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Thymic CD8$^+$ T Cell Production Strongly Influences Tumor Antigen Recognition and Age-Dependent Glioma Mortality[1]

Christopher J. Wheeler,[2] Keith L. Black, Gentao Liu, Han Ying,[3] John S. Yu, Wenxuan Zhang, and Paul K. Lee[4]

For unknown reasons, advanced age remains a dominant predictor of poor clinical outcome for nearly all cancers. A decrease in the production of T cells by the thymus accompanies normal aging and parallels the age-dependent increase in cancer progression, but the specific impact of immunity on tumor progression in general is unknown. Glioblastoma multiforme (GBM), the most common primary brain neoplasm, is characterized by rapid age-dependent rates of progression and death. In this study, we show levels of CD8$^+$ recent thymic emigrants (RTEs) accounted for the prognostic power of age on clinical outcome in GBM patients. CD8$^+$ RTEs, typically a tiny proportion of CD8$^+$ T cells, remarkably accounted for the majority of tumor Ag-binding small precursor cells in PBMC from these patients and from healthy individuals. Large blasting tumor Ag-binding cells comprised of CD8$^+$ RTEs and phenotypically related cells were predominantly expanded following experimental vaccination of GBM patients. Quantification of CD8$^+$ RTE expansion in vivo correlated strongly with vaccine-elicited cytokine responses, and estimated numbers of expanding CD8$^+$ RTEs were consistent predictors of clinical outcome in vaccinated GBM patients. Targeted mutant (CD8$\beta^{-/-}$) mice specifically deficient in thymic CD8$^+$ T cell production uniquely displayed an age-specific decrease in glioma host survival as well as a strong correlation between host survival and thymus cellular production. These findings suggest that levels and function of newly produced CD8$^+$ T cells critically influence age-dependent cancer mortality and exert one of the strongest known influences on GBM outcome by predominantly mediating clinically beneficial antitumor immune responses. ***The Journal of Immunology,*** **2003, 171: 4927–4933.**

Glioblastoma multiforme (GBM)[5] is the most common and most deadly primary brain tumor (glioma), accounting for 50% of all intracranial gliomas and 25% of intracranial tumors in adults (1). Prognosis for GBM patients remains dismal, and age at diagnosis best predicts its clinical outcome despite therapeutic intervention (2). In this respect, GBM is representative of the overwhelming majority of human cancers, in which advanced age is the dominant predictor of poor clinical outcome (3). Although the basis for the age-dependent increase in cancer morbidity and mortality remains poorly understood, T cell immune activity represents an attractive potential contributor, because CD8$^+$ T cell function in particular both contributes to antitumor immunity (4, 5) and is substantially depressed with age (6, 7).

The property of immunity most sensitive to aging is the production and export of T cells from the thymus. This is manifested as a decrease in peripheral levels of naive recent thymic emigrant T cells (RTEs) with age (8–10), which could influence functional CTL precursor frequency if a proportion of RTEs was tumor specific. We therefore examined RTE levels and tumor Ag specificity in GBM patients and correlated these parameters with clinical outcome and antitumor immune responsiveness. We also directly tested the influence of host T cells on age-dependent glioma survival by implanting glioma cells intracranially into aging wild-type and mutant mice.

We demonstrate in this study that age-dependent GBM outcome is more accurately CD8$^+$ RTE dependent, and that the prognostic power of age is derived primarily from its loose association with CD8$^+$ RTE levels. CD8$^+$ RTEs also accounted for the majority of precursor cells capable of recognizing any of a number of tumor epitopes and appeared to predominate in responses to tumor Ags. Decreased thymic CD8$^+$ T cell production in CD8$\beta^{-/-}$ mice elicited decreased age-dependent survival of intracranial glioma hosts, uniquely reflecting the clinical pattern exhibited in human GBM. The data support an overwhelming and direct influence of newly produced T cells on age-dependent tumor outcome.

Materials and Methods

Patients and clinical parameters

Newly diagnosed or recurrent GBM patients (55 years average, 32–78 range) received standard radiation therapy after surgery. Vaccinated patients were steroid free during blood collection and vaccinations, as described (11), and received three vaccines, 2 wk apart, of 10–40 $\times$ 10^6 autologous dendritic cells (DC) loaded with 150 μg/ml autologous tumor freeze-thaw lysate, starting ~15 wk postsurgery. A fourth identical vaccination followed 6 wk later only in phase II trial patients (10 of 17). Serial magnetic resonance imaging scans were performed every 2 mo (66%), every 3 mo (11%), or variably, but at least annually (23%). Tumor recurrence was the time from diagnosis to the first new scan enhancement, if verified by subsequent scans or by histology, or time from diagnosis to death due to tumor progression.

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[5] Abbreviations used in this paper: GBM, glioblastoma multiforme; DC, dendritic cell; FSC, forward light scatter; pHLA^{tum}, tumor peptide-loaded HLA tetramer; qPCR, quantitative real-time PCR; RTE, recent thymic emigrant T cell; TREC, TCR excision circle.

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Cell isolation and lysis

PBMC were prepared with Ficoll from patients' blood obtained at the time of surgery and/or from banked leukapheresis. CD4$^+$ and CD8$^+$ T cells were purified from PBMC using MACS bead separation (Miltenyi Biotec, Auburn, CA). A total of 10^7 CD4$^+$ or CD8$^+$ cells/ml was prepared for quantitative real-time PCR (qPCR) by lysis in 100 μg/ml proteinase K (Boehringer Mannheim, Indianapolis, IN) 1 h, 56ºC, with inactivation at 95ºC, 10 min.

