Comparative evaluation of T-cell receptors in experimental glioma-draining lymph nodes

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Abstract

Background. Glioblastomas, the most common primary malignant brain tumors, are considered immunologically cold malignancies due to growth in an immune sanctuary site. While peptide vaccines have shown to generate intra-tumoral antigen-specific T cells, the identification of these tumor-specific T cells is challenging and requires detailed analyses of tumor tissue. Several studies have shown that CNS antigens may be transported via lymphatic drainage to cervical lymph nodes, where antigen-specific T-cell responses can be generated. Therefore, we investigated whether glioma-draining lymph nodes (TDLN) may constitute a reservoir of tumor-reactive T cells.

Methods. We addressed our hypothesis by flow cytometric analyses of chicken ovalbumin (OVA)-specific CD8+ T cells as well as T-cell receptor beta (TCRβ) next-generation-sequencing (TCRβ-NGS) of T cells from tumor tissue, TDLN, spleen, and inguinal lymph nodes harvested from experimental mouse GL261 glioma models.

Results. Longitudinal dextramer-based assessment of specific CD8+ T cells from TDLN did not show tumor model antigen reactivity. Unbiased immunogenomic analysis revealed a low overlap of TCRβ sequences from glioma-infiltrating CD8+ T cells between mice. Enrichment scores, calculated by the ratio of productive frequencies of the different TCRβ-CDR3 amino-acid (aa) rearrangements of CD8+ T cells derived from tumor, TDLN, inguinal lymph nodes, and spleen demonstrated a higher proportion of tumor-associated TCR in the spleen compared to TDLN.

Conclusions. In experimental glioblastoma, our data did not provide evidence that glioma-draining cervical lymph nodes are a robust reservoir for spontaneous glioma-specific T cells highlighting the requirement for detailed analyses of glioma-infiltrating T cells for the discovery of tumor-specific TCR.

Key points

1. In experimental gliomas, cervical lymph nodes are not a robust reservoir for putative glioma-reactive CD8^{+} T cells.

2. TCRß sequencing reveals broad interindividual diversity in TIL and cervical lymph nodes of inbred mice.

Importance of the study

Malignant gliomas are one of the most immunosuppressive and immunologically cold solid tumors with low mutational burden, only few tumor-infiltrating T cells (TIL) and a profound heterogeneity facilitating immune evasion. Recent studies have shown intra-tumoral infiltration of glioma antigen-specific T cells. Given that the lymphatic system drains CNS antigens to the cervical lymph nodes, this prompted us to analyze whether glioma-draining cervical lymph nodes may harbor putative tumor-reactive T cells and constitute a reservoir of tumor-associated TCR. TCRβ-NGS of different immune compartments of GL261 tumor-bearing C57BL6 mice revealed surprising heterogeneity of CD8^{+} TIL between mice albeit the fact that this cell line-based glioblastoma model is responsive to immune checkpoint inhibition (ICI) and therefore considered immunogenic. When calculating individual enrichment scores by the ratio of productive frequencies of the different TCRβ-CDR3 amino acid (aa) rearrangements of CD8^{+} T cells derived from tumor, cervical lymph nodes, inguinal lymph nodes, and spleen, a higher proportion of tumor-associated TCR was found in the spleen compared to TDLN.

Gliomas are the most common primary tumors of the central nervous system (CNS) with poor median overall survival (mOS) of 14–15 months despite surgery and radiochemotherapy.[1] The CNS has long been considered an immune-privileged site as a consequence of the tight blood-brain barrier (BBB) and a lack of CNS-draining lymphatic vessels.[2,3] Recent discoveries of a functional CNS lymphatic system challenged this concept by providing evidence of effective lymphatic drainage and presentation of CNS antigens in deep cervical lymph nodes.[4–6] Compared to other tumor entities, gliomas display immunologically distinct features resulting in resistance to current immunotherapeutic approaches. First, with very few exceptions, gliomas have a comparatively low mutational load, which leads to a reduced number and repertoire of glioma-specific T cells.[7,8] Second, in gliomas, the BBB constitutes a considerable barrier for the transmigration of peripheral T effector cells into the tumor tissue.[9,10] Third, the glioma microenvironment is characterized by profound immunosuppressive mechanisms limiting the activity of intratumoral T-cell responses.[11] Anecdotal evidence from single patients suggests that unleashing endogenous antigen-specific peripheral T cells by ICI is sufficient to induce regression of glioblastoma, provided there is a sufficiently high neoepitope load.[12,13] There are only very few shared neoepitopes that derive from common genomic aberrations, such as the variant III of the epidermal growth factor receptor (EGFRvIII), a frequent mutation in the gene for isocitrate dehydrogenase type 1 (IDH1[R132H]),[14] or a common point mutation in the gene for the H3 histone H3.3[K27M].[14–16] Thus, there is considerable effort to identify patient-individual, so-called private (neo)epitopes in glioma patients. Recent trials, which aim to integrate personalized neoepitope-specific vaccines into standard of care, not only showed feasibility of a clinically relevant workflow of private neoepitope discovery and subsequent vaccine manufacturing for individual patients, but also indicated efficient induction of antigen-specific T cells that are capable to home into gliomas.[16–18] The coordinated assessment of private (neo)epitopes and their corresponding TCR and particularly the longitudinal monitoring of response to immunotherapy, however, is facing limitations due to limited accessibility of tumor tissue from pre- and post-treatment time points. Hence, a surrogate tissue reflecting intra-tumoral antigen presentation and accumulation of glioma-associated T cells would greatly facilitate both, discovery and monitoring of glioma-associated T-cell responses. If, and if so to what extent, the intra-tumoral TCR repertoire overlaps with the TCR repertoire within the glioma-draining lymph nodes (TDLN), has not been investigated so far. Here we probed this hypothesis by applying dextramer-based flow cytometry and TCRβ-NGS in a mouse glioma model using a model antigen to systematically analyze the TCR repertoire in orthotopic gliomas and secondary lymphoid organs systematically to guide functional high-throughput retrieval of glioma-associated T cells and effective immune monitoring of glioma immunotherapy trials.

