Therapeutic targeting of TLR9 inhibits cell growth and induces apoptosis in neuroblastoma

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ABSTRACT

The Toll-like receptor TLR9 evolved to cope with pathogens, but it is expressed in a variety of tumors for reasons that are unclear. In this study we report that neuroblastoma (NB) cells express functional TLR9. Liposome-complexed CpG oligonucleotides (L-CpG) inhibited the proliferation of TLR9-expressing NB cells and induced caspase-dependent apoptotic cell death. Inhibitory oligonucleotides (iODN) abrogated these effects. RNA interference reduced TLR9 expression but not to the level where functional responses to CpG were abolished. Compared to free CpG, liposomal formulations of NB-targeted CpG (TL-CpG) significantly prolonged the survival of mice bearing NB tumor xenografts. While CpG alone lacked anti-tumor efficacy in NOD/SCID/IL2rg-/mice, TL-CpG retained significant efficacy related to direct effects on tumor cells. TLR9 expression in primary human NB specimens was found to correlate inversely with disease stage. Our findings establish functional expression of TLR9 in NB and suggest that TLR9 may represent a novel theranostic target in this disease.
INTRODUCTION

Toll Like Receptors (TLRs) represent a family of highly conserved pattern recognition receptors, evolved by the immune system to recognize extracellular pathogen associated motifs known as pathogen-associated molecular patterns (1). TLRs are expressed predominantly by dendritic cells, macrophages, NK and B cells. Recently, it has been reported that epithelial and endothelial cells (2, 3) as well various tumors, such as melanoma, colon, breast, prostate and lung cancer (4, 5) can express TLRs. Although understanding of the functional impact of TLRs expression on tumor cells warrants further investigation, current evidence indicates that TLRs on cancer cells can either promote or inhibit tumor progression (4).

TLR9 recognizes unmethylated CpG dinucleotides, very common in bacterial DNA, but not in vertebrate genome in which CpG are often methylated (6). Differently from most TLR members, expressed at the cell surface, TLR9 has cytoplasmic localization and resides in the endoplasmic reticulum (ER) of resting cells (6, 7). It has been shown that CpG DNA binds directly to TLR9. After internalization of CpG DNA into a subcellular lysosomal compartment, TLR9 translocates from ER to this CpG DNA-containing lysosomal compartment where TLR9/CpG DNA binding occurs and signal transduction is initiated (7). Synthetic CpG oligonucleotides (CpG ODN) can be generated with specific CpG sequence motifs, as well as with backbone modifications. So far, three different classes of CpG ODN have been synthesized, differing each other for their ability to stimulate and activate different immune cell populations (8).

Neuroblastoma (NB) is the most common extracranial solid tumor of childhood; despite aggressive treatment approaches, the outcome for high risk NB-affected patients remains poor (9, 10). Thus, the identification of new molecular candidates to be developed as novel therapeutic targets represents a priority area of research.
We previously reported that administration of liposomes targeted to NB cells via disialoganglioside GD2 and encapsulating CpG-containing c-myb specific antisense oligonucleotides led to long term survival of NB-bearing mice (11, 12).

Based on previous studies on the expression of TLRs on tumor cells of different lineages, including melanoma that shares the neuroectodermal origin with NB, we hypothesized that NB cells could express TLR9 and, consequently, respond to treatment with CpG DNA. Herein, we investigated the expression of TLR9 on a wide panel of NB cell lines and primary NB specimens and we assessed its functionality and mechanism of action.

This study demonstrates for the first time, to our knowledge, the functional expression of TLR9 in NB and suggests that TLR9 may represent a novel prognostic and/or therapeutic target.

**MATERIALS AND METHODS**

**Liposomes preparation**

Anti-GD2-targeted CpG entrapping liposomal formulation was prepared using the method already described by us for the tumor selective delivery of antisense oligonucleotides (11, 13-15).

**Cell Lines and culture conditions**

The human NB cell lines IMR-32, SH-SY5Y, SK-NSH, SK-NMC, SK-NAS, SK-NF-I, SK-NBE(2), SK-NBE(2c), and the malignant B cell line Raji, were obtained from the ATCC (USA); the HTLA-230 cell line was a gift of Dr. E. Bogenmann (16) (Los Angeles Children’s Hospital, CA, USA); the other human NB cell line GI-LI-N, GI-ME-N, ACN, LAN-1, LAN-5, IMR-5 were obtained from the Biological Bank and Cell Factory (National Cancer Institute, Genoa, Italy). All cell lines were tested for mycoplasma contamination, and characterized by cell proliferation,
morphology evaluation and multiplex short tandem repeat profiling test, both after thawing and within eight passages in culture and used in this time-frame.

