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Impact of alemtuzumab pharmacokinetics on T-cell dynamics, graft-versus-host disease and viral reactivation in patients receiving allogeneic stem cell transplantation with an alemtuzumab-based T-cell-depleted graft

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ABSTRACT

Administration of alemtuzumab (targeting the CD52 antigen) to the patient (in-vivo) or to the graft (in-vitro) before allogeneic stem cell transplantation (alloSCT) decreases the incidence of graft-versus-host disease (GvHD). Effectiveness of this treatment relies on depletion of donor T cells. Currently, no data are available on alemtuzumab pharmacokinetics and pharmacodynamics in patients who received combined in-vivo and in-vitro alemtuzumab-based T-cell depletion. In this prospective study, we analyzed alemtuzumab pharmacokinetics and its effect on the circulating T cells in 36 patients who received an allogeneic T-cell-depleted graft by addition of 20 mg alemtuzumab "to the bag" with or without prior alemtuzumab (30 mg cumulative dose intravenously) as part of the conditioning regimen. Effective T-cell depletion was shown for all patients, even though alemtuzumab plasma levels varied considerably. Peak alemtuzumab levels were observed directly after graft infusion and were not associated with the number of circulating T cells pre-infusion, but with plasma volumes of the patients. All patients engrafted, confirming feasibility of this transplantation protocol. Only three patients with low alemtuzumab levels developed acute GvHD (grade II in 2 patients and grade III in 1 patient). Persistence of circulating alemtuzumab at 3 weeks after transplantation had prevented reconstitution of CD52-positive T cells when alemtuzumab plasma levels were above 0.7 μg/mL. However, overall T-cell reconstitution did not correlate with the levels of alemtuzumab exposure, due to early reconstitution of CD52-negative alemtuzumab-resistant T cells. The protective effect of these cells likely explains the low incidence of Epstein-Barr-virus- and cytomegalovirus-related disease despite circulating alemtuzumab.

1. Introduction

Allogeneic stem cell transplantation (alloSCT) is a potentially curative treatment for a variety of malignant and non-malignant hematological diseases. However, treatment related morbidity and mortality as a result of graft-versus-host disease (GvHD) remains a challenge. GvHD is caused by an immune reaction against non-hematopoietic healthy tissue mediated by donor T cells infused with the graft. Depletion of donor T cells can prevent the development of GvHD. The therapeutic graft-versus-tumor effect, which results from recognition of residual malignant cells by the donor T cells, is also diminished by donor T-cell depletion [1], but can be restored by postponed application of donor lymphocyte infusions [2–5].

Alemtuzumab (Campath-1H) is a humanized IgG1 antibody which targets the glycophosphatidylinositol (GPI)-anchored protein CD52 that is expressed on lymphocytes, but not (or only marginally) on hematopoietic stem cells [6,7]. Alemtuzumab efficiently depletes CD52-expressing cells through antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and potentially by direct activation of pro-apoptotic pathways. Addition of alemtuzumab

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to the pre-transplant conditioning regimen to deplete donor T cells upon their infusion with the graft (in-vivo depletion), decreases the incidence of severe acute GvHD from 35-50% to 10–20% [8–11]. Even more efficient prevention of GvHD is achieved when alemtuzumab is added directly to the stem cell graft (in-vitro depletion, or alemtuzumab "in the bag") [5,12–15]. The efficacy of alemtuzumab-induced depletion of potentially alloreactive T cells depends on their CD52 expression in relation to the amount of alemtuzumab. After graft infusion, persistence of lytic plasma levels of alemtuzumab will prevent T-cell reconstitution and thereby increase the risk for patients to develop infections [16,17]. Previously, we have shown that T cells that lost expression of CD52 due to loss of GPI-anchor expression have antiviral activity and expand early after alemtuzumab-based in-vitro T-cell-depleted alloSCT [18]. These CD52-negative T cells can contribute to the antiviral protection early after alemtuzumab-based T-cell-depleted alloSCT irrespectively of alemtuzumab levels.

Over the years, alemtuzumab pharmacokinetics have been evaluated in various conditioning regimens using in-vivo alemtuzumab-based T-cell depletion in combination with post-transplantation cyclosporine as GvHD prophylaxis [8,9,19–21]. However, the effect of combined in-vivo and in-vitro T-cell depletion on alemtuzumab pharmacokinetics has not been established. During in-vitro alemtuzumab incubation donor T cells are exposed to a high alemtuzumab concentration and upon graft infusion residual unbound alemtuzumab is co-infused into the circulation. In this study, we performed a comprehensive analysis of alemtuzumab pharmacokinetics in relation to T-cell depletion, T-cell reconstitution, and the occurrence of GvHD and viral infections in 36 patients who had received an allogeneic graft depleted of T cells by addition of alemtuzumab "to the bag" with or without prior in-vivo patient T-cell depletion.

2. Material and methods

2.1. Patients and samples

A cohort of 36 adult patients who received alemtuzumab-based T-cell-depleted alloSCT following one of four transplantation protocols (Table 1) at Leiden University Medical Center (LUMC, Leiden, The Netherlands) between November 2015 and February 2017 were included. Informed consent was obtained for treatment and scientific evaluation (LUMC protocol numbers P03.114, P04.003, P03.173). Sixteen patients received myeloablative (MA) conditioning, consisting of cyclophosphamide and either total body irradiation (TBI, $n = 13$) or busulfan ($n = 3$). In the case of an unrelated donor (UD, $n = 8$) 15 mg alemtuzumab (MabCampath, Sanofi Genzyme, Naarden, The Netherlands) intravenously (IV) at days −6 and − 5 (relative to the day of graft infusion) was added to the conditioning regimen. Twenty patients received non-myeloablative (NMA) conditioning consisting of 15 mg alemtuzumab IV at days −4 and − 3, fludarabine, and busulfan. In case of an UD ($n = 11$) 1 mg/kg anti-thymocyte globulin (ATG; thymoglobulin, Sanofi Genzyme) was added at day −2. All patients received an allogeneic, G-CSF-mobilized peripheral blood stem cell (PBSC, $n = 35$) or bone marrow (BM, n = 1) graft subjected to T-cell depletion by addition of 20 mg alemtuzumab "to the bag" [22]. Cyclosporin as prophylactic immune suppression was only given to patients with an UD after MA conditioning, starting day −1 and tapered from day 30. GvHD was graded according to modified Glucksberg and Shulman criteria [23]. Peripheral blood (PB) samples were scheduled for collection immediately before, 30 min and 1 day after each alemtuzumab cycle and the graft infusion, and at weeks 1, 2, 3, 6, 9 after graft infusion. PB was collected in cell preparation tubes (CPT, BD, Becton Dickinson B.V., Breda, The Netherlands) to isolate mononuclear cells (MNC) and in EDTA tubes (BD) for direct flow cytometric analysis and for plasma isolation. MNC and plasma samples were cryopreserved at −180 °C and − 80 °C, respectively.

