]. ABO incompatibility in

allogeneic HSCT is associated with an increased risk of delayed erythroid reconstitution, pure RBC aplasia, and acute and delayed hemolysis; however, ABO incompatibility has not been identified as a risk factor for BSI. The ABO blood group antigens consist of oligosaccharide glycoproteins and are expressed in erythrocytes as well as in neutrophils, platelets, and vascular endothelial and epithelial cells. The ABO antigens could be immunologic targets for ABO-incompatible donor or recipient lymphocytes, thereby affecting GVHD and engraftment [16]. These phenomena may contribute to the development of BSI.

In conclusion, because the 5-year overall survival rate without BSI exceeded 90%, even in patients who underwent HSCT from an unrelated donor, controlling BSI is very important for a successful outcome of HSCT in patients with pediatric AA.

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First-line treatment for severe aplastic anemia in children: bone marrow transplantation from a matched family donor *versus* immunosuppressive therapy

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ABSTRACT

The current treatment approach for severe aplastic anemia in children is based on studies performed in the 1980s, and updated evidence is required. We retrospectively compared the outcomes of children with acquired severe aplastic anemia who received immunosuppressive therapy within prospective trials conducted by the Japanese Childhood Aplastic Anemia Study Group or who underwent bone marrow transplantation from an HLA-matched family donor registered in the Japanese Society for Hematopoietic Cell Transplantation Registry. Between 1992 and 2009, 599 children (younger than 17 years) with severe aplastic anemia received a bone marrow transplant from an HLA-matched family donor (n=213) or immunosuppressive therapy (n=386) as first-line treatment. While the overall survival did not differ between patients treated with immunosuppressive therapy or bone marrow transplantation [88% (95% confidence interval: 86-90) *versus* 92% (90-94)], failure-free survival was significantly inferior in patients receiving immunosuppressive therapy than in those undergoing bone marrow transplantation [56% (54-59) *versus* 87% (85-90); $P<0.0001$]. There was no significant improvement in outcomes over the two time periods (1992-1999 *versus* 2000-2009). In multivariate analysis, age <10 years was identified as a favorable factor for overall survival ($P=0.007$), and choice of first-line immunosuppressive therapy was the only unfavorable factor for failure-free survival ($P<0.0001$). These support the current algorithm for treatment decisions, which recommends bone marrow transplantation when an HLA-matched family donor is available in pediatric severe aplastic anemia.

Introduction

Aplastic anemia is defined as peripheral blood pancytopenia caused by bone marrow failure; the pathogenesis of this disease is thought to involve autoimmune processes.[1-3] The principal interventions responsible for improved survival in aplastic anemia are bone marrow transplantation (BMT) and immunosuppressive therapy (IST). In children, BMT from an HLA-matched family donor (MFD) is the treatment of choice for severe aplastic anemia (SAA).[1,4-6] For children lacking an MFD, IST with a combination of antithymocyte globulin and cyclosporine has been used as a therapeutic option.[6-10] However, this treatment approach is based on the results of comparative studies between these therapies that were conducted mainly in the 1980s, and there have been few recent studies that compare the outcome of BMT recipients with comparable patients receiving IST.

The largest pediatric series in previous studies was reported by the European Group for Blood and Marrow Transplantation (EBMT) and included 304 children treated from 1970 to 1988; that study indicated survival was better following first-line BMT than after first-line IST (63% *versus* 48%; $P=0.002$) but did not compare failure-free survival after the two therapies.[6] Our previous analysis showed a significant advantage for patients receiving BMT from an MFD as first-line treatment in a study of 100 children with SAA who were treated between 1984 and 1998.[1] In patients who received first-line IST, 10-year overall and failure-free survival rates were 55% and 40%, respectively, both of which were markedly inferior to the rates in patients who initially underwent BMT, which was associated with 10-year overall survival and failure-free survival rates greater than 90%. Since the 1980s, the outcomes of both BMT and IST have improved, likely due to better supportive care and advanced treatment and transplantation protocols. A recently published Cochrane review regarding BMT from an MFD and IST as first-line treatment also pointed out that all studies included in the analysis

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had a high risk of bias due to their study design and were conducted more than 10 years ago and may not be applicable to the standard of care of today.[11] Updated evidence to aid treatment decisions in pediatric SAA is, therefore, required.

In children, the choice of an appropriate treatment is particularly influenced by the long-term sequelae of the disease and its therapy. Thus, failure-free survival is much more important than survival alone when analyzing the long-term outcomes of children with aplastic anemia. Lack of response, relapse, and clonal evolution are problematic in the IST setting, whereas graft failure, acute and chronic graft-*versus*-host disease (GVHD), and infectious complications limit the success of BMT. In the present study, we compared the outcomes of children with SAA who received IST or BMT from an MFD as first-line treatment using data from nationwide IST and BMT registries.

Methods

Patients

Between 1992 and 2009, a total of 599 consecutive children (younger than 17 years) with acquired SAA underwent BMT from an MFD or received IST as first-line treatment in Japan; 213 patients with an MFD underwent BMT and were registered in the Transplant Registry Unified Management Program (TRUMP) conducted by the Japanese Society for Hematopoietic Cell Transplantation, and 386 patients without an MFD were enrolled in two consecutive prospective multicenter trials (AA-92/97) conducted by the Japanese Childhood Aplastic Anemia Study Group and were initially treated with IST (Table 1). The disease severities were defined as previously reported.[12,13] Underlying inherited marrow failure disorders were excluded clinically and by chromosome fragility testing. Marrow cytogenetic studies were performed for all patients, and patients with clonal cytogenetic abnormalities were excluded from this study. Patients with paroxysmal nocturnal hemoglobinuria with clinical symptoms and positive findings on the Ham test/sucrose test were also excluded from this analysis. All treatments were performed after obtaining written informed consent from patients or their parents in accordance with the Declaration of Helsinki.

Immunosuppressive therapy and bone marrow transplantation procedures

The characteristics of the treatment procedures are detailed in Table 2. Three hundred and eighty-six patients were enrolled in the AA-92 (n=84) and AA-97 (n=302) trials, and all the patients were initially treated with a combination of antithymocyte globulin and cyclosporine A. Response to IST and disease relapse were evaluated as previously reported.[12] Transplantation data were collected with the use of standardized forms provided by the TRUMP. A total of 213 patients underwent BMT from an MFD as first-line treatment following the local protocols for conditioning regimens and GVHD prophylaxis. Patients who did not reach neutrophil counts $>0.5\times10^9$/L for 3 consecutive days after transplantation were considered to have had primary graft failure. Patients with initial engraftment in whom absolute neutrophil counts subsequently declined to $<0.5\times10^9$/L were considered to have had secondary graft failure. Acute and chronic GVHD were evaluated according to standard criteria.[14-16] More details on methods are provided in the *Online Supplementary Methods section*.

Statistical analyses

The date of analysis was July 30, 2012. Survival probabilities were estimated by the Kaplan-Meier method and compared between different groups of patients using the log-rank test. The influence of potential risk factors on overall survival and failure-free survival was assessed according to first-line treatment (BMT or IST), time period of treatment (1992-1999 or 2000-2009), age and other variables related to each treatment. Overall survival was defined as the time from diagnosis to death or last follow-up. Failure-free survival was defined as survival with treatment response. Death, primary or secondary graft failure, and secondary malignancy in the BMT group, and death, relapse, disease progression requiring stem cell transplantation (SCT) from an alternative donor or second IST, clonal evolution and evolution to paroxysmal nocturnal hemoglobinuria in the IST group were consid-

Table 1. **Patients' characteristics.**

	First-line treatment		
	BMT n=213	IST n=386	*P*
Age at diagnosis, year, median (range)	10 (0-16)	9 (0-16)	NS
Age at treatment, year, median (range)	11 (0-16)	9 (0-16)	NS
Gender			
Male / female	119/94	217/169	NS
Etiology, n. of patients (%)			
Idiopathic	204 (96)	312 (81)	<0.0001
Hepatitis	7 (3)	67 (17)	
Others	2 (1)	7 (2)	
Severity, n. of patients (%)			
Very severe aplastic anemia	–	227 (59)	–
Severe aplastic anemia	–	159 (41)	
Interval diagnosis-treatment, days, median (range)	84 (14-4605)	15 (1-180)	<0.0001
Time periods of treatment, n. of patients (%)			
1992-1999	121 (57)	155 (40)	0.0001
2000-2009	92 (43)	231 (60)	

BMT: bone marrow transplantation; IST: immunosuppressive therapy; NS: not significant.

ered treatment failures. For multivariate analyses, the Cox proportional hazard regression model was used. *P* values less than 0.05 were considered statistically significant. This study was approved by the institutional ethics committee of the Japanese Red Cross Nagoya First Hospital.

