Rituximab therapy after pediatric hematopoietic stem cell transplantation can cause prolonged B-cell impairment and increases the risk for infections - a retrospective matched cohort study

Rituximab, the monoclonal antibody directed against CD20, is established in treatment regimens against CD20+ non-Hodgkin lymphomas and is used increasingly in refractory systemic autoimmune disorders.[1-4] Besides, it is applied as treatment in patients with Epstein-Barr virus (EBV) infection/reactivation, post-transplant lymphoproliferative disease (PTLD) and autoimmune complications following hematopoietic stem cell transplantation (HSCT).[5-8] Documentation of immunological consequences and impact on immune reconstitution in pediatric HSCT patients is, however, sparse. Previously published studies on small cohorts suggest delayed B-cell recovery, need for prolonged immunoglobulin substitution and an increased risk for secondary infections.[9,10] In order to further elucidate rituximab implications in this setting, we performed a retrospective analysis of 44 pediatric patients who received allogeneic HSCT in our center between 2015 and 2020, and who were treated with rituximab within 365 days after HSCT. We compared this cohort with matched HSCT patients who didn't receive rituximab within 4 weeks before, or after HSCT. Despite similar overall survival, we observed that rituximab therapy significantly delayed B-cell recovery, extended immunoglobulin deficiency and led to longer rehospitalization durations and more bacterial infections despite immunoglobulin replacement therapy. In a subgroup (9 patients, 38%), we observed prolonged immunoglobulin deficiency >365 days after rituximab treatment, suggesting that rituximab harbors a significant risk for prolonged B-cell impairment.

Rituximab patients were matched in a best-match approach with control patients. For matching, eight transplant-relevant parameters from the Joint Accreditation Committee of the International Society for Cellular Therapy and the European Group for Blood and Marrow Transplantation (JACIE) essential data list were chosen. The pool of possible matches was then filtered for one parameter after another which led to high correct-match rates for top priority parameters (HSCT matching 100%; graft source 95%) but lower success in low-priority parameters (Figure 1A). After matching, the control group showed a longer cumulative observation duration. This can be explained by a more frequent use of rituximab in later years. No difference in the overall outcome was observed (Figure 1B; *Online Supplementary Table S1A*). Most patients received the first rituximab dose before day +100 (82%) and indication for initiating rituximab treatment was mainly EBV infection/reactivation (41 patients, 84%) (*Online Supplementary Table S1B*). Analysis of EBV blood level development showed that rituximab was highly effective against EBV infection with 95% treatment success (Figure 1C; *Online Supplementary Table S1B*). Both patients who did not respond completely, developed PTLD. In total, PTLD occurred in 6.1% (3/49) of patients with EBV levels >2,000 copies/mL measured via polymerase chain reaction (PCR) and all three patients had received preemptive rituximab treatment (rituximab 7.9% [3/38] *vs*. control 0% [0/11]; *P*>0.99) (Table 1A). At the same time, we observed that rituximab patients generally were at a higher PTLD risk due to significantly earlier EBV infection/reactivation after HSCT and higher maximal viral loads (Figure 1C and D).[11] The EBV level at the start of rituximab treatment, however, was quite variable (0-1,770,000 copies/mL). For 14 (37%) patients, rituximab treatment was initiated at an EBV load <10,000 copies/mL. The 11 control patients (25%) with EBV infection/reactivation (all <20,000 copies/mL) were treated with ganciclovir and/or foscarnet only, and all resolved their EBV reactivations. Regarding immune reconstitution, our data shows that rituximab treatment delayed B-cell recovery by a median of 162 days (day +282 [range, 43–716] *vs*. day +120 [range, 36–645]; hazard ratio 2.2; *P*=0.008) and led to significantly lower B-cell numbers and B-cell to T-cell ratios at day +365. However, by day +720, the majority of patients had recovered to similar B-cell levels (Figure 2A to D; *Online Supplementary Figure S1*; Table 1A; *Online Supplementary Table S1C*). In order to evaluate B-cell damage beyond normal B-cell count recovery, we analyzed immunoglobulin levels and IgG substitution dependence. Rituximab treatment led to reaching IgG blood levels >5g/L without IgG substitution significantly later (median day +278 [range 4– 1,095] *vs*. day +118 [range, 4-722]; hazard ratio 4.2; *P*<0.001) and the last documented IgG substitution happened significantly later (median day +254 [range, 0-1,095] *vs*. +109 [range, 0-324]; hazard ratio 6.25; *P*<0.001). While IgG recovery and B-cell recovery coincided in both groups (rituximab: last IgG recovery median day +278 and B-cell recovery +282; control: day +118 and +120), significant differences in IgM levels were still measurable even 2 years after HSCT,