Cells were grown in complete medium (Dulbecco’s modified Eagle medium [Sigma], supplemented with 10% fetal bovine serum [FBS; GIBCO-Invitrogen Srl, Carlsbad, CA, USA] and 50 IU/mL sodium penicillin G, 50 µg/mL streptomycin sulphate, and 2 mM L-glutamine [all reagents from Sigma]), as already described (17).

RNA isolation and gene expression analysis

The isolation of total RNA from cell lines was performed by chemical extraction in combination with a silica-based membrane immobilization, using QIAzol and RNeasy mini and micro kit (Qiagen, S.p.A, Milan, Italy). The mRNA expression levels of TLR9 target genes and the GAPDH positive control were analyzed by a two-step real-time RT-qPCR using a random priming-based reverse-transcription (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Austin, TX, USA) and SYBR® Green I binding dye (Platinum® SYBR® Green qPCR SuperMix-UDG, Invitrogen S.r.l.) and data were normalized as previously reported (18).

The comparative Ct method was used for relative quantification of gene expression in wild type and transfected cell lines. Analysis of the data was performed by qGene software (19). (For details see SM).

Cell proliferation Assay

NB cells were plated, in quadruplicate for each treatments, in 96-well plates in complete medium, cultured for 24 hours and then treated with CpG at a concentration of 4 µg/mL. Peripheral blood mononuclear cells (PBMNC) were treated immediately after isolation. CpG were administered to the cells either free or complexed with Lipofectamine™ RNA-iMAX (0.3 µL/well; Invitrogen S.r.l.) for additional 24 hours. Lipofectamine and CpG were complexed according to
manufacturer’s instruction. Hereafter, complexes formed by CpG and Lipofectamine™ RNA-iMAX will be referred to as L-CpG. As controls, cells were also treated with L-ODN-scrambled at the same concentration of L-CpG and Lipofectamine alone. ^H-Thymidine incorporation was quantified as described (20).

**Cell Viability Assay**

NB cells were cultured for 24 hours and then treated with CpG and L-CpG as above for additional 48 hours. At the end of treatment, cells were harvested by scraping, washed with complete medium, and incubated with trypan blue (0.4%; Invitrogen; 1:1) for 1 min at 37°C. Cells were then counted using the Countess™ Automated Cell Counter (Invitrogen S.r.l.) as reported (18, 20).

**Apoptosis Assays**

**Phosphatidylserine detection.** NB cells were plated and treated for 24 hours, as described above. The positivity for Annexin V was detected by the use of a human Annexin V-FITC kit (Bender MedSystems, Vienna, Austria), according to manufacturer’s instructions (18, 20).

**Caspase 3/7 activation.** For the detection of caspase 3-7 cleavage activity, the Apo-ONE® Homogeneous Caspase 3-7 Assay was used (Promega, Madison, WI, USA), according to manufacturer’s instructions.

**Mitochondrial membrane potential assay.** The mitochondrial permeability transition event was evaluated using a MitoPT kit (Immunochemistry Technologies, LLC, Bloomington, MN) according to manufacturer’s instructions as described (20).

(For details see SM).

**Inhibition of TLR9 functionality**
SH-SY5Y and GI-LI-N cells were seeded in 96-well plate (7-9×10^3 cells/well). Inhibitory oligonucleotides class two [iODN (ttaggg)4 (TLRgrade™), Alexis Biochemicals)] were used to block TLR9 functionality. Cells were treated simultaneously with CpG (4μg/mL) and iODN (20 μg/mL). In both cases the administration was performed via Lipofectamine™ RNA-iMAX. Cells were observed after 48 hours, by the use of a contrast phase microscope (Olympus Optical Co LTD, Tokio, Japan), to define morphological changes and photographed. Treated cells were also subjected to evaluation of cell proliferation at 24 hours, as described above.

Silencing of TLR9

NB and Raji cells were seeded in 6-well plate in complete medium. The day after, cells were transfected with siRNA specific for TLR9 (Select Pre-Designed siRNA; Applied Biosystems/Ambion) for 14 hours in serum-free medium. Three different sequences of Select Pre-designed siRNA were tested in silencing experiments; they will be referred hereafter as siRNA1-TLR9, siRNA2-TLR9, siRNA3-TLR9. A siRNA specific for the housekeeping gene GAPDH (siRNA Custom, Applied Biosystems) and one siRNA negative control (Silencer Negative Control #1, Applied Biosystems) were also tested. For details on transfection and evaluation of efficiency of gene silencing see SM.