Material and Methods

Cell Culturing and Testing

The murine glioma cell line GL261 was purchased from the National Cancer Institute Tumor Repository. Cells were

cultured in DMEM (Sigma-Aldrich; D6429) supplemented with 10% FBS (Sigma-Aldrich; F0804), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Sigma-Aldrich; P4333) at 37 °C, 5% CO_2. The purity of cell lines was confirmed using the Multiplex cell Contamination Test by Multiplexion (Heidelberg, Germany). No Mycoplasma or interspecies contamination was detected.

Mice

Female C57BL/6J wild-type mice were purchased from Charles River (Sulzfeld, Germany) or Janvier Laboratories (Le Genest-Saint-Isle, France) at the age of 6-8 weeks and were kept under pathogen-controlled conditions in the center for preclinical studies at German Cancer Research Center (DKFZ, Heidelberg, Germany). Animal experiments were performed according to the rules of the German Animal Welfare Act and were licensed by the local authorities (Regierungspräsidium Karlsruhe).

Generation of GL261-OVA Cell Line

Full-length Ovalbumin cDNA was a gift from R. Offringa (DKFZ) and cloned into pMXS-IRES- Blasticidin (kindly provided by S. Pusch, DKFZ) using the Gateway cloning system (Thermo Fisher). The construct was transfected into GL261 cells using Fugene (Promega) and cells were selected with 9 µg/ml blasticidin (Gibco). The stably transfected cell line was stained for MHC class I-bound Ova CD8 epitope (H-2Kb-SIINFEKL) surface expression using an anti-mouse Kb-SIINFEKL antibody (Biolegend), together with fixable viability dye eFluor 780 (1:1000, Thermo Fisher, 65-0865-14) and sorted for medium expression of OVA on a FACSAria II system (BD Biosciences) using FACS Diva software.

GL261 Tumor Inoculation and MRI Monitoring

One x 10^5 GL261 or GL261-OVA cells in 2 µL PBS were stereotactically implanted into the right hemisphere 2 mm right lateral of the bregma and 1 mm anterior to the coronal suture with an injection depth of 3 mm below the dural surface using a 10 µl Hamilton micro-syringe driven by a fine step stereotactic device (Stoelting, Wood Dale, IL). Mice were examined for tumor-related symptoms on a daily basis. Tumor monitoring with Magnetic Resonance Imaging (MRI) was performed by T2-weighted MR Imaging on a 9.4 Tesla horizontal bore small animal NMR scanner with a four-channel phased-array surface receiver coil (BioSpec 94/20 USR, Bruker BioSpin GmbH, Ettlingen, Germany) as previously described.[19]

Isolation of Lymphocytes from Tumor, Spleen, and Lymph Nodes

Mice were sacrificed by overdosing anesthesia and subsequent cardial perfusion with PBS. The right tumor-bearing hemisphere was excised and mechanically dissected. Tissue was enzymatically digested with 50 µg/ml Liberase

DL (Roche; 05401160001) in HBSS (Sigma-Aldrich; 11088866001) at 37 °C for 30 min with gentle shaking. Cells were consecutively meshed through 100 µm and 70 µm cell strainers (Greiner Bio-One; 542000 and 542070). Isolation of lymphocytes was performed by density gradient centrifugation as previously described.[20] Density gradient was centrifuged at 1250x g at room temperature (RT) for 45 min without brake and lymphocytes were collected from the interface of the 1.072 mg/ml and 1.088 mg/ml layer. Cells were washed with PBS and processed for further analysis. Spleens and lymph nodes were excised and meshed twice through a 70 µm cell strainer to obtain a single-cell suspension. Erythrocytes were lysed with ACK lysis buffer containing 150 mM NH_4Cl, 10 mM $KHCO_3$, and 100 µM Na_2EDTA for 2 min. Cells were washed with PBS and processed for further analysis.

Flow Cytometry

Isolated TIL from GL261-OVA tumors were blocked with αCD16/32 antibody (Biolegend) and stained with PE/APC H-2Kb-SIINFEKL dextramer (Immudex) according to the manufacturer's staining protocol. TIL were subsequently stained with fixable viability dye eFluor 780 (1:1000, Thermo Fisher, 65-0865-14), CD45-BV510 (clone 30-F11, Biolegend), CD11b-PE/Dazzle594 (clone M1/70, Biolegend), CD3-FITC (clone 17A2, Biolegend), and CD8-PercpCy5.5 (clone 53-6.7, Thermo Fischer). TIL were analyzed on a FACSAria II system (BD Bioscience).

