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Epstein-Barr virus related post-transplant lymphoproliferative disorder prevention strategies in allogeneic hematopoietic stem cell transplantation

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Abbreviations:

allo-HSCT; Allogeneic hematopoietic stem-cell transplantation
ATG; Anti-Thymocyte Globulin
CTL; Cytotoxic T Lymphocyte
EBV; Epstein-Barr Virus
GVHD; Graft versus Host Disease
PBMC; Peripheral blood mononuclear cells
PTLD; Post-Transplant Lymphoproliferative Disorder
TCD; T-cell depleted
WB; Whole blood

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Running Head:

EBV PTLD prevention strategies in alloHSCT

Summary

Epstein-Barr virus associated post-transplant lymphoproliferative disorders (EBV PTLD) are recognized as a significant cause of morbidity and mortality in patients undergoing allogeneic hematopoietic stem cell transplantation (alloHSCT). The number of patients at risk of developing EBV PTLD is increasing, partly as a result of highly immunosuppressive regimens, including the use of anti-thymocyte globulin (ATG). Importantly, there is heterogeneity in PTLD management strategies between alloHSCT centres worldwide. This review summarises the different EBV PTLD prevention strategies being utilized including the alloHSCT and T-cell depletion regimes and the risk they confer; monitoring programs, including the timing and analytes used for EBV virus detection, as well as pre-emptive thresholds and therapy with rituximab. In the absence of an institution specific policy, it is suggested that the optimal pre-emptive strategy in HSCT recipients with T-cell depleting treatments, acute graft vs. host disease (GVHD) and a mismatched donor for PTLD prevention is, i) monitoring of EBV DNA post-transplant weekly using plasma or WB as analyte and, ii) pre-emptively reducing immune suppression (if possible) at an EBV DNA threshold of >1,000copies/mL (plasma or WB), and treating with rituximab at a threshold of >1000 copies/mL (plasma) or >5,000copies/mL (WB). There is emerging evidence for prophylactic rituximab as a feasible and safe strategy for PTLD, particularly if pre-emptive monitoring is problematic. Future management strategies such as prophylactic EBV specific CTLs have shown promising results and as this procedure becomes less expensive and more accessible, it may become the strategy of choice for EBV PTLD prevention.

Introduction

Epstein-Barr virus associated post-transplant lymphoproliferative disorders (EBV PTLD) are recognised as a significant cause of morbidity and mortality in patients undergoing allogeneic hematopoietic stem cell transplantation (alloHSCT).[1] Before the year 2000, an attributable mortality for EBV PTLD after HSCT was reported as 86% at 1 year post-transplant.[2] With the introduction of new approaches to prevention and treatment of EBV PTLD such as monitoring for EBV DNA levels using nucleic acid testing (NAT) of peripheral blood, pre-emptive therapy and timely treatment with rituximab or cytotoxic T lymphocytes (CTLs), patient outcomes have improved substantially. However, current mortality rates associated with EBV-PTLD following an allogeneic HSCT (allo-HSCT) are still of concern, at 30%-55%.[3,4] Moreover, there is heterogeneity in management strategies used between alloHSCT centres internationally.[5,6] With a recent increase in the use of anti-thymocyte globulin (ATG) in alloHSCT to reduce the risk of GVHD, the number of patients at risk of developing EBV PTLD is increasing.[7-10] This review summarises, and provides a contemporary perspective of the management strategies to prevent EBV PTLD in an era of increasing alloHSCT using T-cell depleted regimens.

Background

EBV belongs to the genus *Lymphocryptovirus*, subfamily *Gammaherpesvirinae*, family *Herpesviridae*.[11] In immunocompetent hosts, primary EBV infection is predominantly asymptomatic, occasionally causing an infectious mononucleosis in adolescents or young adults. EBV establishes a life-long latent infection in B-cells, controlled by T-lymphocytes and

NK cells. However, in immunocompromised hosts, particularly with T-cell depletion, EBV initiates a latent growth, causing naïve B-cells to transform into proliferating blasts.[11]

EBV PTLD are lymphoid and/or plasmacytic proliferations that occur following a solid organ transplantation or alloHSCT due to immunosuppression.[1] Although the vast majority of PTLD are related to the presence of EBV, EBV-negative disease does occur. Also referred to as EBV lymphoproliferative disease (LPD), EBV PTLD is the result of the outgrowth of EBV-infected B-cells that would normally be controlled by an effective EBV-specific cytotoxic T-cell response.[12] This occurs due to a disruption to the normal balance between latently infected B-cell proliferation and an EBV-specific T-cell response, in which the increased number of latently infected B-cells develop into PTLD, typically presenting with lymphadenopathy or nodules, which may be localized or present as diffuse disease. Non-specific constitutional symptoms such as fever, weight loss, and fatigue are also common.[13,14]

PTLD in alloHSCT recipients is most commonly donor-derived and thought to be due to the proliferation of EBV-infected B-cells in the absence of active T-cell immune surveillance. In a alloHSCT donor infected with EBV, there is an estimated one per million transformed B-cells carrying the virus that are "held in check" by cytotoxic T-cells, resulting in an equilibrium between cell division and death of EBV-infected B-cells.[12] However, when mature T-cells are depleted from a graft, EBV-transformed B-cells may escape from cytotoxic T-cell surveillance, increasing the risk of PTLD. Risk factors for the development of PTLD post HSCT are shown in Table 1.

