Cancer Research

TLR9 Agonists Oppositely Modulate DNA Repair Genes in Tumor versus Immune Cells and Enhance Chemotherapy Effects

Michele Sommariva[1], Loris De Cecco[2], Michelandrea De Cesare[3], Lucia Sfondrini[1], Sylvie Ménard[4], Cecilia Melani[5], Domenico Delia[6], Nadia Zaffaroni[3], Graziella Pratesi[3], Valentina Uva[1], Elda Tagliabue[4], and Andrea Balsari[1,4]

Abstract

Synthetic oligodeoxynucleotides expressing CpG motifs (CpG-ODN) are a Toll-like receptor 9 (TLR9) agonist that can enhance the antitumor activity of DNA-damaging chemotherapy and radiation therapy in preclinical mouse models. We hypothesized that the success of these combinations is related to the ability of CpG-ODN to modulate genes involved in DNA repair. We conducted an *in silico* analysis of genes implicated in DNA repair in data sets obtained from murine colon carcinoma cells in mice injected intratumorally with CpG-ODN and from splenocytes in mice treated intraperitoneally with CpG-ODN. CpG-ODN treatment caused downregulation of DNA repair genes in tumors. Microarray analyses of human IGROV-1 ovarian carcinoma xenografts in mice treated intraperitoneally with CpG-ODN confirmed *in silico* findings. When combined with the DNA-damaging drug cisplatin, CpG-ODN significantly increased the life span of mice compared with individual treatments. In contrast, CpG-ODN led to an upregulation of genes involved in DNA repair in immune cells. Cisplatin-treated patients with ovarian carcinoma as well as anthracycline-treated patients with breast cancer who are classified as "CpG-like" for the level of expression of CpG-ODN modulated DNA repair genes have a better outcome than patients classified as "CpG-untreated-like," indicating the relevance of these genes in the tumor cell response to DNA-damaging drugs. Taken together, the findings provide evidence that the tumor microenvironment can sensitize cancer cells to DNA-damaging chemotherapy, thereby expanding the benefits of CpG-ODN therapy beyond induction of a strong immune response. *Cancer Res; 71(20); 6382–90. ©2011 AACR.*

Introduction

The mammalian innate immune system identifies the presence of infection through recognition of pathogen-associated molecular patterns expressed by a diverse group of infectious microorganisms. Various pattern recognition receptors are involved in pathogen-associated molecular pattern identification, among which the Toll-like receptor (TLR) family of at least 10 different members in humans (1, 2) is probably the best known. These receptors are considered sensors for microbial infections or other danger signals and, together with other molecular sensors, serve as a first line of defense, inducing soluble and cellular mediators of innate immunity and initiating key steps of the adaptive immune response (3). Particular clinical interest now revolves around TLR9, which is expressed not only on cells of the immune system but also on endothelial cells, fibroblasts, and epithelial cells (3–6) and which recognizes bacterial and viral DNA with unmethylated CpG motifs. Synthetic oligodeoxynucleotides (ODN) expressing CpG motifs mimic the immunostimulatory activity of bacterial DNA and are commonly used to activate TLR9 for therapeutic applications (3). CpG-ODN has shown antitumor activity in different animal models (7, 8) and in patients with malignant melanoma, renal carcinoma, and recurrent or refractory lymphoma (9–12); however, experimental studies suggest that CpG-ODN may be more useful as a component of multiagent therapy for cancer rather than as a single agent (13). Because chemotherapy is known to be immunosuppressive, it may seem counterintuitive to combine it with TLR9 stimulation. Nevertheless, different chemotherapeutic drugs have been reported to improve the efficacy of CpG-ODN in mouse tumor models (14, 15). In immunocompetent mice, paclitaxel and cyclophosphamide enhanced CpG-ODN effects by depleting regulatory T cells, increasing the immunogenicity of tumor cells, and changing T-cell homeostasis; moreover, the presence of $CD8^+$ T cells was found to be required (16). In immunocompromised athymic mice, the mechanisms

Authors' Affiliations: [1]Department of Human Morphology and Biomedical Sciences "Città Studi", Università degli Studi di Milano; and [2]Functional Genomic Core Facility, [3]Molecular Pharmacology Unit, [4]Molecular Targeting Unit, [5]Scientific Directorate, and [6]Molecular Mechanisms of Cell Cycle Control Unit, Fondazione IRCCS—Istituto Nazionale Tumori, Milan, Italy

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Corresponding Author: Andrea Balsari, Department of Human Morphology and Biomedical Sciences "Città Studi", Università degli Studi di Milano, via Mangiagalli 31, Milan 20133, Italy. Phone: 39-0223902564; Fax: 39-0223902692; E-mail: andrea.balsari@unimi.it

doi: 10.1158/0008-5472.CAN-11-1285

underlying the improved antitumor effect when CpG-ODN was combined with gimatecan (17), gemcitabine (6), or topotecan (18, 19) have to be different from those in immunocompetent mice and remain unclear. It is noteworthy that, although these 3 chemotherapeutic agents differ in mechanisms of action, their cytotoxic activity is generally the consequence of DNA damage. Even ionizing radiotherapy (RT), generally administered locally to the tumor site or draining lymph nodes, kills cancer by damaging DNA, and experiments in murine models suggest that CpG-ODN can enhance the response to RT in both immunogenic (20, 21) and nonimmunogenic tumors (22). For nonimmunogenic tumors, the mechanism remains undefined, as the rationale for even exploring the combination of CpG-ODN with radiation, that is, that dendritic cells acquire antigens released from tumor cells after RT and migrate to regional lymph nodes where they encounter and activate tumor-specific cytotoxic cells, does not apply.

Unlike observations in tumor cells where CpG-ODN seems to increase the activity of RT, studies on the combination of RT and CpG-ODN in mice have shown that TLR9 engagement on $CD4^+$ T lymphocytes reduces apoptosis and enhances their capacity to repair DNA damage induced by γ-radiation (23).

On the basis of these disparate observations, we hypothesized that CpG-ODN modulates genes involved in DNA repair, increasing their expression in TLR9-expressing immune cells but downregulating their expression in tumor cells and thereby increasing sensitivity to DNA-damaging chemotherapeutic agents. To test this hypothesis, we analyzed the effect of CpG-ODN treatment on DNA repair gene expression in immune and tumor cells *in silico* and in our previously described model of IGROV-1 ovarian tumor–bearing athymic mice (24) and assessed the antitumor effect of CpG-ODN associated with DNA-damaging chemotherapy in this mouse model.

