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Split Immunity: Immune Inhibition of Rat Gliomas by Subcutaneous Exposure to Unmodified Live Tumor Cells

Ilan Volovitz,*,† Yotvat Marmor,* Meir Azulay,* Arthur Machlenkin,* Ofir Goldberger,* Felix Mor,* Shimon Slavin,‡ Zvi Ram,† Irun R. Cohen,* and Lea Eisenbach*

Gliomas that grow uninhibited in the brain almost never metastasize outside the CNS. The rare occurrences of extracranial metastasis are usually associated with a suppressed immune system. This observation raises the possibility that some gliomas might not grow outside the CNS due to an inherent immune response, We report in this study that the highly malignant F98 Fischer rat undifferentiated glioma, which grows aggressively in the brain, spontaneously regresses when injected live s.c. We found that this regression is immune-mediated and that it markedly enhances the survival or cures rats challenged with the same tumor intracranially either before or after the s.c. live-cell treatment. Adoptive transfer experiments showed the effect was immune-mediated and that the CD8 T cell fraction, which exhibited direct tumor cytotoxicity, was more effective than the CD4 T cell fraction in mediating resistance to intracranial challenge of naive rats. Brain tumors from treated rats exhibited enhanced $CD3^+CD8^+CD4^-$ and $CD3^+CD4^+CD8^-$ T cell infiltration and IFN-γ secretion. The results in the F98 glioma were corroborated in the Lewis rat CNS-1 astrocytoma. In both tumor models, s.c. treatment with live cells was significantly better than immunization with irradiated cells. We propose in this study a location-based immunotherapeutic phenomenon we term "split immunity": a tumor that thrives in an immune-privileged site may be inhibited by injecting live, unmodified tumor cells into a site that is not privileged, generating protective immunity that spreads back to the privileged site. Split immunity could explain several long-standing paradoxes regarding the lack of overt extracranial metastasis in patients with primary brain tumors. ***The Journal of Immunology*****, 2011, 187: 5452–5462.**

The CNS is known to be an immune-privileged site; tumor allografts and xenografts, rejected at other sites in the body, are accepted in the CNS in immunocompetent hosts (1–3), and tumors in the brain are either ignored by the immune system or their growth is not controlled (1, 4).

A peculiar phenomenon relating to brain tumors, especially to the highly malignant glioblastoma multiforme (GBM), is the rarity of detectable systemic metastases. Approximately 10 cases of spontaneous metastasis of unresected primary GBM were reported in the medical literature in 50 y (5, 6); other cases of metastasis amounting to <0.5% of patients (7) followed resection of the primary CNS tumor and occurred mostly in immune-compromised hosts (8, 9).

In this paper, we studied the F98 rat brain tumor that is an anaplastic glioma, syngeneic to the Fischer (F344) rat, and exhibits an aggressive infiltrative behavior similar to that of human GBM. The tumor was induced chemically and therefore does not carry any artificially introduced Ags. This tumor was shown to be refractory to most therapeutic modalities and is uniformly lethal following an inoculum of as few as 1–10 tumor cells (10, 11).

In the current study, nevertheless, we observed that the F98 tumor injected s.c. was rejected in 97% of the rats. This observation suggested that the spontaneous peripheral rejection of an otherwise lethal brain tumor might be exploited against intracranial tumors. We report in this study that the antitumor immune response generated by the injection of live F98 tumor cells outside the CNS is not limited to the periphery only, but markedly inhibits the growth of tumors inside the CNS; complete tumor eradication was noted in about half of the rats.

Vaccination is classically defined as the exposure of an individual to an attenuated or inactivated pathogen (e.g., irradiated, fixed, genetically manipulated, etc.) that protects the host against a virulent pathogen (12). In this article, we define a location-based immune phenomenon we term "split immunity," a nonattenuated live tumor that when situated in one location is lethal to the host, but when situated in another location, the tumor generates immunity that can spread back to the original site (e.g., the brain), thereby protecting the host.

Materials and Methods

Animals

Female Fischer (F344) or Lewis rats were obtained from Harlan Israel. The rats were maintained in a specific pathogen-free environment in the Weizmann Institute of Science animal facilities. The rats were used at age 6–10 wk unless specified otherwise. The animal experiments were supervised by institutional animal welfare authorities.

Cells and culture

The F98 undifferentiated glioma is a chemically induced glioma syngeneic to the Fischer rat (13). The cells were obtained from the American Type Culture Collection and maintained as suggested by the supplier.

The CNS-1 astrocytoma is a chemically induced glioma syngeneic to the Lewis rat (14). The CNS-1 line was provided by its originator, William F.

*Department of Immunology, Weizmann Institute of Science, Rehovot 76100, Israel; †Cancer Immunotherapy Laboratory, Department of Neurosurgery, Tel Aviv Sourasky Medical Center, Tel Aviv 64239, Israel; and ‡International Center for Cell Therapy and Cancer Immunotherapy, Weizmann Center, Tel-Aviv 64239, Israel

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Address correspondence and reprint requests to Dr. Ilan Volovitz, Cancer Immunotherapy Laboratory, Department of Neurosurgery, Tel Aviv Sourasky Medical Center, 6 Weizmann Street, Tel Aviv 64239, Israel. E-mail address: volovitz@yahoo.com

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Abbreviations used in this article: GBM, glioblastoma multiforme; IC, intracranially; IT, intratumoral; LU30, number of cells that could generate 30% lysis of the 5000 target cells; MHC-II, MHC class II; TBI, total body irradiation.

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Hickey (Dartmouth Medical Center, Lebanon, NH). The line was grown, as suggested by Prof. Hickey, in RPMI medium supplemented with 4 mM L-glutamine, combined antibiotics, and 10% FCS. Care was taken to use similar cells of a low passage. All cultures were mycoplasma negative.

F98-luc is a subclone of F98 cells we transfected with the firefly luciferase gene (pcDNA-III; Invitrogen, Carlsbad, CA) using the Lipofectamine reagent (Invitrogen). The cells were grown in selection medium, the F98 medium supplemented with 2 mg/ml G-418 antibiotics (Sigma-Aldrich, St. Louis, MO). The F98-luc subclone was selected for its high photon efficiency and ability to express luciferase without a selection agent both in vitro and in vivo for ≥3 mo.

Optical imaging of bioluminescent cells

In vivo bioluminescence imaging was performed using the In Vivo Imaging System IVIS-100 (Xenogen, Almeda, CA). After i.p. injection of the reporter probe D-luciferin, 40 mg/kg body weight (Hopkinton, MA), rats were anesthetized with 80–125 μl 1:2 ratio of xylazine 2% (Vitamed, Benyamina, Israel) and ketamine 100 mg/ml (Fort Dodge, Fort Dodge, IA). The rats were shaved and placed in a supine position in the IVIS (Xenogen) and repeatedly imaged for 2-min intervals until luminescence declined.

Bioluminescence was quantified in units of photon flux ($P \times s^{-1}$) per similar area measured using the Living Image 2.5 software overlay (Xenogen) and IGOR image analysis software (V 4.09A; WaveMetrics, Lake Oswego, OR). Only metabolically active cells contribute to the bioluminescence obtained by luciferase-transfected cells (15, 16). Bioluminescence of a different luciferase-transfected F98 clone injected intracranially (IC) was shown to be highly correlative to the viable IC tumor size measured by micro–positron-emission tomography (16) and serial histological sections (15).

Separation of lymphocytes on MACS magnetic beads

The MACS technique (Miltenyi Biotec, Bergish Gladbach, Germany) was used to separate $CD4^+$ or $CD8^+$ cell populations for various assays. $CD4^+$- or $CD8^+$-depleted or purified populations were typically >99% free from unwarranted cells as ascertained by FACS.

In vitro cytotoxicity assays

In vitro cytotoxicity assays (5 and 16 h) were conducted as described before (17). Target cells were labeled overnight with [^{35}S]methionine 5 mCi/ml (PerkinElmer, Waltham, MA). The supernatants were transferred to TopSeal-A, 96-well microplates (Packard Instruments, Downers Grove, IL), added to with 150 μl microscint 40 (Packard Instruments) scintillation fluid, and counted in a TopCount microplate scintillation and luminescence β-counter (Packard Instruments). An LU30 is defined as the number of cells that could generate 30% lysis of the 5000 target cells (18). The number is calculated from the regression line of the four different E:T ratios (5:1–40:1). The graph depicts the number of LU30/10^7 cells.

