eventually progressive CNS-1 growth was noted in most animals. This indicated a deleterious effect of the CY on the endogenous immune cell component that was initially engendered, sensitized to and keeping the CNS-1 tumor cell growth suppressed. Further substantiating this conclusion, the CY-treated rats, alone as well as in combination therapy, also showed a recurrence of their primary tumor, which apparently was not completely resolved (**Fig. 5F and** G, respectively). In both groups, seven out of eight animals showed progressive tumor growth.

Safety. In our studies we did not observe any signs of toxicity in rats treated with the systemic resiquimod.

Discussion

In the present study we demonstrate that immunotherapy based on the innate immune cell activator resiquimod, is effective as a treatment modality for eradication of established CNS-1 glioma tumors.

Our CNS-1 glioma cell implants are syngeneic, haplotype RT-1[l], for Lewis rats and represent an excellent in vivo glioma model, because of its glial phenotype, reproducible in vivo growth rates and histological features that closely resemble human glioma.[31] It has been demonstrated that CNS-1 tumor cells are immunoreactive for glial fibrillary acidic protein (GFAP), S100 and vimentin, as well as neuronal adhesion molecule, retinoic acid receptor α, intracellular adhesion molecule and neuron specific enolase.[31] This model therefore provides an excellent in vivo model in which to investigate immunotherapeutic intervention strategies against glioblastoma multiforme in immunocompetent hosts.

Natural immune responses against glioma tumors are often elicited as demonstrated by histological evidence of local inflammation and tumor-specific lymphocytes, likely directed against tumor specific antigens. However, the GBM tumor microenvironment is characterized by the presence of a variety of immunosuppressive cells and their inhibitory products, which may eventually result in the escape of the tumor from immune surveillance.[32-35] However, when an effective therapeutic dose of resiquimod was injected three times per week, we observed a dramatic reduction in tumor volume. While most untreated or control tumor-bearing animals had to be sacrificied, either due to massive tumor volumes or due to ulceration of the tumor, the groups receiving a dose of more than 10 μg resiquimod per injection eventually showed complete regression of the tumor volumes. When therapeutic treatment was arrested, at day 49 after implantation, the tumor had shrunk to minute or non-measurable sizes. In vitro studies revealed that resiquimod (0.01 or 0.1 mg/ml), in contrast to CY, did not directly inhibit CNS-1 tumor cell growth.

These results may seem contradictory with other data showing that when tumor cells express TLR7/8, activation of this TLR type leads to cell survival and chemo resistance.[36] We have therefore checked expression of TLR7/8 by CNS-1 cells by RT-PCR, but where unable to detect receptor expression by PCR. However, even if TLR7/8 activation by resiquimod would have stimulated tumor growth the net effect in vivo would apparently still be tumor regression.

Interestingly, all rats proved immune to re-challenge with CNS-1 glioma cells (**Fig. 5**) as evidenced by complete inhibition of tumor development. Immune memory against rechallenge was confirmed for rats which received the additional tumor inoculation even after three months of treatment arrest, while naive rats developed tumors. In view of the short half-life of the imiquimod family members of only few hours it is very unlikely that resiquimod had some remnant activity after a three month resting period before administration of a tumor rechallenge. The complete inhibition of secondary tumor growth suggest that immunotherapeutic treatment during the first tumor growth, using resiquimod, a known innate immune response agonist activating TLR7/8, results in tumor regression that results in the development of T cells with immune memory. Hence this innate immune triggering acts as an in situ therapeutic vaccine, alerting the adaptive immune system to recognize and eliminate the syngeneic secondary CNS-1 brain tumor. In future studies we will set out to decipher the exact mechanism underlying this intriguing observation of in situ immune memory priming.