T-cell depletion (TCD) and EBV PTLD

Initial studies in recipients of alloHSCT that were selectively T-cell depleted (TCD) to prevent GVHD demonstrated EBV reactivation in 65% of recipients, with approximately 20% of these developing EBV PTLD.[15] The study suggested that an elevated EBV DNA load was predictive for developing EBV PTLD, as no patients with a viral load ≤1,000gEq/mL developed PTLD. The threshold of 1,000gEq/mL however, provided a relatively poor positive predictive value for the development of PTLD, with only 39% of patients developing PTLD above this viral load.[15] A follow up study which included a broader range of HSCT recipients showed that only 50% of patients with elevated EBV DNA (defined as >4,000copies/μg PBMC EBV DNA) subsequently developed PTLD, however there were no patients that developed PTLD with negative PCR results.[16]

ATG has been widely incorporated into alloHSCT conditioning regimens as part of GVHD prophylaxis in many transplant centres internationally.[17] ATG is used as a form of *in vivo* donor T-cell depletion as well as depleting host T-cells that remain after the conditioning regimen.[18] Therefore, a major consideration when using ATG is the increased risk of EBV viremia and subsequent risk of EBV PTLD due to a reduction in active T-cell immune surveillance against EBV-infected B-cells. In a large phase 3 trial by Walker *et al.* comparing rabbit ATG versus no ATG in alloHSCT conditioning to reduce the incidence of GVHD, 33% of patients in the ATG arm had EBV reactivation compared with 3% who did not receive ATG.[7] However, other similar randomised controlled studies such as Kroger *et al.* reported fewer EBV reactivations (3.6% with ATG vs 1.4% without ATG).[19] This effect may be due to the type of ATG used, with Walker *et al.* using 4.5mg/kg Thymoglobulin™ and Kroger *et al.* using 30mg/kg of Fresenius™ ATG. Finke *et al.* also used Fresenius™ ATG at a total dose of

60mg/kg, in which 5% of patients in the ATG arm developed EBV PTLD, of which 3 of 5 patients died.[20] Routine EBV monitoring was not mandatory and subsequently the EBV reactivation rate was not reported. Increased EBV reactivation has also been noted following the use of the anti-CD52 monoclonal antibody alemtuzumab for in vivo TCD.[21] Burns *et al.* demonstrated a 48% incidence of EBV reactivation and 4.3% EBV PLTD in consecutive alloHSCT recipients receiving a total of 50mg of alemtuzumab during conditioning.[21]

Strategies to prevent EBV PTLD

There are currently no effective antiviral agents against EBV. Therefore, prevention or pre-emptive therapy is vital in reducing morbidity/mortality caused by EBV PLTD. Recent evidence-based guidelines from the European Conference in Infections in Leukemia (ECIL) recommend weekly screening of EBV DNA for at least three months in high-risk allogeneic HSCT recipients.[1] Monitoring of EBV viral load using quantitative NAT assay, usually by polymerase chain reaction (PCR) amplification assays is recommended. When EBV DNA is detected to a certain threshold, reduction of immunosuppression and pre-emptive treatment with rituximab is recommended.[1] However, with heterogeneity in the assays used for assessing EBV viral load, recommendations for intervention vary. Other novel emerging strategies include prophylactic rituximab with alloHSCT conditioning chemotherapy or the use of EBV CTLs.[1]

Measurement of EBV DNA biomarkers

With different assays using serum, whole blood (WB), or peripheral blood mononuclear cells (PBMCs) to measure EBV DNA, results are difficult to generalise and require differing

interpretation. With no universal standard for PCR assays, the ECIL guidelines do not recommend a specific threshold value of EBV viremia for initiating pre-emptive therapy. Studies have defined an EBV threshold of 1,000copies/mL, 10,000copies/mL, or 40,000copies/mL when determined in plasma, whole blood, or serum, respectively.[1] The rate of increase of EBV copies is also clinically significant given that increases in EBV viremia are due to the expansion of EBV-infected memory B-cells in the peripheral blood.

A recently study that used receiver operating characteristic (ROC) curves to predict PTLD among 2642 European recipients of both solid organ and alloHSCT transplants was able to develop a model to predict the development of EBV viremia measured in plasma.[22] EBV viremia was observed in 331/1784 recipients (18.6%, 95% CI 16.8–20.4). The area under the curve (AUC) of the ROC for EBV viremia predicting the development of PTLD was 59% (51–68%) amongst alloHSCT recipients. Recipients with a negative EBV DNA were less likely to develop PTLD [0.09 (0.05–0.16)] compared to those with EBV DNA at the lower limit of detection. Those with an EBV DNA of 501-5,000copies/mL had a non-significant increased risk of PTLD [2.03 (0.83–4.95)] while a viral load >5,000copies/mL was significantly associated with PTLD [5.78 (1.57–21.25)]. The inclusion of clinical predictors such as age, gender, transplant year and type, high-risk EBV serostatus, and routine biochemistry in addition to EBV viremia in the ROC model increased AUC to 84% (79–89%) among HSCT. Additional factors such as TCD treatment, acute GVHD and donor match increased AUC to 85% (78–91%). These results demonstrate that there is little benefit for the routine monitoring of all alloHSCT recipients, but rather only those with risk factors such as TCD treatment (including the use of ATG or alemtuzumab), acute GVHD and a mismatched donor.

