Biol Blood Marrow Transplant 25 (2019) 712–719

Biology of Blood and Marrow Transplantation

journal homepage: www.bbmt.org

Phase I/II Trial of a Combination of Anti-CD3/CD7 Immunotoxins for Steroid-Refractory Acute Graft-versus-Host Disease

Christoph Groth[1], Lenneke F.J. van Groningen[2,3], Tiago R. Matos[4], Manita E. Bremmers[2], Frank W.M.B. Preijers[5], Harry Dolstra[5], Christian Reicherts[1], Nicolaas P.M. Schaap[2,3], Eric H.G. van Hooren[6], Joanna IntHout[3,7], Rosalinde Masereeuw[8], Mihai G. Netea[9], John E. Levine[10], George Morales[10], James L. Ferrara[10], Nicole M.A. Blijlevens[2,3], Ypke V.J.M. van Oosterhout[6], Matthias Stelljes[1], Walter J.F.M. van der Velden[2,3,*]

[1] *Department of Medicine A/Hematology and Oncology, University Hospital of Muenster, Muenster, Germany*
[2] *Department of Hematology, Radboud University Medical Center, Nijmegen, The Netherlands*
[3] *Radboud Institute of Health Sciences, Radboud University Medical Center, Nijmegen, The Netherlands*
[4] *Department of Dermatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands*
[5] *Department of Laboratory Medicine, Laboratory for Hematology, Radboud University Medical Center, Nijmegen, The Netherlands*
[6] *Xenikos B.V., Nijmegen, The Netherlands*
[7] *Section of Biostatistics, Department for Health Evidence, Radboud University Medical Center, Nijmegen, The Netherlands*
[8] *Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, The Netherlands*
[9] *Department of Internal Medicine, Radboud University Medical Center, Nijmegen, The Netherlands*
[10] *Tisch Cancer Institute, The Icahn School of Medicine at Mount Sinai Hospital, New York, NY*

Article history:
Received 26 August 2018
Accepted 25 October 2018

Key Words:
Immunotoxin
Acute graft-versus-host disease
Allogenic hematopoietic stem cell transplantation

A B S T R A C T

Effective therapies for treating patients with steroid-refractory acute graft-versus-host-disease (SR-aGVHD), particularly strategies that reduce the duration of immunosuppression following remission, are urgently needed. The investigated immunotoxin combination consists of a mixture of anti-CD3 and anti-CD7 antibodies separately conjugated to recombinant ricin A (CD3/CD7-IT), which induces in vivo depletion of T cells and natural killer (NK) cells and suppresses T cell receptor activation. We conducted a phase I/II trial to examine the safety and efficacy of CD3/CD7-IT in 20 patients with SR-aGVHD; 17 of these patients (85%) had severe SR-aGVHD, and all 20 patients had visceral organ involvement, including 18 (90%) with gastrointestinal (GI) involvement and 5 (25%) with liver involvement. A validated 2-biomarker algorithm classified the majority of patients (11 of 20) as high risk. On day 28 after the start of CD3/CD7-IT therapy, the overall response rate was 60% (12 of 20), with 10 patients (50%) achieving a complete response. The 6-month overall survival rate was 60% (12 of 20), including 64% (7 of 11) classified as high risk by biomarkers. The 1-week course of treatment with CD3/CD7-IT caused profound but transient depletion of T cells and NK cells, followed by rapid recovery of the immune system with a diverse TCR Vβ repertoire, and preservation of Epstein-Barr virus- and cytomegalovirus-specific T cell clones. Furthermore, our results indicate that CD3/CD7-IT appeared to be safe and well tolerated, with a relatively low prevalence of manageable and reversible adverse events, primarily worsening of hypoalbuminemia, microangiopathy, and thrombocytopenia. These encouraging results suggest that CD3/CD7-IT may improve patient outcomes in patients with SR-aGVHD.

INTRODUCTION

Acute graft-versus-host disease (aGVHD) is a major complication that can occur following allogenic hematopoietic stem cell transplantation (HSCT). The prognosis for patients who develop aGVHD is poor, particularly in cases of severe steroid-refractory aGVHD (SR-aGVHD) with gastrointestinal (GI) and/or liver involvement [1,2]. At present, no standard second-line therapy is approved for SR-aGVHD, and none of the available treatment options seems to provide convincingly superior results with on average only 30% complete responders [1,3,4]. Six-month survival approximates 50%, but long-term survival is achieved in only 1 out of 5 patients [2].

The underlying core of a graft-versus-host immune reaction is the proliferation and differentiation of alloreactive donor

Financial disclosure: See Acknowledgments on page 718.

* Correspondence and reprint requests: Walter J. F. M. van der Velden, Department of Hematology, Radboud University Medical Center, PO Box 9101, 6500 HB Nijmegen, The Netherlands.
E-mail address: Walter.vanderVelden@radboudumc.nl (W.J.F.M. van der Velden).

https://doi.org/10.1016/j.bbmt.2018.10.020
1083-8791/

T cells in response to the host's antigen-presenting cells, which induce tissue damage and the propagation of inflammation during the effector phase [5–7]. Therefore, many of the currently used therapies consist of antibodies that cause the depletion of T cells or either biologicals or small-molecule inhibitors designed to suppress T cell function [1,3,6,8]. The obvious challenge of such approaches is that the induced immunosuppression should be as selective and as brief as possible, to avoid infectious complications and relapse of the underlying hematologic malignancy, which otherwise could counterbalance the immediate benefit of controlling the aGVHD reaction [9–12].

As a novel approach to achieving this goal, we developed a combination of 2 anti-T cell immunotoxins designed to induce a synergistic in vivo depletion and suppression of T cells while allowing for rapid post-treatment reconstitution of the immune system [13,14]. This combination product consists of a 1:1 mixture of 2 murine monoclonal antibodies against CD3 and CD7, each of which is separately conjugated to a recombinant ricin toxin A chain [15] (T-Guard, designated CD3/CD7-IT hereinafter) [16,17]. Preclinical studies have shown that CD3/CD7-IT induces apoptosis of both T cells—particularly activated T cells—and natural killer (NK) cells by inhibiting protein synthesis, and also reduces T cell activation by blocking and modulating the TCR/CD3 complex (supplementary 1 etcetera; Figure 1) [17]. In a dose-escalation study, 5 out of 7 patients with SR-aGVHD responded to CD3/CD7-IT as third-line therapy [17]. The promising outcome of that study led to the phase I/II study of CD3/CD7-IT for the treatment of SR-aGVHD reported here.

METHODS

This prospective single-arm phase I/II study was approved by the Ethics Committees and Institutional Review Boards at the Radboud University Medical Center Nijmegen and the University Medical Center Muenster. Informed consent was obtained from all patients. This trial has been registered at www.ClinicalTrials.gov (NCT02027805).