Western Blot Analisys

Protein lysates were prepared from various NB cell lines (GI-LI-N, SK-NAS, IMR-32, SH-SY5Y, HTLA-230, LAN-5, GI-ME-N) and from either untreated or treated with siRNAs against TLR9, GI-LI-N and HTLA-230 cells, as described (13). (For details see SM).

In Vivo Therapeutic Studies
All experiments involving animals were reviewed and approved by the licensing and ethical committee of the National Cancer Research Institute, Genoa, Italy and by the Italian Ministry of Health. Five-week old female, athymic (nude-nude), SCID-bg mice were purchased from Harlan Laboratories S.r.l. (S.Pietro al Natisone, Italy). NOD/SCID/IL2rg<sup>-/-</sup> lacking the gene for the common gamma chain, a component of the receptor for IL2 and related cytokines were purchased from The Jackson Laboratory (Bar Harbor, Main, USA). Mice were intravenously (i.v.) injected with 2.5×10<sup>6</sup> HTLA-230 as previously described (11). HTLA-230 NB cell line have been chosen because it presents high levels of both GD<sub>2</sub> and TLR9, and thus can be considered as a good target for in vivo studies. Mice were given i.v. injection with CpG (50 μg) either free or encapsulated within NB-targeted liposomes (TL-CpG). Control mice received HEPES-buffered saline. Treatment started 4 hours after cell challenge and continued for two weeks, two days per week, with a three-day interval between courses. Mice were monitored and sacrificed when signs of poor health, such as abdominal dilatation, dehydration, or paraplegia, became evident.

Patients and Collection of Tumor Samples

Fifty NB specimens at the onset, collected from 1987 to 2000, were retrieved from the Italian Neuroblastoma Tissue Bank by the Ethical Committee of the G. Gaslini Children’s Hospital. All patients or their parents gave informed consent. Tumor cell content was consistently at least 80% as assessed by histological analysis. Disease extension was classified according to the International Neuroblastoma Staging System criteria (21). The specimens were from 10 patients with stage 1-2, age <12 months, 10 patients with stage 1-2, age >12 months, 10 patients with stage IVS, and 20 patients with stage IV tumors (10 no NMYC amplified and 10 NMYC amplified).

Immunohistochemistry
Immunohistochemical analyses were performed using a staining method for sections of formalin-fixed, paraffin-embedded tissue on the Bond automated system (Vision BioSystems, Leica, Germany) as detailed in SM.

**Statistical Analyses**

All *in vitro* data are from at least three independent experiments. All the *in vivo* experiments were performed at least twice with similar results. Results are expressed as mean values ± 95% Confidence Interval (CI) for quantitative variables and as numbers and percentages for qualitative ones. For continuous variables, the statistical significance of differential findings between experimental and control groups was determined by ANOVA with the Tukey’s multiple comparison test. The association between categorical variables was assessed by the Fisher’s exact test. Survival curves were constructed using the Kaplan-Meier method and compared by the Peto’s log-rank test. ANOVA and log-rank test were performed by Graph-Pad Prism 3.0 software (Graph-Pad Software, Inc, El Camino Real, San Diego, CA). The remaining analyses were performed by Stata for Windows statistical package (release 9.2, Stata Corporation, College Station, TX, USA). All tests were two-sided and a *P*-value < 0.05 was considered as statistically significant.

**RESULTS**

**Effects of CpG on cell proliferation, cell death and apoptosis**

Data in Figure 1 show that NB cells express TLR9 at both mRNA and protein levels as assessed by RTqPCR, flow cytometry and western blot analysis (Figure 1A, B and C respectively). Thus, to investigate the functionality of TLR9 in NB cells, PBMNC and NB cells were treated with CpG oligonucleotides, at a concentration (4 µg/mL) already shown to be stimulatory on
immunocompetent cells (22). CpG was delivered to cells either free (CpG) or via Lipofectamine™ RNA-iMAX (L-CpG), here used to allow the uptake of CpG into NB cells (23). Figure 2A shows that L-CpG stimulated cell proliferation of PBMNC but inhibited that of GI-LI-N NB cells. Free CpG showed activity only on PBMNC, likely due to the different membrane permeability to oligonucleotides presented by cells of hematopoietic origin with respect to solid tumor-derived cells (23, 24). Furthermore, the effect of L-CpG was specific: indeed, either Lipofectamine alone or L-ODN-scr did not affect cell proliferation.