Flow cytometry

Purified T cells stained on ice with Abs recognizing CD4, CD8, and CD3 were analyzed by three-color flow cytometry (FACScan II; BD Biosciences, San Jose, CA) to assess purity. A total of 1 μg PE-labeled tetramers for Her-2/HLA-A2.1, MAGE-1/HLA-A1.1, or gp100/HLA-A2.1 (Beckman Coulter, San Diego, CA), or TRP-2 180–188 SVYDFFVWL peptide/HLA-A2.1 (National Institute of Allergy and Infectious Diseases Tetramer Core Facility, Emory University, Atlanta, GA) was incubated with monocyte-depleted PBMC (10^6 cells/50 μl) in PBS, 5% FCS, at 25ºC, 30 min, followed by 30-min incubation at 25ºC with paired combinations of anti-CD8, anti-CD45RO, and/or anti-CD103 mAb (Immunotech, Marseille, France), and 100,000–300,000 flow events were acquired. Tetramer specificity and gating were established by staining epitope-specific T cell clones.

TCR excision circle (TREC) quantification

TRECs were quantified in duplicate or triplicate by qPCR using the 5′ nuclease (TaqMan) method, as previously described (12), and detected on an iCycler system (Bio-Rad, Hercules, CA). qPCR was performed on 5 μl cell lysate (from 50,000 cells) with primers: 5′-CACATCCCTTTCAACCATGCT-3′ (forward), 5′-GCCAGCTGCAGGGTTTAGG-3′ (reverse), and FAM-5′-AC ACCTCTGGTTTTTGTAAAGGTGCCCACT-TAMRA-3′ (probe; MegaBases, Chicago, IL). PCR, including 0.5 U Platinum *Taq* (Life Technologies, Grand Island, NY), 3.5 mM $MgCl_2$, 0.2 mM dNTPs, 500 nM of each primer, and 150 nM probe, were amplified at 95ºC for 5 min, 95ºC for 30 s, and 60ºC for 1 min for 45 cycles. Control β-actin reactions were performed to ensure nucleic acid content, and negative samples were excluded from further analysis. TREC values were adjusted for T cell purity.

CTL assays

DC were prepared by incubating loosely adherent PBMC in RPMI + 10% human AB serum, 500 U/ml IL-4, 800 U/ml GM-CSF for 8 days, 37ºC , 5% CO_2. A total of 2×10^6 DC/ml was pulsed with autologous tumor freeze-thaw lysate (150 μg/ml) 18 h and irradiated. Autologous pre- and postvaccine PBMC (1×10^6 cells/ml) were stimulated in 10% human AB serum with 1×10^6 irradiated lysate-pulsed DC/ml, with IL-2 (300 IU/ml) added on day 2, and 2-h restimulation with 150 μg/ml tumor lysate on day 11. RNA was isolated using TRIzol (Life Technologies Invitrogen, San Diego, CA), and transcribed using random hexamers. Quantified plasmid DNA standards and cDNAs were amplified using qPCR primers and probes (Qiagen Operon, Alameda, CA), as previously described (13, 14). A ≥1.5-fold increase in CD8-normalized IFN-γ production following vaccination indicated a positive response (14). IFN-γ primers: 5′-AGCTCT GCATCGTTTTGGGTT-3′ (forward), 5′-GTTCCATTATCCGCTACAT CTGAA-3′ (reverse), and 5′-FAM-TCTTGGCTGTTACTGCCAGGACC CA-TAMRA-3′ (probe). Reference (CD8) primers: 5′-CCCTGAGCAAC TCCATCATGT-3′ (forward), 5′-GTGGGCTTCGCTGGCA-3′ (reverse), and 5′-FAM-TCAGCCACTTCGTGCCGGTCTTC-3′ (probe). Reactions were amplified in 25 μl, 10 mM dNTP, 400 nM primers, 200 nM TaqMan probe, and 0.5 U Platinum *Taq* polymerase, at 95ºC, 5 min; 95ºC, 30 s; 60 ºC, 30 s for 45 cycles, and detected on an iCycler (Bio-Rad). Patients responsive to TRP-2, Her-2, MAGE-1, or gp100 were identified by postvaccine increases in IFN-γ production by PBMC to peptide-pulsed T2 cells (1 μM peptide, 2 h, 37ºC) using ELISA and/or ELISPOT kits (R&D Systems, Minneapolis, MN), according to manufacturer's instructions.

Statistical analyses

Statistical analyses included two-tailed Mann-Whitney log rank tests for disease-free and overall survival, binomial distribution probability, two-tailed *t* tests (*p* values), and Pearson's correlation coefficients (*r* values) calculated with SAS and Excel software. Each cohort patient was matched for analogous sample collection time and magnetic resonance imaging scan frequency, newly diagnosed or recurrent GBM status, similar postradiation therapies (observation, vaccination, or chemotherapy), and either age (age matched; 36- to 66-year range in each cohort; $n = 10$/cohort; $p = 0.96$) or CD8$^+$ TRECs (CD8$^+$ TREC matched; 1.5–4309.5 and 0.6–5530.4 ranges in old and young cohorts, respectively; $n = 11$/cohort; $p = 0.86$), to a counterpart in the opposing cohort with distinct CD8$^+$ TRECs (age matched; $p < 0.05$) or age (CD8$^+$ TREC matched; $p < 0.008$).

Tumor cell implantation in mice

C57BL/6 (Jackson ImmunoResearch Laboratories, West Grove, PA) and CD8$\beta^{-/-}$ mice (D. Littman, New York University, New York, NY) were housed in a pathogen-free vivarium. Identically sex-matched groups of both middle-aged C57BL/6 (10–15 mo, average = 11.1 mo) and CD8$\beta^{-/-}$ (12–15 mo, average = 13.8 mo) mice, or aged C57BL/6 (18–24 mo, average = 21 mo) and CD8$\beta^{-/-}$ (18–21 mo, average = 20.1 mo) mice were used for tumor implantation. Age ranges within older (18–24 mo) C57BL/6 and CD8$\beta^{-/-}$ groups were statistically identical ($p = 0.5$, two-tailed *t* test). Cultured murine GL26 glioma cells were harvested by trypsinization, and 5000 GL26 tumor cells in 2 μl 1% methylcellulose were implanted intracranially using a stereotactic rodent frame, with injection 1 mm posterior and 2.5 mm lateral to the junction of the coronal and sagittal sutures (bregma), at a depth of 2 mm. Thymuses were removed from terminally symptomatic mice, and thymocytes were counted. Survival in days was compared with thymocyte numbers, and Pearson's correlation coefficients (*r* values) were determined. Survival differences were assessed by two-tailed Mann-Whitney log rank.