Coculture proliferation assays

Inactivated tumor cells (irradiated 5000 cGy and treated with mitomycin C at 80 μg/ml/10^7 cells) were used as stimulators in coculture proliferation assays. The cells were dispensed in stimulation medium (DMEM, 1% normal rat serum, 2 mM glutamine, combined antibiotics, 1 mM sodium pyruvate, 5×10^{-5} M 2-ME, and 1% nonessential amino acids) in quadruplicates in 96-well plates. Effector cells were either splenocytes (2.5×10^5 cells/well) or MACS-purified CD4 T cells (2.5×10^4 cells/well). Where indicated, 5000 cGy-irradiated naive rat thymocytes (2.5×10^5) served as APC.

After 2 d in culture, the wells were pulsed overnight with medium containing methyl [^{3}H]thymidine (5 mCi/ml; GE Healthcare). Cultures were harvested into a 96 GF/C Unifilter (PerkinElmer) and Microscint-20 scintillation fluid (PerkinElmer) added. The plates were read using a Topcount microplate scintillation and luminescence counter (Packard Instruments).

IFN-γ secretion assays

Two days after coculture of immunocytes with tumor cells, as outlined above, assay supernatant from triplicate or quadruplicate microcultures was assayed by ELISA using standard manufacturer protocols (BD-OptEIA, Franklin Lakes, NJ).

Disruption of IC and s.c. tumor to single cells

Brain and s.c. tumors were surgically removed from rats, cut into small pieces, and resuspended in $HBSS^{2+}$ at 200 mg dry tissue/ml. Trypsin 2.5% (Life Technologies-Invitrogen, Carlsbad, CA) was added at a 1:1 ratio to the tumor pieces and incubated at 37°C for 30 min. Cells were then triturated using a Pasteur pipette, and the digestion was stopped by adding FCS at a 1:1 ratio. The cell slurry was then passed through a 40-μm cell strainer (BD Biosciences, Franklin Lakes, NJ) to remove coarse debris.

FACS analysis and Abs

Brain tumor cells were stained and analyzed using a FACS intracellular cytokine secretion protocol similar to Lamoreaux et al. (19). Briefly, excised and disrupted brain tumor cells were incubated for 5 h with PMA/ionomycin (Sigma-Aldrich) and then stained with ViViD, a violet LIVE/DEAD viability dye (Molecular Probes-Invitrogen). Cells were fixed, permeabilized, and washed using the Fix/Perm kit (BD Biosciences, Franklin Lakes, NJ) and then stained with the following anti rat Abs CD4-PE (W3/25), CD8a-PerCP (OX-8), CD3-Ax488 (1F4), IFN-γ–Ax647 (DB1) (all from BioLegend, CA). Single color indirect staining was done using anti-rat MHC class II (MHC-II)–RT1D monomorphic: (OX-17; Serotec, Oxford, U.K.); anti-rat TCR (R73; BioLegend) and a secondary donkey anti-mouse Cy5 Ab (Jackson Immunoresearch Laboratories, West Grove, PA). The cells were monitored by LSRII (BD Biosciences) and analyzed using FlowJo flow cytometry analysis software (Tree Star).

Tumor cell purification using Percoll density gradient separation

Density of F98 cells was determined to be 1.06 to 1.07 g/ml by running the tissue-cultured F98 cells in a stepwise Percoll gradient made by overlaying different ratios of isotonic Percoll (9:1 Percoll [Pharmacia-Sigma] to PBS × 10) mixed with $PBS^{-/-}$.

Trypsinized tumor cells (from IC or s.c. tumors) were run on a continuous Percoll gradient made by mixing 60% isotonic-Percoll mixed with 40% $PBS^{-/-}$ run at 26,000 × *g* for 40 min on a fixed-angle rotor ultracentrifuge (Beckman Coulter, Brea, CA). The single cells were carefully loaded onto one premade gradient, whereas another identical gradient was loaded with density beads (GE Healthcare, Buckinghamshire, U.K.). The two gradients were then centrifuged at 2600 rpm, for 30 min, 20°C, brake off. Cells found at 1.06 to 1.07 g/ml density were collected and counted for viable cells. The percent of the live cells found at the 1.06 to 1.07 density was calculated as part of the total live cells found on the entire gradient. Percoll-purified live cells, 1.8×10^5 (30% of total gradient cells), and unsorted live trypsinized cells (6×10^5) consisting of similar amounts of cells found at the 1.06 to 1.07 g/ml density were both injected s.c. to two groups of rats to determine growth dynamics.

Inoculation with tumor cells s.c.

Rats were injected s.c. in the lower flank with 100 μl PBS containing live or irradiated F98 tumor cells. The average of two perpendicular diameters of the tumor was used to calculate the circular tumor area. Moribund rats exhibiting signs of severe illness (immobility, difficulty to breathe, etc.) or rats with tumors >3.5 cm in mean diameter were euthanized by CO_2. Time to s.c. tumor rejection was calculated as the number of days between the initial recording of an observable or palpable tumor to the day the tumor could no longer be detected.

Inoculation with tumor cells IC

Rats were anesthetized with xylazine and ketamine, and their heads were shaved and the skin opened to reveal the bregma. A hole was drilled with a microdrill at a point 2 mm anterior to the bregma and 4 mm to the right of the midline. The head was fixed in a stereotactic apparatus, and tumor cells (5×10^3, unless specified otherwise), suspended in 2 μl PBS, were slowly infused (2 μl/min) at a depth of 4 mm into the skull with a fine Hamilton syringe. Following the injection and needle removal, the opened skull was swabbed with 70% alcohol, and the scalp was closed with 9-mm Autoclips wound clips (Clay Adams, Parsippany, NJ). All surgical procedures were performed inside a sterile hood.

Adoptive transfer of splenocytes and tumor challenge

Spleens were harvested from IC-rejector rats. RBCs were lysed using RBC lysis buffer (Sigma-Aldrich), and the remaining splenocytes were restimulated with inactivated tumor cells at a tumor/splenocyte ratio of 1:50 for 3 d in lymphocyte medium.

The rats were challenged either s.c. or IC with tumor cells; the next day (s.c.) or the previous day (IC), the rats were irradiated with total body irradiation (TBI) 500 cGy, and, 2 d following irradiation, groups were injected i.v. with 10^7 splenocytes unsorted, MACS depleted of either CD4 or CD8 cells, or unsorted naive rat splenocytes. Naive rat splenocytes were

not restimulated prior to injection, as repeatedly, hardly any viable naive splenocytes remained following the 3-d restimulation.

Statistical analysis

A Student's unpaired two-tailed *t* test (for two groups) or ANOVA and Tukey–Kramer tests (for three or more groups) were used for statistical analysis of quantitative normally distributed data in cytotoxicity, proliferation, or cytokine secretion assays. Results in these assays are expressed as mean ± SEM, unless specified otherwise. Nonparametric survival results were analyzed using the log-rank test. Nonnormal distributed flow cytometric data were analyzed using Mann–Whitney *U* test.

Results

Glioma cells grow progressively in the brain but are rejected spontaneously in an s.c. site

F98 cells injected IC, in doses of 5×10^2 to 5×10^5 cells, uniformly killed untreated rats (n = ~200) within 3–5 wk; the survival time was dose dependent. To follow in vivo the growth kinetics and cell viability of F98 in a noninvasive manner, the parental F98 line was stably transfected with the firefly luciferase gene and subcloned. The bioluminescence of the F98-luc tumor cells could be visualized and quantified either s.c. or IC (Fig. 1*A*) using the IVIS imaging system (Xenogen). The F98-Luc tumor grew both IC and s.c. with growth kinetics similar to the parental F98 (not shown).

F98-luc cells injected IC exhibited progressive growth in the brains of untreated rats (Fig. 1*B*; n = 6); the rats injected with F98-luc IC died after 23 ± 1 d (mean ± SD). Intracranial bioluminescence of another luciferase-transfected F98 clone was shown to be highly correlative to the number of viable tumor cells IC (15).

Although the F98 tumors grew uninhibited when injected IC, the same cells injected s.c. grew initially, but then spontaneously regressed in 191 out of 197 (97%) of naive rats injected once or twice s.c. (cumulative of all experiments quoted in this manuscript). A dose of 2×10^5 F98 cells grew to an area of 25–30 mm^2, but by 1 to 2 wk started to spontaneously regress. By 3 to 4 wk, the tumors were not visible or palpable (Fig. 1*C*). Lower (10^5) or higher (2×10^6) doses of live F98 cells produced tumors of different peak sizes but grew and regressed with similar kinetics (not shown).

