

## Successful combination of local CpG-ODN and radiotherapy in malignant glioma

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Oligodeoxynucleotides containing CpG motifs (CpG-ODN) display broad immunostimulating activity and are currently under clinical trial in various malignancies, including recurrent glioblastomas. Combining CpG-ODN with another therapy that could induce antigen release might enhance tumor-specific immune response. We investigated whether radiotherapy (RT) could be associated advantageously to intratumoral injections of CpG-ODN. Fisher rats bearing 9L glioma were treated with various combinations of RT and CpG-28, an oligonucleotide with good immunostimulating activity. RT and CpG-28 induced complete tumor remission in one-third of the animals. When both treatments were combined, complete tumor remission was achieved in two-thirds of the animals ( $p < 0.001$  when compared to non-treated rats,  $p < 0.03$  when compared to CpG-28 alone). Such efficacy was not observed in nude mice, underlying the role of T cells in antitumor effects. The combination of both treatments appeared optimal when the delay between RT and CpG-28 administration was <3 days (from 100% survival for a 3 days delay, to 57% survival for a 21 days delay,  $p < 0.05$ ). Tumor infiltration by immune cells and expression within tumors of the CpG receptor, TLR9, were not modified by irradiation. These results support an attractive strategy of sequential radiotherapy and immunotherapy by CpG-ODN and have potential implications for future clinical trials with CpG-ODN.

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**Key words:** cancer immunotherapy; CpG motif; oligodeoxynucleotides zTLR9; cancer model; glioma; radiotherapy; CpG-ODN

Oligodeoxynucleotides containing CpG motifs (CpG-ODN) are strong immunostimulating agents, activating both innate and specific immunity. Biological effects of CpG-ODN are mediated by the Toll-like receptor 9 (TLR9), a receptor mainly expressed by B-lymphocytes and plasmacytoid dendritic cells in humans, and also macrophages in rats.<sup>1,2</sup>

In 1999, we demonstrated the potent antitumor activity of CpG-ODN when injected around an established neuroblastoma tumor in mice.<sup>3</sup> Subsequent studies have shown that CpG-ODN were efficient in most tumor models including glioma,<sup>4,5</sup> and several clinical trials using CpG-ODN as a single agent are currently ongoing in melanomas, lymphomas, renal carcinomas or glioblastomas. Activation of both innate and specific immunity (especially NK and CD8 positive cells) is required to achieve optimal antitumor effects in the animal models.<sup>3,6</sup> As CpG-ODN are usually more efficient when injected at the tumor site, the ability of this local treatment to trigger a specific immunity is critical to control distant metastasis. Strategies aiming to enhance tumor-specific immune responses will be important for clinical applications of CpG-ODN.

We investigated whether irradiation could enhance the efficacy of CpG-ODN immunotherapy. Several reasons suggest that this association could be synergic: (i) radiotherapy induces cell necrosis and apoptosis, leading to release of tumor antigens; (ii) radiotherapy can reduce the tumor burden to be eliminated by the immune system; and (iii) radiotherapy can promote local inflammation within the tumor mass, perhaps leading to an increased number of CpG-ODN sensitive cells. The antitumor effects of a CpG-ODN selected for its good antitumor activity was therefore studied in combination with radiotherapy in a glioma model.

### Material and methods

#### Oligodeoxynucleotides

Purified single-stranded phosphorothioate oligodeoxynucleotides were purchased from Eurogentec (Seraing, Belgium). The sequences used in our study were ISS 5'-TGACTGTGAACGTTGAGATGA,<sup>7</sup> IMM 5'-TGACTGTGAAGGTTAGAGATGA,<sup>7</sup> CpG-28 5'-TAAACGTTATAACGTTATGACGTCAT<sup>5</sup> and G3139 5'-TCTCCCAGCGTGCCCAT.<sup>8</sup>

#### Tumor inoculations and CpG-ODN treatment

The rat glioma cell lines 9L and RG2 (kindly given by Dr. Guilamo, Créteil, France, and Dr. Barth, Duke University, USA) were maintained in DMEM with 10% FCS (Boehringer, Meylan, France). Five-week-old male Fisher 344 rats and nude mice were obtained from Charles River Laboratories (Saint-Germain sur l'Arbresles, France). Rats were anesthetized with 45 mg/100 mg body weight intraperitoneal (i.p.) Chloral hydrate (Sigma, Steinheim, Germany).