Results

We quantified CD4$^+$ and CD8$^+$ RTEs in 24 newly diagnosed and 18 recurrent GBM patients by TREC analysis, which measures the concentration of nonreplicating TCR DNA excised from the genomes of ~70% of developing human T cells (8). Seventeen of these patients were enrolled onto an approved phase I (recurrent) or phase II (newly diagnosed or recurrent) vaccine trial for high-grade glioma patients, 11 of whom were also tested for antitumor immune activity. This allowed us to examine the role of thymus output in age-dependent GBM outcome and antitumor immunity. As in healthy individuals (8, 15), CD4$^+$ and CD8$^+$ TRECs in GBM patients decreased with age, albeit loosely (Fig. 1*a*). Because age is the strongest established prognostic factor for GBM (16, 17), it was somewhat surprising that CD4$^+$ and particularly CD8$^+$ TRECs correlated better with recurrence and survival than did patient age (Fig. 1*b*). High CD8$^+$ TRECs also predicted longer recurrence-free and overall survival at least as well as younger age and more significantly than high CD4$^+$ TRECs, whereas age and CD4$^+$ TRECs were similar in this regard (Fig. 2). This suggested that CD8$^+$ TRECs might affect GBM outcome in an independent, but age-associated manner. We identified patient cohorts with identical age ranges, but distinct CD8$^+$ TRECs, and those with identical CD8$^+$ TRECs, but different ages, to address the ability of CD8$^+$ TRECs and age to predict GBM outcome independent of each other. Patient age could not be similarly dissociated from CD4$^+$ TRECs. High CD8$^+$ TRECs predicted longer recurrence-free and overall survival in age-matched cohorts, whereas lower patient age failed to predict either outcome in CD8$^+$ TREC-matched cohorts (Fig. 2). Thus, CD8$^+$ TRECs largely accounted for the prognostic power of age in these patients.

High CD8$^+$ TREC conditions could coincide with tumor-slowing processes independent of T cell function or could directly affect GBM outcome by encouraging antitumor T cell responses. In support of the latter, all vaccinated GBM patients with high prevaccine CD8$^+$ TRECs (5 of 5) exhibited positive IFN-γ responses after vaccination ($p = 0.048$ relative to overall responders; Fig. 3*a*). In contrast, only one vaccinated patient with low CD8$^+$ TRECs (1 of 6) exhibited a positive IFN-γ response after vaccination ($p = 0.0001$ relative to high CD8$^+$ TREC responders; Fig. 3*a*). Because IFN-γ production in this system could be due to either CD4$^+$ T cell and/or CD8$^+$ T cell reactivity, the direct involvement of TREC-bearing CD8$^+$ T cells in this process was uncertain. Nevertheless, high CD8$^+$ TREC patients were significantly more likely to respond to tumor Ags upon vaccination. This could be because high CD8$^+$ TRECs reflect general host immune

