TITLE: TNFRSF14 aberrations in follicular lymphoma increase clinically significant allogeneic T-cell responses

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Key Points

1. *TNFRSF14* gene aberrations, common in follicular lymphoma, increase the ability of lymphoma cells to stimulate allogeneic T-cell responses

2. *TNFRSF14* lesions were associated with increased acute GvHD supporting stratified transplantation approaches in the allogeneic setting
ABSTRACT

Donor T-cell immune responses can eradicate lymphomas after allogeneic hematopoietic stem-cell transplantation, but can also damage healthy tissues resulting in harmful Graft-versus-Host Disease (GvHD). Next-generation sequencing has recently identified many new genetic lesions in follicular lymphoma. One such gene, TNFRSF14, abnormal in 40% of follicular lymphoma patients, encodes the herpes-virus-entry mediator (HVEM) which limits T-cell activation via ligation of B- and T-lymphocyte attenuator. As lymphoma B-cells can act as antigen-presenting cells, we hypothesized that TNFRSF14 aberrations that reduce HVEM expression could alter the capacity of follicular lymphoma B-cells to stimulate allogeneic T-cell responses and impact the outcome of allogeneic hematopoietic stem-cell transplantation. In an in vitro model of alloreactivity, human lymphoma B-cells with TNFRSF14 aberrations had reduced HVEM expression and greater alloantigen-presenting capacity than wild-type lymphoma B-cells. The increased immune stimulatory capacity of lymphoma B-cells with TNFRSF14 aberrations had clinical relevance, associating with higher incidence of acute GvHD and in patients undergoing allogeneic hematopoietic stem-cell transplantation. Follicular lymphoma patients with TNFRSF14 aberrations may benefit from more aggressive immunosuppression to reduce harmful GvHD after transplantation. Importantly, this study is the first to demonstrate the impact of an acquired genetic lesion on the capacity of tumor cells to stimulate allogeneic T-cell immune responses which may have wider consequences for adoptive immunotherapy strategies.
INTRODUCTION

Follicular lymphoma (FL) is a common germinal center B-cell malignancy characterized by slow-progression but inevitable relapse after conventional chemo-immunotherapy. However, some patients can be cured by the graft-versus-lymphoma (GvL) effect provided by donor T-cells in the setting of allogeneic hematopoietic stem-cell transplantation (AHSCT).

FL B-cells carry the hallmark t(14;18) translocation which results in cytoplasmic overexpression of the Bcl-2 protein. Two recent studies have reported that additional tumor-specific genetic aberrations of Tumor Necrosis Factor Receptor Superfamily 14 (TNFRSF14) gene, which encodes the herpes virus entry mediator (HVEM), occur at high frequencies (18-46%) in FL patients. However these studies reported conflicting impact of TNFRSF14 aberrations on clinical outcome, suggesting that their functional effects might be influenced by factors such as differing treatment approaches.

HVEM is a type I transmembrane molecule which acts as a molecular switch through interactions with several different ligands including B and T lymphocyte attenuator (BTLA), LIGHT, CD160, lymphotxin A and glycoprotein D to regulate a range of immune responses. Interaction between HVEM expressed on antigen-presenting cells (APC) and the co-inhibitory receptor BTLA on T-cells limits T-cell activation and proliferation. BTLA has intracellular immunoreceptor tyrosine-based inhibition motifs consistent with immune inhibitory function, and BTLA-deficient animal models display exaggerated immune responses. Importantly, BTLA is expressed by naive CD4+ and CD8+ T-cells, the T-cell compartments known to be enriched for alloreactive specificity, and agonistic antibody-mediated BTLA stimulation reduces donor T-cell
mediated acute GvHD in murine transplant models, consistent with a functional role for BTLA in controlling donor T-cell alloresponses in this setting.\textsuperscript{8-10}

Activated FL B-cells can act as potent alloantigen-presenting cells \textit{in vitro}\textsuperscript{11} and patients with FL often undergo AHSCT with significant residual lymphoma. We hypothesized that \textit{TNFRSF14} aberrations would reduce expression of HVEM and increase the ability of FL B-cells to stimulate allogeneic T-cell responses. We therefore determined the functional effect of \textit{TNFRSF14} aberrations on the alloantigen-presenting capacity of human FL B-cells \textit{in vitro}. We also determined the impact of \textit{TNFRSF14} aberrations on clinical alloreactivity in FL patients after HLA-matched reduced-intensity conditioning AHSCT.
MATERIALS AND METHODS

Patient Samples
Lymph node biopsies were obtained from FL patients after written consent. The study was approved by the London Research Ethical Committee (05/Q0605/140) and was conducted in accordance with the Declaration of Helsinki.

**TNFRSF14 mutation and deletion analysis of FL B-cells**
Tumor DNA from pre-AHSCT lymph node biopsies from FL patients was screened for *TNFRSF14* mutations by PCR amplification/ Sanger sequencing and for deletions by multiplex ligation-probe amplification as previously described. Primers used for Sanger sequencing are summarized in Supplementary Table 1.

