TIGIT is a key inhibitory checkpoint receptor in lymphoma

James Godfrey, Xiuwen Chen, Nicole Sunseri, Alan Cooper, Jovian Yu, Arina Varlamova, Dmitry Zarubin, Yuriy Popov, Connor Jacobson, Ekaterina Postovalova, Zhongmin Xiang, Krystie Nomie, Aleksander Bagaev, Girish Venkataraman, Yuanyuan Zha, Sravya Tumuluru, Sonali M Smith, Justin P Kline

ABSTRACT

Background PD-1 checkpoint blockade therapy (CBT) has greatly benefited patients with solid tumors and lymphomas but has limited efficacy against diffuse large B-cell lymphoma (DLBCL). Because numerous inhibitory checkpoint receptors have been implicated in driving tumor-specific T cell dysfunction, we hypothesized that combinatorial CBT would enhance the activity of anti-PD-1-based therapy in DLBCL. T cell immunoglobulin and mucin domain containing 3 (TIGIT) is a coinhibitory receptor expressed on dysfunctional tumor-infiltrating T cells, and TIGIT blockade has demonstrated encouraging activity in combination with PD-1 blockade in murine tumor models and in clinical studies. However, the degree to which TIGIT mediates T cell dysfunction in DLBCL has not been fully explored.

Results Here, we demonstrate that TIGIT is broadly expressed on lymphoma-infiltrating T cells (LITs) across a variety of human lymphomas and is frequently coexpressed with PD-1. TIGIT expression is particularly common on LITs in DLBCL, where TIGIT+/PD-1+ LITs often form distinct cellular communities and exhibit significant contact with malignant B cells. TIGIT+/PD-1+ LITs from human DLBCL and murine lymphomas exhibit hypofunctional cytokine production on ex vivo restimulation. In mice with established, syngeneic A20 B-cell lymphomas, TIGIT or PD-1 mono-blockade leads to modest delays in tumor outgrowth, whereas PD-1 and TIGIT co-blockade results in complete rejection of A20 lymphomas in most mice and significantly prolongs survival compared with mice treated with monoblockade therapy.

Conclusions These results provide rationale for clinical investigation of TIGIT and PD-1 blockade in lymphomas, including DLBCL.

BACKGROUND

Immune checkpoint blockade therapy (CBT) with monoclonal antibodies that target the programmed death-1 (PD-1)/programmed death-ligand 1 (PD-L1) axis have demonstrated significant clinical activity in relapsed/refractory (r/r) classical Hodgkin lymphoma (cHL) and primary mediastinal B-cell lymphoma (PMBL).1-3 However, most responses induced by anti-PD-1 antibody therapy in cHL and PMBL patients are not durable, which is a major problem in a largely young population with few remaining therapeutic options. Furthermore, the efficacy of anti-PD-1 CBT in most non-HL (NHL), such as r/r diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL) has been disappointing.4,5 Therefore, identifying combinatorial immunotherapies that improve the efficacy of anti-PD-1 CBT in lymphoid malignancies is an important goal.

One such approach is to co-target other highly expressed immune checkpoint receptors, as tumor-specific T cells typically upregulate multiple immune checkpoints during periods of chronic antigen stimulation.5 A clearly successful example of this strategy has been the combination of PD-1 and CTLA-4 blockade therapy in solid tumors, such as melanoma, mesothelioma, and non-small cell
lung cancer (NSCLC), where combination CBT is now a standard front-line treatment option. Moreover, a recent phase 3 study demonstrated that co-blockade of PD-1 and the immune checkpoint, lymphocyte-activation gene 3 (LAG-3), improved progression-free survival compared with anti-PD-1 monotherapy in patients with previously untreated metastatic or unresectable melanoma. Thus, co-targeting dominant immune checkpoints on tumor-reactive T cells is a promising approach to more effectively stimulate antitumor immunity and improve clinical outcomes.

T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain (TIGIT) is a potent inhibitory immune checkpoint receptor upregulated on tumor-reactive T cells in a variety of malignancies. TIGIT expression has been reported on all major T cell subsets, including conventional CD4+ and CD8+ T cells and regulatory T (Treg) cells. On conventional T cells, TIGIT is expressed in the context of chronic antigen encounter and directly inhibits T cell function via its C-terminal domain immunoreceptor tyrosine-based inhibitory motif (ITIM) when engaged with its cognate ligands, poliovirus receptor (PVR) and PVRL2, expressed on dendritic cells, macrophages, and on tumor cells in some cases. TIGIT can also indirectly inhibit T cell function by preventing PVR and PVRL2 from binding with the positive T cell costimulatory receptor, CD226, similar to CTLA-1/CD28 interactions with CD80/80. Recent studies have identified that TIGIT inhibits tumor-specific T cell function through pathways that are complementary to PD-1 and clinical trials have demonstrated encouraging results for TIGIT/PD-1 co-blockade in cancer patients.

Interestingly, TIGIT is also highly expressed on lymphoma-infiltrating T cells (LITs) in cHL and NHL, frequently in combination with PD-1 on LITs. Dual TIGIT+/PD-1+ LITs are dysfunctional with reduced effector cytokine production. These data suggest that combined TIGIT/PD-1 blockade therapy may help restore effector T cell function and promote endogenous antitumor immune responses in lymphoma. Here, we explore the expression and spatial relations of TIGIT+ and PD-1+ LITs in the tumor microenvironment of primary NHL and cHL samples. Across samples, we identified particularly prominent expression of TIGIT in DLBCL, where TIGIT+ LITs frequently coexpress PD-1, are commonly juxtaposed to malignant B cells, and demonstrate hypofunctional effector responses ex vivo. Finally, we show the potency of combined TIGIT/PD-1 CBT in mice with established B-cell lymphomas.