In addition, we evaluated the effects of CY on CNS-1 tumor development. Cyclophosphamide (CY), although primarily used as cytotoxic therapy and expected to suppress the immune system, has been shown to abrogate immunosuppressive T reg function, and beneficially synergize with active immunotherapy when used at an appropriate dose and timed correctly.[29,37,38]

CNS-1 tumors regressed, as a result of CY treatment, and even faster after combined CY-R848 chemo-immunotherapeutic treatment. Importantly, the administration of CY, after the animals had developed immunity to CNS-1, was deleterious (**Fig. 4**). The explanation for why, after tumor rechallenge, the animals that were treated with CY only or by the CY-R848 combination were not able to inhibit secondary tumor development relates to the CY also causing damage to the CTL that had developed in situ at the beginning of the treatment. Additionally, it provides an explanation for why the CY-treated rats also exhibited recurrence of the primary tumor. These data highlight the need to carefully arrange the administration of combined therapeutics involving cytotoxic chemotherapeutic agents with immunotherapeutic agents so one agent does not interfere with the effects derived from the other. However, the delay in tumor growth after rechallenge of the cyclophosphamide group, suggests that there is an immune effect, which is most likely dependent on T cells, although a memory response by B cells cannot be excluded formally. In both senarios T cells are necessary for T help and likely also for T cell effector function. The effect of T cell depletion will be subject of follow-up studies addressing the biological mechanism of action responsible for rechallenge immunity.

These results provoke two intriguing questions. How does R848 eradicate CNS-1 tumors, and how does immune memory develop during this treatment? In addition, it is of interest to know how CY hampers antitumor immunity. The exact mode of action and associated immune pathway responsible for the observed resiquimod-mediated anti-tumor immunity needs to be defined in detailed follow-up studies. Most likely resiquimod-based immunotherapy is able to activate a spontaneous, natural, innate anti-tumor immune response, that under normal circumstances is

unable to control tumor growth, likely as a result of delayed or actively suppressed immune control. Non-specific immune attack of the tumor evoked by TLR7/8 activating resiquimod, but not by poly I:C treatment activating TLR3 (data not shown), may release tumor antigens into the surrounding tumor environment which are sampled by locally attracted antigen presenting cells and which allow presentation to and priming of adaptive immune lymphocytes, in the draining lymph nodes. Alternatively, or in parallel, an in situ "vaccination" occurs as a result of R848 therapy. TLR7 activation by the related imiquimod causes human and rodent dendritic cells to become tumoricidal.[39] Eventually, a sufficient number of tumor-specific naive adaptive immune cells, such as cytolytic T cells, are triggered and expanded in draining lymph nodes as a result of parenteral R848 immunotherapy and enabled by activated antigen-presenting cells. These presumed cytolytic T cells selectively recognize and eliminate the tumor and provide immunological memory, as illustrated by the rejection of secondary tumor cell implants. However, dedicated follow-up studies need to address to involvement of anti-tumor killer macrophages or NK cells, or IFNs for the resiquimod-induced glioma growth regression and immune memory.

In conclusion, our data show that injection of the innate immune cell receptor agonist resiquimod as a therapeutic TLR7/8 activating stand-alone therapy, is able to cure established CNS-1 tumor growth in Lewis rats. They suggest that immunotherapeutic parenteral treatment of established glioma tumors by resiquimod, as defined in the protocol, significantly improves anti-brain tumor immunity in a way that leads to immune memory, which is superior to CY treatment alone. Our studies have thereby identified a promising novel antitumor immunotherapy which may lead to clinical benefit.

Materials and Methods

Tumor Model. Rat CNS-1 cells (2×10^5 cells/200 ul) were implanted subcutaneously (SC) using a 21 gauge needle into the right flank of 8–12 week-old (300 g body weight) male Lewis rats. For each treatment group and control, 4–8 rats/group were used. The same tumor implantation procedure was performed during re-challenge experiments, on the contralateral side, for rats which had controlled the tumor growth after first exposure. All animal studies were approved by an independent ethical committee.