Table 1
Protocols, and patient and graft characteristics.

Protocol	Patient-donor relation	Number of patients
Myeloablative, n	Sibling donor	8
	Unrelated donor	8
Non-myeloablative, n	Sibling donor	9
	Unrelated donor	11
Patients		
Age at alloSCT, y		54 (26–74)[a]
Gender, n	Male	27
	Female	9
BSA, m^2		2.06 (1.57–2.42)[a]
Weight, kg		85 (58–115)[a]
Initial diagnosis, n	AML	15
	MM	5
	T-NHL	4
	ALL	3
	MDS	3
	Myelofibrosis	2
	B-NHL	1
	MCL	1
	CMML	1
	WM	1
Grafts		
Stem cell source, n	G-CSF stimulated PB	35
	BM	1
HLA-match, n	10 out 10	32
	9 out 10	4
CD34 cells, 10^6 cells		496.2 (178.6–1414.4)[a,b]
TNC, 10^9 cells		63.8 (7.7–139.2)[a]

Abbreviations: AML, acute myeloid leukemia; MM, multiple myeloma; T-NHL, T-cell non-Hodgkin lymphoma; ALL; acute lymphoblastic leukemia; MDS, myelodysplastic syndrome; B-NHL, B-cell non-Hodgkin lymphoma; MCL, mantle cell lymphoma; CMML, chronic myelomonocytic leukemia; WM, Waldenström macroglobinemia; TNC, total nucleated cells.

[a] Median and range are given.

[b] One patient received a BM graft for whom the number of CD34 cells was not quantified.

2.2. Analysis of circulating alemtuzumab plasma levels

Alemtuzumab plasma levels were measured using a flow cytometry-based indirect immunofluorescence assay modified from the methodology described by Rebello and Hale [24]. The CD52-positive B-lymphoblastic leukemia cell line Leiden-ALL-HP [25] was seeded in a 96 wells plate (20.000 cells/well) and incubated with 10 µL of patient plasma at 0, 5, 10, 20, and 40 fold dilutions (in PBS) or as a reference (in duplicate) with 10 µL healthy donor plasma containing serially diluted alemtuzumab ranging from 5.0–0.03 µg/mL. After incubation for 30 min at 4 °C, cells were washed 4 times with PBS and incubated with phycoerythrin (PE)-conjugated $F(ab')_2$-goat anti-Human IgG Fc (Thermo Fisher Scientific, Bleiswijk, The Netherlands) for 30 min at 4 °C to detect bound alemtuzumab. Cells were washed twice with PBS and kept on ice until analysis. Fluorescence was measured on an LSRII analyzer (BD) and analyzed using FlowJo software (Treestar, Ashland, OR, USA). The alemtuzumab concentrations within the dilutions of the patient plasma sample were interpolated using the reference samples and multiplied by the respective dilution factor. Alemtuzumab plasma levels in patient samples were calculated from the average concentration from at least two sample dilutions. The lower detection limit of this assay was 0.03 µg/mL alemtuzumab. In 6 patients, non-specific (e.g. anti-HLA antibodies, as determined by testing the samples against a GPI-anchor-deficient subculture of Leiden-ALL-HP that lacks CD52 membrane expression [26]) low background levels (< 0.176 µg/mL) were detected in the pre-treatment samples which were subtracted from subsequent measurements from the same patient. One patient who had received treatment with rituximab up to two months before alloSCT was excluded from this analysis as rituximab is also detected in this assay.

2.3. Flow cytometry

Absolute numbers of circulating CD4 T, CD8 T, B, and NK cells were determined by the clinical Laboratory for Specialized Hematology (LUMC) as part of routine immune monitoring after transplantation on anticoagulated fresh venous blood using Trucount tubes (BD, Becton Dickinson, Breda, The Netherlands) following the manufacturer's instructions. Samples were stained with allophycocyanin (APC)-conjugated anti-CD3, PacificBlue-conjugated anti-CD4, fluorescein isothiocyanate (FITC)-conjugated anti-CD8, APC-H7-conjugated anti-CD14, R-phycoerythrin (PE)-conjugated anti-CD16, PE-Cy7-conjugated anti-CD19, V500-conjugated CD45, and PE-conjugated CD56 (all from BD). Fluorescence was analyzed using a FACSCanto (BD). The lower detection limit for this analysis was 0.5×10^6 cells/L.

Absolute numbers of T cells in the graft were determined in samples taken before and after 30 min incubation with alemtuzumab. Cells were kept at 4 °C throughout the following steps to prevent additional cell loss due to processing. For each sample, the absolute number of nucleated cells was determined using a Sysmex KX-21 N (Sysmex, Etten-Leur, The Netherlands). Samples were pelleted and resuspended in red blood cell lysis buffer (8.4 g/L NH_4CL and 1 g/L $KHCO_3$, pH 7.4) (LUMC pharmacy). After 10 min incubation, cells were pelleted and resuspended in 10% *v*/v inactivated human serum at a concentration of 1×10^6–$2x10^6$cells/mL. For logistic reasons samples were stored overnight at 4 °C. Afterwards, the percentages of viable cells were determined using a Bürker cell counter chamber and eosin staining. Cells were pelleted and resuspended in 600 μL PBS with pasteurized plasma proteins 2% *w/v* (GPO, Sanquin, Amsterdam, The Netherlands) of which 150 μL was used to determine the percentages of T cells within the samples by flow cytometry after with APC-H7-conjugated anti-CD3 (BD). The total numbers of viable T cells in the grafts were calculated by multiplying the Sysmex measurement with the graft volume subtracting the percentage of non-viable cells and multiplying by the percentage of T cells.