Results

Patients' characteristics

The characteristics of the 599 children are detailed in Table 1. The groups treated first-line with BMT (n=213) or IST (n=386) were similar with regards to age at diagnosis, age at treatment and male/female ratio. The majority of patients in both groups had a diagnosis of idiopathic disease, although the proportion of patients with non-idiopathic disease was higher in the IST group. Seven patients (3%) in the BMT group and 67 patients (17%) in the IST group suffered from hepatitis-associated disease. Nine patients had drug-induced or virus-associated disease. Information on the proportion of very severe disease was not available for 141 patients who underwent BMT because the severity of the SAA was not a required item for the registry. The clinical features of these patients were similar to those of the remaining patients. In the IST group, details regarding the severity of disease were provided for all patients: 227 (59%) had very severe disease and 159 (41%) suffered from severe disease. As expected, the time to treatment was significantly longer in the BMT group; the median interval between diagnosis and treatment was 15 days (range, 1-180 days) and 84 days (range, 14-4605 days) for those treated with IST and BMT, respectively. In accordance with decisions taken by the patients and the parents, ten patients underwent BMT more than 5 years after diagnosis. None of the patients who received IST before BMT from an MFD were included in the BMT group.

Table 2. **Treatment characteristics.**

Bone marrow transplantation	**213**
Conditioning regimen, n.	
High-dose CY (200 mg/kg) -based	158
CY ± low-dose irradiation	86
CY + ATG ± low-dose irradiation	72
FLU + CY (100-120 mg/kg) -based	44
FLU + CY ± low-dose irradiation	29
FLU + CY + ATG ± low-dose irradiation	15
Myeloablative	11
CY + TBI (10-12 Gy)	7
BU + CY	4
GVHD prophylaxis, n.	
CyA + MTX	174
CyA alone	23
Tacrolimus + MTX	6
Others	10
Immunosuppressive therapy	**386**
IST trial, n.	
AA-92	84
AA-97	302
IST regimen, n.	
CyA + ATG	140
CyA + ATG + G-CSF	246

CY: cyclophosphamide; ATG: antithymocyte globulin; FLU: fludarabine; TBI: total body irradiation; BU: busulfan; CyA: cyclosporine; MTX: methotrexate; G-CSF: granulocyte colony-stimulating factor.

Immunosuppressive therapy

Response to IST at 6 months was not evaluable in 11 patients for the following reasons: early death (n=7) or BMT from an alternative donor within 6 months of IST (n=4). The causes of the early deaths were sepsis (n=3), interstitial pneumonia (n=2), hemolysis of unknown cause (n=1) and accidental ingestion (n=1). Of the patients who underwent BMT from an alternative donor within 6 months, two patients died of graft failure or cardiac toxicity related to the preconditioning regimen. Overall, 238 of the 375 evaluable patients (63%) improved with first-line IST and achieved a partial response (n=151) or complete response (n=87) at 6 months. All of these patients achieved transfusion independence.

For all 386 patients who received IST initially, the 10-year overall survival rate was 88% [95% confidence interval (CI): 86-90], as shown in Figure 1A, and the median follow-up time for living patients was 106 months (range, 22-224 months). In contrast to the high rate of overall survival, the result regarding survival with response was unsatisfactory, the 10-year failure-free survival rate being 56% (95%

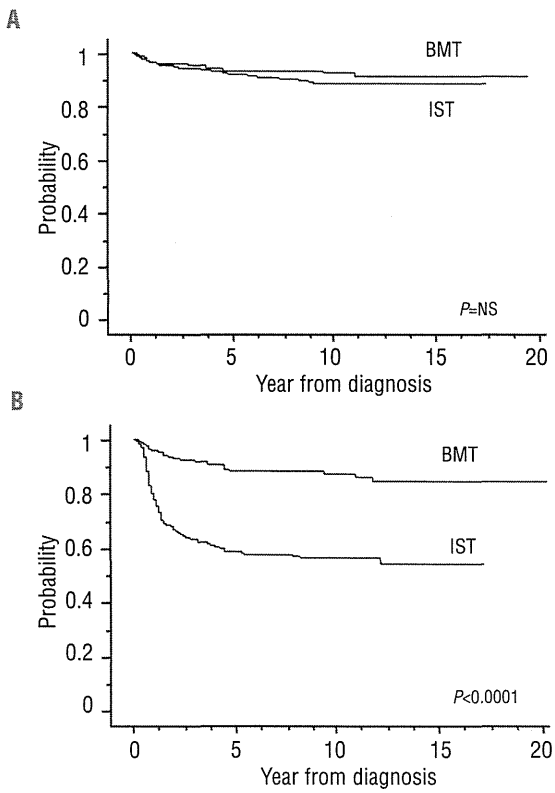

Figure 1. **Survival of 599 children with severe aplastic anemia according to first-line treatments with immunosuppressive therapy (IST) (n=386) or bone marrow transplantation (BMT) (n=213). (A) Overall survival. The 10-year overall survival was 88% (95% CI: 86-90) in the IST group and 92% (95% CI: 90-94) in MFD BMT recipients (*P*=NS). (B) Failure-free survival. The 10-year failure-free survival was 56% (95% CI: 54-59) in the IST group and 87% (95% CI: 85-90) in the BMT group (*P*<0.0001).**

CI: 54-59) (Figure 1B). The cause of treatment failure included death in 12 patients [due to intracranial hemorrhage (n=2), pneumonia (n=1), traffic accident (n=1) and sudden death (n=1) in addition to the seven early deaths], relapse in 23 patients, disease progression requiring second-line treatment in 109 patients, evolution to myelodysplastic syndrome in 15 patients, and appearance of paroxysmal nocturnal hemoglobinuria in two patients. After failed IST, a total of 113 patients underwent SCT from an alternative donor as second- or third-line treatment. The 10-year overall survival of these patients who received a transplant after failed IST was 79% (95% CI: 75-83) with a median of 435 days from diagnosis and SCT. We then analyzed the influence of potential risk factors for survival in the IST group. The prognostic significance of the clinical parameters is shown in Table 3. In the univariate analysis, age younger than 10 years at diagnosis was associated with a favorable overall survival rate [93% (95% CI: 91-95) *versus* 82% (95% CI: 78-86); *P*=0.012], and this was confirmed in a multivariate model. However, the rate of failure-free survival did not differ between patients in the two age groups. No other variables were significantly associated with survival after IST in either univariate or multivariate analyses.

Bone marrow transplantation

In the BMT group, 209 patients (98%) achieved primary engraftment at a median of 16 days after transplantation. As shown in Figure 1A and 1B, the 10-year overall survival and failure-free survival rates for all 213 patients who were treated initially with BMT from an MFD were 92% (95% CI: 90-94) and 87% (95% CI: 85-90), respectively. When the analysis was applied to the patients who underwent BMT within 180 days from diagnosis, similar results were observed; the 10-year overall survival and failure-free survival rates were 94% (95% CI: 92-96) and 89% (95% CI: 86-92), respectively. The median follow-up time for living patients was 101 months (range, 18-213 months). The cause of treatment failure included primary graft failure in two patients, secondary graft failure in ten patients, second malignancy in one patient, and death due to other complications in 12 patients. Although both patients without primary engraftment died, nine of the ten patients with secondary graft failure remain alive; eight were saved by a second transplant, and one recovered spontaneously. Twenty-five of 209 patients (12%) who had achieved primary engraftment developed grade II to IV acute GVHD, and extensive chronic GVHD was observed in 13 of 209 patients (6%) alive 100 days after BMT.

The prognostic significance of the clinical parameters, including variables related to transplantation, was then assessed. We found no association between age, gender, etiology, interval between diagnosis and BMT, or time period of treatment and treatment outcome (Table 3). Of particular interest with regards to conditioning regimens is the fact that the addition of antithymocyte globulin produced an improvement of overall survival [96% (95% CI: 92-99) *versus* 87% (95% CI: 84-91); *P*=0.021], whereas the rate of failure-free survival was comparable. A fludarabine-based regimen did not affect outcome after BMT from an

Table 3. **Univariate analysis of 10-year overall survival (OS) and failure-free survival (FFS), according to first-line treatment.**

	IST					BMT				
Variable	N. of patients	OS % (95% CI)	OS *P*	FFS % (95% CI)	FFS *P*	N. of patients	OS % (95% CI)	OS *P*	FFS % (95% CI)	FFS *P*
Age at diagnosis										
Younger than 10 years	219	93 (91-95)	0.012	57 (54-61)	0.754	89	95 (93-98)	0.163	92 (89-95)	0.200
10 years or older	167	82 (78-86)		55 (51-59)		124	90 (87-93)		84 (81-88)	
Gender										
Male	217	87 (84-90)	0.628	60 (56-64)	0.089	119	91 (87-94)	0.383	87 (83-90)	0.679
Female	169	90 (87-92)		52 (48-56)		94	94 (91-97)		88 (84-91)	
Etiology										
Idiopathic	312	88 (86-91)	0.661	54 (51-57)	0.185	204	92 (90-95)	0.568	87 (85-90)	0.934
Other	74	87 (83-92)		66 (60-71)		9	88 (76-99)		88 (76-99)	
Severity										
Very severe	227	90 (88-92)	0.600	57 (53-60)	0.965		–		–	
Severe	159	85 (82-89)		56 (52-60)						
Interval diagnosis-treatment										
Less than median days	187	91 (88-93)	0.537	60 (57-64)	0.170	105	95 (92-97)	0.322	91 (88-94)	0.362
Median days or more	199	86 (83-89)		53 (49-56)		108	90 (87-94)		85 (82-89)	
Time periods of treatment										
1992-1999	155	85 (82-88)	0.119	54 (50-58)	0.545	121	91 (89-94)	0.510	87 (84-90)	0.801
2000-2009	231	92 (90-94)		59 (56-63)		92	95 (93-98)		89 (85-93)	
Conditioning regimen										
With ATG		–		–		87	96 (92-99)	0.021	86 (83-90)	0.648
Without ATG						126	87 (84-91)		85 (82-89)	
GVHD prophylaxis										
CyA + MTX		–		–		174	93 (90-95)	0.924	88 (85-91)	0.809
Others						39	93 (88-98)		86 (80-93)	

ATG: antithymocyte globulin; CyA, cyclosporine; MTX, methotrexate.