The 4 mg/m^2 T-Guard starting dose of the phase I/II study was selected on basis of the outcome of the dose escalation study [17]. A Bryant-Day 2-stage design was applied [18], with a prescheduled interim analysis after 8 patients, to protect patients from unnecessary exposure to an ineffective or toxic treatment. If after the first 8 patients, 2 or fewer ($\leq$25%) day 28 responders and/or 4 or more ($\geq$50%) dose-limiting toxicities (ie, adverse drug reactions of grade 3 or higher) were observed (phase I), the trial would be terminated for futility and/or toxicity; otherwise, the trial would be extended to a total of 20 patients (phase II) (sample size estimation, S2).

Adult patients (age $\geq$18 years) who developed grade II-IV aGVHD following HSCT or following post-transplantation donor lymphocyte infusion [19] were eligible for participation; aGVHD grade was defined according to the criteria established by Harris et al. [20]. Diagnosis of aGVHD was confirmed with a tissue biopsy. SR-aGVHD was defined as aGVHD that progressed after 3 days or did not improve after 7 days on systemic corticosteroid therapy ($\geq$2 mg/kg/day prednisolone or equivalent) [3,4]. Patients who had already received additional therapy for SR-aGVHD were excluded, as were patients with manifestations of moderate or severe chronic GVHD (cGVHD), severe organ dysfunction, uncontrolled infection, serum creatinine level >266 μmol/L (1.87mg/dL), and/or serum albumin level $\leq$1.5 g/dL.

The treatment schedule for CD3/CD7-IT (S3) consisted of four 4-hour i.v. infusions of 4 mg/m^2 administered at 48-hour intervals. GVHD prophylaxis, which consisted primarily of cyclosporine A either alone or in combination with mycophenolate mofetil, was continued during with CD3/CD7-IT therapy. The recommended taper for systemic corticosteroids in patients responding to CD3/CD7-IT was 10% of the starting dose at 3- to 5-day intervals. After study day 28, the rate of steroid tapering was left to local protocols. The use of antimicrobial prophylaxis, preemptive and/or empirical treatment for infection, and clemastine pretreatment (2 mg i.v.) was left to the discretion of the physician and established local protocols.

Patients were included in the analysis of toxicity and efficacy if they received at least 1 dose of CD3/CD7-IT. The primary endpoints were the overall response rate (ORR; defined as the sum of partial response [PR] and complete response [CR] rates) on day 28 and the occurrence of possible drug-related adverse events (AEs) up to 6 months following treatment with CD3/CD7-IT. The secondary endpoints were the day 28 CR rate, 6-month overall survival (OS), and the incidence of cGVHD. ORR, CR on day 28, and 6-month OS were compared with data recorded for our institutions' historical controls who received either inolimomab-etanercept (n = 21) or infliximab (n = 21) (S4; Table 1) [21]. CR was defined as the resolution of all signs and symptoms associated with aGVHD. PR was defined as an improvement in GVHD stage in all initial GVHD target organs, without complete resolution or emergence of GVHD in any new organ. No response was defined as no change, a mixed response, progressive disease, or the need for salvage therapy before day 28 [22]. The 2014 National Institutes of Health diagnostic criteria were used to assess and score cGVHD [23]. Hematologic and nonhematologic AEs, including cytokine release syndrome (CRS), were graded based on the Common Terminology Criteria for AEs, version 4.0. Capillary leak syndrome was graded as follows using previously defined criteria [24]: grade 1, asymptomatic, not requiring therapy; grade 2, symptomatic but not requiring fluid support; grade 3, respiratory compromise or requiring fluids; grade 4, life-threatening, requiring vasopressor support and/or mechanical ventilation. In the event of a grade 3 AE, subsequent doses with CD3/CD7-IT were to be given only if the patient's toxicity parameters improved or when judged to be in the patient's interest, at the investigator's discretion. Invasive fungal disease (IFD), Epstein-Barr virus (EBV) infection, and cytomegalovirus (CMV) infection were defined in accordance with established guidelines [25–27].

Manufacturing of CD3/CD7-IT

CD3/CD7-IT consists of the murine monoclonal antibodies SPV-T3a (anti-CD3) and WT1 (anti-CD7), each of which is conjugated to recombinant ricin toxin A (RTA). CD3/CD7-IT was manufactured following Good Manufacturing Practices as described previously [15], with the addition of a step to block residual linkers with cysteine and the replacement of deglycosylated plant-derived RTA with recombinant RTA [17,28]. The immunotoxins were formulated at a concentration of 0.2 mg/mL in an isotonic buffered solution (pH 6.5) and stored frozen at -20°C or below.

In Vitro Laboratory Analyses

Peripheral blood samples were collected before and after treatment to analyze predictive GVHD biomarkers, cytokine levels, immune reconstitution, pharmacokinetics, and the development of human anti-drug antibodies (ADAs).

Levels of the biomarkers ST2 (suppression of tumorigenicity 2) and Reg3α (regenerating islet-derived protein 3-α) were measured at the Icahn School of Medicine at Mount Sinai, New York. A probability score was determined for each patient based on a validated algorithm [29] used to predict the risk for treatment failure and nonrelapse mortality among patients with aGVHD. Patients were considered at high risk at $\hat{p}$ > .291 after 1 week $\pm$ 3 days of treatment with systemic corticosteroids.

Serum cytokine levels were measured at Myriad RBM (Austin, TX) using quantitative, multiplexed immunoassays (S5).

Lymphocytes were analyzed by immunophenotyping using flow cytometry. Lymphocytes were gated on CD45$^+$ and low side-scatter cells, and enumeration of helper T cells (CD5$^+$ and CD4$^+$), cytotoxic T cells (CD5$^+$ and CD8$^+$), NK cells (CD56$^+$ and CD5$^-$), and B cells (CD19$^+$) was recorded for each phenotype per microliter of blood. CD5 was used instead of CD3 to identify and quantify T cells because of potential CD3 modulation by the CD3/CD7-IT treatment. For TCR sequencing, DNA was isolated from whole blood collected in PAXgene tubes (PreAnalytiX, Hombrechtikon, Switzerland). The TCRβ CDR3 region was then amplified and sequenced using ImmunoSEQ (Adaptive Biotechnologies, Seattle, WA). Bias-controlled V and J gene primers were used to amplify the rearranged V(D)J segments for high-throughput sequencing (HTS) analysis at approximately 20x coverage [30]. After correcting for sequencing errors using a clustering algorithm, CDR3 segments were annotated using the International ImMunoGeneTics information system, thereby identifying which V, D, and J genes contributed to each rearrangement [31]. The absolute numbers of EBV-associated and CMV-associated T cells were determined by comparing the patients' TCRβ data with TCRβ sequences reported to be specific for EBV and CMV antigens [32].

The serum concentrations of SPV-T3a-RTA and WT1-RTA, as well as the presence of ADAs against either of these immunotoxins, were measured at Celonic AG (Basel, Switzerland) using validated bioluminescence assays. Pharmacokinetics analyses were performed as described previously (S6 and S7) [17].

