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Risk Factors, Treatment, and Immune Dysregulation in Autoimmune Cytopenia after Allogeneic Hematopoietic Stem Cell Transplantation in Pediatric Patients

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A B S T R A C T

Autoimmune or alloimmune cytopenia (AIC) is a known rare complication of hematopoietic stem cell transplantation (SCT). AIC after SCT is considered difficult to treat and is associated with high morbidity and mortality. In this retrospective study in pediatric patients we evaluated incidence, outcome, potential risk factors, and current treatment strategies. A nested matched case-control study was performed to search for biomarkers associated with AIC. Of 531 consecutive SCTs at our center between 2000 and 2016, 26 were complicated by the development of AIC (cumulative incidence, 5.0%) after a median of 5 months post-SCT. Autoimmune hemolytic anemia was the most common AIC with 12 patients (46%). We identified nonmalignant disease, alemtuzumab serotherapy pre-SCT, and cytomegalovirus (CMV) reactivation as independently associated risk factors. The cytokine profile of patients at the time of AIC diagnosis appeared to skew toward a more pronounced Th 2 response compared with control subjects at the corresponding time point post-SCT. Corticosteroids and intravenous immunoglobulin as first-line treatment or a wait-and-see approach led to resolution of AIC in 35% of cases. Addition of step-up therapies rituximab (n = 15), bortezomib (n = 7), or sirolimus (n = 3) was associated with AIC resolution in 40%, 57%, and 100% of cases, respectively. In summary, we identified CMV reactivation post-SCT as a new clinical risk factor for the development of AIC in children. The cytokine profile during AIC appears to favor a Th 2 response. Rituximab, bortezomib, and sirolimus are promising step-up treatment modalities.

INTRODUCTION

Autoimmune or alloimmune cytopenia (AIC) is a relatively rare but serious complication after hematopoietic stem cell transplantation (SCT). Autoimmune or alloimmune hemolytic anemia (AIHA) is the most common AIC, followed by immune thrombocytopenic purpura (ITP) and autoimmune or alloimmune neutropenia (AIN) [1]. Although most patients develop a single AIC, some present with immunity to multiple cell lines. This so-called Evans syndrome, however, mostly combines ITP and AIHA [2].

Treatment of AIC after SCT is challenging, because the disease is often resistant to first-line therapy with steroids and intravenous immunoglobulins (IVIGs) [3], with response rates varying between 36% and 48% [1,4]. Although overall mortality of patients developing AIHA was once as high as 53% [3], second-line therapies with anti-CD20 therapy [1,5] and bortezomib [6,7] have significantly improved survival, with an overall mortality of 15% [1]. Still, little is known about the optimal treatment protocol for AIC after SCT.

AIC post-SCT has mostly been described in case reports, although some retrospective cohort studies have been published [1,4,8]. Cumulative incidences between 2.3% and 6.0% have been reported [1,3]. So far, nonmalignant disease as an indication for SCT and the use of an unrelated donor have been

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identified as clinical risk factors by multiple groups [1,3]. Infectious complications post-SCT, such as cytomegalovirus (CMV) or Epstein-Barr virus (EBV) reactivation, have additionally been hypothesized to play a role in the development of AIC but have not yet been investigated in cohort studies [9]. Furthermore, in-depth analysis of the immune status in SCT patients with AIC and its role in the pathogenesis of this severe complication is lacking. In non-SCT patients with AIHA, elevated levels of both Th 2 cytokines and Th 1 cytokines, as well as an increased number of Th 17 cells, have been reported [10]. The aim of this retrospective study was to evaluate the incidence, potential risk factors, current treatment strategies, and outcome of AIC at our center and to explore the immune dysregulation predisposing to AIC.

METHODS

Patients

Leiden University Medical Center is a national referral center for pediatric SCT. For this study we included all allogeneic SCTs between January 2000 and December 2015 at our center. All patients included in this study and their parents gave consent to register essential patient information about disease state, treatment, and complications in the European Society for Blood and Marrow Transplantation database in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Immune reconstitution analyses were performed with approval of the medical ethical committee of the Leiden University Medical Center (application no. P01.028). Exclusion criteria were absence of informed consent and active AIC as indications for a second SCT. Baseline information regarding diagnosis, transplantation, engraftment, and post-SCT complications were retrieved from the database. A search within patients' complication histories and charts was done to select patients who were diagnosed with AIC after SCT.