**METHODS**

**Patient samples**

Freshly frozen and formalin-fixed, and paraffin-embedded (FFPE) lymphoma and reactive tonsillar samples were obtained from the IRB-approved lymphoma biorepository at the University of Chicago.
neighborhoods of cells or communities. To describe clusters, cell compositions were calculated for each community. Communities were grouped based on the dominant cell type.

**Flow cytometric analysis of lymphoma specimens and ex vivo restimulation**

Fresh human and A20 murine lymphoma tissue was dissociated into single cell suspensions. Single cell isolates were then subjected to flow cytometry to assess the expression of TIGIT and PD-1 on LITs. The antibodies used for flow cytometric analyses are provided in online supplemental table S2. Function of LITs was assessed by determining the extent of effector cytokine production following ex vivo restimulation with PMA/Ionomycin.

**In silico data analysis**

Correlations between TIGIT and PDCD1 gene expression in DLBCL, FL, and cHL tumors were performed using independent large external genomic datasets containing RNAseq or NanoString data. Three independent DLBCL datasets comprising a total of 1200 samples were also combined to determine associations between TIGIT mRNA expression, biological subsets of DLBCL, and clinical outcomes following R-CHOP therapy. CIBERSORTx immune deconvolution was used to calculate inferred proportions of immune cells in the tumor environment.

**In vivo antibody treatment and tumor rechallenge**

To test the efficacy of TIGIT blockade, balb/c mice-bearing subcutaneous A20 lymphomas received intraperitoneal injections of anti-TIGIT, anti-PD-1, or combinations of these antibodies. Treatments began once tumors reached a diameter of 10 mm and were continued every 3 days for five doses. Anti-PD-1 (BioXCell, clone Rmp1-14) and anti-TIGIT (iTeos, mIgG2a, clone 31296) antibodies were administered at a dose of 200 µg. Tumor growth was monitored and compared with A20-bearing mice treated with isotype control antibodies (anti-rat IgG2a, clone 2A3). Mice that achieved complete tumor rejection following single or dual CBT were rechallenged with A20 cells to investigate immunological memory responses.

**RESULTS**

**TIGIT is ubiquitously expressed on LITs and is frequently coexpressed with PD-1**

To investigate the extent to which TIGIT may contribute to promoting T cell dysfunction in lymphoma, TIGIT expression was assessed on LITs from 28 fresh lymphoma samples compared with T cells in 10 reactive tonsil specimens (figure 1A,B). Lymphoma subtypes analyzed included DLBCL (n=12), cHL (n=13), FL (n=2), and chronic lymphocytic leukemia (CLL, n=1). Interestingly, many of the lymphoma samples analyzed demonstrated significantly higher proportions of TIGIT+ T cells compared with reactive tonsil samples, especially among CD8+ T cells (figure 1B). LITs from DLBCL samples exhibited particularly broad TIGIT expression on the majority of T cells analyzed, including CD8+ T cells (71.4%±14.4%), Treg cells (89.3%±8.1%), and conventional CD4+ T cells (63.1%±21%) (figure 1B). In contrast, cHL samples displayed similar frequencies of TIGIT+ CD8+ LITs and Treg cells compared with tonsillar tissue and reduced frequencies of TIGIT+ CD4+ LITs. Across lymphoma and reactive tonsil samples, TIGIT expression was particularly common on Treg cells, as previously reported.

Chronic T cell receptor signaling is known to induce a progressive state of effector dysfunction among tumor antigen-specific T cells—a differentiation state also referred to as exhaustion—which is accompanied by the upregulation of numerous inhibitory immune checkpoint receptors, including PD-1 and TIGIT. We, therefore, sought to examine the extent to which PD-1 and TIGIT were coexpressed on LITs compared with tonsillar tissue. As shown in figure 1C, D, TIGIT and PD-1 coexpression was common on LITs. We observed particularly prominent coexpression of PD-1 and TIGIT on LITs in DLBCL, where dual PD-1/TIGIT expression was significantly more common on conventional CD4+, CD8+, and Treg cells compared with reactive tonsils. For indolent NHL, there was also an apparent increase in the proportion of dual PD-1/TIGIT positive T cells compared with tonsillar tissue, although numbers were small. In cHL, dual PD-1/TIGIT expression was significantly more common on CD8+ T cells compared with reactive tonsils but was less frequent among Treg cells and conventional CD4+ T cells (figure 1D). The majority of lymphoma samples analyzed, therefore, exhibited increased frequencies of dual PD-1/TIGIT LITs within the CD8+ T cell compartment. There was also frequent coexpression of PD-1 and TIGIT on T follicular helper (Tfh) cells across all lymphomas and tonsillar tissues (95.5%±4.02%) (figure 1E). While many of the lymphoma samples demonstrated high frequencies of dual TIGIT+ PD-1+ LITs, the number of LITs that were TIGIT+ PD-1+ or PD-1+ TIGIT low (data not shown).