Materials and Methods

Cell lines

The IGROV-1 tumor (gift from Dr. J. Benard, Institute Gustave Roussy, Villejuif, France) and OVCAR-5 (American Type Culture Collection) were adapted to grow intraperitoneally (i.p.) and maintained by serial intraperitoneal passage of ascitic cells into healthy mice as previously described (25). Every 6 months, cells were authenticated by morphologic inspection and by the presence of specific markers with fluorescence-activated cell-sorting analysis. For *in vitro* experiments, cells were maintained in RPMI-1649 medium supplemented with 10% fetal calf serum (Sigma) and 2 mmol/L glutamine (Cambrex) at 37°C in a 5% CO_2 air atmosphere.

In vitro and *in vivo* experiments for microarray analyses

For microarray experiments, mice were injected i.p. with 2.5×10^6 ascitic cells in 0.2 mL of saline and treated, starting 11 to 12 days later, when mice showed evident and established ascites, with CpG-ODN delivered i.p. at a dose of 20 μg per mouse daily for 3 days. Control mice received saline. Ascites-bearing mice were sacrificed by cervical dislocation 24 hours after the last treatment with saline or CpG-ODN. Tumors adherent to omentum were removed and immediately frozen in liquid nitrogen until RNA or protein extraction.

For microarray experiments, to evaluate a direct action of CpG-ODN on tumor cells, 1×10^6 IGROV-1 cells were seeded in 6-well plates and treated with 10 μmol/L of CpG-ODN in complete culture medium for 24 hours. At the end of treatment, cells were collected and RNA was extracted.

To define whether local treatment at the tumor site is critical to downregulate DNA repair genes, mice were injected i.p. with IGROV-1 tumor cells as described earlier and treated i.p. or s.c. with CpG-ODN at a dose of 20 μg per mouse daily for 3 days. Twenty-four hours after the last treatment with saline or CpG-ODN, ascites-bearing mice were sacrificed and tumors adherent to the peritoneal wall were removed and immediately frozen in liquid nitrogen until RNA extraction.

Results

DNA repair gene modulation in immune and nonimmune normal cells by CpG-ODN

To evaluate the effect of CpG-ODN on DNA repair genes in immune cells, we conducted a comprehensive *in silico* expression analysis of genes implicated in DNA repair (GSE11202) in immune spleen cells from mice treated i.p. with CpG-ODN (26). Spleen cells were chosen for this analysis on the basis of previous studies establishing that the spleen accurately reflects the breadth of immunity induced by CpG-ODN *in vivo* (27–32). Expression levels of mRNA in mouse spleen cells were monitored by microarray at different times after *in vivo* CpG-ODN treatment. From a list of 209 genes retrieved according to the "DNA repair" term from Gene Ontology (GO:0006281 mouse), 189 were present in the GSE11202 and 49 genes were found to be significantly modulated [false discovery rate (FDR) < 0.05] during the course of CpG-ODN treatment, 43 of which were upregulated (Fig. 1). Accordingly, analysis of a published gene expression data set (E-TABM-823) in immune mucosal lung tissue of mice 48 hours after intranasal administration of CpG-ODN, when CpG-ODN-induced recruitment of natural killer and dendritic cell into the bronchoalveolar spaces peaked (33), identified 29 genes in the DNA repair pathway that were significantly modulated by CpG-ODN treatment (FDR < 0.05), 21 of which were upregulated (Supplementary Fig. S1). It is noteworthy that 13 of 29 modulated genes were shared with those modulated in immune spleen cells. Thus, the presence of microbial DNA seems to induce upregulation of genes involved in DNA repair in immune cells.

To test whether CpG-ODN similarly affects nonimmune TLR9-negative normal cells, we conducted expression analysis of DNA repair genes using a whole-mouse genome microarray data set (E-TABM-506), obtained from murine quadriceps muscle injected with CpG-ODN in experiments to evaluate this biodrug as a vaccine adjuvant (34); only 2 genes, NBR and FAM175, for which the specific function of the encoded proteins is unknown, were modulated (FDR < 0.05; data not shown). Thus, CpG-ODN does not induce significant modulation of DNA repair genes in nonimmune normal cells.

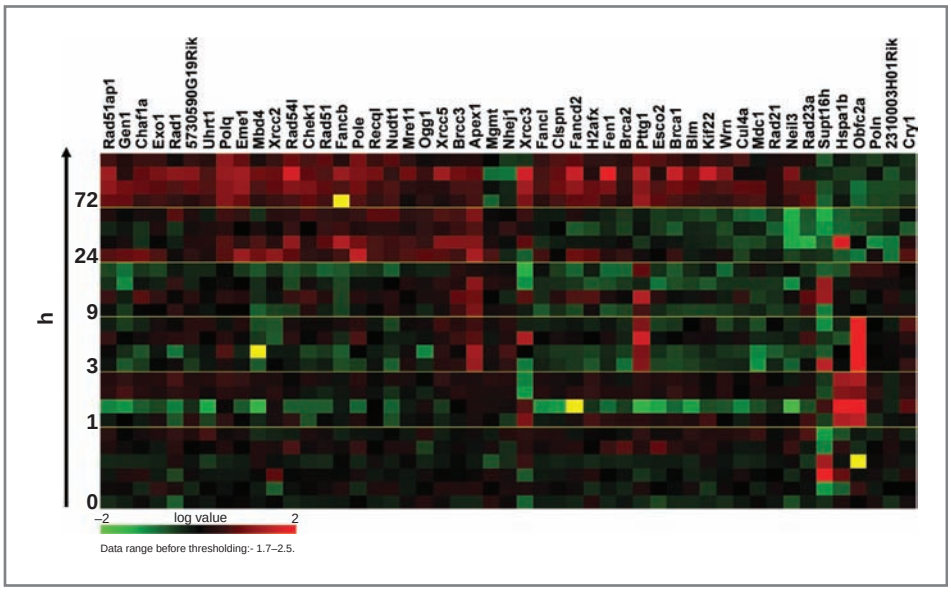


Figure 1. Microarray analysis of DNA repair pathway genes in spleen cells from CpG-ODN–treated mice. Mice were treated i.p. with CpG-ODN. RNA was extracted from spleen cells 1, 3, 9, 24, and 72 hours after treatment and analyzed in-house–assembled oligonucleotide microarray platform. Of 209 genes involved in the DNA repair pathway (GO:0006281 mouse), 189 were present in the GSE11202 data set and 49 of these genes showed significant modulation (FDR < 0.05) compared with that in untreated control mice (0 hour). Color coding for each gene is normalized to the mean of the arrays for untreated controls (0 hour). Black represents no change compared with controls and green and red represent down- and upregulation with respect to the first time point, respectively. Changes from green to red to green indicate initial downregulation, increased expression, and final downregulation, respectively. Missing values are in yellow. Each row represents a sample and each column, a gene.