Statistical Analysis

Patient characteristics were analyzed using descriptive statistics. The estimated aGVHD response rates along with the 95% Clopper-Pearson exact confidence interval (CI) are presented. Toxicity was analyzed by tabulating the incidence of AEs and/or infections with a Common Terminology Criteria for AEs grade $\geq$2. Kaplan-Meier curves were used to analyze OS. The chi-square test was used to compare the ORR and the rates of CR and PR on day 28 after initiation of CD3/CD7-IT therapy, with the corresponding results obtained from institutional historical controls who received either

inolimomab-etanercept (n = 21) or infliximab (n = 21) [21]. The 6-month OS rate was compared using the log-rank test.

Within-patient differences in immunoreconstitution were analyzed in the pretreatment, 1-month, 3-month, and 6-month samples using the Wilcoxon matched-pairs signed-rank test. A 2-sided *P* value <.05 was considered statistically significant. Expanded and enriched T cell clones were identified using differential abundance analysis as described by DeWitt et al. [33]. A given clone was determined to be significantly expanded or contracted in 2 samples based on its proportion in each repertoire or time point and was analyzed using the Fisher exact test with Benjamini-Hochberg correction at the 5% level.

RESULTS

Patient and GVHD Characteristics

Twenty patients were enrolled in the study between June 2014 and September 2016. Patient, donor, and GVHD characteristics are presented in Table 1. At the time of enrollment, 3 patients (15%) had grade II aGVHD and 17 had grade III or IV aGVHD (85%). Sixteen patients (80%) had involvement of 2 organs, with the GI tract and liver involved in 18 (90%) and 5 (25%) cases, respectively. Baseline albumin levels were low, particularly in the patients with GI GVHD (median, 2.3 g/dL; range, 1.6 to 3.4 g/dL; normal range, 3.5 to 5.0 g/dL). A validated algorithm using serum concentrations of ST2 and Reg3α demonstrated a significant risk for all patients with a mean $\hat{p}>$ value of .345; the majority of patients (11 of 20) were classified as high risk for treatment failure and nonrelapse mortality [29]. Treatment with CD3/CD7-IT was initiated after a median interval of 8 days (range, 5 to 16 days) after the initial corticosteroid treatment.

GVHD Response and Patient Outcomes

The median duration of follow-up after therapy with CD3/CD7-IT was 292 days (range, 3 to 889 days). Two patients died due to progressive SR-aGVHD before completing the treatment schedule. The remaining 18 patients (90%) received all 4 scheduled doses at 48-hour intervals. On day 28, ORR was 60% (12 of 20 patients), with a 95% CI of 36% to 81%; 10 patients (50%; 95% CI, 27% to 73%) achieved CR (Figure 1). In the 12 responding patients, corticosteroids could be tapered according to protocol (S8; Figure 7). ORR was 55% (6 of 11) in patients with a high-risk biomarker profile. At the 6-month time point, 12 patients had survived, corresponding to an OS of 60% (95% CI, 36% to 78%) (Figure 1); survival was 64% (7 of 11) in patients with a high-risk biomarker profile (S9; Figure 8). Ten of the 12 surviving patients had achieved a PR or CR. Causes of death for the 8 patients who died during the trial were refractory aGVHD in 4 patients, refractory GVHD with infection in 3 patients, and pseudomembranous colitis in 1 patient.

The outcomes achieved with CD3/CD7-IT were favorable compared with the outcomes reported for the cohort of 42 patients included immediately adjacent to the start of the trial. Specifically, the CR rate was 50% versus 19% (*P* = .012), and the 6-month OS was 60% versus 29% (*P* = .021). To compensate for differences in aGVHD severity at the start of treatment, the foregoing analysis was repeated after adjustment for overall aGVHD grading. After adjustment for aGVHD grade [20], the CR and OS rates remained significant (*P* = .032 and .034, respectively) (S10). At the 2-year follow-up, OS was still better in the study cohort compared with the historical controls (35% versus 16.7%; *P* = .047 and .09, respectively). Three of the 12 patients (25%) who survived to the 6-month time point developed cGVHD, which was mild in 2 patients and severe in 1 patient. Relapse occurred in 3 patients who underwent transplantation for acute myelogenous leukemia with adverse risk features at a median of 4 months after CD3/CD7-IT therapy.

Table 1
Patient Characteristics and HSCT and GVHD Features

Characteristic	Value
Number of patients	20
Age, yr, median (range)	53 (18-74)
Sex, male/female, n (%)	9 (45)/11 (55)
Diagnosis, n (%)	
Myeloid malignancy	15 (75)
Lymphoid malignancy	5 (25)
Donor type, n (%)	
Matched unrelated donor	13 (65)
Matched related donor	5 (25)
MMUD	1 (5)
Haploidentical related	1 (5)
Stem cell source	
Peripheral blood stem cells	19 (95)
Bone marrow	1 (5)
Disease Risk Index, n (%)	
Low	0
Intermediate	5 (25)
High	15 (75)
Conditioning regimen, n (%)*	
MAC	6 (30)
RIC	5 (25)
NMA	9 (45)
GVHD prophylaxis, n (%)	
CyA	5 (25)
CyA/MTX	1 (5)
CyA/MMF (post-CyA)	13 (1) (65)
aGVHD, n (%)	
Post-HSCT	19 (95)
Post-donor lymphocyte infusion	1 (5)
aGVHD grade at enrollment, n (%)	
II	3 (15)
III	11 (55)
IV	6 (30)
Organ involvement, n (%)	
Skin	15 (75)
Liver	5 (25)
Intestinal	18 (90)
2 organs involved	16 (80)
Biomarker score at start of CD3/CD7-IT therapy, high risk ($\hat{p}>.291$), n (%)	11 (55)
Time to aGVHD, d, median (range)	40 (10-308)
Time to treatment with CD3/CD7-IT, d, median (range)†	8 (5-16)

MAC, myeloablative conditioning; NMA, nonmyeloablative conditioning; RIC, reduced-intensity conditioning; CyA, cyclosporin A; MTX, methotrexate; MMF, mycophenolate mofetil.

* NMA conditioning consisted of fludarabine (Flu)-total body irradiation (TBI), RIC regimens were Flu-busulfan (Bu)- and Flu-melphalan (Mel)-based, and MAC regimens were Cy-TBI-, Flu-Mel-TBI-, or Flu-AraC-Amsa/Cy-TBI-based.

† Relative to the initial corticosteroid treatment.

Safety

The Data and Safety Monitoring Board reviewed the preplanned interim analysis of the first 8 patients, based on which they concluded that no major safety concerns had arisen and that the observed risk-benefit balance warranted continuation of the study. In general, CD3/CD7-IT was well tolerated and found to be safe, with no suspected unexpected serious adverse reactions or serious AEs related to the study drug reported. Although no clinically significant infusion-related reactions were recorded, 2 patients who had not received pretreatment experienced chills that resolved quickly after clemastine treatment (grade 2 AE). Most of the patients had elevated levels of markers of macrophage activation/recruitment (MCP-1 and MIP-1β), and this increase was most prominent after the first infusion; however, only the 2 aforementioned patients who experienced chills also had an increase in IL-6 levels [34,35]. The remaining patients had no increase in IL-6, IL-8, IL-10, or IFN-γ concentrations, nor did they develop clinical signs corresponding to CRS (S5).