An analysis of bulk gene expression data derived from several human lymphoma subtypes was next performed to characterize patterns of TIGIT and PDCD1 expression. Here, we again identified that TIGIT and PDCD1 expression were highly correlated in DLBCL (r=0.55, p=1.9×10−36), cHL (r=0.49, p=1.2×10−7) and to a lesser extent, in FL (r=0.29, p=0.034) (figure 1F). Together, these data confirm findings that TIGIT is broadly expressed on LITs and is frequently coexpressed with PD-1 across a variety of lymphoma subtypes and suggest that TIGIT and PD-1 may function together to orchestrate and reinforce a T cell dysfunctional state within the lymphoma environment.

**Topographical distribution of TIGIT+ LITs in the DLBCL environment**

Given the notable expression of TIGIT on LITs in DLBCL (figure 1A,B), we next sought to examine the spatial distribution of TIGIT+ LITs in the DLBCL environment using multiparameter immunofluorescence microscopy (figure 2A–C). Similar to flow cytometric...
analysis of human DLBCL samples (figure 1A,B), we observed that TIGIT was commonly expressed on LITs in almost all of the DLBCL samples analyzed (n=15) (online supplemental figure 1). The mean percentage of TIGIT+ LITs among all cells in the tumor environment was 19.6±10.85%. TIGIT was also expressed on a subset of CD3-negative cells, possibly NK cells and/or B cells (figure 2B). In contrast, PD-1 expression was only observed in 20% of cases (data not shown), which may have reflected suboptimal antibody staining. Regardless, 85% of PD-1+ LITs were also TIGIT+, again demonstrating that PD-1 is frequently coexpressed with TIGIT on LITs in DLBCL (data not shown).

We next performed spatial distribution analyses of TIGIT+ and TIGIT− LITs in the 15 DLBCL cases. Three representative regions from each DLBCL sample were examined (n=44 regions). When all 44 regions were evaluated together, we observed that, on average, TIGIT+ and TIGIT− CD8+ and CD4+ LITs were found at similar distances from CD20+ B cells in the lymphoma environment, while TIGIT+ Tregs resided in closer proximity to B cells than TIGIT− Treg cells (figure 2D). That said, when each individual region was analyzed separately, we identified that more regions exhibited significant contact interactions (cellular interactions existing within five pixels of each other that occur more frequently than by random chance) between TIGIT+ LITs and B cells compared with TIGIT− LITs and B cells (figure 2E). In fact, there were no significant contact interactions identified between TIGIT+ LITs and B cells in any of the regions analyzed, whereas 27.3% (13 of 44) of regions analyzed showed significant contact interactions between TIGIT+ CD4+ LITs and B cells, and 13.6% (6 of 44) of regions analyzed showed significant contact interactions between TIGIT+ CD8+ LITs and B cells (figure 2F). Thus, while CD4+ and CD8+ TIGIT+ LITs are, on average, found at similar distances from B cells compared with TIGIT− LITs, a proportion of DLBCLs exhibited significant contact interactions between TIGIT+ LITs and B cells in the lymphoma environment.

Lastly, our multispectral analysis revealed the existence of 15 unique cellular communities within the DLBCL environment that were defined by similar cell types, proportions, densities, and distributions (online supplemental figure 2). TIGIT+ and TIGIT− LITs were represented in the majority of these cellular niches, but their prevalence in each community varied significantly. TIGIT+ LITs tended to cluster together within these communities,

![Figure 1](https://example.com/figure1.png)

*Figure 1* TIGIT is broadly expressed on LIT cells in multiple lymphoma subtypes and is frequently coexpressed with PD-1. (A) Representative flow cytometry of TIGIT expression on T cells from a tonsillar and DLBCL specimen. (B) Frequency of TIGIT expression on CD4+ T cells, CD8+ T cells, and Treg cells in samples from tonsils, DLBCL, cHL, and indolent non-Hodgkin lymphomas (iNHL) (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). (C) Representative flow cytometric data demonstrating high frequency of dual expression of TIGIT and PD-1 on LITs from a DLBCL sample. (D) Frequency of dual TIGIT and PD-1 expression on CD4+ T cells, CD8+ T cells, and Treg cells in samples from tonsils, DLBCL, cHL, and iNHL. (E) Representative flow cytometric data demonstrating high frequency of TIGIT expression on T follicular helper cells (CD4+CXCR5+PD-1+) in a DLBCL sample. (F) Correlation of PDCD1 and TIGIT expression from bulk gene-expression data in DLBCL, cHL, and FL. cHL, classical Hodgkin lymphoma; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; LIT, lymphoma-infiltrating T cell; TIGIT, T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain.
as did TIGIT LITs, but very few communities harbored high proportions of both (online supplemental figure 2). These data were consistent with the results from the individual neighborhood analyses (figure 2E), in which there were more regions demonstrating significant contact interactions between TIGIT+ TIGIT+ and TIGIT− TIGIT− LITs compared with TIGIT+ TIGIT− LITs (figure 2E). These analyses suggested that TIGIT+ and TIGIT− LITs each form distinct cellular niches composed of unique cell types and distributions. In support of this notion, we observed TIGIT+ CD4+ LITs were frequently localized to cellular communities characterized by a high prevalence of B cells, whereas TIGIT− CD8+ LITs often resided in communities consisting of large proportions of TIGIT− CD4+ LITs and a paucity of B cells (online supplemental figure 2). Collectively, these data suggest that TIGIT could be an important immune checkpoint to target, particularly in DLBCL cases that exhibit significant contact interactions between TIGIT+ LITs and malignant B cells.