To check whether residual tumors remained s.c. following their regression, F98-luc tumor cells were injected in 100 μl (2×10^5 cells) or 1 ml (2×10^6 cells). Tumor volumes as small as 0.2 mm^3 in vivo are detectable by IVIS (Xenogen) (15). Fig. 1*D* shows that s.c. tumors detected by IVIS (Xenogen) on days 10–16 could no longer be detected by day 23. The loss of tumor luminescence (viable tumor cells) preceded the physical disappearance of the tumors. A high tumor dose (2×10^6) and inoculum volume (1 ml) resulted in a larger initial tumor; but in this case, too, tumor luminescence was not detectable by day 23. Following their spontaneous regression s.c., the parental F98 tumors never relapsed ($n > 180$). These findings are compatible with complete spontaneous eradication s.c. of the live tumor cells.

S.c. rejection of glioma cells leads to enhanced survival of rats subsequently challenged intracranially

Because F98 regressed from s.c. sites, we tested whether the s.c. regression could affect the growth of the tumor injected into the brain. Rats were initially injected with 2×10^5 live F98 cells s.c.;

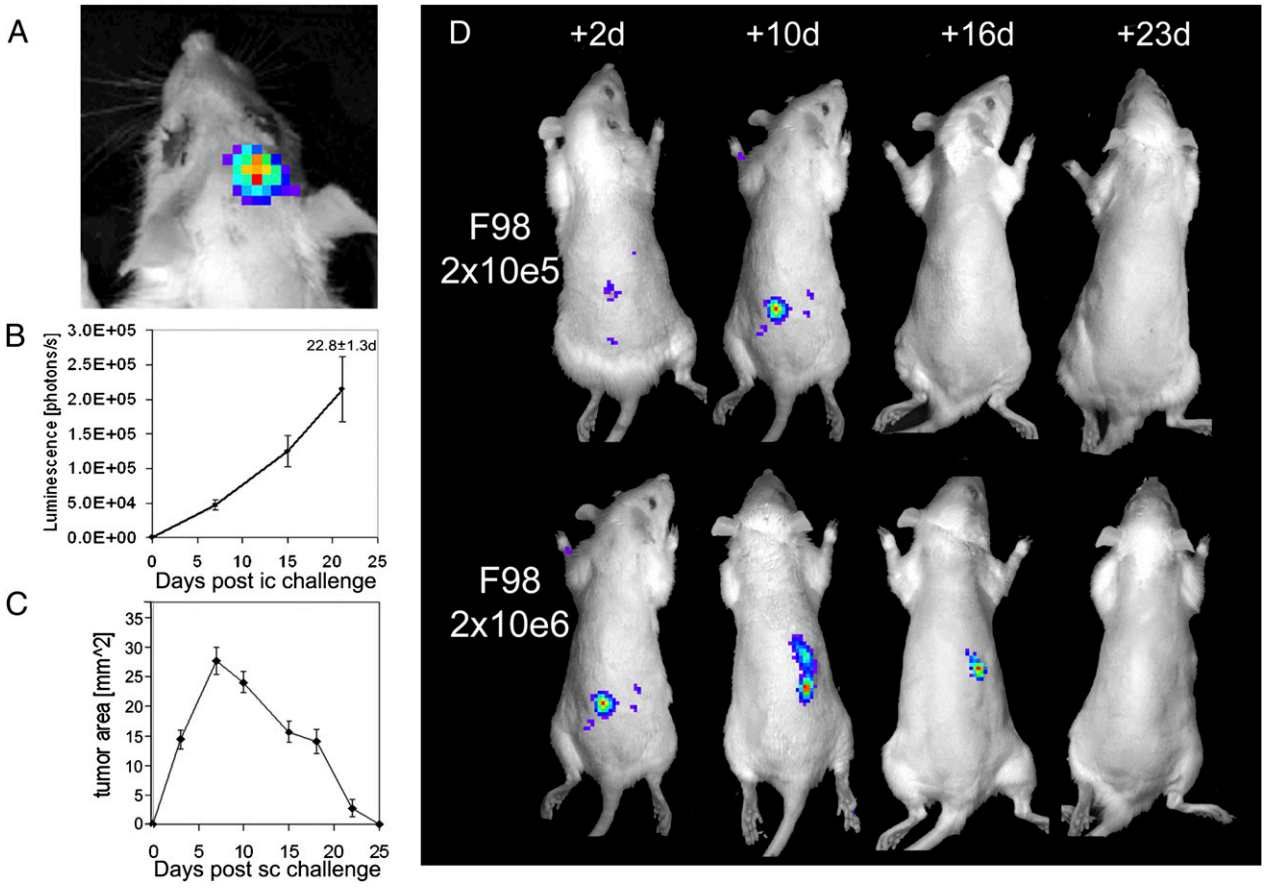

FIGURE 1. F98 glioma cells grow uninhibited IC and rejected s.c. *A*, IVIS imaging (Xenogen) of an F98-luc IC-injected rat 21 d postimplantation. *B*, Mean luminescence of F98-luc tumor cells implanted IC (5×10^3) to rats (n = 6). Rats survived a mean of 22.8 ± 1.3 d. *C*, Mean tumor area with SEM of 2×10^5 F98 tumor cells injected s.c. in the flanks of rats (n = 9). *D*, IVIS imaging (Xenogen) of 2×10^5 or 2×10^6 live F98-luc tumor cells injected s.c. All experiments were repeated two to eight times with similar results.

1 wk to 10 d after all the rats had rejected their s.c. tumors (~3 wk postinjection), the rats were challenged IC with 5×10^3 live F98 tumor cells.

Fig. 2*A* combines three similar protection experiments followed for ≥9 mo; each experiment reached statistical significance. The combined treated group ($n = 20$) survived significantly longer ($p < 0.0001$) than the combined untreated group ($n = 23$). Half ($50 \pm 7\%$) of the treated rats in the combined three experiments survived long-term (≥9 mo) following the IC tumor inoculation and could be considered tumor free.

Preimplanted F98 IC tumor can be treated by live F98 inoculation s.c.

To test whether tumor regression in the periphery might also affect tumors previously inoculated into the brain, F98 cells (5×10^3) were injected IC 3 d (Fig. 2*B*) or 6 d (Fig. 2*C*) before the rats were injected s.c. with 2×10^5 live F98 cells. The s.c. tumors grew and were spontaneously rejected with similar kinetics to rats without brain tumors (not shown). Rats treated 3 d after the IC challenge showed enhanced survival with a median of 186% compared with the control untreated group ($p < 0.005$). Rats treated 6 d after the IC challenge also showed enhanced survival with a median of 138% compared with control ($p < 0.005$). The results indicate that live-cell inoculation s.c. can also enhance the survival of rats harboring established brain tumors.

S.c. rejection of live glioma cells generates long-term tumor resistance

The injection of live glioma cells in a peripheral site affects the rejection of the same cells implanted IC, but does this rejection generate memory?

Fig. 3*A* shows that rats ($n = 7$) rechallenged s.c. as soon as they had rejected a similar dose of s.c. tumor cells (2×10^5) exhibit a shorter tumor rejection time (19 ± 4 d versus 4 ± 2 d; $p < 0.00005$). The peak tumor area (31 ± 13 mm^2) recorded 10 d after the initial challenge was reduced to hardly palpable tumors (8 ± 4 mm^2) 7 d after the second tumor challenge ($p < 0.005$).

To learn whether this memory is sustained, rats that had survived an IC challenge following an s.c. F98 live-cell inoculation (hence termed IC-rejectors) were rechallenged s.c. ~300 d after their IC challenge. Fig. 3*B* shows that all of the rats (six out of six) in the untreated control group accepted the s.c. tumors and spontaneously rejected them with typical kinetics as shown also in Fig. 1*C*. In contrast, two out of four of the IC-rejectors did not develop detectable s.c. tumors. The mean s.c. tumor size in the IC-rejectors when the tumor did grow was smaller compared with the untreated group throughout the experiment ($p < 0.005$), and the time to tumor rejection was shorter (13 versus 19 d). These results are compatible with the acquisition of long-term tumor-rejection memory.

Fig. 3*A* and 3*B* show that memory is maintained when the tumor rechallenge was done in a peripheral site, but is rejection-memory maintained when the challenge site is IC? To answer this, rats (group $n = 6$ to 7) were injected s.c. with live F98 cells and challenged IC 18, 32, or 67 d later with F98 cells. Fig. 3*C* shows that the survival of all three treated groups was enhanced in comparison with the untreated control group. ($p < 0.015$ or less). Small nonsignificant differences were observed among the treated groups.