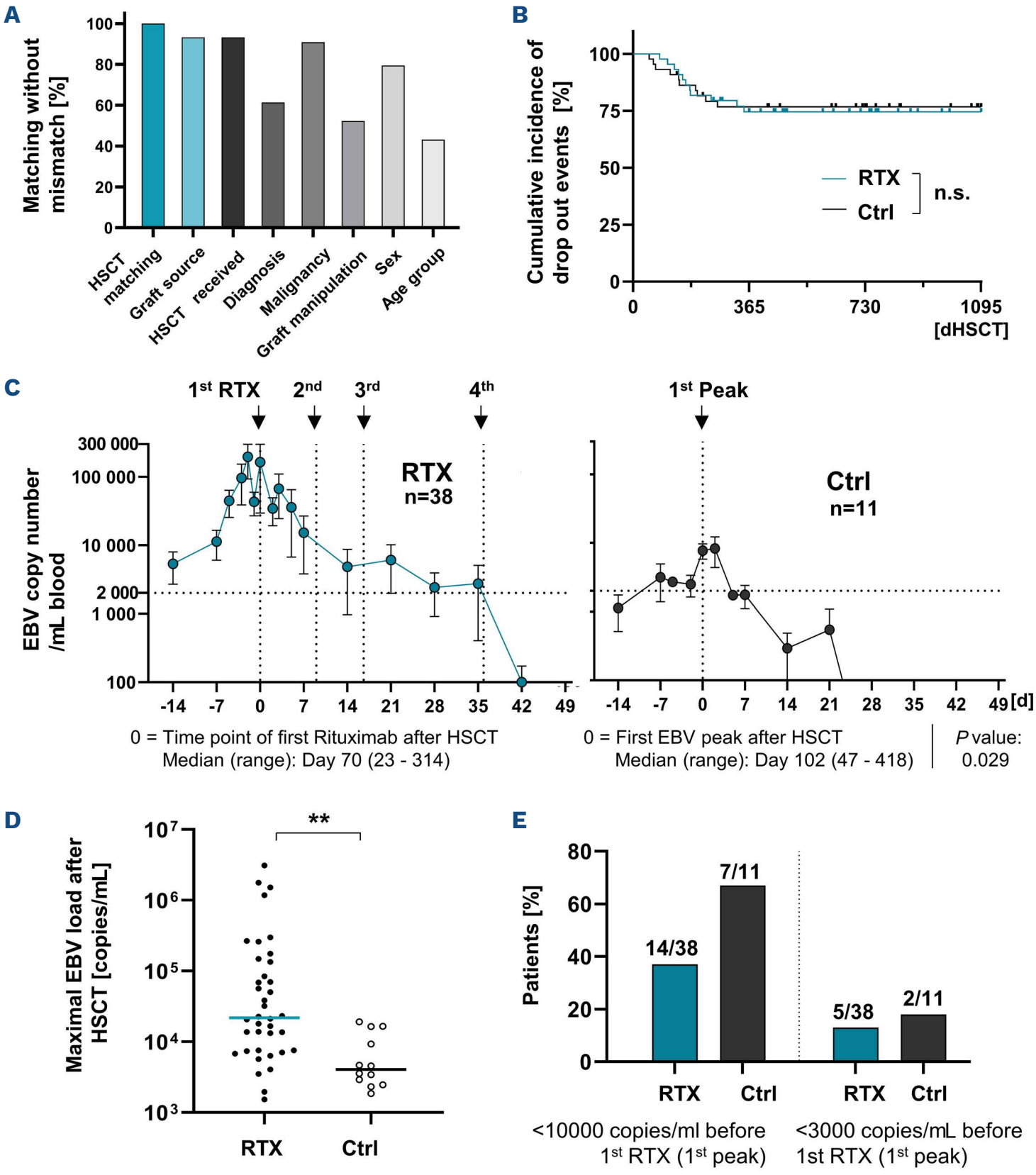

Figure 1. Rituximab treatment is efficient for patients with high Epstein-Barr virus viral load after hematopoietic stem cell transplantation but cutoff viral load for rituximab treatment initiation is unclear. (A) Graphical overview of matching success for different matching categories. From highest matching priority to lowest: hematopoietic stem cell transplantation (HSTC) matching (matched unrelated donor, matched sibling donor or mismatch related donor), graft source (bone marrow or peripheral blood stem cells [PBSC]), HSCT received (number of HSCT received before + 1), exact diagnosis, malignancy (benign or malign disease as indication for HSCT), graft manipulation, sex and age group (<1, 1-5, 6-11, 12-17, 18+ years) (*Online Supplementary Table S1A*). (B) Kaplan-Mayer survival curves and log-rank test for cumulative incidence of drop out events (relapse, non-relapse-related mortality, rejection and retransplantation). (C) Epstein-Barr virus (EBV) copy number/mL EDTA blood development over time in patients with EBV infections (n=38) from the rituximab (RTX) and control (Ctrl) groups (n=11) relative to the date of the first rituximab dose in the RTX group or the first peak in the Ctrl group. The median time points of the 2nd, 3rd and 4th RTX doses were marked with dotted lines for the RTX group. (D) Maximal EBV load after HSCT in