Isolation of CD8+ T Cells for TCRβ Sequencing

For purification of GL261 TILs, myelin removal was performed. Myelin removal beads II (Miltenyi Biotec; 130-096) were added to a tumor single-cell suspension and processed according the manufacturer's protocol. Cells from splenocytes, draining cervical lymph nodes, inguinal lymph nodes, and GL261 infiltrating TIL were stained with Propidium iodide (PI) (Sigma-Aldrich; P4864) for exclusion of dead cells, CD45-BV510 (clone 30-F11, Biolegend), CD11b-PE/Dazzle594 (clone M1/70, Biolegend), CD3-FITC (clone 17A2, Biolegend), and CD8-PercpCy5.5 (clone 53-6.7, Thermo Fischer). CD8$^+$ T cells were isolated by sorting for PI$^-$CD45$^+$CD3$^+$CD8$^+$ cells on the FACSAria II system (BD Biosciences) using FACSDiva software.

TCRβ Next Generation Sequencing

Total genomic DNA (gDNA) from sorted CD8$^+$ T-cells was extracted using QIAamp DNA Micro Kit (Qiagen; 56304). Amplification and sequencing of TCRβ CDR3 regions were performed using the ImmunoSEQ platform at Adaptive Biotechnologies.[21] Samples were sequenced on the Illumina NextSeq500 platform (Illumina Inc, San Diego, CA) using MID output flow cell (156 nt reads + 15 nt Index). Data were analyzed with the ImmunoSEQ analyzer toolset and presented as productive amino acid sequences. To calculate enrichment scores of CD8$^+$ T-cell TCRβ-CDR3 sequences within each immune compartment, the productive frequency from each individual

TCRβ-CDR3-sequence was taken into account. First, a dataset of CD8+ T-cell TCRβ sequences that were putatively enriched after tumor antigen encounter was created for every individual animal. This was achieved by eliminating those TCRβ-CDR3 amino acid rearrangements from the detected CD8+ TIL TCRβ sequences that were also detected in the inguinal lymph nodes, which is considered a glioma antigen-naïve compartment. For each identified putatively tumor-reactive TCRβ sequence, the productive frequency in spleen and cervical lymph nodes was determined. The individual ratio of productive frequencies of TCRβ sequences from spleen and cervical lymph nodes to that from TIL was calculated to quantify the enrichment of every TCRβ-CDR3 amino acid rearrangement in these lymphoid organs.

Bioinformatics

Data processing was done with RStudio v1.0.136 (R Studio, Inc.) and the tcR package. Circos Plots illustrating V- and J-gene pairing were created using VDJviz as described recently.[22] The DeepTCR sequencing analysis was done using the algorithm of Sidhom et al. as described.[23]

Graphical Representation and Statistical Analysis

All data are presented as means ± standard deviations (SD) or as individual data points as indicated in the figure legends. Graphical representation and statistical testing were performed with GraphPad Prism 7.0 using either unpaired two-tailed student's t test, paired two-tailed student's t test, or one-way ANOVA in combination with Tukey's Multiple Comparisons test as stated in the figure legends. P-values < 0.05 were considered significant (*P < .05, **P < .01, ***P < .001, ****P < .0001).

Results

OVA-Specific CD8+ T Cells are Not Enriched in Draining Cervical Lymph Nodes of GL261-OVA-Bearing Mice

To determine whether tumor-specific T cells are enriched in TDLN compared to the periphery, we made use of a syngeneic murine glioma model using GL261 cells expressing the model antigen chicken ovalbumin (GL261-OVA),containing a highly immunogenic CD8+ T-cell epitope, sequence SIINFEKL. As the kinetics of T-cell priming in TDLN and T-cell homing are dynamic, we quantified spontaneous anti-OVA CD8+ T-cell immune responses *in vivo* by flow cytometry using H-2K^b-SIINFEKL-dextramers at day 5, day 12, and day 20 after tumor inoculation (Figure 1A, B). Starting from day 12, spontaneous SIINFEKL-specific CD8+ T cells were detectable in GL261-OVA tumors but not in TDLN or inguinal lymph nodes (Figure 1B, C). To assess shared and unique features of tumor-infiltrating lymphocyte (TIL) and TDLN T-cell repertoires in a more sensitive way

and independent of an immunodominant epitope, we next characterized spontaneous T-cell responses using TCRβ-NGS.

Glioma-Associated TCR are Not Enriched in Draining Cervical Lymph Nodes

In many solid tumor entities there is a considerable overlap of the peripheral and TIL TCR repertoire.[24–26] Despite evidence of a lymphatic CNS drainage in mice and humans,[6,27–29] thus far it is unclear whether and to what extent CNS antigens shape TDLN T-cell repertoires. We addressed this question by employing the syngeneic orthotopic genetically unmodified murine GL261 glioma model and characterized the TIL TCR repertoire, as well as the repertoires of lymphocytes within the cervical and inguinal lymph nodes and spleen (n = 4 C57BL/6J mice). Immune cells were harvested once large tumors had formed (28 days post-inoculation) and analyzed using TCRβ-NGS.