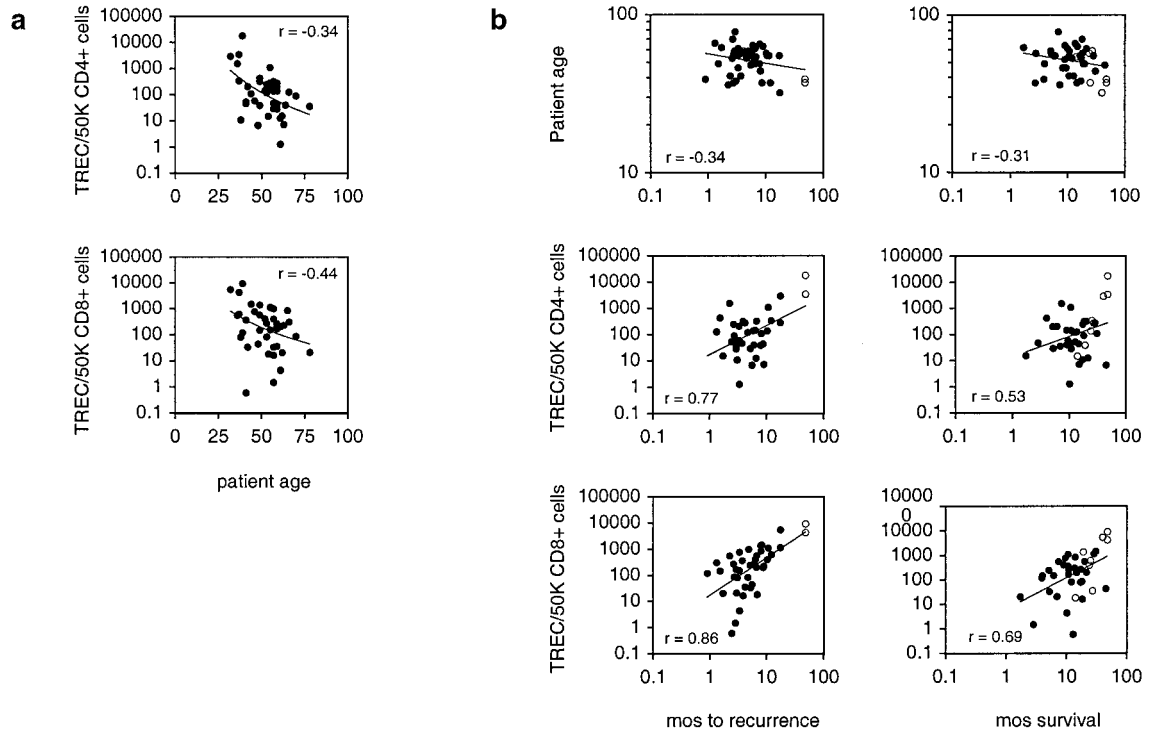

FIGURE 1. TRECs in $CD8^+$ T cells account for age-dependent GBM recurrence and survival. *a*, TRECs within $CD4^+$ and $CD8^+$ T cells generally decrease with age in GBM patients. The number of TREC molecules within 50,000 purified T cells derived from distinct individual GBM patients is represented by each filled circle. *b*, TRECs correlate with clinical outcome of GBM better than patient age. ●, Represent individual GBM patients who recurred or died. ○, Reflect censured clinical outcome data (i.e., minimal estimates from patients with no recurrence and/or death to date). Pearson's correlations were assessed for the indicated parameters, and all correlation coefficients were significant ($p < 0.05$).

competence or because TREC-bearing $CD8^+$ T cells directly influence antitumor responses.

To begin to distinguish between these possibilities, we examined the relationship between IFN-γ response magnitude and either prevaccine $CD8^+$ TRECs or vaccine-induced $CD8^+$ RTE proliferation. We reasoned that the degree of vaccine-induced $CD8^+$ RTE proliferation should be more closely related to the magnitude of vaccine-induced IFN-γ responses than are prevaccine $CD8^+$ TRECs only if $CD8^+$ RTEs directly influence antitumor responses. In this context, $CD8^+$ TRECs of many patients were substantially diluted, whereas their $CD4^+$ TRECs were relatively static after vaccination (Fig. 3, *b* and *c*). This suggested a specific reaction of TREC-bearing $CD8^+$ RTEs upon vaccination, and allowed tracking of $CD8^+$ TREC dilution after normalization to $CD4^+$ TREC changes as a potential measure of $CD8^+$ RTE proliferation (8, 18). The degree of postvaccine $CD8^+$ TREC dilution correlated very well ($r = 0.96$; Fig. 3*d*), whereas prevaccine $CD8^+$ TREC levels correlated poorly ($r = 0.33$; Fig. 3*e*) with vaccine-induced IFN-γ response magnitude. Thus, IFN-γ production is highly coordinated with vaccine-elicited $CD8^+$ TREC dilution. This could reflect IFN-γ production by $CD4^+$ T cells that supports a proportional $CD8^+$ RTE-dependent proliferative response, or IFN-γ production by proliferating $CD8^+$ RTEs themselves. In either case, this supports the notion that $CD8^+$ TREC dilution reflects a proliferative response by $CD8^+$ RTEs themselves that closely parallels cytokine production upon vaccination. If this notion is valid, a measurable proportion of $CD8^+$ RTEs should be specific for tumor Ags.

To directly examine this, we analyzed binding to soluble HLA multimers loaded with tumor-associated Ags ($pHLA^{tum}$) in lymphocytes from GBM patients and healthy subjects. Intriguingly, expression of CD103, a marker on a population of $CD8^+$ RTEs (19), defined a population of small (forward light scatter $(FSC)^{low}$) lymphocytes that was highly enriched for binding to any of four $pHLA^{tum}$ (Figs. 4 and 5). This population consistently represented less than 0.7% of the entire PBMC population (data not shown), but surprisingly included the majority (56–76%) of small $pHLA^{tum+}$ lymphocytes (Fig. 4). Moreover, these cells were indistinguishable from $CD8^+$ RTEs (19), in that they expressed CD8 and CD3, but not CD45RO and were at least 58-fold enriched for TRECs relative to small $CD103^-CD45RO^-CD8^+$ naive T cells from the same patient (Fig. 6 and data not shown). This suggested that $CD8^+$ RTEs comprised most tumor Ag-specific naive precursor cells in patients and healthy subjects, and might be expected to dominate primary immune responses to tumor Ags.

To test this, we identified vaccinated GBM patients exhibiting responses to tumor epitopes (Fig. 5). In patients responding to TRP-2 or to Her-2, small (FSC^{low}) $CD103^+pHLA^{tum+}$ cells were selectively decreased upon vaccination, further supporting these cells' correspondence to the TREC-bearing $CD8^+$ RTEs diluted upon vaccination (Fig. 5). Flu-specific small $pHLA^+$ cells were not decreased upon vaccination (data not shown). We reasoned that $CD8^+$ RTEs that were proliferating upon vaccination should simultaneously depart from the small precursor cell pool and expand within the blasting lymphocyte (FSC^{high}) pool of the same patients. Accordingly, FSC^{high} $CD103^+pHLA^{tum+}$ cells were increased concomitantly with loss of small $CD103^+pHLA^{tum+}$ RTEs in the same patients (Fig. 5). These large cells were evident before vaccination only in GBM patients (Fig. 5), in which they often represented >1% of the entire PBMC population (data not shown), further suggesting that they represented an expanded tumor-reactive population. Large $CD103^+pHLA^{tum+}$ cells were phenotypically similar to small $CD103^+pHLA^{tum+}$ cells (i.e., $CD3^+CD8^+$), except that most of them expressed the effector/memory cell marker, CD5RO (Fig. 6). The only other substantial

