CD8+ Lymphocyte Intratumoral Infiltration as a Stage-Independent Predictor of Merkel Cell Carcinoma Survival

A Population-Based Study

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ABSTRACT

Objectives: Intratumoral CD8+ lymphocytes (IT-CD8s) have shown promise as a prognostic indicator for Merkel cell carcinoma (MCC). We tested whether IT-CD8s predict survival among a population-based MCC cohort.

Methods: One hundred thirty-seven MCC cases that had not previously been analyzed for IT-CD8s were studied.

Results: Three-year MCC-specific survival rates were 56%, 72%, and 100% for patients with absent (n = 46), low (n = 85), and moderate or strong (n = 6) IT-CD8s, respectively. Increased IT-CD8s were associated with improved MCC-specific survival in a multivariate competing risk-regression analysis including stage, age, and sex (hazard ratio [HR] = 0.5; 95% confidence interval [CI] = 0.3-0.9). Although a similar trend was observed for overall survival, statistical significance was not reached (HR = 0.8; 95% CI = 0.6-1.0), likely because of the high rate of non-MCC deaths among older patients.

Conclusions: This study of prospectively captured MCC cases supports the concept that cellular immunity is important in MCC outcome and that CD8+ lymphocyte infiltration adds prognostic information to conventional staging.

Merkel cell carcinoma (MCC) is a skin cancer with a reported incidence rate that has almost quadrupled in the past two decades in the United States[1] and has an often-aggressive disease course (disease-associated mortality of 46%).[2] Although recently adopted consensus staging guidelines[2] have brought consistency to MCC staging and improved MCC prognostication, there remains a significant need for biomarkers to further refine MCC risk.

Patients with immune dysfunction such as human immunodeficiency virus (HIV)/AIDS (before effective antiviral therapy), solid organ transplantation and associated immunosuppressive regimens, and chronic lymphocytic leukemia have long been known to be at increased risk of developing MCC.[3-5] In 2008, a virus (Merkel cell polyomavirus, MCPyV or MCV) was found to contribute to the etiology of most MCCs.[6] It has subsequently been demonstrated that 80% of MCCs express viral oncoproteins[7] and that many MCC cell lines require persistent viral oncoprotein expression.[8] Furthermore, these nonhuman oncoproteins are recognized by the adaptive immune system in patients with MCC.[9,10]

Initially in unbiased microarray studies, and then through an independent validation cohort, we demonstrated that intratumoral infiltration by CD8α+ lymphocytes is associated with improved MCC-specific survival.[11] Patients with the highest levels of CD8+ infiltration represent a minority of cases but enjoy outstanding disease-specific survival. This association is biologically plausible because CD8α+ lymphocytes (which may include both cytotoxic T cells and natural killer cells) are mediators of antiviral immune responses. CD8+ lymphocyte infiltration is a standard immunohistochemistry assay that may be performed by most

pathology laboratories and has appeal both as a prognostic marker and as a potential determinant of which patients may benefit from immunotherapies. However, our initial validation cohort of patients was limited in that it represented patients from a tertiary referral center and, as such, was biased toward patients who are more likely to seek expert consultation (younger and/or with more complicated or advanced disease). We therefore explored CD8+ infiltration and survival in an independent sample that more closely represented the MCC general population.

Kaiser Permanente Northern California (KPNC) is a large, integrated nonprofit health care delivery system caring for more than 3 million persons who are broadly representative of the local and statewide population. Using data from KPNC health plan databases combined with tissue microarray immunohistochemistry findings, we tested the hypothesis that increased CD8+ lymphocyte infiltration is an independent predictor of improved MCC-specific survival.