In total, 4,965 unique productive TCRβ sequences of fluorescence-activated cell sorted CD8+ TIL from 4 mice were obtained. Albeit the fact that this glioblastoma model is responsive to ICI and therefore considered immunogenic,[30,31] little overlap of the CD8+ TIL TCRβ repertoire was found between mice, with only 1.02 % of productive TCRβ sequences being found in more than one mouse, demonstrating a surprising heterogeneity of the CD8+ TIL TCR repertoire despite strict syngeneity and shared immunogenic (neo)epitopes. This observation is underscored by a multidimensional scaling (MDS) analysis showing a considerable overlap of the TCRβ repertoires of the secondary lymphoid tissues while the TCRβ repertoire within the tumor shows great interindividual differences, albeit a higher TCRβ clonality (Figure 2A). We next aimed at comparing the TCRβ repertoire of CD8+ TIL and CD8+ T cells from TDLN in tumor-bearing mice. CD8+ T cells from inguinal lymph nodes (iLN) were used as an internal control, as these T cells are considered not to have encountered tumor antigens. Notably, we observed an overlap of the exact TCRβ-CDR3 amino acid rearrangements of CD8+ TIL and the CD8+ lymphocytes from the TDLN (~24.0% ± 0.028%) compared to iLN (~19.8% ± 0.007%), which suggests that the TDLN may harbor glioma-associated TCR (Figure 2B). However, the greatest overlap with the TIL TCR repertoire was observed in the spleen with 31.3% ± 0.017% of the TIL TCR sequences identified in the splenic TCR repertoire (Figure 2B). When calculating the morisita overlap index (MOI), however, there was no significantly increased overlap of the splenic TCRβ repertoire with the TIL TCRβ repertoire compared to the TCRβ repertoire of the TDLN, indicating that there is no considerable enrichment of glioma-associated T cells within the TDLN compartment (Figure 2B).

To quantify the overlap of only putative tumor-associated clones, rather than all clones, in spleen and TDLN, TCRβ-CDR3 sequences identified in both, tumors and inguinal, non-draining lymph nodes (iLN) were excluded from further analysis. We subsequently determined the ratio of the productive frequency of identified TCRβ-CDR3 sequences from spleen and TDLN in relation to tumor samples. To quantify the enrichment of each TCRβ-CDR3 amino acid

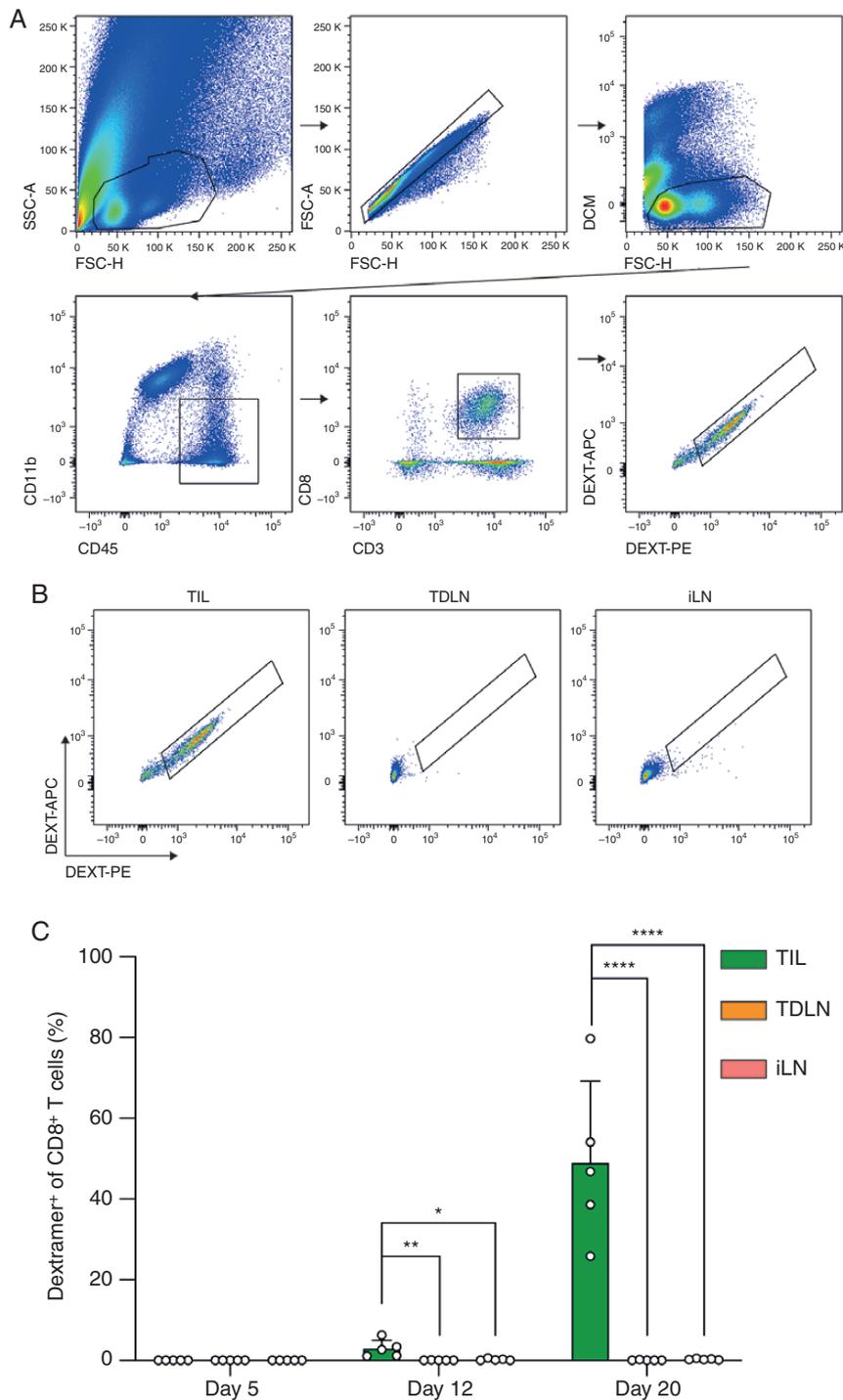**Figure 1.** H-2K^b-SIINFEKL-specific T cells in GL261-OVA tumor-bearing mice. TIL from GL261-OVA tumors from C57B6/J mice (n = 5 for each time point) and corresponding TDLN and iLN were isolated at day 5, 12, and 20 after tumor inoculation. (A) Gating strategy for the identification of SIINFEKL-reactive CD8$^+$ T cells. (B) Exemplary H-2K^b-SIINFEKL-dextramer staining in TIL, TDLN and iLN at day 20. (C) Quantification of (A), (B). Statistical significance was assessed by one-way ANOVA with Tukey's multiple comparisons test for each time point.