Definitions and Diagnosis

Engraftment-related variables were defined as follows: absolute neutrophil count > $.5 \times 10^9$/L at 2 consecutive measurements without neutrophil administration was considered absolute neutrophil count recovery; thrombocyte recovery was achieved when thrombocyte count was >50×10^9/L 1 week after the last transfusion; and full donor chimerism was defined as >95% peripheral blood mononuclear cells of donor origin. Diagnosis of AIHA was made when patients developed anemia with evidence of hemolysis, demonstrated by a positive direct antiglobulin test; elevated reticulocyte count, lactate dehydrogenase, or bilirubin levels; and/or decreased haptoglobin levels. The direct antiglobulin test was used to determine whether IgG, IgM, IgA, or C3b was present on patient RBCs. When a positive screening was present, antibody specificity and the presence of warm and cold antibodies was determined when possible. With the available RBC phenotypes of the patient and donor determined before the SCT, it was concluded whether antibodies were directed against recipient or donor. ITP and AIN were defined as thrombo- or neutropenia with concurrent autoantibodies to thrombocytes or granulocytes, respectively, or when other causes of cytopenia were excluded. Charts were reviewed to identify treatment decisions and efficacy. AIC remission was defined as improvement or stabilization of cytopenia, no active hemolysis in case of AIHA, and with no need for additional therapy for at least 1 year.

Transplantation Procedure and AIC Treatment

Stem cell source, conditioning regimen, pre-SCT serotherapy (with antithymocyte globulin [ATG] or alemtuzumab), and method of graft-versus-host disease (GVHD) prophylaxis were dependent on patient condition, diagnosis, and donor availability and were generally decided on according to European Society for Blood and Marrow Transplantation recommendations for the various underlying diseases. Post-SCT, patients were treated in strict isolation until engraftment had taken place. Patients with positive CMV serology or with a CMV-positive donor received no prophylaxis. EBV, CMV, and adenovirus DNA loads in serum were monitored once weekly for the first 2 months post-SCT and thereafter during every planned visit to the outpatient clinic. Viral reactivation was defined as measurement of a DNA load ≥ 1000 copies/mL in 2 consecutive samples.

Treatment decisions regarding AIC were made at the discretion of the attending physicians and were based on what was considered best practice at the time. There was no specific treatment protocol during the study period. However, preferred first-line treatment of AIC was prednisone, starting at 1 to 2 mg/kg, and/or IVIG (1 g/kg). When the initiated treatment did not induce remission, step-up therapies were initiated or added depending on AIC severity. Step-up therapies consisted of rituximab (375 mg/m^2, 1 to 3 infusions), bortezomib (1.3 mg/m^2, once weekly for 1 to 4 weeks), and sirolimus (.5 to 2 mg/day, depending on weight, aiming at trough levels between 4 and 12 μg/L, until long-term remission). Therapies of last resort were splenectomy, stem cell boost, or a second allogeneic transplantation.

Nested Matched Case-Control Study

For an in-depth analysis of possible immune dysregulation at the time of diagnosis, a nested matched case-control study was performed on all patients with AIC. The AIC patients were matched with 2 non-AIC patients for the following variables: diagnosis (malignant/nonmalignant), donor type, stem cell source, age (difference ≤ 3 years), conditioning regimen (myeloablative or nonmyeloablative), and CMV reactivation. To allow comparison of patients and control subjects at identical time points post-SCT, samples of control subjects had to be available at the time point after SCT at which the corresponding patient was diagnosed with AIC. When there were no suitable control subjects available, we accepted omission of age as a matching factor. Charts of patients and control subjects were reviewed for absolute counts of peripheral blood $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, $CD19^+CD20^+$ B cells, $CD3^-CD16/56^+$ natural killer cells, and $CD3^+TCR\gamma\delta^+$ T cells measured at the post-SCT time point of interest by flow cytometry.

For patients and control subjects with available frozen serum, several cytokines and chemokines were measured using a commercial multiplex cytokine assay (Bio-Rad, Hercules, CA). Furthermore, we analyzed sera of 6 patients with AIHA and 6 matched control subjects obtained at 4 different time points post-SCT: taken at 1 month before the initial decrease in serum hemoglobin, at the day the initial decrease in hemoglobin was first detected, and at 1 month after the initial decrease in hemoglobin and at the time the patients' anemia was resolved. We calculated Th 2/Th 1 cytokine ratios to minimize interindividual variation. In this calculation IL-4, IL-5, IL-6, IL-10, and IL-13 were regarded as Th 2 cytokines, whereas INF-γ, tumor necrosis factor (TNF)-α and IL-12 were regarded as Th 1 cytokines [11].

Statistics

Descriptive statistics were calculated to summarize baseline patient characteristics. Survival was estimated by using the Kaplan-Meier method, and a log-rank test was used on patients with survival > 6 months after the first SCT. To identify potential risk factors for AIC development, univariate Cox regression analysis was performed using the following variables: gender, diagnosis, donor type, stem cell source, conditioning regimen, T cell depletion, GVHD prophylaxis, ATG serotherapy, alemtuzumab serotherapy, ABO match, acute and chronic GVHD, CMV or EBV reactivation or adenovirus infection/reactivation post-SCT, neutrophil recovery, platelet recovery, and peripheral blood mononuclear cell chimerism 100 days post-SCT. Multivariate Cox regression analysis was performed and included all variables with $P < .10$ in the univariate analysis.