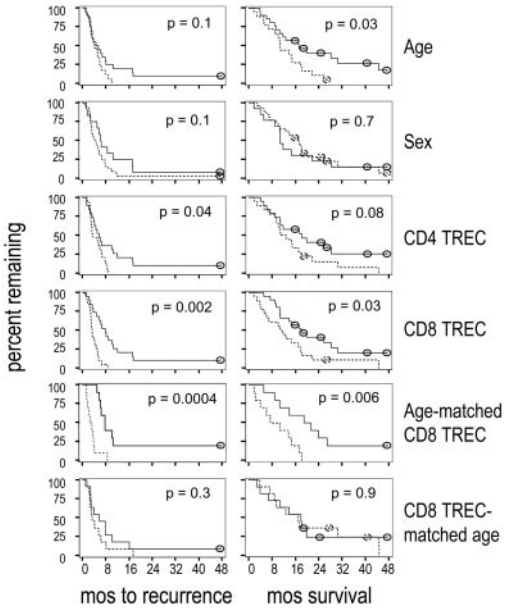

FIGURE 2. CD8+ TRECs account for age-dependent GBM outcome. The entire population of patients (age, sex, CD4 TREC, and CD8 TREC plots) or patient cohorts (age-matched and CD8 TREC-matched plots) was separated into groups based on the parameters indicated at *right* and Kaplan-Meier analysis performed. Patients were separated for analyses by: age above (broken lines) or below (filled lines) the median of the entire population (first row), and age above (filled lines) or below (broken lines) the median of CD8 TREC-matched cohorts (sixth row); TRECs above (filled lines) or below (broken lines) the median of the entire population (third and fourth rows) or cohorts (fifth row); female (filled lines) or male (broken lines) in the entire population (second row). ○, Reflect censured clinical outcome data. Females exhibited a tendency (nonsignificant) toward slower GBM recurrence (second row). Each cohort patient was matched for either age (36- to 66-year range in each cohort; $n = 10$/cohort; $p = 0.96$) or CD8+ TRECs (1.5–4309.5 and 0.6–5530.4 ranges in old and young cohorts, respectively; $n = 11$/cohort; $p = 0.86$), to a counterpart with distinct CD8+ TRECs ($p < 0.05$) or age ($p < 0.008$), respectively. Two-tailed Mann-Whitney log rank tests for disease-free and overall survival for populations segregated by median values of the indicated parameters (age, sex, etc.) were calculated with SAS software.

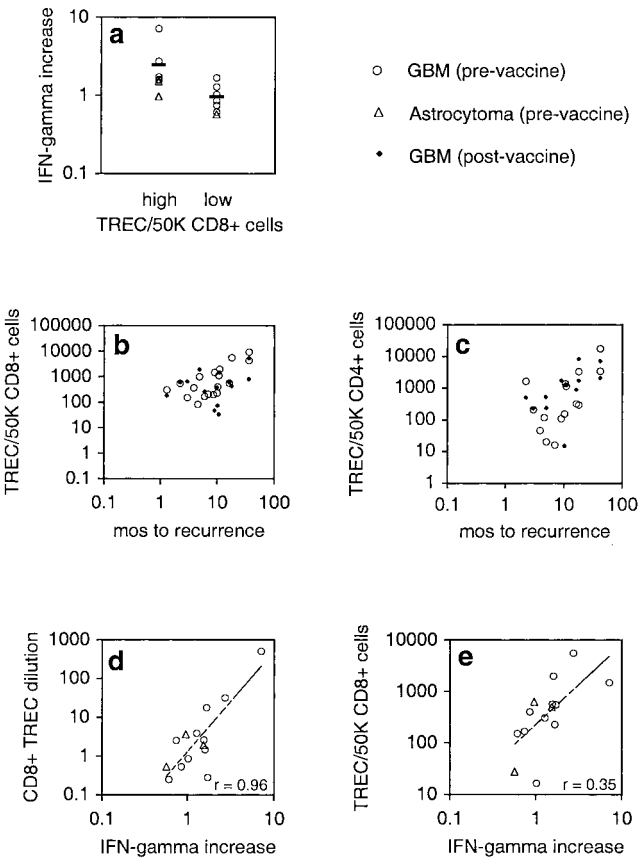

FIGURE 3. CD8+ TRECs are associated with in vitro responses to tumor Ags and are specifically modulated in vivo, upon vaccination. *a*, High and low CD8+ TREC levels correlated with increased incidence of IFN-γ production after vaccination in 11 recurrent GBM (grade IV glioma; ○) patients ($p < 0.05$). Findings were identical with the addition of data from three vaccinated anaplastic astrocytoma (grade III glioma; △) patients. IFN-γ response = postvaccine IFN-γ transcripts normalized to CD8 transcripts in the presence of Ag – no Ag control/prevaccine IFN-γ transcripts normalized to CD8 transcripts in the presence of Ag – no Ag control. Binomial distribution probability was determined for IFN-γ production by patients with high and low CD8+ TREC levels. *b*, Prevaccine CD8+ TRECs (○) from vaccinated GBM patients correlated strongly with recurrence ($r = 0.85$), and were decreased in some patients upon vaccination (◆). *c*, Prevaccine CD4+ TRECs (○) from the same recurrent GBM patients correlated strongly with recurrence ($r = 0.73$; $p < 0.01$), but were not substantially decreased upon vaccination (◆). *d*, The degree of specific CD8+ TREC dilution upon vaccination (prevaccine CD8+ TRECs/postvaccine CD8+ TRECs) was normalized to changes in CD4+ TRECs (divided by prevaccine CD4+ TRECs/postvaccine CD4+ TRECs from the same patient) and Pearson's correlations determined for the indicated parameters. Specific CD8+ TREC dilution upon vaccination correlated with IFN-γ response ($r = 0.96$; $p < 0.001$). Correlations were minimally affected ($r = 0.95$; $p < 0.001$) by the addition of anaplastic astrocytoma data (△). *e*, Pearson's correlations were determined for the indicated parameters. Prevaccine CD8+ TREC levels correlated poorly with IFN-γ response ($r = 0.35$; $p > 0.05$). Correlations were minimally affected ($r = 0.38$; $p > 0.05$) by the addition of anaplastic astrocytoma data (△). Data were derived from all vaccinated GBM or anaplastic astrocytoma patients for whom CTL and TREC results were available, except for the exclusion of a single GBM patient who had been administered independent therapeutic vaccinations before revaccination and analysis.

population of CD103+CD45RO+ T cells resides predominantly within intestinal mucosa (20, 21). Because the CD103+pHLA^{tum+} large cell population expanded after peripheral rather than mucosal vaccination, it is likely that it originates from peripheral CD103+ precursors such as CD8+ RTEs. In support of this, a small population of CD45RO− cells was consistently observed within the CD103+pHLA^{tum+} large cell pool after vaccination (Fig. 6). This feature was not consistently observed before vaccination (data not shown), and indicates that some of the cells that expanded upon vaccination possess a CD8+ RTE phenotype. This in turn suggests that CD103+pHLA^{tum+} large cells originate from peripheral CD45RO−CD8+ RTEs, and that CD8+ RTEs preferentially respond to tumor Ags in vivo.