**FL B-cell sorting, activation and phenotyping**
FL B-cells were stained with CD10- fluorescein isothiocyanate (FITC) (clone 97C5) and CD20- Peridinin chlorophyll (PerCP) (clone LT20) antibodies (both from Miltenyi Biotec) and purified by fluorescence-activated cell sorting (FACS) of dual-positive events on a FACSaria device (Becton Dickinson). Dead cells were excluded using DAPI. Purity of sorted FL B-cells was routinely more than 90% and sorted FL B-cells were routinely more than 95% light chain-restricted assessed with anti-Ig light chain kappa-AlexaFluor700 (clone MHK-49) and anti-Ig light chain lambda-Allophycocyanin (APC) (clone MHL-38) antibodies (Supplementary Figure 1). Following sorting, FL B-cells were activated for 48 hours with 1µg/ml soluble CD40L (InVivoGen), 5µg/ml AffiniPure F(ab’)2 Fragment Goat anti-human IgA+IgG+IgM (H+L) (Jackson Immunoresearch), 5µg/ml CpG (RnD Systems), and 50ng/ml IL-4 (RnD Systems) to
optimally upregulate expression of molecules involved in antigen presentation as previously described.\textsuperscript{13,14} Immunophenotyping of CD10\textsuperscript{+}CD20\textsuperscript{+} FL B-cells was performed by flow cytometry using the following antibodies HVEM-Phycoerythrin (PE) (clone 122), CD58-PE (clone TS2/9), MHC Class I-Pacific Blue (clone W6/32) HLA-DR- APC (clone L243), CD80-PE-Cyanine (Cy)7 (2D10), CD86-APC (clone IT2.2), and their corresponding isotype controls (all from Biolegend).

**Measurement of FL -B-cell-stimulated T-cell alloresponses**

Untouched CD3\textsuperscript{+} T-cells were purified by negative selection from healthy allogeneic donor peripheral blood mononuclear cells (PBMC) using the Pan T-cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Post-sort purity assessed by flow cytometry was routinely greater than 95%. T-cells were stimulated with activated irradiated (30Gy) FL B-cells at a ratio of 3:1 (T: B-cell) for primary allogeneic co-cultures. For secondary allogeneic co-cultures, T-cells received a second round of stimulation on day 3 with irradiated FL B-cells. Flow cytometry was used to measure expression of CD4-AlexaFluor700 (clone RPA-T4), CD8-PerCP (clone SK1), CD25- APC (clone 4E3), CD127-PE (clone MB15-18C9) and FOXP3-PE-Cy7 (clone) on allogeneic T-cells. Proliferation of allogeneic T-cells was measured by thymidine incorporation as previously described, and T-cell subset-specific proliferation measured by CFSE dye dilution assay.\textsuperscript{15} Cytokine secretion in allogeneic co-cultures was measured by ELISA on culture supernatant harvested at day 5 of primary and secondary co-cultures using Ready-Set-Go ELISA kits (eBioscience). Intracellular cytokine staining (IFN-\textgamma-FITC (clone 4S.B3), TNF-\textalpha-PerCP Cy5.5 (clone MAb11), IL-2-PE (clone MQ1-17H12)) and CD107a-PE-Cy5 (clone eBioH4A3) assays were performed on T-cells in allogeneic co-cultures as previously described.\textsuperscript{16} Dead cell exclusion was performed with DAPI, V450-50 or R780/60 fixable viability (from eBioscience).
For allospecific proliferation and cytokine secretion, values for T-cells stimulated with autologous B-cells were subtracted from the values for T-cells in allogeneic cultures. For some allogeneic co-culture experiments, BTLA agonistic (clone MIH26, Biolegend) or antagonistic antibodies (clone 3B1, Genentech) or their respective isotype controls were added at 50µg/ml.

**Clinical outcome of FL patients undergoing allogeneic hematopoietic stem-cell transplantation**

Patients underwent T-replete allogeneic hematopoietic stem-cell transplantation from fully HLA-matched related or unrelated donors using reduced intensity conditioning with fludarabine and cyclophosphamide. Seventeen of the 23 patients in this cohort were included in our previous series. Patient and donor demographics and details of conditioning are in Table 1. GvHD prophylaxis was methotrexate (5mg/m² on days +3, +6 and +11), and cyclosporine (3mg/kg/day) until day +90-120. Remission status prior to allogeneic hematopoietic stem-cell transplantation was assessed using Cheson criteria, modified for CT PET status where appropriate. Acute and chronic GvHD was defined using updated NIH criteria and graded by consensus criteria confirmed when possible by tissue biopsy. All patients with grades 2-4 acute GvHD were treated initially with 1-2 mg/kg methylprednisolone. Acute GvHD was defined as steroid-refractory if progression occurred after 3 days of steroid therapy or there was no response after 7 days.22

**Statistical Considerations**

Data for *in vitro* experiments were analyzed using Prism version 5.0. (GraphPad Software, Inc.). Two-tailed tests are used throughout. Equal variance was not assumed for unpaired t-tests and Welch’s correction was used where appropriate. Clinical outcome data were analyzed using
SPSS Statistics 22 (IBM, New York) and STATA 12 (Statacorp, Texas). The statistical significance of differences in frequencies of categorical variables was assessed using Fisher’s Exact Test. Cumulative incidence of death from acute GvHD was calculated using the method of Fine and Gray with competing risks. Patients transplanted in partial remission were deemed to have persistent disease if detectable at day +100 (or earlier if indicated) and were censored at this date. Overall survival curves were constructed using the method of Kaplan and Meier and differences between groups were assessed using the log-rank statistic.
RESULTS