For tumor implantation, animals were inoculated subcutaneously (s.c.) into the right flank or the right leg with  $10^5$  cells resuspended in 50  $\mu$ l PBS. All rats inoculated s.c. with 9L cells developed rapidly growing tumors. Histological analysis of these tumors showed a glioblastoma pattern with numerous mitosis, and low GFAP expression (not shown). On the indicated days, animals were injected into the tumor bed either with 50  $\mu$ l isotonic sodium chloride or CpG-ODN dissolved in 50  $\mu$ l of saline. Tumor volumes were assessed twice a week with a caliper using the formula:  $\pi/6 \times \text{length} \times \text{width}^2$ .<sup>3</sup> Animals were sacrificed when the tumors reached 3 cm in diameter or monitored for at least 3 months if tumors were rejected.

#### ELISA

TNF $\alpha$  release was tested on the murine RAW 264.7 cell line (ATCC TIB-71), using an ELISA assay (kit Mouse TNF- $\alpha$ , Becton-Dickinson, Le Pont de Claix, France). RAW cells (200,000 cells/well) were seeded on a 96-well microplate in DMEM with 10% endotoxin-free FCS (Invitrogen Life Technologies, Cergy-Pontoise, France). Cells were incubated for 48 hr in the presence of various concentrations of oligonucleotides in triplicate. Supernatants were harvested and TNF $\alpha$  release was measured by optical density following the manufacturer's instructions.

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Dr. A. Carpentier holds a patent position on CpG-ODN for cancer immunotherapy.

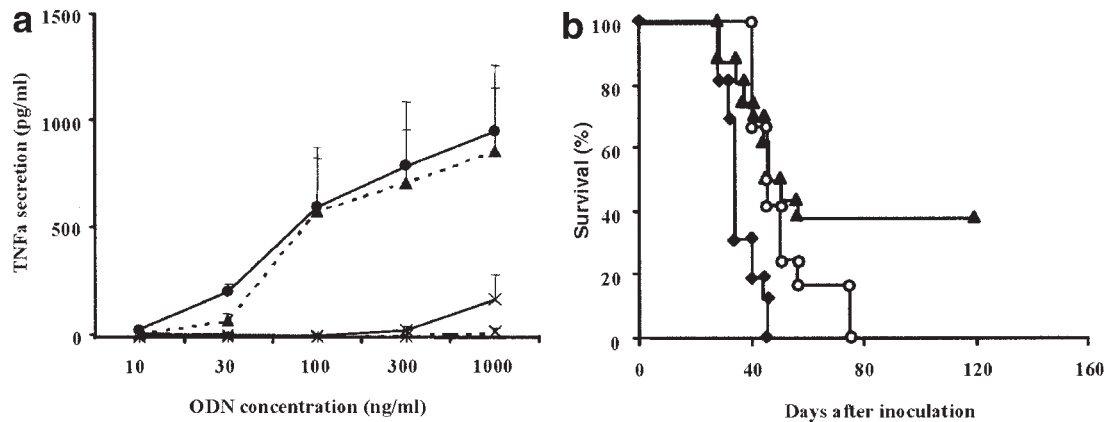
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**FIGURE 1** – Dose response analysis of various oligonucleotides on TNF $\alpha$  release. (a) RAW cells were incubated for 48 hr with various concentrations of CpG-28 (●), ISS (▲), G3139 (-x-) and IMM (--x-). TNF $\alpha$  release was measured using an ELISA assay (graph is the mean of 3 different experiments carried out in triplicate). (b) To test the antitumor effects of CpG-28, 9L cells were inoculated into the flank of Fisher rats. Rats were injected on Day 2 into the tumor bed either with 50  $\mu$ g ISS (○;  $n = 12$ ), 50  $\mu$ g CpG-28 (▲;  $n = 16$ ), or 50  $\mu$ l saline (◆;  $n = 16$ ). Data presented are pooled from 4 experiments with similar results.

