Prophylactic Rituximab after Allogeneic Transplantation Decreases B cell Alloimmunity with Low Chronic GVHD Incidence

Sally Arai1, Bita Sahaf1, Balasubramanian Narasimhan2, George L Chen1, Carol D Jones3, Robert Lowsky1, Judith A Shizuru1, Laura J Johnston1, Ginna G Laport1, Wen-Kai Weng1, Jonathan E Benjamin1, Joanna Schaenman1, Janice Brown1, Jessica Ramirez1, James L Zehnder3, Robert S Negrin1 and David B Miklos1

1Division of Blood and Marrow Transplantation, 2Department of Health Research and Policy – Biostatistics, 3Department of Pathology, Stanford University Medical Center, Stanford, CA, USA

Corresponding author:
David B Miklos, MD, PhD
Department of Medicine, Division of Blood and Marrow Transplantation
Stanford University School of Medicine
269 West Campus Drive CCSR 2205, MS5642
Stanford, CA 94305
e-mail: dmiklos@stanford.edu.
Abstract

B cells are involved in the pathogenesis of chronic graft-versus-host disease (cGVHD). We hypothesized that prophylactic anti-B cell therapy delivered two months after transplantation would decrease allogeneic donor B cell immunity and possibly the incidence of cGVHD. Patients with high risk chronic lymphocytic leukemia (CLL) (n=22) and mantle cell lymphoma (MCL) (n=13) received total lymphoid irradiation 80 cGy x 10 days and anti-thymocyte globulin 1.5mg/kg/day x 5 days. Rituximab (375 mg/m²) was infused weekly, on days 56, 63, 70, and 77 post-transplant. The incidence of acute GVHD was 6%. The cumulative incidence of cGVHD was 20%. Nonrelapse mortality (NRM) was 3%. Rituximab treatment following allogeneic transplantation significantly reduced B cell allogeneic immunity with complete prevention of alloreactive H-Y antibody development in male patients with female donors (p=0.01). Overall survival and freedom from progression at 4 years for CLL patients was 73% and 47%, respectively; for MCL patients, 69% and 53%, respectively. This study was registered at clinicaltrials.gov as NCT00186628.
Introduction

Chronic graft-versus-host disease (cGVHD) remains a significant cause of late morbidity and mortality after allogeneic hematopoietic cell transplantation (alloHCT). Strategies to prevent cGVHD, however, have been largely disappointing (1-4). Although traditionally thought to be mediated by alloreactive T lymphocytes (5-6), increasing evidence supports a role for B cells in the pathogenesis of cGVHD (7). Autoantibody and alloantibody associations with cGVHD have been reported (8). Specifically, alloreactive antibodies against H-Y antigens (9-10) and coordinated B and T cell responses (11) strongly associate with the occurrence of cGVHD in sex-mismatched allogeneic transplantation. A murine study demonstrated allogeneic antibodies deposit in cGVHD affected tissues and cGVHD was prevented when the donor graft was genetically prevented from secreting IgG (12). Other evidence comes from studies showing dysregulated B cell reconstitution (13) and increased B-cell activating factor (BAFF) levels in cGVHD patients (14). B cells collected from cGVHD patients were more responsive to TLR-9 signaling and exhibit increased CD86 expression (15). Furthermore, established steroid-refractory cGVHD patients have reduced numbers of naïve B cells and increased activated CD27+ B cells (13-14), further supporting a role for B cells in cGVHD pathogenesis. Clinically, anti-B cell directed therapy with rituximab has been shown to be effective treatment for established cGVHD, with several groups reporting clinical response rates of 40-70% in steroid-refractory cases (16-20).

Evidence for the potential use of rituximab as cGVHD prophylaxis comes from clinical observations that rituximab added to fludarabine and cyclophosphamide conditioning resulted in a low rate of cGVHD in 10 chronic lymphocytic leukemia patients (21).
Others have shown a decrease in acute and/or cGVHD in patients with B cell malignancies treated with rituximab within six months prior to allogeneic transplantation (22-23).

Taken together, these findings suggest that rituximab depletion of donor B cells following alloHCT may reduce cGVHD. We hypothesized that prophylactic anti-B cell therapy with rituximab following allogeneic transplantation would deplete adoptively transferred alloreactive donor B cells, and thus decrease cGVHD. We present our findings on the effect of rituximab treatment infused two months after allogeneic transplantation focusing on safety, feasibility, B cell immune reconstitution, and overall clinical outcomes. Cognizant that allogeneic B cell responses may also have anti-tumor benefits, the study piloted in vivo B cell depletion strategy in patients with CD20-expressing B cell malignancies. We modified our institution’s total lymphoid irradiation-anti-thymocyte globulin (TLI-ATG) allogeneic transplant regimen to study in vivo B cell depletion following rituximab treatment and show decreased allogeneic H-Y antibody development with promising low chronic GVHD incidence.