We then investigated if L-CpG could also induce cell death. As showed in Figure 2B, only treatment with L-CpG induced GI-LI-N cell death, while (L-CpG vs Control, P<0.001). Lipofectamine or L-ODN-scr were not effective.

Cells treated with L-CpG displayed rounding-up, retraction of pseudopodes, reduction of cellular volume and blebbing of the plasma membrane (Figure 2C, left panel). All of these changes are hallmarks of apoptotic cells. The apoptotic cell death was further confirmed by an Annexin-V assay, in which Annexin-V binds to externalized phosphatidylserine on the surface of apoptotic cells. As showed in Figure 2C, right panel, L-CpG treated GI-LI-N contained a significantly higher percentage of Annexin V⁺ apoptotic cells than control (L-CpG vs Control, P<0.001). The addition of a pan-Caspase inhibitor (Z-VAD-FMK) almost completely reverted the L-CpG induced cell apoptosis (L-CpG vs Z-VAD-FMK + L-CpG, P<0.01), indicating the involvement of a caspase-dependent mechanism of programmed cell death. Figure S1 shows the results obtained by treating IMR-32, GI-ME-N, HTLA-230 and LAN-5 as above already described. As confirmed by the Annexin-V assay, all the cell lines tested, responded to treatment with L-CpG undergoing apoptosis.

We next investigated whether the administration of L-CpG induced cleavage of caspase 3 and 7. As clearly showed in Figure 2D, left panel, treatment of GI-LI-N cells with L-CpG for 24 hours resulted in caspase 3 and 7 activation by cleavage. The proteasome inhibitor Bortezomib was used as positive control (20). Pre-treatment of NB cells with Z-VAD-FMK completely prevented
CpG-induced caspase 3 and 7 cleavage. However, pre-treatment of NB cells with the anti-oxidant NAC (inhibitor of reactive oxygen species generation) was not effective in inhibiting caspase 3 and 7 cleavage.

Recently, caspases 3 and 7 have been reported to be key mediators of some mitochondrial events of apoptosis (25). Thus, we investigated if treatment of NB cells with L-CpG was associated to the activation of the intrinsic apoptotic signaling pathway. Treatment of GI-LI-N cells with L-CpG caused a statistically significant decrease in mitochondria membrane potential, with respect to control (L-CpG vs Control, *P*<0.01; Figure 2D, right panel). Pre-treatment with either Z-VAD-FMK or NAC increased the percentage of cells with polarized mitochondria to the level of control. These results indicate that TLR9 triggering with CpG-ODNs inhibit NB cell proliferation and induces cell death by apoptosis.

**TLR9 silencing and functionality**

To try to demonstrate that the abrogation of TLR9 protein result in CpG ODN loss of efficacy, we tested inhibitory ODNs (iODN). The class two iODN here used are entirely composed of TTAGGG multimers designed to block the co-localization of CpG ODNs with TLR9 within endosomal vesicles and to abrogate the signaling cascade downstream to TLR9 (26, 27). This suppressive activity correlates with the ability of TTAGGG motifs to form G-tetrads (26); these iODN have been initially synthesized with the aim to block the activation of immune cells by CpG ODNs (28). They exert the inhibitory effect when administered in molar excess with respect to CpG ODNs. A 5-fold molar excess of iODN, with respect to L-CpG, was able to almost completely revert the anti-proliferative effect obtained by the use of L-CpG on SH-SY5Y NB cells (iODN + L-CpG vs L-CpG, *P*<0.01; Figure 3A). The iODN administered alone, as control, did not affect cell growth. Similar results were also obtained for GI-LI-N cells (data not shown). These results are further supported by the findings showed in Figure 3B. Indeed, while SH-SY5Y cells treated with
L-CpG underwent apoptotic cell death, cells co-treated with iODN, in molar excess with respect to L-CpG, had a morphological aspect similar to untreated control cells.