Additionally, it was shown that a plasma viral load >5,000copies/mL significantly increased the risk of developing EBV PTLD. This must be taken into consideration for the timing of initiating pre-emptive treatment, with early intervention required prior to the EBV DNA threshold of 5,000copies/mL.

For detection of EBV, the optimal choice of assay sample between plasma, WB and PBMCs, is debatable.[23,24] Despite partial correlation between WB and PBMC ($r = 0.755$, $P < 0.001$),[25] detection with PBMCs appears to be the most sensitive technique, however this technique may be too sensitive for indicating the onset of a rising EBV level as a surrogate to predict PTLD.[26] It has also been suggested that, by using serum, the cell-free viral DNA detected may reflect either the release of EBV material due to cell lysis or a lytic infection, which may be more closely associated with the onset of PTLD.[25,27,28] Care must also be taken when interpreting results due to a wide inter-laboratory variation in results, however the standardization and reproducibility of these results are improving.[29,30] Because of such variation in assays sample types used and DNA levels reported in the literature, it is difficult to assess the exact DNA levels required for preventative management interventions.

Pre-emptive therapy with rituximab

The two most common pre-emptive strategies associated with a reduction in progression to PTLD after EBV viremia are treatment with rituximab and reduction of immunosuppression.[1] The ECIL guideline recommends that the primary method for pre-emptive therapy is rituximab, 375 mg/m^2, once weekly until EBV viremia negativity.[1] The number of doses administered should be assessed locally on the basis of changes in EBV viremia and an assessment of the patient's immune function; this usually ranges from one to four doses. In addition, the use of

rituximab should be combined with reduction in immunosuppression if possible, particularly as there is a significant survival advantage in patients who have progressed to PTLD when both rituximab therapy and a reduction of immunosuppression are applied (39% cumulative incidence of PTLD related mortality for rituximab alone, compared to 16% with combined rituximab and reduction in immunosuppression).[3] The utility of pre-emptive therapy with rituximab is well established, with a rate of cure or EBV negativity using this method of 90%, versus using rituximab for the treatment of PTLD at 63%.[31] The optimal EBV viral load threshold to initiate pre-emptive therapy is currently unknown, though various studies have used EBV viral loads thresholds as low as 1,000copies/mL (plasma) to 40,000copies/mL (WB). A summary of studies which describes a pre-emptive approach is shown in Table 2.

A recent publication from Jain *et al.* evaluating 488 patients who underwent myeloablative or non-myeloablative alloHSCT's described the use of pre-emptive rituximab in one of the largest cohorts to date.[32,33] The institutional protocol for initiation of rituximab was EBV measured on WB >2,000copies/ml with increasing viral load (clinically relevant EBV reactivation); alternatively, if EBV copy number was >5,000copies/mL, immediate therapy was considered with repeat monitoring after 3-5 days; alternatively, for patients with <5,000copies/mL, repeated EBV testing every 3-5 days with treatment instituted in patients considered at a higher risk for EBV reactivation. Rituximab was given at 375mg/m^2 weekly until EBV levels were undetectable or at provider's discretion. Sixty-seven (14%) patients had clinically relevant EBV reactivation after alloHSCT. Of these, 60 (90%) received rituximab for EBV reactivation which resolved without any additional clinical consequence, 1 (1.5%) received rituximab but later developed PTLD and 6 (9%) developed PTLD prior to initiation

of rituximab. Median time to EBV reactivation was at 34 days (range, 13-602) post alloHSCT. Levels of EBV copies/mL at the time of initiation of rituximab were 2,000-3,999 in 14 (23%), 4,000-5,999 in 11 (18%), 6,000-7,999 in 3 (5%) and ≥ 8,000 in 32 (53%) patients. The median of the highest level of EBV load was 10,300copies/ml (range, 2,000-645,000). Doses of rituximab required to clear viremia were 1 in 31 (53%), 2 in 15 (26%), 3 in 5 (9%) and 4 in 7 (12%) patients. It took a median of nine days (range, 1-41) from the initiation of rituximab to resolve viremia. The median level of EBV viral load in blood was higher in patients who required more than one dose of rituximab (1 dose: 8,060copies/mL; >1 dose: 13,100copies/mL; P=0.03). Immunosuppression was reduced in 15 (47%) patients whose viremia cleared with one dose of rituximab compared to 4 (15%) patients who required >1 dose to clear (P=0.005). This finding suggests early intervention may reduce the total cumulative dose of rituximab required to pre-emptively treat the EBV reactivation. Whilst the pre-emptive strategy used by Jain *et al.* of treating with rituximab when EBV DNA level on WB was between 2,000-5,000copies/mL did result in a small number of break-through PTLDs, 1.4% is among the lowest rate demonstrated by a large-scale study to date. The low rate of PTLD in this study is particularly remarkable given that over half of the study cohort had received ATG.