### Radiotherapy

Local radiotherapy was carried out using a 4-MV linear accelerator (Orion type, General Electric). Rats were anesthetized and placed on the LINAC couch in prone position with laser alignment. A 15-mm thickness of equivalent tissue was positioned on the leg of the rat to improve dose distribution around the tumors. Depending upon the protocols, the irradiation delivered either 30 Gy in 10 fractions of 3 Gy over 12 days, or a single dose (2, 6 or 10 Gy). Anterior and posterior fields were equally weighted and treated each time. For irradiation, the rats were anesthetized as described previously.

### PCR for TLR9

Total RNA were extracted from freshly resected established 9L tumors with the kit Nucleospin RNA II (Macherey-Nagel Bioprobe, Montreuil, France). One microgram of RNA were reverse-transcribed with random hexameric primer (Promega, Charbonnières, France) together with M-MLV reverse transcriptases (Gibco, Invitrogen, Cergy Pontoise, France). Five microliters of the reverse-transcriptase reaction were then amplified by PCR (30 cycles) using the specific primers for actin or TLR9 and the following conditions for each cycle: 45 sec at 94°C, 45 sec at the annealing temperature (60°C), and 1 min elongation at 72°C. TLR9 primers, spanning the first intron, were 5'-gggagccttgga-gaatctt and 5'-ccatgagccttcagttcaca.<sup>9</sup> Actin primers were 5'-GAG ACCTTCAACACCCAGCC and 5'-GGCCATCTCTTGCTC-GAAGTC. The calculated cDNA size was 154 bp for TLR9 fragment, and 275 bp for the actin fragment. The PCR products were separated on a 2% agarose gel and stained by ethidium bromide. Spleen cDNA and 9L cells cDNA were used as positive and negative controls.

### Histological analysis

Subcutaneous tumors were surgically removed and embedded in paraffin for staining with hematoxylin and eosin, or snap-frozen and stored at -80°C. Frozen sections (10- $\mu$ m thick) were fixed for 10 min in cold acetone. The following primary antibodies were used: CD45 (1:10; BD Pharmingen, Le pont de Clax, France) directed against the leucocyte common antigen (LCA); and ED1 (1:200; Serotec, Cergy, France) directed against macrophages. Sections were further incubated with a secondary anti-mouse IgG antibody (Amersham Bioscience, Orsay, France) diluted 1/1,000. The reaction was revealed through an avidin-biotin system (Vectorlab, Burlingame, CA) and examined under a Leica fluorescent microscope. Quantitative analysis of labeled cells was carried out

by an investigator who was blinded to the animal's history using 2 different sections for each sample.

### Western blot studies for anti-tumoral antibodies

Crude 9L and RG2 cell extracts were subjected to electrophoresis on a 10% polyacrylamide SDS gel, and transferred to nitrocellulose filters (Amersham Bioscience). Filters were blocked in 5% non-fat milk for 1 hr at room temperature, cut into strips and incubated for 12 hr at room temperature with the rat sera diluted 1/200 in 5% goat serum. The strips were then incubated with a secondary biotinylated goat anti-rat IgG antibody (Amersham Bioscience) diluted 1/2 000. The reaction was revealed through an avidin-biotin system (Vectorlab).

### Statistics

Statistical significance for survival was assessed using the Kaplan-Meier analysis. Differences in tumor size among the various groups were determined by the ANOVA repeated-measures test. Correlations were assessed by linear regression analysis.

