γδ T Cells in Merkel Cell Carcinomas Have a Proinflammatory Profile Prognostic of Patient Survival

Nicholas A. Gherardin1,2, Kelly Waldeck3, Alex Caneborg4, Luciano G. Martelotto4, Shiva Balachander4, Magnus Zetheven5, Pasquale M. Petrone5, Andrew Pattison6, James S. Wilmott5,6, Sergio M. Quiñones-Parral1, Fernando Rossello4, Atara Posner6, Annie Wong4, Alison M. Wepppler7, Kerwin F. Shannon5,6,8, Angela Honn5,6,8, Peter M. Ferguson5,6,8, Valerie Jakrot5, Jeanette Raleigh3, Athena Hatzimihalis5, Paul J. Neeson3,9, Paolo Delesio10, Meredith Johnston10,11, Margaret Chua10, Juergen C. Becker12, Shahneen Sandhu7,9, Grant A. McArthur7,9, Anthony J. Gill13, Richard A. Scolyer5,6,8,14, Rodney J. Hicks9,15, Dale I. Godfrey1,2, and Richard W. Tothill4,9

Merkel cell carcinomas (MCC) are immunogenic skin cancers associated with viral infection or UV mutagenesis. To study T-cell infiltrates in MCC, we analyzed 58 MCC lesions from 39 patients using multiplex-IHC/immunofluorescence (m-IHC/IF). CD4+ or CD8+ T cells comprised the majority of infiltrating T lymphocytes in most tumors. However, almost half of the tumors harbored prominent CD4/CD8 double-negative (DN) T-cell infiltrates (>20% DN T cells), and in 12% of cases, DN T cells represented the majority of T cells. Flow cytometric analysis of single-cell suspensions from fresh tumors identified DN T cells as predominantly Vδ2γδ T cells. In the context of γδ T-cell inflammation, these cells expressed PD-1 and LAG3, which is consistent with a suppressed or exhausted phenotype, and CD103, which indicates tissue residency. Furthermore, single-cell RNA sequencing (scRNA-seq) identified a transcriptional profile of γδ T cells suggestive of proinflammatory potential. T-cell receptor (TCR) analysis confirmed clonal expansion of V81 and V63 clonotypes, and functional studies using cloned γδ TCRs demonstrated restriction of these for CD1c and MR1 antigen-presenting molecules. On the basis of a 13-gene γδ T-cell signature derived from scRNA-seq analysis, gene-set enrichment on bulk RNA-seq data showed a positive correlation between enrichment scores and DN T-cell infiltrates. An improved disease-specific survival was evident for patients with high enrichment scores, and complete responses to anti–PD-1/PD-L1 treatment were observed in three of four cases with high enrichment scores. Thus, γδ T-cell infiltration may serve as a prognostic biomarker and should be explored for therapeutic interventions.

Introduction

Merkel cell carcinoma (MCC) is a rare neuroendocrine skin cancer caused by either oncoviral infection or UV carcinogenesis. In viral-positive MCC (VP-MCC), integration of the Merkel cell polyomavirus (MCPyV) genome into the host cell causes cellular transformation via the early transforming genes, which encode the small and large T-antigens (1, 2). In contrast, viral-negative MCC (VN-MCC) is associated with sun-induced somatic hypermutation (3–5). For both subtypes of MCC, immune evasion is likely important for tumor initiation and progression. Immunosuppression is a known risk factor for MCC, and age-related immunosenescence is also likely responsible for increased incidence primarily in the elderly. Spontaneous tumor regression and MCC of unknown primary origin are also well documented, suggesting immune clearance of the primary tumor has occurred (6, 7). Importantly, almost half of patients with advanced MCC respond to anti–PD-1/PD-L1 immune checkpoint inhibitor (ICI) therapies with durable objective responses (8–10).

There is strong evidence to support the role of host-adaptive immune responses in MCC. Heavy T-cell infiltrates are observed in approximately 20% of cases and have prognostic and predictive significance (11–13). T cells reactive to MCPyV peptides have been isolated from VP-MCC patients’ blood and tumors, showing likely tumor specificity (14–17), while VN-MCC also exhibit T-cell responses, presumably due to recognition of neoantigens (5). A loss of antigen-presenting machinery in tumor cells, including the

1Department of Microbiology and Immunology, Peter Doherty Institute for Infection and Immunity, University of Melbourne, Melbourne, Victoria, Australia. 
2Australian Research Council Centre of Excellence in Advanced Molecular Imaging, University of Melbourne, Melbourne, Victoria, Australia. 
3Research Division, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia. 
4Department of Clinical Pathology and Centre for Cancer Research, University of Melbourne, Melbourne, Victoria, Australia. 
5Melanoma Institute Australia, The University of Sydney, Sydney, New South Wales, Australia. 
6Faculty of Medicine and Health, The University of Sydney, Sydney, New South Wales, Australia. 
7Medical Oncology Department, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia. 
8Royal Prince Alfred Hospital, Camperdown, New South Wales, Australia. 
9Sir Peter MacCallum Department of Oncology, University of Melbourne, Melbourne, Victoria, Australia. 
10Radiation Oncology Research, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia. 
11Liverpool Hospital, Sydney, New South Wales, Australia. 
12German Cancer Consortium (DKTK), Translational Skin Cancer Research, University Medicine Essen, Essen and DKFZ, Heidelberg, Germany. 
13Cancer Diagnosis and Pathology Group, Kolling Institute of Medical, Research and The University of Sydney, Sydney, New South Wales, Australia. 
14New South Wales Health Pathology, Sydney, New South Wales, Australia. 
15Cancer Imaging Department, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia.