To determine whether CD8+ RTE antitumor responses contributed to the association between CD8+ TRECs and GBM outcome, we separated the 11 vaccinated GBM patients into two groups based on age above or below the median. The same 11 patients were separated into similar paired groups, based on medians of vaccine-induced IFN-γ response magnitude, prevaccine CD4+ or CD8+ TRECs, degree of postvaccine CD8+ TREC dilution, or number of CD8+ TRECs diluted after vaccination. When recurrence and survival times were compared within each group pair, only those distinguished by numbers of CD8+ TRECs lost after vaccination exhibited significantly different recurrence-free and overall survival (Fig. 7). Thus, the most accurate correlate of clinical outcome in these patients was the number of CD8+ RTEs

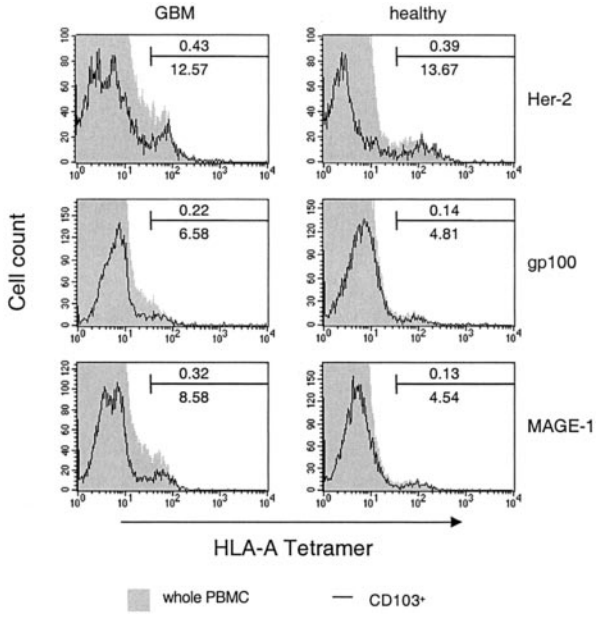

FIGURE 4. Most nonblasting cells capable of binding tumor Ag/HLA tetramers (pHLA^{tum+}) are CD103$^+$. Blasting cells and nonlymphocytes were electronically excluded by gating on low forward (FSC^{low}) and side light scatter. Tumor Ag-loaded HLA tetramer (pHLA^{tum}) and CD103 staining was analyzed simultaneously. The proportion of pHLA^{tum+} cells within all FSC^{low} lymphocytes in PBMC (shaded; top of gate) and within the FSC^{low} CD103$^+$ lymphocyte subpopulation (solid line; bottom of gate) is shown. Staining characteristics are representative of at least three individuals for each pHLA^{tum}.

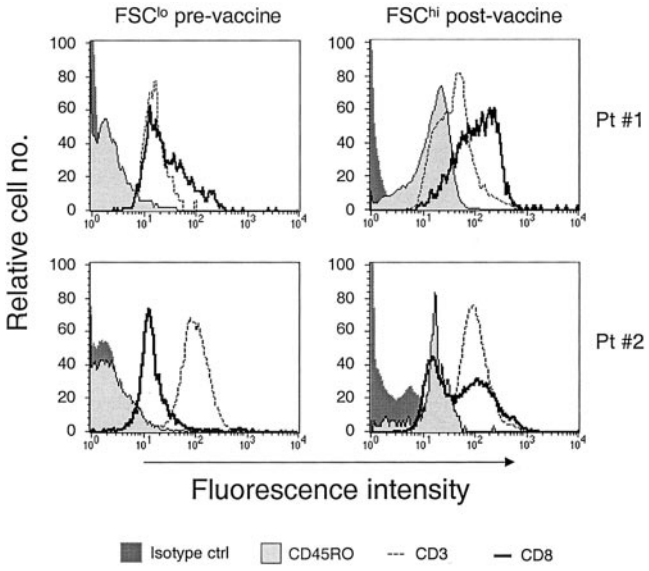

FIGURE 6. All small CD103$^+$pHLA^{tum+} cells and a subset of large blasting CD103$^+$pHLA^{tum+} cells are phenotypically identical to CD8$^+$ RTEs. Electronic gates were set according to cell size (FSC) and expression of both CD103 and pHLA^{tum} and were analyzed for expression of CD8, CD3, and CD45RO. Small and large CD103$^+$pHLA^{tum+} cells expressed equivalent levels of CD3 and CD8, but distinct CD45RO levels: small cells were CD45RO$^-$, whereas large cells were mostly CD45RO$^+$. Large prevaccine CD103$^+$pHLA^{tum+} cells were phenotypically similar to large postvaccine CD103$^+$pHLA^{tum+} cells, except that they did not consistently possess a CD45RO$^-$ component (data not shown).

proliferating over a relatively short time span. Because this proliferation was tightly associated with antitumor responses after vaccination (Fig. 3*d*), this suggests that the reason prevaccine CD8$^+$ TRECs predict GBM outcome is that they reflect the potential for ongoing antitumor responses mediated directly by CD8$^+$ RTEs. In this context, segregating patients by any criteria (median or higher) for IFN-γ responsiveness itself failed to significantly correlate with recurrence-free or overall survival. This additionally suggests that the clinical manifestations of antitumor activity by CD8$^+$ RTEs may be more directly related to their proliferation than to any associated IFN-γ production.