Materials and Methods

Patient and Data Characteristics

All studies were approved by the relevant institutional review board (IRB; Fred Hutchinson Cancer Research Center IRB#6585; KPNC IRB# CN-09MAsga-03-H) and performed in accordance with Declaration of Helsinki principles. All patients receiving care at KPNC who were diagnosed with MCC between the years of 1995 and 2009 were considered for inclusion. A waiver of consent was obtained from the KPNC IRB for patients with MCC. A total of 137 patients were included based on the following criteria: MCC diagnosis as assessed by two pathologists, known age at diagnosis, known sex, known diagnosis date, known local-regional-distant stage, presence of follow-up, and availability of adequate formalin-fixed, paraffin-embedded pathology tissues for CD8+ immunohistochemical study. A single patient was identified whose results overlapped with our prior CD8+ infiltration report[11]; results were unchanged with this patient excluded.

CD8 Immunohistochemistry

Formalin-fixed, paraffin-embedded sections (5-μm) were used. CD8α immunohistochemistry was performed using previously reported methodologies.[11] In brief, we used antibody 4B11 (Novocastra, Bannockburn, IL) at 1:200 dilution with heat-induced epitope retrieval and unmasking with pH 8 buffer. Intratumoral CD8+ (IT-CD8+) infiltration was scored semiquantitatively using six bins (scores ranged from 0 to 5) by a single pathologist who was blinded to patient information or outcome. Although the current

study was carried out by only one pathologist, a previous study using the same scoring system tested reproducibility between scorers in a blinded fashion and found them to be reproducible. The observed agreement was 85% and calculated κ was 0.65, consistent with substantial agreement between observers.[11] A score of 0 to 5 represented average infiltration into the tumor taken as a whole, as opposed to only the densest region of intratumoral infiltration. Only CD8 cells that had infiltrated into the tumor and were not directly in contact with a vessel were counted. Effort was made to avoid counting areas with necrosis to minimize false or nonspecific reactions. Ideally, eight to 10 representative fields of tumor would be assessed when possible. To provide a more quantitative assessment of these six levels of infiltration, we previously[11] determined the approximate numbers of CD8+ cells per mm^2 that corresponded to the 0 to 5 scores, representative examples of which are shown in ▮Image 1▮. Although microscopes vary, a typical high-powered field (×10 ocular and ×40 objective) is 0.15 mm^2, meaning there are approximately 7 high-power fields per mm^2. Our prior attempt[11] to quantitate the number of CD8+ cells in each semiquantitative category yielded the following breakdown: score 0: 0 cells/mm^2, score 1: 1 to 179 cells/mm^2, score 2: 180 to 433 cells/mm^2, score 3: 434 to 582 cells/mm^2, score 4: 583 to 731 cells/mm^2, and score 5: more than or equal to 732 cells/mm^2.