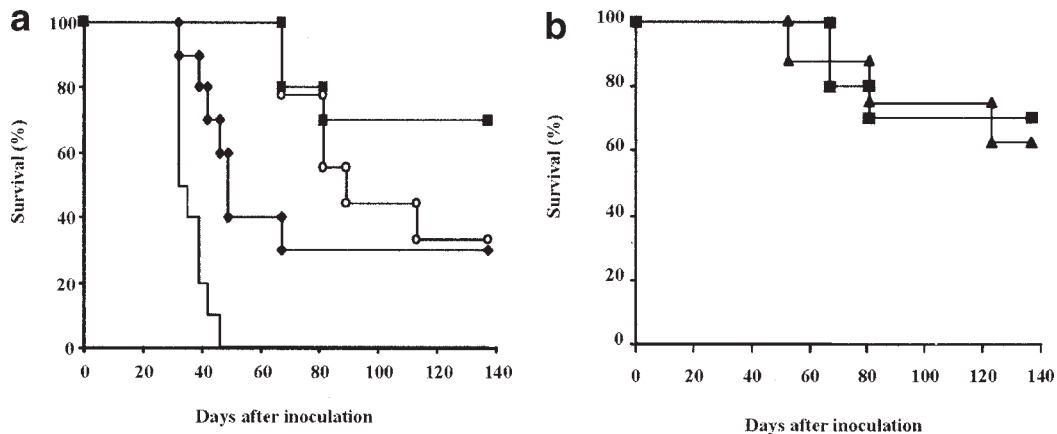
## Results

### Immunostimulating properties of different CpG-ODN

The immunostimulating activity of a new ODN containing 3 CpG motifs (CpG-28) was compared to various ODN reported in the literature using an ELISA assay for TNF $\alpha$  release from RAW cells. As shown in Figure 1a, CpG-28 was very potent to activate murine cells and compared favorably to the ISS reported previously.<sup>7</sup> As expected, IMM that has no CpG-motifs was not immunostimulant and G3139 that is an antisense oligonucleotide having non-conventional CpG-motifs<sup>8</sup> displayed moderate activity on RAW cells.

### CpG-28 displays strong antitumor effects

To test the antitumor effects of CpG-28, 9L cells were inoculated into the flank of Fisher rats. Rats were injected on Day 2 into the tumor bed either with 50  $\mu$ g ISS, 50  $\mu$ g CpG-28 or saline. The animals were sacrificed when the tumors reached 3 cm (Fig. 1b). When compared to controls, rats treated with ISS or CpG-28 showed an increased survival (median survival 46 days for ISS and 50 days for CpG-28, vs. 33 days for controls;  $p < 0.001$  for both). Interestingly, complete tumor remission and long-term survival was seen in the CpG-28 treated group (37% of the rats), but not in the rats treated with ISS ( $p < 0.02$ ).



**FIGURE 2** – Combination of radiotherapy and CpG-ODN in the 9L glioma model. (a) Fisher rats bearing 5-mm 9L tumors were treated either with saline (—;  $n = 10$ ); a local injection of CpG-28 (◆;  $n = 10$ ), local irradiation (30 Gy, 10 fractions in 12 days) (○;  $n = 9$ ), or CpG followed by irradiation (■;  $n = 10$ ). The rats treated with CpG-28 or RT showed an increased survival ( $p < 0.0001$  for both groups when compared to controls). Combination of RT and CpG-28 further improved survival with a long-term survival increased to 70% ( $p < 0.0001$  when compared to controls;  $p < 0.03$  when compared to the CpG-ODN group). (b) Combination of RT and CpG-28 was equally efficient whether CpG-28 was injected after (■;  $n = 10$ ) or before RT (▲;  $n = 8$ ). (a) and (b) issued from the same experiment, split for the clarity of the presentation.

#### Combined effects of radiotherapy and CpG-28

To investigate whether RT could be advantageously combined to CpG-28, rats bearing 5-mm tumor were treated either with local injection of saline, CpG-28, local irradiation with 10 fractions of 3 Gy over 12 days (total dose 30 Gy) or local injection with CpG-28 followed by local irradiation. All rats injected with saline had to be sacrificed within 7 weeks (median survival = 32 days), whereas animals treated with CpG-28 or RT showed an increased survival (median survival = 49 days and 93 days respectively,  $p < 0.0001$  for both groups when compared to controls). In addition, 3 rats (30%) in the CpG-28 group and 3 rats (33.3%) in the RT group rejected the established tumors and remained tumor-free for at least 7 months. Combination of CpG-28 and RT further improved survival as the percentage of animals rejecting the tumors and showing long-term survival was increased to 70% ( $p < 0.0001$  when compared to controls;  $p < 0.03$  when compared to the CpG-28 group) (Fig. 2a).