Cumulative incidence was calculated using a competing risk analysis where patient death from another cause and another SCT procedure were considered competing risks for the development of AIC. For our matched nested case-control analysis, we performed a paired Wilcoxon signed-rank analysis. Furthermore, for our longitudinal analysis of 6 AIHA patients, we analyzed the separate time points using a Mann-Whitney U test. *P* values were computed using 2-sided tests, and $P < .05$ was considered significant. IBM SPSS Statistics version 23 (IBM, Armonk, NY) was used for statistical testing.

RESULTS

Patients

A total of 479 children underwent 531 consecutive allogeneic SCTs between January 2000 and December 2015. The median follow-up was 48 months. Of these, 26 SCTs were complicated by AIC. The 3-year cumulative incidence was 5.0% (95% confidence interval, 3.4% to 7.3%), as shown in Figure 1. AIHA was the most common AIC with 12 patients (46%), followed by ITP ± AIN (9, 34%), AIHA + ITP ± AIN (4, 15%), and AIN (1, 4%). Cold antibodies were detected in 7 patients with AIHA. In 3 patients no antibodies were detected by screening technique. Importantly, specific antibodies were in 4 patients directed against recipient rhesus antigens (rhesus C, D, E) and in 2 patients against donor antigens (rhesus B, D, E). In 6 patients only panreactive warm autoantibodies without specificity were detected. AIC was diagnosed at a median of 5 months post-SCT (range, 1 to 36). Baseline SCT characteristics of the 2 groups are summarized in Table 1. More detailed

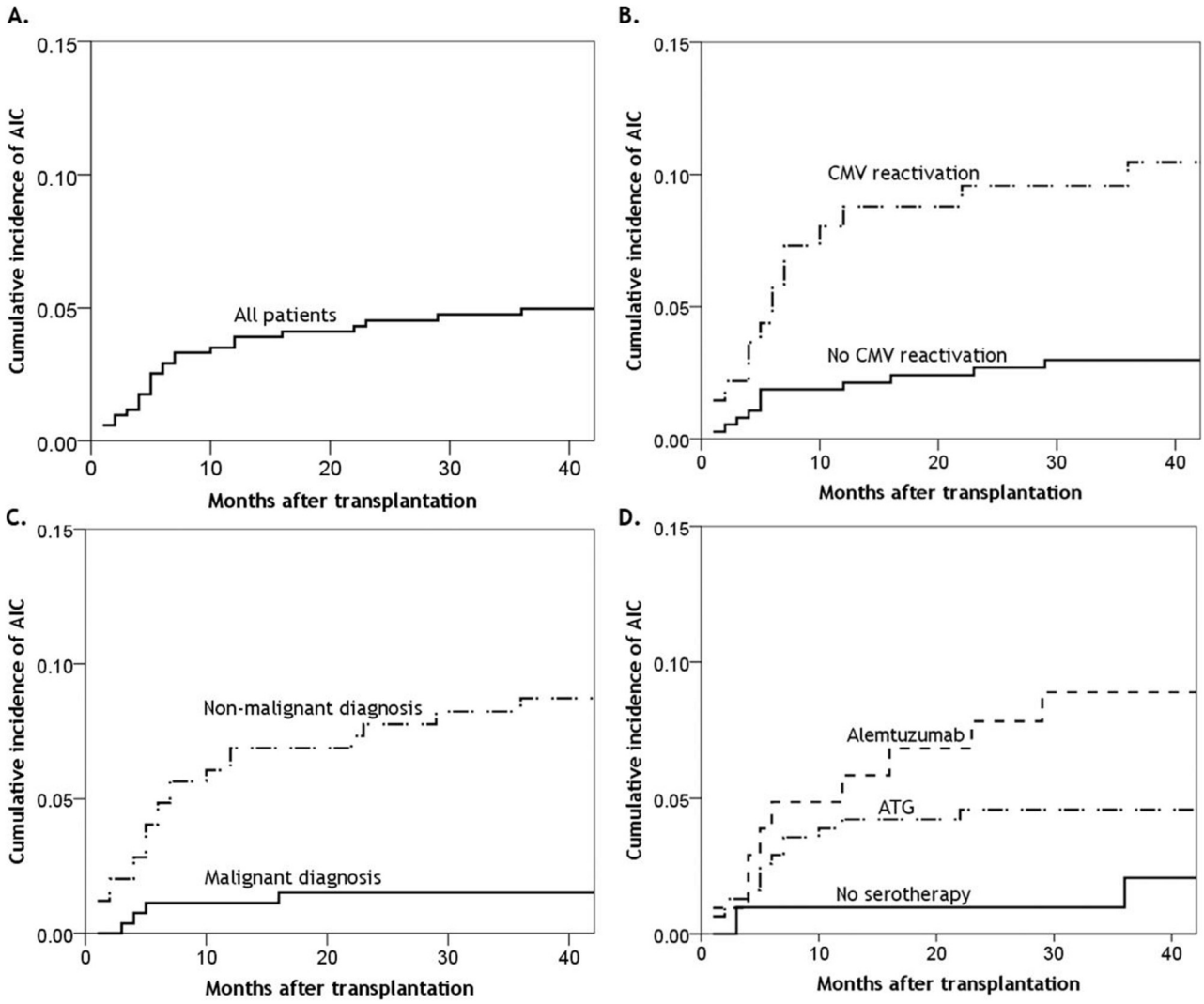

Figure 1. Cumulative incidence of the development of AIC after SCT. (A) All patients. (B) Patients with CMV reactivation versus patient without CMV reactivation. (C) Patients with malignant disease versus patients with non-malignant disease as SCT indication. (D) Patients who received alemtuzumab versus patients who received ATG versus patients who received no serotherapy.

characteristics of the AIC patients are shown in Supplementary Table S1.