The kinetics of s.c. and IC tumor rejection followed using luciferase-transfected F98 cells

We used F98-luc to follow in real time the in vivo growth and rejection kinetics of tumor cells injected either s.c. or IC. IC-rejectors (−250 d, $n = 3$) and untreated rats ($n = 2$) were s.c. challenged with 1×10^6 F98-luc cells. Fig. 3*D* shows parallel measurements of tumor area and tumor bioluminescence. Whereas the untreated group manifested tumors that grew both in size and luminescence that peaked at 7 d, the IC-rejector group exhibited its maximal tumor size and luminescence 1 d after challenge. Complete loss of tumor luminescence occurred by 4 d in the IC-rejectors and only 7 d later in the untreated rats. In both treated and untreated groups, tumor luminescence could no longer be detected 3 to 4 d after the tumors started diminishing in size. In both groups, only 10–12 d after the luminescence was undetected, the tumors were physically cleared from the rats' flanks. The rapid tumor clearing observed in the IC-rejectors is compatible with a memory response.

To follow growth kinetics of the F98 tumors IC, rats were injected with 2×10^5 ($n = 4$) or 2×10^6 ($n = 3$) F98-luc cells or were left untreated ($n = 6$). Following s.c. tumor regression, the rats were challenged IC with 5×10^3 F98-luc. Fig. 3*E* shows that whereas all of the untreated rats developed progressively growing tumors in the brain, some of the treated animals exhibited IC

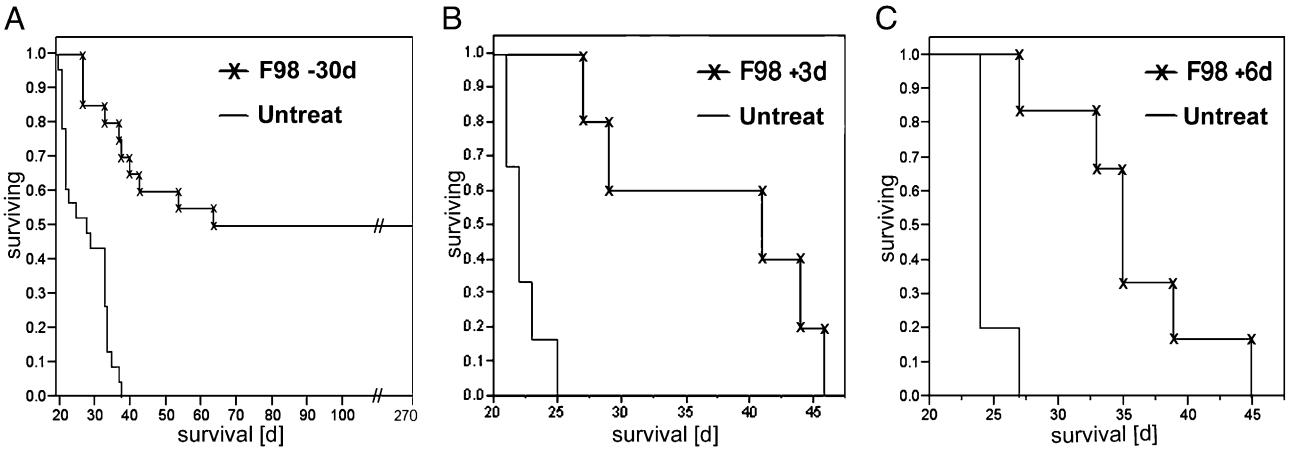

FIGURE 2. S.c. live tumor rejection enhances survival of rats challenged IC. *A*, Kaplan-Meier survival curves of data accumulated from three statistically significant experiments followed for at least 270 d. Rats injected s.c. with 2×10^5 F98 live tumor cells ($n = 20$) were challenged IC with 5×10^3 F98 tumor cells 7–10 d after the s.c. tumors spontaneously regressed or were left untreated ($n = 23$). The survival of combined treated group was prolonged in comparison with the combined control untreated group ($p < 0.0001$). Representative results from eight repeats. Survival of rats initially challenged IC with live F98 tumor cells (5×10^3) and treated with live F98 tumor cell (2×10^5) inoculation s.c. 3 (*B*) or 6 (*C*) d later. The survival of the treated group was prolonged in comparison with the control untreated group. *B* and *C* are representative results of five repeats. $p < 0.005$ for both *B* and *C*.

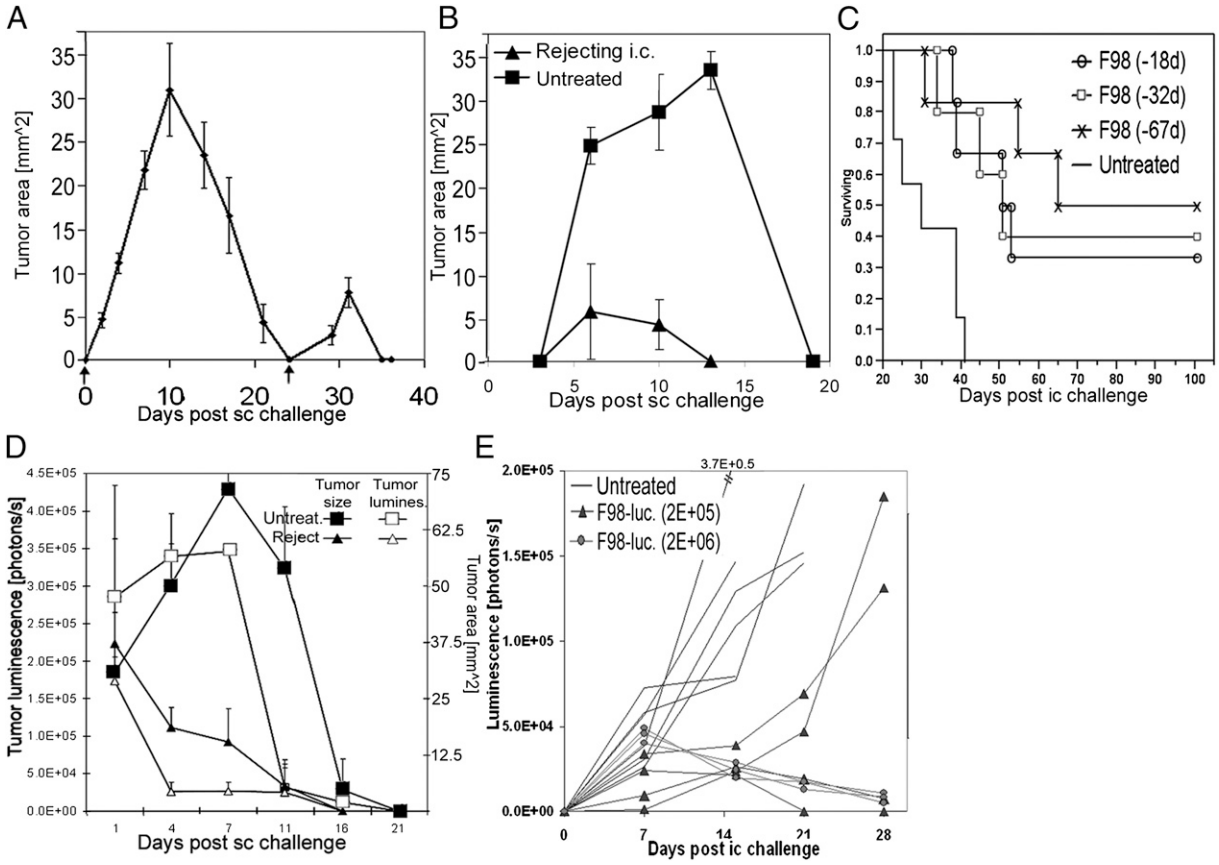

FIGURE 3. S.c. F98 rejection generates tumor-rejection memory. *A*, Rats rejecting a live s.c. tumor (2×10^5) challenge were rechallenged with live (2×10^5) tumor cell s.c. in the opposite flank. Time to tumor rejection and peak tumor area were reduced in the second versus the first challenge ($p < 0.005$ or less). Repeated three times with similar results. *B*, A group of IC-rejectors 300 d post-IC challenge and an untreated group (naive) were challenged s.c. with live F98 tumor cells (2×10^5). In the IC-rejector group, only two out of four rats developed tumors versus all six rats developing tumors in the naive group; these tumors were smaller than those recorded in the untreated group ($p < 0.005$ or less). Repeated twice with similar results. *C*, Groups of six to seven rats were either untreated or injected s.c. with 2×10^5 live F98 tumor cells 18, 32, or 67 d prior to IC challenge with 5×10^3 F98 tumor cells. The survival of treated groups was prolonged in comparison with the untreated control group ($p < 0.015$ or less). *D*, A group of three IC-rejectors 250 d following IC challenge (Reject) and two control untreated rats were challenged s.c. with live F98-luc tumor cells (1×10^6). Tumor area and luminescence were measured. Mean tumor area is different between the groups at days 7 and 11 ($p < 0.02$ or less, *t* test); the mean tumor luminescence is different at day 7 ($p < 0.0001$). *E*, Luminescence of the head area of individual rats challenged IC with 5×10^3 F98-luc tumor cells. Rats were either untreated (black line, $n = 6$) or injected with 2×10^5 (grey triangle, $n = 4$) or 2×10^6 (grey circle, $n = 3$) live F98-luc tumor cells s.c. Treated groups exhibit significantly less tumor luminescence ($p < 0.025$ or less) on days 7–21 for the group treated with 2×10^5 cells ($p < 0.025$) and on days 15–21 for group treated with 2×10^6 cells ($p < 0.025$). Repeated twice with similar results.

tumor growth and then regression. In the group injected with 2×10^5 live cells s.c., two out of four rats developed tumors that grew slower than those of the untreated rats. The other two out of four rats in this group reached maximal tumor size by day 7 and then exhibited gradual loss of tumor luminescence to no luminescence by days 21–28. The rats treated with the higher dose (2×10^6) of live cells s.c. had mean IC tumor luminescence by 7 d, similar to that of the untreated rats. The luminescence of their tumors gradually decreased to no luminescence by 28 d. Significant differences in tumor luminescence to untreated rats were recorded for the 2×10^5 treated rats on days 7–21 and for the 2×10^6 treated rats on days 15–21 ($p < 0.025$ or less). The results indicate that although all live cell-treated animals supported initial IC growth of tumors, tumor growth IC was either delayed or the tumors were rejected.