MFD, although the number of patients treated with such regimens was too small to draw any conclusions. Multivariate analysis showed that none of the variables significantly influenced survival.

Survival and prognostic factors

The overall outcomes of the 599 children with SAA, stratified according to their first-line treatment, are shown in Figure 1A and 1B. Our data clearly showed a significant advantage for children receiving BMT from an MFD as first-line treatment; the failure-free survival was significantly superior in patients treated with BMT than in those in whom IST was used ($P<0.0001$), whereas the overall survival of patients in these two treatment groups did not differ. Figure 2A and 2B show survival curves in all patients treated in the two sequential time periods, 1992-1999 and 2000-2009: results were comparable over time [10-year overall survival: 88% (95% CI: 86-90) *versus* 93% (95% CI: 91-95); 10-year failure-free survival: 67% (95% CI: 65-70) *versus* 68% (95% CI: 66-71)], indicating no significant improvement in the last two decades. When age groups were considered, overall survival at 10 years in the younger group (<10 years old) was significantly better than that in the other age groups [93% (95% CI: 92-95) *versus* 85% (95% CI: 83-88); $P=0.007$], although no difference in failure-free survival was observed (Figure 3A and 3B). The favorable overall survival in the younger group may be mostly due to that observed in the first-line IST group. In multivariate analysis, age younger than 10 years at diagnosis was identified as a favorable factor for overall survival ($P=0.007$), and choice of first-line BMT from an MFD was confirmed as an independent favorable factor for failure-free survival ($P<0.0001$), as shown in Table 4.

Discussion

For children with SAA, BMT and IST have been accepted as standard treatments during the past three decades. The current guideline recommends BMT from an MFD as the treatment of choice for pediatric SAA[17-19] based on the results of comparative studies performed in the 1980s.[1,5,6,20,21] On the other hand, recent prospective studies with intensified IST for pediatric SAA have resulted in dramatic improvements in survival.[22,23] For example, a study from the EBMT showed a 100% overall survival rate at 6 years after first-line IST in 31 SAA patients younger than 20 years treated from 2002 to 2008.[22] These excellent overall survival results after IST have led to discussion about

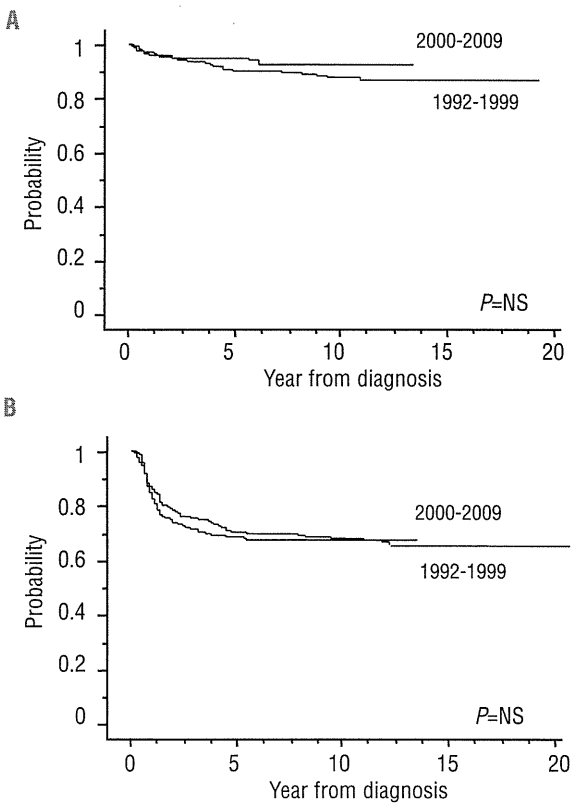

Figure 2. Survival of patients according to time periods of treatment: 1992-1999 (n=276) or 2000-2009 (n=323). (A) Overall survival. The 10-year overall survival was 88% (95% CI: 86-90) in 1992-1999 *vs.* 93% (95% CI: 91-95) in 2000-2009. (B) Failure-free survival. The 10-year failure-free survival was 67% (95% CI: 65-70) in 1992-1999 *vs.* 68% (95% CI: 66-71) in 2000-2009.

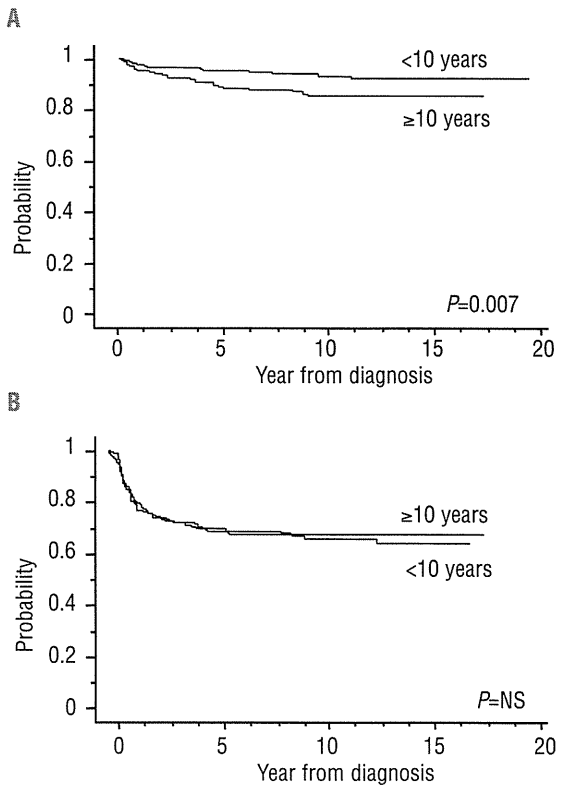

Figure 3. Survival of patients according to age at diagnosis: <10 years (n=308) or ≥10 years (n=291). (A) Overall survival. The 10-year overall survival in the younger group (<10 years) was significantly better than that in the other group [93% (95% CI: 92-95) *vs.* 85% (95% CI: 83-88); $P=0.007$]. (B) Failure-free survival. No difference in failure-free survival at 10 years was observed [67% (95% CI: 65-70) *vs.* 63% (95% CI: 59-67)].

the first-line treatment in children with SAA. To obtain solid evidence on which to base treatment decisions, ideally, a randomized controlled trial is required. However, because of the rarity of the disease, no randomized controlled trials comparing IST with BMT from an MFD as first-line treatment for SAA exist, and only retrospective studies using data from registries or relatively small cohorts of patients are available. Following the previous report of 304 children treated from 1970 to 1988,[6] the EBMT SAA Working Party (SAAWP) reported a consecutive study of 911 children younger than 16 years initially treated with IST (n=304) or BMT (n=607) between 1991 and 2002, which indicated that first-line IST gave an overall survival rate comparable to that of first-line BMT (81% *versus* 79%).[10] Unfortunately, the analyses had several limitations, because the drugs used for IST varied (e.g., antithymocyte globulin only, cyclosporine A only, or a combination of antithymocyte globulin and cyclosporine A) and the donor types used for BMT were not consistent (15% of the donors were mismatched family donors or matched/mismatched unrelated donors, although the majority of those were MFD). In addition, neither EBMT study provided results on failure-free survival,[6,10] which seems to be much more important than survival alone. Recent advances in supportive care and salvage therapies have effectively rescued non-responders to IST.[24] On the other hand, relapse, clonal evolution in the IST group and secondary graft failure and late malignancy in the BMT group are serious problems in long-term survivors. That is the reason why overall survival is no longer the only endpoint to determine optimal first-line treatment in children with SAA. In Japan, we have conducted consecutive prospective trials with a unified IST regimen consisting of antithymocyte globulin and cyclosporine A since 1992, enrolling 386 SAA patients younger than 17 years. During the same period, 213 SAA patients younger than 17 years underwent BMT from an MFD and were registered into the TRUMP, which provided a unique opportunity to investigate updated evidence for treatment decisions in pediatric SAA, although this study also had limitations due to its retrospective nature.

This study confirmed the excellent outcomes obtained in Japanese children with SAA treated with BMT from an MFD or IST. Consistent with the EBMT studies,[10,22] the survival of children with SAA initially treated with IST has improved markedly since the 1980s, when first-line IST gave greatly inferior survival (with overall survival rates of around 40-50%) when compared with first-line BMT[1,5,6,20,21]; in the current analyses, the probability of overall survival at 10 years in the patients treated first-line with IST reached 88%, which was comparable to that of the group treated first-line with BMT. Recent significant advances in second-line SCT, especially with a matched unrelated donor, may contribute to this marked improvement in survival after first-line IST.[25-27] In our series, a certain number of patients underwent SCT from an alternative donor after failed IST as a second- or third-line treatment. When patients were subdivided into three groups (first-line BMT from an MFD, IST only, and SCT after failed IST groups), the 10-year overall survival rates in these groups were 91%, 93% and 79%, respectively (P<0.0001), confirming that, in the case of failure of IST, SCT from an alternative donor is a very good salvage option, whereas MFD BMT and IST are excellent first-line treatments for children with SAA.