Risk Factors

To identify risk factors associated with AIC development, we performed univariate Cox regression analyses (Table 1). Additional stratification by AIC type can be found in Supplementary Table S2. Univariate analysis showed that nonmalignant disease was associated with AIC. Further subclassification of nonmalignant pre-SCT diagnosis, shown in Supplementary Table S3, revealed that AIC was common in β-thalassemia patients (n = 9; cumulative incidence, 11.8% [95% confidence interval, 6% to 22%]) followed by hemophagocytic lymphohistiocytosis (n = 3). Furthermore, non–total body irradiation based conditioning regimen and CMV reactivation post-SCT were associated with a higher risk of developing AIC. Patients who developed AIC were, on average, 2 years younger at the time of transplantation than patients who did not develop AIC ($P = .076$).

Serotherapy with alemtuzumab pre-SCT was not significantly associated with AIC development ($P = .053$) in univariate analysis of all patients. However, alemtuzumab serotherapy was associated with a higher risk of ITP ± AIN ($P = .011$). Gender, donor type, stem cell source, conditioning regimen, T cell depletion, GVHD prophylaxis, ATG serotherapy pre-SCT, ABO match, HLA mismatch, acute and chronic GVHD, EBV reactivation, adenovirus infection/reactivation, platelet recovery, absolute neutrophil count recovery, and chimerism in peripheral blood mononuclear cells were not associated with AIC. We subsequently performed multivariate Cox regression analysis to control for confounding factors. CMV reactivation (hazard ratio, 3.4; $P = .02$), nonmalignant diagnosis pre-SCT (hazard ratio, 3.5; $P = .031$), and alemtuzumab use (hazard ratio, 2.5; $P = .028$) were independently associated with the occurrence of AIC (Table 2). For patients with CMV reactivation, diagnosis of AIC was made at a median of 4.5 months (interquartile range, 1 to 9) after detection of the maximum viral load.

Nested Matched Case-Control Study

To study the immunologic background of this complication we performed a nested matched case-control analysis

Table 1
SCT Characteristics and AIC Risk Factors—Univariate Cox Regression Analysis

	No AIC (n = 505)	AIC (n = 26)	*P*-value
Gender, male	336 (66.5)	18 (69.2)	.782
Age (mean [range])	8.3 [0-19]	6.3 [1-15]	**.076**
Diagnosis			**.001**
Malignant	273 (54.1)	4 (15.4)	
Non-malignant	232 (45.9)	22 (84.6)	
Donor type			.368
Unrelated	288 (57.0)	17 (65.4)	
Identical related	147 (29.1)	5 (19.2)	
Other related	70 (13.9)	4 (15.4)	
HLA match			.555
10/10	263 (52.2)	12 (46.2)	
≤ 9/10	121 (24.0)	8 (30.8)	
Not registered	121 (24.0)	6 (23.1)	
Stem cell source			.739
Bone marrow	353 (69.9)	19 (73.1)	
Peripheral blood	107 (21.2)	7 (26.9)	
Cord blood	45 (8.9)	-	
Conditioning regimen			.667
Myeloablative	451 (89.3)	22 (84.1)	
Reduced intensity	36 (7.1)	3 (11.5)	
Non-myeloablative	18 (3.6)	1 (3.8)	
Irradiation, any	145 (28.7)	2 (7.7)	**.032**
T-cell depletion	93 (18.4)	4 (15.4)	.927
GVHD prophylaxis			.550
CSA + MTX ± other	322 (63.8)	20 (76.9)	
CSA ± other	133 (26.3)	4 (15.4)	
Other/none	50 (9.9)	2 (7.7)	
Serotherapy			
ATG	321 (63.6)	15 (57.7)	.606
Alemtuzumab	105 (20.8)	9 (34.6)	.053
No serotherapy	102 (20.2)	2 (7.7)	.124
ABO match	257 (50.9)	14 (53.8)	.914
Acute GVHD, any	102 (20.2)	3 (11.5)	.257
Gr I–II	66 (13.1)	2 (7.7)	
Gr III-IV	36 (7.1)	1 (3.8)	
Chronic GVHD, any	56 (11.1)	3 (11.5)	.779
Limited	26 (5.1)	-	
Extensive	30 (5.9)	3 (11.5)	
CMV reactivation	126 (25.0)	14 (53.8)	**.001**
CMV status*			**.006**
Serology+/reactivation+	126 (25.0)	14 (53.8)	
Serology+/reactivation–	199 (39.4)	7 (26.9)	
Serology–/reactivation–	180 (35.6)	5 (19.2)	
EBV reactivation	103 (20.4)	5 (19.2)	.875
Adenovirus infection/ reactivation	98 (19.4)	6 (23.1)	.378
absolute neutrophil count recovery (n = 391) (median days [IQR])	22 [18-27]	22 [17-29]	.989
Platelet recovery (n = 486) (median days, [IQR])	31 [24-42]	27.5 [20-42]	.457
Chimerism† (n = 482)			.724
Full donor	376 (74.6)	21 (77.8)	
Mixed	80 (15.9)	5 (18.5)	