A similar strategy was reported by Michallet *et al.* who evaluated 359 allo-HSCTs using EBV PCR monitoring on WB.[34] When a patient exceeded 1,000copies/ml, the primary therapeutic intervention was a gradual weaning of immunosuppression, and then rituximab infusions (375mg/m², four injections weekly) were administered when the viremia exceeded 10,000copies/ml. Two hundred and twenty-two (62%) patients reactivated EBV after a median time of 1.3 months (0.7-2.5) after allo-HSCT with a cumulative incidence of 48 % (47-50) at

3 months. Among the 222 patients with EBV reactivation, only 35 (15.7%) needed treatment with rituximab. Rituximab was introduced after a median time of 55 days post transplantation with a median number of five infusions and a median dose of 2025mg/m². EBV treatment was successful in all patients; none progressed to PTLD. Interestingly, this study also demonstrated that the presence of EBV reactivation (with multivariate analysis) was associated with a significant lower relapse rate, HR= 0.52 [0.35-1], P=0.05 on multivariate analysis . This pre-emptive strategy used by Michallet *et al.* was successful in preventing PTLD and provides a useful technique of defining an early EBV DNA level on WB to reduce immune suppression. However, the results are difficult to extrapolate to other studies as the ATG usage rate and dose is not reported and therefore the patients' baseline risk of developing PTLD may not be the same as other studies.

Other novel approaches in pre-emptive treatment involve treating only when EBV load is >40,000copies/mL (WB) and circulating CD4+ T-cells $<0.3x10^9$/L,[35] or when the first signs of clinical symptoms of PTLD (eg, adenopathy or fever) appeared, in addition to >4,000copies/μg (PBMC)[16]. However, these strategies were associated with the development of PTLD and required more intensive patient monitoring than the more conservative EBV viral load monitoring. By using the latter method, Wagner *et al.* demonstrated that the detection of 2 or more levels of EBV DNA levels >4,000copies/μg (PBMC) had a sensitivity of 100% for the prediction of early PTLD but a specificity of only 50% (in 8/16 patients).[16] Despite initial concerns about the safety of rituximab following alloHSCT, the impact of pre-emptive rituximab has been consistently shown to have no significant deleterious effect on overall survival or non-relapse mortality.[21]

Jain *et al.* showed that when using pre-emptive management with rituximab, patients who demonstrate a higher level of EBV viremia before rituximab intervention, required a greater number of subsequent doses of rituximab to clear EBV viremia.[32] As it has been demonstrated that the majority of patients who exceed EBV viral loads of 5,000copies/mL will also reach 10,000copies/mL (serum),[28] it is hypothesised that pre-emptive treatment with rituximab at a lower threshold, may reduce the treatment required to clear viremia and be advantageous both economically and for the patients' quality of life.

Prophylactic therapy with rituximab

Given the apparent safety and efficacy of rituximab administered post alloHSCT for EBV viremia, as well as previous studies suggesting that rituximab administered prior to alloHSCT for B-cell malignancy lowered the incidence of EBV reactivation post-transplant,[21] some investigators have assessed the role of rituximab prophylaxis either immediately before, or after the day of stem cell infusion.[36-39] Studies using rituximab prophylactically are outlined in Table 2.

Dominietto *et al.* used a 200mg total dose of rituximab on day +5 in 55 alloHSCT patients compared to a control group of 68 patients without rituximab, which resulted in a significantly lower rate of EBV viremia [31/55 (56%) vs 58/68 (85%), P=0.0004] and a significantly lower risk of exceeding 1,000copies/10^5 (PBMC) [8/55 (14%) vs 33/68 (49%), P=0.0001]. There were no patients in the rituximab prophylaxis group who died following the development of PTLD vs 2/68 (3%) in the control group (P=0.3). Additionally, the cumulative incidence of grade II-IV acute GvHD was significantly reduced in those receiving rituximab

(20 vs 38%, P=0.02). There was a trend for a survival advantage for patients receiving rituximab prophylaxis (46 vs 40%, P=0.1), mainly because of lower transplant related mortality (25 vs 37%, P=0.1). With all patients in this study considered at high risk of EBV PTLD due to ATG use, the approach used by Dominietto *et al.* is particularly attractive because it may result in a reduced requirement for monitoring and follow-up, which can be difficult for some patients. However, with 14% of patients still requiring a pre-emptive dose of rituximab with EBV DNA >1,000/10^5 cells on PBMC, some degree of monitoring is still required.