**Methods and Materials**

**Patient selection**

Between July 15, 2005 and November 30, 2007, 35 patients with high risk chronic lymphocytic leukemia (CLL) (n=22) and mantle cell lymphoma (MCL) (n=13) enrolled on the protocol, which was approved by the Stanford Institutional Review Board (Table 1). High risk CLL eligibility included: 1) FISH with 17p deletion or 11q deletion; 2)
unmutated VH-IgG (<2% nucleotide change compared to germline sequence); or 3) fludarabine-refractory disease (24). MCL patients in CR or PR were eligible.

**Treatment Plan**

The reduced intensity conditioning (RIC) regimen of TLI-ATG was adapted for this trial (25-26). As shown schematically in Figure 1, TLI was administered at 80cGy x 10 days and rabbit ATG (Thymoglobulin, Genzyme) at 1.5mg/kg x 5 days, followed by infusion of unfractionated granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood progenitor cells (PBPC) on day 0. The experimental treatment of this study was the infusion of rituximab 375 mg/m² weekly x 4 on days 56, 63, 70 and 77. The infusion of rituximab 2 months post-transplant was timed to coincide with peripheral blood donor B cell reconstitution after TLI-ATG conditioning (25). Primary GVHD prophylaxis included cyclosporine (CSP) and mycophenolate mofetil (MMF). Figure 1 shows the immunosuppression taper schedule and timing of research sample collection.

**HLA Typing and Matching**

Patients and donors were HLA-typed by high-resolution techniques (27). All matched related recipients were matched with their donors for 10 of 10 HLA alleles. Of the 16 unrelated recipients, 3 had single HLA allele level (9 out of 10) mismatches (Table 1a, 1b).
Supportive Care

Patients were hospitalized for the ATG infusion for the first five days only. Antimicrobial, antiviral and antifungal prophylaxis was administered as previously described (25). Monitoring for cytomegalovirus (CMV) and Epstein-Barr virus (EBV) by polymerase-chain-reaction assay was performed starting day 7 post-transplant and continued weekly for at least the first 90 days after transplantation. IgG levels were assessed pre-rituximab, day 90, day 180 and at 1 year.

Fluorescence-activated cell sorting (FACS) analyses

B cell reconstitution was assessed in donors and patients pre-, day 56, 90 and 180. Subsequent analyses of B cell recovery post-rituximab will be reported separately (BS, manuscript in preparation). For comparison, we measured B cell recovery in 19 TLI-ATG patients who had never received rituximab. Cryopreserved peripheral blood mononuclear cells (PBMC) were thawed and washed (28). Cells were stained with a cocktail of fluorochrome conjugated antibodies against cell surface markers: CD3 Qdot605 (Invitrogen, Carlsbad, USA), CD19-PE (phycoerythrin), CD5-APC (Allophycocynin), CD23-APC-Cy7, CD20 CY5-PerCP (Becton Dickinson [BD] BioSciences, San Jose, CA). Staining proceeded for 15 min on ice (29). Cells were then washed and resuspended in staining media containing 0.4% formaldehyde before analysis on a LSRII flow cytometer (BD). Gates were set using the fluorescence minus one (FMO) samples as negative controls. Data was analyzed using FlowJo (TreeStar Inc. Ashland, OR). Significance was calculated using the non-parametric Wilcoxon/Kruskal-Wallis test.
Rituximab quantitation

Rituximab levels were measured by ELISA (30) on pre-transplant and day 56 serum samples.

GVHD grading and therapy

Diagnosis and scoring of acute GVHD was based on standard criteria (31) and cGVHD was based on the National Institutes of Health (NIH) Consensus guidelines (32). Scoring was performed by a designated GVHD team member. Initial therapy for acute and chronic GVHD was prednisone 1mg/kg per day.

Analysis of donor chimerism and disease responses

Chimerism analyses were performed on whole blood and blood mononuclear cells separated into CD3, CD19, CD15, and CD56 populations using Dynal coated immunomagnetic beads. Donor engraftment employed DNA genotyping of simple sequence-length polymorphic markers that encode short tandem repeats as previously described (33). Chimerism analyses were performed after transplant at 30, 56, and 90 days post-transplant. Full donor chimerism was defined as ≥ 95% donor peripheral blood CD3+ T cells.