The above data are consistent with a direct influence of thymus CD8$^+$ T cell production on age-dependent GBM outcome. We sought to unequivocally verify this in a rodent model of intracranial glioma. Based on the sufficiency of Ag-pulsed professional APC administration to elicit clinically beneficial antitumor immunity in rodent glioma models (22–24), it was considered unlikely that CD8$^+$ RTEs limited such immunity in wild-type mice. This led to the prediction that age-dependent glioma outcome similar to that observed in GBM patients would not be evident in mice unless CD8$^+$ RTE production was specifically diminished. CD8$\beta^{-/-}$ mice exhibit a partial reduction in thymic production of CD8$^+$ T cells, with retention of peripheral CD8$^+$ T cell activity levels comparable to wild-type mice (25, 26). This allowed us to test whether age-dependent glioma survival was directly influenced by CD8$^+$ T cells by implanting GL26 glioma cells (27) intracranially into middle-aged and aged (to optimally model human GBM patients) wild-type and CD8$\beta^{-/-}$ mice. Survival was prolonged in aged relative to young (data not shown) or middle-aged GL26-bearing wild-type mice (Fig. 8), reflecting a general trend in aged murine

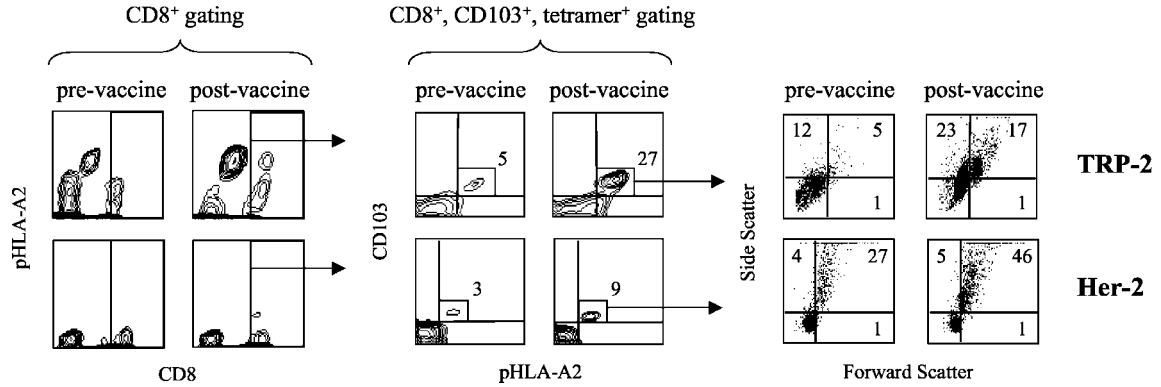

FIGURE 5. CD8$^+$CD103$^+$pHLA^{tum+} cells are expanded upon vaccination in Ag-responsive GBM patients. Electronic gates were set according to experiment-specific negative controls (isotype-matched mAb). Similar expansions were observed in CD8$^+$PBMC recognizing gp100- or MAGE-1 epitopes (2- to 3.8-fold; data not shown). Numerical values indicate percentages of analyzed cells within the indicated gates.

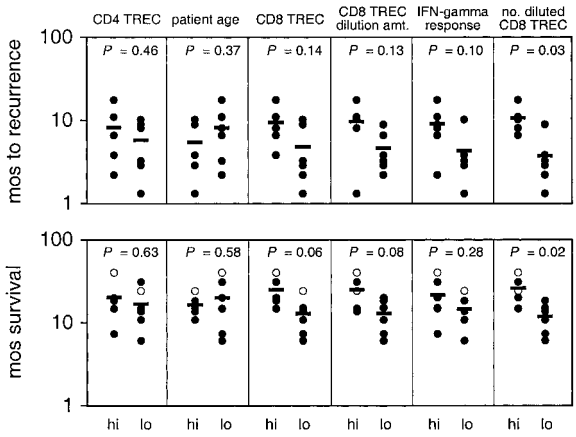

FIGURE 7. Numbers of responding CD8$^+$ RTEs consistently predict GBM clinical outcome. Recurrent vaccinated GBM patients (11) were separated into high and low groups according to median values of the indicated parameters (*above*), and tested for significant differences in recurrence and survival times by two-sided *t* test. ○, Reflect censured outcome data (two surviving patients; all had recurred). CD8 TREC, Indicates prevaccine CD8$^+$ TREC levels. CD8 TREC dilution amt., Indicates prevaccine CD8$^+$ TRECs/postvaccine CD8$^+$ TRECs divided by prevaccine CD4$^+$ TRECs/postvaccine CD4$^+$ TRECs. IFN-γ response, Indicates postvaccine IFN-γ with Ag – no Ag control/prevaccine IFN-γ with Ag – no Ag control. No. diluted CD8 TREC, Indicates prevaccine − postvaccine CD8$^+$ TREC levels (identical groupings were obtained by multiplying CD8 TREC dilution amt. by CD8 TREC). No. diluted CD8 TREC also correlated strongly (Pearson's correlation) with recurrence and survival times ($r = 0.7$ and 0.87, respectively; both $p < 0.05$), as did CD8 TREC ($r = 0.8$ and 0.78, respectively; both $p < 0.05$). CD8 TREC dilution amt. and IFN-γ response correlated well with survival only ($r = 0.7$ and 0.6, respectively; both $p < 0.05$). All other correlations were relatively weak ($r < 0.39$; $p > 0.05$).