Statistical Analysis

All statistical analyses were performed on Stata software version 11.0 for Macintosh (StataCorp, College Station, TX). A P value of .05 was considered to be statistically significant. Univariate and multivariate analyses were performed. All multivariate analyses included age at diagnosis, sex, and local-regional-distant stage in addition to degree of CD8 infiltration and used robust standard errors. For cause-specific survival analyses, competing risks regression was performed with deaths from MCC (n = 36) or probable MCC (n = 10) considered to be events and deaths from known non-MCC causes (n = 37) considered to be competing events. Patients who were alive at last follow-up were censored on their last day of follow-up (n = 38; median follow-up among this group, 7 years) and patients who died of unknown causes (n = 16) were censored on their day of death. For overall survival analyses, Cox regression was performed; deaths from any cause (including unknown) were considered to be events. In both the competing risk regression (disease-specific survival [DSS]) and standard (overall survival [OS]) Cox models, the semiquantitative CD8 score (which ranges from 0 to 5) was treated as a continuous variable. In this case, the hazard ratio (HR) measures each single point increase (eg, from 0 to 1, or from 1 to 2). An HR in which the 95% confidence interval (CI) did not cross 1.0 and the P value was less than .05 was
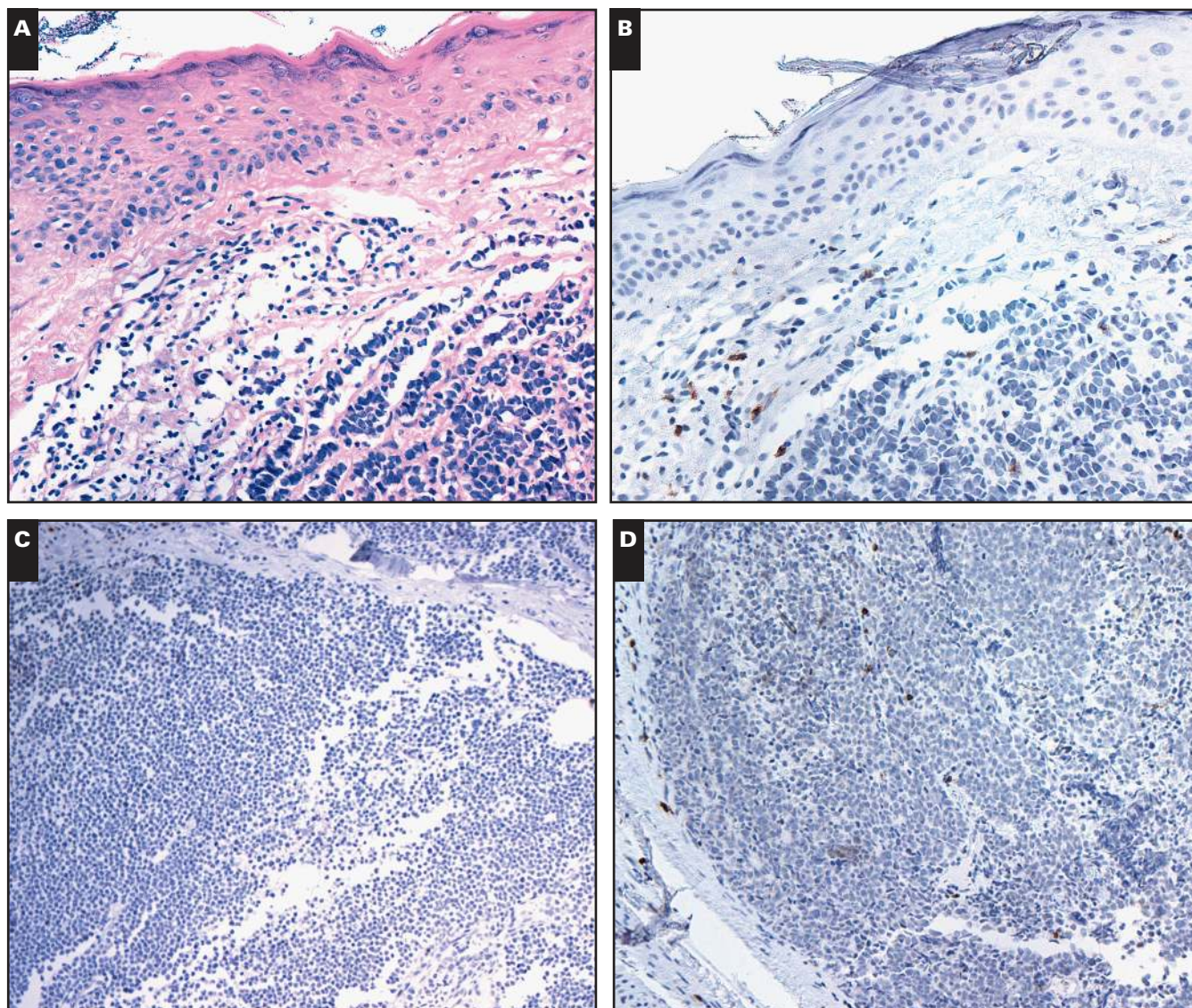
Image 1 Merkel cell tumor (MCC) tumor scores for intratumoral (IT) CD8+ cells. **A**, H&E staining of MCC tumor. **B**, Anti-CD8 staining of the same tumor[11] demonstrating peritumoral and IT-CD8+ cells: IT-CD8+ cells must be surrounded by tumor cells and not have direct contact with stroma. MCC tumors showing varying scores of IT-CD8+ cell infiltration per high-power field (hpf) scored semiquantitatively as follows: score 0: 0 cells/mm^2 per hpf (**C**), score 1: approximately 1-179 cells/mm^2 per hpf (**D**).

considered to be statistically significant. For the purposes of survival data visualization, Kaplan-Meier graphics were created; groupings of absent (CD8 infiltration score of 0), low infiltration (score of 1 or 2), or moderate-strong infiltration (score of 3-5) were selected a priori.