All values are expressed as number (n) and percentage (in between brackets), unless otherwise specified.
Abbreviations: ATG, anti-thymocyte globulin; CSA, cyclosporine A; MTX methotrexate; IQR, interquartile range.
* Donor and/or recipient CMV serology pre-SCT/CMV reactivation post-SCT.
† Chimerism in peripheral blood mononuclear cells (PBMC) evaluated 100 days post-SCT.

for immune status, cytokines, and chemokines. Twenty-five patients qualified for inclusion, but 1 patient was excluded because of a lack of stored serum samples.

Immune status regarding the counts of $CD3^+CD4^+$ T cell subset, $CD19^+CD20^+$ B cells, $CD3^-CD16/56^+$ natural killer cells, and $CD3^+TCR\gamma\delta^+$ cells did not differ significantly between patients at the time of AIC diagnosis and control subjects at the corresponding time point after SCT. However, AIC patients had a significantly lower $CD3^+CD8^+$ T cell count ($P = .002$). Further stratification showed significantly higher $CD3^+CD8^+$ T cell counts of patients with CMV reactivation post-SCT (Supplementary Figure S1). Median cytokine serum levels of AIC patients and control subjects are summarized in Supplementary Table S4. AIC patients had significantly lower serum levels of IL-4 (median difference, .62 pg/mL; $P = .004$), eotaxin (difference, 22 pg/mL; $P = .015$), platelet-derived growth factor BB (difference, 464 pg/mL; $P < .001$), and RANTES (difference, 951 pg/mL; $P < .001$) and higher levels of IL-6 (difference, 7.6 pg/mL; $P = .020$), IL-16 (difference, 117 pg/mL; $P = .001$), and IL-2 ($P = .027$).

Table 2
Multivariate Cox Regression Analysis of Risk Factors Associated with the Development of AIC

Risk factor	HR [95% CI]	*P*-value
Age at SCT (per year)	.96 [.9-1.0]	.232
Malignant vs. non-malignant	3.49 [1.1-10.9]	**.031**
Irradiation vs. no irradiation	.44 [.1-2.0]	.289
Alemtuzumab vs. no alemtuzumab	2.5 [1.1-5.7]	**.028**
CMV status		
Serology +, reactivation+ vs. serology–, reactivation–	3.42 [1.2-9.6]	**.020**
Serology +, reactivation– vs. serology–, reactivation–	.93 [.3-3.0]	.900

Abbreviations: HR: hazard ratio, CI: confidence interval.

To test our hypothesis that AIC after SCT is a Th 2 mediated disease, we focused on comparison of Th 2/Th 1 ratios between patients and control subjects. Ratios are summarized in Figure 2. The Th 2/Th 1 ratios IL-5/IFN-γ ($P = .044$), IL-5/TNF-α ($P = .017$), IL-5/IL-12 ($P = .016$), IL-6/IFN-γ ($P < .001$), IL-6/TNF-α ($P = .001$), IL-13/IFN-γ ($P = .002$), IL-13/TNF-α ($P = .003$), IL-13/IL-12 ($P = .007$), and IL-10/TNF-α ($P = .029$) were significantly higher in AIC patients compared with control subjects, whereas the IL-4/IFN-γ ratio was slightly lower ($P = .049$). In the longitudinal analysis of 6 patients with AIHA, there appeared to be an increase in median serum IL-5 concentration, median IL-5/TNF-α ratio, and median IL-5/IL-12 ratio (Figure 3). However, these differences were not statistically significant compared with the 6 control subjects.