Regarding survival with response after first-line treatment, we found that the failure-free survival rate in patients treated with IST plateaued over the past two decades after having slightly improved since the 1980s (from 40% in the 1980s to 56% currently).[1] Thus, unlike the overall survival results, failure-free survival in the IST group was significantly inferior to that in the MFD BMT group. Consistent with our observations, the EBMT group also demonstrated no significant improvement in outcomes in response to IST since the 1990s.[10] This may suggest that the IST regimen has not improved over time. Over the past decade, with the hypothesis that more intense IST might produce better outcomes, the addition of newer immunosuppressive agents, such as mycophenolate mofetil and sirolimus to antithymocyte globulin and cyclosporine A, has been tested, but has failed to improve responses.[28-31] The combination of antithymocyte globulin and cyclosporine A is, therefore, still regarded as the standard IST regimen. Another possibility is that we have reached a ceiling in the percentage of patients with the capacity to respond to IST.[18] In patients refractory to IST, the pathophysiology of the disease may be different from that in patients responsive to IST, which is thought to involve autoimmune processes, although there are no good markers to routinely or reliably distinguish non-responders from responders.[13,32-34] Further studies are needed to identify patients refractory to IST, because these patients might benefit from prompt alternative donor SCT.

Importantly, all patients in the current analyses were treated with horse antithymocyte globulin (Lymphoglobulin), which has recently been withdrawn from Asian and European markets and replaced by rabbit antithymocyte globulin. To date, there are only limited studies using rabbit antithymocyte globulin as first-line IST for pediatric aplastic anemia, and thus, the effectiveness of this form of antithymocyte globulin for pediatric patients remains controversial.[35-38] The change of product might result in different outcomes in response to IST for children with SAA.

Survival after BMT from an MFD in children with SAA has exceeded 90% for the past two decades, and this has remained unchanged when compared with our previous observation in the 1980s. In this study, the major causes of treatment failure were primary and secondary graft failure, but notably, most patients with secondary graft failure were rescued by second transplantation or careful observation. In addition to short-term complications, long-term sequelae, such as chronic GVHD and late malignancy, should be taken into consideration to make optimal treatment decisions, especially in children. Our results showed that acute and chronic GVHD were relatively uncommon

Table 4. **Multivariate analysis of favorable factors for survival in all 599 patients with SAA.**

Overall survival	Hazard ratio	95% CI	P
First-line treatment: BMT	1.619	0.881-2.977	NS
Treatment period: 2000-2009	1.536	0.556-2.753	NS
Age: <10 years	2.207	1.240-3.927	0.007
Failure-free survival	**Hazard ratio**	**95% CI**	**P**
First-line treatment: BMT	4.497	2.935-6.891	<0.0001
Treatment period: 2000-2009	1.090	0.812-1.464	NS
Age: <10 years	1.113	0.833-1.488	NS

BMT: bone marrow transplantation; NS: not significant.

in the setting of BMT from an MFD for pediatric SAA, which is consistent with recently reported results from the EBMT SAAWP, with 11% of grade II to IV acute GVHD and 4% of extensive chronic GVHD after BMT from an MFD for SAA in all age groups.[39] Regarding late malignancy, Kikuchi *et al.* recently published data from 329 Japanese children with SAA from the nationwide registry, confirming a low incidence of late malignancy after BMT from an MFD; the cumulative incidence of late malignancy was 0.8% at 10 years and 2.5% at 20 years, respectively, which was much lower than the cumulative incidences in reports from western countries.[40] In the present series, only one patient developed a late malignancy (myelodysplastic syndrome), and was saved by second BMT. These observations suggest that this approach has been already established as first-line treatment for children with SAA.

In conclusion, our updated data clearly demonstrate that children receiving BMT from an MFD as first-line treatment have a significant advantage over children managed with first-line IST, given the dramatically better failure-free survival and the lower incidence of associated long-term sequelae in the BMT group, which supports the current algorithm for treatment decisions that recommends BMT for pediatric SAA when an MFD is available. On the other hand, IST using the combination of antithymocyte globulin and cyclosporine A is the treatment of choice for children with SAA without an MFD considering the comparable overall survival with BMT from an MFD, which could possibly be ascribed to recent improvements in outcomes after SCT from an alternative donor. In other words, patients have an excellent chance of survival, even after failed first-line IST, when they undergo second-line SCT from an alternative donor.

Acknowledgments

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Authorship and Disclosures

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Anti–IL-12/23 p40 Antibody Attenuates Experimental Chronic Graft-versus-Host Disease via Suppression of IFN-γ/IL-17–Producing Cells

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Chronic graft-versus-host disease (GVHD) is a major cause of late death and morbidity after allogeneic hematopoietic cell transplantation. Recently, in addition to Th2 cells, Th1 and Th17 cells have been shown to contribute to chronic GVHD progression. IL-12 induces Th1 cells and IL-23 plays a role in stabilizing and/or amplifying Th17 cells, as well as in inducing IFN-γ/IL-17 double-producing cells. Because mAb targeting the p40 subunit common to both IL-12 and IL-23 can inhibit both IL-12R and IL-23R-mediated signaling, we investigated the effects of anti-p40 mAb on a well-defined chronic GVHD mice model. Treatment of anti-p40 mAb in allogeneic recipients significantly reduced the severity of clinical and pathological chronic GVHD. Intracellular staining revealed that IFN-γ single-positive (IL-17$^-$) and IFN-γ/IL-17 double-positive cells were suppressed in anti-p40 mAb–treated allogeneic recipients compared with control recipients. The cytokine levels of IFN-γ and IL-17 were also decreased in serum from anti-p40 mAb–treated allogeneic recipients. T-bet expression of donor IL-17$^+$ CD4$^+$ T cells was reduced significantly in anti-p40 mAb–treated recipients, and this reduction in T-bet expression was associated with IL-22 production by donor T cells. These results suggested that anti-p40 mAb attenuated chronic GVHD via suppression of IFN-γ/IL-17–producing cells, and that targeting the IL-12/IL-23 pathway may represent a promising therapeutic strategy for preventing and treating chronic GVHD.
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Recent progress with various sources of hematopoietic stem cells has enabled many more patients to receive treatment, and a number of patients have now survived for years posttransplant. However, such patients still experience development of graft-versus-host disease (GVHD), a major cause of late death and morbidity (1–3). Chronic GVHD occurs in approximately half of long-term survivors of allogeneic hematopoietic cell transplantation and presents with clinical manifestations similar to those typically observed in autoimmune disease, such as scleroderma and Sjögren syndrome. Although steroids remain the standard initial treatment of chronic GVHD, and half of patients respond to first-line treatment, steroid-refractory chronic GVHD, especially generalized scleroderma, carries a poor prognosis (4, 5).

The pathogenesis of chronic GVHD remains elusive, but recent studies have provided some insights. Donor T cells play a central role in the immunologic attack on host tissues in both acute and chronic GVHD. It has traditionally been assumed that the predominant cytokines produced during acute GVHD are Th1 cytokines, whereas those produced during chronic GVHD are Th2 cytokines. In addition to Th2 cells, recent studies have suggested that multiple cytokines secreted by Th1 and Th17 cells are involved in the pathogenesis of chronic GVHD (6–10). Our previous study showed that Th1 cell and Th17 cell expansion occurred during chronic GVHD and contributed to chronic GVHD progression using a mouse model (10). These results were consistent with clinical studies showing that Th1 and Th17 cells increased in patients with active chronic GVHD (11–15). We also identified a population of donor-derived IFN-γ/IL-17 double-positive cells after only allogeneic transplantation, not syngeneic transplantation, suggesting that this population is generated by allogeneic stimulation (10). These IFN-γ/IL-17 double-producing cells are found in both mice and humans, and much attention is currently focusing on elucidating the role of those cells in various inflammatory diseases (16–25).

The Th17 cell spectrum has been shown to range from "classical" to "alternative" Th17 cells (25). McGeachy et al. (22) showed that Th17 cells generated via TGF-β and IL-6 are nonpathogenic, whereas "alternative" Th17 cells produce IFN-γ and are more pathogenic (16–26). IL-12 induces Th1 cells, and IL-23 promotes the generation of "alternative" Th17 cells (22–28). mAb targeting the p40 subunit common to IL-12 (a heterodimer of p35 and p40) and IL-23 (a heterodimer of p19 and p40) can inhibit both IL-12R and IL-23R-mediated signaling. Anti-p40 mAb is

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S.O. and H.F. conducted the experiments, analyzed the data, and wrote the manuscript; Y.M. designed the experiments, supervised the research, and wrote the manuscript; H.N. performed the research; T.T. performed histopathologic analyses of the organs; A.Y. provided mAbs for the study; and K.-i.M., N.F., E.K., and M.T. supervised the research.