FL B-cells with TNFRSF14 aberrations have reduced HVEM expression but retain expression of other molecules important in alloantigen-presentation

We initially sought to determine the impact of TNFRSF14 aberrations in human FL B-cells on the expression of HVEM and other molecules important to stimulation of T-cell alloreponses. We used flow cytometry to compare surface HVEM expression on TNFRSF14 wild-type FL B-cells and FL B-cells with single or dual TNFRSF14 aberrations. The individual genetic lesions in FL B-cells used for in vitro experiments are detailed in Supplementary Table 3. Samples with dual TNFRSF14 aberrations were selected whose genetic lesions were predicted to result in loss of expression of HVEM (e.g. homozygous deletions, nonsense mutations plus deletions).

In wild-type cases, approximately 50% of FL B-cells expressed HVEM at levels above isotype control, whereas HVEM expression was virtually undetectable in FL B-cells with dual TNFRSF14 aberrations. As expected, FL B-cells from patients with single TNFRSF14 aberrations had HVEM expression intermediate between patients with wild-type and dual aberrations (Figure 1A-B). In contrast, molecules associated with antigen-presenting capacity (including MHC class I, MHC class II, CD80, CD86 and CD58 (LFA-3) were expressed at similar levels on TNFRSF14 wild-type FL B-cells and those with dual TNFRSF14 aberrations both before and after in vitro activation which models the pro-inflammatory post-transplant in vivo environment. Importantly, in vitro activation had had no significant effect on HVEM expression levels (Figure 1C).
FL B-cells with dual TNFRSF14 aberrations stimulated greater frequencies of alloreactive effector T-cells

After confirming that aberrations in TNFRSF14 FL B-cells resulted in a significant reduction in expression of HVEM we chose to investigate whether this impacted upon the ability of FL B-cells to stimulate allogeneic T-cell responses in vitro. As HVEM expression was more markedly and consistently reduced in FL B-cells with dual rather than single TNFRSF14 aberrations we compared TNFRSF14 wild-type FL B-cells to those with dual aberrations in subsequent functional experiments.

Allostimulation with FL B-cells with dual TNFRSF14 aberrations resulted in a significantly higher frequencies of alloreactive responder CD4+ T-cells identified by upregulation of the activation marker CD25 when compared to TNFRSF14 wild-type FL B-cells (median 17% versus 8%, p=0.01 (primary allostimulation) and 28% versus 13%, p=0.02 (secondary allostimulation). A smaller and less consistent increase of activated alloreactive CD8+ T-cells was seen after allostimulation with FL B-cells with dual aberrations when compared with TNFRSF14 wild-type FL B-cells with a trend towards significance after secondary allostimulation (median 10% versus 7%, p=0.08, Figure 2A and 2B). The increase in the proportion of activated CD4+ T-cells after allostimulation with FL B-cells with dual TNFRSF14 aberrations was not due to an increase in CD4+ T-cells with a regulatory T-cell phenotype identified either by surface CD25+CD127lo expression pattern or by expression of intracellular FOXP3 (Figure 2C-D). These data are consistent with an increase in the frequency of activated alloreactive effector T-cells after allostimulation with FL B-cells with dual TNFRSF14 aberrations when compared to stimulation with wild-type FL B-cells.
Frequencies of polyfunctional alloreactive T-cells are increased after stimulation with FL B-cells with dual TNFRSF14 aberrations

We next measured the generation of the pro-inflammatory cytokines IL-2, IFN-γ and TNF-α in allogeneic co-culture supernatants. When analyzed individually, there was a trend towards only significantly increased IL-2 and IFN-γ levels in culture supernatants after allostimulation with FL B-cells with dual aberrations compared to TNFRSF14 wild-type FL B-cells. However, allostimulation with FL B-cells with dual aberrations resulted in significantly higher combined levels of all three pro-inflammatory cytokines when compared to allostimulation with wild-type FL B-cells (Figure 3A).

We next used intracellular cytokine cytometry to measure allospecific cytokine production at a cellular level. Primary allostimulation with FL B-cells with dual aberrations resulted in significantly greater frequencies of CD4+ T-cells accumulating IL-2 and a trend to greater frequencies of CD8+ T-cells, accumulating IL-2 and IFN-γ. In contrast, allostimulation with FL B-cells with dual aberrations did not significantly increase frequencies of T-cells accumulating TNF-α consistent with our ELISA data (Figure 3B).