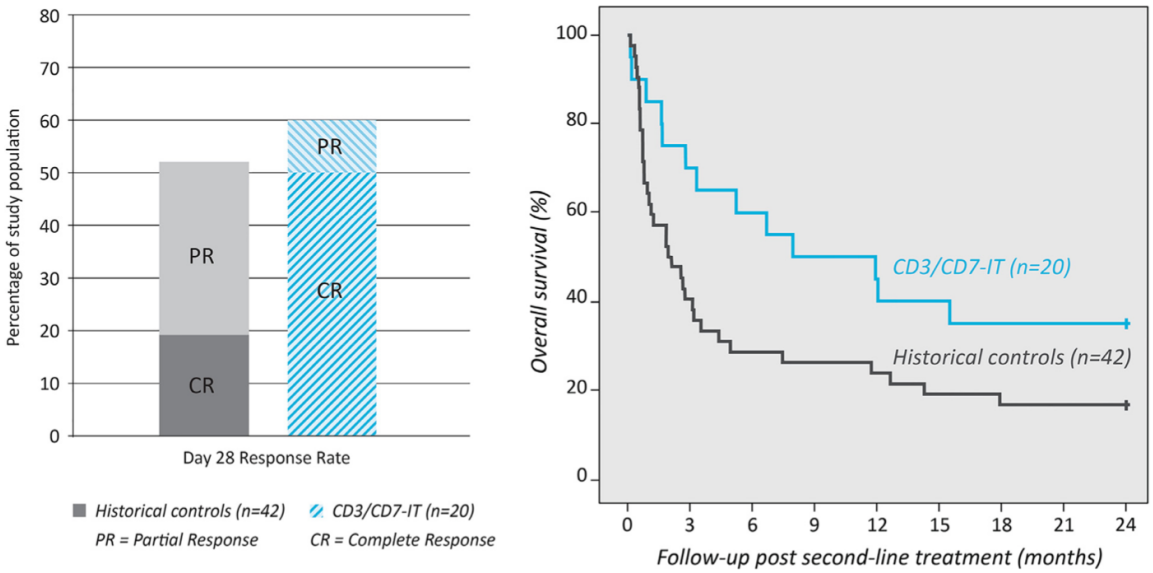

Figure 1. Overview of the response rate at day 28 (top) and OS after treatment with CD3/CD7-IT (bottom) compared with historical controls. The difference between patients who received CD3/CD7-IT and the historical controls was statistically significant, with improvements in both the CR rate (P = .012) and 6-month OS (P = .021). All survivors reached the 24 month milestone.

Several of the 20 patients developed a limited number of possible treatment-related AEs, including hypoalbuminemia, microangiopathy, and/or thrombocytopenia (Table 2). Hypoalbuminemia was present in all 20 patients at baseline (grade 2 or 3 in 80% of the patients) and may have worsened in 8 patients due to treatment with CD3/CD7-IT. These 8 patients developed mild peripheral edema, which could be easily managed with diuretics in all but 1 patient. One patient required treatment with an albumin infusion and diuretics for generalized edema and marked weight gain; thus, this patient was classified as having grade 2 capillary leak syndrome. Fifteen patients (75%) had a preexisting low platelet count (grade 3 or 4 in 25% of cases), and thrombocytopenia either occurred or worsened in 14 patients (70%). Although various other causes might have contributed to the development of thrombocytopenia, the time course was at least suggestive of a possible relationship with CD3/CD7-IT in 9 patients. Nevertheless, the thrombocytopenia was transient, did not result in a bleeding event, and rarely required platelet transfusion. Early EBV and CMV infections (within 3 months) were observed in 3 patients each (with 2 patients positive for both EBV and CMV); however, no EBV or CMV disease occurred. Although only 40% of patients received mold-active antifungal prophylaxis, IFD was not observed in any of the patients. Nevertheless, as expected in this setting, the number of infections and AEs was relatively high. Two patients developed a *Clostridium difficile* infection, and 1 of them died due to pseudomembranous colitis. Moreover, although 5 patients developed bacteremia (with infection by enterococci in 2 patients, staphylococci in 2 patients, and *Klebsiella oxytoca* in 1 patient), the incidence rate (25%) was not higher than that reported in historical controls [21].

After treatment with CD3/CD7-IT, ADAs against SPV-T3a-RTA and/or WT1-RTA were detected in 10 out of 20 patients (50%). In 4 of these 10 patients, the titers were ≥20,000 at any given point (S7); nonetheless, no cases of serum sickness were reported. The emergence of ADAs was considered of little clinical relevance, because ADAs typically form after 9 to 10 days, whereas CD3/CD7-IT is currently offered as a 1-week treatment option only, and its serum half-life is only 9 hours.

Pharmacokinetics

Pharmacokinetics analysis revealed a mean serum half-life and mean maximum concentration of CD3/CD7-IT of 8.59 ± 3.04 hours and 1231 ± 671 μg/L, respectively (S6), which is consistent with previously published data [17].

Immune Reconstitution and Antiviral Immunity

Consistent with its intended effect, treatment with CD3/CD7-IT led to a profound depletion of T cells and NK cells, with

Table 2
Summary of AEs Potentially Related to Treatment

Grade 2*	Grade 3	Grade 4
Anemia (1)	Thrombocytopenia (3)	Thrombocytopenia (5)
Abdominal pain (1)	Neutropenia (1)	
Thrombocytopenia (1)	Elevated bilirubin (2)	
Neutropenia (1)	Myopathy (1)	
Microangiopathy (1)	Microangiopathy (1)	
Chills (2)	Hypoalbuminemia (1)	
Capillary leak syndrome (1)		
Hypoalbuminemia (1)		

The numbers in parentheses refer to the number of patients who experienced the indicated AE.
* Grading of each AE is based on version 4.0 of the Common Terminology Criteria for AEs, with the exception of capillary leak syndrome, which was graded using the system described by Messmann et al. [24].

rapid recovery starting as early as the second week after treatment (Figure 2A and B). Importantly, no significant effect on the absolute B cell count was observed (Figure 2C). No apparent patterns were seen in terms of treatment-induced changes in the relative proportions of naïve, memory, effector, and effector memory T cells before and after treatment start, as well as no decrease in or reversal of the CD4:CD8 ratio. In addition, the absolute count of regulatory T cells (Tregs) and the percentage of Tregs in the $CD4^+$ cell population showed normal variation, with no obvious upward or downward trends observed at 28 days after initiation of treatment or during the remainder of the follow-up period.