copies/mL EDTA blood in RTX and Ctrl groups. (E) Depiction of patient numbers for which RTX treatment was initiated at <10,000 or <3,000 EBV copies/mL in the RTX group or where the peak was <10,000 or <3,000 EBV copies/mL in the Ctrl group. dHSCT: HSCT treatment timeline day (d0 = day of HSCT). Significance levels: ***P<0.001; **P<0.01; *P<0.05; n.s.: not significant

pointing towards a prolonged impairment not only of B-cell numbers but also function (*Online Supplementary Figure S1A*; Table 1A). Additionally, we evaluated the question of a correlation between “rituximab doses received” and “rituximab initiation time point” with primary endpoints, but no correlation could be found (*Online Supplementary*

Figure S2A). Investigating secondary complications, we found a significantly higher cumulative duration of rehospitalizations in the rituximab group (median 20 *vs*. 9 days). In line, we noted significantly more non-EBV viral infections in the rituximab group, but as this was true independently from rituximab initiation, we suggest a general increased risk for viral infections in the rituximab group (Table 1A; *Online Supplementary Table S1D*). However, despite similar use of myelotoxic antiviral agents (e.g., foscarnet) after rituximab treatment initiation, we found significantly more neutropenia relapses, initiations of intravenous antibiotic treatment and a higher rate of pa-