Monitoring Tumor Growth. The sizes of the CNS-1 tumor volumes were measured using a caliper three times per week on Mondays, Wednesdays and Fridays to monitor the effects of each treatment group.

Completion of Experiment. Tumor implanted rats were sacrificed if they showed unfavorable signs of discomfort, as defined by the ethical committee. For example if they appeared moribund due to weight loss, lethargy, ruffled fur, or when tumors showed ulceration. A mixture of Rompun and ketamine was used for anesthesia, followed by a dose of sodium pentobarbital for euthansia.

Chemicals and reagents. *Immunomodulators and potentiators.* Rats were subcutaneously (SC) injected in the flank, contralateral to the tumor-implanted side, with resiquimod (R848) (purchased from Invivogen, catalog number tlrl-r848), a Toll-like receptor 7/8 agonist, in a range of 3.3–166.6 μg/kg, corresponding to 1–50 μg/dose, three times per week on Mondays, Wednesdays and Fridays. Resiquimod(R-848, S-28463) was shown to be more soluble and more potent in inducing cytokine expression than its family member imiquimod which has a half-life of 2–3 h in humans[40].

In a parallel arm of the experiment we evaluated the effect of cyclophosphamide administration on CNS-1 glioma development. Cyclophosphamide (CalBiochem, cat. no. 239785) was given at 30–100mg/kg. CY was injected once every two weeks on Fridays.

Cytotoxicity Assay. The direct cytotoxicity of resiquimod, CY and Poly I:C (Invivogen, cat. no. Tlrl-pic, tlrl-pic-5), which was included as a reference TLR-3 agonist, was determined by exposing CNS-1 cells at a concentration of 200,000 cells per well in a 96-well plate in DMEM culture medium (cat. no. 30–2002, ATCC), supplemented with 10% fetal bovine serum (FBS; Lonza, cat. no. DE14–801E), for 24 h. The viability of CNS-1 cells, measured in triplicate, was measured in a standard (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) bromide assay, absorbance was read at 590 nm, and was expressed as a percentage of viability measured for cells cultured in medium only.

TLR 7 and TLR 8 detection by RT-PCR. *Samples collection.* Normal spleen tissue was obtained by surgical resection from a male non-treated Lewis rat and cut in pieces of 1 mm^3 with a sterile surgical blade. CNS-1 and GL-261 cell lines were cultured as described above and a pellet of 1×10^6 cells was used. Cells or tissue sample were put in lysis buffer using the SV Total RNA Isolation System (Promega Corp., Leiden, The Netherlands).

© 2012 Landes Bioscience.
Do not distribute.

RNA extraction and reverse transcription. After extraction of total RNA it was reverse-transcribed by using the Thermoscript RT-PCR System (Life Technologies, Inc., Paisley, UK) as previously described[41].

Oligonucleotide primers used for PCR amplification: Primers for the PCR amplification were obtained by Real Time Primers LLC, PA, USA, according to successful approach for TLR-7,[42] or as customized primers for TLR-8 obtained from Real Time Primers LLC, PA, USA.

PCR. PCR was performed according to the manufacturer's recommendations, with Platinum® PCR SuperMix (Life Technologies, Inc., Paisley, UK). Aliquots of the RT products were subjected to PCR in a total volume of 50 μl, with 100 nM adequate paired primers. PCR products were visualized on a 2% agarose gel with GelRed™ Nucleic Acid Gel Prestaining Kit (Biotium, CA, USA), visualized on an UV transilluminator and photographed using a Canon Powershot G10 photograph, equipped with a conversion lens 032 LA-DC58K.

Statistical analysis. ANOVA followed by the students t-test was used to compare groups, with a p value of < 0.05 (*), $p < 0.01$ (**), $p < 0.001$ (***), considered statistically significant.

Disclosure of Potential Conflicts of Interest

All authors affiliated to *Epitopoietic Research Corporation (*ERC) are financially supported by ERC.