As lytic function of alloreactive T-cells is associated with clinically significant acute GvHD we also measured surface CD107a expression on alloreactive T-cells stimulated with FL B-cells as a surrogate marker of degranulation and lytic capacity. We observed significantly higher frequencies of CD4+ T-cells and a trend toward higher frequencies of CD8+ T-cells expressing CD107a following primary allostimulation with FL B-cells with dual aberrations compared to TNFRSF14 wild-type FL B-cells (Figure 3C).
Polyfunctional T-cell responses *in vitro* have been shown to correlate with clinical immunity.\(^{28}\) Therefore we next used multiparameter flow cytometry combining intracellular measurement of IFN-\(\gamma\), IL-2 and TNF-\(\alpha\) accumulation and surface expression of CD107a to assess the frequencies of polyfunctional alloreactive T-cells after stimulation with FL B-cells. We observed significantly higher frequencies of dual function T-cells expressing CD107a and accumulating IFN-\(\gamma\), IL-2 or TNF-\(\alpha\) after allostimulation with FL B-cells with dual aberrations compared to *TNFRSF14* wild-type FL B-cells (*Figure 3D*). Finally we used SPICE software to compare the proportion of alloreactive T-cells with a polyfunctional phenotype (those positive for two or more of surface CD107a, and intracellular IFN-\(\gamma\), IL-2 and TNF-\(\alpha\)) after stimulation with allogeneic FL B-cells (*Figure 3E*). We observed significantly higher frequencies of polyfunctional alloreactive T-cells after allostimulation with FL B-cells with dual aberrations compared to wild-type *TNFRSF14* FL B-cells (9.1% versus 3.7%, \(p=0.03\)).

**FL B-cells with dual *TNFRSF14* aberrations stimulate greater allogeneic T-cell proliferation than wild-type FL B-cells**

As alloreactive effector T-cells must expand *in vivo* to exert clinically effects, we measured allospecific T-cell proliferation after stimulation with FL B-cells. Proliferative responses of allogeneic responder T-cells measured by thymidine incorporation were significantly greater after allostimulation with FL B-cells with dual aberrations when compared to *TNFRSF14* wild-type FL B-cells after both primary and secondary allostimulation (*Figure 4A*). We next used CFSE dye dilution to determine whether this difference was due to a selective effect on proliferation of either CD4\(^+\) or CD8\(^+\) responder T-cells. The proportion of responder CD4\(^+\) T-cells proliferating after allostimulation with FL B-cells with dual aberrations was consistently
and significantly greater than after allostimulation with TNFRSF14 wild-type FL B-cells (median 14% versus 7%, p= 0.01 (primary allostimulation) and 37% versus 10%, p=0.008 (secondary allostimulation). Consistent with our data for activation marker expression, we also saw a smaller and less consistent increase in proliferation of allogeneic CD8+ T-cells after allostimulation with FL B-cells with dual aberrations compared to TNFRSF14 wild-type FL B-cells with a trend towards significance after secondary allostimulation (median 50% versus 40%, p=0.10, Figure 4B-D).

Collectively these data demonstrate that the capacity of FL B-cells with dual TNFRSF14 aberrations to stimulate T-cell alloresponses in vitro is both quantitatively and qualitatively enhanced when compared to TNFRSF14 wild-type FL B-cells.

**BTLA ligation reduces T-cell alloresponses stimulated by FL B-cells with TNFRSF14 aberrations**

To provide further evidence that the presence of TNFRSF14 aberrations increase the capacity of FL B-cells to stimulate T-cell alloresponses via a reduction in HVEM-BTLA signaling, we next examined the effect of antibody-mediated modulation of BTLA signaling on B-cell stimulated T-cell alloresponses in vitro. BTLA blockade increased T-cell alloresponses stimulated with TNFRSF14 wild-type FL B-cells demonstrating that interaction of HVEM and BTLA regulates the magnitude of T-cell alloresponses stimulated by FL B-cells, as has previously been shown with dendritic cell-stimulated T-cell alloresponses.29 Importantly, addition of agonistic BTLA antibody to co-cultures of FL B-cells with TNFRSF14 dual aberrations and allogeneic T-cells reduced the proportion of alloproliferative CD4+ and CD8+ T-cells (Figure 4E-F). Taken together, these results are consistent with BTLA signaling contributing to control of proliferation
of alloreactive T-cells after stimulation with \textit{TNFRSF14} wild-type FL B-cells, and reduced BTLA signaling contributing to increased proliferation of alloreactive T-cells after stimulation with FL B-cells with \textit{TNFRSF14} aberrations.

\textit{TNFRSF14} aberrations are associated with increased acute GvHD in FL patients undergoing allogeneic hematopoietic stem-cell transplantation

Finally we sought to assess whether the increased alloantigen-presenting capacity we had observed in FL B-cells with \textit{TNFRSF14} aberrations had clinical impact after allogeneic transplantation. We assessed the \textit{TNFRSF14} status in lymph node biopsies performed prior to transplantation in a cohort of patients with FL (Table 1). We observed a similar frequency of \textit{TNFRSF14} aberrations to previously published studies\textsuperscript{4}\textsuperscript{5} with aberrations detected in 12 of 23 patients (52%). Seven patients had mutations (all within the region encoding the extracellular domain, \textbf{Supplementary Figure 2}), 9 had deletions (with 4 patients possessing both a mutation and a deletion) and 1 had a homozygous deletion of both \textit{TNFRSF14} alleles.

To assess the impact of \textit{TNFRSF14} aberrations on clinical alloreactivity after transplantation, we determined the incidence of acute GvHD in evaluable patients who engrafted. Ten of 23 evaluable patients developed significant acute GvHD (Grades 2-4). Using a categorical approach the only pre-transplant risk factor associated with significantly increase incidence of acute GvHD was tumor \textit{TNFRSF14} status (8 of 11 patients with \textit{TNFRSF14} aberrations developed acute GvHD compared to only 2 of 11 \textit{TNFRSF14} wild-type patients (p=0.03, \textbf{Figure 5A}). There was no significant difference in the incidence of acute GvHD in patients grouped by age, prior histological transformation, prior rituximab therapy, prior lines of therapy, prior autologous transplantation, remission status pre-transplant or donor type, despite there being a higher
frequency of unrelated donors in patients with \textit{TNFRSF14} aberrations (Table 2). We also analyzed the impact of pre-transplant factors on acute GvHD using the time-dependent approach of cumulative incidence (CI) with competing risks. Confirming our categorical analysis, \textit{TNFRSF14} status was also the only variable associated with significantly increased CI of acute GvHD (Figure 5B and Table 1).