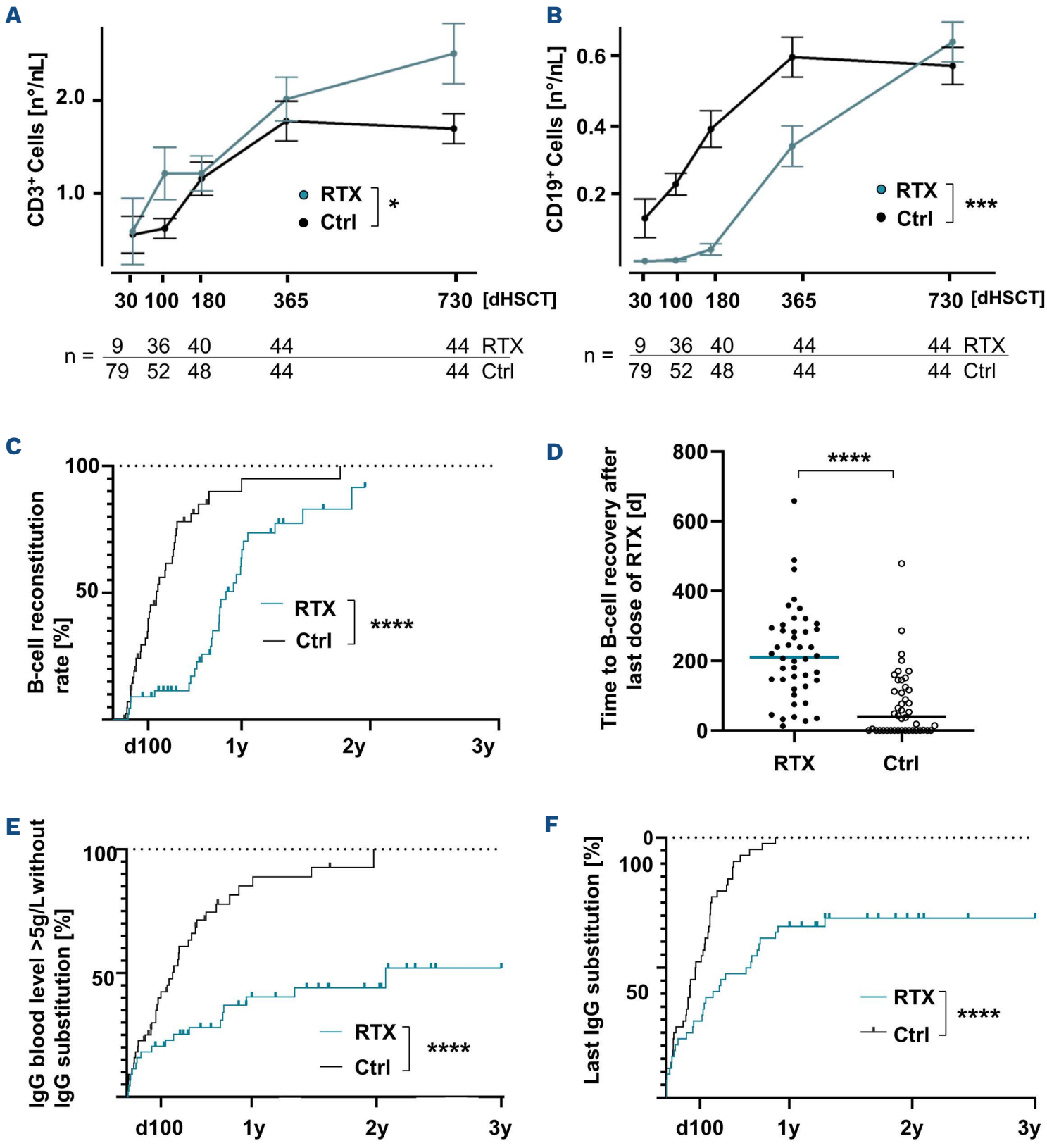

Figure 2. B-cell recovery and function is impeded by rituximab treatment after pediatric hematopoietic stem cell transplantation. (A and B) T-cell (CD3+) and B-cell (CD19+) recovery over time after hematopoietic stem cell transplantation (HSCT) for rituximab (RTX) and control (Ctrl) groups depicted as mean and standard error of mean per group and day after HSCT. Patients were allocated to either RTX or Ctrl group for each time point depending on RTX therapy initiation and a time and group matched mixed model analysis was computed in R version 1.4.1717 (R foundation) for group comparison. (C, E and F) Inverse Kaplan-Meier curves depicting the rate of patients at a certain time point that achieved either (C) B-cell reconstitution, (E) IgG levels >5 g/L without IgG substitution or (F) receiving no more IgG substitutions for RTX or control groups. (D) Comparison of the elapsed time to B-cell recovery after the last dose of RTX was administered in the RTX group and the elapsed time until B-cell recovery after a time point identical to the time point of the last RTX dose for each individual matched patient for the Ctrl group. Data depicted as single patient values and median. dHSCT: HSCT treatment timeline day (d0 = day of HSCT), BW: body weight. Significance levels: ***P<0.001; **P<0.01; *P<0.05; n.s.: not significant.

tients who had positive bacterial blood culture findings (17 [39%] *vs*. 6 [14%]) after rituximab therapy. While we saw no difference regarding acute graft-*versus*-host disease (GvHD) incidence, that necessitated systemic treatment, moderate-severe chronic GvHD only occured in the rituximab group (5 [11%]). Four of these patients had received rituximab before GvHD onset or steroid treatment. A possible explanation could be that these patients were

Table 1. Comparison of study cohorts.