Acknowledgments

Supported in part by NIH R01 CA154256 to C.A.K..

References

1. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 2006; 313:1960-4; PMID:17008531; http://dx.doi.org/10.1126/science.1129139
2. Naito Y, Saito K, Shiiba K, Ohuchi A, Saigenji K, Nagura H, et al. CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. Cancer Res 1998; 58:3491-4; PMID:9721846
3. Zhang L, Conejo-Garcia JR, Katsaros D, Gimotty PA, Massobrio M, Regnani G, et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. N Engl J Med 2003; 348:203-13; PMID:12529460; http://dx.doi.org/10.1056/NEJMoa020177
4. Nakano O, Sato M, Naito Y, Suzuki K, Orikasa S, Aizawa M, et al. Proliferative activity of intratumoral CD8(+) T-lymphocytes as a prognostic factor in human renal cell carcinoma: clinicopathologic demonstration of antitumor immunity. Cancer Res 2001; 61:5132-6; PMID:11431351
5. Vesalainen S, Lipponen P, Talja M, Syrjanen K. Histological grade, perineural infiltration, tumour-infiltrating lymphocytes and apoptosis as determinants of long-term prognosis in prostatic adenocarcinoma. Eur J Cancer 1994; 30:1797-803; PMID:7880609; http://dx.doi.org/10.1016/0959-8049(94)E0159-2
6. Marrogi AJ, Munshi A, Merogi AJ, Ohadike Y, El-Habashi A, Marrogi OL. Freeman, S. M. Study of tumor infiltrating lymphocytes and transforming growth factor-beta as prognostic factors in breast carcinoma. Int J Cancer 1997; 74:492-501; PMID:9355970; http://dx.doi.org/10.1002/(SICI)1097-0215(19971021)74:5<492::AID-IJC3>3.0.CO;2-Z
7. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol 2002; 3:991-8; PMID:12407406; http://dx.doi.org/10.1038/ni1102-991
8. Dunn GP, Old LJ, Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. Immunity 2004; 21:137-48; PMID:15308095; http://dx.doi.org/10.1016/j.immuni.2004.07.017
9. Kaplan DH, Shankaran V, Dighe AS, Stockert E, Aguet M, Old LJ, et al. Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. Proc Natl Acad Sci USA 1998; 95:7556-61; PMID:9636188; http://dx.doi.org/10.1073/pnas.95.13.7556
10. Beckhove P, Feuerer M, Dolenc M, Schuetz F, Choi C, Sommerfeldt N, et al. Specifically activated memory T cell subsets from cancer patients recognize and reject xenotransplanted autologous tumors. J Clin Invest 2004; 114:67-76; PMID:15232613
11. Hofman FM, Stathopoulos A, Kruse CA, Chen TC, Schijns VEJC. Immunotherapy of Malignant Gliomas Using Autologous and Allogeneic Tissue Cells. Anticancer Agents Med Chem 2010; 10:462-70; PMID:20879986
12. Zitvogel L, Tesniere A, Kroemer G. Cancer despite immunosurveillance: immunoselection and immunosubversion. Nat Rev Immunol 2006; 6:715-27; PMID:16977338; http://dx.doi.org/10.1038/nri1936
13. Dannull J, Su Z, Rizzieri D, Yang BK, Coleman D, Yancey D, et al. Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells. J Clin Invest 2005; 115:3623-33; PMID:16308572; http://dx.doi.org/10.1172/JCI25947
14. Thomas DA, Massague J. TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. Cancer Cell 2005; 8:369-80; PMID:16286245; http://dx.doi.org/10.1016/j.ccr.2005.10.012