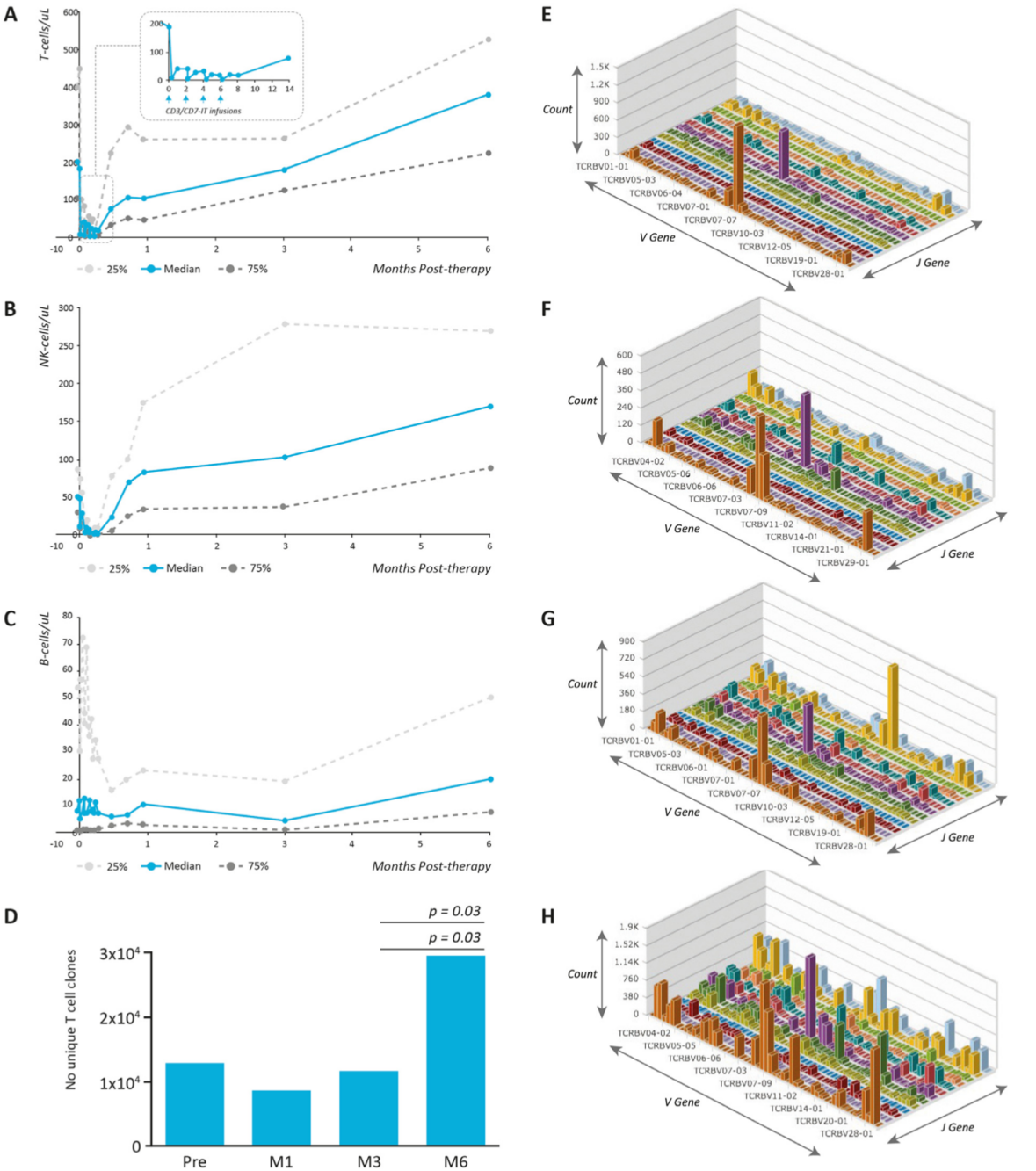

Figure 2. CD3/CD7-IT induces rapid immune reconstitution with a diverse T cell repertoire. (A-C) Time course of the median T cell count (A), median NK cell count (B), and median B cell count (C) for all patients. In each plot, the blue line represents the median value, and the lower and upper gray dotted lines represent the 25th and 75th percentiles, respectively. (D) Summary of the absolute number of unique T cell clones before administration of CD3/CD7-IT (Pre) and at 1, 3, and 6 months after treatment. The number of unique T cell clones was measured using the total number of unique CDR3 sequences. The *P* values are based on the Wilcoxon matched-pairs signed-rank test. The significant increase in unique T cell clones at 6 months after CD3/CD7-IT therapy reflects an increase in the diversity of expanded T cells. (E-H) Representative histograms showing the T cell repertoires in a single patient before CD3/CD7-IT therapy (E) and at 1 month (F), 3 months (G), and 6 months (H) after therapy.

HTS was performed on the CDR3 region of the TCRβ genes in PBMCs before and, when possible, at 1, 3, and 6 months after treatment with CD3/CD7-IT. HTS can determine the total T cell count, the diversity of the T cell repertoire, and the sequences of the TCR CDR3 regions in all T cells in a given sample. The T cell diversity in a sample is characterized by the number of unique T cell clones present in the sample, which is reflected by the number of unique CDR3 sequences identified using HTS. Before the start of treatment with CD3/CD7-IT, the patients had low T cell diversity that further decreased after the first month, most likely due to a reduction in the absolute number of T cells. T cell diversity rebounded steadily by 6 months post-treatment, with a diverse T cell repertoire that included several new polyclonal T cell populations (Figure 2D-H).

We next examined whether CD3/CD7-IT treatment affects antivirus T cell clones. To do so, we analyzed the development of EBV- and/or CMV-specific T cell clones in patients following treatment with CD3/CD7-IT. Antiviral T cell clones were identified by screening for a validated list of 164 and 854 TCRβ sequences encoding receptors that recognize CMV- and EBV-specific antigens, respectively (S11) [32].

Serology was positive for EBV in 95% of patients and 85% of donors and for CMV in 40% of patients and 35% of donors. Infections occurred only in those patients with positive serology. Four patients experienced EBV and/or CMV infection after treatment with CD3/CD7-IT, including 2 patients with either EBV or CMV infection and 2 patients with both EBV and CMV infections) (Figure 3A and C). All these patients demonstrated increased numbers of postinfection EBV- and CMV-associated clones, suggesting that the antiviral T cell response was not negatively affected by treatment with CD3/CD7-IT.

Finally, we performed a differential analysis of unique antiviral T cell clones by performing pairwise comparisons between samples taken directly before treatment with CD3/CD7-IT and samples obtained at 1 month and 3 months after treatment in patients who tested positive for a viral infection before the start of treatment. This analysis revealed that at the start of treatment, the EBV- and CMV-associated T cell clones were distributed equally throughout the entire T cell population in terms of clonal abundance; moreover, these clones did not expand or contract as a result of therapy with CD3/CD7-IT (Figure 3B-D). Similar results were obtained when we analyzed samples from patients who had antiviral T cells at the start of treatment but did not develop a viral infection; our data (not shown) suggest that these patients may have acquired these antivirus clones from a seropositive donor. Taken together, these results indicate that CD3/CD7-IT does not negatively affect the proportions of anti-EBV or anti-CMV T cell clones, suggesting that this treatment does not appear to put these patients at greater risk of acquiring an infection with these opportunistic viruses.