Disease responses for CLL were assessed using the updated NCI Working Group Criteria (34). Minimal residual disease (MRD) was monitored by quantitative allele-specific oligonucleotide-IgH PCR (ASO-Q-PCR) (35-36) for those CLL patients in whom a clone was detected pretransplant. For MCL patients, disease response was assessed using the Revised Response Criteria for Malignant Lymphoma (37).
Disease progression was managed with immunosuppression withdrawal and donor lymphocyte infusion (DLI).

**H-Y antibody assays**

Plasma samples from the 10 male patients with female grafts treated on the trial were tested by enzyme-linked immunosorbent assay (ELISA) for antibodies to five H-Y antigens (10-11). Samples were diluted 1:50 and quantified for H-Y specific IgG by ELISA with absorption at 550 nm -450 nm (optical density [OD] units). An OD of 0.1 was established as the cut-off value for positive antibody reactivity for all antigens (10-11). For comparison, plasma samples were similarly tested for H-Y antibodies from 25 males with female donors undergoing concurrent TLI-ATG allogeneic transplantation and never received rituximab, and who had a one year post transplant blood sample collected.

**Statistical analysis**

Overall survival (OS) and freedom from progression (FFP) were estimated by the Kaplan-Meier method (38). Cumulative incidence estimates were calculated for acute and cGVHD, relapse, and nonrelapse mortality. Death and relapse were treated as competing events in analyses of GVHD. Factors considered in the univariate analyses of relapse/progression and cGVHD included pretransplant rituximab level, graft CD34 dose, graft CD3 and CD19 composition, absolute CD19 B cell count at day 56, donor T cell (CD3) chimerism at 30 and 90 days, MCL versus CLL, matched related donor
versus unrelated donor, and prior acute GVHD. All P values were derived from logrank statistics.

**Results**

**Patient characteristics**

The high risk features of the 22 CLL patients are described in Table 1a. Fifteen out of 22 patients (68%) were fludarabine-refractory, and 18/20 (90%) had an unmutated VH-IgG. Eight of 16 CLL patients (50%) had a 17p deletion and 5/16 (31%) had an 11q deletion. Only four CLL patients were in clinical remission pre-transplant (34). The MCL group characteristics are shown in Table 1b.

**Hematopoietic recovery**

The median CD34 cell dose for the 35 patients on study was 7.5x10^6/kg (range 2.3-19.2x10^6/kg) and the median CD3 cell dose was 2.7x10^8/kg (range 1.6-6.1x10^8/kg). All patients had hematopoietic recovery except for one patient with primary graft failure who had autologous recovery 30 days after transplant and remains alive with CLL. Ten patients (7 CLL, 3 MCL) had neutropenia (<500/µl) before graft infusion (day 0). Twenty-five patients (71%) never reached a platelet nadir below 20,000/µl.

**B cell reconstitution and rituximab infusion**

Our prior studies showed donor B cell engraftment by donor chimerism analysis occurred ~60 days after TLI-ATG transplantation (25). Rituximab infusion days 56, 63, 70, 77 was timed to coincide with this donor B cell recovery. We quantified blood
CD19+ B cells present before rituximab infusion on day 56, and confirmed rituximab mediated B cell depletion at 90 and 180 days after HCT by FACS analysis. CD19+CD5- cells were reported in order to exclude persistent CD5+ malignant B cells from donor B cell reconstitution. Figure 2a shows that, while normal donors have 175-500 CD19+CD5- cells/µl blood, CLL and MCL patients had significantly fewer absolute donor B cells at day 56 (median 7 cells/µl), with 13 patients having no detectable CD19+CD5- B cells. In comparison, the B cell recovery at day 56 in 19 TLI-ATG patients who had never received rituximab (Table S1), was significantly higher (median 70 CD19+CD5- cells/µl; p<0.01). We presumed this reduced donor B cell recovery at day 56 and the similarly low number of B cells observed pre-HCT in the CLL/MCL group resulted from extensive rituximab therapy before transplant that persisted in the patient’s blood after transplant, depleting donor B cells. To test this directly, pre-transplant and day 56 serum samples were measured for rituximab by ELISA (30). All patients who received rituximab within 6 months of transplant had detectable drug pre-transplant. The patients with detectable rituximab pretransplant had fewer day 56 donor B cells than those who had no detectable rituximab at the time of transplant (Figure 2b). Alemtuzumab treatment before transplant also appeared to impair day 56 donor B cell engraftment (Figure S1).

**CLL decreased following day 56 rituximab**

Immunophenotyping detected persistent CLL 56 days after transplant in most CLL patients; ten out of 16 (63%) patients had >10 CD19+CD5+CD23+ cells/µl in the peripheral blood (Figure 2c). However, following the completion of 4 weekly rituximab
doses, 6 of 20 (30%) CLL patients had any detectable CLL cells by flow cytometry, suggesting rituximab provided anti-tumor benefit. There was negligible MCL detected by flow cytometry 56 days after HCT or after rituximab on day 90 (Figure 2c).