Treatment and Outcome

Patients were treated with a median of 3 treatment modalities (range, 0 to 6). A complete list of treatment modalities and achieved remission percentages is shown in Table 3. Treatment and remission data for separate AICs and patients can be found in Supplementary Tables S5 and S6. First-line treatment, usually with prednisone and/or IVIG, or a wait-and-see approach was sufficient in 9 patients (35%). The wait-and-see approach was sometimes chosen for non–life-threatening AIC. Prednisone and IVIG as first-line therapy generally did not induce remission, with achieved remission percentages of 13% (n = 2, after 2 and 8 weeks) and 9% (n = 1, after 4 weeks), respectively. Additionally, step-up treatments were initiated in 17 patients after a median of 2.5 weeks (range 1 to 8), usually with continuation of prednisone. Rituximab was initiated in 14 patients and associated with resolution of AIC in 5 patients (36%) in 2 to 12 weeks (median, 5.5). Step-up treatment with bortezomib or sirolimus was given to, respectively, 7 and 3 patients and was associated with remission in 57% and 100% of patients. A second SCT or stem cell boost was performed in 4 patients of which 3 AICs resolved. In summary, with the mentioned therapies 24 patients (92%) eventually achieved remission. Patients with

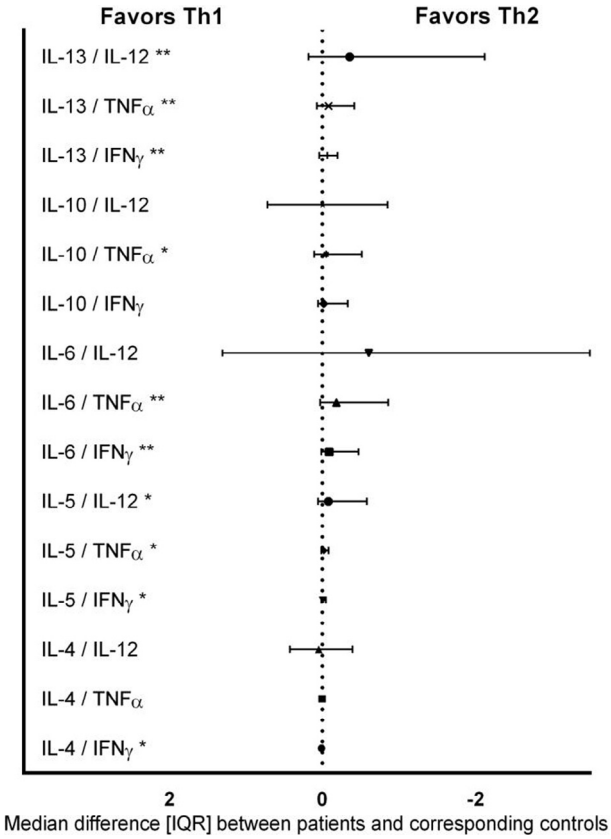

Figure 2. Th2/Th1 cytokine ratios in serum. Visual representation of the median differences between AIC patients (n = 25) and their corresponding control subjects (n = 50) for several Th2/Th1 ratios. Cytokines were measured in sera obtained at the time of AIC diagnosis and at the corresponding time point after SCT in the respective control subjects. $^{*}P < .05$, $^{**}P < .01$.

AIHA or AIHA + ITP ± AIN were transfused with a median of 3.5 units of RBCs (range, 0 to 19) during their treatment.

Of 24 AIC patients, 19 (79%) were still alive at last follow-up (Supplementary Table S6). One patient died due to multiorgan failure attributable to AIHA treatment. Another patient, who was diagnosed with AIC after 2 separate SCTs, developed a lethal engraftment syndrome after a third SCT to treat graft failure and ITP + AIN. Of the 3 other patients, 1 patient developed cerebral vasculitis; another patient died of a neurologic disorder, possibly because of toxicity of the SCT conditioning regimen; and 1 patient died of relapse of the original disease. When comparing all-cause mortality after the median follow-up of 48 months, there was no significant difference between AIC and non-AIC patients ($P = .887$).