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Abbreviations used in this article: BMT, bone marrow transplantation; GVHD, graft-versus-host disease; PLN, peripheral lymph node; TCD-BM, T cell–depleted bone marrow.

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clinically available as ustekinumab and showed marked efficacy for the treatment of chronic inflammatory disorders such as psoriasis, psoriatic arthritis, and Crohn's disease (29–32). We hypothesized that Th1 and "alternative" Th17 play a role in the pathophysiology of chronic GVHD. We show in this study that blockade of IL-12/IL-23 by anti-p40 mAb reduces chronic GVHD damage using a well-defined mouse model of chronic GVHD.

Materials and Methods

Bone marrow transplantation

Female B10.D2 (H-2^d) donor mice were purchased from Japan SLC (Shizuoka, Japan). Female BALB/c (H-2^d) recipient mice were purchased from Charles River Japan (Yokohama, Japan). All mice were maintained under specific pathogen-free conditions and used at 8–12 wk of age. All animal experiments were performed according to the regulations of the Institutional Animal Care and Research Advisory Committee, Okayama University Advanced Science Research Center. Mice received transplants according to standard protocols described previously (10). In brief, recipient BALB/c mice received a single dose of 5.8-Gy X-ray total body irradiation and were injected with 2×10^6 spleen T cells and 8×10^6 T cell–depleted bone marrow (TCD-BM) cells from B10.D2 donors. T cell depletion and purification were performed using anti-CD90.2 microbeads and an AutoMACS system (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Donor cells were injected i.v. into the recipients on day 0.

Evaluation of chronic GVHD

After bone marrow transplantation (BMT), animals were weighed twice a week and scored for skin manifestations of GVHD. The following scoring system was used (10): healthy appearance, 0; skin lesions with alopecia <1 cm^2 in area, 1; skin lesions with alopecia 1–2 cm^2 in area, 2; and skin lesions with alopecia >2 cm^2 in area, 3. In addition, animals were assigned 0.3 point for skin disease area (lesions or scaling) on each of the ears, tails, and paws. The minimum score was zero, and the maximum was 3.9.

Tissue histopathology

Shaved skin from the interscapular region (~2 cm^2), liver, and salivary gland specimens of recipients were fixed in 10% formalin, embedded in paraffin, sectioned, mounted on slides, and stained with H&E. Skin slides were scored based on dermal fibrosis, fat loss, inflammation, epidermal interface changes, and follicular dropout (0–2 for each category; the maximum score was 10) (10). Liver slides were scored based on bile duct injury and inflammation (0–4 for each category), and the maximum score was 8 (33). Salivary slides were scored on mononuclear cell infiltration and follicular destruction, and the maximum score was 4 (34). All slides were scored by a pathologist (T.T.) blinded to the experimental group.

Intracellular cytokine staining and cytokine analysis

Peripheral lymph nodes (PLNs) were removed from the mice and processed into single-cell suspensions. Skin-infiltrating cells were obtained from skins of the recipient interscapular region (2 cm^2) with a gentleMACS Dissociator (Miltenyi Biotec) and Whole Skin Dissociation Kit (Miltenyi Biotec) according to the manufacturer's protocol. Cells were stimulated in vitro with 50 ng/ml PMA (Sigma-Aldrich, St. Louis, MO) and 100 ng/ml ionomycin (Sigma-Aldrich) at 37°C for 3 h or with anti-CD3 (1 μg/ml) at 37°C overnight (24). Cells were then incubated with GolgiStop (BD Biosciences, Franklin Lakes, NJ) for an additional 2 h. mAbs conjugated to FITC, PE, peridinin–chlorophyll protein complexes, allophycocyanin, Alexa Fluor 647, or Brilliant Violet 421 were used to assess the cell populations and were purchased from BD Pharmingen (Franklin Lakes, NJ) or eBioscience (San Diego, CA). Cells were analyzed on a MACS Quant flow cytometer (Miltenyi Biotec) with FlowJo software (TreeStar, Ashland, OR) that was housed in the Central Research Laboratory, Okayama University Medical School. The total PLN cells were adjusted to 1×10^6/ml in culture. The supernatants were removed, and the cytokine levels were measured by ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol.

Anti-p40 mAb

mAbs (clone C17.8) against p40 (a common subunit of IL-12 and IL-23), provided by Dr. Akihiko Yoshimura, Keio University School of Medicine, were purified by protein G-column chromatography from ascites of nude mice transplanted with hybridoma. Mice were injected i.p. with anti-p40 mAb (500 μg/mice) or rat IgG (Sigma-Aldrich) every 3 d from day 0 after BMT.

Real-time PCR

Total RNA from snap-frozen skin tissues was extracted using the TRIzol reagent (Life Technologies, Tokyo, Japan) according to the manufacturer's protocol. cDNA was synthesized using oligo(dT) primers and SuperScript II reverse transcriptase (Invitrogen). Target cDNA levels were quantified using real-time quantitative PCR with an ABI Prism 5300 system (Applied Biosystems, Tokyo, Japan). TaqMan Universal PCR Master mix, primers, and the fluorescent TaqMan probe specific for murine Tbx21 (Mm00450960_m1), murine ifng (Mm00801778_m1), murine Rorc (Mm00441139_m1), and a housekeeping gene, mGAPDH (Mm99999915-g19), were purchased from Applied Biosystems. The mRNA levels of individual genes were normalized relative to GAPDH, using the cycle threshold (Ct) equation: $\Delta Ct = Ct_{target} - Ct_{GAPDH}$.

Statistical analyses

Group comparisons of skin chronic GVHD scores and pathology scores were performed using the Mann–Whitney *U* test or Kruskal–Wallis test. Cell populations, cytokine levels, mean weights, and gene expression data were analyzed by unpaired Student *t* tests. Survival was evaluated using the log-rank test. All data were analyzed using GraphPad Prism software (version 5.0). The p values <0.05 indicate statistical significance.

Results

Anti–IL-12/23 p40 mAb attenuates murine chronic GVHD

To examine whether anti–IL-12/23 p40 mAb can alleviate chronic GVHD, we used a common chronic GVHD model, the MHC-compatible, murine minor histocompatibility Ag–incompatible allogeneic BMT model (B10.D2 into BALB/c). Sublethally irradiated (5.8 Gy) BALB/c mice were transplanted with 2×10^6 spleen T cells and 8×10^6 TCD-BM cells from B10.D2 mice. As previously reported (10), full donor chimerism (<5% recipient cells) of CD3$^+$ T cells and B220$^+$ B cells were recognized in the spleens, PLNs, and peripheral blood from both the control IgG and the anti-p40 groups on day 14 (Fig. 1A). Anti-p40 mAb was injected peritoneally on every third day from day 0 of BMT. Allogeneic recipients treated with control IgG showed significantly increased clinical chronic GVHD scores compared with syngeneic recipients (Fig. 1B). Allogeneic recipients also showed obvious histopathological damage to the skin and other organs, such as the salivary gland and liver (Fig. 2A). However, we found that anti-p40 mAb significantly ameliorated the clinical score compared with the controls ($p = 0.002$; Fig. 1B). Histopathological examination of the skin on day 28 showed significantly reduced chronic GVHD damage in anti-p40 mAb–treated animals (2.8 ± 0.4 versus 6.0 ± 0.3; $p < 0.01$; Fig. 2B). A dry mouth is one of the distinctive features of chronic GVHD, and lymphocytic inflammation, fibrosis, and atrophy of acinar tissue were observed in the salivary glands of control-treated allogeneic recipients. Histopathological examination of the salivary glands showed reduced chronic GVHD pathology in the anti-p40 mAb–treated recipients (2.0 ± 0.4 versus 3.2 ± 0.2; $p < 0.05$; Fig. 2B). Pathological scores of the liver also tended to be less in anti-p40 mAb–treated recipients compared with control-treated recipients, although it was not statistically significant (Fig. 2B). These findings suggest that anti-p40 mAb attenuated clinical and pathological chronic GVHD. We also examined whether anti-p40 mAb could be used for the treatment of chronic GVHD. Anti-p40 mAb was injected i.p. to mice from day 21 of BMT, when mice had developed clinical signs of chronic GVHD, and anti-p40 mAb significantly improved the clinical scores ($p = 0.03$; Fig. 2C).

