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**Locoregional delivery of unmethylated
CpG-oligodeoxynucleotides to cancer therapy:
preclinical studies**

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ABSTRACT

Tumor cell growth, even in advanced stages of ovarian cancer, is nearly always restricted to the peritoneal cavity, therefore repeated intraperitoneal injections of CpG-ODN recruiting and activating innate effector cells throughout the abdominal cavity to the tumor site might control tumor cell growth and ascites formation. After a single CpG-ODN treatment, in IGROV-1 ovarian tumor ascites-bearing athymic mice, the number of tumor cells declined rapidly and markedly, and ascites volumes declined shortly after treatment (5 h), increasing thereafter at a slower rate than in controls. When administered every 7 days for 4 weeks, CpG-ODN had only a marginal effect on survival time, whereas administration 5 days/week for 3 or 4 weeks led to a significantly increased survival-time as compared to controls and completely controlled ascites growth without apparent toxicity, although a disorganization of lymphoid organs was observed. Depletion of NK or monocytes/macrophages only slightly influenced the CpG-ODN-induced reduction of ascites tumor cells, indicating that the antitumor activity might not be related to a specific cell/cytokine but rather to the repertoire of cells and cytokines accumulated in the peritoneal cavity. Thus, our data suggest a relevant role for repeated activation of cells and cytokines of innate immunity in the therapy of ovarian cancer patients with malignant ascites. However, daily i.p. administration of CpG-ODN induced a significant increase of survival-time but no cure of a single mouse, therefore we screened the effectiveness of CpG-ODN in combination with different agents, including bevacizumab, Poly(I):Poly(C) and cisplatin. Our data indicate that the combination of repeated i.p. CpG-ODN treatments plus bevacizumab and Poly(I):Poly(C) do not significantly increase median survival time, while the association of CpG-ODN and cisplatin revealed a significant increase in mice lifespan. Based on these results, we performed several experiments in order to gain insight into the molecular mechanisms by which CpG-ODN improves the therapeutic efficacy of cisplatin, a DNA-damaging drug. We demonstrate that an immunostimulatory Toll-like receptor-9 (TLR9) agonist CpG-oligodeoxynucleotide (CpG-ODN) oppositely modulates expression of DNA repair genes in

tumor and immune cells. Analyses *in silico* and in a human ovarian tumor model by microarray revealed downregulation of these genes in tumors and upregulation in immune cells, with no detectable modulation in normal non-immune tissue. CpG-ODN induced activation of cells present in the tumor microenvironment was critical in inducing DNA-repair gene modulation in tumors. In conclusion, the combination of cisplatin with CpG-ODN was effective and well tolerated, and might represent a preclinical basis for the design of clinical studies, even considering the ability of CpG-ODN to down-modulate DNA repair genes in tumor cell.

INTRODUCTION

OVARIAN CANCER

Ovarian cancer causes more deaths in the United States than any other type of female reproductive tract cancer, with an estimated 22,430 new cases and 15,280 deaths in 2007 (1). Approximately 70% of ovarian cancers are diagnosed at advanced stage and only 30% of women with such cancers can expect to survive 5 years. Analysis of trends in overall five-year survival rates for women with ovarian cancer indicates some recent improvement for those diagnosed between 1996 and 2002, compared to the 1970's and 1980's (1). Nonetheless, these gains are rather modest and there clearly remains a need to better understand the molecular pathogenesis of ovarian cancer so new drug targets and biomarkers that facilitate early detection can be identified. Approximately 90% of primary malignant ovarian tumors are epithelial (carcinomas), and are thought by most investigators to arise from the ovarian surface epithelium (OSE) or more likely from surface epithelial inclusion cysts (2,3). Some investigators have suggested they may develop from the secondary Müllerian system, which includes paraovarian and paratubal cysts, the rete ovarii, endosalpingiosis, and endometriosis (4). The classification of ovarian epithelial tumors currently used by pathologists is based entirely on tumor cell morphology. The four major types of epithelial tumors (serous, endometrioid, clear cell, and mucinous) bear strong resemblance to the normal cells lining different organs in the female genital tract. For example, serous, endometrioid, and mucinous tumor cells exhibit morphological features similar to non-neoplastic epithelial cells in the fallopian tube, endometrium, and endocervix, respectively. Representative examples of serous, endometrioid, clear cell, and mucinous ovarian carcinomas are shown in Figure 1.

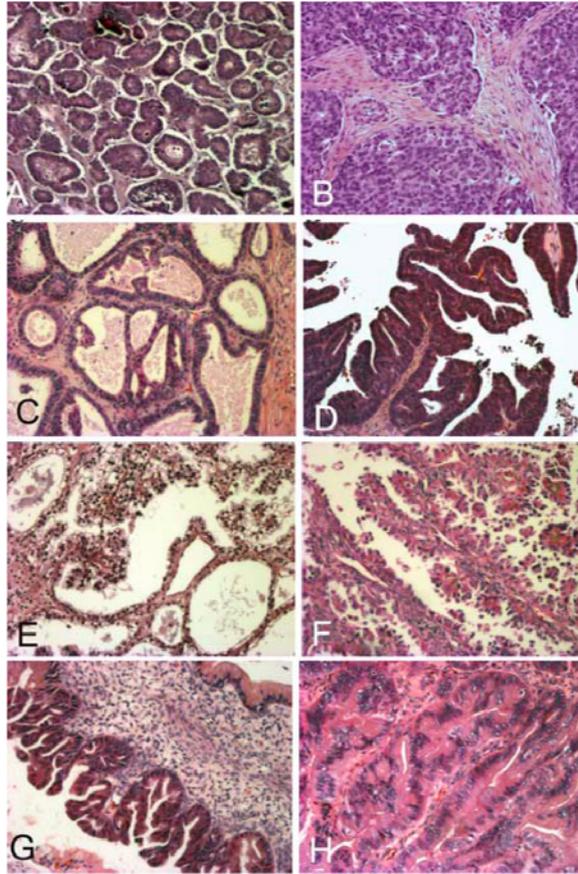


Figure 1. Pictures of the four most common histologic types of ovarian cancer, stained with hematoxylin and eosin. A, Ovarian serous carcinoma showing papillae formation. B, Ovarian serous carcinoma with predominant solid growth pattern. C, Ovarian endometrioid tumor of low malignant potential showing glands similar to the complex hyperplasia of the uterine endometrium. D, High-power view of ovarian endometrioid carcinoma that is morphologically similar to endometrial carcinoma of the uterus. E, Ovarian clear carcinoma showing cellular clearing and cystic growth pattern. F, High-power view of ovarian clear cell carcinoma with hobnail growth pattern. G, Ovarian mucinous tumor of low malignant potential. H, Well-differentiated ovarian mucinous carcinoma.