A - Rituximab cohort characteristics	**Rituximab (N=44)**	**Control (N=44)**	***P* value[a]**
Patients with EBV who developed PTLD, N (%)	3/38 (7.9)	0/11 (0)	>0.99
B-cell count at day 365, N/nL (range)[b]	0.2 (0-1.3)	0.5 (0.1-2.0)	0.0003
B-cell to T-cell ratio at day 365 (range)[b]	0.2 (0-1.1)	0.4 (0-1.0)	0.0005
B-cell recovery, days after HSCT (range)[b]	282 (43-716)	120 (36-645)	0.0001
Time to B-cell recovery after last RTX (Ctrl: equivalent time point), days (range)[b]	211 (13-658)	37 (0-479)	<0.0001
IgG serum level at day 365, g/L (range)[b]	6.2 (1.3-14)	9.1 (3.2 -22)	0.03
IgM serum level at day 365, g/L (range)[b] at day 730, g/L (range)[b]	 0.5 (0.1-1.7) 0.6 (0.2-1.1)	 0.8 (0.2-3.6) 0.9 (0.3-2.7)	 0.0002 0.01
Last IgG substitution, days after HSCT (range)[b]	254 (0-1,095)	109 (0-324)	<0.0001
Cumulative rehospitalization duration, days (range)[b] Before first RTX After first RTX	20 (1-293) 3 (2-293) 16 (1-141)	9 (1-107) 6 (1-22) 9 (1-107)	0.0123 0.77 0.033
Patients with any non-EBV viral infection (>2,000 copies/mL in blood), N (%) Before first RTX After first RTX	28 (63.6) 23 (52.3) 9 (20.5)	16 (36.4) 15 (34.1) 5 (11.4)	0.008 0.08 0.22
Initiations of intravenous antibiotic treatment (range)[b] Before first RTX After first RTX	3 (1-14) 1 (0-5) 1 (0-11)	1 (1-6) 1 (0-3) 0 (0-6)	0.0004 0.049 0.006
Patients with positive blood cultures, N (%) Before first RTX After first RTX	26 (59.1) 15 (34.1) 17 (38.6)	13 (29.55) 11 (25.0) 6 (13.64)	0.021 0.5 0.029
Moderate - severe chronic GvHD, N (%)	5 (11.4)	0 (0)	0.025
B - Prolonged B-cell damage subgroup characteristics	**PBD (N=9)**	**RTX-Ctrl (N=15)**	***P* value[a]**
Time until EBV viral load drops below <50% of value at RTX initiation (Ctrl: 1st peak), days (range)[b]	1 (1-2)	2 (1-42)	0.049
B-cell recovery, days after HSCT (range)[b]	471 (50-716)	301 (43-460)	0.026
Time to B-cell recovery after last RTX, days (range)[b]	306 (144-658)	214 (127-350)	0.029
IgG level day 365, g/L (range)[b]	6.0 (2.2-9.7)	9.0 (3.0-14)	0.025
IgM level day 365, g/L (range)[b]	0.1 (0.1-0.5)	0.6 (0.4-1.0)	<0.0001
Cumulative IgG dose, g/kg BW (range)[b]	7.9 (1.0-28)	0.9 (0.02-3.8)	0.0002
IgG substitution after B-cell recovery, N (%)	6 (66.7)	3 (20)	0.036
Rehospitalizations per patient (range)[b,c] Before first RTX After first RTX	5 (0-34) 0 (0-1) 4 (0-34)	1 (0-9) 0 (0-3) 1 (0-8)	0.036 0.56 0.021
Initiations of intravenous antibiotic treatment (range)[b] Before first RTX After first RTX	4 (1-14) 2 (1-3) 2 (1-11)	2 (1-4) 1 (0-3) 1 (0-3)	0.034 0.053 0.04

[a]In order to compare cohorts the Wilcoxon signed rank test was used for continuous data and the McNemar test for binary data. When matching was impossible, the Mann-Whitney U and Fishers exact tests were used. Test statistics were created using SPSS version 28.0 (IBM SPSS Statistics, Armonk, USA), GraphPad PRISM 8 & 9 (GraphPad Software, San Diego, USA).[b]Median (range), [c]rehospitalizations for rituximab application only were not included. EBV: Epstein-Barr virus; PTLD: post-transplant lymphoproliferative disease; RTX: rituximab; Ctrl: control (group); HSCT: hematopoietic stem cell transplantation; GvHD: graft-*versus*-host disease; PBD: prolonged B-cell damage (subgroup); RTX-Ctrl: non-PBD rituximab control group (patients from rituximab group that were observed longer than 365 days after initiation of rituximab treatment).