Silencing experiments were also performed firstly on Raji cell to identify the more efficient TLR9-specific si-RNA sequence and to determine the time point at which the highest TLR9 silencing was reached. As showed in Figure 3C, left panel, the GAPDH housekeeping gene was drastically silenced, in a time dependent manner, reaching its maximum effect at 72 hours. Similarly, TLR9 silencing was time dependent. Nevertheless, a high silencing efficiency was not obtained for all the siRNA sequences used. Then, the three siRNA sequences were used to test their efficiency on a panel of NB cell lines. Also in this case the GAPDH gene was almost completely silenced at 72 hours (Figure 3C, right panel), while the best sequence of siRNA against TLR9 (the number 1) reduced TLR9 expression of about 60%. The silencing efficiency was further investigated by Western blot analysis. GI-LI-N and HTLA-230 cells were transfected with siRNA1-TLR9 and siRNA2-TLR9 and harvested at 72 hours. Both siRNA sequences failed to completely shutdown the expression of the expected full-length TLR9 protein (140 KDa; Figure 3D), as well as of the cleaved isoforms (roughly 70-90 KDa). These last proteins have been reported recently to be functional also in macrophages (29). The expression of these low molecular weight isoforms has been confirmed in NB cells by a different Ab against TLR9 (data not shown).

Furthermore, as showed in Figure 3E, we demonstrated that the amount of TLR9 protein remaining after silencing was still functional and sufficient to respond to L-CpG. Indeed, transfection of GI-LI-N cells for 72 hours with siRNA1-TLR9, only marginally reverted the inhibition of cell proliferation induced by a 24-h treatment with L-CpG.

These results clearly demonstrate that low level expression of TLR9 in NB cells is sufficient to allow full functionality of this receptor.

In vivo effects of CpG

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We next investigated whether CpG had anti-tumor activity in a biologically and clinically relevant pseudo-metastatic mouse model of human NB (11). The \textit{in vitro} delivery of ODNs to solid tumor-derived cells is usually accomplished by carriers such as lipidic vesicles and cationic polymers (23, 24), whose utilization, in turn, can lead to \textit{in vivo} limitations, due mostly, to the lack of specificity for target cells and some systemic toxicity (30, 31).

We have previously developed a standardized method to efficiently deliver ODNs to neuroectodermal tumors via disialoganglioside, GD\textsubscript{2} (13). GD\textsubscript{2} is abundantly expressed at the cell surface of NB cells, while its expression in normal tissues is very limited (32). MAbs recognizing GD\textsubscript{2} bind to NB cells with high affinity and specificity and are rapidly internalized after binding, thus becoming an excellent tool for liposome-mediated tumor targeting (33). We therefore decided to use GD\textsubscript{2}-targeted liposomes as vehicles for the \textit{in vivo} intracellular delivery of CpG into GD\textsubscript{2}+ human NB cells. The high selectivity of this approach is guaranteed by the lack of GD\textsubscript{2} expression in any mouse tissue (34).

NB-targeted liposomes containing CpG (TL-CpG) were thus administered \textit{in vivo} for therapeutic purposes. As showed in Figure 4A, both CpG and TL-CpG significantly prolonged the survival of tumor-bearing Nude mice ($P<0.0001$). Interestingly, mice treated with TL-CpG lived longer than those treated with CpG ($P=0.0154$), being all animals cured six month after NB cell inoculation. This impressive anti-tumor effect obtained with TL-CpG is likely due to a dual attack of tumor cells. First, liposomal formulations containing CpG-ODNs activate the host immune system to kill NB cells through a NK cell-dependent mechanism (11). Second, TL-CpG act directly on NB cells binding to TLR9 and causing their apoptosis. In SCID-bg mice, lacking of B and NK cells (11), similar results were unexpectedly obtained. Indeed, at 6 moths after tumor challenge, animals treated with TL-CpG were all alive (TL-CpG \textit{vs} Control $P=0.0004$), while about 40% of long term survival was obtained for those treated with free CpG (Figure 4B; TL-CpG \textit{vs} CpG $P=0.023$). Although in our previous work (11), administration of liposomal CpG-containing
antisense oligonucleotides to tumor-bearing SCID-bg mice lost its anti-tumor effectiveness, the present finding is in agreement with other studies showing that, upon activation with CpG, macrophages acquired cytotoxic potential against tumor cells (35). To definitively overcome the influence of the immune system in our therapeutic results, and to distinguish the direct effects of TL-CpG on tumor cells, we thus used NOD/SCID/IL2rg⁻/⁻ mice. This mouse model lacks of B and NK cells and presents macrophages with an impaired functionality (36). In this mouse model, treatment with CpG alone was completely ineffective with respect to control animals, while, TL-CpG treatment still maintained anti-tumor efficacy leading to an increased life span with respect to controls (Figure 4C, P=0.0002).