**Prophylactic rituximab infusion two months after transplant is associated with low cGVHD**

Grade II-IV acute GVHD was observed in 2 out of 35 patients (6%; Table 2). The two affected patients had grade II skin involvement, which resolved with a short course of prednisone. Chronic GVHD developed in seven of the 35 patients, including the two patients with prior acute GVHD (Table 2). The cumulative incidence of cGVHD at 4 years was 20% (95%CI: 6 - 34%; Figure 3).

The median time to onset of cGVHD was 200 days (range 146-413 days). Using NIH Consensus guidelines for scoring cGVHD severity (32), two patients had mild cGVHD, four had moderate, and one had severe cGVHD. All 5 surviving patients were successfully tapered off prednisone and their disease remains quiescent. Two patients (SPN 3723, 3879) died from complications of infection following cGVHD, one with H1N1 infection.

**Rituximab prophylaxis prevents allogeneic antibody development**

We hypothesized that prophylactic rituximab infusion following alloHCT would deplete alloreactive B cells and alloantibodies. In order to measure B cell alloimmunity, we evaluated H-Y antibody development in male HCT patients with female donors (F→M
HCT). The frequency and intensity of antibody development to 5 H-Y antigens are shown by heat map presentation (Figure 4). None of the ten F→M HCT patients who received prophylactic rituximab in this study developed H-Y antibodies and none developed cGVHD. As a comparison, 25 F→M HCT patients who underwent the same TLI-ATG conditioning during this time period without post transplant rituximab were evaluated, and fourteen of the 25 (56%) developed H-Y antibodies, with 13 of the 25 (52%) patients developing cGVHD. Table S2 shows the concordance of H-Y antibody development in association with cGVHD (p<0.005), consistent with what we have previously reported (11). While rituximab infusion two months after F→M HCT prevented H-Y antibody development (p=0.01), rituximab infusion before F→M HCT did not. Twelve of the 25 comparison patients had received rituximab within 6 months prior to alloHCT, and 5/12 (42%) developed H-Y antibodies with 3 of the 5 developing cGVHD. In summary our study confirms that H-Y antibody develops in association with cGVHD following TLI-ATG conditioning, and rituximab infusion following this reduced intensity conditioning regimen has achieved our goal of reducing B cell alloimmunity with no H-Y antibody development. We believe, but have not proven, reduced B cell alloimmunity may decrease cGVHD incidence.

**Allogeneic HCT with post-transplant rituximab maintains disease control**

The median clinical follow-up is 4 years. For the CLL patients, the 4-year overall survival was 73% (95% CI: 57%-94%) and 4-year freedom from progression was 47% (95% CI: 30%-75%). For the MCL patients, the 4-year overall survival was 69% (95% CI: 48%-99%) and 4-year freedom from progression was 53% (95% CI: 31%-89%)
(Figure 5a, b). We further assessed CLL disease by measuring, minimal residual disease (MRD) employing allele-specific oligonucleotide quantitative PCR (ASO-PCR) assays in 19 of the 22 patients who had measurable clones. Ten of 19 (53%) of the VH-IgG unmutated CLL patients achieved MRD negativity by one year following HCT. Of the 10 patients who were MRD-negative at 1 year post-transplant, eight (80%) are still alive and in remission.

Twelve patients (11 CLL; 1 MCL) received DLI following disease progression. Six of the CLL patients achieved MRD negativity and remain in clinical remission. The one MCL patient who has received three DLI is alive with persistent mixed CD3 chimerism and no GVHD. Ten trial patients have died. Seven died from relapse, two from infection and cGVHD, and one from donor lymphocyte infusion-related GVHD.

Predictors for cGVHD, disease progression, and overall survival: univariate analysis

Graft CD34 cell dose, CD3 and CD19 cell composition, absolute CD19 B cell count at day 56, donor T cell (CD3) chimerism at day 30 and day 90, disease type (MCL versus CLL), matched related versus unrelated donor, prior acute GVHD, and pretransplant rituximab level were explored in univariate analysis. Although none of the variables was significant for development of cGVHD, relapse/progression, or overall survival, there was a trend toward an association of relapse with CD3 chimerism at day 90 post-transplant (p=0.07) and a trend toward more cGVHD (p=0.056) in patients with no detectable rituximab at the time of transplant.
Rituximab infusion post-transplant was well tolerated with low infection rate

Non-relapse mortality at day 100 and 1-year was 0% and 3%, respectively.