rearrangement in the different immune compartments, we developed an enrichment score, calculating the individual ratios of the productive frequencies of splenic and TDLN TCRβ sequences to the TIL sequences, respectively.

Interestingly, in line with our previous findings, a higher enrichment of tumor-associated TCRβ-CDR3 amino acid rearrangements was observed in the spleen (0.047) compared to TDLN (0.026), further supporting the notion that
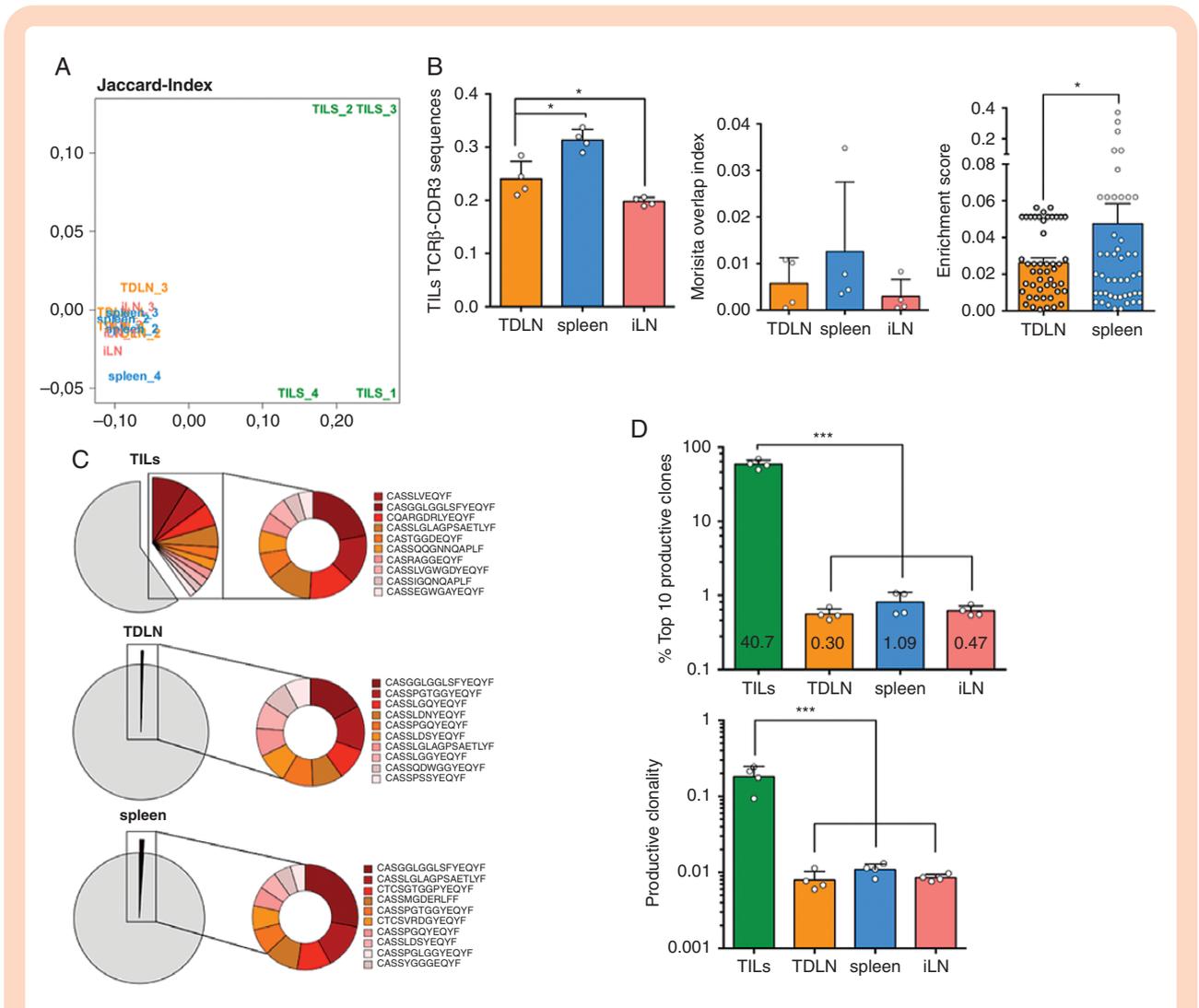
Figure 2. Interindividual heterogeneity and diversity of the TCRβ repertoire of different immune compartments. (A-D) T-cell receptor sequencing was performed on different immune compartments of GL261wt tumor-bearing C57BL6/j mice (*n* = 4). (A) *Multidimensional scaling* (MDS) plot of the TCRβ repertoire of the distinct immune compartments using the Jaccard index. Data was analyzed using the VDJtools algorithm. (B) Left, frequencies of TIL TCRβ sequences within the TCRβ repertoires of secondary lymphoid organs. Middle, Morisita overlap index representing the overlap of TCRβ repertoires of secondary lymphoid organs with the TIL TCRβ repertoire. Right, relative enrichment scores representing the ratio of productive TCRβ frequencies between indicated lymphoid organ and TIL frequencies after removal of TIL TCRβ sequences that were detected in iLN. (C) Frequencies of top 10 productive TCRβ-CDR3 rearrangements within the TCRβ repertoires of indicated immune compartments (numbers in percent). The donut chart illustrates the top ten productive TCRβ-CDR3 amino acid sequences. Data shown for *n* = 1 representative animal. (D) Frequencies of top ten productive clones (top) and their productive clonality (bottom) in the tumor (TILs), TDLN, spleen and inguinal lymph nodes. TILs, tumor-infiltrating lymphocytes; TDLN, tumor-draining lymph nodes; iLN, inguinal lymph nodes. B, D, data represented as mean ± SD. Statistical significance was determined by a one-way ANOVA with Tukey's multiple comparisons test. (**P* < .05; ***P* < .01; ****P* < .001).