DISCUSSION

Here we report the results of a multicenter phase I/II trial to study the in vivo safety and efficacy of using CD3/CD7-IT therapy in patients with SR-aGVHD. Our results show that CD3/CD7-IT has promising efficacy, with an ORR of 60% on day 28; specifically, 50% of our patients achieved a CR, and the 6-month OS rate was 60%. These results are superior to the outcomes reported for our institutional historical controls (Figure 1) and are notable given the patients' high-risk profile: 85% with severe SR-aGVHD, 90% with GI involvement, and 55% with a high-risk biomarker profile. A pooled analysis of second-line therapies showed that only 32% of patients achieve CR, with a corresponding 6-month survival rate of 49% [1]. In addition, our phase II results closely match those reported for other drugs

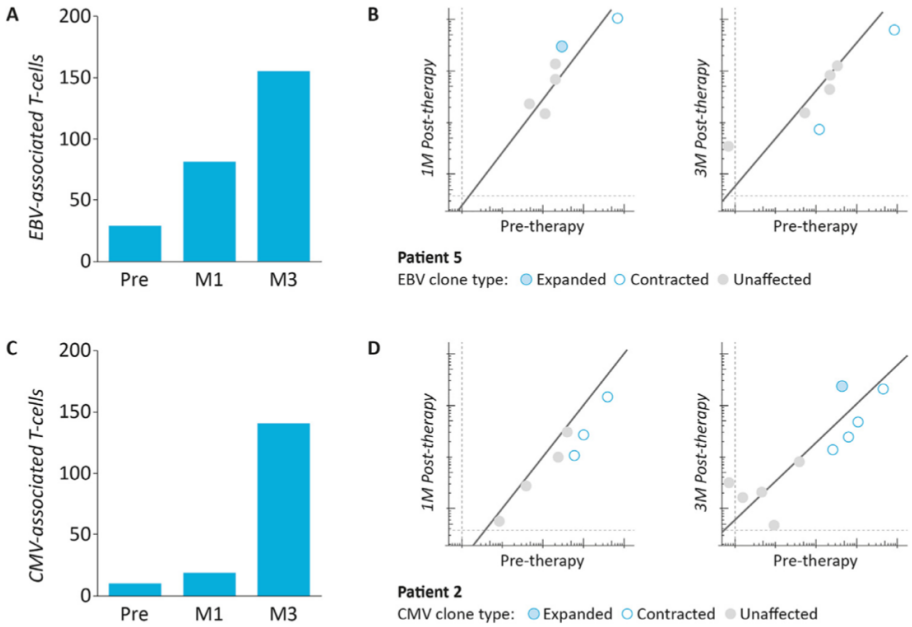

Figure 3. CD3/CD7-IT does not affect the fraction of anti-virus EBV- and CMV-associated T cell clones. (A and C) Summary of the absolute numbers of anti-EBV (A) and anti-CMV (C) T cells in patients who tested positive for viral infection after treatment. In each patient group, the number of virus-associated T cells was measured before and after treatment. (B and D) Plots showing the differential abundance analysis of unique anti-EBV (B) and anti-CMV (D) T cell clones. Shown are representative graphs of 2 patients who tested positive for the respective viral infection before treatment. Screening samples were compared with samples obtained at 1 month and 3 months after therapy with CD3/CD7-IT. This pairwise comparison confirms that the majority of the respective CMV- and EBV-associated clones neither expanded nor contracted as a result of therapy. In each plot, the solid gray diagonal line indicates equal numbers of clones in both samples (no change). Clones positioned between the dotted gray lines and the respective *x*- or *y*-axis were not present in other samples (eg, present before therapy but not after therapy).

currently under investigation for SR-aGVHD, including brentuximab vedotin and ruxolitinib, which have been shown to achieve CR in approximately 30% of patients [36].

This study has several limitations that should be acknowledged. First, the sample size was relatively small, and we did not include a randomized comparator arm. In addition, the study population was heterogeneous with respect to age, conditioning regimen, donor type, and GVHD prophylaxis regimens used. Nonetheless, the study population is representative of patients with SR-aGVHD treated at our institutions and consisted primarily of patients with underlying high-risk features.

CD3/CD7-IT therapy appears to be safe. Despite the presence of the anti-CD3 mAb SPV-T3a, CD3/CD7-IT induced a mild infusion reaction in 2 patients, neither of whom had received preinfusion clemastine. In addition, we observed no toxicity related to CRS or rhabdomyolysis as has been reported with other RTA-based immunotoxins [37,38]. We did consider hypoalbuminemia, microangiopathy, and thrombocytopenia as possibly related to CD3/CD7-IT; however, these AEs primarily involved worsening of preexisting conditions, and we considered these events as likely related to the underlying SR-GVHD and/or the concomitant use of a calcineurin inhibitor. Nonetheless, given the potential toxic effects of immunotoxins, it remains possible that CD3/CD7-IT may have contributed to these events, and this possibility merits consideration in future studies.

As expected in the clinical setting of this study, infections were relatively common; however, the incidence of infection did not differ substantially from that in previous reports or in our institutional controls [21,39,40]. The multifaceted immune defects due to the presence—and treatment—of GVHD itself, the disruption in the mucosal barrier due to GI GVHD, and/or dysbiosis can explain the majority of these infections, particularly the *C difficile* infections and enterococcal bacteremia [41]. Although only one-half of our patients received mold-active antifungal prophylaxis, we observed no cases of IFD. More importantly, despite the profound depletion of T cells and NK cells, the incidence of EBV/CMV infections was relatively low (15%) [21,39], and no cases of post-transplantation lymphoproliferative disorder or CMV disease occurred in our patients. This may be explained by the fact that virus-specific T cells were relatively spared by the treatment, and that immune reconstitution occurred within 6 months after the start of treatment. In the second week of treatment, the T cell and NK cell counts began to rise, particularly in patients who achieved remission of SR-aGVHD; at 3 months, these cell counts were similar to those normally seen following HSCT [42]. This increase in cell numbers was also accompanied by a simultaneous and significant increase in the diversity of T cell clones. Thus, therapy with CD3/CD7-IT allows the patient's immune system to recover after remission is achieved, and the immune reconstitution after therapy seems favorable compared with other treatment modalities that rely on in vivo T cell depletion, such as antithymocyte globulin and alemtuzumab [43,44].

Other immunotoxin-based treatments, such as H65-RTA (anti-CD5, ricin A chain) and denileukin diftitox (CD25, diphtheria toxin), have been clinically evaluated for treating aGVHD [13,45]. CD3/CD7-IT may offer advantages compared with these previous therapies. First, the combination targets multiple antigens on the same target cell, a strategy that tends to be more efficacious than the use of single immunotoxins [46–53]. In addition, CD3/CD7-IT has a clear preference for recently activated T cells, as well as the NK cells that may play a role in the efferent phase of aGVHD [17]. Finally, CD3/CD7-IT has a dual mechanism of action, in that the anti-CD3 mAb SPV-T3a provides added immunosuppression by binding to the CD3/TCR complex via a mechanism independent of RTA-induced cell killing (S1; Figure 1) [17].