We took a similar approach to determine the impact of pre-transplant factors on the incidence of death from acute GvHD. Death from acute GvHD was limited to patients with \textit{TNFRSF14} aberrations, occurring in 5 of 11 patients compared to 0 of 11 \textit{TNFRSF14} wild-type patients and \textit{TNFRSF14} status was statistically significantly risk factor for death from acute GvHD in using both categorical and CI approaches (Figure 5C). However, death from acute GvHD was also significantly associated with pre-transplant remission status (Table S3) and all patients who died from acute GvHD had both \textit{TNFRSF14} aberrations and residual nodal lymphoma at the time of transplantation. Although we cannot separate the effects of \textit{TNFRSF14} aberrations and pre-transplant remission status on death from acute GvHD in this small cohort, this observation is consistent with the hypothesis that FL B-cells with \textit{TNFRSF14} aberrations increase clinically significant alloresponses that result in acute GvHD after AHSCT.

In contrast to acute GvHD, the incidence of chronic GvHD was not significantly associated with \textit{TNFRSF14} status, or any other pre-transplant factor using both categorical and CI approaches, Table S4. The overall incidence of relapse/progression of lymphoma post-transplant was low in our cohort with only four patients relapsing/progressing, precluding an assessment of the impact of \textit{TNFRSF14} status on GvL effects, which needs to be addressed prospectively in a larger cohort of patients.
DISCUSSION

We have shown that aberrations in the *TNFRSF14* gene, which occur in up to half of FL patients, increase the capacity of tumor cells to stimulate clinically significant allogeneic donor T-cell immune responses. These findings have implications for allogeneic hematopoietic stem-cell transplantation strategies for FL patients. The study also has wider significance in demonstrating for the first time that genetic alterations in tumor cells may impact the outcome of allogeneic immunotherapy.

Although we had demonstrated that FL B-cells with *TNFRSF14* aberrations stimulate allogeneic T-cell responses more effectively than wild-type FL B-cells *in vitro*, our finding that this tumor-specific genetic lesion was associated with severe acute GvHD after AHSCT was somewhat unexpected. We anticipated *TNFRSF14* lesions would have little effect on allogeneic T-cell responses that initiate GvHD early post-transplant where alloantigen might be presented predominantly by non-malignant tissue APC, but would result in increased alloantigen-presentation by small numbers of residual tumor cells resulting in increases GvL effects and less lymphoma relapse post-transplant. The increase in acute GvHD in patients with *TNFRSF14* aberrations may have reflected two different mechanisms. FL B-cells present in the early post-transplant period could contribute to direct alloantigen presentation, with tumor cells with *TNFRSF14* aberrations priming alloresponses more efficiently than their wild-type counterparts. This is supported both by our observation that steroid-refractoriness and death from acute GvHD was restricted to patients with *TNFRSF14* aberrations with persistent nodal lymphoma pre-transplant and by our *in vitro* experiments. Alternatively, *TNFRSF14* aberrations in lymphoma cells might modulate the recipient microenvironment, establishing pro-inflammatory conditions which increase alloantigen-presentation by non-malignant host APC thereby increasing donor T-
cell alloresponses. Since reduced BTLA ligation on dendritic cells and tissue macrophages increases pro-inflammatory cytokine release\textsuperscript{30,31} this mechanism could further amplify alloresponses in patients with \textit{TNFRSF14} aberrations to increase severe acute GvHD.

It is likely that increased T-cell alloresponses stimulated by lymphoma cells with \textit{TNFRSF14} aberrations occurred predominantly as a result of reduced ligation of BTLA on donor T-cells. FL B-cells with \textit{TNFRSF14} aberrations induced greater alloproliferation in CD4\textsuperscript{+} T-cells than in CD8\textsuperscript{+} T-cells \textit{in vitro}, consistent with retention of high levels of BTLA expression on both naive and memory CD4\textsuperscript{+} T-cells, whereas BTLA expression is progressively lost with CD8\textsuperscript{+} T-cell differentiation.\textsuperscript{29} Although HVEM also ligates a second co-inhibitory T-cell receptor, CD160, it is unlikely that this receptor plays a significant role in this setting, as CD160 is expressed at very low levels on human CD4\textsuperscript{+} T-cells in the tumor micro-environment.\textsuperscript{32}