To see whether CpG-28 administration would be more efficient after than before RT, one additional group received CpG-28 after completion of the RT regimen (Fig. 2b). The survival curve (63% long-term survival) was clearly improved when compared to non-treated rats ( $p < 0.0001$ ), but was not statistically different from the group treated with CpG-28 before RT. As treatment was equally efficient whether CpG-28 was injected before or after RT, the sequential utilization of RT followed by CpG-28 was selected for subsequent studies.

#### Combination of RT and CpG-ODN in nude mice

To investigate the role of T cells in the association of RT and CpG ODN, nude mice were inoculated into the right leg with 9L cells. When the tumors reached 5-mm in diameter (approximately 10 days), the mice were treated with local irradiation with 10 Gy at one time, or with local irradiation with 10 Gy followed by a local injection with 100  $\mu$ g CpG-28 ( $n = 6$  per group). All animals developed rapidly growing tumors at similar rates in both groups. On Day 24, the mean tumor volume in the group treated with RT and CpG-28 were slightly lower than in the group treated with RT only, but the difference was not significant (mean tumor volumes  $\pm$  SEM;  $2,390 \pm 370$  mm<sup>3</sup> for RT + CpG-28 vs.  $2,790 \pm 600$  mm<sup>3</sup> for RT treated rats,  $p = 0.78$ ). This underlies the role of T cells in the combination of RT and CpG ODN.

#### Optimal time schedule for combining RT and CpG-ODN

To define the optimal schedule for combining RT and CpG-ODN, Fisher rats bearing 5-mm tumor were irradiated locally with

10 Gy at one time, then divided in several groups for subsequent treatment with CpG-28 at different days. As control, an additional group was treated with RT only (Fig. 3a). All groups treated with both RT and CpG-28 showed an increased in survival when compared to the group treated with RT only. As shown in Figure 3b, the benefit of the association appeared optimal when the delay between RT and CpG-28 administration was <3 days (no delay = 86% survival; 3-day delay = 100% survival; 7-day delay = 69% survival; 14-day delay = 67% survival; 21-day delay = 57% survival;  $p < 0.05$  by linear regression analysis).

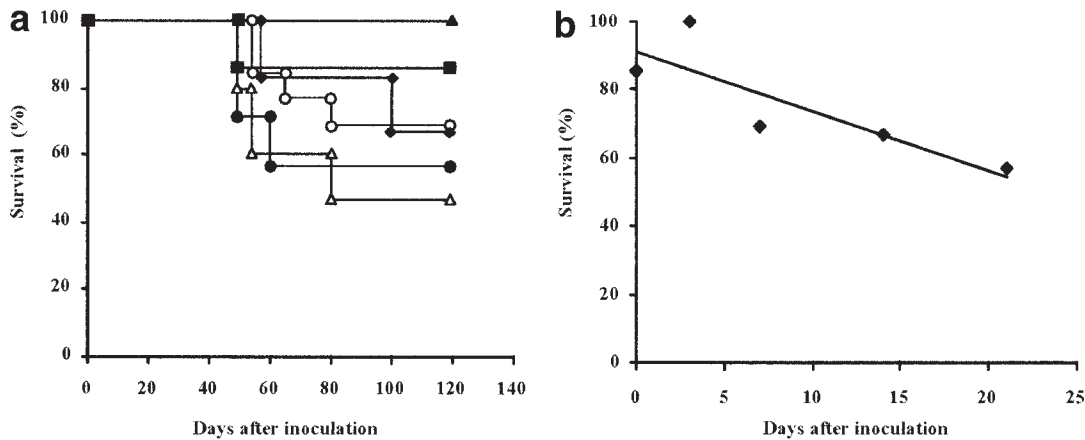
All groups treated with both RT and CpG-28 showed reduced tumor growth when compared to the group treated with RT only. This was particularly striking in the group treated with CpG-28 on Day 3, in which complete tumor regression was achieved in all animals within 1 month after treatment ( $p < 0.001$  when compared to RT alone).