No rituximab-related infusional toxicities occurred. However, rituximab-related neutropenia (defined as any absolute neutrophil count (ANC) <500/µl detected subsequent to day 56 rituximab infusion) developed in 14 (8 CLL; 6 MCL) of the 35 patients (40%). Figure S2 graphically illustrates when neutropenia was detected in patients on the trial. Fourteen patients were neutropenic one or more times after the day 56 rituximab infusion, but ten of these 14 were neutropenic before day 56 rituximab as well. Rituximab-related neutropenia was treated with G-CSF 5mcg/kg QOD for an average of 5 doses under the discretion of the treating physician. In general, the neutropenia resolved without infectious complications. Only one patient was hospitalized with infection and neutropenia after day 56 rituximab. The average duration of neutropenia was 2 weeks to 2 months. One patient (SPN 3489) had persistent neutropenia and required intermittent GCSF support for one and a half years until resolution. This patient’s MCL disease remains in remission with normal blood counts five years following HCT. Univariate analysis of rituximab related neutropenia was not significant for survival outcome.

Documented Infections

Cytomegalovirus (CMV) reactivation occurred in 12 of 21 patients at risk (57%) and is detailed in Figure S3. The median time to CMV reactivation was 10 days post-transplant (range -4 to 83 days), which was before rituximab infusion, suggesting that TLI-ATG conditioning alone leads to early CMV reactivation, as has been previously
described (39). Only one patient (SPN 3975) developed pulmonary CMV disease which resolved with intravenous ganciclovir and immunoglobulin (IVIG). Overall, 15 of the 35 patients (43%) had severe grade 3 infectious complications in the first year post-transplant, none of which were fatal. Among the 15 patients there were 5 bacterial, 9 viral, and 5 fungal infections with 3 patients having a combination. IgG concentrations remained unchanged during the first year.

**Discussion**

In this first prospective study of rituximab as cGVHD prophylaxis following allogeneic transplantation, we show that rituximab infusion 56, 63, 70, and 77 days after alloHCT is well-tolerated and reduces B cell allogeneic immunity. Our study confirms that H-Y antibodies develop in association with cGVHD following TLI-ATG conditioning, and rituximab infusion following this reduced intensity conditioning regimen has achieved our goal of reducing B cell alloimmunity with no H-Y antibody development. We believe, but have not proven, reduced B cell alloimmunity may decrease cGVHD incidence. Our institution’s standard TLI-ATG reduced intensity regimen has already reported a low incidence of acute GVHD of 2-10% (25-26) and our current study again supports this finding. The addition of rituximab to the TLI-ATG regimen was associated with a low cGVHD cumulative incidence of 20% (95%CI: 6-34%). We recognize that our group has previously reported a low cGVHD cumulative incidence of 27% for TLI-ATG without rituximab (26), however, it should be noted that 60% of the patients reported had a diagnosis of non-Hodgkins lymphoma and had received prior rituximab. The reduction of allogeneic antibody against H-Y antigens in the F→ M HCT patients was correlated
with the clinical reduction in cGVHD, providing biological support for rituximab prophylaxis decreasing B cell alloimmunity with low cGVHD incidence. We believe, but have not proven, reduced B cell alloimmunity may decrease cGVHD.

Investigators at the Dana Farber Cancer Institute are studying an alternative rituximab prophylaxis dose schedule infusing 375 mg/m² at 3 months, 6 months, 9 months, and 12 months (40). The 1-year cumulative incidence of cGVHD was 40% compared with 65% for their historical controls, adding support to the hypothesis that B-cell depletion with rituximab may be effective in preventing or controlling cGVHD (40). Reduction of extensive cGVHD has also been observed in patients treated with rituximab within six months prior to reduced intensity transplant in a retrospective series (23). Our prospective study took this observation a step further by quantifying rituximab levels pre-transplant. Patients with no detectable rituximab at the time of transplant appeared to have a trend toward more cGVHD (p=0.056), however, these results must be interpreted with caution due to the small numbers of patients.