In the setting of haploidentical alloHSCT, Peccatori *et al.* administered a single 500mg rituximab dose on day -1 in 121 patients in combination with sirolimus for GVHD prophylaxis. No cases of PTLD or "high-titer" EBV viremia were documented, although the definition of this EBV level was not reported. The authors comment that this result was may have been due to the combined effect of rapid T-cell recovery and the inclusion of rituximab in the conditioning regimen. In addition, sirolimus may have contributed to the reduction in EBV viremia, as it has been previously demonstrated to inhibit the of growth of human EBV-transformed B-lymphocytes in vitro with reports of its use in combination with rituximab to prevent EBV viremia successfully in renal transplantation.[40,41]

Van Besien *et al.* administered a single dose of rituximab 375mg/m^2 two weeks prior to haplo-cord alloHSCT unless the patient has a recent prior exposure to rituximab due to treatment for a primary B-cell malignancy. When this cohort was compared to a control group without prior rituximab exposure, EBV reactivation occurred in 1/51 (2%) with rituximab exposure vs 27/146 (18%) without prior rituximab exposure (P=0.004). PTLD developed in 16/146 (12%) without prior rituximab exposure vs 0/51 with rituximab exposure. This study

suggests that the routine use of rituximab post transplant can prevent EBV PTLD, as well as suggesting that prior rituximab within 6 months of HSCT can lower the risk of developing EBV PTLD and might be used to help inform risk stratification.

Prophylaxis or pre-emptive therapy with CTLs

Another novel strategy for the prevention of EBV PTLD is the use of donor or third party EBV-specific CTLs. The utility of CTLs has been demonstrated both in the pre-emptive setting, with approximately 95% successful elimination of EBV viremia, and in the treatment of PTLD, with 65%-88% success, even after the failure of rituximab based therapy.[31,42-46]. However access to these products and the requirement for HLA matching poses a challenge.[1] Heslop *et al.* report on the largest cohort receiving CTLs for prophylaxis for EBV PTLD with 101 alloHSCT in which 90% were CD6+ and CD8+T-cell–depleted allografts.[46] The majority of patients received a single dose of $2x10^7$ cells/m^2 EBV-specific CTLs followed by weekly EBV monitoring. Of these, 12/101(12%) patients developed elevated levels of EBV DNA, though the exact viral load was not reported. Prophylactic infusions decreased the EBV load in 11/12 patients, with no other evidence of EBV PTLD before infusion. In the one exception, EBV DNA remained elevated at more than 1,500copies/μg (PBMC) for more than a year despite an additional dose of CTLs, but then normalized without the development of PTLD. The authors commented that these results, compared to 42 patients who did not receive CTLs but who were enrolled on the same transplantation protocol, were superior in preventing PTLD with an incidence of 5/42 (11%) in the group who did not receive CTLs.

Evidence for EBV PTLD management with the use of EBV CTLs is also a rapidly expanding area, with a number clinical trials emerging in recent years, including a large phase

III study evaluating the use of commercially available EBV CTL in alloHSCT (MATCH)[47] as well as a number of phase I studies completed or in progress.[44,48]

Potential future strategies

Peccatori *et al.*, demonstrated that sirolimus, a macrolide antifungal antibiotic isolated from *Streptomyces hygroscopicus*, may have a beneficial effect against EBV viremia and PTLD.[38] The mechanism of action for this effect has previously been described, with sirolimus not only demonstrating inhibitory effects on normal cells of the immune system, but also inhibiting the proliferation of transformed cell lines.[49] Experimental studies have demonstrated inhibition of growth of human EBV-transformed B lymphocytes by sirolomus, including an inhibitory effect on the growth of PTLD-like EBV cells xenotransplanted into severe combined immunodeficiency mice, with cell death via apoptosis induction.[40,50] Potential mechanisms underlying the inhibitory effect of sirolimus include: i) inhibition of IL-10 production, an autocrine growth factor for EBV+ B-cell lymphomas, ii) inhibition of constitutive activation of the Janus kinase/signal transducer and activator of transcription pathway and iii) direct effects on the expression of cell-cycle proteins, potentially inducing arrest of EBV+ B-cell lymphomas in the G1 phase.[40] Reddy *et al.* have proposed that a combination of low-dose rituximab (100-150 mg/m^2) and sirolimus GVHD prophylaxis (with other immunosuppressive agents) may be the optimal EBV PTLD management strategy to significantly reduce the expense, morbidity, and mortality in high-risk populations, however the use of this regime has not yet been reported.

T-cell manipulation with post-transplant cyclophosphamide (PTCy) may also be a potential strategy to prevent EBV PTLD. A large retrospective analysis of PTLD incidence in 785 adult alloHSCT recipients given PTCy as GVHD prophylaxis demonstrated no PTLD at 1 year post HSCT.[51] This finding is consistent with recent reports on the use of PTCy in haploidentical HSCT.[52,53] Several hypotheses for this effect have been described including destruction of donor and host EBV-infected B-cells, relative sparing of EBV specific memory T-cells, and rapid immune reconstitution.[51] Recent studies describing dual T lymphocyte suppression with ATG and PTCy in haploidentical transplantation however, have demonstrated a significant EBV PTLD risk, with 64% reactivating EBV and 8% developing PTLD, although all patients were successfully treated with rituximab and reduction in immune suppression.[54]

Conclusions

EBV PTLD prevention is an essential component of care in the post alloHSCT setting, especially in T-cell depleted allograft settings. The most commonly used strategies have utilized weekly EBV PCR monitoring, usually by quantifying viral load in plasma or WB. Due to the lack of standardization of assays and sample types used, it is difficult to assess the exact DNA level required for preventative management interventions and therefore pre-emptive rituximab should be administered depending on an institute-specific threshold.