Results

Patient and Tumor Characteristics

A total of 137 patients were included, with a median follow-up of 2.3 years (7.0 years among patients alive at last contact date) and a total follow-up of 493 years. Of these, 87 (63.5%) were male and 50 (36.5%) female. Age at diagnosis ranged from 31 to 96 years, with a median and mean age of 78 and 75 years, respectively. These demographics are similar to those of recently reported US national registry data (61.5% male, mean age 76 years for women and 74 years for men).[1] Of these patients, 85 (62%) had local disease, 41 (30%) had regional/nodal disease, and 11 (8%) had distant metastases. As is typical for MCC, 18 (13%) patients had nodal or distant MCC spread but no identifiable skin primary site.

CD8+ Lymphocyte Infiltration

Tumors from 46 patients (34%) lacked appreciable CD8+ lymphocyte infiltration. Among the 66% of patients with CD8+ infiltration, most had low (n = 77) or low-moderate (n = 8) infiltration and only a few (n = 6) had robust infiltration
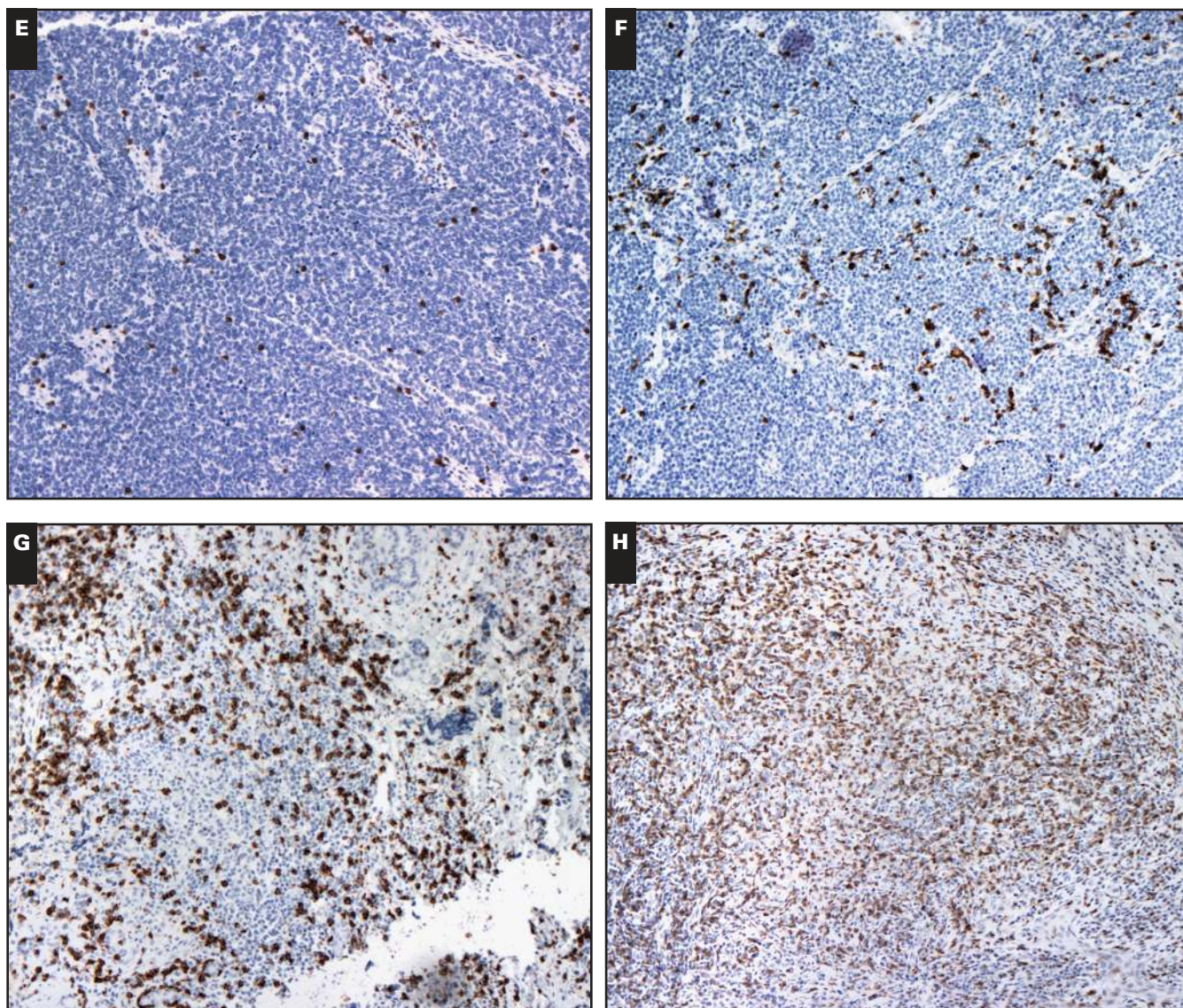
Image 1 (cont) Score 2: approximately 180-433 cells/mm^2 per hpf (**E**), score 3: approximately 434-582 cells/mm^2 per hpf (**F**), score 4: approximately 583-731 cells/mm^2 per hpf (**G**), and score 5: ≥732 cells/mm2 per hpf (**H**).