The design of our study developed from our hypothesis that prophylactic anti-B cell therapy delivered two months after transplantation would decrease allogeneic donor B cell immunity and possibly the incidence of cGVHD. In normal B cell development, CD20, the target of rituximab, is first expressed after heavy and light chain gene rearrangement, and CD20 is no longer expressed on the majority of mature plasma cells. Thus, B cell depletion two months after allogeneic transplantation was expected to: 1) deplete donor derived alloreactive B cells, 2) permit reconstitution of newly generated tolerant B cells derived from donor hematopoietic stem cells, and 3) maintain
CD20 negative plasma cells and immunoglobulin levels to protect against infection. Using H-Y antibody development as a biomarker for alloreactivity, we directly demonstrated that rituximab infusion two months after allogeneic transplantation depleted B cells that are alloreactive for the recipient. None of the F→M HCT patients receiving rituximab two months after transplantation developed H-Y antibodies or cGVHD. In contrast, 14 of 25 (56%) F→M HCT patients who never received rituximab after alloHCT did develop H-Y Ab in strong association with cGVHD (P<0.005), further supporting our hypothesis that alloreactive B cells play a central role in cGVHD pathogenesis. The prevention of H-Y antibody development and cGVHD is in agreement with a recent murine study which shows donor B cell alloantibody deposition and germinal center B cell infiltration affected cGVHD liver and lung tissue (12). Further, Srinivasan et al. (12) demonstrated that bone marrow grafts obtained from mice genetically incapable of somatic hypermutation or undergoing IgG isotype switching significantly decreases cGVHD development. Consistent with this murine study, our low cGVHD incidence may result from rituximab prophylaxis 2 months after alloHCT decreasing/preventing allogeneic antibody development.

Another interesting observation of our study was that rituximab within 6 months prior to alloHCT did not prevent H-Y antibody development although our rituximab prophylaxis 2 months after alloHCT did. Patients receiving rituximab within 6 months prior to alloHCT did have decreased early donor B cell engraftment, but this pre-HCT rituximab did not prevent allogeneic H-Y antibody development. In contrast, rituximab prophylaxis two months after alloHCT decreased, and possibly prevented, allogeneic H-Y antibody development with low chronic GVHD incidence. With the recent suggestion that
allogeneic antibodies are pathogenic for cGVHD development (12), the prevention of allogeneic antibody development may be a worthwhile pharmacodynamic goal for cGVHD prevention.

Extensive bone marrow immunophenotyping studies of patients from the study confirmed that rituximab treatment depleted adoptively transferred immunoglobulin expressing mature donor B cells. After rituximab prophylaxis, B cells developed from donor hematopoietic stem cells and lymphoid progenitor cells by one year after transplantation (BS, manuscript in preparation). Long-lived CD20 negative plasma cells were unaffected by rituximab in the TLI-ATG transplant, thereby explaining the relatively unchanged IgG concentrations in our study patients.

Our study of prophylactic rituximab was restricted to patients with CD20+ malignancies so that loss of potential graft-versus-tumor (GVT) effects by allogeneic B cell depletion would be offset by direct anti-tumor effects of the monoclonal antibody. In doing so, however, we recognize that donor B cell reconstitution could be impacted by patient’s pretransplant rituximab therapy. Compared to the patients with myeloid malignancies undergoing the same TLI-ATG conditioning but never receiving rituximab, the CLL and MCL patients reconstituted fewer CD19+ B cells at day 56 after HCT (Figure 2a). Thus, future studies of post-transplant rituximab in patients with non-B cell malignancies will be required to further elucidate the potential impact of prophylactic anti-B cell directed therapy on cGVHD incidence and severity.

The survival and freedom from progression at four years for related and unrelated donors in high risk CLL patients with reduced intensity conditioning is comparable to
other published studies from Sorror et al. (41) and Khouri et al. (21) albeit with low transplant related risk. Moreover, MRD durability was achieved with associated GVT responses in 53% of patients, with low incidence and severity of cGVHD. Only 4 of the 10 MRD-negative patients developed cGVHD, and all surviving patients have been tapered off immune suppression medications. These results compare favorably with those of the CLL3X trial performed by the GCLLSG (German CLL Study Group) (42). With mantle cell lymphoma, the patients were more varied between CR and PR status, but overall survival and freedom from progression were comparable to prior reports (43). The non-relapse mortality of only 3% at one year and the low incidence of acute (6%) and cGVHD (20%) compare favorably.

Neutropenia following rituximab infusion was observed in 40% of patients and should be a caution when using rituximab post-transplant. Neutropenia has been reported in other rituximab studies, including our own institution (44-46). Often neutropenia will resolve with short-course G-CSF support without serious infectious complications. While infections were generally not increased in our cohort, one important consideration in using post-transplant rituximab is the impaired ability to mount a humoral immune response against neoantigens, such as H1N1, as was observed in one of the fatalities among the cGVHD patients. The long-term clinical follow-up and immunologic reconstitution studies are critical to fully evaluate rituximab prophylaxis following alloHCT.
Because this trial employed a nonmyeloablative regimen, mixed chimerism was expected, and provision was made for patients with progressive disease post-transplant to receive dose-escalated DLI. Thirteen patients received DLI for disease progression and eight of the patients are alive. Mixed chimerism at day 90 had a trend of association with an increased relapse rate in univariate analysis (p=0.07) Rituximab was not felt to have an effect on mixed chimerism in this study as the rate was comparable to that seen in our standard TLI-ATG regimen (26). There was also the observation that 53% of the CLL patients achieved CR to the level of molecular remission from PR without DLI which must be attributed to potent GVT effects, even with the minimal-intensity conditioning of TLI-ATG.