The histological similarity of ovarian epithelial tumors to epithelia in other portions of the female genital tract is not surprising, given that all of these epithelia, as well as the cells lining the peritoneal cavity, are thought to be derived from a common embryological precursor, the coelomic mesothelium (5). Of note, provocative recent studies suggest the distal fallopian tube may actually be the site of origin of at least some serous carcinomas previously thought to arise in the ovary or pelvic peritoneum (6,7). Once grouped by cell type, tumors can be further subdivided into those that are clearly benign (cystadenomas), those that are frankly malignant (carcinomas), and those that have features intermediate between these two (variably called “atypical proliferative” tumors, tumors of “low malignant potential” or tumors of “borderline” malignancy). The present clinical

management of ovarian carcinoma patients is not significantly influenced by the histological subtype of the tumor, although accumulating clinical pathological and molecular data suggest the major subtypes likely represent distinct disease. In addition to type of differentiation, ovarian carcinomas can be sub-classified based on degree of differentiation (tumor grade). Historically, the most commonly used grading systems have been those proposed by the International Federation of Gynecology and Obstetrics (FIGO), the World Health Organization (WHO), and the Gynecologic Oncology Group (GOG) (8). The FIGO system uses 3 grades based on architectural criteria, i.e., the proportion of glandular or papillary structures relative to areas of solid tumor growth. Grades 1, 2, and 3 correspond to <5%, 5–50%, and >50% solid growth, respectively. The WHO system incorporates both architectural and cytological features, but these are not assigned based on quantitative criteria and as a consequence, this system can be considered rather subjective. In the GOG system, the grading method varies depending on the histological type of the tumor. For example, endometrioid adenocarcinomas are graded using FIGO criteria, while clear cell carcinomas are not assigned a grade at all. More recently, a 3 grade system has been proposed that can be applied to all ovarian carcinomas (9), and two binary grading systems have been proposed for ovarian serous carcinomas, the most common type (10,11). Review of both clinicopathological and molecular studies to date has led to a model in which ovarian carcinomas can be generally divided into two broad categories designated Type I and Type II tumors, akin to the division of endometrial carcinomas into two major types as recently reviewed by Di Cristofano and Ellenson (12). Tumor grade is an important, albeit not sole factor, distinguishing Type I from Type II tumors.

CLINICOPATHOLOGICAL FEATURES OF THE MAJOR TYPES OF OVARIAN CARCINOMAS

During the 1990's, a number of advances were made in the histopathological classification of ovarian carcinomas (13). These include better recognition of patterns of metastatic carcinoma

previously misinterpreted as primary ovarian tumors, establishment of improved criteria for distinguishing invasive from non-invasive endometrioid and mucinous ovarian carcinomas, and interpretation of carcinosarcomas (malignant mixed mesodermal/Müllerian tumors) as carcinomas with areas of “sarcomatous” differentiation (or epithelial-mesenchymal transition) rather than sarcomas. In addition, primary peritoneal serous carcinomas and ovarian serous carcinomas are now considered essentially interchangeable for the purposes of diagnosis and treatment. These and perhaps other factors have altered the current histologic type and stage distribution of ovarian carcinomas compared to earlier case series. Seidman and colleagues recently analyzed the histologic type and stage distribution of 220 consecutive ovarian and peritoneal carcinomas (13). In Seidman’s series, nearly 70% of tumors were serous and fewer than 5% of these were confined to one or both ovaries (Stage 1) at diagnosis. Serous carcinomas typically display papillary or solid growth with slit-like spaces. Nuclear atypia is usually marked and mitotic activity abundant. Endometrioid adenocarcinomas account for 10–20% of ovarian carcinomas in most older reports, but in the more recent series of Seidman and colleagues, only 7% were endometrioid. These tumors have morphological features similar to their endometrial counterparts, showing varying quantities of overt gland formation, sometimes accompanied by squamous differentiation. In contrast to the serous carcinomas, over 50% of endometrioid adenocarcinomas are confined to the ovaries at diagnosis and nuclear atypia is usually less pronounced. Approximately 10% of ovarian carcinomas in the Seidman series showed clear cell differentiation. Several growth patterns (e.g., solid, papillary, tubulocystic) for clear cell carcinoma have been recognized. Although nearly one-third are Stage 1 at diagnosis, some studies have noted a relatively unfavorable prognosis of these tumors, even when corrected for tumor stage (14,15). Finally, it is worth noting that mucinous carcinomas comprised fewer than 3% of primary ovarian carcinomas in the Seidman series and were almost always confined to the ovary at diagnosis. Mucinous adenocarcinomas also show overt gland formation, but in contrast to endometrioid adenocarcinomas, the tumor cell cytoplasm is mucin-rich. It should be kept in mind that many previous clinical and molecular analyses of

mucinous adenocarcinomas were almost certainly compromised by inadvertent inclusion of metastatic adenocarcinoma to the ovaries (frequently from the gastrointestinal tract) misclassified as primary ovarian carcinomas (16). For many types of common adult solid tumors, such as those of the colon, breast, and uterine cervix, the stages of neoplastic progression are fairly well defined and reflected by morphologically recognizable entities that represent a continuum including normal epithelium, preinvasive lesions, invasive carcinoma, and metastatic disease. In contrast, our understanding of the progression of ovarian carcinoma is incomplete, perhaps because we have only recently begun thinking about the different histological types of ovarian carcinomas as largely distinct disease entities.