15. Strauss L, Bergmann C, Szczepanski M, Gooding W, Johnson JT, Whiteside TL. A unique subset of CD4+CD25highFoxp3+ T cells secreting interleukin-10 and transforming growth factor-beta1 mediates suppression in the tumor microenvironment. Clin Cancer Res 2007; 13:4345-54; PMID:17671115; http://dx.doi.org/10.1158/1078-0432.CCR-07-0472
16. Shankaran V, Ikeda H, Bruce AT, White JM, Swanson PE, Old LJ, et al. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature 2001; 410:1107-11; PMID:11323675; http://dx.doi.org/10.1038/35074122
17. Takeda K, Kaisho T, Akira S. Toll-like receptors. Annu Rev Immunol 2003; 21:335-76; PMID:12524386; http://dx.doi.org/10.1146/annurev.immunol.21.120601.141126
18. Schijns VEJC, Lavelle EC. Trends in vaccine adjuvants. Expert Rev Vaccines 2011; 10:539-50; PMID:21506650; http://dx.doi.org/10.1586/erv.11.21
19. Gollnick H, Barona CG, Frank RG, Ruzicka T, Megahed M, Tebbs V, et al. Recurrence rate of superficial basal cell carcinoma following successful treatment with imiquimod 5% cream: interim 2-year results from an ongoing 5-year follow-up study in Europe. Eur J Dermatol 2005; 15:374-81; PMID:16172048
20. Gaitanis G, Kalogeropoulos C, Bassukas ID. Imiquimod can be combined with cryosurgery (immunocryosurgery) for locally advanced perocular basal cell carcinomas. Br J Ophthalmol 2011; 95:890-2; PMID:21378006; http://dx.doi.org/10.1136/bjo.2010.195800
21. Gaspari A, Tyring SK, Rosen T. Beyond a decade of 5% imiquimod topical therapy. J Drugs Dermatol 2009; 8:467-74; PMID:19537370
22. Vasilakos JP, Smith RM, Gibson SJ, Lindh JM, Pederson LK, Reiter MJ, et al. Adjuvant activities of immune response modifier R-848: comparison with CpG ODN. Cell Immunol 2000; 204:64-74; PMID:11006019; http://dx.doi.org/10.1006/cimm.2000.1689
23. Risini D, Weeratna RD, Shawn R. Makinen, McCluskie MJ, Davis HL. TLR agonists as vaccine adjuvants: comparison of CpG ODN and Resiquimod (R-848). Vaccine 2005; 23:5263-70; PMID:16081189; http://dx.doi.org/10.1016/j.vaccine.2005.06.024
24. Hamm S, Heit A, Koffler M, Huster KM, Akira S, Busch DH, et al. Immunostimulatory RNA is a potent inducer of antigen-specific cytotoxic and humoral immune response in vivo. Int Immunol 2007; 19:297-304; PMID:17289655; http://dx.doi.org/10.1093/intimm/dxl146
25. Xiong Z, Ohlfest JR. Topical imiquimod has therapeutic and immunomodulatory effects against intracranial tumors. J Immunother 2011; 34:264-9; PMID:21389872; http://dx.doi.org/10.1097/CJI.0b013e318209eed4
26. Jurk M, Heil F, Vollmer J, Schetter C, Krieg AM, Wagner H, et al. Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. Nat Immunol 2002; 3:499; PMID:12032557; http://dx.doi.org/10.1038/ni0602-499
27. Candolfi M, Curtin JF, Nichols WS, Muhammad AG, King GD, Pluhar GE, et al. Intracranial glioblastoma models in preclinical neuro-oncology: neuropathological characterization and tumor progression. J Neurooncol 2007; 85:133-48; PMID:17874037; http://dx.doi.org/10.1007/s11060-007-9400-9
28. Berd D, Mastrangelo MJ. Effect of low dose cyclophosphamide on the immune system of cancer patients: reduction of T-suppressor function without depletion of the CD8+ subset. Cancer Res 1987; 47:3317-21; PMID:2953413