In summary, we show that rituximab prophylaxis after reduced intensity conditioning transplantation is feasible and associates with a low cGVHD incidence while maintaining disease control. Furthermore, post-transplant rituximab depletes alloreactive B cells as shown by H-Y antibody testing. In this first prophylactic study of rituximab after alloHCT, we provide important insights into donor B cell depletion by applying rituximab pharmacokinetic measurements, B cell phenotyping, and allogeneic antibody assessment of 35 patients receiving day 56, 63, 70, and 77 rituximab. The optimal timing of rituximab prophylaxis may be suggested by comparing clinical outcomes using three dosing schedules, 1) rituximab included conditioning (21), 2) rituximab depletion of alloreactive B cells 2 months after alloHCT, or 3) sustained and repeated B cell depletion infusing rituximab every 3 months through a year following alloHCT(40). Correlative laboratory studies of rituximab PK, donor B cell reconstitution,
and both allogeneic and protective anti-infection antibodies will aid the design of a well-powered and informative randomized trial to test the efficacy and safety of rituximab prophylaxis for cGVHD prevention.

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Author contributions and Disclosures of Conflicts of Interest

SA, BS, and DBM designed the experiments, analyzed the data and wrote the manuscript. CDJ and JZ designed experiments and analyzed data. GLC, and JR performed experiments, BN analyzed data. SA, RL, JAS, LJJ, GGL, WKW, JEB, JS, JB, RSN, and DBM treated patients on protocol, and all authors revised the manuscript. The authors have no conflicts of interest to disclose.
References


Table 1a. Patient characteristics- chronic lymphocytic leukemia (CLL), n=22

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<td>Gender</td>
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SPN= Stanford patient number, dx= diagnosis, Mos= months, R= rituximab, Cyto by FISH= Cytogenetics determined by fluorescent in situ hybridization, Flud.Refract.= fludarabine refractory*, VH-IgG = heavy chain immunoglobulin, Unmut=unmutated VH-IgG CLL clone (CLL VH-IgG sequence varies by <2% from germ line), LN= lymph node, CLL= chronic lymphocytic leukemia, HCT= hematopoietic cell transplantation, RD= matched related donor, URD= unrelated donor, MM= mismatch, M= male, F= female, N= no, Y= yes, NA= not available, ND= not determined, CR= complete response, PR= partial response, PD= progressive disease

*defined as failure to achieve a partial response or complete response to at least one fludarabine-containing regimen, disease progression while on fludarabine treatment, or disease progression within 6 months of the last dose of fludarabine
Table 1b. Patient characteristics- mantle cell lymphoma (MCL), n=13

<table>
<thead>
<tr>
<th>Patient Characteristics</th>
<th>Mantle Cell Lymphoma Disease Features</th>
<th>Mantle Cell Lymphoma Transplant Features</th>
</tr>
</thead>
<tbody>
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SPN= Stanford patient number, dx= diagnosis, Mos= months, R= rituximab, MCL= mantle cell lymphoma, HCT= hematopoietic cell transplantation, RD= matched related donor, URD= unrelated donor, MM= mismatch, M= male, F= female, NA= not available, PET= positron emission tomography, pos= positive, neg= negative, CR= complete response, PR= partial response
Table 2. Acute and chronic GVHD manifestations of the patients.

<table>
<thead>
<tr>
<th>SPN</th>
<th>RD/URD</th>
<th>CD34 cell dose per kg (10^6)</th>
<th>Full donor chimerism (PB CD3&gt;95%) first achieved</th>
<th>aGVHD onset</th>
<th>aGVHD grade</th>
<th>aGVHD organs involved</th>
<th>cGVHD onset</th>
<th>cGVHD severity</th>
<th>cGVHD organs involved</th>
<th>Days on prednisone</th>
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<tr>
<td>3431</td>
<td>RD</td>
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<td>D28</td>
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<td></td>
<td></td>
<td>D322</td>
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<td>mouth, liver, fasciitis</td>
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<td>D90</td>
<td>D70</td>
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<td>skin</td>
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<td>D180</td>
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<td>D180</td>
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<td>378</td>
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<td>3723</td>
<td>URD</td>
<td>4.8</td>
<td>D28</td>
<td>D25</td>
<td>2</td>
<td>skin</td>
<td>D146</td>
<td>severe</td>
<td>skin, gut, liver</td>
<td>Never stopped-death (562 days on steroids)</td>
</tr>
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<td>3879</td>
<td>RD</td>
<td>6.2</td>
<td>D90</td>
<td></td>
<td></td>
<td></td>
<td>D413</td>
<td>mod</td>
<td>skin, fasciitis</td>
<td>Never stopped-death (425 days on steroids)</td>
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<td>D28</td>
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<td>D168</td>
<td>mod</td>
<td>mouth, gut, skin</td>
<td>644</td>
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<tr>
<td>3969</td>
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<td>D180</td>
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<td>mild</td>
<td>skin, gut</td>
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RD=matched related donor; URD=unrelated donor; aGVHD=acute GVHD; cGVHD=chronic GVHD