TDLN are not enriched for glioma-associated T cells compared to the peripheral lymphoid organs (Figure 2B).

Clonality of TDLN T Cells is Not Increased

As different TCRβ-chains can recognize the same antigen, we analyzed the immunological properties of the TCR repertoires in the respective immune compartments in more detail. Tumor-specific T-cell responses and proliferation of tumor-reactive T cells shape the TCR repertoire and thus lead to enhanced clonality. We hypothesized that, if tumor-reactive T cells and TCR can be found in secondary lymphoid organs such as the TDLN, TCR clonality is enriched in these peripheral tissues. The ten most frequent TCRβ sequences represented 40.7% (range 30.5 – 49.7) of the entire TCRβ repertoire of CD8+ TIL (Figure 2C, D). This might indicate an expansion of putative tumor-reactive clones in the tumor microenvironment. Surprisingly, despite genetically identical, cell line-derived tumors in an inbred mouse strain, TCRβ repertoires showed a great inter-individual heterogeneity with only 1.02% of all CD8+ TIL TCRβ amino acid rearrangements being present in more than one mouse. In addition, productive clonality of

TDLN T cells (7.9 x 10⁻³) did not differ remarkably from the spleen (1.1 x 10⁻²) or the iLN (8.5 x 10⁻³) T cells, which all were significantly lower than that of TILs (Figure 2D).

Overlapping TCRβ variable (V) and TCRβ joining (J) gene usage is considered another surrogate parameter for shared antigen specificity between TCRs. Following the hypothesis that TCRβV-TCRβJ-genes are shared between the tumor microenvironment and the secondary lymphatic tissues, we employed TCRβV and TCRβJ gene usage analysis to analyze whether distinct CD8⁺ TIL were expanded in TDLN. This revealed significant differences in *TCRβV04-01 (8.9%)*, *TCRβV13-02 (13.3%)*, *TCRβV19-01 (13.4%)*, *TCRβJ01-03*01 (12.2%)* and *TCRβJ02-07*01 (40.4%)* utilization in CD8⁺ TIL compared to T cells in the periphery (Figure 3A-C). However, TCRβ V- or TCRβ J-gene usage within TDLN was similar to spleen and iLNs, arguing against a relevant enrichment of putative tumor-associated CD8⁺ T-cells in TDLN (Figure 3B, D). From a set of 22 identified productive *TCRβV-* and 13 productive *TCRβJ*-genes, there were 286 possible *TCRβV-TCRβJ* combinations within the TIL. The most frequent pairing was *TCRβV13-02* to *TCRβJ02-07*, accounting for 3.9% of all pairings and 79.0% of all productive TCRβ CDR3 amino acid rearrangements. When investigating the same V-J gene pairing in TDLN and spleen, we found this pairing much less abundant with only 1.9% and 2.1% of all pairings and 2.5% and 3.6% of all productive TCRβ CDR3 amino acid rearrangements, respectively (Figure 3C). To reduce the complexity of highly diverse CDR3 sequence datasets, we determined TCR features by using the DeepTCR algorithm.[23] As shown in Figure 3E, TCR features in iLN and TDLN, respectively, are dominated by peripheral TCR features also found in the spleen (Figure 3E). Moreover, these peripheral TCR features are also found in healthy animals (Figure 3E), suggesting non-association with GL261 tumors. In line with the above-mentioned observation of inter-individual heterogeneity within the TIL TCR repertoire on a CDR3 amino acid level and also on a feature level, we observed an intra-individual enrichment, but no shared tumor-associated TCR features. DeepTCR learning of the TCR repertoire in TDLN revealed a strong domination of peripheral TCR features observed in the spleen and iLN. However, these abundant peripheral TCR features might inhibit the discovery of subclonally expanded tumor-associated TCR on a feature level.