#### Combination of low doses RT and CpG-ODN

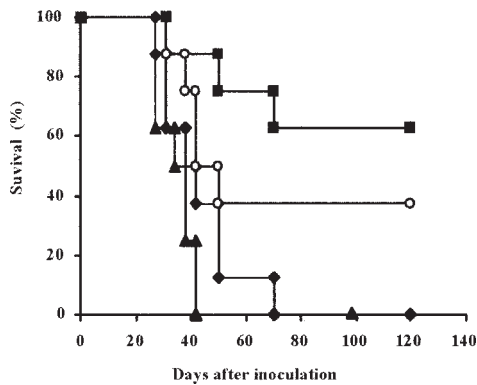
We then questioned whether minimal dose of RT could be advantageously combined to CpG-28. Rats bearing 5-mm tumor were treated either with a local injection with saline, or a local injection with 100  $\mu$ g CpG-28, or a local irradiation with one fraction of 2 Gy, or a combination of radiotherapy and CpG-28 (Fig. 4). All rats injected with saline or treated with RT had to be sacrificed within 7 weeks (median survival = 38 days). The animals treated with CpG-28 showed an increased survival and 3 rats (38%) rejected the established tumors (median survival = 45 days,  $p < 0.05$  when compared to controls). Combination of RT and CpG-28 further improved survival and complete tumor rejection was achieved in 63% of the animals ( $p < 0.05$  when compared to controls or RT alone), but the association did not reach statistical significance over CpG-28 alone.

#### TLR9 expression and cellular infiltrates within tumors after RT

Biological effects of CpG-ODN are mediated by TLR9-positive immune cells infiltrating *in vivo* the tumors. TLR9 expression within established 9L tumors was studied at different times after RT to see whether modulation of its expression could be induced by RT (Fig. 5). Fisher rats bearing 5-mm tumor were irradiated locally (10 Gy non fractionated). The tumors were surgically removed 1 hr, 3 days, 7 days, 14 days or 21 days after RT (3 rats/group). Non-irradiated tumors were used as controls for basal expression. RNA was extracted and TLR9 expression was examined by RT-PCR. RNAs from *in vitro* cultivated 9L cells, which



**FIGURE 3** – Optimal time schedule for combining RT and CpG-ODN. (a) Fisher rats bearing 5-mm 9L tumors were irradiated locally with 10 Gy in one fraction and divided into several groups for subsequent treatment with 100  $\mu$ g CpG-28, either 1 hr ( $\blacksquare$ ;  $n = 7$ ), 3 days ( $\blacktriangle$ ;  $n = 13$ ), 7 days ( $\circ$ ;  $n = 13$ ), 14 days ( $\blacklozenge$ ;  $n = 6$ ); or 21 days ( $\bullet$ ;  $n = 7$ ) after RT. All rats treated with both RT and CpG-28 showed an increased survival over rats treated with RT only ( $\triangle$ ;  $n = 15$ ). (b) Linear regression analysis of the percentage of long-term surviving animals and the delay between RT and treatment with CpG-28 ( $p < 0.05$ ).



**FIGURE 4** – Combination of low-doses RT and CpG-ODN. Fisher rats bearing 5-mm 9L tumors were treated with local injections with saline on Day 3 ( $\blacklozenge$ ;  $n = 8$ ); local injections with 100  $\mu$ g CpG-28 on Day 3 ( $\circ$ ;  $n = 8$ ); 2 Gy local irradiations on Day 0 ( $\blacktriangle$ ;  $n = 8$ ); or 2 Gy local irradiations on Day 0 followed by local injections with 100  $\mu$ g CpG-ODN on Day 3 ( $\blacksquare$ ;  $n = 8$ ). Treatment with CpG-28, but not RT, increased survival. Combination of CpG-ODN and RT further increased survival, but the difference did not reach statistical significance over the CpG-28 arm ( $p < 0.05$  when compared to controls or RT alone,  $p = 0.2$  when compared to CpG-28).

do not express TLR9<sup>5</sup> were used as negative controls. TLR9 expression was easily detected in every tumor and no modulation of its expression over time after RT could be detected.