multimorbid patients with a high coincidence of complications (Table 1A; *Online Supplementary Table S1D*). We then followed previous reports of prolonged B-cell impairment after rituximab treatment in non-HSCT-related situations.[4,10,12] Out of 24 patients who were observed longer than 365 days after rituximab treatment ended, we identified nine (38%) who had unresolved immunoglobulin deficiency. We compared these patients (i.e., the prolonged B-cell damage group [PBD]) to the other 15 and found that B-cell recovery and function were severely impeded in the PBD group (*Online Supplementary Figure S2B* to *D*; Table 1B; *Online Supplementary Table S1E*). Three (33%) PBD patients had unmeasurable B-cells counts at day +365. Regarding prolonged functional impairment, we observed significantly lower IgG and IgM levels despite receiving more IgG substitutions and significantly more PBD patients received IgG substitutions after B-cell recovery. They also developed more complications after initiation of rituximab treatment as suggested by significantly more rehospitalizations, a higher rate of non-EBV viral infections and more initiations of intravenous antibiotic treatments. Apart from significantly faster rituximab therapy response and a tendency towards younger age (8 years [range, 2-19] *vs*. 12 years [range, 4-21]), no significant differences were found when looking for possible risk factors (*Online Supplementary Figure S2*; Table 1B; *Online Supplementary Table S1E*). Follow-up on IgG substitution beyond the observation period on 06/30/2022 showed that three of the nine PBD patients had become independent of IgG substitutions. This leaves six (25%) patients with a prolonged B-cell damage with continuous dependence on IgG substitutions beyond 2 years after HSCT. A complication that has so far been described only in case reports.[9,10]

Although age, graft manipulation and cGvHD mismatches create potential bias as immune reconstitution influencing confounders, our study confirms for the first time in a large pediatric cohort that rituximab therapy <365 days after HSCT leads to a delay in B-cell recovery of both B-cell numbers and function.[5,9,12,13] In line with Ottaviano *et al*., who observed prolonged hypogammaglobinemia after rituximab treatment in a non-HSCT-related setting, the faster rituximab therapy response in the PBD subgroup supports the hypothesis of an increased rituximab sensitivity at the time point of first rituximab application.[4] This also fits with our finding that B-cell impairment did not correlate with the number of rituximab doses received. Regarding secondary infections, our results clearly point towards an increased risk for secondary bacterial infections after rituximab initiation which is in line with Petropoulou *et al*., although no increase in mortality could be observed in our pediatric cohort.[14] In contrast to the findings of Arai *et al*., we could not confirm a decreased alloimmunity after rituximab treatment.[6] We conclude that rituximab harbors a significant risk for prolonged B-cell impairment and bacterial infections when administered shortly after HSCT. It remains unclear whether regular IgG substitution can completely mitigate the adverse side effects, but similar overall survival suggests that IgG substitution and appropriate treatment of complications can compensate the damage. We postulate that rituximab treatment within 365 days after HSCT poses a 20-40% risk to develop especially prolonged B-cell impairment. This risk should be discussed in a shared decision-making process with caretakers when considering initiation of rituximab treatment. In order to propose a solution for prolonged B-cell impairment, donor stem cell boosts could be evaluated further in cases without GvHD.[9] Our findings furthermore support the need to research factors predisposing for rituximab sensitivity. Exact determination of each patients risk to develop prolonged B-cell damage after rituximab therapy could help to identify those patients that could qualify for an alternative treatment, e.g., EBV-specific T-cell transfer.[15] Finally, we urge physicians to carefully consider the initial indication for rituximab treatment and recommend to generally not start rituximab therapy too early at low EBV levels, but instead to monitor EBV levels daily in these situations.

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Contributions
ML conceptualized the study, collected and analyzed data and prepared the manuscript. DT collected and analyzed data and prepared the manuscript. EO collected data and reviewed the manuscript. BM performed the mixed-model and fine and grey analysis, gave input on statistical aspects and possible sources of bias and reviewed the manuscript. FZ, LO, HED, AGH, AK, PH, HvB, AE, AvS and PL participated in designing the study, discussing the data and reviewed the manuscript. AP contributed data and reviewed the manuscript. JHS conceptualized the study and reviewed the data, results and manuscript. All authors have read and agreed to the published version of the manuscript.

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Data-sharing statement
All data collected and analyzed in this study as well as detailed test statistics can be received upon request. This excludes data that falls under data privacy restrictions (e.g., exact date of transplantation).

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