29. Zitvogel L, Apetoh L, Ghiringhelli F, Kroemer G. Immunological aspects of cancer chemotherapy. Nat Rev Immunol 2008; 8:59-73; PMID:18097448; http://dx.doi.org/10.1038/nri2216
30. Kempf RA, Mitchell MS. Effects of chemotherapeutic agents on the immune response. Cancer Invest 1984; 2:459-66; PMID:6509357; http://dx.doi.org/10.3109/07357908409048519
31. Kruse CA, Molleston MC, Parks EP, Schiltz PM, Kleinschmidt-DeMasters BK, Hickey WF. A rat glioblastoma model, CNS-1, with invasive characteristics similar to those of human gliomas: a comparison to 9L gliosarcoma. J Neurooncol 1994; 22:191-200; PMID:7760095; http://dx.doi.org/10.1007/BF01052919
32. Avril T, Vauleon E, Tanguy-Royer S, Mosser J, Quillien V. Mechanisms of immunomodulation in human glioblastoma. Immunotherapy 2011; 3(Suppl):42-4; PMID:21524170; http://dx.doi.org/10.2217/imt.11.39
33. Gajewski TF, Meng Y, Blank C, Brown I, Kacha A, Kline J, et al. Immune resistence orchestrated by the tumor microenvirenment. Immunol Rev 2006; 213:131-45; PMID:16972901; http://dx.doi.org/10.1111/j.1600-065X.2006.00442.x
34. Roszman T, Elliott L, Brooks W. Modulation of T-cell function by gliomas. Immunol Today 1991; 12:370-4; PMID:1958290; http://dx.doi.org/10.1016/0167-5699(91)90068-5
35. Ge L, Hoa N, Bota DA, Natividad J, Howat A, Jadus MR. Immunotherapy of brain cancers: the past, the present and future directions. Clinical and Developmental Immunology2010.
36. Cherfils-Vicini J, Platonova S, Gillard M, Laurans L, Validire P, Caliandro R, et al. Triggering of TLR7 and TLR8 expressed by human lung cancer cells induces cell survival and chemoresistance. J Clin Invest 2010; 120:1285-97; PMID:20237413; http://dx.doi.org/10.1172/JCI36551
37. Kruse CA, Mitchell DH, Kleinschmidt-DeMasters BK, Bellgrau D, Eule JM, Parra JR, et al. Systemic Chemotherapy Combined with Local Adoptive Immunotherapy Cures Rats Bearing 9L Gliosarcoma. J Neurooncol 1993; 15:97-112; PMID:8509824; http://dx.doi.org/10.1007/BF01053931
38. Gomez GG, Hutchison R, Kruse CA. Chemo-immunotherapy and chemo- adoptive immunotherapy of cancer. Cancer Treat Rev 2001; 27:375-402; PMID:11908930
39. Stary G, Bangert C, Tauber M, Strohal R, Kopp T, Sting G. Tumoricidal activity of TLR7/8-activated inflammatory dendritic cells. J Exp Med 2007; 204:1441-51; PMID:17535975; http://dx.doi.org/10.1084/jem.20070021
40. Soria I, Myhre P, Horton V, Ellefson P, McCarville S, Schmitt K, et al. Effect of food on the pharmacokinetics and bioavailability of oral imiquimod relative to a subcutaneous dose. Int J Clin Pharmacol Ther 2000; 38:476-81; PMID:11073288
41. Westfall B, Sitaraman K, Solus J, Hughes J, Rashtchian A. High specificity and robustness of AccuPrime™ Taq provides the ideal tool for demanding miniaturized, multiplex, and high-throughput PCR. Focus 1997; 19:46.
42. Xiong Z, Ohlfest J. Topical Imiquimod has therapeutic and immunomodulaotory effects against intracranialt tumors. J Immunother 2011; 34:264-9; PMID:21389872; http://dx.doi.org/10.1097/CJI.0b013e318209eed4

© 2012 Landes Bioscience.
Do not distribute.