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N.A. Gherardin, K. Waldeck, and A. Caneborg contributed equally to this article.

Current address for S.M. Quiñones-Parral: Department of Molecular Biology, University of California, San Diego, La Jolla, California.

Corresponding Author: Richard W. Tothill, Clinical Pathology, University of Melbourne, Parkville, Victoria 3010, Australia. E-mail: rtothill@unimelb.edu.au

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downregulation of the MHC class I complex, which is frequent in MCC (18), may facilitate immune escape and has been implicated in cell-based immunotherapy resistance (19).

Conventional MHC-restricted CD8 T cells are important for cancer immune surveillance; however, there is also growing interest in "unconventional" T cells that lack MHC-restriction (20). CD8 T cells are a major subset of such cells and are defined by expression of a T-cell receptor (TCR) encoded by the TCR-αβ repertoire. Upon activation, CD8 T cells rapidly proliferate and are proinflammatory, producing cytokines such as IFN-γ and IL-17, and releasing cytotoxic granules of granzymes and perforin. CD8 T cells are emerging as key players in tumor immune surveillance, and have been identified in tumors of the skin, colon, breast, and pancreas (21–27). However, their role in MCC is currently unknown.

Here, we studied tumor-infiltrating T cells in MCC using multiplex IHC/immunofluorescence (m-IHC/IF), flow cytometry, and single-cell RNA sequencing (scRNA-seq). We observed abundant CD4/CD8 double-negative (DN) T cells in some tumors, indicating the presence of unconventional T cells. These cells were characterized as CD8 T cells using flow cytometry, and scRNA-seq revealed a proinflammatory gene-expression signature that was prognostic in an expanded cohort of patients.

## Materials and Methods

### Patient cohorts

Patient cohorts were collected from three centers according to protocols approved by their respective institutional review boards (IRB) according to the Helsinki Declaration. These centers were the Sydney Local Health District Ethics Committee (Protocol No. X15–0454; 2015; Protocol No. X17–0312; 2015; and Northern Sydney Local Health District Protocol HREC/11/RPAH/32 (n = 12)), the Melanoma Institute of Australia were dissociated into single-cell suspensions using the human Tumor Dissociation Kit (Miltenyi Biotec, catalog no. 130–095–929) and gentleMACS Dissociator (Miltenyi Biotec, catalog no. 130–093–235), according to the manufacturer’s instructions. Pelleted cells were resuspended in RPMI1640 containing 10% FBS and used for FACS analysis or resuspended in 10% DMSO/90% FBS and frozen at −80°C.

### Cell staining

Thawed samples were incubated in DNAse I (Sigma-Aldrich, catalog no. 4716728001) for 20 minutes at 37°C. Cells were filtered, washed in PBS and incubated in PBS + 50 nmol/L dasatinib (StemCell Technologies, catalog no. 73082) and DNAse I at 37°C for 1 hour. Cells were stained with viability dye LIVE/DEAD Fixable Near Infrared (Thermo Fisher Scientific, catalog no. L34975) followed by blocking with Fc block (BD Biosciences, catalog no. 564219; 500 ng/test) and 5% normal mouse serum (generated in-house). Cells were then stained in FACs buffer (2% FBS in PBS) with surface mAbs and tetramers (see Supplementary Table S6 for the reagents used and the dilutions of the reagents). Intracellular staining was performed using the BD Cytofix/Cytoperm Kit (BD Biosciences, catalog no. 554717). Remaining samples were fixed in 2% paraformaldehyde (PFA), washed, filtered, and immediately analyzed using a BD LSR Fortessa. Analysis of case P007 was performed independently where cells were labeled with mAbs in FACs buffer for 30 minutes at 4°C, washed twice in PBS, fixed in 2% PFA, washed, and analyzed on a BD LSRII.

### Flow cytometry analysis

Data were analyzed using Flowjo software (Treestar Inc.). For sorting and analysis of the 11-sample cohort, T cells were defined as LIVE/DEAD−, CD19+CD14−CD45+ and CD3+ after doublet exclusion. CD8+ T cells were also TCRγδ+κ−. Example gating is shown in Supplementary Fig. S1.
Plate-based single-cell TCR analysis

cDNA from single T cells sorted into 96-well PCR plates was generated using Superscript VILLO (Thermo Fisher Scientific, catalog no. 11754050) and 0.1% Triton X-100. TCR-γ and -δ transcripts were amplified by two rounds of semi-nested multiplexed PCR as described previously (28). TRGC or TRDC internal reverse primers were added to PCR products and then sent for Sanger sequencing at the Australian Genome Research Facility (ARGF; primer sequences were as in ref. 28). Results were analyzed using the IMGT database.