GPI-anchor and CD52 membrane expression on CD4 and CD8 T cells was analyzed by flow cytometry on thawed MNC by staining with the GPI-anchor-specific inactivated toxin pro-aerolysin coupled to Alexa Fluor 488 (FLAER-AF488, Sanbio, Uden, The Netherlands), APC-conjugated anti-CD52 (ITK diagnostics, Uithoorn, The Netherlands), APC-H7-conjugated anti-CD3, v500-conjugated anti-CD4 (BD), PacificBlue-conjugated anti-CD8 (BD), and AlexaFluor700-conjugated anti-CD45 (ITK diagnostics). Fluorescence was analyzed using an LSRII (BD).

2.4. Population pharmacokinetics model building

Pharmacokinetic parameters were estimated using a developed population pharmacokinetic model based on the alemtuzumab plasma concentration-time data by non-linear mixed-effects modeling using NONMEM (V7.3, Icon Development Solutions, Ellicot City, MD, USA). The Perl-speaks-NONMEM toolkit version 4.7.0 (ref [27]) and Pirana version 2.8.0 (ref [28]) were used as modeling environment. Results were plotted using R (v2.25.2, Boston, MA, USA) and RStudio (v0.97.248). First-order conditional estimation method with interaction was used throughout the analysis. One and two compartmental pharmacokinetic models with first order elimination or non-linear elimination were compared with the observed alemtuzumab plasma concentration-time data to find the optimal fit. Model selection was based on statistical significance, goodness of fit and stability. Throughout the model building process, an altered model was chosen over a pre-cursor model if the difference in the objective functions (-2 log likelihood) was > 6.63 ($P < .01$, with 1 degree of freedom, assuming $\chi 2$ distribution). To identify possible covariates influencing alemtuzumab pharmacokinetics, diagnostic plots were constructed of the random effects of clearance (Cl), distribution volume (V_1) versus the demographic (gender, age, body weight, height, body surface area, body mass index,) and clinical covariates (initial diagnosis, absolute number of circulating patient T cells per liter of blood before IV alemtuzumab infusions, absolute number of donor leukocytes in the graft, hematocrit, and plasma volume. Plasma volume was calculated from blood volume using the Nadler method [29] and corrected for the patients hematocrit (ht) (formulas in Table S1). The final population pharmacokinetic model was validated by means of a prediction corrected visual predictive check (pcVPC, 500 runs) and bootstrap (1000 runs) [30].

2.5. EBV and CMV detection and management

Epstein-Barr virus (EBV)-reactivation was defined as a detectable EBV DNA load in the serum by qPCR. Rituximab treatment (375 mg/m2) for EBV-reactivation was initiated if EBV DNA load was above 10^3 copies/mL. Cytomegalovirus (CMV)-reactivation was defined as a DNA CMV load higher than 10^3 copies/mL in serum by qPCR [31]. Treatment for CMV-reactivation was initiated if the load was higher than 10^3 copies/mL at two consecutive measurements and consisted of oral valganciclovir (900 mg twice daily) or IV ganciclovir (5 mg twice daily) for 2 weeks. CMV-disease was defined as CMV-reactivation with proven organ involvement.

2.6. Statistical analysis

Statistical analyses were performed using Prism 6 (GraphPad, La Jolla, CA, USA) using tests as indicated.

3. Results

3.1. In-vivo depletion of patient T cells by alemtuzumab before graft infusion

All 20 NMA-conditioned patients and all 8 MA-conditioned patients with an unrelated donor received two intravenous (IV) infusions of 15 mg alemtuzumab as part of the conditioning regimen. To examine the effectiveness of alemtuzumab-induced in-vivo patient T-cell depletion, we determined the absolute numbers of circulating T cells directly before the first alemtuzumab infusion and 30 min after the second alemtuzumab infusion using flow cytometry. 6 patients were excluded from this analysis as either the pre- or post-alemtuzumab infusion sample was absent. The median T-cell count before the alemtuzumab infusions was 50×10^6 cells/L (range 4×10^6–829×10^6 cells/L). After the alemtuzumab infusions, complete depletion of circulating T cells was observed in 18 out of 22 patients (Fig. 1A), whereas in the remaining 4 patients minimal numbers of residual T cells were found (range 0.6×10^6–1.0×10^6 cells/L).

To establish the alemtuzumab levels after the IV alemtuzumab infusions, we measured alemtuzumab plasma concentrations in available samples taken from patients before and 30 min after each infusion. After infusion 1, the median alemtuzumab plasma level was 2.5 μg/mL (range 1.1–6.5 μg/mL, $n = 25$, Fig. 1B). No correlation was found between the alemtuzumab plasma levels and the numbers of circulating T cells ($r = 0.11$, $p = .63$, Fig. 1C) or the total numbers of circulating lymphocytes ($r = 0.13$, $p = .58$, data not shown) before infusion. Alemtuzumab plasma levels were comparable between patients diagnosed with different malignancies (data not shown). The median alemtuzumab plasma level before the second infusion was 1.7 μg/mL (range 0.8–3.4 μg/mL, $n = 23$, data not shown) and increased to 3.9 μg/mL (range 2.4–9.7 μg/mL, $n = 26$, Fig. 1B) after the second infusion. In patients with detectable T cells after the two alemtuzumab infusions (Fig. 1A red symbols), alemtuzumab plasma levels were within the normal range (Fig. 1B red symbols). The increase in the alemtuzumab plasma levels by infusion 2 correlated with the alemtuzumab plasma levels that had been reached in individual patients after infusion 1 ($p < .0001$, both samples available for 22 patients, Fig. 1D). Because therapeutic antibodies are initially distributed in the blood plasma volume [32], we analyzed whether the alemtuzumab plasma levels in