High TIGIT expression is associated with an ‘inflamed’ DLBCL environment

We next sought to determine the extent to which TIGIT expression in DLBCL was associated with outcome to initial treatment or with defined genomic or biological subtypes of the disease. TIGIT expression was analyzed from bulk transcriptomic data from two large DLBCL datasets, and an internal dataset that collectively comprised a total of 1200 diagnostic samples. We then compared the clinical, biological, and genomic features of low (bottom quartile), intermediate, and high (top quartile) TIGIT-expressing DLBCLs. No significant differences in progression-free or overall survival to standard front-line chemotherapy were observed among the three groups (online supplemental figure 3A–D). Among recently defined NCI DLBCL genomic clusters, TIGIT− low DLBCLs were significantly more likely to be classified as EZB-MYC+, whereas TIGIT+ high DLBCLs were more likely to fall into the genomically unclassifiable cluster (online supplemental figure 3E).

We next compared transcriptomes of TIGIT+ high versus TIGIT− low DLBCLs. Overall, there were 169 differentially

Figure 2  Topographical distribution of TIGIT+ T cells in DLBCL. (A) Representative low power magnification of multiparameter immunofluorescence staining demonstrating the distribution of TIGIT expression in a DLBCL sample. (B) Heatmap and UMAP plots demonstrating the distribution of TIGIT and PD-1 expression in DLBCL (n=15). (C) Representative DLBCL sample demonstrating the conversion of immunofluorescence data to a cell typing plot and the downstream rendering of defined cellular subtypes. (D) Distance of TIGIT+ T cells and TIGIT− CD4+ T cells, CD8+ T cells, and Treg cells to CD20+ B cells in DLBCL (n=15, distance in microns, 1–3 representative cores were chosen from each DLBCL case for distance calculations). Student’s t-test, **p<0.01. (E) Heatmap demonstrating DLBCL samples (columns) that display significant contact interactions between specific cell types (rows). Again 1–3 representative cores were chosen for each DLBCL case for neighborhood analyses (n=44 total regions). Specific cell type interactions of interest are shown in red brackets. The sequencing of the cellular interaction informs the specific contact. For example, CD4 T cells TIGIT+→ B cells indicates whether CD4 TIGIT+ T cells have more frequent contact interactions (within 5 pixels of each other) with B cells than is expected to occur more by random chance, whereas B cells → CD4 T cells TIGIT+ indicates whether B cells contact CD4 TIGIT+ T cells more frequently than by random chance. (F) Proportion of DLBCL regions where there is significant contact between TIGIT+ or TIGIT− LITs and CD20+ B cells in DLBCL (*p<0.05, ****p<0.0001). DLBCL, diffuse large B-cell lymphoma; LITs, lymphoma-infiltrating T cells; TIGIT, T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain.
expressed genes (DEGs) identified between the two groups using a false discovery rate of 0.05 and log fold change of ≥1.5 or ≤−1.5 (figure 3A). Interestingly, the majority of the DEGs enriched in TIGIT-high DLBCLs were immune-related transcripts, including those related to T cells (CD2, CD3, CD8A), effector cytokines (IFNG), cytotoxic granules (GZMA, GZMK), costimulatory molecules (CD28, ICOS), and other immune checkpoint proteins (PDCD1, LAG3, CTLA4) (figure 3B). Concordant with these results, gene-set enrichment analysis (GSEA) confirmed that top gene-sets enriched in TIGIT-high DLBCLs were immune-related pathways such as interferon gamma response, inflammatory response, and JAK/STAT signaling (figure 3C). In contrast, gene-sets enriched in TIGIT-low DLBCLs included MYC-target gene-sets (figure 3C), consistent with the enrichment of TIGIT-low DLBCLs in the EZB-MYC genomic cluster (online supplemental figure 3E). We next compared inferred immune cell numbers in TIGIT-low and TIGIT-high DLBCLs through an immune cell deconvolution analysis and observed that TIGIT-high DLBCLs exhibited significantly increased inferred numbers of CD4+ T cells, CD8+ T cells, Treg cells, Tfh cells, and macrophages when compared with TIGIT-low cases (figure 3D,E). Overall, these data indicate that high TIGIT expression identifies DLBCLs with an ‘inflamed’ tumor environment and suggests that TIGIT is upregulated on LITs in the context of an endogenous host anti-lymphoma immune response.

Dual TIGIT+ and PD-1+ LITs exhibit hypofunctional immune responses

Coexpression of TIGIT and PD-1 has been associated with effector dysfunction in tumor-infiltrating T cells in various solid and hematologic tumors. Moreover, the degree of tumor-specific CD8+ T cell dysfunction has been directly correlated with the number of simultaneously expressed inhibitory checkpoint receptors. To determine the extent to which TIGIT+/PD-1+ LITs in DLBCL would exhibit a dysfunctional phenotype, the production of various effector cytokines was assessed in TIGIT+/PD-1− versus TIGIT−/PD-1+ LITs from DLBCL samples following brief ex vivo stimulation with PMA and ionomycin (figure 4A). Significantly lower proportions of CD8+ TIGIT+/PD-1+ LITs produced IL-2 compared with
CD8+ TIGIT+/PD-1+ LITs, and there were trends toward decreased TNFα production in both CD4+ and CD8+ TIGIT+/PD-1+ LITs as well (figure 4A). Conversely, CD8+ and CD4+ TIGIT+/PD-1+ LITs more frequently expressed IFNγ compared with CD8+ and CD4+ TIGIT+/PD-1+ LITs (figure 4A). Thus, TIGIT+/PD-1+ LITs exhibit a partially dysfunctional phenotype that may be reversible through TIGIT and/or PD-1 receptor blockade.