OVARIAN SEROUS CARCINOMA

The serous histotype is the most common type of ovarian carcinoma. It is classified as low grade or high grade on the basis of the extent of nuclear atypia and mitosis (10). Morphologically, low-grade serous carcinoma has minimal nuclear atypia, and mitoses are rare (12 per 10 high-power fields); high-grade serous carcinoma, on the other hand, is characterized by marked nuclear atypia and more mitoses (≤ 12 per 10 high-power fields) (10). Low-grade and high-grade carcinomas are different at the genomic and molecular levels. For instance, low-grade serous carcinoma shows fewer molecular abnormalities by both cytogenetic analysis (17,18) and single nucleotide polymorphism analysis (18,19) than high-grade carcinoma. Comparative genomic hybridization (CGH) studies have demonstrated that high-grade serous carcinoma has a significantly higher frequency of copy number abnormalities than low-grade tumors (20-22). Furthermore, high-grade carcinoma showed underrepresentation of 11p and 13q and overrepresentation of 8q and 7p, while low-grade carcinomas showed 12p underrepresentation and 18p overrepresentation more frequently (23). High-grade serous carcinoma commonly involves p53 mutations, but such mutations are rare in low-grade carcinoma (24). Accumulating data suggest that loss of BRCA1/2 function may

predispose to the development of both sporadic and hereditary high-grade serous carcinomas (25). However, the exact mechanism by which BRCA1/2 deficiency triggers tumorigenesis is still not clear. It has been demonstrated that cells with defective BRCA1 are hypersensitive to DNA-damaging agents, are slower to repair double-stranded DNA breaks, and show impairment in transcription-coupled repair (26,27). BRCA1 has been shown to cooperatively bind to p53 and stimulate transcription of the cyclin-dependent kinase inhibitor p21WAF/Cip1 (28). We recently demonstrated that BTAK, a mitotic phase regulatory protein, is overexpressed in ovaries of women with a BRCA mutation or history of ovarian or breast cancer (29). Furthermore, BTAK overexpression was strongly associated with p53 overexpression, suggesting that p53 may be a physiological substrate of BTAK (29), although the underlying mechanisms of how the interaction of BRCA, p53, and BTAK regulates the initiation of ovarian tumorigenesis are not clear. Other genetic alterations detected in high-grade ovarian cancer include epidermal growth factor receptor (12–82%), Her2/neu (5–66%) (30), AKT2 (36%) (31), phosphoinositide-3 kinase (PI-3K) (31), and c-myc (70%) (32). Low-grade serous carcinoma is characterized by mutations in the KRAS or BRAF pathway, as mutations in KRAS or its downstream mediator BRAF have been detected in 68% of low-grade and 61% of low-malignant-potential (LMP) serous carcinomas (33,34). RAS encodes the highly homologous and evolutionarily conserved 21,000-kD GTP-binding protein that is often activated in low-grade ovarian serous carcinoma, mucinous ovarian cancer, and endometrioid ovarian cancer. Ras exerts its effects through three downstream effector pathways, namely PI-3K, RAF, and RAL-GEFs. Much of the existing knowledge of these pathways was based on studies of murine cells, which showed that Raf is an effector used by Ras to induce murine cell transformation (35). Recent studies suggest, however, that human cells require more genetic changes in neoplastic transformation than do their murine counterparts. Several types of human primary cells, fibroblasts, embryonic kidney cells, and breast epithelial cells have been successfully transformed by using a set of genetically defined elements (36-38), suggesting that different cell types may require the combination of distinct genetic elements to achieve full transformation. The

Ras pathway may also be activated by the elimination of regulatory proteins such as Dab2 (39). Dab2 could sequester Grb2 from binding to SOS, and the dissociation of the Grb2/SOS complex may reduce Ras activation, which is thought to be a feedback mechanism for Ras downregulation (40). Dab2 has been found to be widely expressed in normal human tissues, particularly in ovarian surface epithelial cells (40). In contrast, Dab2 mRNA and protein expression have been found to be absent or suppressed in most ovarian cancers. Hence, the loss of Dab2 expression may be one of the general changes associated with cell transformation (40). Alternatively, loss of Dab2 may contribute to tumor cell growth, as Dab2 transfection suppressed the expression in morphologically normal epithelium adjacent to ovarian cancer suggests that Dab2 functions as a tumor suppressor and that its expression is an early event in ovarian cancer progression (40). The clinical presentation, morphological features, and molecular data indicate that low- grade and high-grade serous carcinomas arise via different genetic pathways (19, 41-45). Singer et al. designated them as type I tumors and type II tumors (46). Type I tumors are low-grade neoplasms that develop in a stepwise fashion from “adenoma–borderline tumor–carcinoma” progression. Type II tumors, however, develop de novo from the surface epithelium and grow rapidly without morphologically recognizable precursor lesions. Mutational analysis of low-grade serous carcinoma showed high frequency of KRAS and BRAF mutations, suggesting that this group of tumors develops through a dysregulated RAS–RAF signaling pathway. High-grade serous carcinoma has a high frequency of mutations in the p53 and BRCA1/2 genes, and thus these tumors most probably arise via TP53 mutations and BRCA1 or BRCA2 dysfunction (45-47). Schematic models for the development of low- and high-grade serous carcinomas are shown in Figure 2.

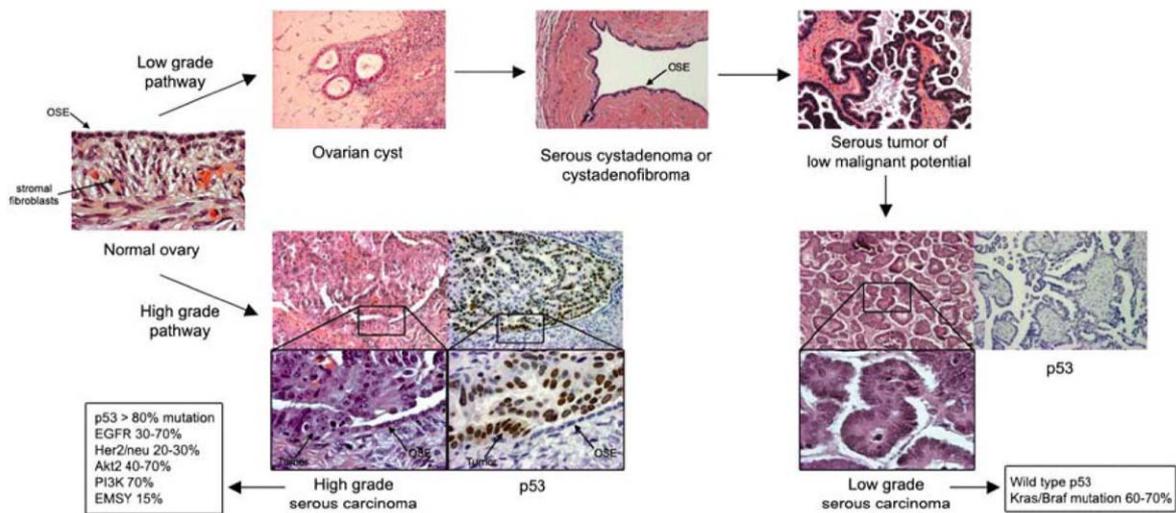


Figure 2. Dualistic models of serous ovarian cancer development. Development of low-grade serous carcinoma proceeds through morphologically recognizable intermediates, from inclusion cystadenoma or cystadenofibroma to serous tumor of low malignant potential and low-grade serous carcinoma, which is characterized by a high frequency of KRAS/BRAF mutations; the high-grade serous tumor develops de novo, with no recognizable intermediates, and is characterized by a high frequency of p53 mutations and an absence of KRAS/BRAF mutations. OSE: ovarian surface epithelial cells.