This dose-dependent response observed with F98-luc was noted also using F98 parental cells; whereas treatment with 2×10^5 F98 cells usually produced 50% long-term survivors, treatment with 10^5 cells significantly enhanced survival, but produced considerably fewer (range 0–17%) long-term survivors (not shown).

Splenocytes obtained from IC-rejectors mediate tumor cell rejection

The observation of long term-tumor resistance suggests an immune mechanism. To test whether tumor rejection is immune mediated, rats were injected s.c. with live F98 tumor cells and 1 d later underwent lymphocyte ablation by TBI 500 cGy to facilitate adoptive transfer (20); 2 d later, the rats were injected i.v. with 10^7 splenocytes from untreated rats or IC-rejectors.

Fig. 4*A* shows that the group receiving the IC-rejector splenocytes had a significantly smaller mean s.c. tumor area than did the group that had received naive rat splenocytes; the rejecting rat splenocytes restored in three out of four rats the capacity to reject the s.c. tumor. In the naive splenocyte-injected group, only one out of four rats ultimately rejected its s.c. tumor.

These results indicate that the mild lymphoablation by TBI compromised the rats' ability to spontaneously reject a s.c. tumor. The replenishment of the irradiated recipients with IC-rejecting rat splenocytes, but not with naive splenocytes, restored the rats' ability to reject the s.c. tumor. Similar results were observed using F98-luc cells (not shown).

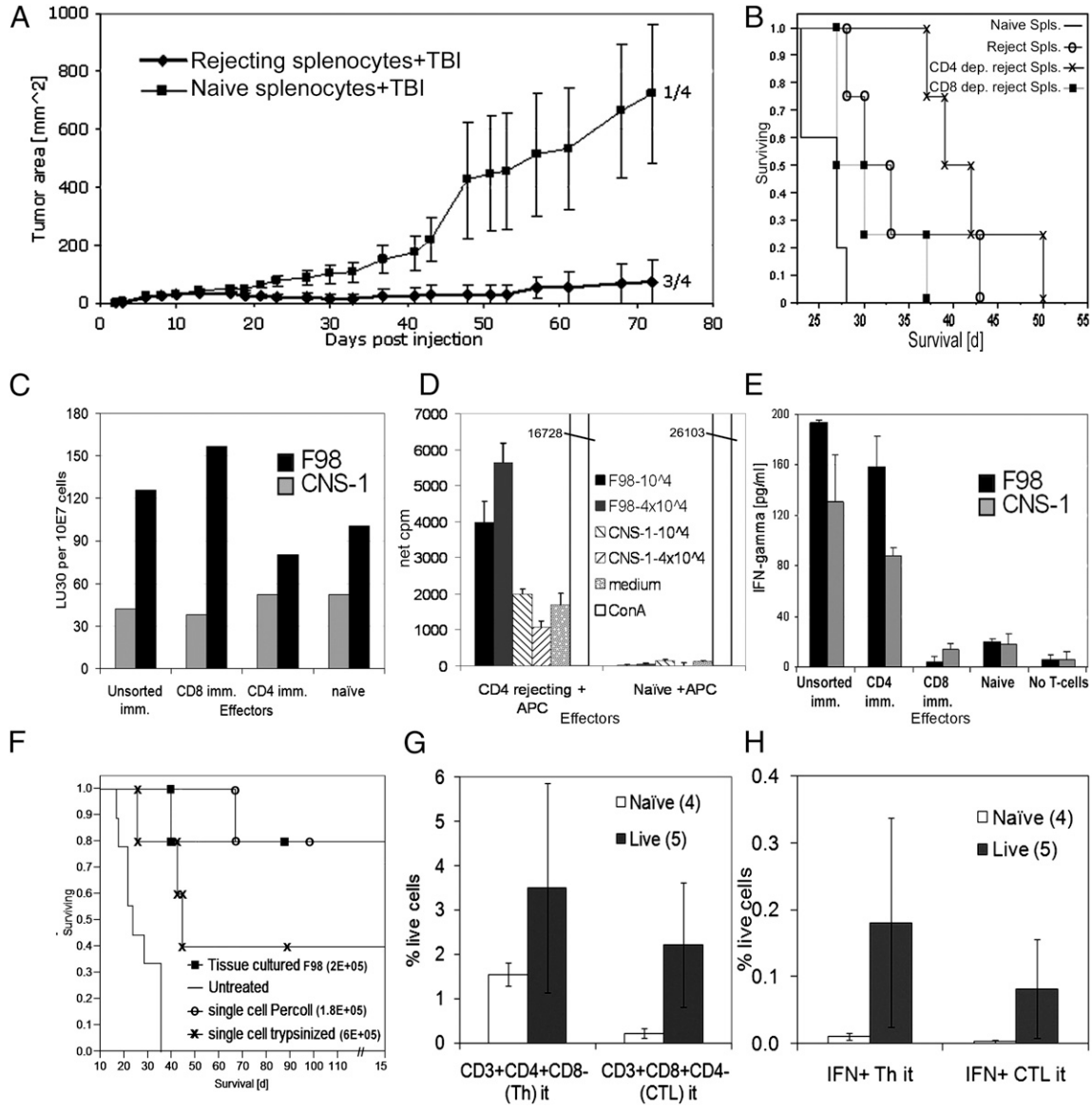

FIGURE 4. T cells from s.c. rejectors mediate tumor cell rejection. *A*, Rats were injected in the flank with live F98 tumor cells (2×10^5). The next day, the rats received TBI of 500 cGy. Two days later, the groups were injected with splenocytes obtained from an IC-rejector or naive rat. Significant differences in five out of seven time points (days 19–37) ($p < 0.05$ or less). The fraction of tumor-free animals by day 72 is displayed. *B*, One day after receiving TBI 500 cGy, rats were injected IC with live F98 tumor cells (5×10^3). The next day, groups were injected with total splenocytes or CD4/CD8 MACS-depleted splenocytes taken from an IC-rejector. Survival of the rejecting-rat splenocytes and CD4-depleted rejecting-rat splenocyte-treated groups was extended compared with the control group receiving naive-rat splenocytes ($p < 0.02$, $p < 0.005$, respectively). *C*, Tumor-restimulated splenocytes from an IC-rejector rat were sorted for CD4 cells (CD4 imm.), CD8 cells (CD8 imm.), or unsorted (Unsorted imm.). These and control naive splenocytes were assayed for cytotoxicity. Depicted LU30/10^7 cells calculated from 40:1 up to 5:1 E:T ratio. *D*, Tumor-restimulated $CD4^+$ splenocytes were cocultured either with the inactivated F98 or CNS-1 cells. Net cpm depicted was calculated by subtracting the thymidine incorporation cpm of the irradiated cells from the corresponding group. The proliferation to F98 cells in the CD4 rejecting + APC group was higher than the background proliferation to medium ($p < 0.05$). *E*, Tumor-restimulated IC-rejector splenocytes (imm.) or MACS-purified CD4/CD8 groups from it and naive splenocytes group were cocultured with irradiated tumor cells for cytokine secretion assay. IFN-γ secretion to F98 in the Unsorted imm. and CD4 imm. groups was higher than naive ($p < 0.05$). Secretion of CD4 imm. to F98 was higher than to CNS-1 tumor cells ($p < 0.05$). *F*, Rats (five to nine per group) were injected s.c. with 6×10^5 viable single cells obtained from a harvested F98 brain tumor or 1.8×10^5 cells of the same preparation collected from the 1.06 to 1.07 g/ml density of a continuous Percoll density gradient (both preparations contained equal amount of cells from the 1.06 to 1.07 g/ml, a density previously determined to be the density of cultured F98 tumor cells), 2×10^5 of tissue-cultured F98 cells, or left untreated. Following s.c. rejection, all groups were challenged IC with 5×10^3 F98 cells (tissue cultured). Survival of all treated groups is significantly enhanced versus the untreated group ($p < 0.01$ or less). *G*, Percent CTLs ($CD3^+CD8^+CD4^-$) and Th ($CD3^+CD4^+CD8^-$) of total live ($ViViD^-$) cells found IT in brain tumors of live cell-treated ($n = 5$) and untreated rats ($n = 4$). Significantly more CTLs are found IT in brain tumors of treated rats than in untreated rats ($p < 0.05$). *H*, Percent IFN-γ^+ CTLs and IFN-γ^+ Th of total live cells found IT in brain tumors of treated and untreated rats. More IFN-γ^+ CTLs are found IT in brain tumors of treated rats than in untreated rats ($p = 0.05$).