scRNA-seq and scTCR-seq

FACS-sorted cells were loaded on a Chromium Single Cell Controller (10x Genomics) using the Single-Cell Immune-ProFiling Solution (Chromium Single Cell 5’ Library & Gel Bead Kit, catalog no. PN-1000002), following the manufacturer’s recommendations for GEM Generation and Barcoding. Post GEM-RT clean up, cDNA Amplification and quality control. TCR enrichment was carried out using the Chromium Single Cell V(D)J Enrichment Kit (catalog no. PN-1000005), human T cell (αβ transcripts), or custom-designed primers for γδ TCR transcripts enrichment (Supplementary Fig. S2). Gene expression and TCR libraries (αβ and γδ) were prepared following manufacturer’s recommendations. All libraries were sequenced using 150 bp paired-end sequencing on the Illumina NovaSeq 6000. Five thousand and 50,000 read-pairs/cell were generated for TCR and gene expression libraries, respectively (see Supplementary Table S7 for scRNA-seq run summary statistics and Supplementary Data for gene expression and TCR-seq annotations, respectively).

Bulk tissue RNA-seq

RNA extraction was done using the RNeasy FFPE RNA extraction kit according to manufacturer’s protocols (Qiagen, catalog no. 35047). RNA-seq library preparation and hybridization capture enrichment were done using either Illumina RNA-Access (Illumina, catalog no. 15049525) or Agilent RNA-Direct (Agilent, catalog no. G7564A) kits according to manufacturers’ protocols. Total RNA-seq libraries were prepared using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (rRNA depletion workflow; NEB, catalog no. E7760L) as previously described (29). Sequencing was done using Illumina HiSeq 2000, NextSeq and NovaSeq 6000 (Illumina; see Supplementary Table S8 and Supplementary Data for bulk RNA-seq statistics and processed count data, respectively). Raw read-level sequencing data cannot be made publicly available owing to restrictions on patient consent preventing sharing of potentially identifiable genetic data.

Bioinformatics and statistical analysis

scRNA-seq and scTCR-seq

scRNA-seq binary base call (BCL) files were demultiplexed and converted into FASTQ files using BCLToFastq prior to alignment to hg38 with CellRanger (v3.1.0) and analyzed in R studio with Seurat (v3.1.1). Cells with a mitochondrial proportion greater than 10% and/or a feature count < 800 or > 4,000 were discarded. Doublet finding was performed with Scrivnet (v0.2.1). Sample batches were combined, normalized and scaled with SCTransform. Cell cycle–associated genes were recessed from the data using the Seurat workflow. Uniform Manifold Approximation and Projection (UMAP) clustering was performed selecting the first nine principal components. scTCR-seq αβ and γδ TCR FASTQs were aligned with CellRanger (v3.0.2) to the default CellRanger VDJ reference and a custom γδ reference constructed from the IMGT database, respectively. Output contig annotations were filtered and analyzed in R studio with Seurat.

Bulk RNA-seq and microarray analysis

RNA-Seq FASTQ files were aligned to hg19 and raw counts were quantified with featurecount via bioinfo-nexgen (v1.6a-b). Count data were filtered for lowly expressed genes, TMN-normalized and logCPM converted with limma (v3.42.0) and edger (v3.28.1). A previously published MCC microarray dataset was selected because of the availability of clinical data kindly supplied by the authors of the original study (Supplementary Table S1; ref. 11). The microarray data was sourced from the NCBI Gene Expression Omnibus (GEO) database (GSE22396; ref. 11) and read into R with GEOquery package (v2.52.0), filtered, and RNA-normalized with affy (v1.62.0). MCC cell line (GSE124857; ref. 30) and innate immune cell and T cell (GSE124731; ref. 31) RNA-seq datasets were sourced from NCBI GEO database.

Gene-set variance analysis

Gene-set variance analysis (GSVA) using RNA-seq or microarray data was performed using GSVA (v1.34.0). Prior to GSVA, RNA-seq data sets were filtered and converted to log2-CPM, and the microarray data was filtered and RNA-normalized. RNA-seq cohorts were integrated and batch corrected with limma (v3.42.0). Signature genes in the RNA-seq set were scaled by subtracting the mean gene logCPM values and microarray expression values were converted to z-scores for visualization of gene expression compared with GSVA scores.

Statistical analysis

Analysis of the cell phenotype populations and comparison across tumors was performed in GraphPad PRISM and R. Survival analysis was performed using survival package (v3.1.8). For MCC-specific overall survival, non-MCC–related events were censored. Statistical significance was calculated with a log-rank test. Multiple Cox regression analysis was performed, and Kaplan–Meier and forest plots were produced with the survminer package (v0.4.8).

Jurkat-76 and CIR cell lines

The Jurkat-76 MG2 cell line has previously been described (32). For MGI, MG3, and MG4, matched TCR-γ and -δ genes were cloned into the pMIG expression vector as per MG2 (32). Vectors were then transduced, along with a pMIG vector encoding full-length CD3e, δ, γ and ζ subunits, into Β2m-deficient, ΒTCR-deficient jurkat-76 cells as previously described (33). CIR-MRI (34) CIR.CD1a (35), CIR.CD1b (36), CIR.CD1c (37), and CIR.CD1d (38) lines are previously described. Jurkat-76 cells provided by Zhenjun Chen (University of Melbourne) who received them in 2015 from Mirjam H.M. Heemskerk (ref. 39; Leiden University Medical Center, Leiden, the Netherlands).