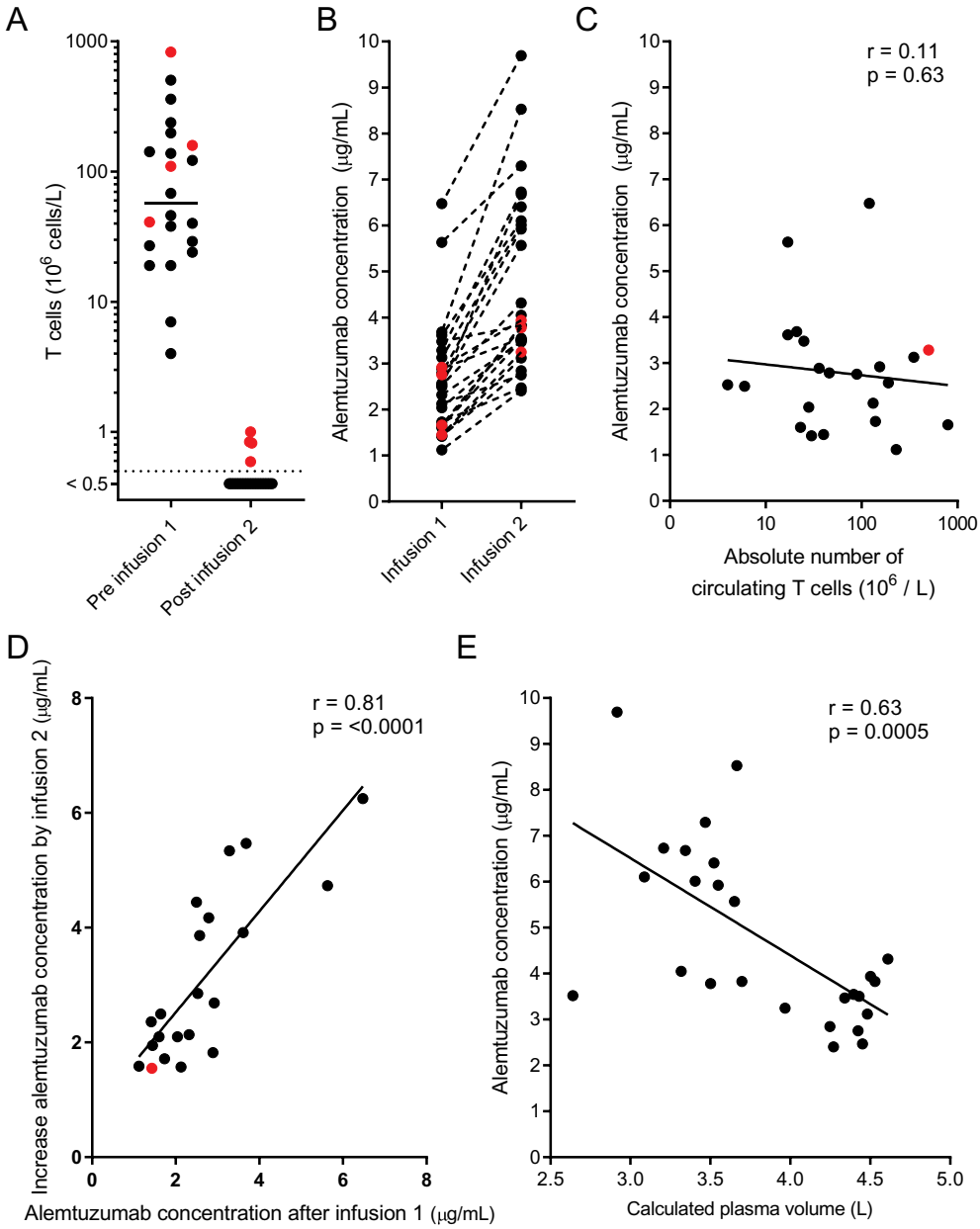

Fig. 1. Efficient in-vivo patient T-cell depletion following IV alemtuzumab.
(A) Absolute numbers of circulating T cells before IV alemtuzumab infusion 1 and after IV alemtuzumab infusion 2. Red symbols depict patients that displayed low numbers of residual T cells following infusion 2. The dotted line represents the detection limit for this analysis. (B) Alemtuzumab plasma levels 30 min after IV alemtuzumab infusion 1 and 2. Dotted lines connect measurements from the same patient. (C) Relation between the number of circulating T cells before IV alemtuzumab infusion 1 and the alemtuzumab plasma level directly after IV alemtuzumab infusion 1. (D) Correlation between the alemtuzumab plasma levels 30 min after IV alemtuzumab infusion 1 and the increase in alemtuzumab plasma levels by IV alemtuzumab infusion 2. The increase in alemtuzumab plasma levels following infusion 2 was calculated by subtracting the alemtuzumab plasma levels before (max 8 h) infusion 2 and 30 min after infusion 2. (E) Correlation between the alemtuzumab plasma levels directly after IV alemtuzumab infusion 2 and the calculated patients' plasma volume. The solid line depicts a linear regression analysis and *P* values were generated by F test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

individual patients correlated with their calculated plasma volumes. A strong negative correlation was found between the calculated plasma volumes and the alemtuzumab plasma levels measured after infusion 1 ($r = 0.58$, $p = .002$, data not shown) and infusion 2 ($r = 0.63$, $p = .0005$, Fig. 1E). Weaker or no significant correlation was found between body surface area and weight and alemtuzumab plasma levels after infusion 2 ($r = 0.48$, $p = .01$ and $r = 0.31$, $p = .13$, respectively; Fig. S1). In conclusion, the plasma volumes and not the numbers of circulating lymphocytes correlated with the alemtuzumab plasma levels in patients after IV alemtuzumab infusions.

3.2. Alemtuzumab-induced lysis of donor T cells

To prevent acute GvHD, 20 mg alemtuzumab was added to the stem cell grafts. Due to the limited graft volumes (median 360 mL, range 115–730 mL), high alemtuzumab concentrations were reached in the bags (estimated median 55 µg/mL, range 27–177 µg/mL), thereby promoting efficient coating of target cells. Since grafts are only incubated with alemtuzumab for 30 min before infusion, we expected partial lysis of donor T cells "in the bag" followed by ongoing in-vivo lysis after infusion of the stem cell grafts into the patients. To estimate the magnitude of in-vitro T-cell depletion, absolute numbers of T cells before and after 30 min alemtuzumab incubation were determined in five grafts. The median T-cell count before alemtuzumab infusion was 14.6×10^9 T cells (range 8.3×10^9–17.3×10^9 T cells) and after incubation 0.5×10^9 T cells (range 0.2×10^9–1.3×10^9 T cells), indicating a 30 fold reduction (range 11–44 fold). After alemtuzumab incubation, the stem cell grafts including residual alemtuzumab-coated donor T cells and unbound alemtuzumab were infused into the patients. We determined the absolute numbers of circulating T cells in patient PB samples taken 30 min after graft infusion (available for 30 patients). In 22 patients, no circulating T cells were found. In the remaining 8 patients, low numbers of T cells (range 0.2×10^6–7×10^6 cells/L) were detected at that time, but these were no longer found in subsequent samples (taken between days 1 and 7 after graft infusion) in 7 out of 8 patients (data not shown). The remaining patient continued to have low numbers of circulating T cells with the lowest number (1.04×10^6 cells/L) at day 14 after graft infusion. Insufficient cells were available to