Anti-p40 mAb treatment reduced IFN-γ and IL-17 production in donor T cells of PLNs

Cells isolated from PLNs were harvested on day 28 after BMT and analyzed for cytokine expression of those stimulated with PMA and ionomycin (Fig. 3A). Intracellular staining showed that IFN-γ$^+$ (IL-17A$^-$) CD4$^+$ T cells from PLNs of allogeneic recipients were increased and were detected more frequently than PLNs from

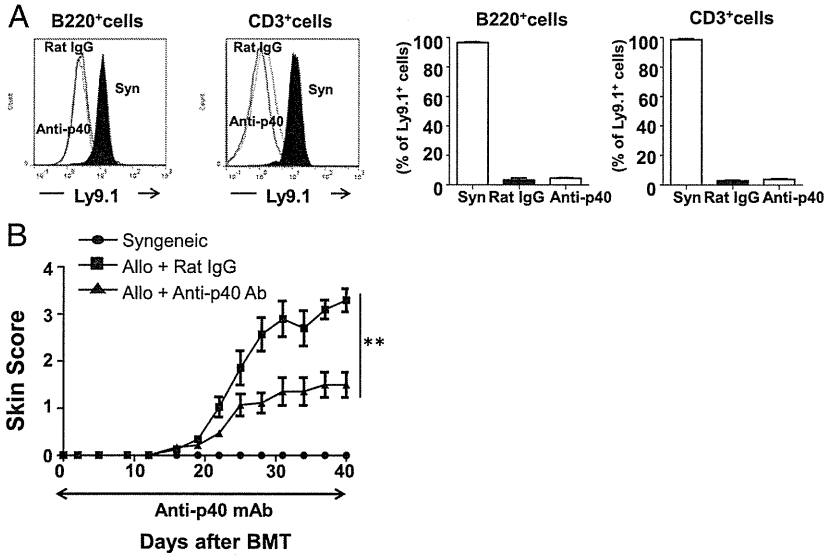

FIGURE 1. Anti-p40 mAb attenuates murine chronic GVHD. Sublethally irradiated (5.8 Gy) BALB/c mice were transplanted with 2×10^6 spleen T cells plus 8×10^6 TCD-BM from WT B10.D2 mice. The syngeneic group received transplantation of the same dose of splenocytes and TCD-BM from BALB/c mice. The allogeneic recipients were treated i.p. with anti-p40 mAb (500 μg/mice) or rat IgG (Sigma-Aldrich) every 3 d from day 0 after BMT. (**A**) Representative histograms of Ly9.1 expression on $B220^+$ cells and $CD3^+$ cells from peripheral blood are shown (*left panels*). Percentages of $Ly9.1^+$ cells among $B220^+$ and $CD3^+$ cells from peripheral blood from day 14 after BMT are shown (*right panels*). Data shown are from one representative of three independent experiments (n = 4–6 in each group). (**B**) Clinical GVHD skin scores are shown. Anti-p40 mAb or rat IgG were administered every 3 d from day 0; data shown are from one representative of three independent experiments (n = 6 in each group). The means ± SE of each group are shown. Data are representative of at least two independent experiments. $^{**}p < 0.01$.

syngeneic recipients, as expected (58 ± 6 versus 3.3 ± 0.3%; p = 0.0005; Fig. 3A). IFN-γ^+ (IL-17A$^-$) CD4$^+$ T cells of anti-p40 mAb–treated recipients tended to be decreased compared with control-treated allogeneic recipients (38 ± 9 versus 58 ± 8%; p = 0.1; Fig. 3A). The IFN-γ/IL-17A double-positive cells were reduced and detected significantly less frequently in anti-p40 mAb–treated recipients (1.5 ± 0.2 versus 4.0 ± 0.4%; p = 0.0003; Fig. 3A). In contrast, IL-17A$^+$ (IFN-γ^-) CD4$^+$ T cells of anti-p40

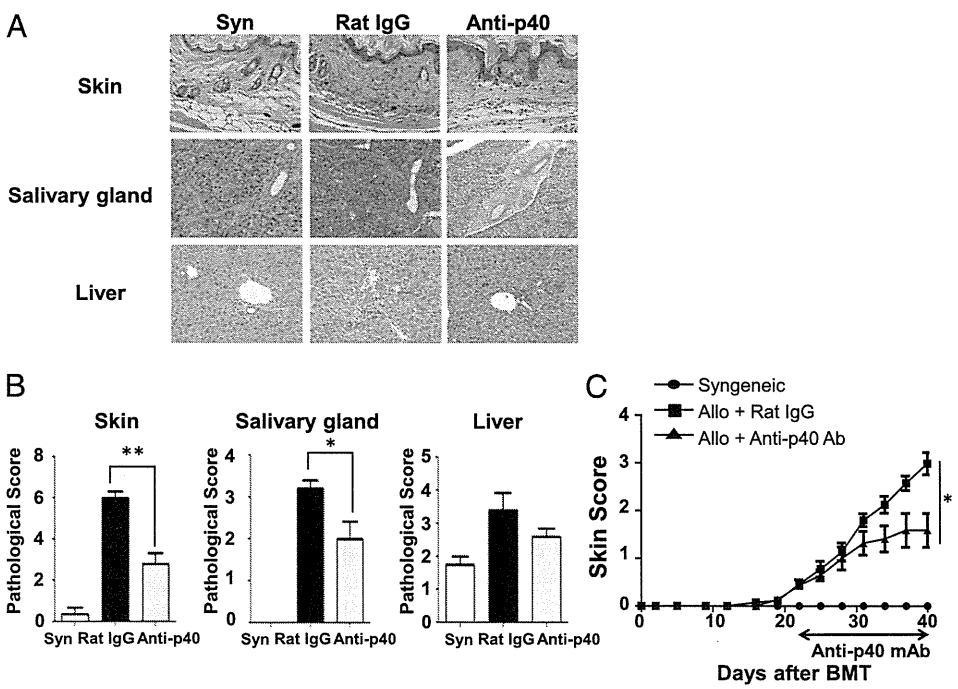

FIGURE 2. Anti-p40 mAb reduces pathological GVHD damages. Sublethally irradiated BALB/c recipients were transplanted from B10.D2 or syngeneic BALB/c donors. The allogeneic recipients were treated i.p. with anti-p40 mAb or rat IgG every 3 d from day 0 after BMT. (**A**) Histopathology of the skin, salivary gland, and liver of syngeneic and allogeneic recipients 28 d after BMT (H&E staining, original magnification ×100). (**B**) Skin, salivary gland, and liver pathology score from syngeneic (white bar), control-treated allogeneic (black bar), or anti-p40 mAb–treated allogeneic (gray bar) recipients are shown. Data shown are from one representative of three independent experiments (n = 6 in each group). (**C**) Clinical GVHD skin scores are shown. Anti-p40 mAb or rat IgG were administered every 3 d from day 21 after BMT. Three to six mice per group were used. The means (±SE) of each group are shown. Data are representative of at least two independent experiments. $^*p < 0.05$, $^{**}p < 0.01$.

mAb–treated recipients did not decrease compared with control-treated allogeneic recipients (7.7 ± 0.9 versus 4.9 ± 1%; $p = 0.1$; Fig. 3A). We also determined intracellular stainings of IFN-γ^+ (IL-17A$^-$), IL-17A$^+$ (IFN-γ^-), and IFN-γ/IL-17A double-positive cells isolated from PLNs stimulated with anti-CD3. Results with anti-CD3 stimulation were similar to those with PMA and ionomycin, and confirmed that anti-p40 mAb treatment reduced both IFN-γ^+ (IL-17A$^-$) cells and IFN-γ/IL-17A double-positive cells (IFN-γ^+ [IL-17A$^-$] cells: 0.33 ± 0.039 versus 3.33 ± 0.97%, $p < 0.05$; IFN-γ/IL-17A double-positive cells: 0.86 ± 0.10 versus 1.72 ± 0.33%, $p < 0.05$; Fig. 3B). Next, we evaluated Th1 and Th17 cell cytokine development after BMT. Cells isolated from PLNs of allogeneic recipients secreted significantly greater amounts of IL-17, IFN-γ, and IL-23 than syngeneic recipients after stimulation with PMA and ionomycin. However, cells isolated from PLNs of anti-p40 mAb–treated allogeneic recipients secreted significantly less IFN-γ and IL-17 than controls (IFN-γ: 1000 ± 200 versus 1500 ± 30 pg/ml, $p = 0.04$; IL-17: 280 ± 50 versus 620 ± 50 pg/ml, $p = 0.002$; Fig. 4A). Although levels of cytokine, especially IFN-γ, from PLNs with anti-CD3 stimulation were lower than those with PMA and ionomycin, results of anti-CD3 stimulation revealed similar results showing that cells isolated from anti-p40 mAb–treated groups secreted less IFN-γ and IL-17 (IFN-γ: 199.4 ± 74 versus 654.1 ± 215 pg/ml, $p = 0.04$; IL-17: 327 ± 135 versus 497 ± 279 pg/ml, $p = 0.58$; Fig. 4A). IL-23 productions from anti-p40 mAb–treated groups also tend to be suppressed, but with no significance. These cytokine levels were also decreased in serum from anti-p40 mAb–treated allogeneic recipients 28 d after BMT (IFN-γ: 10.0 ± 0.6 versus 35 ± 7 pg/ml, $p = 0.03$; IL-17: 2.8 ± 2 versus 7.5 ± 2 pg/ml, $p = 0.2$; Fig. 4B). Collectively, these findings indicated that anti-p40 mAb reduced IFN-γ and IFN-γ/IL-17A double-positive cells, leading to alleviation of chronic GVHD.