The cell line has not been authenticated. The cell line has returned negative Mycoplasma tests. Cells were used for up to approximately 10 passages. Cells were maintained in RF10 complete media consisting of RPMI-1640 base (Thermo Fisher Scientific, catalog no. A1049101) supplemented with 10% FBS (Thermo Fisher Scientific, catalog no. 10099133), penicillin (100 U/mL), streptomycin (100 μg/mL), Glutamax (2 mmol/L), sodium pyruvate (1 mmol/L), nonessential amino acids (0.1 mmol/L), HEPES buffer (15 mmol/L), pH 7.2 to 7.5 (all from Thermo Fisher Scientific, catalog numbers: 15140163, 35050061, 11360070, 11140050, and 15630080, respectively), and 2-mercaptoethanol (50 μmol/L, Sigma, catalog no. M-7522).

Jurkat assays

5 × 10⁴ Jurkat-76 TCR cells were cocultured with 5 × 10⁶ cell-trace violet (CTV; Thermo Fisher Scientific, catalog no. CS4557)–labeled CIR cells for approximately 18 hours. To label CIR cells with CTV, cells were washed twice with PBS before being resuspended in 500 μL
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Results
Characterizing T-cell infiltrates by mIHC/IF
To study T cells in MCC, we performed quantitative analysis using mIHC/IF. FFPE tissue sections were stained with antibodies targeting T-cell epitopes (CD3, CD4, CD8, FOXP3), PD-L1, synaptophysin (tumor), and DAPI to detect cell nuclei (mIHC/IF). FFPE tissue sections were stained with antibodies targeting BioLegend, catalog no. 361102 and catalog no. 331502, respectively) pelleted and resuspended in RF10 ready for counting. For blocking RF10 complete media (as above) was then added before cells were stained with antibodies targeting markers (cytotoxic T cells were defined as CD3+CD8+; helper T cells were defined as CD3+CD4+; regulatory T cells were defined as CD3+FOXP3+; DN T cells were defined as CD3+CD4−CD8−). T-cell populations were enumerated within intratumoral regions or within complete ROIs (including the stroma), and cell counts were averaged across ROIs within individual tissue sections.

We used m-IHC/IF to analyze samples from a cohort of 39 patients treated at three institutions. Of the 39 patients, 28 had VN-MCC and 11 VP-MCC by MCPyV large T-antigen IHC staining. A total of 58 FFPE tumor samples were stained, including 34 primary and 24 metastatic lesions. Matched primary and metastatic lesions were available for 17 cases (Supplementary Table S2). Primary VP-MCC had significantly higher intratumoral CD8+ (P < 0.001) and CD4+ (P = 0.0456) cell infiltrates compared with primary VN-MCC. Among metastatic lesions, only CD4+ T cells were significantly higher in VP-MCC tumors in total ROIs (P = 0.03; Fig. 1B). No significant differences in PD-L1 staining were observed between VP-MCC and VN-MCC (Supplementary Fig. S3).

Tumors were classified into four groups based on the dominant CD4/CD8 staining profile (Fig. 1C). Among 34 primary tumors, the single largest group had a predominance of CD4+ T cells (50%) followed by CD8+ and DN T cells (with 12% each), while 26% had no dominant subset. Among the matched primary–metastatic pairs, the dominant T-cell subset was consistent in 10 of 17 (59%) cases (Supplementary Fig. S4). In 40% of MCC at least 20% of all infiltrating T cells were DN T cells, and a subset of MCC showed a dominant DN T-cell infiltration pattern.

Flow cytometry analysis of fresh MCC tumors
Flow cytometry was used to characterize immune cells in 11 MCC tumors (3 primary and 8 metastatic) from 10 patients (Supplementary Table S3). Consistent with the m-IHC/IF data, CD4+ and CD8+ cells were the most abundant T cells, ranging from 14% to 82% and 16% to 77%, with medians of 57.1% and 39.4%, respectively. DN T cells ranged from 1% to 25% of CD3+ cells, with a median of 4.0% (Fig. 2A). DN T cells are a heterogeneous collection of unconventional T cells including CD1d-restricted Type I natural killer T (NKT) cells, mucosal-associated invariant T (MAIT) cells, and γδ T cells. Analysis of MCC samples revealed no appreciable NKT cells and very few MAIT cells (median 0.3%) as determined by CD1d–αGalCer and MR1–5-OP-RU tetramer staining. In one tumor, MAIT cells represented 16.9% of T cells; however, this was a liver metastasis, and the liver is known to be enriched for MAIT cells (Fig. 2A; Supplementary Fig. S5; ref. 39). In contrast, γδ T cells accounted for a median of 2.8% of total T cells, ranging from 0.3% to 27.5%.