DNA repair gene modulation in tumor cells by CpG-ODN

The potential effect of CpG-ODN in tumor cells was evaluated on the basis of the expression of the corresponding GO:0006281 mouse genes in a data set obtained from MC38 murine colon carcinoma cells in mice injected intratumorally with CpG-ODN (GSE18203; ref. 35). CpG-ODN treatment was found to modulate DNA repair gene expression in tumors, and 50 genes were modulated at a threshold of $P < 0.05$. However, unlike observations in immune cells, CpG-ODN treatment induced mainly a downregulation of DNA repair genes in tumor cells (40 of 50 modulated genes were downregulated; Fig. 2A and B). These findings were experimentally proofed by microarray analyses of human IGROV-1 ovarian carcinoma xenografts in mice treated daily i.p. with CpG-ODN or saline beginning 3 days after evidence of ascites. Twenty-four hours after the final treatment, tumors adhering to omentum were collected, and RNA extracted from the tumors cells was

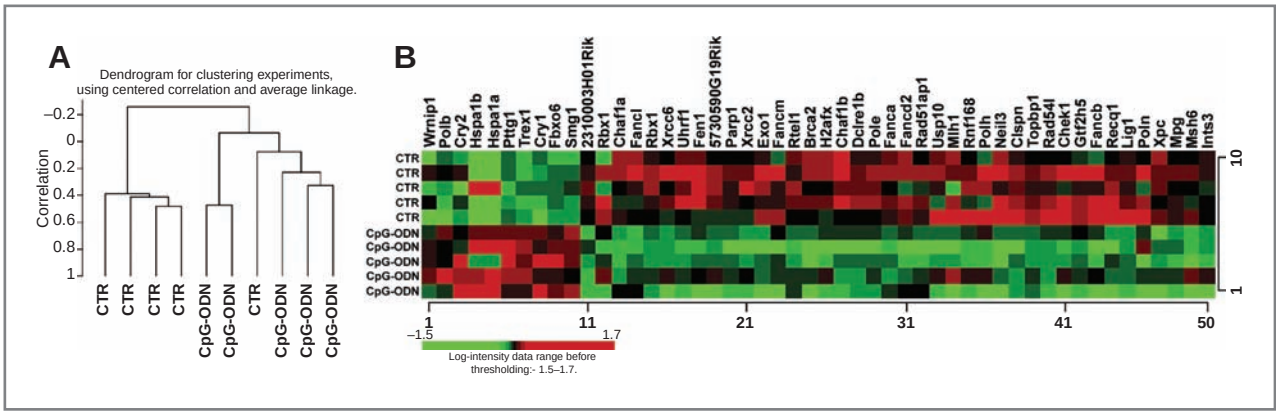
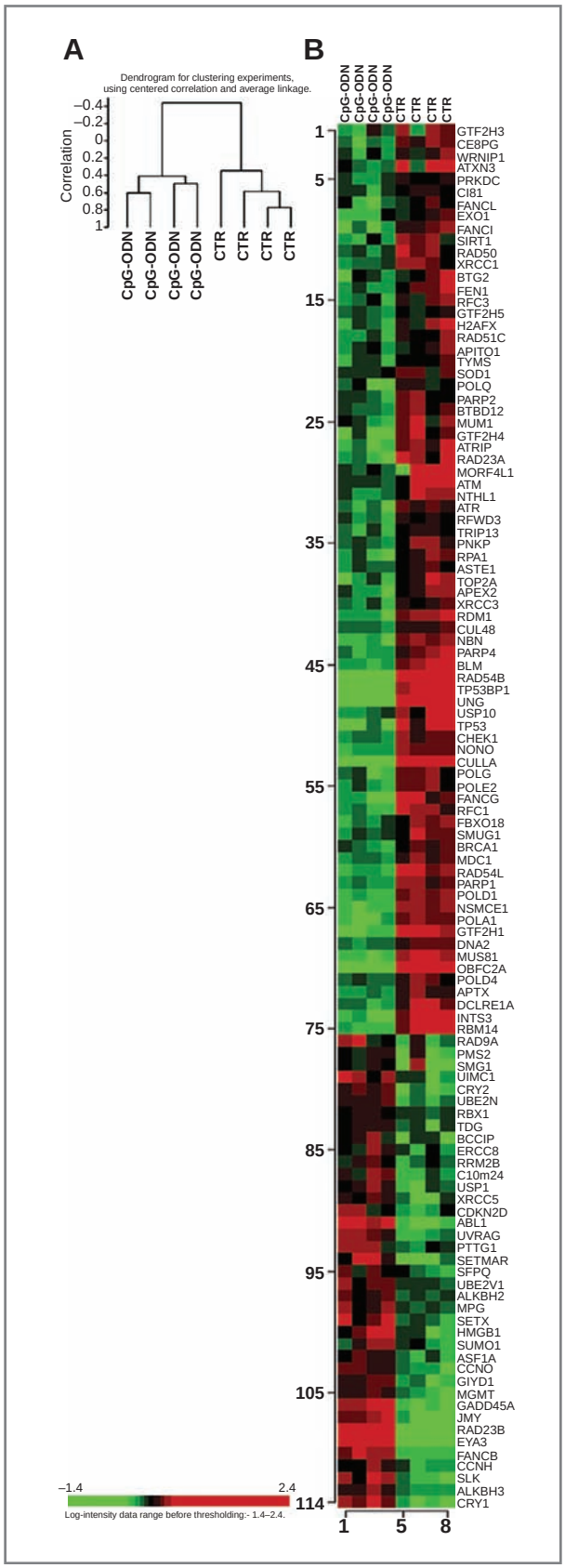


Figure 2. Microarray analysis of DNA repair pathway genes in MC38 murine colon tumors from CpG-ODN–treated mice. Mice bearing s.c. MC38 tumors were injected intratumorally with CpG-ODN or saline (control group). RNA extracted from tumors 6 hours after treatment was analyzed on Affymetrix Platform using Affymetrix Mouse gene 1.0 ST chips; 201 genes in the DNA repair pathway (GO:0006281 mouse) were detected in the Affymetrix Mouse Array (GSE18203). A, unsupervised hierarchical clustering of tumors according to the expression levels of 201 DNA repair genes. B, heat map of modulated genes, 40 down- and 10 upmodulated (threshold $P < 0.05$), in CpG-ODN–treated mice (red, upregulated genes; green, downmodulated genes). Each row represents a sample and each column, a gene.



analyzed for gene expression profile. Among the 232 genes belonging to GO:0006281 human, 227 genes available in our microarray platform clustered tumors according to saline or CpG-ODN treatment (GSE23441; Fig. 3A), and the pattern of this gene modulation in CpG-ODN–treated mice reflected an increased susceptibility to DNA damage (75 of 114 genes modulated at a threshold of $P < 0.05$ were downregulated; Fig. 3B). The power of CpG-ODN in modulating cancer cell DNA repair genes was confirmed by Ingenuity Pathway Analysis (IPA), as IPA of 1,765 up- or downmodulated genes by CpG-ODN compared with controls revealed that the cell death, embryonic development, DNA replication, recombination, and repair pathway were the most influenced (Supplementary Fig. S2). Quantitative real-time PCR (qRT-PCR) of *RAD51C, SIRT1, RAD54B,* and *RAD23B* genes validated microarray data (Supplementary Fig. S3).