OVARIAN ENDOMETRIOID CARCINOMA

Ovarian endometrioid carcinoma comprises 10–20% of all epithelial ovarian cancer cases. These tumors are most common in women aged 50–59 years (mean, 56 years). Approximately 15–20% of these women also have endometriosis, which may be outside of the ovary, in the ipsilateral or contralateral ovary, or within the tumor itself. Approximately 14% of women with this cancer have synchronous endometrial carcinoma of the uterus. Endometrioid tumors have a smooth outer surface. An examination of the cut section usually reveals solid and cystic areas; the cysts contain friable soft masses and bloody fluid. Less commonly, the tumor is solid, with extensive hemorrhage and necrosis. Endometrioid carcinoma has a 5-year survival rate of 40–63%, and the relatively good prognosis is due mostly to the high percentage of patients presenting with early stage disease. When patients with endometrioid tumor of the ovary are matched with those with a serous tumor by age and tumor grade, stage, and level of cytoreduction, no significant difference is found in the 5-year survival rate or survival duration (48). Relatively little is known about the molecular events that

lead to development of ovarian endometrioid carcinoma, and no molecular markers have been identified as prognostic indicators. Mutation of the β -catenin gene is one of the most common molecular alterations in endometrioid carcinoma (49) and thus may be a useful molecular marker. β -catenin has been implicated in two important biologic processes: cell-cell adhesion and signal transduction (50,51). At the junctions of epithelial cells, association of β -catenin with the cytoplasmic domain of cadherins plays an important role in Ca^{2+} -dependent cell adhesion. In the nucleus, β -catenin participates in signal transduction, binding to the DNA to activate transcription. Deregulation of the cadherin/catenin complex has been implicated in the development, progression, differentiation, invasion, and metastasis of several malignancies (50,51). Deregulation of β -catenin may be caused by an oncogenic mutation in the β -catenin gene (CTNNB1), mutations in the APC gene, or alterations of the Wnt signal transduction pathway. Endometrioid carcinoma arising in the uterine cavity and that arising in the ovaries are morphologically similar but differ at the molecular level. For example, frequency of β -catenin mutation is different in these tumor subtypes (49). Moreover, ovarian endometrioid cancers exhibit microsatellite instability and PTEN alterations less frequently than their uterine counterparts (52). PTEN mutations are found more frequently in endometrioid carcinomas (approximately 43%) than other histologic types, indicating that they may play a role in the development of this subtype (53).

OVARIAN MUCINOUS CARCINOMA

Primary mucinous tumors are classified as benign, borderline, or malignant, depending on their histopathologic features. Mucinous tumors may be endocervical-like or intestinal-like, and mixtures of both cell types do occur. Intestinal-like epithelium is most easily recognized when it contains goblet cells; these may be seen in benign tumors but are more prominent in borderline and malignant tumors. Other types of intestinal cell differentiation may be found in ovarian mucinous tumors, however, including features typical of gastric superficial/foveolar and pyloric cells,

enterochromaffin cells, argyrophil cells, and Paneth cells (55,56). Unlike serous tumors, which are generally homogeneous in their cellular composition and degree of differentiation, mucinous tumors are often heterogeneous, particularly the intestinal type. Mixtures of benign, borderline, and malignant elements (including noninvasive and invasive carcinomas) are often found within a single neoplasm. Tumor heterogeneity in these intestinal-type mucinous tumors suggests that malignant transformation is sequential, progressing from a cystadenoma or borderline tumor to noninvasive, microinvasive, and invasive carcinoma. Analyses of KRAS mutations lend molecular genetic support to this theory (56,57). KRAS mutations are common in mucinous ovarian tumors. Interestingly, some microdissected mucinous tumors were found to have the same KRAS mutation in histologically benign, borderline, and malignant areas of the same tumor (56,57). Thus, KRAS mutation may be an early event in ovarian mucinous carcinogenesis. Mucinous carcinomas are classified according their extent of invasion (58). Mucinous tumors of intestinal type that contain glands with the architectural and cytologic features of adenocarcinoma but lack obvious stromal invasion are classified as noninvasive carcinomas. Microinvasion has been found in approximately 9% of intestinal-type borderline tumors (59,60). In general, individual infiltrative foci with a maximum dimension of < 3.0 mm or a maximum area of < 10 mm² (provided neither of two linear dimensions exceeds 3.0 mm) are considered microinvasive (59,61,62). Other investigators have used a cutoff of 2.0 mm (39) or 5.0 mm (30). Individual microinvasive foci commonly are < 1.0 or 2.0 mm (60,62). The number of invasive foci in a tumor is variable. More than half of these tumors may have more than five foci (62). The histologic criteria for microinvasion include the presence of irregular jagged glands and small strips or nests of tumor cells accompanied by reactive fibroblastic stroma. Chronic inflammatory infiltrate may be also present. Recently, an expansile type of invasion was defined (59). This is characterized by an architecturally complex, arrangement of glands, cysts, or papillae lined by malignant epithelium with minimal or no intervening normal ovarian stroma. However, the extent, depth, and number of microinvasive foci, and their clinical significance, still need to be scientifically validated. Invasive mucinous carcinoma is uncommon,

accounting for fewer than 10% of all primary ovarian carcinomas (59,63). The prognosis of invasive mucinous carcinomas of intestinal type depends on the FIGO stage and the histologic pattern of stromal invasion (59,61,62,64) but is favorable compared with that of serous carcinomas; this is because 80% of invasive mucinous carcinomas are stage I at diagnosis. Carcinomas with an infiltrative pattern of invasion are more aggressive than those with an expansile pattern. In two recent series, all 27 cases with expansile invasion were stage I, and none of the 21 for which follow-up data were available had metastasized (59,62,64). The molecular mechanisms that lead to the progression of benign mucinous tumors are still largely unknown. In a recent study, Wamunyokoli et al. profiled gene expression in 25 microdissected mucinous tumors (6 cystadenomas, 10 LMP tumors, and 9 adenocarcinomas) and described the pathway analysis used to identify gene interactions that may influence ovarian mucinous tumorigenesis and genes that may mediate the phenotypes typically associated with these tumors (65). These latter include genes that regulate multidrug resistance (ABCC3 and ABCC6), signal transduction (SPRY1 and CAV-1), cytoskeleton rearrangement/signal transduction (RAC1, CDC42, RALA, IQGAP2, cortactin), cell cycle regulation and proliferation (CCND1, ERBB3, transforming growth factor (TGF)- α , and transformation (c-JUN, K-ras2, ECT2, YES1).