γδ T cells can be broadly subdivided on the basis of TCR-δ usage, with Vδ2+ and Vδ2−γδ T cells representing functionally distinct populations. Vδ2 mAb staining revealed that most γδ T cells were Vδ2+, with Vδ2− and Vδ2+ cells accounting for medians of 1.3% and 0.2% of total T cells, with ranges of 0.1% to 27.3% and 0% to 2.4%, respectively (Fig. 2A). Overall, γδ T cells accounted for a median of 73.1% of tumor-infiltrating DN T cells.

In one tumor (P055), >25% of total infiltrating T cells were DN T cells, and these were almost exclusively Vδ2−γδ T cells (Fig. 2B). Phenotypic analysis of total Vδ2−γδ T cells in this tumor demonstrated that although 90% were DN, approximately 10% were CD8+ (Fig. 2C). Moreover, the DN cells displayed an enrichment of terminally differentiated cells (CD45RA+, CD27−) relative to CD4+ and CD8+ T cells in the same tumor, and they expressed markers of activation and tissue retention such as CD69 and CD103, including co-expression of these markers by 27% of cells, which is indicative of a tissue-resident memory (TRM) phenotype (Fig. 2D). Similar to their CD8+ T-cell counterparts, these cells had high expression of immune checkpoint markers PD-1 and LAG3, suggesting an exhaustion phenotype (Fig. 2D).

TCR repertoire and antigen discovery for tumor-infiltrating γδ T cells
To gauge the clonality of γδ T cells from P055, we performed TCR analysis on sorted single cells. Sequencing of 59 cells revealed an oligoclonal repertoire dominated by four clonotypes. The Vδ1/Vγ4 TCR (clone MG1), Vδ1/Vγ3 TCR (clone MG2), Vδ1/Vγ8 TCR (clone MG3) and Vδ1/Vγ4 TCR (clone MG4) clones represented 21%, 18%, 18%, and 12% of total γδT cells, respectively (Supplementary Table S9). The oligoclonal repertoire indicated the possibility of antigen-driven clonal expansion, which was consistent with the cells expressing checkpoint markers and having an activated, resident phenotype.

We previously showed that gene transfer of the TCR expressed by the second most abundant clone (MG2) into a Jurkat-76 reporter T-cell line imbed TCR-mediated reactivity to the monomorphic antigen-presenting molecule MHC-related protein-1 (MR1), suggesting that the self-protein MR1 may be the molecular target for this tumor-infiltrating γδ T-cell clone (32). To extend this analysis, we generated three more Jurkat-76 lines expressing the TCRs from the MG1, MG3, and MG4 clones. These lines were challenged with C1R antigen-presenting cells (APC) engineered to express distinct candidate γδ TCR ligands: the MHC-like molecules MR1, CD1a, CD1b, CD1c and CD1d (Supplementary Fig. S6). As expected, the MG2 line reacted strongly to MR1. MG3 and MG4 did not reproducibly respond to any of the C1R APCs, albeit with some nonreproducible low-level basal responsiveness to C1R cells that varied between repeated assays. In contrast, MG1 responded strongly to CD1c-expressing APCs, and this response was blocked by an anti-CD1c mAb, confirming the reactivity. Thus, the two most frequent γδ T-cell clones observed in P055 were CD1c- and MR1-reactive, respectively, and these two clones collectivley represented 39% of the γδ T cells and 11% of the total T cells in that tumor.
Figure 1.
m-IHC/IF analysis of 58 MCC tumors from 39 patients for canonical T-cell markers and PD-L1. A, Representative m-IHC/IF images of a single MCC tissue section (P007 retroperitoneal lymph nodes (RPLN)). Tissues were stained using mAbs targeting CD3, CD4, CD8, FOXP3, and PD-L1 and the MCC-specific marker synaptophysin. ROI, distribution of five ROIs within a single MCC tissue section (P007 RPLN). Composite, layered images depicting the distribution of each fluorescent marker within the tissue section. Tissue segmentation, intratumoral regions discerned from stromal regions via synaptophysin and DAPI+ staining. Phenotype mapping, phenotyping of the individual cell subsets permeating the section. B, Box and whisker plot representing the distribution of mean T-cell infiltrates, CD4/CD8 DN T cells, CD4+, CD8+, and regulatory T cells (Treg) per ROI—classified on viral status and primary/secondary site of disease. Mann-Whitney U test was performed on mean ROI values between matching cell types by viral status (*, P < 0.05; **, P < 0.005; ***), P < 0.0005). (i) Intratumoral infiltrates; (ii) total infiltrates. Whiskers display minimum and maximum values within the IQR + IQR*1.5; “+” refers to mean. C, Details of individual tumors stained by m-IHC/IF, showing total infiltrate numbers per ROI, tumor location, MCPyV status, immunophenotyping, as well as PD-L1 staining. Tumors grouped based on the predominate T-cell infiltrate population (>50% of T cells).
scRNA-seq analysis

We next applied scRNA-seq to sorted CD3⁺ (2,651) and γδ⁺ (1,357) cells from tumor P055. All cells were combined for dimensionality reduction and UMAP clustering (Fig. 3A). UMAP cell clusters corresponded to CD8α-expressing T cells (C1), CD4-expressing T cells (C2), FOXP3-expressing T cells (C4), and two distinct populations of γδ T cells (C0 and C3). Differential gene-expression analysis was performed contrasting each cell cluster to the remaining clusters (P < 0.05, fold change > 0.25; Fig. 3B). The majority of γδ T cells clustered within C0 and were characterized by high expression of TCRD and TCRG genes (e.g., TRDV1, TRDC, and TRGC2); markers of innate immunity (NKG7, GLNY, KLRC3, and FCGR3A, which encodes CD16); markers of cytotoxic cell function, including granymes (GZMA, GZMH, and GZMB); perforin (PRF1); and markers of T-cell exhaustion (PDCD1, which encodes PD-1, LAG3, and HAVCR2, which encodes TIM3; Fig. 3C). Cells in cluster C3 had high expression of CD69 and innate-like T-cell markers, but lacked the exhausted and tissue-resident characteristics of cluster C0.