CD8 T cells are cytotoxic, and CD4 T cells proliferate and secrete IFN-γ in response to the specific tumor cells

To check which of the T cell subsets obtained from IC-rejectors confers tumor protection, we adoptively transferred CD4 or CD8 MACS-depleted splenocytes into IC tumor-challenged hosts.

Groups of naive rats ($n = 4$ out of 5) were injected IC with F98 cells, and 1 d later, the rats were irradiated TBI 500 cGy; 2 d later, rats were replenished i.v. with 1×10^7 naive splenocytes, unsorted rejecting rat splenocytes, CD8-depleted rejecting-rat splenocytes ($CD8^-CD4^+$), or with CD4-depleted rejecting-rat splenocytes ($CD8^+CD4^-$).

Fig. 4*B* shows that immunity to the IC tumor challenge was transferable either by the $CD8^+CD4^-$ rejecting rat splenocytes (mean survival 164% to control) or by the unsorted rejecting rat splenocytes (mean survival 131% to control). Both groups manifested significantly prolonged survival compared with the group replenished with naive splenocytes ($p < 0.005$ and $p < 0.02$, respectively). These results indicate that the bulk of the immune effect is mediated by $CD8^+$ T cells.

Nonetheless, in all of the experiments we observed, a consistent minor effect of the $CD4^+CD8^-$ subset. A $CD4^+CD8^-$ T cell line obtained from an IC-rejector and raised on inactivated F98 tumor cells significantly enhanced the survival of IC-challenged rats versus controls (137% mean survival to control; $p < 0.05$, not shown).

Fig. 4*C* shows the LU30 (LU 30 per 10^7 cells) summarizing 5:1 to 40:1 E:T ratio of a 16-h cytotoxicity assay using the MACS-purified CD4 or CD8 fractions of an IC-rejector or total splenocytes of an IC-rejector or naive rat splenocytes. Both the unsorted splenocytes and the CD8-purified fraction of the IC-rejector lysed the F98 tumor cells more than they did an MHC-matched control, the CNS-1 astrocytoma.

Fig. 4*D* shows that CD4 cells of an IC-rejector but not naive splenocytes proliferated in response to inactivated F98 cells, more than to the control, CNS-1 astrocytoma cells. The response to F98 tumor cells required APC; without APC, no proliferation was detected in any of the cultures (<600 cpm, not shown).

Fig. 4*E* shows that total splenocytes and the purified $CD4^+$ cell fraction of IC-rejectors secreted higher amounts of IFN-γ in response to F98 compared with the response of naive splenocytes ($p < 0.05$). IFN-γ secretion of the CD4-purified IC-rejector group to F98 cells was higher than to the CNS-1 tumor cells ($p < 0.05$). Specific cytoxicity mediated by CD8 T cells and specific IFN-γ production and proliferation by CD4 T cells are known to be associated with tumor rejection (21, 22).

T cells and CD8-expressing cells were immunohistochemically shown to penetrate the s.c. and the IC tumors; macrophages were observed to penetrate the s.c. tumors only (Supplemental Fig. 1). The immune cell infiltration to the IC tumors was limited to the tumor area only; no cells were observed beyond 1 mm of the tumor or in the contralateral parallel brain area.

Vaccination s.c. with glioma cells obtained from IC-harvested tumors induces protective immunity; nontumor stromal cells moderate rejection

To address the interaction between tumor and stroma cells inside the brain tumor, we compared the protection induced following s.c. vaccination with viable glioma cells obtained from a rat brain tumor disrupted enzymatically using trypsin to single cells (containing the entire stromal cellular milieu) compared with the trypsinized single cells purified on a Percoll density gradient. Fig. 4*F* exhibits a protection experiment using equal amounts of brain-harvested tumor cells. The rats were injected either with 1.8×10^5 Percoll-purified tumor cells (found at the 1.06 to 1.07 g/ml density, the density of tissue-cultured F98 cells) or injected with 6×10^5 unsorted trypsinized single cells (including 1.8×10^5 of 1.06 to 1.07 g/ml density cells [30%]). To verify that both mixtures had similar amounts of tumor cells, s.c. tumor growth was monitored (Supplemental Fig. 3). Both cell mixtures grew and were spontaneously rejected in identical growth curves, suggesting that the s.c. tumor growth and rejection was not affected by stromal cells accompanying the tumor cells.

Following s.c. tumor rejection, both groups and a group of rats previously vaccinated with 2×10^5 tissue-cultured F98 were challenged IC with F98 (5×10^3). Both the unsorted trypsinized single-tumor cells and Percoll-purified tumor cells protected rats against an IC challenge significantly better than the untreated group ($p < 0.01$ and $p < 0.002$, respectively). In contrast, although the proportion of rats cured following vaccination with 1.8×10^5 Percoll-purified tumor cells (80%) was similar to the group of the 2×10^5 tissue-cultured F98 cells (80%), the group injected with 6×10^5 unsorted trypsinized tumor cells cured only 40% of the rats ($p = 0.15$ trypsinized to the Percoll-purified group).

Taken together, the s.c. and IC experiments suggest that stromal cells exert a moderately suppressive effect on the IC immune response generated following s.c. live-cell treatment. Although this suppressive effect did not change the growth and rejection of tumor cells injected peripherally, it reduced, to a certain extent, the proportion of rats cured from brain-residing tumors. These results might suggest that in future clinical applications of this method, the stromal constituents of the brain tumor should either be reduced or entirely removed.

These results are compatible with another set of results in which we found that a cyclooxygenase inhibitor (indomethacin) given to a group of live cell-treated rats did not change their survival compared with rats treated with live cells not given this cyclooxygenase inhibitor (data not shown). The results suggest that the effect of live-cell treatment in the F98 model is not suppressed by PGE_2, a pivotal, mostly stromal-secreted, immune-suppressive factor found in rat gliomas (23) as well as in human gliomas (24).

Live-cell vaccination increases intratumoral T cell infiltration and IFN-γ secretion

To follow the changes occurring in the intratumoral immune milieu following live-cell treatment, untreated and s.c. live cell-treated rats were injected IC with 5×10^5 F98 cells. Twelve days after IC inoculation (approximately the peak of the immune response) (Fig. 3*E*), brain tumors were excised and disrupted enzymatically to single cells. Following disruption, the cells were incubated for 5 h with PMA-ionomycin and then monitored using five-color flow cytometry. Supplemental Fig. 2 shows representative dot plots of untreated and live cell-treated rats. All samples were serially gated for nondoublet cells/nondebris (singlets) > live cells (live) > lymphocyte size and granularity (lymphs) > and then for their CD3/CD4/CD8 marker expression. Live CTLs ($ViViD^-CD3^+CD8^+CD4^-$) and Th cells ($ViViD^-CD3^+CD4^+CD8^-$) were further gated for the secretion of IFN-γ.

As can be observed in Fig. 4*G* and 4*H* and Supplemental Fig. 2 summarizing the data from four naive rats and five live cell-treated rats, the treated rats had, on average, more than twice the number of Th cells (1.6–3.5%) and >10 times the number of CTLs (0.2–2.2%, respectively) infiltrating the tumor ($p = 0.05$). In addition, more of the infiltrating Th and CTL in the live cell-treated rats were capable of IFN-γ secretion ($p < 0.05$).

An experiment in which brain tumors were harvested at a similar time point but the cells stained using one-color flow cytometry showed similar results. Significantly larger amounts of T cells (TCR^+) infiltrated the IC tumors of the live cell-treated rats ($n = 5$)

compared with those of untreated rats ($n = 5$) ($p = 0.05$, not shown). Single cells obtained from s.c. tumors ($n = 3$) exhibited similar T cell infiltration frequency as the IC tumors of live cell-treated rats (4 to 5% either, not shown).