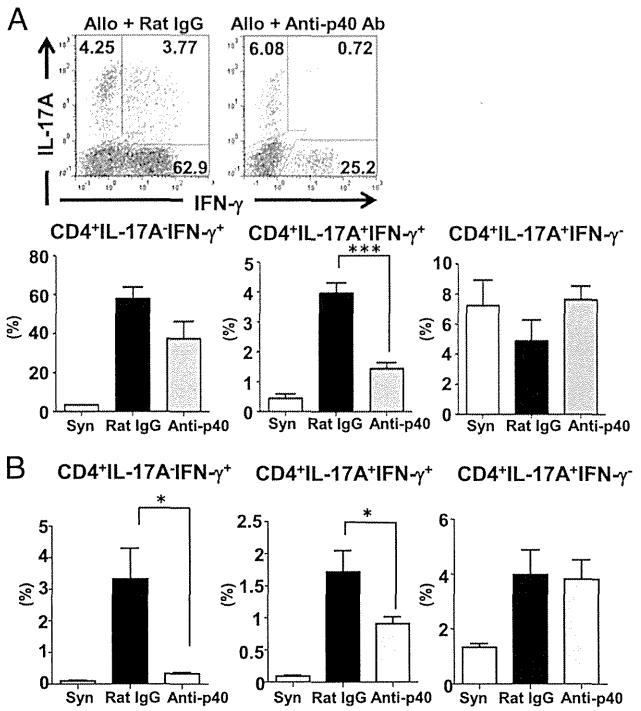

FIGURE 3. Anti-p40 mAb treatment downregulates IFN-γ/IL-17 double-positive cells. Sublethally irradiated BALB/c recipients were transplanted as in Fig. 1. (**A**) Representative staining for intracellular IFN-γ and IL-17A on CD4$^+$ T cells on day 28 for control-treated allogeneic or anti-p40 mAb–treated allogeneic mice stimulated with PMA/ionomycin are shown. PLN cells from syngeneic (white bar), control-treated allogeneic (black bar), or anti-p40 mAb–treated allogeneic (gray bar) recipients were stained for intracellular IFN-γ and IL-17A on day 28 after BMT. The percentages of IL-17A$^-$/IFN-γ^+ cells, IL-17A$^+$/IFN-γ^+ cells, and IL-17A$^+$/IFN-γ^- cells stimulated with PMA/ionomycin (A) and with anti-CD3 (**B**) are shown. Four to seven mice per group were used. The means (± SE) of each group are shown. Data are representative of at least two independent experiments. $*p < 0.05$, $***p < 0.001$.

Anti-p40 mAb treatment suppressed IFN-γ/IL-17A double-positive cells

Because IFN-γ/IL-17A double-positive cells are enriched in the target organs of several autoimmune disease models, it has been suggested that these double producers are particularly pathogenic in tissue inflammation and autoimmunity. These double-positive cells show higher expression of T-bet than IL-17A single-positive T cells (24, 35). Therefore, we examined ROR-γt and T-bet expression in donor IL-17A$^+$ CD4$^+$ T cells isolated from PLNs harvested on day 28 after BMT. ROR-γt and T-bet$^+$ cells in Th17 cells were significantly higher in allogeneic recipients than in syngeneic recipients (Fig. 5A, 5B). Anti-p40 mAb–treated recipients showed significantly lower T-bet expression than controls (PMA/ionomycin: 0.77 ± 0.2 versus 1.6 ± 0.3%, $p = 0.03$; CD3: 0.038 ± 0.012 versus 0.88 ± 0.35%, $p = 0.043$; Fig. 5A); however, both anti-p40 mAb–treated and control-treated allogeneic recipients displayed similar levels in ROR-γt expression after stimulation with either PMA/ionomycin or CD3 (Fig. 5B). This reduction in T-bet expression was associated with IL-22 production by CD4$^+$ T cells from anti-p40 mAb–treated recipients (42 ± 18 versus 110 ± 17 pg/ml, $p = 0.03$; Fig. 5C). IL-22 was reported to be secreted by pathogenic Th17 cells, $\gamma\delta$ T cells, NK cells, and innate lymphoid cell 3 (36). IL-22$^+$ cells were mainly CD4$^+$ cells (>70%) in this allogeneic model, and IL-22$^+$ CD4$^+$ T cells of anti-p40 mAb–treated recipients were decreased compared with control-treated allogeneic recipients (3.07 ± 0.54 versus 7.65 ± 0.70%, $p < 0.001$; Fig. 5D). The levels of IL-22 were also decreased in serum from anti-p40 mAb–treated allogeneic recipients 28 d after BMT (19 ± 5 versus 206 ± 78 pg/ml, $p = 0.04$; Fig. 5E). These results suggested that anti-p40 mAb treatment suppressed Th1 cells and "alternative" Th17 cells, but not "classical" Th 17 cells, during chronic GVHD.

Skins targeted by GVHD showed direct clues of GVHD alleviation

Cytokine production profiles of CD4$^+$ T cells in the PLNs and skin might differ. Analysis of lymphocyte extraction from skin homogenates revealed that anti-p40 mAb treatment reduced IFN-γ^+ (IL-17A$^-$) cells, but not significantly, and reduced IFN-γ/IL-17A double-positive cells significantly (IFN-γ^+ [IL-17A$^-$] cells: 1.08 ± 0.41 versus 1.94 ± 0.28%, $p = 0.16$; IFN-γ^+/IL-17A$^+$ double-positive cells: 0.34 ± 0.072 versus 0.67 ± 0.10%, $p < 0.05$; Fig. 6A). To confirm cytokine gene expression, we also checked gene expression of ROR-γt, Tbx21, and ifng by RT-PCR from skins (Fig. 6B). Tbx21 and ifng expression significantly decreased in the anti-p40 mAb group compared with the allogeneic group. In contrast, ROR-γt expression did not differ in both groups. ELISA analyses of skin homogenates showed that only IFN-γ increased significantly in the control IgG group. IL-17 and IL-23 were similar levels in both control IgG and anti-p40 groups (Fig. 6C). These results suggested that anti-p40 mAb treatment suppressed mainly skin-infiltrating Th1 cells and "alternative" Th17 cells.

Discussion

The results of this study show that anti–IL-12/IL-23 p40 mAb attenuated clinical and pathological chronic GVHD using a well-

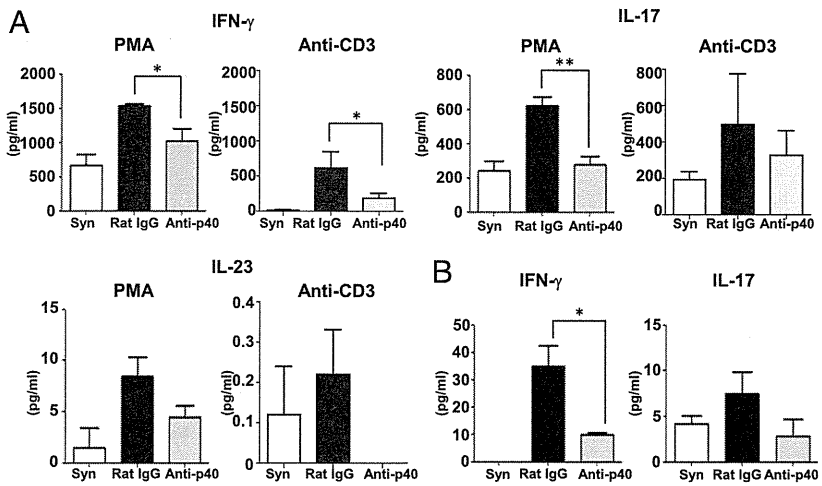

FIGURE 4. Anti-p40 mAb treatment downregulates Th1 and Th17 cell cytokine development. Sublethally irradiated BALB/c recipients were transplanted as in Fig. 1. (**A**) PLN cells from syngeneic (white bar), control-treated allogeneic (black bar), or anti-p40 mAb–treated allogeneic (gray bar) recipients on day 28 were stimulated with PMA/ionomycin or with anti-CD3 in vitro. The supernatants were collected and the cytokine (IFN-γ, IL-17, and IL-23) levels determined by ELISA. Graphs indicate the levels of cytokines secreted per 1×10^6 total stimulated PLN cells. (**B**) Levels of these cytokine were also determined in serum from syngeneic, control-treated allogeneic, or anti-p40 mAb–treated allogeneic recipients on day 28 after BMT. Four to seven mice per group were used. The means (±SE) of each group are shown. Data are representative of at least two independent experiments. $*p < 0.05$, $**p < 0.01$.

defined mouse model of chronic GVHD. Anti-p40 mAb suppressed IFN-γ single-positive (IL-17$^-$) and IFN-γ/IL-17 double-positive cells, whereas IL-17 single-positive (IFN-γ$^-$) cells were not altered compared with control-treated allogeneic recipients. Donor IL-17$^+$ CD4$^+$ T cells of anti-p40 mAb-treated recipients tended to show similar ROR-γt expression compared with control-treated allogeneic recipients. By contrast, T-bet expression of IL-17$^+$ CD4$^+$ T cells was abrogated significantly in anti-p40 mAb–treated recipients. These results suggested that anti-p40 mAb treatment attenuated chronic GVHD via suppression of IFN-γ and IL-17 production.