The effector function of LITs isolated from established A20 syngeneic murine lymphomas was next assessed. Like human DLBCLs, conventional CD4+ and CD8+ T cells, and Treg cells from A20 lymphomas frequently expressed TIGIT along with other immune checkpoint proteins such as PD-1 and CTLA-4 (figure 4B). Moreover, the frequency of TIGIT expressing LITs correlated directly with tumor volume suggesting that upregulation of TIGIT and PD-1 on LITs was associated with disease progression. Interestingly, other immune checkpoint receptors, such as LAG-3, were also expressed on LITs, but to a lesser degree than PD-1 and TIGIT, suggesting that TIGIT and PD-1 may be particularly important in mediating T cell dysfunction in this context (figure 4B).

Ex vivo restimulation of A20 LITs yielded similar results to those observed in human DLBCLs. CD8+ TIGIT+/PD-1+ A20 LITs again more frequently expressed IFNγ compared with CD8+ TIGIT+/PD-1+ A20 LITs but were significantly less likely to produce TNFα on ex vivo stimulation (figure 4C). Together, these data indicate that in contrast to other solid and hematologic cancers, TIGIT+/PD-1+ tumor infiltrating T cells in DLBCL retain the expression of some critical effector cytokines. However, polyfunctional cytokine production is compromised.

**Combinatorial TIGIT and PD-1 blockade therapy decreases tumor growth and improves survival in lymphoma-bearing mice**

Given the prominent expression of TIGIT and PD-1 on hypofunctional LITs, we next examined whether TIGIT and PD-1 blockade therapy would impact A20 lymphoma growth in vivo (figure 5A). Of note, A20 lymphomas exhibited moderate PD-L1 expression, but no significant expression of TIGIT ligands CD155 (PVR) and CD112 in vitro (online supplemental figure 4). In mice with established A20 lymphomas, both anti-TIGIT and anti-PD-1 monotherapy led to modest delays in tumor outgrowth when compared with A20-bearing mice treated with isotype control antibodies (figure 5B). Strikingly, however, combined PD-1 and TIGIT blockade resulted in complete rejection of A20 lymphomas in most mice and led to significantly prolonged survival compared with mice treated with either single anti-PD-1 or anti-TIGIT antibody therapy (figure 5B,C). Interestingly, TIGIT blockade also improved the survival of A20-bearing mice when coupled with costimulatory immune checkpoint therapies such as a 4-1BB agonist (figure 5B,C). Finally, mice treated with combined PD-1/TIGIT blockade therapy demonstrated...
immunologic memory, with no mice having rejected primary A20 lymphomas demonstrating tumor growth on A20 cell rechallenge (figure 5D). Together, these results suggest that TIGIT functions as a critical immune checkpoint in A20 lymphomas, and that TIGIT expression impairs LIT function in a manner that is complementary to inhibitory signals delivered by PD-1.

DISCUSSION
Clinical outcomes to anti-PD-1 immunotherapy in lymphoma are currently suboptimal. The majority of lymphoma subtypes are poorly responsive to anti-PD-1 therapy and disease relapse remains a challenge for those that are.1–5 Therefore, identifying additional immune checkpoint receptors that mediate T cell dysfunction in the lymphoma environment has been a major goal toward improving clinical outcomes to PD-1 blockade therapy. Here, we demonstrate the inhibitory immune checkpoint receptor, TIGIT, is commonly expressed on LITs and represents a promising therapeutic target in combination with PD-1 blockade therapy for the treatment of lymphoid malignancies.

Chronic antigen stimulation of tumor-reactive T cells is associated with the progressive upregulation of inhibitory immune checkpoints, including PD-1 and TIGIT, that contribute to the development of T cell dysfunction.6 Recent studies in multiple myeloma, for example, have demonstrated that TIGIT and PD-1 are coordinately upregulated on bone marrow resident CD8+ T cells during periods of disease progression, and that TIGIT+CD8+ T cells exhibit a dysfunctional phenotype characterized by decreased proliferation and cytokine production.31 Dual expression of TIGIT and PD-1 has also been previously characterized on dysfunctional LITs in various human lymphomas.20 21 We similarly observed that TIGIT was frequently co-expressed with PD-1 on LITs, with particularly prominent coexpression in DLBCL. Our data also suggests that CD8+ TIGIT+PD-1 LITs in DLBCL were less likely to exhibit polyfunctionality with regard to effector cytokine production, despite their maintained expression of IFNγ. We therefore speculate that TIGIT+PD-1 LITs exhibit a hypofunctional phenotype, which ultimately leads to impaired immunologic control of tumor growth. Interestingly, analogous hypofunctional responses have been described among exhausted tumor-specific T cells in solid tumors that respond poorly to PD-1 mono-blockade.32 This may explain the limited efficacy we observed with anti-PD-1 monotherapy against A20 lymphoma, as many of the A20 LITs were hypofunctional and expressed both TIGIT and PD-1 (figure 4C,D). In contrast, when TIGIT blockade therapy was combined with anti-PD-1 or anti-4-1BB therapy, significantly improved tumor control and survival of lymphoma-bearing mice was observed (figure 5B,C), suggesting that co-targeting TIGIT with other CBTs more potently reactivates productive anti-lymphoma immune responses.