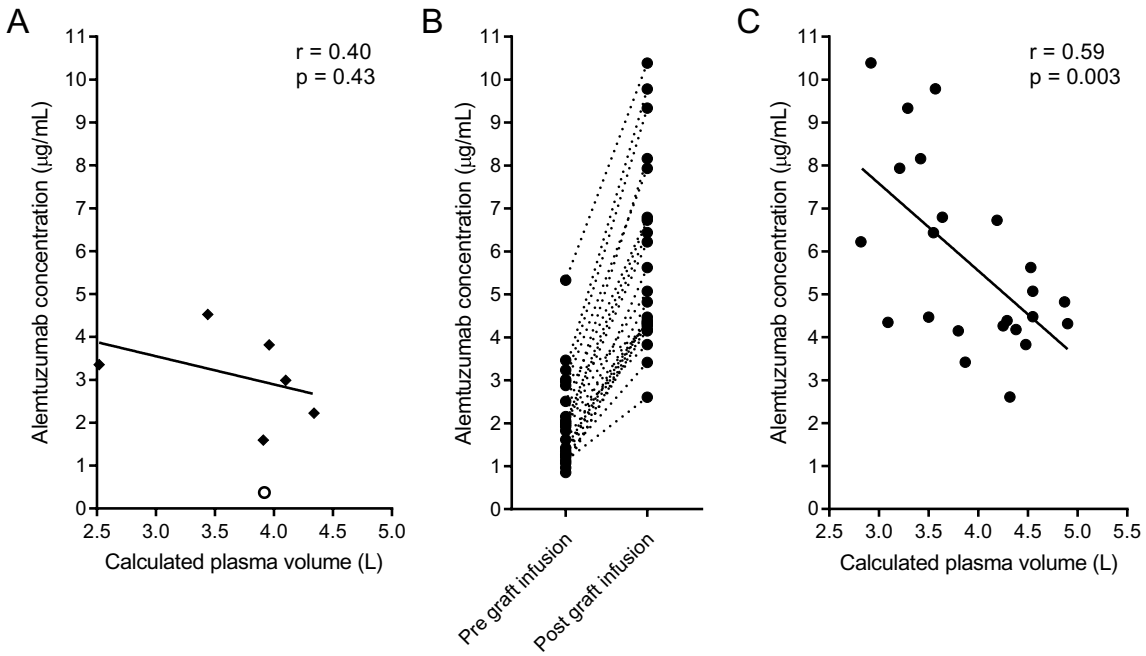

Fig. 2. Increase in patients alemtuzumab plasma levels following graft infusion.
(A) Relation between the alemtuzumab plasma levels directly after graft infusion and the patients' plasma volumes in patients who had not received alemtuzumab as part of the patient conditioning regimen. (B) Alemtuzumab plasma levels directly before and after graft infusion in patients who had already received alemtuzumab as part of the patients conditioning regimen. Dotted lines connect measurement from the same patient. (C) Correlation between the alemtuzumab plasma levels directly after graft infusion and the patients' plasma volumes in patients who had already received alemtuzumab as part of the patients conditioning regimen. The solid line depicts a non-linear regression analysis and *P* values were generated by F test.

determine whether these cells were of patient or donor origin. These data show that in the majority of patients no circulating T cells are found after the infusion of a stem cell graft to which 20 mg alemtuzumab is added.

To evaluate the effect of alemtuzumab infused with the graft on the alemtuzumab plasma levels in the patients, we analyzed the alemtuzumab plasma levels in samples taken before (maximum 8 h) and 30 min after graft infusion. In MA-conditioned patients with a sibling donor who had only received alemtuzumab "in the bag", alemtuzumab plasma levels reached a median of 3.0 µg/mL (range 0.4–4.5 µg/mL, samples were available for 7 out of 8 patients). Since in these patients alemtuzumab was not part of the patient conditioning regimen, residual circulating patient T cells were present before graft infusion (range 1×10^6–64×10^6 cells/L), but these did not correlate with alemtuzumab plasma levels after graft infusion ($r = 0.30$, $p = .51$, data not shown). A trend was observed between higher calculated plasma volumes and lower alemtuzumab plasma levels ($r = 0.40$, $p = .43$, Fig. 2A). In one patient, alemtuzumab levels were particularly low (0.4 µg/mL, Fig. 2C open circle). This patient had a history of hairy cell leukemia and splenomegaly at the time of transplantation, which may have contributed to absorption of alemtuzumab. For patients who had already received alemtuzumab as part of the patient conditioning regimen, pre and post graft infusion samples were available for 23 out of 28 patients. Before graft infusion, a median alemtuzumab concentration of 2.0 µg/mL (range 1.0–5.3 µg/mL, Fig. 2B) was present. After graft infusion, alemtuzumab plasma levels increased to a median of 4.8 µg/mL (range 2.6–10.4 µg/mL, Fig. 2B). Similar alemtuzumab plasma levels were found between NMA-conditioned patient who had (median 4.8 µg/mL, range 3.4–10.4 µg/mL) or had not (median 4.6 µg/mL, range 2.6–9.8 µg/mL) received additional ATG during in-vivo conditioning. A strong negative correlation was found between the calculated patients' plasma volumes and the alemtuzumab plasma levels before ($r = 0.67$, $p = .0001$, data not shown) and after graft infusion ($r = 0.59$, $p = .003$, Fig. 2C). Together these data show that the increase in alemtuzumab levels by graft infusion was independent of pre-treatment with alemtuzumab during patient conditioning. The increase in alemtuzumab levels were in line with an increase expected from direct IV injection, indicating presence of an excess amount of alemtuzumab "in the bag" whereby the bulk of alemtuzumab remained unbound.