To complement Sanger sequencing–based TCR analysis, we adapted the same primer reagents for γδ TCR-seq to be compatible with 10x Genomics. Consistent with the Sanger sequencing–based data, the γδ T cells were mostly Vδ1 and Vδ3 clonotypes (Fig. 3D). Furthermore, the same top four clonotypes represented 80% of all γδ T cells (Fig. 3E). In contrast, scTCR-seq of γδ cells revealed a diverse repertoire of γδ TCRs in the CD4⁺ and CD8⁺ populations and no dominant clones (Supplementary Fig. S7).

Derivation of a γδ T cell–gene-expression signature

We next sought to identify a gene signature from the scRNA-seq data what would reflect the proinflammatory and innate profile of γδ T cells in MCC (Fig. 4A). Because γδ T cells and CD8⁺ T cells shared the most common gene-expression patterns, we contrasted γδ clusters (C0, C3) and CD8α-expressing cells (C1) after excluding other T-cell clusters (log fold change > 0.25, minimum percentage of cells expressing >30%). Genes highly expressed (logCPM > 0.5) in MCC cell lines (30) were removed to exclude genes potentially expressed by MCC tumor cells, which would confound downstream bulk tumor analysis. Genes not correlated (r < 0.6) in bulk RNA gene-expression data across 34 MCC tumor samples were also removed. γδ TCR constant regions (e.g., TRDC and TRGC2) were removed as we found they are expressed in other T- and NK-cell subsets based on published bulk RNA-seq data, but TRDV1 was retained as it is more specific and reflects the predominant Vδ1 infiltrate in MCC (Supplementary Fig. S8; ref. 31). Correlation between mRNA expression and the intratumoral DN T-cell count (detected by mHIC/IF) showed that 7 of 13 genes were ranked above the 90th percentile (Fig. 4B; Supplementary Table S10). The expression of the
individual genes comprising the 13-gene γδ T-cell signature in the scRNA-seq data is shown in Supplementary Fig. S9.

GSVA was next used to calculate enrichment scores in MCC tumors subject to bulk RNA-seq (Fig. 4C). As expected, the GSVA 13-gene γδ T-cell signature score correlated with intratumoral DN T cells (mean cells/ROI) detected by m-IHC/IF ($r_s = 0.6$). Lower positive correlations were observed for CD8$^+$ ($r_s = 0.27$) and FOXP3$^+$ cells ($r_s = 0.27$), but no correlation to CD4$^+$ cells ($r_s = 0.02$; Fig. 4D). Similar results were observed for total T-cell infiltrates with respect to T-cell subset. The cell-type specificity of the GSVA γδ T-cell enrichment was further tested using published bulk RNA-seq data for sorted adaptive and innate immune cell types, including CD8, CD4, NK, Vδ1, Vδ2, NKT, and MAIT cells (Supplementary Fig. S10; ref. 31). High enrichment scores were observed not only for Vδ1$^+$ and Vδ2$^+$ γδT cells, but also for NK cells. NKTs, MAITs, CD4$^+$ and CD8$^+$ T cells all had negative median GSVA scores.

### Clinical associations of the γδ T-cell signature in MCC

GSVA using the 13-gene γδ T-cell score was next applied to available gene-expression datasets with clinical annotation (Supplementary Table S11). Two datasets represented patients treated before the introduction of ICI therapies, including an unpublished RNA-seq cohort from Melbourne (RNA-Access/RNA-Direct; $n = 39$) and a published dataset representing patients treated in the United States and Europe (GSE22396; $n = 29$; ref. 11). A third series included RNA-seq data for patients treated with anti-PD-1/PD-L1 antibodies
**Figure 4.**

γδ T-cell gene-set derivation, gene-set enrichment, and survival analysis. A, Signature derivation workflow. DEG, differentially expressed gene(s). B, Spearman rank permutation tests of logCPM expression values ranked against the mIHC/IF DN T-cell total in comprise: gd for six patients; therefore, they were removed from Cox regression analysis (dataset, classifying tumors and patients into three groups based on the (NEB-Next; n = 21; ref. 29). GSVA was applied independently to each MCC RNA-seq cohort (n = 34). D, Spearman rank correlation between GSVA enrichment and mIHC/IF infiltrates. E, MCC-specific survival in an MCC patient cohort (n = 89) based on rank GSVA 15-gene set enrichment classification (high ≥75th, medium 25th–74th, and low ≥25th percentile). F, Multiple Cox regression of clinical features. Covariates comprise: γδ T-cell signature enrichment, age at diagnosis, sex, MCPyV status, AJCC stage at diagnosis, and immunotherapy. Complete clinical data were incomplete for six patients; therefore, they were removed from Cox regression analysis (*P* < 0.05; **P** < 0.005; ***P*** < 0.0005). CI, confidence interval.