However we need to take into account the limitations of our study. The functional \textit{in vitro} experiments were restricted to FL B-cells with dual rather than single \textit{TNFRSF14} aberrations. Although further studies are required to accurately define comparative effects of single \textit{TNFRSF14} lesions, and the differential impact of specific mutations, our clinical data suggest single \textit{TNFRSF14} lesions may also be clinically relevant in this setting as severe acute GvHD was observed in both patients with single and dual \textit{TNFRSF14} aberrations. Additionally, other potentially immune-modulating genetic lesions have been identified in lymphoma, including mutations in genes encoding β2-microglobulin and CD58 which could result in reduction of antigen-presenting capacity.\textsuperscript{33} Although our present study does not directly examine such mutations, it is unlikely they impacted on our results as we demonstrated similar expression of HLA Class I and CD58 on FL B-cells with and without \textit{TNFRSF14} aberrations. Finally, in the
current study we measured the effect of *TNFRSF14* aberrations on HLA-mismatched alloresponses which are driven predominantly by direct alloantigen-presentation by recipient rather than donor APC. BTLA ligation is known to also limit human minor histocompatibility antigen (mHag)-specific T-cell responses that mediate acute GvHD in the HLA-matched setting and our clinical data is consistent with *TNFRSF14* aberrations impacting on the capacity of FL B-cells to present mHags leading to increased acute GvHD after HLA-matched AHSCT. Although we have not extended our functional assays to measure mHag-specific HLA-matched alloresponses, this will be a focus of future work. The impact of *TNFRSF14* aberrations in FL on alloreactivity in other immunotherapy platforms such as T-depleted approaches or cord blood transplants will need to be further explored.

Given our findings in the allogeneic setting, *TNFRSF14* aberrations which reduce expression of HVEM on FL B-cells might also be expected to potentiate autologous T-cell anti-tumor immunity via reduced BTLA-mediated suppression. However, as previous studies suggest *TNFRSF14* aberrations may confer a poor prognosis in patients treated with chemo-immunotherapy it is likely that additional or alternative HVEM-mediated mechanisms impact on FL B-cell survival in the autologous setting. As BTLA is also expressed on B-cells, and negatively regulates B lymphocyte transformation and malignant B-cell survival, reduction of HVEM-ligation of BTLA on FL B-cells either in *cis* or *trans* (from neighboring tumor cells), could potentiate tumor growth and survival in this setting.

In summary, these data support the development of risk-adapted allogeneic transplant strategies in FL patients with *TNFRSF14* aberrations to reduce harmful acute GvHD, which could include pre-transplant purging of recipient B-cells or augmented post-transplant immunosuppression.
Genetic lesions in tumor cells that modulate allogeneic immune responses may have wider significance, impacting the outcome of allogeneic cellular immunotherapy not only for lymphoma but also for other hematologic cancers.
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Author contributions: E.K, designed and performed experiments, analyzed data and wrote the manuscript, J.O. performed genetic analysis, C.B. performed experiments, S.I. collated patient samples, J.M. provided clinical data, J.F. contributed to the genetic analysis and critically reviewed the manuscript, J.G contributed to experimental design and wrote the manuscript, J.D conceptualized the study, designed and analyzed experiments and clinical data and wrote the manuscript.

Competing interests: None
References


Figure 1: Cell surface expression of HVEM is reduced on follicular lymphoma (FL) B-cells with \textit{TNFRSF14} aberrations but expression of other molecules involved in antigen-presentation is maintained

(A) Cell surface expression of HVEM (filled histograms) and isotype controls (open histograms) on FL B-cells. Representative data are shown from patients with \textit{TNFRSF14} wild-type (left panel), single \textit{TNFRSF14} aberrations (middle panel) and dual \textit{TNFRSF14} aberrations (right panel).

(B) Mean (+/- standard deviation) frequencies of HVEM$^+$ cells, expressed as a percentage of FL B-cells, for wild-type (n=5), single aberration (n=3) and dual \textit{TNFRSF14} aberration cases (n=5). P value is for two-tailed unpaired t-test.

(C) Surface expression of molecules involved in antigen-presentation on FL B-cells shown pre- and post-activation. The mean values are shown for 5 wild-type cases and 5 cases with dual \textit{TNFRSF14} aberrations. Error bars show standard deviation. There were no significant differences in the level of expression of CD58, MHC Class I and II, CD80 or CD86 in wild-type FL B-cells and FL B-cells with dual \textit{TNFRSF14} aberrations either before or after \textit{in vitro} activation. ns, not significant (p>0.05 in two-tailed unpaired t-test).

Figure 2: Follicular lymphoma (FL) B-cells with dual \textit{TNFRSF14} aberrations stimulate allogeneic T-cell activation more effectively than wild-type FL B-cells

(A) and (B) Proportion of CD4$^+$ and CD8$^+$ T-cells expressing the activation marker CD25 after primary (A), n=7) and secondary (B), n=5) allogeneic co-cultures with wild-type (WT) FL B-cells or FL B-cells with dual \textit{TNFRSF14} aberrations. Horizontal bars and adjacent numbers represent median values and p values are for two-tailed paired t-tests.
(C) Proportion of CD4\(^+\) T-cells expressing the CD25\(^{hi}\)CD127\(^{lo}\) regulatory T-cell surface phenotype or (D) FOXP3 after primary (n=4) and secondary (n=4) allogeneic co-cultures with WT FL B-cells or FL B-cells with dual TNFRSF14 aberrations. Mean values are shown and error bars represent standard deviation. P values are for two-tailed paired t-tests. ns, non significant.

**Figure 3:** Frequencies of polyfunctional alloreactive T-cells are increased after stimulation with follicular lymphoma (FL) B-cells with dual TNFRSF14 aberrations.