Determination of an institute-specific threshold needs to take account of sensitivity and specificity of the assay used as well as the turn-around time of results. However, in studies where the lowest incidences of PTLD have been reported, thresholds for initiating treatment have been 1,000copies/mL using plasma or 2,000-10,000copies/mL using WB. This review

suggests that the optimal PTLD prevention strategy in alloHSCT recipients who been offered T-cell-depleting treatments (including the use of ATG or alemtuzumab) for GVHD prophylaxis, have developed acute GVHD or have been transplanted using a mismatched donor is, i) monitoring of EBV DNA weekly post-transplant until day +100 using plasma or WB as analyte and, ii) pre-emptively reducing immune suppression (if possible) at an EBV DNA of >1,000copies/mL (plasma or WB), and at a threshold of >1000 copies/mL (plasma) or >5,000copies/mL (WB), treating with rituximab 375mg/m^2 weekly until clearance of EBV DNA. Although the body of evidence is still growing, prophylactic rituximab appears to be a feasible and safe strategy, particularly if pre-emptive monitoring is problematic. Future management strategies such as prophylactic EBV specific CTLs have shown promising results and as this procedure becomes less expensive and more accessible, it may become the strategy of choice for EBV PTLD prevention.

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Conflict of Interest: None

Table 1. Risk factors for EBV PTLD in HSCT

Factors which INCREASE the risk of developing EBV PTLD[1,9,55-57]
Anti-thymocyte Globulin (ATG) or alemtuzumab
In vivo T-cell Depletion
EBV serology donor/recipient mismatch (recipient-negative/donor-positive)
Cord blood transplantation
Reduced intensity conditioning
HLA mismatch
Splenectomy
Second HSCT
Severe acute or chronic GvHD requiring intensive immunosuppressive therapy
Infusion of mesenchymal stromal cells
Factors which REDUCE the risk of developing EBV PTLD[21,49,51,58]
Rituximab exposure within 6 months pre-HSCT
Post-transplant cyclophosphamide (without ATG)
Sirolimus use for GVHD Px
CD4+ T-lymphocyte count >50 at day +30

Table 2. Summary of reported EBV preventative strategies

Reference	Patients and HSCT Protocol	EBV Monitoring	Sample used	Pre-emptive EBV threshold	Treatment	EBV reactivation and PTLD rates	Other outcomes
Pre-emptive therapy with rituximab							
Van Esser *et al.* 2002[59]	N = 49 Conditioning = CyTBI GVHD Px = CSP, TCD ATG 10mg/kg (Imtix Sangstat, Amstelveen, The Netherlands), with partial ex-vivo T-cell depletion	Weekly until day 180 or longer in patients with chronic graft-versus-host disease (GVHD)	Plasma	>1,000geq/mL	Pre-emptive rituximab 375mg/m^2 and immunosuppressive medication was continued	35% (17/49) EBV >1,000geq/mL 4% (2/49) EBV PTLD	55% (27/49) EBV viremia. 14/15 treated patients without EBV PTLD had a complete and sustained response. EBV DNA in plasma became undetectable after a median of 8 days (range, 1-46 days). Median time to EBV reactivation: 112 days (range, 39-189 days) after HSCT Median EBV DNA level measured 2100geq/mL (range, 500-14,000) prior to admission and a median of 3 days (range, 1-14) elapsed between that day and initiation of pre-emptive therapy. No Recurrent reactivations.
Wagner *et al.* 2004[16]	N = 111 Conditioning = Fully ablative CyAraCTBI, BuCyAraC or CyTBI GVHD Px = Full Px not reported, ATG (type or dose not reported) either alone or with T-cell and/or B-cell depletion, or alemtuzumab (dose not reported).	Second weekly	PBMC	>4,000copies/μg in addition to sign of clinical symptoms of EBV PTLD (eg, adenopathy or fever)	Pre-emptive rituximab 375mg/m^2 weekly or CTLs	29% (25/85) EBV >4,000copies/μg (without clinical symptoms and were NOT pre-emptively treated) 9.4% (8/85) EBV PTLD (developed prolonged fever, lymphadenopathy, or other symptoms or imaging findings)	9/25 patients had EBV load elevated only once and was not accompanied by clinical symptoms, these normalized spontaneously. 16 of these 25 had EBV DNA levels >4,000copies/μg on 2 or more occasions. Of the 8 affected, two received CTLs, 5 received rituximab (1-4 doses of 375mg/m^2), and 1 patient received