**TLR9 expression in primary Neuroblastoma tumors**

We analyzed by IHC, 50 NB specimens at different stages as detailed in Material and Methods. NB specimens were scored as negative, weak, moderate or strong with respect to the expression of TLR9.

Figures 5A,a and b, show a negative control stained with an isotype-matched irrelevant antibody and a TLR9 positive control, respectively, both from a pulmonary adenocarcinoma specimen. TLR9 positivity was graded by the intensity of cytoplasmic staining. Panels c to f show representative micrographs of TLR9 expression in NB specimens. Specifically, Panel c is negative for TLR9 expression and representative of a stage 4, MYCN amplified tumor. Panel e shows weak TLR9 expression in a stage 4, MYCN non-amplified tumor. Panel f shows moderate TLR9 expression in a stage 4S patient and, finally, Panel d represents strong TLR9 expression from a stage 1 tumor.

Histogram representation (Figure 5B) gives a semi-quantitative scoring of the immunostaining, assessed by the Quick Score method, adapted from that commonly used for breast carcinomas (37). This Panel clearly shows that the expression of TLR9 is inversely related to the
stage of the disease. Indeed, the highest expression of TLR9 is present in stage 1-2 and stage 4S NB patients, while about 50% of stage 4 patients are completely negative. The association between TLR-9 and clinico-pathological features is also illustrated in Table 1. High TLR-9 expression was prevalent in stage 1-2 (100%) and stage 4S (80%) patients, while much lower TLR-9 expression was observed in the Stage 4 group (85%, \(P < 0.0001\)). Furthermore, high TLR-9 expression was associated with low age at diagnosis (\(P = 0.018\)) and normal \(MYCN\) status (\(P = 0.030\)).

**DISCUSSION**

The significance of TLR9 expression and its biological impact in tumor cells is still far to be completely understood. The aim of this study was to investigate expression, functionality and biological relevance of TLR9 in Neuroblastoma. Here, we demonstrated, for the first time to our knowledge, that NB cells express TLR9, both at the mRNA and protein levels. In this respect, a previous report showed that human Neuroblastoma cells express also an intracellular form of TLR4 (38).

Previous reports indicate that triggering of TLR9 expressed by different malignant cells can lead to opposite effects, i.e. stimulation of tumor progression or inhibition of tumor growth (4). Indeed, in TLR9-expressing human prostate cancer cells (39), treatment with CpG damped tumor growth and proliferation and induced apoptosis, while it prolonged the survival of glioma-bearing mice (40). Moreover, Ren at al (41) demonstrated that human lung cancer cells expressed functional TLR9 and its stimulation enhanced their metastatic potential.

In this paper, we demonstrate that TLR9 expression has a functional impact on NB cell growth since stimulation of this receptor with CpG triggered tumor cell death. The latter was attributable to apoptosis, as demonstrated by the involvement of caspases 3 and 7. Since apoptosis may occur through activation of different and independent pathway, we also investigated...
mitochondrial cell death. Indeed, treatment of NB cells with CpG resulted in the depolarization of mitochondrial membrane potential; this event is thought to contribute to cell death through the disruption of the normal function of mitochondria (42). Although caspases 3 and 7 have been so far considered as effector caspases downstream to mitochondrial related apoptosis, it was recently reported that the above caspases are crucial for apoptotic cell death (25). We found that pretreatment of NB cells with a pan-caspase inhibitor prevented CpG-induced caspase 3 and 7 cleavage activation as well as the depolarization of mitochondrial membrane potential. Nevertheless, the use of the anti-oxidant NAC prevented mitochondrial membrane depolarization, but not caspase 3 and 7 activation. These findings are consistent with a previous report (25) showing that caspases 3 and 7 work up-stream the mitochondrial pathway and suggest that free radicals could be one of the intermediate in this pathway of apoptosis.

The functionality of TLR9 in NB was further proved with experiments in which inhibitory oligonucleotides abrogated CpG-mediated apoptosis. Silencing experiments were also performed to support this finding. However, none of the three TLR9-specific siRNA sequences completely abrogated TLR9 expression. This finding is consistent with a recent report, in which investigators, by using RNA interference, down-regulated only partially the expression of TLR9 in dendritic cells. Nevertheless, such down-regulation was sufficient to abrogate the effects of CpG ODNs in the induction of dendritic cells maturation (43). In contrast, we observed that all NB cell lines transfected with siRNA against TLR9 retained the ability to respond to CpG, undergoing apoptosis. Thus, also NB cells expressing very low amount of TLR9 may be responsive to treatment with CpG-ODNs. This conclusion is supported by the lack of correlation between TLR9 expression and apoptotic response to L-CpG observed in the various NB cell lines, further underlining that very little TLR9 seems to be needed for maximal CpG responses.