**Figure Legends**

**Figure 1. Trial schema**
Reduced intensity conditioning using 80 cGy TLI x 10 days and ATG 1.5 mg/kg x days 1-5 followed by PBPC infusion on day 0. Rituximab infusion (375 mg/m²) was infused on days 56, 63, 70, 77. CSP and MMF were used as primary GVHD prophylaxis. Triangles (▲) indicate time points for peripheral blood immune analyses.

**Figure 2a. Blood CD19+CD5-CD23- B cell quantification**
Blood samples collected from HLA identical donors (○), and study subjects (●) before TLI-ATG conditioning and 56 days after alloHCT were FACS analyzed to quantify CD19+ B cells. Recipient MCL and CLL cancer cells were excluded from this donor B cell quantification by excluding CD5+ or CD23+ cells. Blood CD19+CD5-CD23- B cell quantification performed 90 and 180 days after alloHCT showing limited donor B cell recovery following rituximab. For comparison, donor B cell recovery 56 days after TLI-ATG alloHCT was determined in 19 patients who never received rituximab (▲) to control for passive transmission of rituximab infused before HCT.

**Figure 2b. Rituximab infused 6 months or less before HCT is detected by ELISA at transplant**
Blood collected immediately before conditioning was measured by ELISA for rituximab concentration (µg/ml; Y-axis). Each pre HCT rituximab level was related to the number of months since their last rituximab infusion (x-axis).

**Figure 2c. CLL decreases following rituximab infusion 56 days after alloHCT**
Immunophenotyping detected persistent CD19+CD5+CD23+ CLL cells 56 days after HCT in most CLL patients. Ten of 16 (63%) patients had >10 CD19+CD5+CD23+ cells/µl peripheral blood. Following rituximab infusion, only four of 20 had CLL detected by flow cytometry on day 90. Twelve MCL patients had negligible CD19+CD5+CD23- cells in blood measured on both days 56 and 90.

**Figure 3. Only 20% of patients receiving rituximab prophylaxis developed chronic GVHD.** The cumulative incidence of cGVHD at 4 years was 20% (95% CI: 6 - 34%).

**Figure 4. Rituximab prophylaxis prevents H-Y allogeneic antibody development**
Blood IgG against 5 H-Y antigens were determined by ELISA in 25 male patients with female donors who never received rituximab following TLI-ATG (left panel) and ten study patients treated with rituximab days 56, 63, 70, and 77. These heat maps show that no alloreactive H-Y antibodies developed in study patients receiving rituximab two months after TLI-ATG alloHCT, whereas one or more H-
Y antibodies developed in 56% (14/25) of patients receiving TLI-ATG without post-transplant rituximab. Considering all patients who survived 9 months, rituximab prophylaxis prevents H-Y antibody development (p=0.01).

**Figure 5a,b. Overall survival following rituximab prophylaxis exceeds 70%**
For the CLL patients, the 4-year overall survival was 73% (95% CI: 57%-94%) and freedom from progression was 47% (95% CI: 30%-75%). For the MCL patients, the 4-year overall survival was 69% (95%CI: 48%-99%) and freedom from progression was 53% (95%CI: 31%-89%).
Figure 1. Trial schema.

ATG: Anti-thymoglobulin 1.5mg/kg x 5 days

TLI: Total lymphoid irradiation 80cGy x 10 days
Figure 2a.

CD19+CD5-CD23-

p<0.01

Rituximab infused
d56, 63, 70, 77

Donor
n=18

Pre
n=25

d56 TLI/ATG
no ritux
n=19

d56
n=24

d90
n=33

d180
n=32

2c.

CLL patients CD19+CD5+CD23+ (n=16)

Rituximab

MCL patients CD19+CD5+ (n=12)

Rituximab
Figure 2b.
Figure 3.
Figure 4. H-Y antibodies measured in male patients with female donors following allogeneic HCT
Figure 5.

a. CLL

b. MCL

Survival Probability

Days post Tx
Prophylactic rituximab after allogeneic transplantation decreases B cell alloimmunity with low chronic GVHD incidence