**Case study of γδ T-cell-inflamed MCC responsive to anti-PD-L1 treatment**

Four patients treated with ICI therapies had high tumor γδ T-cell enrichment scores, of whom three had a complete response to treatment. Case P007 represented VN-MCC of unknown primary presenting initially in a submental lymph node and then serially as metastatic lesions in the axilla, near the lip and finally in a retroperitoneal lymph node (Fig. 5A and B). All of these tumors were surgically resected. Following the removal of a retroperitoneal tumor mass, the patient received adjuvant therapy with the anti-PD-1 therapy pembrolizumab; the patient remained disease free following 3 years of continuous treatment. Archival FFPE tissue was available for three MCC lesions, including the lip, axillary node, and retroperitoneal metastasis confirming the abundance of in situ γδ T cells was similar (49%–70% DN T cells). Flow cytometry analysis of the retroperitoneal metastasis confirmed the presence of DN T cells (Fig. 5D). Moreover, IHC targeting TCRD confirmed heavy infiltration of γδ T cells (Fig. 5E). Similar to our observation in tumor P055, flow cytometry showed DN T cells had high PD-1 expression (Fig. 5F).

**Discussion**

T-cell infiltration is a common feature of MCC tumors and associated with improved survival, reflecting the immunogenic properties of this disease (11–13). Here, we confirmed the abundance of infiltrating CD4+ and CD8+ T cells in MCC and identified a large fraction of T cells without CD4 or CD8 cell-surface expression. This feature would have been missed by previous IHC-based studies as they did not use multiparameter methods. Flow cytometry revealed that γδ T cells...
represented the majority of DN T cells in MCC tumors. In the context of T-cell inflammation, γδ T cells can express markers of T-cell activation, exhaustion and tissue residency, suggestive of an antigen-driven response. We showed that γδ T cells in MCC had a proinflammatory and innate gene-expression profile predictive of better patient survival. These results indicate for what we believe to be the first time that γδ T cells are likely an important effector cell type in MCC tumors.

Tumor-infiltrating γδ T cells have been described in several other cancer types. γδ T-cell infiltrates in melanomas and cutaneous squamous cell carcinomas (cSCC) are higher in tumors than healthy skin or tonsil and associated with a better prognosis (21, 23, 40). There is conflicting data relating to γδ T-cell infiltrates in breast, colorectal, and pancreatic cancers, with variable prognostic significance reported (24–27). Like MCC, other skin cancers have a predominance of Vδ1+ cells, although in melanoma, an increased proportion of Vδ2+ over Vδ1+ cells are associated with improved survival (21). In cSCC, Vδ2+ cells were found to be proinflammatory and IFNγ-expressing, whereas Vδ1+ cells have a predominantly Th17 phenotype (23). In MCC, we detected a dominance of Vδ2+ γδ T-cell infiltrates, but these cell harbored an innate gene-expression pattern, similar to that reported in triple-negative breast cancers (26). Although we did not stimulate γδ T cells from MCC tumors to measure cytokine expression, these cells had detectable IFNG gene expression, and it is known that in the resting state, IFNG mRNA expression in T cells is predictive of IFNγ secretion upon stimulus (31). These data suggest that most γδ T cells in MCC have a proinflammatory function.

Several prior studies have described methods for in silico prediction of γδ T-cell tumor infiltrates. Indeed, the CIBERSORT method showed that γδ T-cell enrichment was the best predictor of survival across 18,000 tumors of 39 cancer types (41). However, the original LM22 reference used for CIBERSORT was criticized for lack of specificity owing to colinearity of γδ T cells with other immune cell types, leading to efforts to improve the accuracy of reference datasets (42). A reliance on reference datasets representing cells from blood is another potential pitfall as functionality of these cells may be different to their counterparts in tumors. Furthermore, focusing on specific Vδ1+ or Vδ2+
subsets may limit the signature specificity given their different gene-expression profiles (43). In this study, we profiled γδ T cells isolated directly from tumor rather than peripheral blood. The 13-gene γδ T-cell GSEA score correlated with DN T cells by mHGC/F, consistent with our assumption that the majority of DN T cells in MCC are γδ T cells. However, it is clear that individual genes comprising the 13-gene signature may also be expressed by other innate cell types, such as NK cells. Previous quantification of NK infiltrates in MCC relied upon CD16 IHC staining (12, 44). That said, CD16 is not a specific marker of NK cells (45), and indeed we showed that the gene encoding CD16 (FCGR3A) was expressed by a substantial fraction of γδ T cells and correlated with DN T cells (Supplementary Table S9). However, given that we cannot exclude the contribution of NK cells to the 13-gene γδ T-cell score, the interpretation of the scoring in individual cases should be treated with the appropriate level of caution.