In addition, because B-lymphocytes and macrophages are known to express TLR9 in rat, frozen sections of the resected tumors were analyzed for ED1 (macrophages) and LCA (leucocytes) positive cells infiltration. All tumors showed a mild to moderate infiltration with ED1 cells, which was heterogeneous depending on the tumor area. Positive CD45 cells were mainly seen at the tumor margins, but were very scattered inside (<5 per 0.135 mm<sup>2</sup> field). No significant modulation of these infiltrations was noted after radiotherapy.

#### Long-term immunity and study of circulating antitumoral antibodies

To determine whether rejection of s.c. implanted 9L tumor cells led to a specific long-term immunity, 7 rats that had rejected 9L cells after treatment with RT and 7 rats that had rejected 9L cells

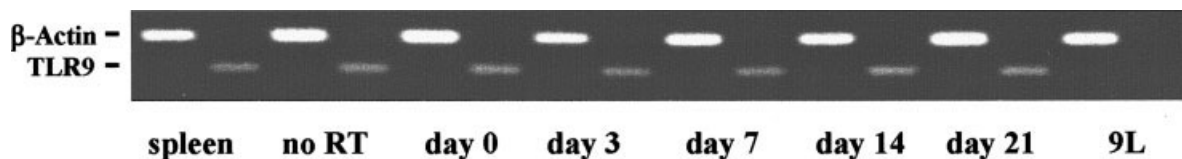
after treatment with RT and CpG-28 on Day 3 were inoculated 5–7 months later with 9L glioma cells into the right flank. None of these rats did develop 9L tumor. These rats were then subjected to an inoculation with RG2 cells, another syngenic glioma cell line in this strain of rats. All animals developed RG2 tumor.

To see whether circulating antibodies specifically targeted against a 9L antigen could explain these selective immunization against 9L but not RG2, sera of 7 rats treated with RT and CpG-28 and 7 rats treated with RT alone were studied by Western blot for reactivity against 9L and RG2 extracts. When tested at a dilution of 1:200, several antigens from 9L and RG2 cells were recognized by all animals' sera. The number of antigens recognized by circulating antibodies was not significantly higher in the group treated with RT and CpG-28 when compared to the group treated with RT only. A faint band against a 135 kDa Ag was seen in 5 of 7 rats treated with RT and CpG-28, but not in the rats treated with RT only, but this reactivity was very weak (data not shown).

#### Discussion

Radiotherapy is a standard treatment for management of high-grade gliomas in humans. Local treatment with CpG-ODN was shown to be efficient in prolonging survival in rats with established gliomas<sup>4</sup> and this approach is currently under clinical trials. Our study shows that RT can enhance the efficacy of CpG-ODN. The combined effects of RT and CpG-ODN appeared optimal when the delay between irradiation and administration of CpG-ODN was <3 days (Fig. 3) suggesting that a temporal association is needed. This temporal association was not related to any modulation of TLR9 expression or infiltration of immune cells within the tumor.

We used an oligonucleotide containing 3 CpG motifs (CpG-28) that seemed to be very potent on murine cells to induce TNF $\alpha$  secretion (Fig. 1a) and B cells proliferation (data not shown). CpG-ODN display slightly different biological effects depending upon the sequences surrounding the CpG motif and the type of backbone's chemical modification. In humans, 3 types of CpG ODN (Type A, B and C) have been described, but the differences between these families are less clear in murine species.<sup>1,2</sup> Some studies have suggested that optimal CpG motif might differ between species, 5'-GTCGTT being more efficient for humans and 5'-GACGTT for murine species.<sup>10</sup> Our oligonucleotide CpG-28 harbors the 5'-AACGTT motif that has been described for its good efficacy in both humans and murine species.<sup>7,11</sup>



**FIGURE 5** – TLR9 and actin mRNA expression in 9L tumors. Fisher rats were inoculated into the right leg with 9L cells. When the tumors reached 5- mm in diameter, the rats were irradiated locally (10 Gy in one fraction). The tumors were surgically removed either 1 hr, 3, 7, 14 or 21 days after radiotherapy. Non-irradiated tumors (no RT) were used as controls. RNA from spleen and 9L cells cultivated *in vitro* were used as a positive and negative control for expression of TLR9.