OVARIAN CLEAR CELL CARCINOMA

Ovarian clear cell carcinoma (OCCA) accounts for fewer than 5% of all ovarian malignancies and 3.0–12.1% of all ovarian epithelial neoplasms (66). Unlike serous carcinoma, OCCA often presents as a large pelvic mass in early stages and thus is diagnosed early. Advanced-stage disease has a poor prognosis and is resistant to cisplatin-based chemotherapy. Little is known about the development and progression of OCCA. Studies have shown that 5–10% of cases are associated with endometriotic lesions; however, little molecular evidence exists to support an ectopic origin. While p53 mutations are common in tumorigenesis and have been found in various epithelial

subtypes, particularly serous ovarian carcinoma, they are conspicuously absent in OCCA (67,68), implying that other anti-apoptotic mechanisms are involved. The target of methylation-induced silencing 1/apoptosis-associated speck-like protein (TMS-1/ASC) is a member of the caspase recruitment domain family of proapoptotic mediators. A high frequency of aberrant methylation that results in transcriptional silencing of TMS-1/ASC has been observed in OCCA tumors, indicating a mechanism for apoptotic escape in tumor development; this may have implications for drug resistance in OCCA as well (69). Mutations in the PTEN gene are common in endometrioid and clear cell ovarian cancers but not in serous or mucinous ovarian cancers. Loss of CD44 splice control has also been observed in OCCA (70,71). CD44 is a membrane glycoprotein and is the major cell-surface receptor of hyaluronate, a glycosaminoglycan that is present on the surface of human peritoneal cells. The presence of the CD44 isoform CD44-10v was associated with recurrence or death in 71% of women with OCCA, whereas only 18% of women without the isoform experienced recurrence or died of disease (72). The CD44-10v isoform was also absent in the contralateral, unaffected ovaries, suggesting that aberrant alternative mRNA splicing of CD44 is involved in the development and progression of OCCA. Several investigators have studied OCCA to determine whether it has a distinct genetic fingerprint. Using conventional CGH, Suehiro et al. found increased copy numbers of 8q11–q13, 8q21–q22, 8q23, 8q24–qter, 17q25–qter, 20q13–qter, and 21q22–qter, and reduced copy numbers of 19p. A molecular signature that distinguishes OCCA from other histologic types was reported by Schwartz et al., who identified 73 genes that were expressed at 2–29 times higher levels in OCCA than in other histologic types (73). However, this study included only eight OCCA specimens and revealed that OCCA had a two times higher level of Her-2 expression than other types. More recently, a comparison of the gene expression profiles of serous, endometrioid, and clear cell types of ovarian cancer with that of normal ovarian surface epithelium revealed 43 genes that were common to all types (71), suggesting that the process of malignant transformation in serous, endometrioid, and clear cell types of ovarian cancer involves a common pathway. Furthermore, the profiles of OCCA were similar to those of renal and

endometrial clear cell carcinomas, implying that certain molecular events are common to clear cell tumors, regardless of the organ of origin (71), and that crossover molecular target exist with which to treat these tumors. Microsatellite instability is caused by defects in DNA mismatch–repair genes. In experimental systems, mismatch repair–deficient cells are highly tolerant to the methylating chemotherapeutic drugs streptozocin and temozolomide, and, to a lesser extent, cisplatin and doxorubicin (74). Thus, these drugs are expected to be less effective against mismatch repair deficient tumors in humans. It has been observed high-level microsatellite instability involvement in the development of a subset of OCCAs and a strong association between alterations in hMLH1 and hMSH2 expression and microsatellite instability in these tumors (75). Significantly elevated mRNA levels of ERCC1 (excision-repair, complementing defective, in Chinese hamster-1) and XPB, two key genes involved in the nucleotide excision repair pathway and in *in vitro* resistance to platinum based chemotherapy (76), have also been observed in OCCA but not in other epithelial ovarian carcinoma subtypes (77). Therefore, altering the expression of DNA-repair genes may provide a possible mechanism of drug resistance against DNA-damaging agents in OCCA. A unique 93-gene expression profile, the chemotherapy response profile, was recently described; this profile was predictive of pathologic complete response to first-line platinum- or taxane-based chemotherapy in 60 patients with epithelial ovarian carcinoma, 92% of whom had the serous histologic type (78). The apoptotic activator BAX was associated with chemoresistance: its expression was reduced in patients with chemoresistant tumors. High levels of BAX protein have previously been associated with paclitaxel sensitivity and improved survival in patients with epithelial ovarian cancer (79). Among the immunohistochemical characteristics of OCCA is the notable overexpression of BAX in stage I and II OCCA tumors (67). Furthermore, the antiapoptotic protein Bcl-2, which inhibits BAX-mediated apoptosis, is expressed at higher levels in metastatic OCCA lesions than in primary OCCA lesions (80). A p53-mediated pathway has been implicated in the induction of cell death after DNA damage by platinum-based chemotherapeutic agents, which results in a decrease in the relative ratio of Bcl-2/Bax, thus favoring apoptosis (81). Hence, the presence of a lower relative

ratio of Bcl-2/BAX in early stage OCCA tumors and a higher relative ratio of Bcl-2/BAX in metastatic OCCA lesions may account for the dichotomy in outcome observed between patients with early stage OCCA, who have a good prognosis, and those with late stage, platinum-resistant OCCA, who have a poorer prognosis than their counterparts with serous carcinomas.