3.3. Influence of circulating alemtuzumab on reconstitution of T cells

After transplantation, neutrophil repopulation indicated engraftment in all patients. To study the influence of alemtuzumab on T-cell reconstitution, we determined the absolute numbers of circulating CD4 and CD8 T cells starting at three weeks after the transplantation. At week three after graft infusion (data were available for 33 out of 36 patients), 23 patients had already reconstituted with CD4 T cells (range 0.9×10^6–106×10^6 cells/L) and 21 patients with CD8 T cells (range 0.7×10^6–848×10^6 cells/L). At week nine after graft infusion (data were available for 31 patients), 30 patients had circulating CD4 T cells (range 1.1×10^6–864×10^6 cells/L) and 29 patients had circulating CD8 T cells (range 4×10^6–3367×10^6 cells/L). Persistence of circulating alemtuzumab after graft infusion was modelled by population pharmacokinetics analysis with non-linear mixed effects modeling using alemtuzumab plasma concentration-time data. Alemtuzumab pharmacokinetics was best described by a two-compartment intravenous model with first-order elimination. Random effect parameters for inter-individual variability in clearance, distribution volume of the central compartment and the peripheral compartment were identified (model parameters in Table 2; pcVPC and goodness of fit plots in Fig. S2 and S3, respectively). The mean half-life of alemtuzumab in the elimination phase was 8.7 days (range 2.4–19.1). No direct correlations were found between the number of CD4 or CD8 T cells and modelled alemtuzumab plasma levels at 3 weeks ($r = 0.23$ and $r = 0.14$, respectively) or 9 weeks ($r = 0.06$ and $r = 0.16$, respectively) post-transplantation. These data show no direct correlation between T-

Table 2
Model parameter estimates.

Clearance (L/day)	0.9 (0.2–14.0)
Half-life distribution phase (hours)	10 (4–23)
Half-life elimination phase (days)	8.7 (2.4–19.1)
Volume central compartment (L)	2.8 (1.1–29.2)
Inter-compartmental clearance (L/day)	2.9
Volume peripheral compartment (L)	6.3 (3.1–12.2)
Peak alemtuzumab concentration (μg/mL)	8.4 (0.7–22.2)
Total alemtuzumab exposure[a] (μg.day/mL)	25.2 (1.4–54.1)

Median values (range) are given unless stated otherwise.
[a] After graft infusion

cell reconstitution and residual circulating alemtuzumab.

We and others have previously shown that after alemtuzumab-based T-cell-depleted alloSCT, reconstituting T cells have frequently lost CD52 membrane expression [18,33] making them resistant to alemtuzumab. We therefore evaluated the effect of residual alemtuzumab on reconstitution of CD52-positive and CD52-negative T cells. CD52 membrane expression was analyzed by flow cytometry and combined with the absolute numbers of circulating CD4 and CD8 T cells determined in samples taken at the same time point. Complete absence of CD52-positive CD4 and CD8 T cells was observed at week 3 when alemtuzumab plasma levels were above 0.7 μg/mL (Fig. 3A and B, respectively). In contrast, no association was found between alemtuzumab plasma levels and the numbers of CD52-negative CD4 and CD8 T cells (Fig. 3C and D, respectively). At week nine, all patients had alemtuzumab plasma levels < 0.34 μg/mL and no effect of alemtuzumab plasma levels was observed on either CD52-negative of CD52-positive T-cell reconstitution (data not shown).

These data show that in-vivo alemtuzumab levels above 0.7 μg/mL prevented reconstitution of CD52-positive T cells.

3.4. Absence of GvHD in the presence of high alemtuzumab levels

Because acute GvHD is mediated by naïve donor T cells infused with the graft [34], we investigated whether modelled alemtuzumab peak concentrations directly after graft infusion correlated with the incidence of GvHD. Two patients who had developed an early relapse (weeks 6 and 7 post graft infusion) and received additional chemotherapy were not included in the analysis. Three patients (one MA- and one NMA-conditioned patient with sibling donors and one MA-conditioned patient with an UD) had developed GvHD grade II or III within the first 10 weeks following graft infusion. All three patients had