(A) Pro-inflammatory cytokines generated from allogeneic T-cells after primary (n=9) and secondary (n=7) co-culture with TNFRSF14 wild-type (WT) or dual TNFRSF14 aberration FL B-cells

(B) Intracellular cytokine accumulation in allogeneic CD4\(^+\) and CD8\(^+\) T-cells after primary (n=8) and secondary (n=7) co-culture with follicular lymphoma B-cells. Cytokine-positive cell frequencies are expressed as a percentage of T-cell subsets.

(C) Surface CD107a\(^+\) cell frequencies (expressed as a percentage of T-cell subsets) in allogeneic CD4\(^+\) and CD8\(^+\) T-cells after primary (n=8) and secondary (n=7) co-culture with FL B-cells. (A)-(C) Mean values +/- standard deviation are shown.

(D) Frequencies of allogeneic bi-functional T-cells (CD107a\(^+\) and cytokine\(^+\)) after primary allogeneic co-culture with FL B-cells. Horizontal lines depict median values.

(E) Multifunctional allogeneic T-cell effectors after primary co-culture with FL B-cells. Cytokine-secreting populations within the alloreactive CD107a\(^+\) T-cell compartment are shown. Arcs show frequencies of T-cells positive for CD107a, IFN-\(\gamma\), TNF-\(\alpha\) and IL-2, and slices show effector populations with combined expression patterns of CD107a, IFN-\(\gamma\), TNF-\(\alpha\) and IL-2. Mean frequencies from 7 independent experiments are shown. P values are for two-tailed t-tests throughout.
Figure 4: Follicular lymphoma (FL) B-cells with dual TNFRSF14 aberrations stimulate greater allogeneic T-cell proliferation than wild-type FL B-cells

(A) Proliferation of allogeneic T-cells measured by thymidine incorporation after primary (n=7) and secondary (n=6) co-culture with TNFRSF14 wild-type (WT) or dual TNFRSF14 aberration FL B-cells. Cpm, counts per minute. Horizontal lines show median values.

(B) Representative histograms of CFSE dye dilution in allogeneic T-cells in secondary co-culture with FL B-cells. Numbers are proportions of proliferating cells.

(C)-(D) Proportion of proliferating allogeneic CD4+ and CD8+ T-cells after primary (C, n=7) and secondary (D, n=5) co-culture with FL B-cells. Horizontal lines show median values.

(E) Proportion of proliferating CD4+ and CD8+ T-cells after primary allogeneic co-culture with TNFRSF14 WT FL B-cells without and with exogenous BTLA antagonist or isotype control antibody.

(F) Proportion of proliferating CD4+ and CD8+ T-cells after primary allogeneic co-culture (n=3) with FL B-cells with dual TNFRSF14 aberrations without and with exogenous BTLA agonist or isotype control antibody. Mean values +/- standard deviation for 3 independent experiments are shown in (E-F).

P values are for two-tailed t-tests throughout. Ns, not significant

Figure 5: TNFRSF14 aberrations in follicular lymphoma (FL) are associated with severe acute GvHD after AHSCT.

(A) Schematic representation denoting TNFRSF14 status of purified FL B-cells from lymph node biopsies from patients prior to allogeneic transplantation and occurrence of clinically significant
acute GvHD (Grades 2-4). Red text denotes patients whose acute GvHD was refractory to steroid therapy. WT, wild type; NE, not evaluable.

(B) Cumulative incidence analysis of acute GvHD (Grades 2-4) after allogeneic transplantation in FL patients with relapse/progression of lymphoma and death from other causes as competing risks. P value is for Gray’s test.

(C) Cumulative incidence analysis of death from acute GvHD (Grades 2-4) after allogeneic transplantation in FL patients with relapse/progression of lymphoma and death from other causes as competing risks. P value is for Gray’s test.
Table 1 Patient and donor demographics and transplant outcome

<table>
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<th>Patient</th>
<th>Age (years)</th>
<th>Prior therapies</th>
<th>Prior autograft</th>
<th>Prior rituximab</th>
<th>Donor</th>
<th>Disease Status</th>
<th>TNFRSF14 mutation</th>
<th>TNFRSF14 deletion</th>
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CR, Complete Remission; PD, Progressive Disease;

*spontaneous acute excluding DLI-induced † progressed D+72 given DLI ‡ progressed D+85 given DLI § Died of MI in CR
Table 2 Effect of pre-transplant variables on incidence of acute GvHD

<table>
<thead>
<tr>
<th>Pre-transplant Factor</th>
<th>Incidence of acute GvHD</th>
<th>Categorical p (FET)</th>
<th>Cumulative p (Gray)</th>
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</table>

CR, Complete Remission; FET, Fisher’s exact test
Figure 1

A

\[ \text{Events} \]

\[ \text{HVEM} \]

Wild-type \quad Single \quad Dual

\text{TNFRSF14 aberration}

B

\[ \text{HVEM, \% positive B cells} \]

\[ \text{Wild-type} \quad \text{Single} \quad \text{Dual} \]

\( p=0.04 \)