OVARIAN CARCINOMA SUBTYPES ARE LARGELY DISTINGUISHABLE BASED ON SPECIFIC GENE MUTATIONS AND GLOBAL GENE EXPRESSION PATTERNS

An important message of is that pathologists' traditional morphology-based classification allows prediction of specific molecular genetic alterations likely to be present in a given ovarian tumor. Figures 1A–1D show that each of the four major histopathologic types of ovarian carcinoma are characterized by rather distinctive, though not necessarily unique, genetic abnormalities. For example, TP53 gene mutations are extremely common in serous carcinomas, while mucinous adenocarcinomas have a high prevalence of KRAS mutations (46,82-84). Mutations of CTNNB1, the gene encoding β -catenin, are common in endometrioid adenocarcinomas but rare in serous, mucinous and clear cell carcinomas (85,86). Mutations of PIK3CA, which encodes the catalytic subunit of PI3K (phosphoinositide 3- kinase), are observed most frequently in clear cell carcinomas (87). The point is that specific genetic defects are likely to be present in certain histologic types of ovarian carcinomas, and unlikely to be present in others. Practicing pathologists and researchers alike are accustomed to thinking about molecular alterations in tumors on a gene-by gene basis. Indeed, pathologists routinely use immunohistochemical staining, with one antibody at a time, to evaluate altered expression of individual gene products in tumor sections. Just a few years ago, the notion of comprehensively and simultaneously evaluating expression or DNA copy number of thousands of genes in a single assay seemed rather far-fetched. Nevertheless, using technologies developed during the past decade, reams of information can now be collected routinely from

individual tumor samples based on single experiments. A previously published study using oligonucleotide microarrays to evaluate gene expression in a sizeable series of primary ovarian carcinomas is illustrative (85,88). In this study, Affymetrix U-133A oligonucleotide microarrays were used to simultaneously interrogate expression of approximately 14,500 well characterized genes in 99 primary ovarian carcinomas (mostly serous or endometrioid with fewer clear cell and mucinous carcinomas). A statistical method called principal component analysis (PCA) was used to compare global gene expression in each tumor by distilling variances in expression across the entire set of genes down to a two-dimensional plot, in which tumors that fall closer together on the plot are more similar in their global gene expression than tumors that are farther apart. Figure 3A shows PCA data from each of the 99 primary tumors. When viewed separately, serous carcinomas (each represented by a green circle, Figure 3B) are mostly clustered together toward the left side of the two-dimensional PCA plot. Note that clear cell and mucinous carcinomas (yellow and blue circles, respectively) are quite distinct from serous carcinomas with respect to gene expression pattern, as shown by their own clustering profiles (Figures 3C and 3D). The divergent gene expression profiles observed amongst serous, clear cell, and mucinous carcinomas likely reflect, at least in part, readily apparent differences in the histopathologic features of these tumor types. The gene expression profiles of the endometrioid adenocarcinomas are more problematic. While a number of these tumors appear to be quite distinct from the serous carcinomas, others overlap directly into the serous carcinoma gene expression space (Figure 3E). These findings prompted an investigation of the basis for the apparent heterogeneity of endometrioid tumors, because each of the four histological groups was originally expected to have clearly distinct gene expression profiles.

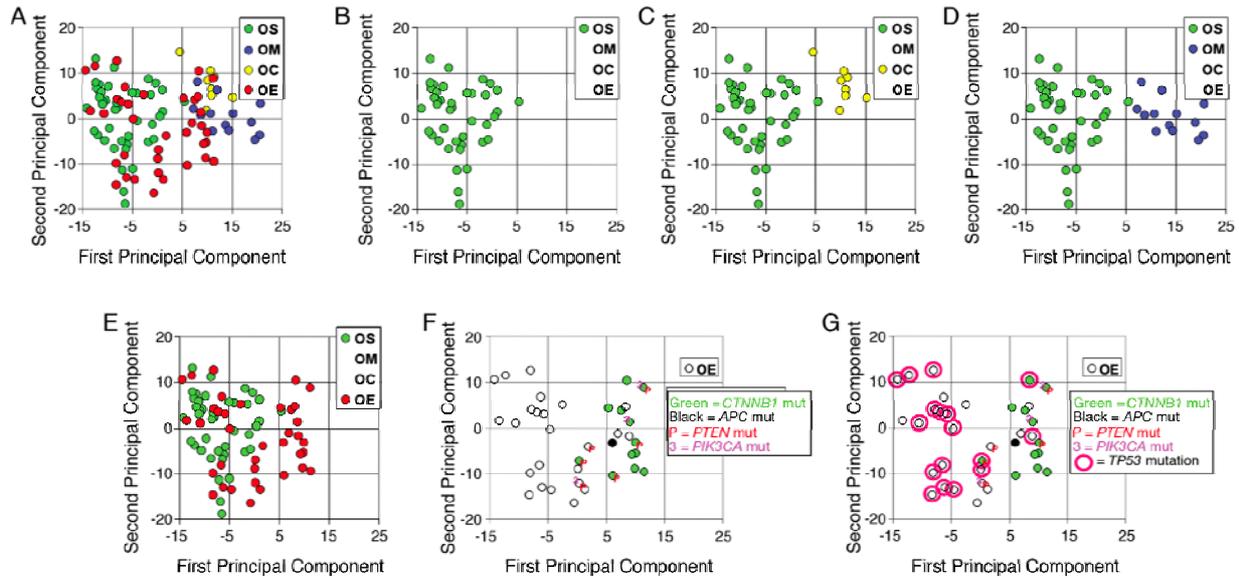


Figure 3. Gene expression profiling of primary ovarian carcinomas. A) Principal component analysis (PCA) of 99 ovarian carcinomas using all probe sets on the U133A array; the first two principal components are shown. Individual tumors are annotated with histopathologic type as indicated (green-OS, serous; blue-OM, mucinous; yellow-OC, clear cell; and red-OE, endometrioid); B) Same PCA plot showing only serous carcinomas; C) Same PCA plot showing only serous and mucinous carcinomas; D) Same PCA plot showing only serous and endometrioid carcinomas; E) Same PCA plot showing only endometrioid carcinomas annotated with mutational status of CTNNB1, APC, PTEN and PIK3CA as indicated; G) Same PCA plot showing only endometrioid carcinomas annotated with mutational status of CTNNB1, APC, PTEN, PIK3CA, and TP53 as indicated.