with scores of moderate or stronger. A breakdown of patients by individual score is presented in **Table 1**.

DSS

Each 1-point increase of CD8+ infiltration on the 0 to 5 point IT-CD8 scale was associated with significantly improved MCC-specific survival in a univariate model (HR = 0.6 per increase; P = .02) **Table 2**. Also significant in the univariate model were regional stage (vs local stage) and distant stage (vs local stage). Age and sex were not predictive of MCC-specific survival. MCC-specific 3-year survival rates among patients with absent (score = 0), low (score = 1-2), and moderate-strong CD8+ lymphocyte infiltration (score = 3-5) were 56%, 72%, and 100%, respectively **Figure 1A** (Table 1). In the multivariate model including stage, age, and sex in addition to

IT-CD8, degree of IT-CD8+ lymphocyte infiltration remained a significant predictor of MCC-specific survival (HR = 0.5 per increase; P = .01), as did stage (Table 2).

OS

In the univariate model, each 1-point increase in CD8+ infiltration was associated with improved survival (HR = 0.8; P = .07) **Table 3**; however, this did not reach statistical significance. Three-year OS rates were 39%, 49%, and 83% among patients with absent, low, and moderate-strong CD8+ lymphocyte infiltration, respectively **Figure 1B**. Age and distant disease were found to be significant predictors of OS, whereas sex and regional stage (vs local stage) were not. In the multivariate analysis, although lower stage and stronger IT-CD8 were associated with better survival, only age was

■Table 1■
One- and Three-Year Disease-Specific Survival (DSS) for Each CD8 Score Group

CD8 Intratumoral Semiquantitative Score	No. of Patients in the Bin	1-y DSS (%)	3-y DSS (%)
0	46	77	56
1	77	90	73
2	8	100	64
3	3	100	100
4	2	100	100
5	1	Not calculable[a]	Not calculable[a]

[a] Patient was alive at last follow-up at 259 days.

found to be a significant predictor of OS (Table 3). This may reflect the increased rate of non-MCC death among older patients (3-year non-MCC death rate of 14% among patients younger than the median age at diagnosis of 78 years vs 38% non-MCC death rate among patients aged ≥ 78 years).

Discussion

MCC is a virus-associated malignancy of increasing clinical impact, and there remains a need for biomarkers to improve prognostication. Here we report that in a population representative cohort of 137 patients with MCC, IT-CD8+ infiltration is an independent predictor of MCC-specific survival and that a greater degree of IT-CD8+ infiltration portends better prognosis.