Results of microarray analyses were also validated by examining the expression of the gene products RAD51 and SIRT1 in tumors from CpG-ODN–treated and control mice. Western blot analysis of tumor cell lysates indicated decreased expression of RAD51 and SIRT1 proteins in treated mice as compared with controls (Fig. 4A and B). Thus, microarray analyses indicate that locally administered TLR9 agonists regulate genes involved in DNA repair in tumor cells in the opposite way from immune cells.

Expression of CpG-ODN–modulated DNA repair genes and survival in ovarian and breast cancer patients treated with DNA-damaging therapy

To evaluate whether CpG-ODN-induced DNA repair gene modulations, observed in IGROV-1 microarray analysis, were relevant to increase the cell sensitivity to DNA damages, among the genes found differentially modulated between the CpG-ODN–treated and untreated IGROV-1 tumors, a set of 27 genes with a level of FDR less than 0.01 and a fold difference of more than 1.5 was selected (Supplementary Fig. S4) and the average expression of both CpG-ODN–treated and untreated tumors was calculated for each gene; the resulting expression pattern was used to analyze an ovarian microarray data set containing the gene expression data of ovarian cancer samples obtained at initial cytoreductive surgery from patients who then received platinum-based primary chemotherapy (36). The Pearson correlation coefficient between expression values of the 27 genes and those available in

Figure 3. Microarray analysis of DNA repair pathway genes in IGROV-1 ovarian tumors from CpG-ODN–treated athymic mice. IGROV-1–bearing mice with established ascites, that is, increased abdominal volume and body weight, were treated i.p. daily for 3 days with CpG-ODN or saline (control group) and sacrificed 24 hours later. RNA extracted from tumors was analyzed on Illumina human whole-genome bead chips; 227 genes in the DNA repair pathway (GO:0006281 human) were detected in our microarray experiment. A, unsupervised hierarchical clustering of tumors according to expression levels of 227 DNA repair genes. B, heat map of modulated genes, 75 down- and 39 upmodulated (threshold $P < 0.05$), in CpG-ODN–treated mice; (red, upregulated genes; green, downmodulated genes). Each column represents a sample and each row, a gene.

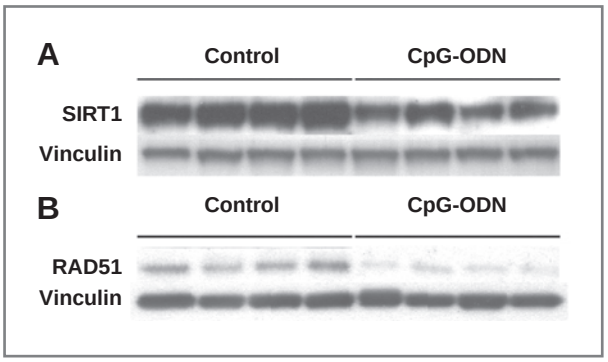


Figure 4. Western blot analysis of DNA repair proteins in IGROV-1 tumor cells adhering to the peritoneal wall after i.p. injection of CpG-ODN. Protein expression levels of SIRT1 (A) and RAD51 (B) in IGROV-1 ovarian cancer cells are shown from athymic mice treated daily for 3 days with CpG-ODN or saline (4 mice per group). Vinculin was used to normalize protein loading per lane.

the ovarian data set was calculated for both the CpG-ODN–treated and untreated conditions; the difference was tested for its association with clinical outcome in Kaplan–Meier survival analysis, with patients grouped depending on correlation values. Two groups were identified as CpG-ODN–treated-like cases (named "CpG-like") and CpG-ODN–untreated-like cases (named "CpG-untreated-like"). Analysis of the data set for available clinical data revealed no association of CpG-like feature with grade and debulking (Supplementary Table S1A).

As shown in Fig. 5A, patients of the CpG-like group showed a significantly increased overall survival compared with the CpG-untreated-like group. Cox proportional hazard analysis confirmed above-mentioned results [HR = 0.5949; 95% confidence interval (CI), 0.3663–0.9661; $P = 0.0367$]. Multivariate Cox proportional model indicated that CpG-like signature resulted in independent association with overall survival (HR = 0.5749; 95% CI, 0.346–0.9553; $P = 0.0335$; Supplementary Table S1B). A cross-validation procedure removing each single gene from the gene set and the Cox analysis on the remaining gene set revealed that the prediction performance is not related to a particular gene, as in all analyses a value of $P < 0.05$ was observed (Supplementary Fig. S5), even though *RAD23B* is the gene that mainly influences the significance of statistical analysis.

To test the relevance of *RAD23B* in the sensitivity to DNA-damaging agents in IGROV-1 cell line, we downregulated RAD23B protein levels by specific siRNA transfection (Supplementary Fig. S6A). *In vitro* cisplatin cytotoxicity was significantly reduced ($P < 0.0001$) by downregulation of RAD23B (Supplementary Fig. S6B).

When Kaplan–Meier survival analysis was conducted on a breast cancer data set of whole-genome expression of patients who received adjuvant DNA–damaging chemotherapy after surgery (37), patients classified as CpG-like showed a significantly increased relapse-free and overall survival compared with CpG-untreated-like patients (Fig. 5B); on the contrary, no outcome difference was observed in the cohort of patients not treated with DNA-damaging adjuvant therapy (Fig. 5C).

In chemotherapy-treated patients, CpG-like signature was significantly associated with grade (1 + 2; $P = 0.0048$), whereas no association was found with the other pathologic parameters (estrogen receptor, tumor size, and node status; Supplementary Table S2A). Multivariate analysis of all covariates (i.e., estrogen receptor, tumor size, grade, node status, and CpG-like signature) indicated that CpG-like signature resulted in an independent strong prognostic factor (HR = 0.3875; 95% CI, 0.1812–0.8287; $P = 0{,}015$) of relapse-free survival (Supplementary Table S2B).

Together, these findings indicate that CpG-ODN modulate DNA repair gene expression that are relevant for cell sensitivity to DNA damages.