*In vivo* experiments were performed in a biologically and clinically relevant mouse model of human NB (11). Since the most frequently used DNA carriers lack specificity to target cells, we
have generated tumor-targeted delivery of CpG-ODNs, specific for NB cells, via anti-GD₂-targeted stealth liposomes (herein called TL-CpG), which have been shown to be the most efficient and safe envelop packaged way to transfer nucleic acid into neuroectodermal tumors cells (11, 13).

TL-CpG showed higher anti-tumor effect in NB animal models than CpG, becoming a candidate for further clinical development. In the last few years, nanomedicine has turned into a rapid growth research area, particularly for anticancer applications. Several nanomedicines, primarily lipid-based drug carriers such as DOXIL®/Caelyx®, have received clinical approval and several lipid-based and polymeric carriers are undergoing clinical evaluation (44). A logical extension of this success is to further improve the anti-tumor effects of liposomal nanomedicines, in a more selective manner, through the use of “active-targeting moieties”, coupled to their external surface. Receptor-mediated internalization of nanomedicines into tumor cells is mandatory for improved therapeutic efficacy of targeted-liposomal drugs (45). To this regard, the disialoganglioside GD₂ is an internalizing receptor selectively expressed by neuroectodermal-derived tumor cells only of human origin (32-34, 46, 47), including the HTLA-230 cell line used in this study. Our results indicated that liposomal CpG were able to enter into the NB cells when targeted via the anti-GD₂ mAb coupled at the external surface of the nanoparticles, but not in the absence of the targeting agent, as already showed for antisense oligonucleotides and different anti-tumor drugs (11, 13). These results support the direct effect of CpG on TLR9 expressed by NB cells.

TL-CpG triggered long term survival of NB-bearing Nude mice, significant superior to that achieved administering CpG alone. The above result could be due to a dual mechanism of action of TL-CpG. Indeed, liposomes containing CpG-ODNs can also work as activators of the immune system, giving rise to a cascade of events that culminate in the activation of NK cells and consequent tumor cells killing, carried out by NK cells themselves (11). Furthermore, CpG and TL-CpG showed both efficacious in SCID-bg mice, even if at different extent, confirming the
implication of the immune system in the anti-tumor result. Since SCID-bg mice lack of B and NK cells our data could be explained by macrophages intervention upon activation with CpG (35). The definitive abrogation of immune system implication in therapeutic results was obtained by the use of NOD-SCID-IL2rg<sup>−/−</sup>, that beside NK and B cells deficiency further present the myeloid lineage with an impaired functionality (48). Indeed, it has been suggested that macrophages in NOD mice are functionally immature. As expected, the only effective treatment in this model was TL-CpG, that led to an increased life span. Thus, the potent anti-tumor effect of TL-CpG seems to be also due to a direct anti-tumor effects of CpG on NB cells that, as demonstrated in this study, express TLR9 as a functional intracellular “death receptor”.

Investigations of TLR9 expression in Neuroblastoma patients has highlighted an inverse correlation between the stage of the disease and the levels of TLR9 expression (<i>P</i>&lt;0.0001). Low stages of NB disease are indeed characterized by moderate to strong TLR9 expression, while high stages are either negative or weakly/moderately positive. The strong expression of TLR9 in stage IV S and stage 1-2 tumors from patients younger than 12 months may lead to speculate that TLR9 could be one of the molecules involved in trans-differentiation, apoptosis and/or spontaneous regression of low stage NB (9, 10). This hypothesis may be supported by the correlation between high TLR9 expression and age lower than 12 months (<i>P</i>=0.018). In contrast, NB cells lacking TLR9 (advanced stages) may be prone to tumor progression.

Studies are ongoing to verify in larger cohort of NB patients whether the expression of TLR9 correlates also with the outcome of the disease. In such case, TLR9 could become a new prognostic marker for NB.