Our results are in agreement with a recent study reporting synergistic activity of CpG ODN combined to RT in another tumor model, but with higher doses (20 Gy in one fraction).<sup>12</sup> Others studies have reported that RT could be combined advantageously to immunotherapy, such as vaccination with tumor cells transfected with cytokines, intratumoral injections with dendritic cells or systemic injections of Flt-3-ligand.<sup>13–16</sup> Local treatment with CpG-ODN, which is known to promote dendritic cells maturation and antigen presentation,<sup>2</sup> might induce the antitumor effect in a similar fashion. Our study is also in line with reports of synergistic effects of CpG-ODN and chemotherapy in some animal models,<sup>5,17,18</sup> in which the role of tumor cell lysis, antigen-release and enhanced T cell immunity has been hypothesized.

A radiosensitizing effect of CpG-ODN cannot explain the enhanced efficacy of the combined treatment in our model, as a similar efficacy was observed when CpG-ODN were administered before or after RT (Fig. 2b). The mechanisms by which tumor cells are rejected are likely immune-mediated. Indeed, NK cells and T-lymphocytes are known to be critical for tumor rejection induced by CpG-ODN.<sup>3,5,6</sup> In our study, the poor efficacy of RT and CpG-ODN over RT alone in nude mice suggests that the enhanced efficacy does not rely on activation of innate immunity, but rather on T cells. Differences in efficacy of CpG-28 between rats and mice cannot explain this lack of activity in nude mice, as CpG-28 promotes TNF- $\alpha$  secretion in the mouse RAW cell line (Fig. 1b). The synergistic or additive activity of RT and CpG-ODN might be surprising at the first glance, as one could speculate that RT might destroy tumor infiltrating lymphocytes or impair dendritic cells functions,<sup>19</sup> thus decreasing the activity of the immune system. Lymphocytes depletion was reported to be transient after RT<sup>20</sup> and tumor infiltrating macrophages are resistant to radiation therapy. This is supported by the positive and non-modified TLR9 expression that we observed in our tumors after irradiation

with 10Gy. As 9L cells do not express TLR9, the positive detection of TLR9 in established tumors are probably related to the presence of tumor-infiltrating immune cells. Macrophages and scattered leukocytes were observed by immunocytochemistry in our tumors. In line with the TLR9 study, we did not observe any significant modulation of these cell infiltrates after RT.

Several mechanisms can explain the synergistic activity of the combination of RT and CpG-ODN. First, local irradiation can reduce the tumor burden to be eliminated by the immune system. Second, ionizing radiation exhibits immunomodulatory properties. Radiation has been reported to upregulate expression of various surface molecules on glioma cells (MHC, costimulatory molecules B7-1, B7-2, death receptors, heat shock proteins)<sup>21</sup> and to generate a proinflammatory environment with cytokines such as TNF- $\alpha$  or IL-1 $\beta$  and expression of ICAM-1.<sup>22</sup> Glioma cells escape immune surveillance by secretion of immunosuppressive factors such as TGF- $\beta$  or IL10.<sup>23</sup> The proinflammatory environment induced by RT may counteract these immunosuppressive cytokines and favor maturation of dendritic cells. Radiation-induced damage leads to cell death by apoptosis and necrosis, the latter being referred as mitotic or clonogenic cell death.<sup>24</sup> Apoptotic cell death was seen in our model for several days after RT, using TUNEL analysis on freshly resected tumors (data not shown). Dendritic cells can use both apoptotic cells and necrotic cells as a source of antigen.<sup>25,26</sup> Priming immunity with apoptotic cells has been successfully used in glioma model, in which immunization with 7-hydroxystaurosporine-induced apoptotic 9L cells can increase survival of intracranial 9L bearing rat.<sup>27</sup>

In conclusion, we showed that CpG-ODN and radiotherapy can be successfully combined to increase tumor regression of established tumors and long-term survival in a glioma model. These results have potential implications for ongoing clinical investigations with CpG-ODN and warrant clinical trials.

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