Antitumor effect of CpG-ODN and cisplatin in human ovarian tumor xenografts

To test for a correlation between DNA repair gene downmodulation and sensitivity to DNA-damage–inducing drugs, we evaluated the effect of CpG-ODN treatment on the antitumor activity of cisplatin, a DNA cross-linking agent (38). IGROV-1 ovarian tumor–bearing athymic mice were used in these experiments, as IGROV-1 cells are sensitive to cisplatin (39) and as CpG-ODN in this model has been shown to prolong survival of mice with bulky disease inducing an activation of different effector cells and cytokines of innate immunity at the site of tumor growth (8, 24). Mice were treated i.p. with CpG-ODN, cisplatin, or both 8 days after tumor cell injection, when ascites start to form. Analysis of the effect of the combined treatment revealed a significant ($P < 0.0001$) increase in life span compared with the use of either reagent alone [treated/control (T/C) values: 200% for cisplatin, 305% for CpG-ODN, and 460% for cisplatin plus CpG-ODN; Fig. 6].

The antitumor efficacy of CpG-ODN in combination with cisplatin was also assayed in mice bearing the human ovarian tumor cells OVCAR-5. As previously observed in IGROV-1 mouse model, mice bearing OVCAR-5 human ovarian cell line and treated with CpG-ODN plus cisplatin survived significantly longer than those treated with CpG-ODN or cisplatin alone (T/C values: 118% for cisplatin, 188% for CpG-ODN, and 267% for cisplatin plus CpG-ODN; Supplementary Fig. S7)

It should be noted that the modulation of DNA repair genes in human ovarian IGROV-1 tumors and the increase in the antitumor effect of cisplatin and CpG-ODN against IGROV-1 human ovarian tumor xenografts in mice were observed in mice injected with a CpG-ODN sequence specific for murine TLR9, making unlikely the possibility that the modulation was related to a direct interaction of CpG-ODN with tumor cells, as different DNA motifs are required for stimulation of mouse and human cells by CpG-ODN (40, 41). Nevertheless, we carried out a microarray analysis on mRNA extracted from IGROV-1 cells stimulated *in vitro* with murine CpG-ODN (GSE23442); none of 16,824 analyzed genes were found to be significantly modulated by CpG-ODN (no genes with FDR < 0.1) compared with IGROV-1 cells cultured in medium alone (data not shown), excluding a direct action of CpG-ODN on tumor cells.

Our evidence of downmodulation in DNA repair genes in tumor cells in the analyses thus far involved the administra-

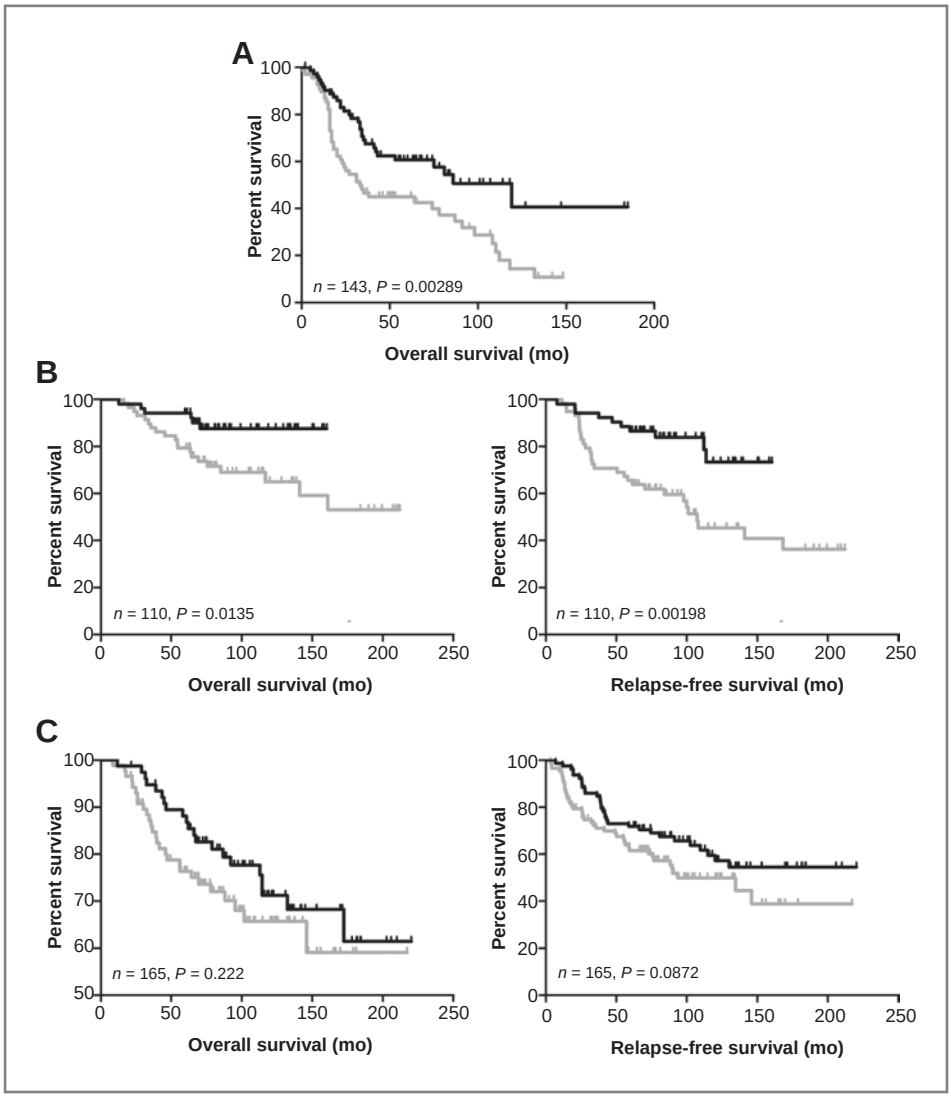


Figure 5. Association between DNA repair genes modulated by CpG-ODN and survival in patients treated with chemotherapy in an adjuvant setting. Two data sets on whole-genome gene expression profiling of ovarian and breast cancer patients treated with chemotherapy were chosen. A Pearson correlation coefficient was calculated to assess the correlation between each tumor and the CpG-ODN signature. The correlation was determined on the basis of expression values of the 27 genes found modulated, comparing the CpG-ODN treatment versus the control condition, and the corresponding genes available in each data set. Tumors were split into 2 groups according to the difference between the correlation obtained for the CpG-ODN treatment and the control condition, with the average used as threshold. Kaplan–Meier curves indicate the survival probability for patients with ovarian cancer (A), patients with breast cancer who received adjuvant systemic chemotherapy (B), and patients with breast cancer who did not receive any systemic treatment (C). Patients with a greater correlation to the CpG-ODN than with the untreated condition showed better outcome. Black curve, CpG-like patients; gray curve, CpG-untreated-like patients.

tion of CpG-ODN at or near the tumor site. Indeed, microarray analyses of mRNA from IGROV-1 tumors adhering to the omentum in mice bearing IGROV-1 ascites and treated subcutaneously daily for 3 days with CpG-ODN revealed no significantly modulated genes (FDR < 0.1) as compared with tumors from saline-treated mice (data not shown); thus, injection of CpG-ODN at the tumor site seems to be critical for DNA repair gene modulation in tumor cells.