In conclusion, this study demonstrated for the first time that TLR9 is expressed by NB, is functional and has a biological impact on tumor growth, thus suggesting that TLR9 can be considered as a new molecular target for Neuroblastoma treatment.
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REFERENCES


### Table 1. Association between clinicopathologic variables and TLR-9 expression in 50 neuroblastoma patients

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<td>3  15.0</td>
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<tr>
<td>&lt;12 months</td>
<td>4 18.2</td>
<td>18 81.8</td>
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<tr>
<td>&gt;12 months</td>
<td>15 53.6</td>
<td>13 46.4</td>
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<tr>
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<td>No amplified</td>
<td>7  70.0</td>
<td>3  30.0</td>
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<sup>a</sup>TLR-9 expression was dichotomized into low (score negative + weak) and high (moderate + strong) groups for analysis.

<sup>b</sup>Fisher exact test (two-sided).

<sup>c</sup>Stage [International Neuroblastoma staging system (30)].
FIGURE LEGENDS

Figure 1: TLR9 expression in NB cell lines. A) mRNA expression of TLR9, expressed as arbitrary units, was evaluated by RT-qPCR on Raji cells and Neuroblastoma cell lines. B) The intracellular protein expression of TLR9 was investigated by flow cytometry. The expression was defined as mean ratio fluorescence intensity (MRFI) over control (cells stained with an isotype matched non specific Ab). MRFI equal to one means no expression. Experiments were performed three times in triplicate. C) TLR9 protein expression was investigated by western blot, on a panel of NB cell lines.

Figure 2: Effects of CpG on NB cell lines. A) Cell proliferation defined as percentage of control level, was determined in PBMNC and GI-LI-N cells after a 24-h treatment with CpG alone, Lipofectamine alone, L-CpG and L-ODN-scr. B) The mean percentage of dead GI-LI-N cells, treated as above, was determined by trypan blue dye exclusion assay. C) Left panel. Microphotographs representative of morphological changes induced in GI-LI-N cells by treatment with L-CpG for 48 hours. Scale bar 100 μm. C) Right panel. The mean percentage of apoptotic GI-LI-N cells was examined by the use of an Annexin-V FITC kit. D) Left panel. Caspase 3/7 activity was determined on GI-LI-N cells treated as above. Data are expressed as Relative Fluorescence Unit (RFU). D) Right panel. The mitochondrial membrane potential was evaluated on GI-LI-N cells treated as above. (*P<0.05; **P<0.01; ***P<0.001). In C and D the anti-oxidant N-acetyl-cysteine (NAC) and the pan-caspase inhibitor Z-VAD-FMK were also used. In D Bortezomib (BTZ) was used as positive control for apoptosis induction.

Figure 3: Silencing of TLR9 and inhibition of TLR9 functionality. A) SH-SY5Y cells were co-treated for 24 hours with L-CpG and iODN. Cell proliferation was assessed by ^3^H-Thymidine incorporation. B) Microphotographs representative of morphological changes induced on SH-
SY5Y cells by the different treatments at 48 hours. Scale bar 150 μm. C) TLR9 mRNA expression was evaluated after transfection with different siRNAs of Raji (left panel) and Neuroblastoma cells (right panel), respectively. D) GI-LI-N and HTLA-230 cells were transfected for 72 hours with siRNA1 and siRNA2 specific for TLR9. The amount of TLR9 protein was determined by Western blot. *, not specific band. E) GI-LI-N cells transfected with siRNA1 were subjected to L-CpG treatment for further 24 hours. Cell proliferation was determined by ³H-Thymidine incorporation. (n.s. means not significant; ***P<0.001).

**Figure 4:** Survival curves of NB-bearing mice treated with TL-CpG. A) Nude (n = 14), B) SCID-bg (n=7) and C) NOD/SCID/IL2rg⁻/⁻ (n=7) mice were injected with HTLA-230 NB cells into the tail vein and treated as reported in the M&M Section.

**Figure 5:** Immunohistochemical analyses of TLR9 expression in NB specimens. A,a) Negative control from pulmonary adenocarcinoma specimen. A,b) Positive control from pulmonary adenocarcinoma specimen. A,c) Stage 4, MYCN amplified, NB specimen, negative for the expression of TLR9. A,d) Stage 1 NB specimen, with strong TLR9 expression. A,e) Stage 4, MYCN non-amplified, NB specimen with weak with weak TLR9 expression. A,f) Stage 4S NB specimen with moderate TLR9 expression. B) Semiquantitative scoring of the immunostaining represented as histograms. Scale bar 200 μm.
(Figure 2)
Figure 3
(Figure 4)
(Figure 5)
Therapeutic targeting of TLR9 inhibits cell growth and induces apoptosis in neuroblastoma

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