Th17 cells were divided into two groups: IL-17 single-positive cells that are nonpathogenic "classical" Th17 cells and IFN-γ/IL-17 double-positive cells that are more pathogenic "alternative" Th17 cells. Previously, we showed that IFN-γ/IL-17 double-positive donor cells were detected only in allogeneic recipients with chronic GVHD, but not in syngeneic recipients (10). Recent studies have revealed that IFN-γ/IL-17 double-producing cells are associated with infection or isolated from sites of inflammation (16–25). IFN-γ/IL-17 double-producing cells are isolated from the gut of patients with Crohn's disease and the skin of patients with psoriasis (19, 20). Dander et al. reported that Th17 cells in the

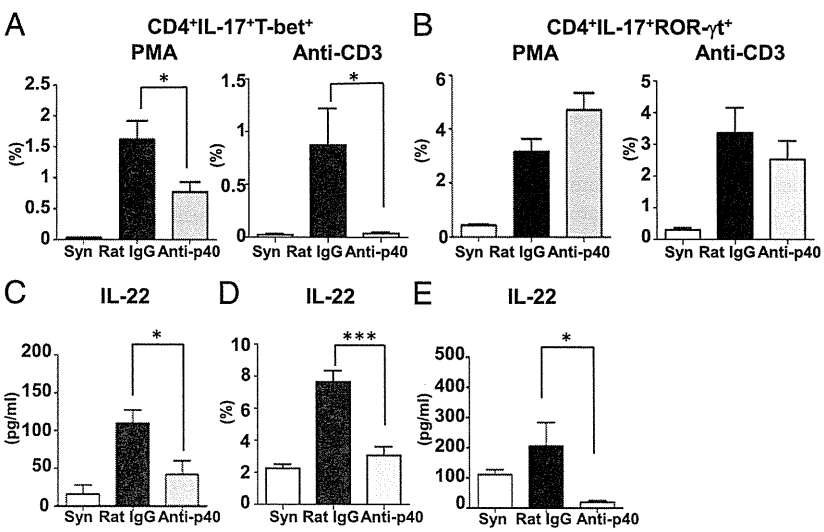

FIGURE 5. Anti-p40 mAb treatment suppresses Th1 cells. Sublethally irradiated BALB/c recipients were transplanted as in Fig. 1. PLN cells from syngeneic (white bar), control-treated allogeneic (black bar), or anti-p40 mAb–treated allogeneic (gray bar) recipients were stained for intracellular IL-17A, T-bet, and ROR-γt of CD4 T cells stimulated with PMA/ionomycin or with anti-CD3 in vivo on day 28 after BMT. The percentages of IL-17A$^+$/T-bet$^+$ cells (**A**) and IL-17A$^+$/ROR-γt$^+$ cells (**B**) are shown. (**C**) PLN cells from syngeneic and allogeneic recipients on day 28 were stimulated with PMA and ionomycin in vitro. Five hours later, the supernatants were collected to determine cytokine levels of IL-22 by ELISA. Graphs indicate the levels of cytokines secreted per 1×10^6 total stimulated PLN cells. (**D**) The percentages of IL-22$^+$ cells on CD4$^+$ cells are shown. (**E**) Levels of IL-22 were also determined in serum from syngeneic, control-treated allogeneic, or anti-p40 mAb–treated allogeneic recipients on day 28 after BMT. Four to seven mice per group were used. The means (±SE) of each group are shown. Data are representative of at least two independent experiments. $*p < 0.05$, $***p < 0.001$.

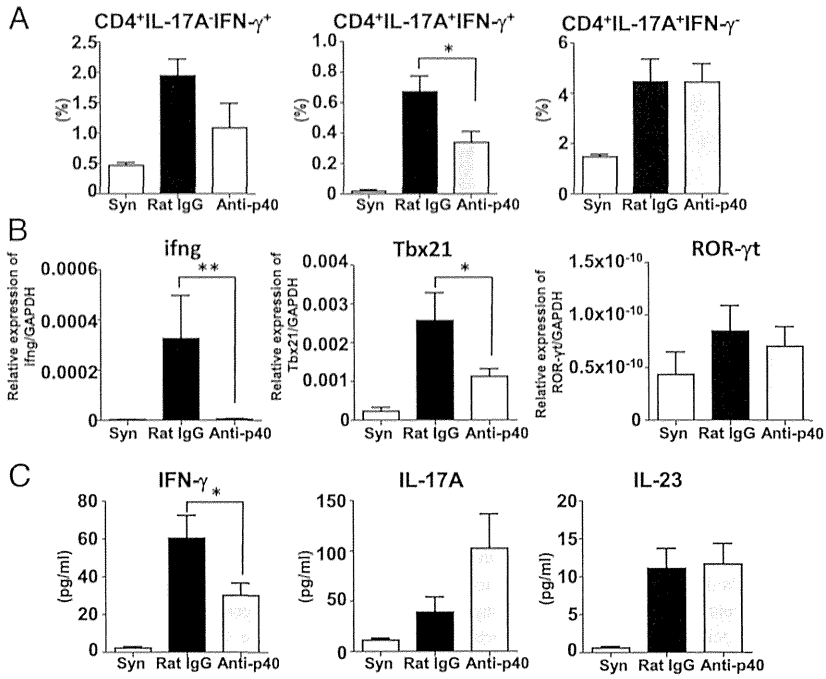

FIGURE 6. Anti-p40 mAb treatment alleviates Th1 cells of the skin. Sublethally irradiated BALB/c recipients were transplanted as in Fig. 1. Cutaneous infiltration cells from syngeneic (white bar), control-treated allogeneic (black bar), or anti-p40 mAb–treated allogeneic (gray bar) recipients were stained for intracellular IFN-γ and IL-17A on day 28 after BMT with PMA/ionomycin. (**A**) The percentages of IL-17A$^-$/IFN-γ$^+$ cells, IL-17A$^+$/IFN-γ$^+$ cells, and IL-17A$^+$/IFN-γ$^-$ cells are shown. (**B**) Ifng, Tbx21, and ROR-γt mRNA from skins of syngeneic, control-treated allogeneic, or anti-p40 mAb–treated allogeneic recipients on day 28 after BMT are shown. (**C**) The supernatants of skin homogenates on day 28 were collected and the cytokine (IFN-γ, IL-17, and IL-23) levels were determined by ELISA. Four to seven mice per group were used. The means (±SE) of each group are shown. Data are representative of at least two independent experiments. $*p < 0.05$, $**p < 0.01$.

peripheral blood of patients with active chronic GVHD expressed IL-23R, and IFN-γ/IL-17 double-positive cells were detected in the liver and skin GVHD lesions (11). IFN-γ/IL-17 double-producing cells arise from Th17 cells and have lower RORγt expression than IL-17 single-positive cells (24). T cells that lack the IL-23R fail to develop into IFN-γ/IL-17 double-producing cells and do not trigger colitis in the T cell transfer model (23). Thus, these cells develop through IL-23–driven upregulation of T-bet and may play an important role in disease pathogenesis (24). In this study, anti-p40 mAb reduced IFN-γ/IL-17 double-positive cells, and donor IL-17$^+$ CD4 T cells showed decreased T-bet expression. Production of IL-22, "alternative" Th17-type cytokine, was also reduced in T cells from anti-p40 mAb–treated recipients. These results suggested that IFN-γ/IL-17 double-producing cells play a role in the pathophysiology of chronic GVHD, and anti-p40 mAb treatment might have shifted the "alternative" Th17 cells to "classical" Th17 cells.

Psoriasis is a chronic, relapsing, immunoinflammatory dermatosis, and Crohn's disease is a chronic inflammatory bowel disease. IL-12 and IL-23, as well as IL-12– and IL-23–mediated Th1 and Th17 cells, are involved in the pathophysiology of psoriasis and Crohn's disease. The human mAb ustekinumab binds the p40 subunit common to IL-12 and IL-23, and shows marked efficacy for the treatment of chronic inflammatory disorders such as psoriasis (29, 30), psoriatic arthritis (31), and Crohn's disease (32). IL-23, rather than IL-12, seems to be essential for the pathogenesis of experimental autoimmune encephalomyelitis, arthritis, and inflammatory bowel disease because IL-23 knockout mice are protected from disease (26, 37, 38). By contrast, Becker et al. (39) reported that because IL-23 cross-regulates IL-12 production, IL-23 knockout mice produce increased levels of IL-12 and were highly susceptible to the development of experimental T cell–mediated 2,4,6-trinitrobenzenesulfonic acid colitis. Blockade of p40 rescued IL-23 knockout mice from lethal colitis. In this study, although blockade of IL-12/IL-23 by anti-p40 mAb effectively suppressed chronic GVHD, further investigation is needed to clarify whether blockade of only the IL-23 pathway can reduce chronic GVHD.

In conclusion, anti-p40 mAb attenuated clinical and pathological chronic GVHD. Anti-p40 mAb suppressed IFN-γ single-positive and IFN-γ/IL-17 double-positive cells, but not IL-17 single-positive cells. T-bet expression in donor IL-17$^+$ CD4 T cells was decreased after anti-p40 mAb treatment, suggesting that anti-p40 mAb suppressed mainly the "alternative" Th17 cells. Modulation of the IL-12/IL-23 pathway may represent a new strategy for the treatment of chronic GVHD, and anti-p40, which is clinically available as ustekinumab, might be a promising therapeutic agent for chronic GVHD.

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Disclosures

The authors have no financial conflicts of interest.

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