In that case, a relevant role for peritumoral TLR9-expressing cells, such as innate immune cells, and/or endothelial cells, fibroblasts, and epithelial cells, in this modulation is plausible. These cells might induce downregulation of DNA repair genes in tumor cells through a direct cell–cell interaction and/or by secreting soluble factors.

To evaluate the involvement of soluble molecule(s) in DNA repair gene modulation, cell-free ascitic fluid supernatants, obtained from mice treated i.p. daily for 3 days with CpG-ODN or saline, were added to cultured IGROV-1 cells. Forty-eight hours later, mRNA was extracted and the mRNA levels of *RAD23B, RAD51C, SIRT1*, and *RAD54B* were determined by qRT-PCR. Incubation with ascitic fluids from CpG-ODN–treated mice induced a downmodulation of *RAD51*C ($P = 0.0443$), *SIRT1* ($P = 0.0374$), and *RAD54B* ($P = 0.00851$) levels, consistent with microarray results, and a trend toward a higher median expression level for RAD23B (Supplementary Fig. S8), which was upmodulated in microarray experiments.

Moreover, preincubation of IGROV-1 cells with ascitic fluids from CpG-ODN–treated mice for 48 hours enhanced cisplatin cytotoxicity, as cell viability quantified by MTT assay was 66.0% ± 4.6% (mean ± SEM) for cells preincubated with ascitic fluid from CpG-ODN–treated mice and 90.1% ± 5.8% for cells preincubated with ascitic fluids from saline-treated mice ($P = 0.0121$).

DNA repair gene modulation in tumors by TLR7 agonist imiquimod

Furthermore, to determine whether modulation of DNA repair genes in tumor cells is restricted to CpG-ODN–induced

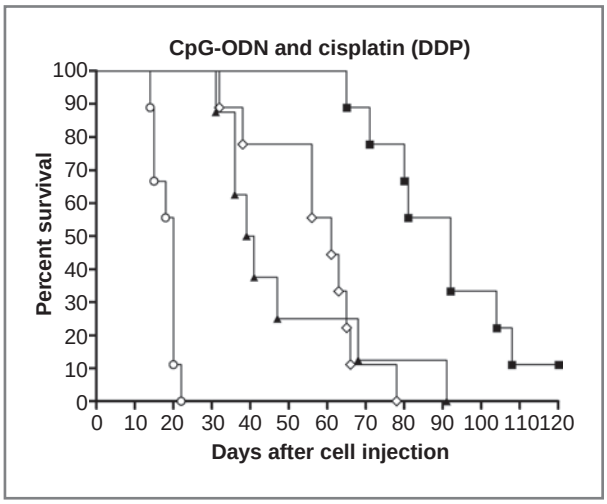


Figure 6. Kaplan–Meier plot of percentage of survivors over time among IGROV-1 ovarian tumor–bearing athymic mice. Mice were treated i.p., starting from 8 days after tumor cell injection, with CpG-ODN (20 μg per mouse, 5 d/wk for 4 weeks), cisplatin (DDP, 3 mg/kg i.p., once per week for 4 weeks), or both. Control mice received saline. Saline-treated mice (open circles); CpG-ODN–treated mice (open diamonds), cisplatin-treated mice (filled triangles); and CpG-ODN plus cisplatin-treated mice (filled squares). Experimental groups consisted of 8 to10 mice group.

TLR9 activation or occurs after activation with the only other TLR agonist currently used in oncologic therapy, imiquimod—an immunomodulator that functions as agonist of TLR7 and/or TLR8 (42)—a comprehensive *in silico* expression analysis of genes implicated in DNA repair (GO:0006281 mouse) on mRNA extracted from spontaneous s.c. tumors from mice treated topically with imiquimod (GSE20032; ref. 43) was conducted. Imiquimod treatment was found to modulate DNA repair gene expression in tumors, which clustered according to treatment (treated vs. control; Supplementary Fig. S9A), and the pattern of gene modulation in imiquimod-treated mice reflected an increased susceptibility to DNA damage (29 of 32 genes modulated at a threshold of $P < 0.05$ were downregulated; Supplementary Fig. S9B).

Discussion

In the present study, we show by microarray analyses that TLR9 ligand CpG-ODN treatment induces downmodulation of DNA repair genes in tumor cells of both murine and human origins. Expression-level analysis of proteins, RAD51, a key protein in the homologous recombination DNA repair pathway (44), and SIRT1, whose activity promotes homologous recombination (45), in human tumor cells confirmed microarray results. These proteins are involved in homologous recombination and, consequently, are relevant for the repair of interstrand cross-links, which are the most cytotoxic lesions induced by cisplatin. Accordingly, the combination of cisplatin and CpG-ODN against IGROV-1 human ovarian tumor xenografts in athymic mice was found to induce a remarkable increase in life span compared with that using either reagent alone ($P < 0.0001$).

The combined data from our analysis and *in silico* analyses revealed modulation of DNA repair genes in both murine colon carcinoma MC38 cells and human ovarian carcinoma IGROV-1 cells, suggesting that this effect is not restricted to a specific cancer cell histotype. The observation that this modulation also occurs after treatment with an agonist of TLR7 and/or TLR8 might suggest that this event can be shared by the functional subgroup of TLRs, consisting of TLR7, TLR8, and TLR9, which reside intracellularly and recognize nucleic acids derived from the genome of viruses and bacteria (42).

The findings that intratumoral delivery of CpG-ODN was critical in inducing DNA repair protein downmodulation in tumors and that CpG-ODN did not interact directly with the tumor cells to induce this downmodulation in IGROV-1 ovarian cancer cells point to the importance of the activation of the TLR9-positive cells (innate immune cells, endothelial cells, fibroblasts, and epithelial cells) present in the tumor microenvironment, in modulating DNA repair gene expression. Moreover, our data suggest that soluble molecules in ascitic fluid, presumably released by the above-mentioned TLR9-positive cells, are involved in the modulation of DNA repair genes. The lower modulation of DNA repair genes observed in *ex vivo* experiments compared with that in *in vivo* might depend on the loss of activity of some factors or on the need for both soluble factors and contact between CpG-ODN–activated cells and tumor cells.

Clinical results from association of CpG-ODN with DNA-damaging drugs in lung tumor patients have so far been disappointing in improving clinical outcome (46).