Two independent US cohorts of patients with MCC (including the present study) used the same CD8 scoring methods and demonstrated an association between CD8+ lymphocyte infiltration and improved MCC-specific survival.[11] An additional population-based cohort study from Finland using a different approach further supports an independent prognostic role for IT-CD8+ as well as CD3+ lymphocytes in MCC.[12] Classic H&E evaluation of tumor-infiltrating lymphocytes (TILs) has previously shown univariate but not independent significance[11,13]; these studies are limited by technical difficulties in differentiating small, round, blue tumor cells from small, round, blue lymphocytes, particularly in the tumor field. We therefore suggest that immunohistochemical evaluation of cytotoxic intratumoral T-cell infiltration is a more sensitive means to assess the antitumor response.

There is no consensus in the literature for how TIL scoring should best be performed, in part because there are many variables that must be considered, such as the tumor type, the stain used, and the region that is assessed (tumor vs stroma vs both). However, a few patterns have emerged in

■Table 2■
MCC-Specific Survival[a]

	Univariate		Multivariate[b]	
	HR (95% CI)	P Value	HR (95% CI)	P Value
Age at diagnosis (per year increase)	1.0 (1.0-1.0)	.57	1.0 (1.0-1.0)	.53
Female sex (vs male)	1.1 (0.6-2.0)	.76	1.2 (0.6-2.2)	.62
Stage at diagnosis				
Regional (vs local)	2.4 (1.3-4.5)	<.01	2.8 (1.4-5.5)	<.01
Distant (vs local)	3.5 (1.2-10.4)	.02	4.7 (1.5-14.4)	.01
CD8 infiltration (per increase on 0-5 scale)	0.6 (0.4-0.9)	.02	0.5 (0.3-0.9)	.01

CI, confidence interval; HR, hazard ratio; MCC, Merkel cell carcinoma.
[a] Results of competing risks Cox regression (deaths from MCC or probable MCC were considered events, deaths from non-MCC causes competing events; n = 137). *P* < .05 was considered statistically significant.
[b] Multivariate analysis considered all listed variables.

■Table 3■
Overall Survival (n = 137)

	Univariate		Multivariate[a]	
	HR (95% CI)	P Value[b]	HR (95% CI)	P Value[b]
Age at diagnosis (per year increase)	1.03 (1.0-1.1)	<.01	1.03 (1.0-1.1)	.01
Female sex (vs male)	0.7 (0.5-1.1)	.14	0.8 (0.5-1.2)	.19
Stage at diagnosis				
Regional (vs local)	1.2 (0.8-1.9)	.42	1.5 (0.9-2.4)	.14
Distant (vs local)	2.1 (1.1-4.3)	.03	1.7 (0.8-3.8)	.16
CD8 infiltration (per increase on 0-5 scale)	0.8 (0.6-1.0)	.07	0.7 (0.5-1.0)	.08