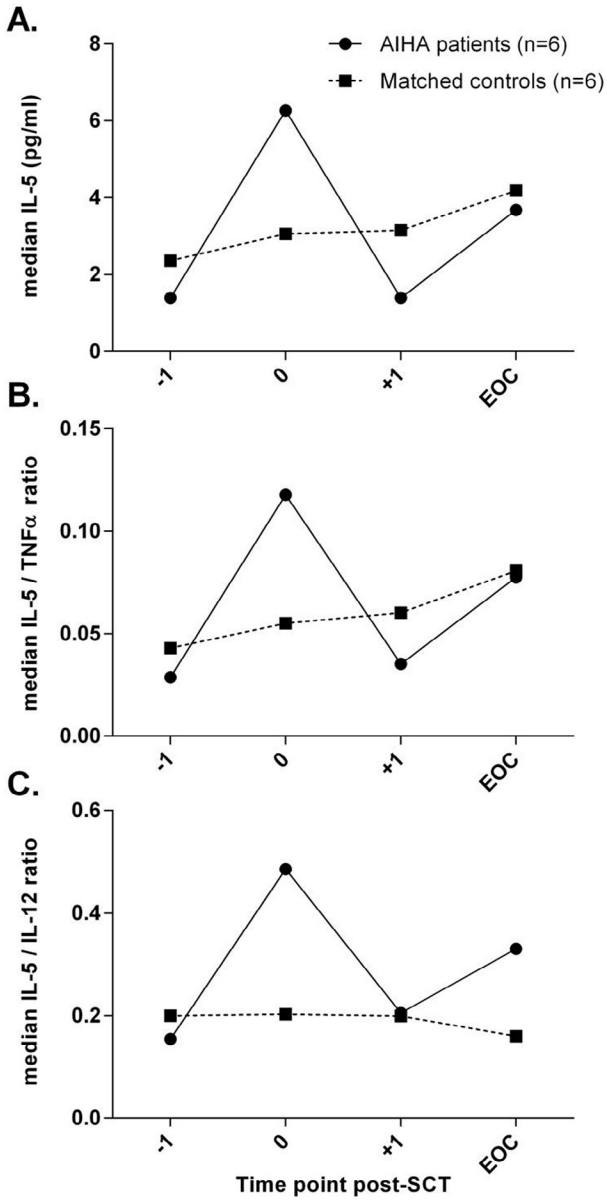

Figure 3. Longitudinal cytokine analysis of AIHA patients. Longitudinal analysis of six patients with AIHA compared with six matched control subjects. Median values of both groups are shown. Time points post-SCT are defined as follows: −1: one month prior to initial decrease in serum hemoglobin; 0: Start of decrease in hemoglobin; +1: one month after the decrease in hemoglobin; EOC: end of cytopenia. Statistics of differences at time point 0: median IL-5 concentration $P = .11$; median IL-5/TNF-α ratio $P = .15$; C. median IL-5/IL-12 ratio $P = .15$.

DISCUSSION

Here we report the incidence, potential risk factors, immune status, cytokine profile, and current treatment strategies in patients with AIC after allogeneic SCT. In our cohort the 3-year cumulative incidence of AIC was 5.0%, which is in line with previously reported incidences [3,4,12]. AIC was diagnosed at a median of 5 months post-SCT, and AIHA and ITP ± AIN were most frequently observed.

We identified nonmalignant disease to be a clinical risk factor for AIC development. The association between nonmalignant disease as SCT indication and AIC has been observed in other cohorts, although the underlying mechanism is unclear [1,3,4]. A major contributor to this association was β-thalassemia (9/26 patients in the present cohort), which has also been reported earlier [8]. Possibly, patients with β-thalassemia are sensitized to the development of AIC because of a history of multiple blood transfusions or another, yet unknown, mechanism.

We have identified CMV reactivation as a major risk factor for the development of AIC. In the past, CMV has been hypothesized as a causative factor in the development of autoimmunity post-SCT [9]. Several mechanisms could contribute to the development of AIC. First, it has previously been reported that CMV reactivation post-SCT can mediate nonspecific polyclonal B cell stimulation resulting in the

Table 3
Treatment Characteristics of AIC patients

	All AIC patients	
	Treatment N (%)	Remission N (% of treated patients)
Total	26 (100)	24 (92)
Wait-and-see/G-CSF	6 (23)	5 (83)
First-line treatment (n = 21)		
Prednisone	15 (71)	2 (13)
IVIG	11 (52)	1 (9)
Rituximab	1 (5)	1 (100)
Second and subsequent-line treatments (n = 17)		
Rituximab	14 (82)	5 (36)
Bortezomib	7 (41)	4 (57)
MPP	3 (18)	0 (0)
SCT/SC boost	4 (24)	3 (75)
Sirolimus	3 (18)	3 (100)
Splenectomy	3 (18)	2 (67)
Plasmapheresis	1 (6)	0 (0)
Prednisone*	1 (6)	1 (100)
IVIG*	5 (29)	3 (60)

Data are presented as number of patients with percentages in between brackets. Therapies are generally initiated while continuing previous treatments. Treatment trajectories of individual patients are summarized in supplementary Table S6.
MPP, methylprednisolone pulse; SCT/SC boost, Stem cell transplantation (n = 3) or stem cell boost (n = 1); G-CSF, granulocyte colony-stimulating factor.
* In combination with other second-line treatment modality.

production of various autoantibodies and monoclonal gammopathies [13]. CMV infection has also been associated with autoantibody production after liver transplantation [14]. Molecular mimicry has been hypothesized as a mechanism. However, CMV epitopes that mimic hematopoietic cells have not yet been identified. Furthermore, CMV reactivation after SCT causes a major expansion of $CD8^+$ T cells and leaves a dynamic imprint on the T cell compartment [15], which can possibly result in a proinflammatory environment, causing nonspecific bystander activation of autoreactive $CD4^+$ T cells. Our subjects with CMV reactivation also showed higher $CD8^+$ T cell counts. However, $CD8^+$ T cell counts in AIC patients were significantly lower compared with control subjects. $CD4^+$ T cell counts did not differ significantly but appeared to be slightly higher in AIC patients with CMV reactivation (Supplementary Figure S1). Other viral infections post-SCT (EBV, adenovirus) were not associated with AIC development. Nevertheless, not all patients with AIC had a previous CMV reactivation, which supports the multifactorial etiology of AIC.