On the basis of our findings, this absence of CpG-induced enhanced chemosensitivity of tumor cells to DNA-damage–inducing agents may rest in the s.c. administration of CpG-ODN, distant from the tumor cells.

On the contrary, we observed by *in silico* analyses that TLR9 ligand CpG-ODN treatment induces essentially an upmodulation of DNA repair genes in immune cells. Moreover, it is noteworthy that 19 of 49 genes modulated in spleen cells after CpG-ODN treatment and even did so in IGROV-1 tumor cells, but for most of them the modulation was in an opposite way (14 genes upregulated in spleen were downregulated in tumor, and 3 genes downregulated in spleen were upregulated in tumor).

Despite numerous previous microarray analyses of immune cells after *in vitro* or *in vivo* stimulation with CpG-ODN (26, 33, 35, 43, 47), to our knowledge, an upregulation of DNA repair genes after CpG-ODN treatment has not been reported. This might reflect a primary focus in previous studies on signaling pathways that induce expression of immune and proinflammatory genes (48), in light of the function of TLRs as innate immunity sensors of microbial products.

CpG-ODN–induced downmodulation of DNA repair genes in tumor cells and upmodulation in immune cells might represent a physiologic phenomenon that occurs locally in the presence of an infectious event. Thus, upon detecting the presence of an infectious agent via endosomal TLRs, immune cells might regulate DNA repair genes to decrease their susceptibility to possible proapoptotic signals during infections and, at the same time, directly and/or indirectly induce

modulation of DNA repair genes in infected (or transformed) cells to facilitate their death. Insignificant modulations of these genes observed in normal muscle cells suggest that CpG-ODN–activated immune cells induce downmodulation of DNA repair genes only in "altered" cells expressing apposite receptors. Moreover, better outcome of cisplatin-treated ovarian carcinoma patients, as well as of anthracycline-treated breast cancer patients, classified as CpG-like, compared with patients classified as CpG-untreated-like, indicates the relevance of these genes in the tumor cell response to DNA-damaging drugs. In subjects who did not receive adjuvant chemotherapy, expression of CpG-ODN–modulated DNA repair genes was not associated with significantly better outcome, indicating that these genes are not prognostic of survival.

Together, our present data provide the first evidence that TLR9-expressing cells present in the tumor microenvironment can sensitize cancer cells to DNA-damaging chemotherapy, underscoring the need for further investigation of the synergistic effect of CpG-ODN in combination with DNA-damaging drugs in cancer treatment.

Disclosure of Potential Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

The authors thank Drs. Silvana Canevari, Delia Mezzanzanica, and Alessandra Meini for fruitful discussions.

Grant Support

This work was supported by AIRC (Associazione Italiana per la Ricerca sul Cancro) and MIUR-FIRB Italy.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 19, 2011; revised August 3, 2011; accepted August 9, 2011; published OnlineFirst August 30, 2011.

References

1. Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol 2004;4:499–511.
2. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nat Immunol 2004;5:987–95.
3. Krieg AM. Antiinfective applications of toll-like receptor 9 agonists. Proc Am Thorac Soc 2007;4:289–94.
4. Krieg AM. Antitumor applications of stimulating toll-like receptor 9 with CpG oligodeoxynucleotides. Curr Oncol Rep 2004;6:88–95.
5. Krieg AM. Development of TLR9 agonists for cancer therapy. J Clin Invest 2007;117:1184–94.
6. Pratesi G, Petrangolini G, Tortoreto M, Addis A, Belluco S, Rossini A, et al. Therapeutic synergism of gencitabine and CpG-oligodeoxynucleotides in an orthotopic human pancreatic carcinoma xenograft. Cancer Res 2005;65:6388–93.
7. Carpentier AF, Chen L, Maltonti F, Delattre JY. Oligodeoxynucleotides containing CpG motifs can induce rejection of a neuroblastoma in mice. Cancer Res 1999;59:5429–32.
8. De Cesare M, Calcaterra C, Pratesi G, Gatti L, Zunino F, Ménard S, et al. Eradication of ovarian tumor xenografts by locoregional administration of targeted immunotherapy. Clin Cancer Res 2008;14:5512–8.
9. Link BK, Ballas ZK, Weisdorf D, Wooldridge JE, Bossler AD, Shannon M, et al. Oligodeoxynucleotide CpG 7909 delivered as intravenous infusion demonstrates immunologic modulation in patients with previously treated non-Hodgkin lymphoma. J Immunother 2006;29:558–68.
10. Krieg AM. Toll-like receptor 9 (TLR9) agonists in the treatment of cancer. Oncogene 2008;27:161–7.
11. Hofmann MA, Kors C, Audring H, Walden P, Sterry W, Trefzer U. Phase 1 evaluation of intralesionally injected TLR9-agonist PF-3512676 in patients with basal cell carcinoma or metastatic melanoma. J Immunother 2008;31:520–7.
12. Leonard JP, Link BK, Emmanouilides C, Gregory SA, Weisdorf D, Andrey J, et al. Phase I trial of toll-like receptor 9 agonist PF-3512676 with and following rituximab in patients with recurrent indolent and aggressive non Hodgkin's lymphoma. Clin Cancer Res 2007;13:6168–74.
13. Weiner GJ. CpG oligodeoxynucleotide-based therapy of lymphoid malignancies. Adv Drug Deliv Rev 2009;61:263–7.
14. Vollmer J, Krieg AM. Immunotherapeutic applications of CpG oligodeoxynucleotide TLR9 agonists. Adv Drug Deliv Rev 2009;61:195–204.
15. Jahrsdorfer B, Weiner GJ. CpG oligodeoxynucleotides as immunotherapy in cancer. Update Cancer Ther 2008;3:27–32.
16. Vicari AP, Luu R, Zhang N, Patel S, Makinen SR, Hanson DC, et al. Paclitaxel reduces regulatory T cell numbers and inhibitory function and enhances the anti-tumor effects of the TLR9 agonist PF-3512676 in the mouse. Cancer Immunol Immunother 2009;58:615–28.
17. Petrangolini G, Tortoreto M, Perego P, Carenini N, De Cesare M, Balsari A, et al. Combination of metronomic gimatecan and CpG-oligodeoxynucleotides against an orthotopic pancreatic cancer xenograft. Cancer Biol Ther 2008;7:596–601.
18. Balsari A, Tortoreto M, Besusso D, Petrangolini G, Sfondrini L, Maggi R, et al. Combination of CpG-oligodeoxynucleotide and a topoisomerase I inhibitor in the therapy of human tumour xenografts. Eur J Cancer 2004;40:1275–81.
19. Weigel BJ, Rodeberg DA, Krieg AM, Blazar BR. CpG oligodeoxynucleotides potentiate the antitumor effects of chemotherapy or tumor resection in an orthotopic murine model of rhabdomyosarcoma. Clin Cancer Res 2003;9:3105–14.
20. Mason KA, Ariga H, Neal R, Valdecanas D, Hunter N, Krieg AM, et al. Targeting toll-like receptor 9 with CpG oligodeoxynucleotides enhances tumor response to fractionated radiotherapy. Clin Cancer Res 2005;11:361–9.
21. Milas L, Mason KA, Ariga H, Hunter N, Neal R, Valdecanas D, et al. CpG oligodeoxynucleotide enhances tumor response to radiation. Cancer Res 2004;64:5074–7.
22. Mason KA, Neal R, Hunter N, Ariga H, Ang K, Milas L. CpG oligodeoxynucleotides are potent enhancers of radio- and chemoresponses of murine tumors. Radiother Oncol 2006;80:192–8.
23. Zheng L, Asprodites N, Keene AH, Rodriguez P, Brown KD, Davila E. TLR9 engagement on CD4 T lymphocytes represses gamma-radiation-induced apoptosis through activation of checkpoint kinase response elements. Blood 2008;111:2704–13.
24. De Cesare M, Sfondrini L, Campiglio M, Sommariva M, Bianchi F, Perego P, et al. Ascites regression and survival increase in mice bearing advanced-stage human ovarian carcinomas and repeatedly treated intraperitoneally with CpG-ODN. J Immunother 2010;33:8–15.
25. Benard J, Da Silva J, De Blois MC, Boyer P, Duvillard P, Chiric E, et al. Characterization of a human ovarian adenocarcinoma line, IGROV1, in tissue culture and in nude mice. Cancer Res 1985;45:4970–9.
26. Klaschik S, Tross D, Klinman DM. Inductive and suppressive networks regulate TLR9-dependent gene expression *in vivo*. J Leukoc Biol 2009;85:788–95.
27. Klinman DM, Yi AK, Beaucage SL, Conover J, Krieg AM. CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete

interleukin 6, interleukin 12, and interferon gamma. Proc Natl Acad Sci U S A 1996;93:2879–83.
28. Takeshita S, Takeshita F, Haddad DE, Ishii KJ, Klinman DM. CpG oligodeoxynucleotides induce murine macrophages to up-regulate chemokine mRNA expression. Cell Immunol 2000;206:101–6.
29. Ishii KJ, Takeshita F, Gursel I, Gursel M, Conover J, Nussenzweig A, et al. Potential role of phosphatidylinositol 3 kinase, rather than DNA-dependent protein kinase, in CpG DNA-induced immune activation. J Exp Med 2002;196:269–74.
30. Zelenay S, Elias F, Flo J. Immunostimulatory effects of plasmid DNA and synthetic oligodeoxynucleotides. Eur J Immunol 2003;33: 1382–92.
31. Datta N, Mukherjee S, Das L, Das PK. Targeting of immunostimulatory DNA cures experimental visceral leishmaniasis through nitric oxide up-regulation and T cell activation. Eur J Immunol 2003;33:1508–18.
32. Ito S, Ishii KJ, Gursel M, Shirotra H, Ihata A, Klinman DM. CpG oligodeoxynucleotides enhance neonatal resistance to Listeria infection. J Immunol 2005;174:777–82.
33. Pesce I, Monaci E, Muzzi A, Tritto E, Tavarini S, Nuti S, et al. Intranasal administration of CpG induces a rapid and transient cytokine response followed by dendritic and natural killer cell activation and recruitment in the mouse lung. J Innate Immun 2010;2:144–59.
34. Mosca F, Tritto E, Muzzi A, Monaci E, Bagnoli F, Iavarone C, et al. Molecular and cellular signatures of human vaccine adjuvants. Proc Natl Acad Sci U S A 2008;105:10501–6.
35. Westwood JA, Haynes NM, Sharkey J, McLaughlin N, Pegram HJ, Schwendener RA, et al. Toll-like receptor triggering and T-cell costimulation induce potent antitumor immunity in mice. Clin Cancer Res 2009;15:7624–33.
36. Bild AH, Yao G, Chang JT, Wang Q, Potti A, Chasse D, et al. Oncogenic pathway signatures in human cancers as a guide to targeted therapies. Nature 2006;439:353–7.
37. Van de Vijver MJ, He YD, Van'T Veer LJ, Dai H, Hart AA, Voskuil DW, et al. A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med 2002;347:1999–2009.
38. Muggia F. Platinum compounds 30 years after the introduction of cisplatin: implications for the treatment of ovarian cancer. Gynecol Oncol 2009;112:275–81.
39. Perego P, Giarola M, Righetti SC, Supino R, Caserini C, Delia D, et al. Association between cisplatin resistance and mutation of *p53* gene and reduced bax expression in ovarian carcinoma cell systems. Cancer Res 1996;56:556–62.
40. Hartmann G, Krieg AM. Mechanism and function of a newly identified CpG DNA motif in human primary B cells. J Immunol 2000;164: 944–53.
41. Hartmann G, Weeratna RD, Ballas ZK, Payette P, Blackwell S, Suparto I, et al. Delineation of a CpG phosphorothioate oligodeoxynucleotide for activating primate immune responses *in vitro* and *in vivo*. J Immunol 2000;164:1617–24.
42. Agrawal S, Kandimalla ER. Synthetic agonists of Toll-like receptors 7, 8 and 9. Biochem Soc Trans 2007;35:1461–7.
43. Lu H, Wagner WM, Gad E, Yang Y, Duan H, Amon LM, et al. Treatment failure of a TLR-7 agonist occurs due to self-regulation of acute inflammation and can be overcome by IL-10 blockade. J Immunol 2010;184:5360–7.
44. Schild D, Wiese C. Overexpression of RAD51 suppresses recombination defects: a possible mechanism to reverse genomic instability. Nucleic Acids Res 2010;38:1061–70.
45. Uhl M, Csernok A, Aydin S, Kreienberg R, Wiesmuller L, Gatz SA. Role of SIRT1 in homologous recombination. DNA Repair (Amst) 2010;9:383–93.
46. Murad YM, Clay TM, Lyerly HK, Morse MA. CPG-7909 (PF-3512676, ProMune): toll-like receptor-9 agonist in cancer therapy. Expert Opin Biol Ther 2007:1257–66.
47. Torri A, Beretta O, Ranghetti A, Granucci F, Ricciardi-Castagnoli P, Foti M. Gene expression profiles identify inflammatory signatures in dendritic cells. PLoS One 2010;5:e9404.
48. Liew FY, Xu D, Brint EK, O'Neill LA. Negative regulation of toll-like receptor-mediated immune responses. Nat Rev Immunol 2005;5: 446–58.