Discussion

Gliomas remain a challenge for immunotherapeutic approaches despite considerable progress in understanding the immunobiology of these tumors. Low mutational load and an immune-excluding phenotype limit the efficacy of ICI. Different vaccination approaches have been translated into early clinical trials including targeting personalized neoepitopes[18,32,33] or shared clonal driver mutations such as
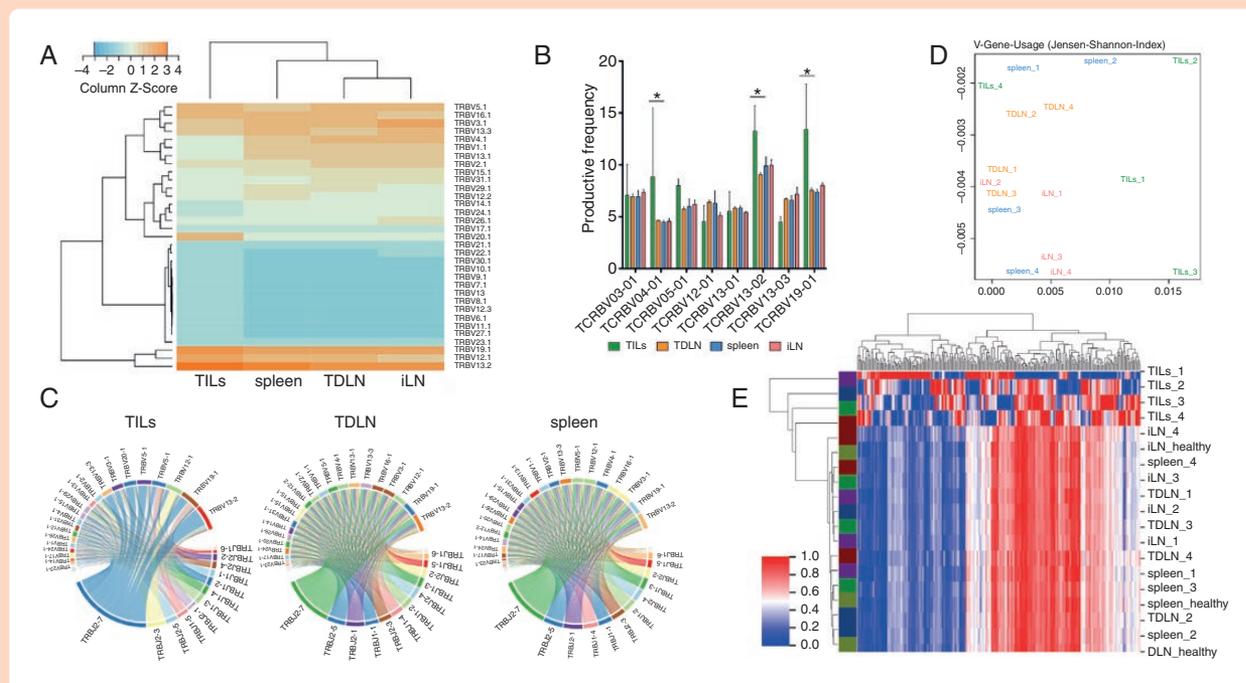

Figure 3. Evaluation of the TCRβ repertoire overlap of different immune compartments. (A-E) T-cell receptor sequencing was performed on different immune compartments of GL261wt tumor bearing C57BL6/j mice (*n* = 4). (A) Cluster-based analysis of the TCRβV gene usage using the VDJtools algorithm. Hierarchical clustering was performed by using the Euclidian distance. (B) Productive frequencies of TCRβV genes present within the tumor (TILs), TDLN, spleen and iLN respectively. (C) Circos maps illustrating the pairing frequencies of V-segments and J-segments from V(D)J-containing reads detected in the tumor (TILs) and secondary lymphatic tissues (TDLN, spleen) of one representative animal. All identified segments are ranked by usage frequencies. (D) Multidimensional scaling (MDS) plot of the TCRβV gene usage of the different immune compartments using the Jensen-Shannon-Index. (E) DeepTCR deep learning analysis revealing structural concepts within the TCR repertoire of the different immune compartments. Statistical significance was determined by a two-tailed student's t-test (*P < .05).

IDH1[R132H] or H3.3[K27M].[14,16,17] However, the number of shared glioma-specific neoantigens is limited and in addition to the identification of private glioma-specific neoantigens, the discovery of corresponding neoepitope-specific T-cell clones remains a challenge. As the frequency of tumor-reactive T-cell clones, especially in the peripheral blood, is low, one of the major challenges in personalized glioma therapy is the choice of the right compartment for discovery of putative glioma-reactive T cells. Moreover, obtaining serial TIL samples to assess the dynamics of glioma-reactive T-cell responses upon immunotherapies like ICI is difficult, supporting the need for surrogate tissue to monitor T-cell responses.

The CNS had long been considered an immune-privileged organ. Recent studies, however, challenged this concept by providing experimental evidence of a sufficient lymphatic drainage by showing that tracers and proteins injected into the brain parenchyma can be detected in CNS-draining lymph nodes.[6,34,35] It has been shown that the meninges surrounding the brain harbor a variety of immune cells providing immune surveillance and affecting brain function. Tumor antigens released during tumorigenesis get phagocytosed and subsequently processed and presented by antigen-presenting cells. Together with soluble macromolecules these cells traffic into the deep cervical lymph nodes where they can induce an anti-tumor T-cell response.[36] In this study, we assessed spontaneous CD8+ T-cell responses to model and endogenous GL261 glioma antigens, in the tumor and secondary lymphoid organs including TDLN mainly to assess whether TDLN may represent a potential surrogate tissue for the retrieval of glioma-associated T cells for the development and monitoring of cellular TCR-transgenic or TIL therapies. Yet, longitudinal flow cytometric analysis of CD8+ T cells from different secondary lymphatic tissues using MHCI-OVA-dextramers did not show OVA-reactive CD8+T cells in TDLN compared to TIL in GL261-OVA-bearing C57BL6 mice (Figure 1).