Our findings support the notion that PD-1 and TIGIT induce T cell dysfunction through unique and non-overlapping mechanisms. This is consistent with a recent study in NSCLC, in which TIGIT and PD-1 were each
observed to inhibit antitumor CD8+ T cell function by impairing CD226 co-stimulation through distinct mechanisms.15 In this study, PD-1 inhibited downstream CD226 intracellular signaling via its ITIM domain, whereas TIGIT restricted CD226 co-stimulation by blocking interactions with their common ligand, PVR. Importantly, full restoration of CD226 signaling and optimal antitumor CD8+ T cell responses required blockade of both TIGIT and PD-1. Given these findings, it seems likely that TIGIT and PD-1 function as dominant immune checkpoints in multiple cancer types, and the results of ongoing TIGIT/PD-1 combination clinical trials in lymphoma and other cancers are therefore highly anticipated.

While combined TIGIT/PD-1 CBT is a promising treatment for lymphoma and other malignancies, moving forward it will be important to dissect the mechanisms by which this treatment promotes antitumor immunity. In particular, a more complete understanding of the specific cellular mediators of response to combined TIGIT/PD-1 CBT is needed. Some studies suggest that CBTs prevent or even reverse the dysfunction of conventional T cells,33 while others demonstrate that CBTs enhance antitumor immunity primarily by inhibiting the suppressive functions of Treg cells.29 34 35 Given that TIGIT is broadly expressed on conventional T cells and Treg cells in the lymphoma environment (figure 1B), it is possible that inhibiting TIGIT/PVR interactions in any of these T cell subsets could be important for enhancing anti-lymphoma immune responses following TIGIT blockade. Thus, any investigations into the cellular mediators of response to TIGIT/PD-1 CBT will require detailed functional experiments and the generation of conditional Tigit knock-out mice. Future studies should also investigate the effects of different Fc domains on the efficacy of anti-TIGIT antibodies, as the varied effector functions of different isotype domains have been shown to significantly impact the efficacy of CBTs.18 36 37

Lastly, several unanswered questions remain regarding TIGIT function in the lymphoma environment. First, given the differences in expression and localization of TIGIT+ LITs across individual lymphomas (figure 1B and figure 2E,F), will the extent to which TIGIT is expressed or spatially distributed correlate directly with response to CBT incorporating anti-TIGIT antibodies? To this end, we find that certain genomic subsets of DLBCL (EZB-MYC) are significantly enriched in cases with low TIGIT expression and these lymphomas might be less responsive to anti-TIGIT therapies. In contrast, we observed that a subset of DLBCLs (~20%) were characterized by increased proximity of TIGIT+ LITs to tumor cells (figure 2F), and these DLBCLs could be particularly vulnerable to TIGIT blockade therapy as anti-TIGIT antibodies may reactivate TIGIT+ LITs to induce killing of adjacent malignant cells. Or perhaps the expression of TIGIT ligands such as PVR and PVRL2 will select for those patients most likely to benefit from treatment. We identify a significant positive correlation between the mRNA expression of CD274 and PVR in large genomic DLBCL datasets, which suggests there is a subset of DLBCLs with increased PD-L1/PVR expression that could derive particular benefit from combination PD-1/TIGIT CBT (figure 3B). TIGIT and CD226 mRNA expression are also tightly correlated, so TIGIT blockade in high TIGIT-expressing DLBCLs may invoke potent LIT activation given concomitant increased CD226 expression. Additionally, is there a potential role of TIGIT-targeted antibody therapy for T-cell lymphomas? Although not addressed in the current study, many T cell lymphomas express TIGIT at high levels38 39 and TIGIT-targeting antibodies could potentially induce therapeutic antibody-dependent and complement-dependent cellular cytotoxicity of these tumors. Lastly, what is the role of TIGIT in inducing the dysfunction of adoptively transferred CAR T cells? CAR T-cell therapy has evolved as a cornerstone in the management of r/r DLBCL, and functional and transcriptomic signatures of T cell exhaustion in CAR T-cell products have been associated with inferior CAR T-cell outcomes in lymphoid malignancies.40–42 Thus, it would be interesting to investigate the contribution of TIGIT to the dysfunctional state of CAR T-cells both at baseline and over time following infusion to determine whether TIGIT blockade could represent a mechanism by which to improve outcomes to CAR T-cell therapy.

In summary, we report that TIGIT is a prominent immune checkpoint expressed on LITs and is a promising therapeutic target in lymphoma. Our data provide strong rationale to investigate the efficacy of combined TIGIT/PD-1 blockade in lymphoma clinical trials.

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Contributors JG performed, analyzed, and interpreted experiments and wrote the manuscript. XC and NS performed and analyzed flow cytometric and in vivo experiments. AC and JY performed computational analyses of genomic datasets. AV, DZ, YP, CJ, EP, ZX, KN, AB and YZ performed and analyzed multispectral immunofluorescence imaging data. GV provided pathologic review and confirmed the biopsy quality of all human lymphoma samples. ST reviewed the manuscript. SMS reviewed the manuscript and established the lymphoma biorepository. JPK designed and supervised the study and wrote the manuscript. JPK is guarantor for this work.

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Patient consent for publication Not applicable.

Ethics approval This study involves human participants and this study included archived and banked human lymphoma tissue. These banked samples were collected on an IRB approved protocol at The University of Chicago—IRB #13-1297. All animal experiments performed for this study were approved by University of Chicago Institutional Animal Care and Use Committee (IACUC) Protocol 

REFERENCES


Methods

Patient samples

Freshly frozen and formalin-fixed, and paraffin-embedded (FFPE) lymphoma and reactive tonsillar samples were obtained from the IRB-approved lymphoma biorepository at The University of Chicago. Informed written consent was obtained from each patient prior to the banking of specimens. Each sample was reviewed by an expert hematopathologist (G.V.) to confirm the accuracy of the lymphoma diagnosis.