In this experiment, we also found increased numbers of MHC-II–expressing cells within the tumors of live cell-treated rats (8 versus 5%). MHC-II is expressed on activated microglia and astrocytes (25) as well as on rat T cells following activation (26).

Taken together, the results indicate that there are significantly more T cells infiltrating brain tumors of live cell-treated rats, whereas there are almost no tumor-infiltrating CTLs in untreated rats and large numbers of CTLs infiltrate the tumors of live cell-treated rats. The cells found inside the tumors of the treated rats were also in a more activated state, as observed by the enhanced secretion of IFN-γ by T cells and by wider cellular expression of MHC-II by tumor stromal cells. Tumor infiltration by T, NK, and NKT cells is a sign of improved prognosis in several human tumors (27).

S.c. inoculation with live tumor cells is more effective than s.c. immunization with irradiated tumor cells

Although we show the efficacy of live-cell treatment s.c. against IC tumors, one could argue that irradiated tumor cells are safer to use and might produce similar results when used for vaccination.

To check the effects of vaccination s.c. with irradiated cells on subsequent growth of live cells injected s.c., rats were either immunized s.c. with 2×10^5 irradiated tumor cells or left untreated ($n = 7$–14/group). Ten days after the irradiated-cell treatment, the groups were challenged s.c. with live F98 cells.

Fig. 5*A* shows that the groups treated with irradiated tumor cells developed significantly smaller s.c. tumors at days 10 and 14 ($p < 0.02$). The mean peak tumor area in the irradiated cell-treated rats was reached 3 d earlier and was smaller ($p < 0.005$) than that of the untreated rats. The time to tumor rejection, however, was not significantly different between the two groups (12 ± 5 d versus 17 ± 5 d irradiated versus untreated).

The same graph (Fig. 5*A*) also depicts the s.c. growth of live F98 tumors cells following two immunizations with irradiated cells (combined data of two similar experiments). Following two irradiated cell immunizations s.c. (−36 d, −14 d), the majority (9 out of 14) of the rats did not develop any detectible tumors. The peak tumor area, reached 7 d postchallenge, was significantly smaller than the peak tumor areas of either of the other two groups ($p < 0.005$). The group also showed a shorter time to tumor rejection (2 ± 3 d; $p < 0.00005$) compared with the group treated once with irradiated cells or to untreated rats.

To determine whether irradiated F98 cells could also be used to immunize against IC tumors, rats ($n = 6$–8) were pretreated either with 2×10^5 or 2×10^6 irradiated tumor cells (Fig. 5*B*, 5*C*, respectively) or with 2×10^5 live tumor cells as a control.

Fig. 5*B* and 5*C* illustrate similar results; whereas immunization with live tumor cells protected 43–50% of the challenged rats, immunization with irradiated cells did not produce any long-term survivors. The live-cell treatment s.c. enhanced the survival both when compared with the untreated rats ($p < 0.002$ for either experiment) and when compared with treatment with a similar dose ($p < 0.0008$, Fig. 5*B*) or with a 10-fold higher dose of irradiated cells ($p = 0.02$, Fig. 5*C*). Taken together, the results suggest that s.c. treatment with live tumor cells is more effective than immunization with irradiated tumor cells.

Protection of IC-challenged rats by s.c. immunization with irradiated tumor cells followed by live tumor cells

Fig. 6 shows that although a single treatment s.c. with live cells produced significant enhancement of survival compared with the control ($p < 0.003$ or less) and 43% long-term survivors, two treatments with live tumor cells s.c. produced 83% long-term survivors. These results were observed in two similar experiments.

A group of rats treated twice with irradiated cells and then twice with live cells showed enhanced survival compared with the control untreated group ($p < 0.0001$). The combined irradiated plus live-cell treatment produced 88–100% long-term surviving rats in two similar experiments. The survival of the irradiated plus live cell-treated group was also significantly enhanced compared with the group treated once with live tumor cells ($p < 0.015$). Thus, prior immunization with irradiated cells, which inhibited the s.c. growth of live tumor cells (Fig. 5*C*), did not abrogate the beneficial effect mediated by the live-cell treatment on the survival of IC-challenged rats. A combined irradiated plus live-cell immunization protocol should be considered when designing a future brain tumor-immunization clinical trial making use of the split immunity concept.

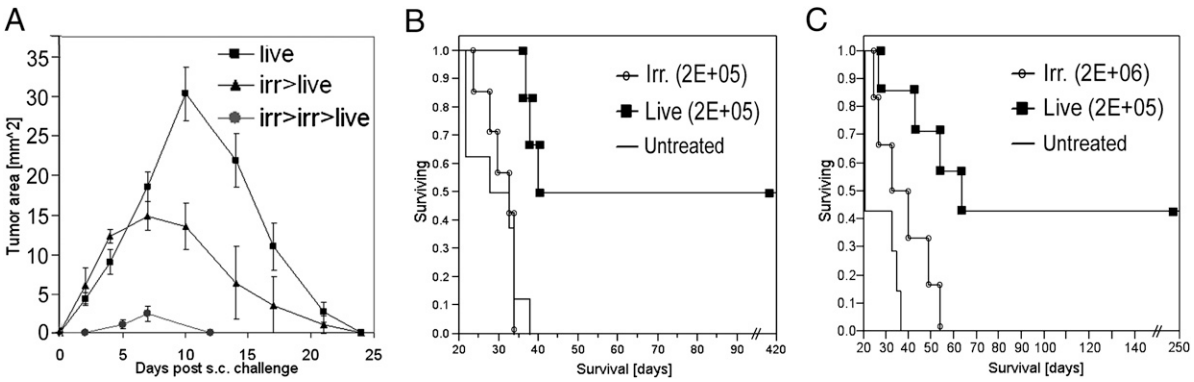

FIGURE 5. S.c. and IC protection produced by live or irradiated tumor cells. *A*, Untreated rats or rats immunized s.c. with 2×10^5 irradiated tumor cells were challenged with live F98 cells (2×10^5) s.c. 10 d after the irradiated tumor cell immunization. Shown are tumor areas of untreated rats, live ($n = 14$), and the irradiated cell-immunized (irr) group, irr>live ($n = 7$). Graph also depicts accumulated data of two experiments in which rats received irradiated cells twice (−36 d, −14 d) before the live-cell challenge s.c., irr>irr>live ($n = 14$). Repeated twice with similar results. *B*, Groups of six to eight rats were injected s.c. with 2×10^5 live or irradiated cells. After 35 d, both groups and an untreated group were challenged IC with 5×10^3 cells. Survival of live cell-treated rats is significantly different from the irradiated cell-immunized rats ($p \leq 0.02$) or the untreated groups ($p < 0.002$). *C*, Rats ($n = 6$ to 7) were injected s.c. with 2×10^5 live or 2×10^5 irradiated cells. After 76 d (live) or 25 d (irr.), both groups and an untreated group were challenged IC with 5×10^3 cells. Survival of live cell-treated rats is significantly different from the irradiated cell-treated or untreated groups ($p < 0.0008$ and $p < 0.002$, respectively). The irradiated cell-immunized group is not significantly different from the untreated group in both *B* and *C*.

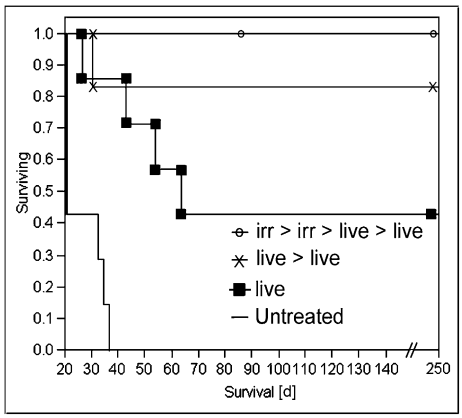

FIGURE 6. Protection from IC glioma by combination of irradiated (irr) and live tumor cell treatment. Groups of six to seven rats were immunized s.c. with live F98 cells once (−38 d) or live cells twice (−38 d and −15 d) or with irradiated cells twice and then live cells twice (−74 d, −52 d, −38 d, and −15 d). The second injection of live cells was done after the complete rejection of live tumors s.c. All groups and a control untreated group were challenged IC with 5×10^3 F98 cells (0 d). Kaplan-Meier survival curves are shown. Survival of live and the live > live cell-immunized rats are significantly different from the untreated group ($p <$ 0.003 or less). Survival of irr > irr > live > live-treated group is significantly different from the untreated group ($p < 0.0001$) and from the live-treated group ($p < 0.015$). Repeated twice with similar results.