Outstanding questions remain regarding the antigens detected by γδ T cells. γδ T-cell infiltration does not correlate with MCC viral status, suggesting that antigens common to both VP-MCC and VN-MCC are recognized by γδ T cells. We observed clonal expansion of Vδ2− (Vδ1+ or Vδ3+) clonotypes by scTCR-seq, which, combined with the expression of activation and tissue retention markers and immune checkpoint molecules, is consistent with antigen-driven expansion. Subsets of Vδ2− cells are known to detect a range of monomeric MHC class I-like molecules including CD1d and CD1c. Here, we demonstrate functional recognition of CD1c by the most abundant clone within the tumor P055. MG1. We previously reported a novel class of γδ T cells that recognize the MHC-like molecule MR1 (32). In that study, we briefly reported the second most abundant TCR clonotype (MG2) derived from MCC lesion P055 was MR1 reactive. The role of MR1 and CD1c in the context of MCC is unclear. Both are antigen-presenting molecules that present small metabolites and lipids, respectively. CD1c is typically expressed by a range of myeloid and lymphoid cell types and can present leukemia-associated lipid antigens (46). MR1 is expressed by all nucleated cells, and appears to present as-yet-undefined tumor-associated antigens from a wide range of tumor types (47, 48). It is thus conceivable that MR1 expression on MCC cells or MR1/CD1c-expressing tumor-infiltrating cells are presenting tumor-associated antigens for surveillance by γδ T cells. Accordingly, MR1 and CD1c may represent novel immunotherapeutic targets, and further studies on this axis are clearly warranted.

The prospect that γδ T cells can be a target of existing ICI therapies is highly relevant to contemporary treatment of patients with MCC, although our understanding is currently limited. We reported complete response to ICI treatment in three patients with MCC where tumors had high γδ T-cell enrichment scores. There is evidence that γδ T cells may influence response to ICI in other diseases. In melanoma patients treated with anti-CTLA4 (ipilimumab), total γδ T cells in peripheral blood had no prognostic relevance but better patient survival was observed on the basis of increased frequency of Vδ2+ over Vδ1+ subsets (22). This is consistent with Vδ2+ cells being cytotoxic effectors in melanoma. Similar to our observations in MCC, γδ T cells found in melanomas and patient blood commonly express immune-checkpoint molecules including Lag3, Tim3, and PD-1 (21). Functional experiments to show the direct effects of ICI on γδ T cells is currently limited but PD-1 blockade has been reported to upregulate IFNγ secretion by γδ T cells in a leukemia model (49). Accordingly, γδ T cells are likely targeted by ICI but the functional outcomes require further investigation. The detection of γδ T cells or a gene-set enrichment score for γδ T cells, as described in this study, should be explored in future biomarker analysis for ICI-responsive MCC.

In summary, we have described γδ T cells as an abundant effector cell in MCC, thereby expanding our understanding of the immune response to this highly immunogenic disease. Future work will require analysis of a larger independent series of MCC and deeper characterization of the function of γδ T cells to fully understand their prognostic and therapeutic predictive value. Given the cytotoxic and proinflammatory effector function of γδ T cells, together with their lack of MHC class I restriction, which makes them impervious to tumor MHC class I downregulation, targeting these cells may represent an alternative approach to cellular therapeutics through adoptive or allogeneic transfer into patients with MCC cancer, and it is an approach being explored in other cancers (50).

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Authors' Contributions

N.A. Gherardin: Formal analysis, investigation, methodology, writing—original draft, writing—review and editing. K. Waldeck: Data curation, formal analysis, investigation, writing—review and editing. A. Caneborg: Formal analysis, writing—review and editing. L. Martelotto: Formal analysis, methodology, writing—review and editing. S. Balachander: Formal analysis, writing—review and editing. M. Zethoven: Formal analysis, writing—review and editing. P.M. Petrone: Formal analysis, writing—review and editing. A. Pattison: Formal analysis, supervision, writing—review and editing. J.S. Wilmott: Formal analysis, writing—review and editing. S.M. Quinones-Parra: Formal analysis, writing—review and editing. A. Posner: Formal analysis, writing—review and editing. A. Wong: Formal analysis, writing—review and editing. A.M. Weppeler: Data curation, writing—review and editing. K.F. Shannon: Data curation, writing—review and editing. A. Hong: Data curation, writing—review and editing. P.M. Ferguson: Data curation, writing—review and editing. J. Raleigh: Data curation, writing—review and editing. A. Hatzimihalis: Data curation, writing—review and editing. R.A. Sculley: Resources, formal analysis, writing—review and editing. P. Deleo: Data curation, writing—review and editing. M. Johnston: Data curation, writing—review and editing. M. Chua: Data curation, writing—review and editing. J.C. Becker: Resources, writing—review and editing. S. Sandhu: Resources, data curation, writing—review and editing. G.A. McArthur: Resources, writing—review and editing. A. J. Gill: Resources, formal analysis, writing—review and editing. R.A. Sculley: Resources, formal analysis, writing—review and editing. R.J. Hicks: Resources, supervision, writing—review and editing. D.I. Godfrey: Resources, formal analysis, supervision, writing—review and editing. R.W. Tothill: Conceptualization, resources, supervision, funding.
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References


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