CI, confidence interval; HR, hazard ratio.
[a] Multivariate analysis included all listed variables.
[b] *P* < .05 was considered statistically significant.
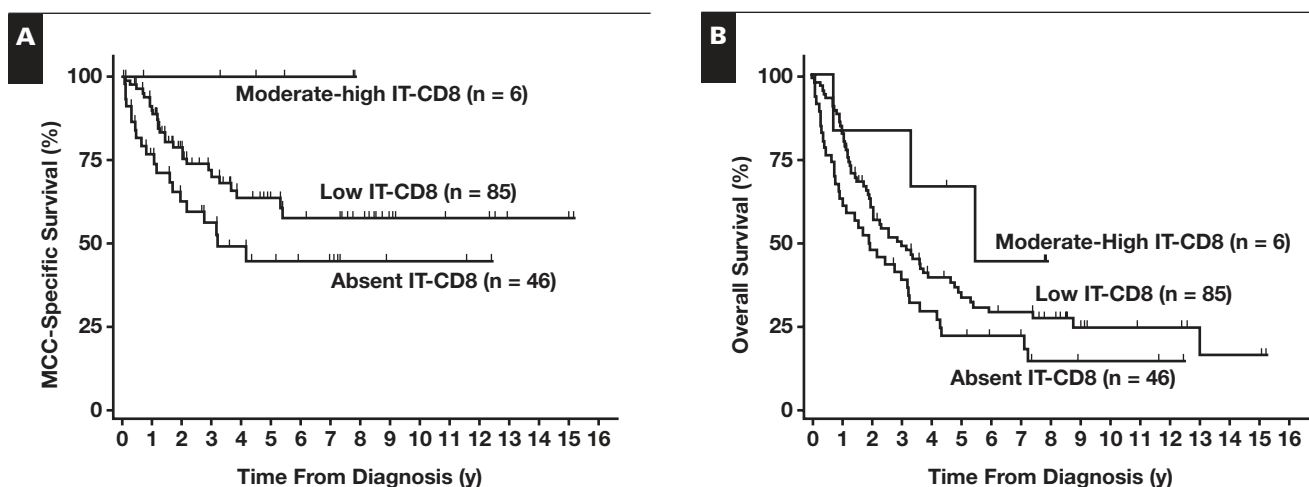
Figure 1 CD8+ lymphocyte infiltration and Merkel cell carcinoma (MCC) survival. **A**, MCC-specific survival rates using Kaplan-Meier curves for patients with absent, low, and moderate-strong intratumoral CD8+ infiltration, respectively, were 77%, 91%, 100% at 1 year; 56%, 72%, 100% at 3 years; and 45%, 64%, 100% at 5 years. **B**, Overall survival rates for patients with absent, low, and moderate-strong intratumoral CD8+ infiltration, respectively, were 63%, 84%, 83% at 1 year; 39%, 49%, 83% at 3 years; and 22%, 34%, 67% at 5 years. Data are shown for purposes of visualization. (See Tables 2 and 3 for univariate and multivariate Cox regression statistical analysis.)

the recent TIL literature such as less prognostic significance of TIL infiltration into stromal or peritumoral regions than for TIL infiltration into the tumor itself.[14,15] Nevertheless, the extent and quality of intratumoral T-cell infiltration has been associated with prognosis in multiple other cancers including colorectal, breast, ovarian, and endometrial carcinomas.[16-23] Studies have demonstrated that colorectal cancer prognosis is influenced by the presence of memory effector T cells in the stroma, within the invasive front and in the parenchyma.[16,24] Immune infiltration of hepatic metastasis from colorectal cancer was also associated with better prognosis.[25] In addition, several studies have shown that cancer prognosis is improved by a Th1 (type 1 adaptive) response of the tumor-infiltrating cells[14,26] whereas a Th2 response tends to be detrimental.[27,28] Seo et al[29] noted that CD8+ TILs independently predict treatment response to systemic therapy in breast cancer (n = 153). A high ratio of IT-CD8 T cells to T-regulatory cells was an independent prognostic factor for both improved disease-specific survival ($P = .001$) and overall survival ($P < .0001$) in hepatocellular carcinoma[30] and had a favorable prognosis in ovarian cancer.[17] Indeed, these findings have led to the proposal for an immune-based parameter (quantifying the extent of cytotoxic and memory T cells at the core and invasive margin of tumors) to be added to tumor staging.[31] We suggest that MCC may also benefit from the addition of an immune score to the standard staging algorithm, and look forward to further developments in these areas.

Our findings, combined with the presence of oncogenic viral proteins in most MCCs, and prior epidemiologic links to immune dysfunction,[3-5] suggest a critical role for cytotoxic T-cell immunity in controlling progression of this cancer. T-cell immune-stimulating therapies will soon be explored in MCC, and evaluation of lymphocyte infiltration may help to determine which patients could benefit most from such treatments. Identifying the underlying CD8+ lymphocyte evasion strategies used by this virus-driven cancer holds exciting potential for both scientific and clinical impact.

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