Discussion

Vaccination, as classically defined, is the immunization of an individual with an attenuated/killed pathogen or to its antigenic determinants, leading to resistance to the live pathogen (12). We define in this study a different, location-based immune phenomenon we term "split immunity." Split immunity refers to a situation in which a nonattenuated tumor that is lethal in one location, when inoculated in another location, generates immunity that can spread back to the otherwise tolerant site, thus protecting the host.

The differences observed in the efficacy of treatment with live compared with irradiated (attenuated) cells might stem from both quantitative and qualitative factors. Quantitatively, live tumor cells might present their Ags for a longer duration to the immune system (28); moreover, a growing tumor might present larger amounts of tumor Ags to the immune system.

Ionizing irradiation has the capacity to induce immunogenic cell death via membrane-expressed factors (e.g., CRT, HSP90) as well as by secreted factors (e.g., HMGB1) (27). Tumor irradiation is a standard part of the treatment given to cancer patients, but although irradiation was shown to significantly enhance the survival of high-grade glioma patients, it almost never provides cure (29).

Qualitatively, the immune system responds differently to the different protein repertoires of live cells versus those of dead or dying irradiated tumor cells. Major changes occur in the gene expression profile of irradiated tumor cells (30–32) and in their expression of MHC molecules, costimulatory molecules, adhesion molecules, death receptors, heat shock proteins, cytokines, and inflammatory mediators (33).

Irradiated cells were extensively trialed for the immunotherapy of most types of human cancers, including gliomas, usually in combination with an adjuvant; these treatments demonstrated poor results (only 18 out of 531 patients exhibited a complete response in 24 trials). This low response rate is similar to those observed in two trials on primary brain tumors (2 out of 29 patients exhibited a complete response) (34).

Graf et al. (35) showed in four rat glioma models, including the F98 model, that vaccination of rats with irradiated cells following an IC challenge mobilizes myeloid suppressor cells into the tumor bed. The myeloid cells inhibited T cell functions, resulting in enhanced tumor growth (35). Chen et al. (36) showed that live glioma cells that were attenuated by transfection with a membrane form of M-CSF induced a protective immune response in rats challenged with the parental glioma cells IC. Vaccination with the transfected cells, freeze-thawed, x-irradiated, or mitomycin C treated, did not produce any protective effect (36).

The enhanced immunogenicity of live infectious pathogens compared with killed pathogens has been repeatedly shown since the experiments of Pasteur (37). In this study, too, the vaccination with unmodified live F98 cells exhibited the best overall survival rates for this tumor model (38). The exact nature of the differences between the response induced following immunization with live cells versus irradiated cells in the context of split immunity is yet unknown and will be the subject of future research.

We have confirmed the split immunity concept in another rat glioma model, the CNS-1 astrocytoma, a carcinogen-induced tumor syngeneic to the Lewis rat (14). Contrary to the initial publications claiming that CNS-1 is "…accepted without rejection in noncentral nervous system sites by Lewis rats," we saw a small but reproducible fraction (~5%, $n > 200$, not shown) of rats in which CNS-1 s.c. tumors spontaneously regressed (14). More importantly, treatment with live CNS-1 cells s.c. 3 d following an IC challenge with CNS-1 cells generated superior survival results (60% of rats surviving >6 mo) compared with immunization with irradiated CNS-1 cells (14% survival) or to nontreatment (0% survival) (Supplemental Fig. 4).

How does the rejection of live glioma cells in the periphery correspond with the well-documented immune suppression induced by glioma cells (39, 40)? Intratumoral and intrabrain immune suppressive milieu is mediated by a myriad of cells (e.g., microglia/macrophages) and factors (e.g., TGF-β, PGE_2, IL-10, gangliosides, MCP-1, Fas/Fas ligand, B7-H1, RCAS1, and CD70) (40). However, this immune suppression is not absolute. Animal tumor models have shown that peripheral vaccination with attenuated glioma cells could induce some effective antiglioma responses (36, 41). In humans, Tang et al. (42) have shown that high-grade (but not low-grade) glioma patients produce a de novo robust CD8 T cell response to their cognate brain tumor cells. Moreover, they have shown that GBM patients exhibit normal adenovirus-specific memory T cell responses and functional dendritic cells (42). Taken together with the data presented in this paper, it is reasonable to conclude that the glioma-induced suppression is not absolute and does not preclude the ability to produce robust peripherally generated immune responses against these tumors.

Two immune phenomena that share some similarity with split immunity are concomitant and sinecomitant tumor immunity. Concomitant immunity describes an immune response in a host with a primary progressive tumor that rejects the same tumor injected in a different location (usually right and left flanks) (43). Sinecomitant tumor immunity describes a similar phenomenon but, in that case, the primary growing tumor is surgically removed before the introduction of the second tumor (44). Split immunity defines a different phenomenon, a tumor that in one tissue kills the host, and in another location it generates protective immunity. Although different proportions of the rats spontaneously rejected s.c. live F98 (~97%) or CNS-1 (~5%) tumor cells, in both cases, peripheral exposure to the tumor cells induced protective immunity, whereas the IC inoculation always led to death. Moreover, differently from sine/concomitant immunity, the effective immunological information flow described by the split immunity concept is unidirectional: the inoculation of brain tumor cells IC

before or after the s.c. challenge did not change the s.c. tumor growth/rejection dynamics, as would be expected if sine/concomitant tumor immunity were the case.

One may ask whether brain tumors generally tend to be rejected when inoculated outside the brain. A recent review examines the eight most widely used brain tumor models in rat neuro-oncology: C6, 9L, T9, RG2, F98, BT4C, RT-2, and CNS-1 (45). Spontaneous rejection of unattenuated live cells was observed in the C6 (46), the 9L (47), and the T9 tumors (48). We add to this list the F98 and, in some cases, the CNS-1 astrocytoma model (14).

In 1979, Maat et al. (49) investigated the fate of 77 different neurocarcinogen-induced CNS and peripheral nervous system tumors inoculated s.c. as tumor pieces; these brain tumors, originating from WAG/Rij or Sprague Dawley rats, were accepted s.c. by only 62% of syngeneic rat hosts. Most of the s.c. growing tumors were characterized histologically as non-CNS tumors (49). It is difficult to assess why certain brain tumors, such as the F98, are rejected spontaneously s.c. at different rates in the hands of different researchers (47). It is, however, apparent that whereas tumor passage efficiency (i.e., the percent of animals accepting without rejection a syngeneic tumor piece obtained from another animal) in many animal brain tumor models is not 100%, passage efficiency of unmodified non-CNS animal tumors is in most, if not in all cases, 100%. Hewitt et al. (50) reported that of 20,000 maintenance transplants of 27 different murine tumors passaged s.c., none failed, and none regressed.

Several explanations were given to the apparent lack of brain tumor metastasis into peripheral sites (7–9). These explanations cannot account for the paradoxical differences in percentages of overt metastasis found in the original brain tumor patient: ~0.5% (51) compared with 23–46% donor-transmitted brain tumors growing inside organs grafted into organ recipients that were pharmacologically immune suppressed (52).

We propose in this study that the introduction of unmodified CNS tumor cells into areas outside the CNS might in itself be an immunogenic event. This notion is partially supported by the observation that inoculation into peripheral sites of unmodified tissues or cells obtained from immune privileged organs, such as brain or testis, could induce experimental autoimmune encephalomyelitis (53) or orchitis (54), respectively, without the use of an adjuvant.

As pointed out above, it is plausible to assume that brain tumor cells reach peripheral sites in most patients (52). Why then do these tumor cells not induce natural split immunity that spontaneously inhibits the IC brain tumors?

We suggest that the induction of effective split immunity requires peripheral exposure to a larger number of tumor cells than occurs during spontaneous metastasis from a brain tumor. We observed that low or high doses of live cells inoculated s.c. (10^5, 2×10^5, or 2×10^6) generate different survival rates (0–17, ~50, and ~100%, respectively). The s.c. localization of the live-cell treatment may also contribute to immunogenicity (55); we observed that i.p. injection of brain tumor cells did not induce effective split immunity (not shown).

The phenomenon of split immunity might explain another paradoxical observation: that the duration from diagnosis to death in glioblastoma patients having extracranial metastasis was 18.2 mo on average (51), significantly longer (~65%) than the average expected life span of 11 mo for glioblastoma patients without extracranial metastases (56).

High-grade brain tumors are almost uniformly lethal (56), and no standard treatments appear to extend patient survival by more than several weeks (57). Our data suggest that the split immunity approach could be useful to treat tumors developing in immune-privileged sites. This approach is planned to be translated from bench to bedside in the near future.

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Disclosures

A patent on the split immunity mode of treatment was filed by the Weizmann Institute of Science and the Tel-Aviv Sourasky Medical Center.

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