	(85/111 had at least 4 EBV results for analysis, 26 excluded)						CTLs and rituximab (1 dose of 375mg/m^2). In all 8 patients, clinical symptoms associated with increased EBV load resolved.
Deeg *et al.* 2006[60]	N = 56 Conditioning = Bu/Cy GVHD Px = MTX, CSP, ATG-T was 4.5-6mg/kg	Twice weekly to day 42	Plasma	>1,000copies/mL	Pre-emptive rituximab 375mg/m^2	1.7% (1/56) EBV >1,000copies/mL (1/15 in 6mg/kg ATG-T) No EBV PTLD	
Carpenter *et al.* 2010[61]	N = 111 Conditioning = RIC FluMel or CyTBI GVHD Px = CSP, RIC: alemtuzumab 100 mg-20mg. CyTBI: 20 mg alemtuzumab "in the bag."	Weekly until day 100, and then at follow up	Whole blood	>40,000 copies/mL	Pre-emptive rituximab 375mg/m^2 (single infusion) and withdrawal of immune suppression	40% 2yr CI of EBV >200 copies/mL 16% (18/111) >40,000 copies/mL 1% (1/111) EBV PTLD	
Worth *et al.* 2011[35]	N = 70 Conditioning = RIC FluMel GVHD Px = CSP alone (sib) or CSP + MMF, alemtuzumab 0.5–1mg/kg.	Weekly until recovery of circulating CD4+ T-cells to >0.3x10^9/L.	Whole blood	>40,000copies/ml on two consecutive occasions with CD4+ T-cells to <0.3x10^9/L	Pre-emptive rituximab 375mg/m^2 weekly for 4 weeks and immunosuppression was reduced if there was no active GVHD	53% (37/70) EBV >1,000copies/mL 27% (20/70) progressed to >40,000copies/ml 1.4% (1/70) EBV PTLD	6/70 patients had viremia >40,000copies/ml but were not treated with rituximab on the basis of having T-cell counts >0.3x10^9/L and none developed EBV PTLD.
Wang *et al.* 2014[62]	N = 224 (haploidentical donors) Conditioning = Ara-C/Bu/Cy/Simustine or TBI/Cy/Simustine GVHD Px = CSP, MMF, MTX, ATG-T (N= 112 6mg/kg vs 112 10mg/kg)	Weekly until day 100, then once every 2 weeks until day 180, followed by once every month until 1 year after HSCT or twice weekly if any positive level	Not reported	>1,000copies/mL	Pre-emptive rituximab (dose and duration not reported)	6mg/kg ATG-T EBV: 10% (11/112) >1,000copies/mL 2% EBV PTLD 10mg/kg ATG-T EBV: 25% (28/112) >1,000copies/mL	

						8% EBV PTLD	
Michallet *et al.* 2016[34]	N = 359 Conditioning= 171 myeloablative, 188 RIC GVHD Px = not reported, ATG type or usage rate not reported however reported that ATG was used.	Weekly for 3 months	Whole blood	>1,000/ >10,000copies/mL	>1,000copies/mL: Reduce immune suppression at >10,000copies/ml: Pre-emptive rituximab 375mg/m² four injections weekly	62% (222/359) EBV >1,000copies/mL 16% (35/222) EBV >10,000copies/mL No EBV PTLD	Median time of treatment was 55 days after transplantation. Median of 5 rituximab infusions.
Burns *et al.* 2016[21]	N = 186 Conditioning = FluMel or CyTBI GVHD Px = CSP +/- MTX or MMF, alemtuzumab 10 mg D-7 to D-3	Weekly to second weekly for the first 6 months, intermittently thereafter	Whole blood	>20,000geq/mL	Pre-emptive rituximab 375mg/m^2 up to 4 weekly infusions	18% (38/186) EBV >20,0000geq/mL 4.3% (8/186) EBV PTLD	48% Cumulative incidence of EBV >500geq/mL. 5/8 EBV PTLD (63%) had B symptoms documented at presentation and 7/8 (88%) had stage ⩾3 disease. Of these, all 30 without evidence of PTLD, and 5/8 (63%) with PTLD responded to therapy. Median time from EBV load >20 000copies/mL and radiographically documented PTLD was 7 days (range 1–16 days). Additionally, only 1/25 (4%) patients who received rituximab within 6 months before transplant (for primary B-cell malignancy) reactivated EBV in the first year.
Jain *et al.* 2019[32,33]	N = 488 Conditioning = myeloablative and non-myeloablative GVHD Px = included a selection of TAC, MMF, MTX frequency not reported. ATG-T 2.5-	Weekly until day 100, or beyond this if GVHD, immunosuppressants or previous EBV PTLD.	Whole blood	>2,000copies/mL and continues to rise on a weekly basis; OR 2,000-5,000copies/mL and considered	Pre-emptive rituximab 375mg/m^2 weekly	14% (67/488) EBV reactivations as per criteria 1.4% (7/488) EBV PTLD	Median time to detection of EBV reactivation from HSCT was 34 (range 13–602) days. Median level of EBV copy number detected was 10,400 copies/mL (range 2,000-645,000).