In conclusion, the results of our phase I/II study involving patients with high-risk SR-aGVHD show that CD3/CD7-IT provides a high rate of clinical remission and rapid immune reconstitution following treatment. Based on these results, a phase III study is currently being designed to examine the potential value of including CD3/CD7-IT in the treatment of SR-aGVHD.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the Data and Safety Monitoring Board members R. Raymakers, B. J. J. W. Schouwenberg, and A. F. J. de Haan for their evaluation and input.

Financial disclosure: T.R.M was supported by a generous contribution from the Fundação para a Ciência e a Tecnologia (FCT SFRH/BD/98980/2013), the Fondation René Touraine, and an European Academy of Dermatology and Venereology Research Fellowship.

Conflict of interest statement: E.H.G.v.H. and Y.V.J.M.v.O are employees of Xenikos and own stock in Xenikos. The remaining authors have no conflicts of interest to report.interests.

Authorship statement: Y.V.J.M.v.O, W.J.F.M.v.d.V., and M.S. designed the research; C.G., L.F.J.v.G, T.R.M., M.E.B., F.W.M.B.P., H.D., C.R., N.P.M.S., E.H.G.v.H., J.i.H., R.M., M.G.N., J.E.L., J.L.F., N.M.A.B., Y.V.J.M.v.O, M.S., and W.J.F.M.v.d.V. performed the research; C.G., L.F.J.v.G, T.R.M., F.W.M.B.P., H.D., N.P.M.S., E.H.G.v.H., J.i.H., R.M., M.G.N., J.E.L., J.L.F., N.M.A.B., Y.V.J.M.v.O, M.S., and W.J.F.M.v.d.V. analyzed and interpreted the data; J.i.H and T.R.M. performed statistical analyses; C.G., L.F.J.v.G, T.R.M., E.H.G.v.H., J.i.H., J.L., J.F., Y.V.J.M.v.O, W.J.F.M.v.d.V., and M.S. wrote the manuscript; and all authors reviewed and approved the manuscript. C.G. and L.F.J.v.G. contributed equally to this work. M.S. and W.J.F.M.v.d.V. contributed equally to this work.

SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at doi:10.1016/j.bbmt.2018.10.020.

REFERENCES

1. Martin PJ, Inamoto Y, Flowers ME, Carpenter PA. Secondary treatment of acute graft-versus-host disease: a critical review. *Biol Blood Marrow Transplant*. 2012;18:982–988.
2. Calmettes C, Vigouroux S, Labopin M, et al. Risk factors for steroid-refractory acute graft-versus-host disease after allogeneic stem cell transplantation from matched related or unrelated donors. *Biol Blood Marrow Transplant*. 2015;21:860–865.
3. Martin PJ, Rizzo JD, Wingard JR, et al. First- and second-line systemic treatment of acute graft-versus-host disease: recommendations of the American Society of Blood and Marrow Transplantation. *Biol Blood Marrow Transplant*. 2012;18:1150–1163.
4. Deeg HJ. How I treat refractory acute GVHD. *Blood*. 2007;109:4119–4126.
5. Socié G, Blazar BR. Acute graft-versus-host disease: from the bench to the bedside. *Blood*. 2009;114:4327–4336.
6. Zeiser R, Blazar BR. Acute graft-versus-host disease - biologic process, prevention, and therapy. *N Engl J Med*. 2017;377:2167–2179.
7. Holtan SG, Pasquini M, Weisdorf DJ. Acute graft-versus-host disease: a bench-to-bedside update. *Blood*. 2014;124:363–373.
8. Blazar BR, Murphy WJ, Abedi M. Advances in graft-versus-host disease biology and therapy. *Nat Rev Immunol*. 2012;12:443–458.
9. Meunier M, Bulabois CE, Thiebaut-Bertrand A, et al. Alemtuzumab for severe steroid-refractory gastrointestinal acute graft-versus-host disease. *Biol Blood Marrow Transplant*. 2014;20:1451–1454.
10. Arai S, Margolis J, Zahurak M, Anders V, Vogelsang GB. Poor outcome in steroid-refractory graft-versus-host disease with antithymocyte globulin treatment. *Biol Blood Marrow Transplant*. 2002;8:155–160.
11. Martinez C, Solano C, Ferrá C, et al. Alemtuzumab as treatment of steroid-refractory acute graft-versus-host disease: results of a phase II study. *Biol Blood Marrow Transplant*. 2009;15:639–642.