H&E analysis and histopathological evaluation

FFPE tissue blocks (n = 18) were cut at 4 μm thickness, mounted on slides, stained for Hematoxylin and Eosin (H&E) and scanned at 40x magnification (0.5 μm per pixel) on a Vectra Polaris scanner in *.qptiff format. The slides were then reviewed by pathologists to select the regions of interest (ROI) based on tumor content. Two tissue microarray (TMA) blocks were made. Each TMA block contained three cores (2.0 mm in diameter) each from nine samples. The TMAs were sectioned at 4 μm thickness and mounted onto 22 x 22 mm square glass coverslips (Electron Microscopy Sciences, 72204-01) precoated with 0.1% poly-L-lysine (Sigma, P8920).

CODEX® (CO-Detection by indEXing) technology (Akoya Biosciences) was used for multiplex immunofluorescence marker detections on FFPE tissue sections. This method requires antibodies to be conjugated to unique DNA barcodes and the signals are revealed by fluorophore-conjugated complementary DNA barcodes (Reporters).

Supplemental material

BMJ Publishing Group Limited (BMJ) disclaims all liability and responsibility arising from any reliance placed on this supplemental material which has been supplied by the author(s) J Immunother Cancer doi: 10.1136/jitc-2022-006582: e006582. 11 2023; J Immunother Cancer, et al. Godfrey J
Antibody conjugation

Aside from the commercially available conjugates from Akoya Biosciences, additional antibody conjugates were made in-house using barcodes and conjugation kits provided by Akoya per the manufacturer’s protocol. In brief, each antibody in purified, carrier-free form was treated with the Reduction Solution (conjugation kit component) to open up the thiol groups, and then incubated with a unique DNA barcode for two hours. The successful conjugations were first confirmed via a gel run showing a shifted-up molecular weight. These conjugates were then tested at different dilutions for staining on a sample tissue expressing the target antigen. The staining was reviewed and approved by pathologists. All the antibody conjugates used in this study, including the conjugates from Akoya, are listed in Table S1.

Tissue staining

CODEX staining and imaging were performed per Akoya Biosciences' detailed instructions. In brief, FFPE sections of 4 μm thickness mounted on coverslips were baked at 70°C for 60 minutes, deparaffinized in two changes of Histoclear (VWR, 97060-934), hydrated in descending series of ethanol (100% x2, 95%, 75%, 50%, 30%) and finally rinsed and incubated in distilled water. The coverslips were then submerged in a glass beaker containing antigen retrieval solution ((AR9) pH 9, Sigma) and treated in a TintoRetriever pressure cooker (BioSB) preset for 120°C for 20 minutes. Once cooled down, the coverslips were rinsed in diH$_2$O, followed by rinses in Codex Hydration Buffer, and then placed in Staining Solution to incubate at room temperature for 30 minutes. The coverslips were rested with tissue side up on a home-made humidified chamber; 200 uL of antibody conjugate mix consisting of Codex Staining Solution, blocking solutions, and antibody conjugates at different dilutions (see Table S1) were added on the tissue section and incubated for three hours at room temperature. The tissues were completely covered
with no bubbles interfering with antibody binding. After incubation, the coverslips were washed in Staining Solution twice, post-fixed with 1.6% paraformaldehyde, cold 100% methanol, and an Akoya Final Fixative with abundant washing steps in 1x PBS in between and after each step. Coverslips were then ready for imaging or short-term storage in Storage Solution at 4°C for up to five days.

**Imaging on Codex instrument**

For imaging, a 96-well reporter plate was prepared containing the corresponding reporters. Each well to be used contained three reporters that correspond to the antibodies used in each cycle, plus a Dapi nuclear stain. Two wells containing only Dapi were used as the first and last cycle for correction of background, known as blanks. The plate was sealed with foil plate seal, blocked from light, and used for imaging.

One coverslip was mounted on a perfusion stage rested on a Keyence fluorescence microscope (Osaka, Japan; model BZ-X800) equipped with a 20x/0.75 objective. The solution exchanges were performed using a microfluidics instrument (Akoya Biosciences) and controlled through a software interface. Light exposure times and the order of markers per cycle are listed in Table S1. After completion of imaging cycles, the raw image tiles in .tiff format were fed through the Codex image processor for stitching. The completed fluorescence images were then segmented and analyzed using a BostonGene proprietary software.

**MxIF image analysis: Object-based segmentation**

CNN with Mask R-CNN\textsuperscript{39} architecture (ResNet-18 as a backbone)\textsuperscript{40} was used to perform segmentation. Model expected 3-channel image as input: first channel corresponded to nuclei
staining (DAPI), second to dominant membrane marker (CD20) and the last one to set of additional markers stacked by maximum pixel value (CD4, CD8).

**MxIF image analysis: Cell typing**

Cell typing was performed based on probabilities of expression. To obtain them two CNNs for two different patterns (membrane, e.g. CD20 and nuclei, e.g. Foxp3) were trained as binary classifiers in order to predict the presence or absence of a marker expression. For preselected cell types we constructed ideal marker signatures (e.g. B-cells were defined as CD20+CD3e-CD4-CD8-PD1-TIGIT-ICOS-Foxp3-CD31-SMA-) and calculated cosine similarity between these reference vectors and vectors of CNNs outputs. The final cell type labels were then assigned as the closest match.