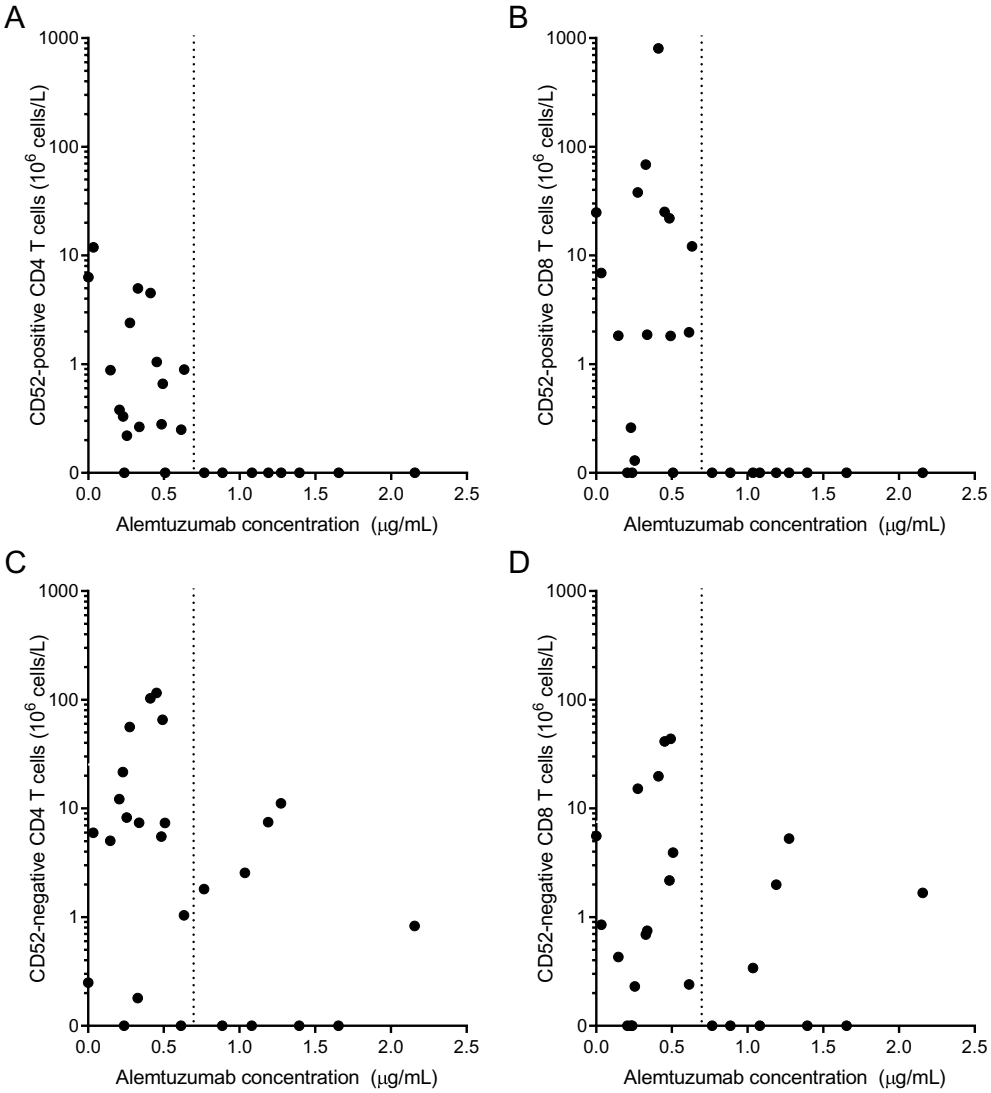

Fig. 3. Reconstitution of CD52-positive T cells is blocked by persistence of lytic alemtuzumab plasma levels.
Absolute number of circulating CD52-positive (A) CD4 and (B) CD8 and CD52-negative (C) CD4 and (D) CD8 T cells at week 3 after graft infusion. Each dot represents the analysis for one patient. The dotted lines indicate the alemtuzumab concentration of 0.7 μg/mL.

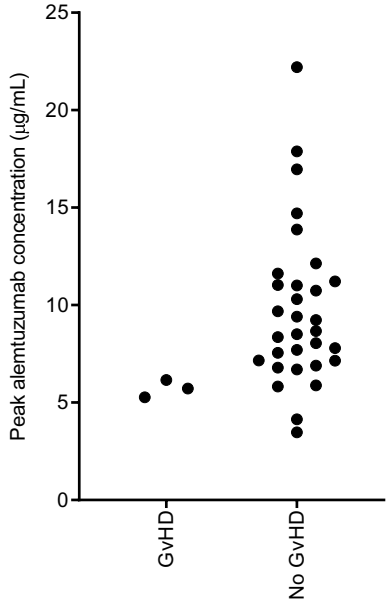

Fig. 4. Absence of GvHD in the presence of high alemtuzumab levels.
Peak alemtuzumab plasma levels and the occurrence of GvHD within the first 10 weeks after graft infusion. Alemtuzumab peak levels were estimated for each patient using pharmacokinetics modeling. Each dot represents the analysis for one patient.

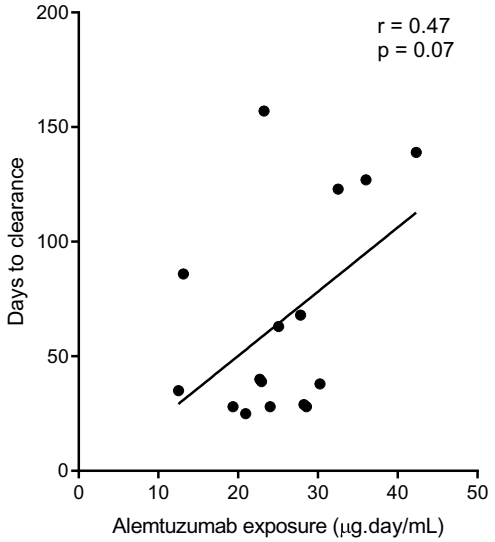

Fig. 5. Effect of alemtuzumab exposure on CMV-clearance.
Relation between alemtuzumab exposure and time to clear a CMV-reactivation. Alemtuzumab exposure was estimated for each patient using population pharmacokinetics modeling. Each dot represents the analysis for one patient. The solid line depicts a non-linear regression analysis and *P* values were generated by F test.

low peak alemtuzumab plasma concentrations (Fig. 4). No GvHD was observed in patients with peak alemtuzumab levels above 6.5 (µg/mL).

3.5. Effect of alemtuzumab exposure on viral complications

Because persistence of alemtuzumab was shown to affect CD52-positive T-cell reconstitution, we analyzed whether differences in alemtuzumab exposure after graft infusion had affected the incidence of viral complications. Alemtuzumab exposure was estimated for each patient using population pharmacokinetics and related to reactivations of EBV and CMV occurring within the first 10 weeks after graft infusion. 5 out of 36 patients were excluded from this analysis as they had developed an early relapse ($n = 2$) or GvHD ($n = 3$) and received additional chemotherapy or steroid treatment. No EBV-reactivation was observed in patients that were EBV-seronegative before transplantation (n = 3). From the 28 pre-transplant EBV-seropositive patients, 7 had an EBV-reactivation (of whom 2 had an EBV-seronegative donor). None of these patients required treatment for the EBV-reactivation and alemtuzumab exposure was comparable to patients without EBV-reactivation (mean area under the curve 28.4 ± 11.9 µg.day/mL compared to mean 31.2 ± 10.4 µg.day/mL, $p = .63$). For CMV, no reactivation was observed in the patients who were CMV-seronegative before transplantation ($n = 14$). From the 17 pre-transplant CMV-seropositive patients, all had detectable CMV-reactivations within the first 10 weeks after graft infusion and 14 patients were consequently treated with antiviral drugs. None of the patients developed CMV-disease. There was a weak correlation between alemtuzumab exposure and the duration of CMV viremia (Fig. 5). Although circulating alemtuzumab levels in this cohort were sufficient to prevent the development of acute GvHD in the majority of patients, no patient developed viral disease in this period.

4. Discussion

This study confirms that the combination of in-vivo and in-vitro alemtuzumab effectively prevents acute GvHD after alloSCT without compromising engraftment [35,36]. Our data demonstrate that plasma volume rather than the number of circulating lymphocytes determines inter-individual variation in alemtuzumab plasma peak. Patients with a plasma peak level after transplantation above 6.5 µg/mL did not develop acute GvHD. Although the post-transplantation T-cell suppression in this cohort was sufficient to prevent acute GvHD in the majority of patients, no cases of EBV-PTLD or CMV-disease were seen. Antiviral immune protection results from an early outgrowth of CD52-negative T cells. Whereas circulating CD52-positive T cells were absent when residual alemtuzumab level were above 0.7 µg/mL, the reconstitution of CD52-negative T cells was not affected by alemtuzumab.