NEW CONCEPTS OF OVARIAN TUMOR CLASSIFICATION

This “two pathway” scheme for ovarian endometrioid carcinoma pathogenesis is reminiscent of one previously proposed for ovarian serous carcinomas (89,90). Low-grade serous carcinomas typically show micropapillary architecture, and often arise in association with recognizable precursors – specifically, serous borderline tumors (serous tumors of low malignant potential; atypical proliferative serous tumors). The low grade serous carcinomas characteristically have mutations of KRAS or BRAF, but TP53 mutations are uncommon in these tumors. Mutations of KRAS or BRAF lead to constitutive activation of the MAPK (mitogen-activated protein kinase) signaling pathway. MAP kinases are serine/threonine-specific protein kinases that respond to extracellular stimuli (mitogens) and regulate various cellular activities, such as gene expression, mitosis, differentiation, and cell survival/apoptosis. Although the low-grade serous carcinomas tend to behave in a more indolent fashion than their high grade counterparts, some investigators have noted poor response of

low-grade serous carcinomas to platinum-based therapeutic regimens (90). The great majority of serous carcinomas are high-grade and precursor lesions for these tumors remain poorly defined. Possible sites of origin include the ovarian surface epithelium, surface epithelial inclusion cysts, and the distal fallopian tube. High-grade serous carcinomas have a high prevalence of TP53 gene mutations, while mutations of KRAS or BRAF are rare. Collectively, studies of the type described above have led to the proposal of a new model for classifying ovarian carcinomas - in which the surface epithelial tumors can be divided into two broad categories designated Type I and Type II tumors based on their pattern of tumor progression and molecular genetic changes (45,91). Importantly, in this model, Type I and Type II refer to tumorigenic pathways and are not specific histopathologic diagnostic terms. Type I tumors include low-grade serous carcinoma, low-grade endometrioid carcinoma, mucinous carcinoma, and a subset of clear cell carcinomas, which develop in a stepwise fashion from well-recognized precursors, in most cases, borderline tumors. The borderline tumors, in turn, appear to develop from the ovarian surface epithelium or inclusion cysts in the case of serous and mucinous tumors and from endometriosis in the case of endometrioid and clear cell tumors. Most Type I tumors are slow growing as evidenced by the observation that they are generally large and often confined to the ovary at diagnosis. In contrast, the Type II tumors are high- grade and almost always have spread beyond the ovaries at presentation. Type II carcinomas include high-grade serous carcinoma, high-grade endometrioid carcinoma, undifferentiated carcinoma, probably some clear cell carcinomas, and malignant mixed mesodermal tumor (carcinosarcoma). Other than their association with endometriosis (in keeping with the Type I pathway), the clinicopathologic and molecular features allowing distinction of Type I from Type II clear cell carcinomas are yet to be defined. Type II carcinomas presumably evolve rapidly, disseminate early in their clinical course and are highly aggressive. In contrast to Type I tumors, Type II tumors are rarely associated with morphologically recognizable precursor lesions; however, Type II tumors may arise from “dysplasia” in inclusion cysts or serous intraepithelial carcinoma in the fallopian tubes (92-94). These precursor lesions may be difficult to recognize because they

presumably undergo rapid transit from the occult lesion to a clinically diagnosed carcinoma. Type I and Type II tumors have very different molecular profiles. Chromosomal instability levels, as reflected by genome-wide changes in DNA copy number, are much higher in Type II tumors than in Type I tumors. Type I tumors often harbor somatic mutations of genes encoding protein kinases including KRAS, BRAF, PIK3CA and ERBB2, and other signaling molecules including CTNNB1 and PTEN. In contrast, Type II tumors generally lack these mutations but are characterized by a high frequency of TP53 mutations which are rare in Type I tumors. The division of ovarian cancer into two broad groups, Type I and Type II, continues to emphasize the heterogeneity of ovarian cancers, but also provides a morphological and molecular framework for future studies aimed at improving our understanding of ovarian cancer pathogenesis and developing more effective strategies for their early detection. Previous attempts to improve early diagnosis of ovarian cancer were based on the assumption that ovarian cancer represents one disease when, from a pathogenetic view, it is at least two - with each having different implications for early detection. Current strategies are largely aimed at detecting low stage tumors. These strategies are most suitable for detecting tumors belonging to the Type I group, which often present as large tumor masses without dissemination at the time of diagnosis. A more useful endpoint for early detection of Type 2 ovarian carcinomas may be low tumor volume rather than low tumor stage. Importantly, it is well recognized that the most important prognostic indicator for Type 2 tumors is not stage at diagnosis, but the volume of residual disease following cytoreductive surgery (95,96). The published studies should prompt practicing pathologists to strongly consider the merits of a two-grade system for assigning tumor grade to ovarian carcinomas, rather than the traditional three-grade system used for most tumors. Indeed, two-tiered systems for grading serous carcinoma, the most common type of ovarian carcinoma, have already been proposed (10,11). Although uncertainties remain regarding the specific criteria that should be used to distinguish Type 1 from Type 2 ovarian carcinomas, careful analysis of both morphological and molecular features should help in determining which criteria are most appropriate.

PHARMACEUTICAL MANAGEMENT OF OVARIAN CANCER

A LONG HISTORY OF THE ADMINISTRATION OF ANTINEOPLASTIC AGENTS IN THE MANAGEMENT OF OVARIAN CANCER

For more than 50 years, epithelial ovarian cancer has been recognized to be one of the most biologically sensitive solid tumours to cytotoxic chemotherapeutic agents (97). During the earliest days of the modern chemotherapeutic era, the newly identified alkylating agents were examined as therapeutic strategies in this malignancy (97,98). Although the definitions of clinical activity were not as clearly delineated during this time period as they are today, it was evident that palliation of distressing symptoms (e.g. abdominal pain resulting from malignant ascites accumulation) was achieved in a substantial percentage of individuals treated with several drugs (melphalan, thiotepa, cyclophosphamide) in this therapeutic class (99). Unfortunately, most of these responses were relatively short lived. Moreover, long-term follow-up revealed that a subset of ovarian cancer patients who received alkylating agents for extended periods of time as a result of impressive control of the malignant process, ultimately died as a direct result of developing treatment-induced secondary acute myelogenous leukaemia (100,102). Not surprisingly, this profoundly disturbing experience has appropriately tempered enthusiasm for any form of ‘maintenance therapy’ in ovarian cancer. Additional cytotoxic agents developed during this era, including doxorubicin, methotrexate, altretamine and fluorouracil (5-FU), were subsequently shown to possess at least a modest degree of biological activity in ovarian cancer (99,103). As a result, single-agent treatment of ovarian cancer (e.g. oral melphalan) was largely replaced with combination chemotherapy regimens, such as Hexa-CAF (altretamine, cyclophosphamide, doxorubicin and 5-FU) and AC (doxorubicin and cyclophosphamide) (103-105). Limited phase III trial data confirmed that combination therapy could improve objective response rates compared with single alkylating agents, but the overall impact on survival was more modest.