**Significant contact interactions identified by MxIF images**

Significant contact interactions between cell types of interest were determined as previously described. Briefly, cell phenotypes were labeled according methods listed above. Pairwise interactions between and within cell phenotypes of interest that occurred within 5 pixels of each other constituted “cellular contact” and were calculated for each single cell with its neighbors. Pairwise interactions between and within cell phenotypes were then compared to a random distribution using permutation testing. The corresponding significance testing represents the likelihood that specific cellular interactions of interest are enriched in comparison to a randomized version of the same tissue.
Community analysis of MxIF images

Community clusters were obtained based on the graph of cells centroids generated via Delaunay triangulation algorithm. Edges with length exceeding 200 pixels were excluded. To each node we assigned corresponding cell type and median length of connected edges. Using an Adversarially Regularized Variational Graph Auto-Encoder\textsuperscript{20} GNN (from Pytorch Geometric python package) was trained to obtain embedding vectors of cell neighborhoods in an unsupervised, generative-adversarial manner. Architecture consists of two models - variational graph autoencoder and adversarial model (3 layered perceptron), the latter model is used for regularization of the main graph autoencoder model.

Model was trained for a fixed length of 100 epochs and the model with lowest loss was selected for downstream analysis. The trained autoencoder predicted embedding vectors for all samples in the cohort and these vectors were then clustered using the K-means algorithm to obtain 15 different clusters, which represent neighborhoods of cells or communities. To describe clusters cell compositions were calculated for each community. Communities were grouped based on the dominant cell type.

Flow cytometric analysis of lymphoma specimens and ex vivo restimulation

Fresh human and A20 murine lymphoma tissue was dissociated into single cell suspensions using collagenase and mechanical digestion. Single cell isolates were then subjected to flow cytometry to assess the expression of TIGIT and PD-1 on LITs. The antibodies used for flow cytometric analyses are provided in Table S2. Function of TIGIT\textsuperscript{+}/PD-1\textsuperscript{+} and TIGIT\textsuperscript{-}/PD-1\textsuperscript{-} LITs
was assessed by determining the extent of effector cytokine production following \textit{ex vivo} restimulation with PMA/Ionomycin.

\textit{In silico data analysis of bulk tumor tissue}

Correlations between \textit{TIGIT} and \textit{PDCD1} gene expression in DLBCL, follicular lymphoma, and cHL tumors were performed using independent large external genomic datasets containing comprehensive RNAseq or NanoString data\textsuperscript{21–24}. Three independent DLBCL genomic datasets comprising a total of 1,200 samples were also combined to determine associations between \textit{TIGIT} mRNA expression, biological subsets of DLBCL, and clinical outcomes following standard front-line R-CHOP therapy\textsuperscript{23,24}. CIBERSORTx immune deconvolution was utilized to calculate inferred proportions of immune cells in the tumor environment\textsuperscript{25}.

\textit{In vivo antibody treatment and tumor rechallenge}

To test the efficacy of TIGIT blockade, balb/c mice bearing subcutaneous A20 lymphomas received intraperitoneal injections of anti-TIGIT, anti-PD-1, or combinations of these antibodies. Treatments began once tumors reached a diameter of 10 mm and were continued every 3 days for 5 doses. Isotype control (anti-rat IgG2a, clone 2A3), anti-PD-1 (BioXCell #BE0146, Rmp1-14), and anti-TIGIT (iTeos, mIgG2a, clone 31296) were each administered at a dose of 200\textmu g. Tumor growth was monitored and compared to that in A20-bearing mice treated with isotype control antibodies. Mice that achieved complete tumor rejection following single or dual CBT were re-challenged with A20 cells to investigate immunological memory responses.
### Table S1. Antibody conjugates utilized for immunofluorescence imaging

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### Table S2. Antibodies utilized for flow cytometric analyses

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Supplemental Figure 1. TIGIT is broadly expressed on LIT cells in DLBCL. Bar graphs indicating the proportion of LITs expressing TIGIT in DLBCL (n = 15) as assessed by multispectral immunofluorescence imaging.
Supplemental Figure 2. TIGIT+ and TIGIT- LITs form unique cellular communities in DLBCL. Community composition analysis of multispectral immunofluorescence imaging data demonstrating the unique cellular composition of communities enriched for TIGIT+ and TIGIT- LITs in DLBCL.
Supplemental Figure 3. Clinical, biological, and genomic associations of TIGIT expression in DLBCL. (A) Histogram and boxplot indicating differential TIGIT expression in TIGIT-low, TIGIT-intermediate, and TIGIT-high DLBCLs. (B and C) Progression-free and overall survival of patients with TIGIT-low, TIGIT-intermediate, and TIGIT-high DLBCLs treated with standard front-line R-CHOP or R-CHOP-like therapy. (D) Association of TIGIT-low, TIGIT-intermediate, and TIGIT-high DLBCLs with cell of origin, IPI, and DHITsig status. (E) Proportion of TIGIT-low, TIGIT-intermediate, and TIGIT-high DLBCL cases amongst the NCI-defined DLBCL genomic clusters.
Supplemental Figure 4. Expression of PD-1 and TIGIT ligands on A20 lymphoma. Cell surface protein expression of TIGIT (CD155 and CD112) and PD-1 (PD-L1) ligands on A20 tumor cells in vitro as assessed by flow cytometry.