To compare the TCR repertoires between the different immune compartments of GL261 tumor-bearing mice in more detail and to investigate whether the CD8+ TCRβ CDR3 amino acid rearrangements of the TDLN serve as a potential indicator for intratumoral immune reactions, we compared the TCRβ-enrichment scores of the TDLN and the spleen, the latter as surrogate for the peripheral circulation in mice. Even though our data suggests that putative tumor-associated TCR can be detected in TDLN, these were not enriched compared to the splenic TCR repertoire (Figure 2B). Consistent with this result, comparison of the productive clonality, V- and J-gene usage, and TCR features of CD8+ TIL across the TDLN and the spleen did not show relevant differences (Figure 3A-E). Anatomically, the TDLN are the site where tumor antigens drain first and tumor-derived dendritic cells migrate to. Therefore, this is a location of critical decision-making between the initiation of an anti-tumor immune response or the induction of tolerance. Accumulating evidence suggests that, under control of the drained tumor, TDLN are converted into a site that favors immune suppression by IDO-expressing DCs and highly activated regulatory T cells, leading to immunological tolerance and an active suppression of an anti-tumor immune response.[37–40] However, it remains speculative if this previously described pathomechanism also occurs in draining lymph nodes of the CNS, which conceptually leads to a higher proportion of naïve unexpanded T cells constituting a heterogenic TCR repertoire in TDLN despite CNS antigen presentation by TDLN-DCs. Here we investigated TCR repertoires in TDLN at late-stage tumors (d28). Considering complex dynamics of T-cell trafficking to and interaction with DCs within TDLN, it is tempting to speculate that the likelihood to identify tumor-associated TCR by assessment of intra-nodal clonal expansion is time-dependent as proliferation and emigration may co-occur.[41] However, by longitudinal flow cytometric analyses we were not able to identify spontaneous model antigen reactive CD8+T cells in TDLN. More investigations including TCR repertoire analysis of TDLN at different stages of tumor formation and additional model systems are required to probe this hypothesis. Conversely, successful and rapid emigration of primed T cells from TDLN in comparison to the TIL compartment might in principle challenge T-cell enrichment as key parameter for therapeutic TCR discovery. In addition, drainage of tumor antigens may be influenced by the tumor microenvironment. Even though CNS-draining lymphatic vessels express all traditional markers of tissue lymphatic endothelial cells like Prox1, CD31, Lyve-1, podoplanin, CCL21, and VEGFR3[6,27] there may be differences in the dynamics of lymphatic drainage compared to the peripheral lymphatics. In peripheral tissues, macromolecules and fluid enter the lymphatic vessels by diffusion through permeable endothelial cell junctions.[42] A discontinuous basement membrane and the lack of pericytes facilitate the drainage of cells and molecules. Anatomically, a distinction is made between initial and collecting vessels, the latter of which contain bi-leaflet valves to prevent backflow in conjunction with surrounding smooth muscle cells to provide anterograde flow.[42–44] The meningeal lymphatics share features of initial lymphatic vessels being smaller in size and less protected by the environmental tissue.[6,45] Hence, this might indicate that the dynamics of the meningeal lymphatics are highly influenced by environmental factors, which may hamper the drainage of tumor antigens and subsequently the expansion of tumor-associated TCR.

Since it is described that the majority of CD8+T-cell clones present in tumors are bystander T cells that lack reactivity, we would expect tumor-associated TCR in the draining lymph nodes at an even lower proportion.[46] Therefore, a pre-selection of putative tumor-reactive T cells based on specific markers such as PD-1, CD137, 4-1BB, OX-40, and CD39 may facilitate TCR discovery and the development of cellular therapies for glioma patients.[46–49] Apart from TCR discovery for patient-tailored therapy approaches, sequential TCRβ deep sequencing could play an important role in therapy monitoring, especially in glioma patients treated with ICI. Recently published data underscores this relevance by showing a considerable contraction of the neoepitope repertoire in responding patients, indicating sufficient T-cell responses against tumor cells. In line with that, Riaz *et al.* reported a simultaneous expansion of T cells and loss of neo-epitopes in a linear manner in ICI responding but not ICI non-responding patients.[50] Therefore, early detection of the expansion of distinct TCRβ-CDR3 amino acid rearrangements by sequential TCRβ deep sequencing in patients treated with ICI could constitute a predictive biomarker for response/non-response and hence prevent patients from severe adverse events. While we have previously demonstrated that clonal expansion of tumor-infiltrating T cells is associated with response in experimental gliomas,[30] it remains to be elucidated whether the TDLN could provide an advantage over the periphery in a therapeutic setting after pre-enrichment for PD-1+T cells.

However, here we assessed TDLN repertoires of treatment-naïve glioma-bearing mice.

In summary, our data suggests that, despite evidence of a lymphatic drainage of the CNS, treatment-naïve TDLN do not constitute a better source of spontaneous glioma-associated T-cell clones than spleen in a syngeneic mouse glioma model. However, further studies are required to assess if the discovery of tumor-associated TCR in TDLN of glioma patients without prior immunotherapy is in principle inapplicable for patient-tailored therapy regimens.

Keywords

glioma draining lymph nodes | glioblastoma | TCR discovery

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