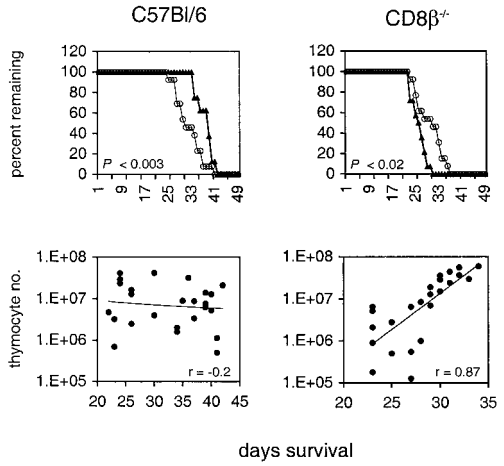

FIGURE 8. Decreased CD8$^+$ T cell production elicits the pattern of age-dependent outcome observed in GBM patients. *Top row*, Intracranial tumor cell implantation into middle-aged (10–15 mo; ○) and aged (18–24 mo; ▲) wild-type C57BL/6 or CD8$\beta^{-/-}$ mice revealed significantly decreased survival in aged CD8$\beta^{-/-}$ relative to both middle-aged CD8$\beta^{-/-}$ mice ($p < 0.02$) and aged wild-type C57BL/6 mice ($p < 0.000001$; Mantel-Cox log rank), with identical survival of middle-aged wild-type and CD8$\beta^{-/-}$ mice ($p = 0.3$; Mantel-Cox log rank). *Bottom row*, Thymocyte numbers were determined in combined middle-aged and aged (●) wild-type C57BL/6 or CD8$\beta^{-/-}$ mice upon acquisition of terminal glioma symptoms, and correlated (Pearson's correlations) with host survival in days. Strong correlation similar to that observed between CD8$^+$ RTEs and GBM patient clinical outcome ($r \geq 0.86$; $p < 0.001$ in both cases) was observed exclusively in CD8$\beta^{-/-}$ mice.

tumor hosts (28). Thymocyte numbers, which are directly proportional to peripheral CD8$^+$ RTE/TREC levels in mice (10), also failed to correlate with host survival after GL26 implantation in wild-type hosts (Fig. 8). Such correlation is an expected consequence of CD8$^+$ RTE levels influencing tumor host survival. In contrast, significantly shorter survival was observed in aged CD8$\beta^{-/-}$ relative to both young CD8$\beta^{-/-}$ as well as aged wild-type GL26 hosts (Fig. 8). CD8$\beta^{-/-}$ mice also exhibited a strong correlation between thymocyte numbers and survival after GL26 implantation (Fig. 8). This suggests that diminished thymic CD8$^+$ T cell production accounts for increased mortality in aged tumor hosts.

Discussion

We show that a T cell parameter predicts clinical outcome in an advanced, nonimmunogenic human tumor better than the strongest established prognostic factor (age). Specifically, CD8$^+$ TREC levels accounted for age-dependent GBM recurrence and survival rates. CD8$^+$ TREC dilution following vaccination was directly proportional to the magnitude of antiglioma lysate responses, suggesting that CD8$^+$ RTEs might account for a substantial proportion of tumor-reactive T cells, a notion supported by the specific enrichment of CD8$^+$ RTEs within an experimental rat glioma (29). Further supporting this notion, CD8$^+$ RTEs comprised the majority of precursor T cells capable of recognizing any of at least four distinct common tumor Ags expressed by gliomas, and dominantly contributed to responses directed toward these Ags. Moreover, the epitopes recognized by CD8$^+$ RTEs can be functionally present on distinct tumors such as melanoma and carcinoma (30–33). This reveals the possibility that CD8$^+$ RTEs may be broadly relevant to cancer immunity. In this context, changes in CD8$^+$ RTE numbers might help explain differences in the outcome of human cancer based on variables that may influence CD8$^+$ RTE production by the thymus, including age, gender, treatment modalities, and endogenous or exogenous hormones.

Numbers of CD8$^+$ RTEs proliferating to tumor Ags in vivo, as estimated by tracking CD8$^+$ TREC dilution, significantly predicted clinical outcome in vaccinated GBM patients, whereas other immunological parameters (enhanced IFN-γ production) did not. In this context, it is interesting that IFN-γ response magnitudes corresponded well with CD8$^+$ TREC dilution factors, but still failed to predict GBM outcome. This implies that IFN-γ response magnitude may accurately reflect proportions, but not numbers of responding CD8$^+$ RTEs, and that the latter is most clinically relevant. In addition, clinically effective antitumor activity by these cells is most likely mediated by a cellular property that is not directly related to IFN-γ production. By default, this implicates conventional granzyme- and/or death receptor-dependent pathways of CTL killing. Because antitumor response enhancement was observed after lysate-pulsed DC vaccination, this also raises the possibility that clinical efficacy of such vaccines is most likely when CD8$^+$ RTE numbers are high.

CD8$\beta^{-/-}$, but not wild-type mice implanted intracranially with GL26 tumors exhibited trends reminiscent of human GBM patients: significantly increased mortality in aged hosts and robust correlation between thymus cellular production and tumor outcome. CD8$\beta^{-/-}$ mice display a specific reduction in CD8$^+$ T cell production by the thymus with retention of peripheral CD8$^+$ T cell function similar to that in wild-type mice (25, 26), suggesting that preferential reduction of thymic CD8$^+$ T cell production dramatically alters age-specific patterns of glioma survival. Moreover, these findings indicate that the age-dependent decrease in glioma

host survival and its strong correlation with thymus cellular product levels are influenced in a concerted manner by $CD8^+$ T cell production and/or function. Taken together, this strongly suggests that an endogenous host immune parameter, namely thymus production of $CD8^+$ T cells, is sufficient to account for age-dependent glioma mortality in mice and in human GBM patients. In wild-type mice, however, the influence of this process is masked, suggesting that at least aged patients and wild-type mice differ with respect to processes critically limiting beneficial antitumor immunity. Because Ag availability and professional APC function appear to be the primary limitations to beneficial antitumor immunity in rodent tumor models (23, 24), this may help explain why APC-based cancer vaccines are at best of limited efficacy in many cancer patients (11).

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