12. Schwartz DM, Kanno Y, Villarino A, Ward M, Gadina M, O'Shea JJ. JAK inhibition as a therapeutic strategy for immune and inflammatory diseases. *Nat Rev Drug Discov*. 2017;16:843–862.
13. Martin PJ, Nelson BJ, Appelbaum FR, et al. Evaluation of a CD5-specific immunotoxin for treatment of acute graft-versus-host disease after allogeneic marrow transplantation. *Blood*. 1996;88:824–830.
14. Byers VS, Henslee PJ, Kernan NA, et al. Use of an anti-pan T-lymphocyte ricin A chain immunotoxin in steroid-resistant acute graft-versus-host disease. *Blood*. 1990;75:1426–1432.
15. Schindler J, Gajavelli S, Ravandi F, et al. A phase I study of a combination of anti-CD19 and anti-CD22 immunotoxins (Combotox) in adult patients with refractory B-lineage acute lymphoblastic leukaemia. *Br J Haematol*. 2011;154:471–476.
16. van Oosterhout YV, van Emst JL, Bakker HH, et al. Production of anti-CD3 and anti-CD7 ricin A-immunotoxins for a clinical pilot study. *Int J Pharm*. 2001;221:175–186.
17. van Oosterhout YV, van Emst L, Schattenberg AV, et al. A combination of anti-CD3 and anti-CD7 ricin A-immunotoxins for the in vivo treatment of acute graft versus host disease. *Blood*. 2000;95:3693–3701.
18. Bryant J, Day R. Incorporating toxicity considerations into the design of two-stage phase II clinical trials. *Biometrics*. 1995;51:1372–1383.
19. Andersen JT, Daba MB, Berntzen G, Michaelsen TE, Sandlie I. Cross-species binding analyses of mouse and human neonatal Fc receptor show dramatic differences in immunoglobulin G and albumin binding. *J Biol Chem*. 2010;285:4826–4836.
20. Harris AC, Young R, Devine S, et al. International, multicenter standardization of acute graft-versus-host disease clinical data collection: a report from the Mount Sinai Acute GVHD International Consortium. *Biol Blood Marrow Transplant*. 2016;22:4–10.
21. van Groningen LF, Liefferink AM, de Haan AF, et al. Combination therapy with inolimomab and etanercept for severe steroid-refractory acute graft-versus-host disease. *Biol Blood Marrow Transplant*. 2016;22:179–182.
22. MacMillan ML, DeFor TE, Weisdorf DJ. What predicts high risk acute graft-versus-host disease (GVHD) at onset?: identification of those at highest risk by a novel acute GVHD risk score. *Br J Haematol*. 2012;157:732–741.
23. Lee SJ. Classification systems for chronic graft-versus-host disease. *Blood*. 2017;129:30–37.
24. Messmann RA, Vitetta ES, Headlee D, et al. A phase I study of combination therapy with immunotoxins IgG-HD37-deglycosylated ricin A chain (dgA) and IgG-RFB4-dgA (Combotox) in patients with refractory CD19(+), CD22 (+) B cell lymphoma. *Clin Cancer Res*. 2000;6:1302–1313.
25. De Pauw B, Walsh TJ, Donnelly JP, et al. Revised definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. *Clin Infect Dis*. 2008;46:1813–1821.
26. Styczynski J, van der Velden W, Fox CP, et al. Management of Epstein-Barr virus infections and post-transplant lymphoproliferative disorders in patients after allogeneic hematopoietic stem cell transplantation: Sixth European Conference on Infections in Leukemia (ECIL-6) guidelines. *Haematologica*. 2016;101:803–811.
27. Ljungman P, Boeckh M, Hirsch HH, et al. Definitions of cytomegalovirus infection and disease in transplant patients for use in clinical trials. *Clin Infect Dis*. 2017;64:87–91.
28. Ghetie V, Swindell E, Uhr JW, Vitetta ES. Purification and properties of immunotoxins containing one vs. two deglycosylated ricin A chains. *J Immunol Methods*. 1993;166:117–122.
29. Major-Monfried H, Renteria AS, Pawarode A, et al. MAGIC biomarkers predict long-term outcomes for steroid-resistant acute GVHD. *Blood*. 2018;131:2846–2855.
30. Matos TR, de Rie MA, Teunissen MBM. Research techniques made simple: high-throughput sequencing of the T cell receptor. *J Invest Dermatol*. 2017;137:e131–e138.
31. Lefranc MP. IMGT, the International ImMunoGeneTics information system. *Cold Spring Harb Protoc*. 2011;2011:595–603.
32. Emerson RO, DeWitt WS, Vignali M, et al. Immunosequencing identifies signatures of cytomegalovirus exposure history and HLA-mediated effects on the T cell repertoire. *Nat Genet*. 2017;49:659–665.
33. DeWitt WS, Emerson RO, Lindau P, et al. Dynamics of the cytotoxic T cell response to a model of acute viral infection. *J Virol*. 2015;89:4517–4526.
34. Lee DW, Gardner R, Porter DL, et al. Current concepts in the diagnosis and management of cytokine release syndrome. *Blood*. 2014;124:188–195.
35. Hay KA, Hanafi LA, Li D, et al. Kinetics and biomarkers of severe cytokine release syndrome after CD19 chimeric antigen receptor-modified T cell therapy. *Blood*. 2017;130:2295–2306.
36. Chen YB, Perales MA, Li S, et al. Phase 1 multicenter trial of brentuximab vedotin for steroid-refractory acute graft-versus-host disease. *Blood*. 2017;129:3256–3261.
37. Schindler J, Sausville E, Messmann R, Uhr JW, Vitetta ES. The toxicity of deglycosylated ricin A chain-containing immunotoxins in patients with non-Hodgkin's lymphoma is exacerbated by prior radiotherapy: a retrospective analysis of patients in five clinical trials. *Clin Cancer Res*. 2001;7:255–258.
38. Stone MJ, Sausville EA, Fay JW, et al. A phase I study of bolus versus continuous infusion of the anti-CD19 immunotoxin, IgG-HD37-dgA, in patients with B cell lymphoma. *Blood*. 1996;88:1188–1197.
39. Socié G, Vigouroux S, Yakoub-Agha I, et al. A phase 3 randomized trial comparing inolimomab vs usual care in steroid-resistant acute GVHD. *Blood*. 2017;129:643–649.
40. Garcia-Cadenas I, Rivera I, Martino R, et al. Patterns of infection and infection-related mortality in patients with steroid-refractory acute graft versus host disease. *Bone Marrow Transplant*. 2017;52:107–113.
41. Taur Y, Xavier JB, Lipuma L, et al. Intestinal domination and the risk of bacteremia in patients undergoing allogeneic hematopoietic stem cell transplantation. *Clin Infect Dis*. 2012;55:905–914.
42. Alho AC, Kim HT, Chammas MJ, et al. Unbalanced recovery of regulatory and effector T cells after allogeneic stem cell transplantation contributes to chronic GVHD. *Blood*. 2016;127:646–657.
43. Mohty M. Mechanisms of action of antithymocyte globulin: T cell depletion and beyond. *Leukemia*. 2007;21:1387–1394.
44. Willemsen L, Jol-van der Zijde CM, Admiraal R, et al. Impact of serotherapy on immune reconstitution and survival outcomes after stem cell transplantations in children: thymoglobulin versus alemtuzumab. *Biol Blood Marrow Transplant*. 2015;21:473–482.
45. Shaughnessy PJ, Bachier C, Grimley M, et al. Denileukin diftitox for the treatment of steroid-resistant acute graft-versus-host disease. *Biol Blood Marrow Transplant*. 2005;11:188–193.
46. Herrera L, Farah RA, Pellegrini VA, et al. Immunotoxins against CD19 and CD22 are effective in killing precursor-B acute lymphoblastic leukemia cells in vitro. *Leukemia*. 2000;14:853–858.
47. Preijers FW, Tax WJ, Wessels JM, Capel PJ, De Witte T, Haanen C. Different susceptibilities of normal T cells and T cell lines to immunotoxins. *Scand J Immunol*. 1988;27:533–540.
48. Preijers FW, De Witte T, Rijke-Schilder GP, et al. Human T lymphocyte differentiation antigens as target for immunotoxins or complement-mediated cytotoxicity. *Scand J Immunol*. 1988;28:185–194.
49. Derocq JM, Laurent G, Casellas P, et al. Rationale for the selection of ricin A-chain anti-T immunotoxins for mature T cell depletion. *Transplantation*. 1987;44:763–769.
50. Yu YH, Crews JR, Cooper K, et al. Use of immunotoxins in combination to inhibit clonogenic growth of human breast carcinoma cells. *Cancer Res*. 1990;50:3231–3238.
51. Crews JR, Maier LA, Yu YH, et al. A combination of two immunotoxins exerts synergistic cytotoxic activity against human breast-cancer cell lines. *Int J Cancer*. 1992;51:772–779.
52. Dean GS, Pusztai L, Xu FJ, et al. Cell surface density of p185(c-erbB-2) determines susceptibility to anti-p185(c-erbB-2)-ricin A chain (RTA) immunotoxin therapy alone and in combination with anti-p170(EGFR)-RTA in ovarian cancer cells. *Clin Cancer Res*. 1998;4:2545–2550.
53. Engert A, Gottstein C, Bohlen H, et al. Cocktails of ricin A-chain immunotoxins against different antigens on Hodgkin and Sternberg-Reed cells have superior anti-tumor effects against H-RS cells in vitro and solid Hodgkin tumors in mice. *Int J Cancer*. 1995;63:304–309.