	7.5mg/kg in 306/488 patients	Repeat testing every 3-5 days after reactivation		high risk of EBV reactivation; OR >5,000copies/mL			Median EBV level at which rituximab was started was 8,360 (range 2,000-645,000) copies/mL. Of the 7 patients who developed EBV PTLD, 1 developed post rituximab. 6 developed prior to initiation of rituximab.
Prophylaxis with rituximab							
Dominietto *et al*. 2012[37]	N = 123 Conditioning = CyTBI or ThioCy GVHD Px = CSP, ATG-T 6–10 mg/kg, rituximab 200mg D+5 in 55 patients (versus 68 patients without rituximab)	Weekly from D+15 to D+100.	PBMC	>1,000/10^5 cells	Prophylactic rituximab at fixed dose of 200mg for all patients was administered on D+5. EBV copies <1,000/10^5 cells had CSP dose reduced by 50% and were monitored. Patients with EBV copies >1000/10^5 cells, had their CSP discontinued and rituximab 375mg/m^2. Patients who did not clear EBV from peripheral blood in 1 week, received a second dose of rituximab 375 mg/m^2	Prophylactic rituximab: 14% (8/55) EBV >1,000 copies/10^5 cells No EBV PTLD Control: 49% (33/68) EBV >1,000 copies/10^5 cells, P=0.0001 2.9% (2/68) developed a lethal EBV PTLD, P=0.3	Prophylactic rituximab demonstrated 56% (31/55) EBV viremia compared to 85% (58/68) in the control group P=0.0004. Additionally, the cumulative incidence of grade II–IV acute GvHD was significantly reduced in rituximab patients (20 vs 38%, P=0.02). Chronic GvHD was comparable. There was a trend for a survival advantage for patients receiving rituximab (46 vs 40%, P=0.1), mainly because of lower transplant mortality (25 vs 37%, P=0.1).
Peccatori *et al*. 2015[38]	N=121 (haploidentical donors) Conditioning = TreoFlu GVHD Px = SIR, MMF, ATG-F (30mg/kg), Rituximab 500mg D-1	Weekly, start date and duration were not reported	Plasma	Definition of EBV "high-titre", or pre-emptive therapy not reported.	Prophylactic rituximab administered as a fixed 500 mg dose on day -1.	No EBV PTLD	No "high-titer" EBV viremia was documented. Authors comment this is possibly because of the combined effect of rapid T-cell recovery and inclusion of rituximab in the conditioning regimen.
Van Besien *et al*. 2019[39]	N = 198 (halpo-cord donor, supplemented by CD34	Twice weekly during	Not reported	Not reported, PTLD reported	Prophylactic rituximab 375mg/m^2 was	Prior rituximab: No EBV PTLD	2% (1/51) EBV viremia (>200 copies/mL) in the prior rituximab

	selected third-party cells to accelerate recovery) Conditioning = most commonly FluMel+/-TBI GVHD Px = TAC, MMF, ATG 4.5-6mg/kg, rituximab 375mg/m^2, 2 weeks prior to HSCT (n=38), prior treatment with rituximab (n=13), no rituximab (n=147)	hospitalization, then weekly for the first 100 days, then second weekly until day180 or as clinically indicated		based on "rapid increasing EBV levels", positive PET scan and biopsy proven PTLD	administered 2 weeks prior to HSCT (n=38) unless prior treatment with rituximab was given for primary B-cell malignancy (n=13).	No prior rituximab: 12% (16/146) EBV PTLD	group, compared to 18% (27/146), P=0.004. Of the 16/146 who developed PTLD, 11/16 were biopsy proven, and 5/16 were treated based on rapidly increasing EBV viremia and positive PET scan. 9/16 responded to single agent rituximab treatment, 7/16 required additional treatment with chemotherapy, and one also received adoptive cellular therapy with EBV directed third-party donor cells. 5/16 patients died from PTLD and one died from chemotherapy-induced sepsis.
Cytotoxic T-Lymphocytes (CTLs)							
Heslop *et al.* 2010 [46]	N = 101 CTL prophylaxis Conditioning = Not reported GVHD Px = CD6+ and CD8+T-cell–depletion in 90/101 (90%)	Weekly, start date and duration were not reported	PBMC	N/A	Prophylactic EBV CTLs were administered with 1 dose of 2x10^7 cells/m^2 to the majority of patients	No EBV PTLD	Prophylactic infusions decreased the EBV load in 11 of the 12 patients, with no other evidence of EBV PTLD before infusion. In the one exception, EBV DNA remained elevated at more than 1500 copies/μg of PBMC-DNA for more than a year despite an additional dose of CTLs, but then normalized without the development of PTLD. 12/101(12%) patients developed "elevated levels" of EBV DNA, however the exact viral load is not reported. The authors commented that these results, compared to 42 patients who did not receive CTLs but who were enrolled on the same transplantation protocol, were superior in preventing PTLD with an incidence of 5/42 (11%)

							in the group who did not receive CTLs.

Abbreviations: Cy: Cyclophosphamide, TBI: Total Body Irradiation, GVHD: Graft Versus Host Disease, Px: Prophylaxis, CSP: Cyclosporin, TCD: T-cell Depletion, ATG: Antithymocyte Globulin, ATG-T: Thymoglobulin™ Antithymocyte Globulin, ATG-F: Fresenius™ Antithymocyte Globulin, AraC: Cytarabine, Bu: Busulfan, MMF: Mycophenolate, MTX: Methotrexate, RIC: Reduced Intensity Conditioning, Flu: Fludarabine, Mel: Melphalan, TAC: Tacrolimus, Thio: Thiotepa, Treo: Treosulfan, SIR: Sirolimus,