We also found alemtuzumab serotherapy pre-SCT to be associated with development of AIC. In the past alemtuzumab has been shown to significantly prolong immune reconstitution post-SCT [16,17]. We hypothesize that the delayed in vivo B cell and T cell recovery after alemtuzumab, as compared with ATG, may result in increased dysregulation during the immune reconstitution, possibly leading to AIC. However, the choice to apply alemtuzumab for all patients who undergo a second SCT and require serotherapy and the more frequent use of alemtuzumab in nonmalignant disease, together with the previously reported association between alemtuzumab and a higher rate of CMV reactivation, may confound this result [18].

In other studies transplantation with non-identical related donor grafts, cord blood transplantation, and extensive chronic GVHD have been associated with development of AIC or AIHA [1,3,19]. These associations were not confirmed in the current cohort. Although we did observe an 8% higher proportion of unrelated donor transplants in the AIC group, the difference was not significant. Our center has a relatively low proportion of cord blood transplantations and a low incidence of chronic GVHD (11%), which may account for a lack of association with AIC in these particular subgroups [20].

We investigated 28 cytokines and chemokines for an association with AIC development in a nested matched case-control analysis at the time of diagnosis (Supplementary Table S4). Because of the autoantibody-mediated nature of AIC, we hypothesized that serum levels of Th 2–specific cytokines IL-4, IL-5, IL-6, and IL-13 would be significantly increased compared with control subjects. We failed to demonstrate this association. Although IL-6 concentrations were higher in AIC patients, concentrations of IL-4 were significantly decreased compared with control subjects, possibly because of high interindividual variation in absolute cytokine concentrations. We subsequently focused on analyzing Th 2/Th 1 cytokine ratios to reduce this variation, as previously performed by other groups [21,22]. In this analysis we found several Th 2/Th 1 ratios to be significantly higher in AIC patients (Figure 2), suggesting that the balance in patients is skewed toward a Th 2 response. The significantly lower concentration of RANTES, which has been identified as a Th 1–related chemokine, in AIC patients is an observation that may support this assertion [23,24]. Furthermore, the results of the longitudinal analysis of 6 AIHA patients (Figure 3) also point toward a Th 2 mediated pathophysiology, although these results were not statistically significant, possibly because of a lack of statistical power.

In our experience the preferred first-line treatment with prednisone and IVIG is often insufficient, leading to AIC remission in only 13% and 9% of patients. In the absence of controlled trials on treatment of AIC, we recommend early addition of second-line therapy with rituximab, bortezomib, or sirolimus for patients with severe and life-threatening AIC. The efficacy of rituximab for AIC, particularly AIHA, has been well established, but the effect usually has to be awaited for several weeks [1,4,5]. Bortezomib, a proteasome inhibitor, targets plasma cells and effectively depletes the antibody-producing compartment. It has shown efficacy for refractory autoantibody-mediated autoimmunity [6,7,25,26]. The mechanistic target of the rapamycin (mTOR) inhibitor sirolimus has shown similar efficacy for refractory cytopenias [27]. Interpretation of efficacy of the single treatment components in our patients is biased, because patients often receive step-up therapies like rituximab, bortezomib, and sirolimus in addition to steroids. However, we consider both bortezomib and sirolimus as promising step-up therapies of AIC in our population. After multiple lines of therapy, remission was achieved in 92% of patients. As a consequence, all-cause mortality of AIC patients (21%) was lower than that reported in other cohorts [3,12].

Strengths of this study are the relatively large number of AIC patients included and the large amount of information that could be retrieved from patient charts and the European Society for Blood and Marrow Transplantation database. Few cohort studies have focused on AIC after SCT, and none has analyzed CMV reactivation as a possible risk factor or immune status at the time of AIC diagnosis.

This study also has several limitations. Because auto-/allo-antibodies were not measured in all patients who presented with mild cytopenia, subclinical AICs may have gone undetected or been attributed to poor engraftment. A further limitation is that donor and patient typing was not so

complete that real autoimmunity or a donor versus patient or vice versa AIC could be differentiated. Mostly, however, the late post-SCT timing of AIC with confirmed complete donor chimerism suggests autoimmune dysregulation as the cause of the AIC. Finally, the design of this nonrandomized cohort study may have introduced confounding of treatment results. A randomized controlled trial would be an ideal setup but might be difficult in a single center because of the relatively low incidence of AIC. We believe future research should focus on prospective, multicenter studies with a larger cohort of AIC cases to confirm risk factors, treatment efficacy, and the proposed Th 2 mediated pathophysiology.

In conclusion, in this single-center retrospective study of 531 consecutive allogeneic SCTs, the cumulative incidence of AIC was 5.0%. CMV reactivation, alemtuzumab serotherapy pre-SCT, and nonmalignant disease as an indication for SCT were identified as independently associated risk factors for the development of AIC. The cytokine profile of patients appears to favor a Th 2 response. Although first-line therapy with corticosteroids and IVIG often gave an insufficient or untimely response, 92% of patients achieved eventual remission of AIC after second-line treatment. For patients with severe or refractory AIC, rituximab, bortezomib, or sirolimus can be regarded as promising step-up therapies.

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SUPPLEMENTARY DATA

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