\text{TNFRSF14 aberration}

C

\[ \text{WT pre-activation} \quad \text{Dual aberration pre-activation} \]

\[ \text{WT post-activation} \quad \text{Dual aberration post-activation} \]

\[ \% \text{positive B cells} \]

\[ \text{MFI} \]

\[ \text{HVEM} \quad \text{CD58} \quad \text{MHC Class I} \quad \text{MHC Class II} \quad \text{CD80} \quad \text{CD86} \]

\( p=\text{ns} \)

\( p=\text{ns} \)
Figure 2

A

% CD25⁺

WT Aberration

CD4⁺

CD8⁺

B

% CD25⁺

WT Aberration

CD4⁺

CD8⁺

C

CD25⁺CD127⁻ % of CD4⁺ cells

WT Aberration

Primary

Secondary

D

FOXP3⁺ % of CD4⁺ cells

WT Aberration

Primary

Secondary
Figure 3

A

\[ p = 0.02 \quad \text{Sum of all three} \quad p = 0.03 \]

\[ p = 0.07 \quad \text{IL-2} \quad p = 0.06 \]

\[ p = 0.08 \quad \text{IFN-\( \gamma \)} \quad p = 0.12 \]

\[ p = 0.10 \quad \text{TNF-\( \alpha \)} \quad p = 0.16 \]

\[ \text{ng/ml} \]

\[ 0 \quad 1 \quad 2 \quad 3 \quad 4 \]

\[ \text{WT Aberration Primary} \]

\[ \text{WT Aberration Secondary} \]

B

\[ \text{Primary} \quad \text{Secondary} \]

\[ p = 0.05 \quad p = 0.10 \]

\[ p = 0.09 \quad p = 0.01 \]

\[ \text{IL-2 cells, \%} \]

\[ 0 \quad 2 \quad 4 \quad 6 \quad 8 \quad 10 \]

\[ \text{WT Aberration} \]

\[ \text{WT Aberration} \]

C

\[ 0 \quad 10 \quad 20 \quad 30 \quad 40 \]

\[ \text{CD4\(^{+}\) CD8\(^{-}\) CD4\(^{-}\) CD8\(^{+}\)} \]

\[ \text{Primary} \quad \text{Secondary} \]

\[ p = 0.01 \quad p = 0.10 \]

D

\[ p = 0.03 \quad p = 0.03 \quad p = 0.02 \]

\[ \text{\% of T cells} \]

\[ 0 \quad 1 \quad 10 \quad 100 \]

\[ \text{WT Aberr} \quad \text{WT Aberr} \quad \text{WT Aberr} \]

\[ \text{CD107a\(^{+}\) IFN-\( \gamma \)} \quad \text{CD107a\(^{+}\) IL-2} \quad \text{CD107a\(^{+}\) TNF-\( \alpha \)} \]

E

\[ 3.7\% \quad p = 0.03 \quad 9.1\% \]

\[ \text{Pie Arcs} \quad \text{Pie Slices} \]

\[ \text{CD107a\(^{+}\)} \quad \text{CD107a\(^{+}\) IFN-\( \gamma \)} \quad \text{IL-2} \quad \text{TNF-\( \alpha \)} \quad \text{CD107a\(^{-}\) IFN-\( \gamma \)} \quad \text{IL-2} \quad \text{TNF-\( \alpha \)} \]

\[ \text{IFN-\( \gamma \)} \quad \text{IFN-\( \gamma \)} \quad \text{IL-2} \quad \text{IL-2} \quad \text{TNF-\( \alpha \)} \quad \text{TNF-\( \alpha \)} \quad \text{TNF-\( \alpha \)} \quad \text{TNF-\( \alpha \)} \]
Figure 4

A

WT Aberration

p=0.006

WT Aberration

p=0.04

Primary

Secondary

B

WT Aberration

61%

86%

14% 17%

77%

p=0.04

p=0.006

CD4+

CD8+

C

WT Aberration

p=0.01

WT Aberration

p=ns

CD4+

CD8+

D

WT Aberration

p=0.007

WT Aberration

p=0.1

CD4+

CD8+

E

WT

WT plus BTLA antagonist

WT plus isotype control

F

Aberration

Aberration plus plus BTLA agonist

Aberration plus isotype control

% CFSE−

ns

p=0.03

% CFSE−

p=0.1

CD4+

CD8+

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Figure 5

A

Acute GvHD

| Patient | NE | Grade 3 | Grade 2 | Grade 4 | Grade 3 | Grade 2 | Grade 2 | None | None | None | Grade 2 | Grade 2 | None | None | None | None | None | None | None | None | None |
|---------|----|---------|---------|---------|---------|---------|---------|------|------|------|---------|---------|------|------|------|------|------|------|------|------|------|------|
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| 22      |    |         |         |         |         |         |         |      |      |      |         |         |     |      |      |      |      |      |      |      |      |      |
| 23      |    |         |         |         |         |         |         |      |      |      |         |         |     |      |      |      |      |      |      |      |      |      |

Key: TNFRSF14

WT
Mutated
Deleted

B

Cumulative Incidence of acute GvHD

C

Cumulative Incidence of death from acute GvHD
TNFRSF14 aberrations in follicular lymphoma increase clinically significant allogeneic T-cell responses

Eleni Kotsiou, Jessica Okosun, Caroline Besley, Sameena Iqbal, Janet Matthews, Jude Fitzgibbon, John G. Gribben and Jeffrey K. Davies