One of the aims of adding of alemtuzumab to the conditioning regimen of a patient before alloSCT is the eradication of patient T cells to avoid graft rejection. We showed that virtually all circulating T cells were efficiently depleted in patients who had received infusion of 30 mg alemtuzumab IV. In contrast, in MA-conditioned patients with a sibling donor who had not received alemtuzumab IV, circulating T cells were found up to the moment of graft infusion. Alemtuzumab plasma levels in our cohort correlated with the patients' plasma volumes. In patients with CLL treated with alemtuzumab, alemtuzumab plasma levels were found to be highly affected by the tumor burden due to target-cell-mediated alemtuzumab absorbtion [37–39]. In our cohort, alemtuzumab plasma levels did not correlate with the numbers of circulating T cells present at time of alemtuzumab infusion, likely because patients in our cohort were in remission for the primary disease when receiving the alloSCT and had received TBI and/or chemotherapy before alemtuzumab, together resulting in low numbers of circulating lymphocytes. One patient had particularly low alemtuzumab plasma levels. This patient had a history of hairy cell leukemia and had splenomegaly at the start of the alloSCT, which was given to treat a therapy related acute myeloid leukemia. We hypothesize that the spleen effectively absorbed alemtuzumab and therefore we suggest that patients with residual bulky disease require higher alemtuzumab dosing to overcome alemtuzumab absorption, as previously suggested by Chakraverty et al. [40]

Alemtuzumab is added to the graft to eradicate donor-derived T cells thereby preventing GvHD. We showed that pre-incubation of the graft with 20 mg alemtuzumab reduced donor T-cell numbers on average by a factor of 30. After infusion of the graft with remaining donor T cells with bound and unbound alemtuzumab, circulating alemtuzumab led to ongoing lysis of circulating T lymphocytes. In order to correlate alemtuzumab pharmacokinetics with clinical outcomes like

the occurrence of acute GvHD, viral reactivation, and T cell reconstitution after alloSCT, alemtuzumab peak concentrations and alemtuzumab exposure were estimated for each patient using population pharmacokinetics analysis with non-linear mixed effects modeling. Three patients in our cohort developed limited GvHD after alloSCT, suggesting that some donor T cells had escaped alemtuzumab treatment. Because GvHD is predominantly mediated by naïve donor T cells infused with the graft [34], which express high levels of CD52 and as a consequence are highly sensitive to alemtuzumab-mediated cell lysis [41], acute GvHD would be expected to occur because of inadequate peak alemtuzumab levels. Indeed, low alemtuzumab peak concentrations were found in all three patients who developed GvHD. Furthermore, no GvHD was observed in patients with alemtuzumab peak levels above 6.5 (μg/mL), illustrating that adequate alemtuzumab peak levels indeed effectively prevent GvHD. These plasma peak levels were the product of alemtuzumab added to the graft and alemtuzumab applied IV as part of the conditioning. Since the plasma peak levels correlated with the patients' plasma volume, modifying the alemtuzumab dose based on this parameter could be considered as a way to dictate the plasma peak level post-transplant. Indeed, the three patients in our cohort who developed GvHD coinciding with low alemtuzumab peak levels had relatively high plasma volumes. However, we are somewhat reluctant to suggest patient-specific adjustment of the alemtuzumab dose based on plasma volume only, due to the low patient numbers and the likelihood that proper prediction of the peak levels post-transplant most likely requires inclusion of additional parameters such as pre-transplant alemtuzumab serum measurements, and factors like splenomegaly. The incidence of GvHD in our cohort was comparable to previous studies in which patients received in-vivo alemtuzumab in combination with post-transplant immune suppression with cyclosporin [40,42].

Persistence of high levels of residual alemtuzumab can also delay reconstitution of protective virus-specific T cells. Here we established an in-vivo lytic alemtuzumab plasma level of 0.7 μg/mL as the lower threshold for reconstitution with CD52-positive CD4 and CD8 T cells. This level is substantially higher than the previously suggested lytic alemtuzumab level of 0.1 μg/mL, which was based on the anticipated combined effect of ADCC and CDC as effector mechanisms [42,43]. Low numbers of effectors cells early after alloSCT may decrease the contribution of ADCC to in-vivo cell lysis. CDC, which is known from in-vitro tests to be efficient only at higher alemtuzumab concentrations starting at levels identical to the minimal lytic alemtuzumab level we found [44], may be the main mechanism causing T-cell depletion after transplantation. Although a correlation between alemtuzumab levels and outgrowth of CD52-positive T cells was found, there was no correlation between total numbers of T cells and alemtuzumab exposure. This discrepancy resulted from rapid outgrowth of large populations of CD52-negative alemtuzumab-resistant T cells early after transplantation. Previously we have reported that these CD52-negative T-cell populations are mainly of a memory phenotype and contain functional virus-specific CD8 T cells, including CMV-specific T cells. These CD52-negaive memory T cells appeared to be mainly of donor origin, but also profound outgrowth of CD52-negative memory T cells of recipient origin was observed in some patients, especially associated with viral clearance shortly after the transplantation [18]. The protective effect of these cells likely explains why no correlation was found between the level of alemtuzumab exposure and the incidence of EBV- or CMV-reactivation, or on EBV-clearance, and only a minimal effect on CMV-clearance. Of note, unlike naïve T cells, these CD52-negative memory T cells are not expected to contribute to GvHD.

We conclude that after alloSCT using in-vitro with or without prior in-vivo T-cell depletion with alemtuzumab, plasma alemtuzumab peak levels above 6.5 μg/mL prevent the development of acute GvHD. After alloSCT, circulating CD52-positive T cells are absent as long as the circulating alemtuzumab level is above 0.7 μg/mL. The reconstitution of CD52-negative cells is not influenced by alemtuzumab level and contributes to the prevention of viral disease after alloSCT.

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Author contributions

F.C.L. designed and performed research, discussed data, and wrote the paper. H.M.E. and C.W. performed research and analyzed data. D.J.A.R.M. performed the population pharmacokinetics analysis. P.A.B. and H.V. consented patients, obtained samples, and recorded clinical data. J.H.F.F., I.J., and C.J.M.H. designed research, discussed data, and wrote the paper. All auteurs reviewed and approved the final manuscript.

Statement of equal authors' contribution

C.J.H. and I.J. share senior authorship.

Conflict-of-interest disclosure

The authors declare no competing interests.

Off-label use of medicine

Alemtuzumab

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