THE CISPLATIN ERA

In the 1970s, cisplatin, one of the most toxic pharmaceutical agents ever delivered to any patient (neurotoxicity, emesis, nephrotoxicity, ototoxicity), was introduced into the clinic (106-109). However, this drug, with its impressive list of distressing adverse effects, was reluctantly accepted (by patients and oncologists) because of the recognized remarkable level of both biological and clinically relevant activity of the agent in multiple tumour types, including ovarian cancer (110-112). Cisplatin was initially revealed to produce objective responses in women with ovarian cancer whose disease was shown to be resistant to alkylating agent therapy (111,112). Of note, during this era, the definition of ‘resistance’ varied, and essentially included all patients whose cancers recurred or progressed following initial therapy. Following this experience, cisplatin was quickly moved to the front-line setting (113-116) and the agent subsequently became established as the cornerstone of the chemotherapeutic management of ovarian cancer. Both individual phase III randomized trials and several meta-analyses involving the results of multiple studies, have revealed the platinum agents to be the single most active class of antineoplastic drugs in this malignancy (113-117).

CISPLATIN-BASED COMBINATION CHEMOTHERAPY

For a period of time there existed considerable controversy regarding the ‘optimal’ cisplatin-based, multi-agent regimen, with individual phase III trial data supporting the two-drug combination of cyclophosphamide plus cisplatin, (118-120) but with several meta-analyses suggesting the superiority of a three-drug regimen of cyclophosphamide, doxorubicin and cisplatin (121-123). Ultimately, most investigators became convinced that any possible small benefit resulting from the addition of an anthracycline to the two-drug cisplatin plus cyclophosphamide regimen was outweighed by the well recognized additional toxicity associated with such a strategy (124).

CARBOPLATIN-BASED CHEMOTHERAPY

Initially proposed as a more active platinum drug, carboplatin has been shown in multiple phase III randomized ovarian cancer trials to be equivalent in efficacy to cisplatin, but to possess a substantially superior adverse effect profile, particularly a lower risk of severe emesis, nephrotoxicity and neurotoxicity (125-129). A specific highly appealing feature of carboplatin compared with cisplatin, is the ability to easily deliver the drug in the outpatient setting, without the requirement for extensive hydration to prevent the nephrotoxic effects of the parent drug. Also, in general, the well recognized dose-limiting haematological toxicity of carboplatin produces less severe clinically relevant consequences to patients and is easier to manage (e.g. dose reduction, use of bone marrow colony-stimulating factors) than are the adverse effects associated with cisplatin. Furthermore, compared with cisplatin, it has proven easier to combine other active antineoplastic agents in ovarian cancer with carboplatin (e.g. paclitaxel) (127-130). However, it is important to again note that the almost universal choice of carboplatin for intravenous administration in the management of ovarian cancer, rather than cisplatin, is based on a more favourable toxicity profile and ease of delivery, and not on any evidence of superior efficacy (127-130).

PLATINUM PLUS TAXANE-BASED PRIMARY CHEMOTHERAPY

In the late 1980s, paclitaxel was demonstrated to be an active agent in platinum-resistant ovarian cancer (131-133). In this era, the definition of 'primary chemotherapy resistance' in ovarian cancer had become reasonably well standardized to include those patients whose cancers had failed to respond to initial treatment (disease progression or 'stable disease' as best response) or where an objective response had occurred, but the disease subsequently progressed within 6 months of discontinuation of platinum-based therapy (132). Of interest, similar to the initial experience with cisplatin in ovarian cancer (108,109), the early experience with paclitaxel suggested the drug was quite toxic (134,135), and its continued use was justified principally by the level of biological and

clinical activity observed (131-133). Also, similar to the drug development process for cisplatin, where evidence of activity in the second-line setting (alkylating-resistant) led to incorporation of the agent into primary chemotherapy trials (111-113), paclitaxel was quickly combined with cisplatin in the front-line setting and directly compared with the ‘standard of care’ at this point in time, which was a platinum agent plus cyclophosphamide (117,136,137). Although several trials subsequently confirmed the superiority of a cisplatin plus paclitaxel combination in improving survival compared with the previous ‘standard’ of cisplatin and cyclophosphamide (136,137), this outcome was not observed in all phase III evaluations (117,138). More recent evidence-based data have documented the equivalence of a cisplatin plus paclitaxel versus a carboplatin plus paclitaxel regimen employed as primary treatment of advanced ovarian cancer (Table I) (127-130).

Table I. Evidence-based platinum-taxane regimens employed as primary chemotherapy of advanced ovarian cancer

Cisplatin (75 mg/m²) + paclitaxel (135 mg/m² over 24 h) q21d × six cycles^[34,40]
Carboplatin (AUC 6–7.5) + paclitaxel (175 mg/m² over 3 h) q21d × six cycles^[31-34]
Carboplatin (AUC 6) + docetaxel (75 mg/m²) q21d × six cycles^[44]
AUC = area under concentration-time curve; q21d = every 21 days.

Again, the carboplatin-based combination is generally preferred by most oncologists because of the ease of administration (simple outpatient regimen) and overall superior toxicity profile (less emesis, nephrotoxicity, neurotoxicity) (127-130,137,139), but the survival of patients treated with paclitaxel plus either carboplatin or cisplatin is equivalent. A large phase III trial has also directly compared the delivery of carboplatin plus paclitaxel versus carboplatin plus docetaxel as primary treatment of advanced ovarian cancer (140). Again, the two carboplatin regimens produced equivalent survival outcomes, but the regimens were associated with quite different toxicity profiles. The paclitaxel-containing programme was associated with a modestly higher risk of peripheral neuropathy, while patients treated with the docetaxel plus carboplatin regimen experienced a moderately greater incidence of potentially clinically relevant neutropenia. There was no difference in therapy-related

mortality between the two carboplatin-based approaches.

ADDITIONAL STRATEGIES EXPLORED TO IMPROVE PRIMARY CHEMOTHERAPY OF ADVANCED OVARIAN CANCER: INTRAPERITONEAL CHEMOTHERAPY

The concept of intraperitoneal (IP) delivery of chemotherapy for the treatment of ovarian cancer has been around for 30 years (141). Key aspects of the biologic behavior of ovarian cancer lend themselves particularly well to the pharmacology of drugs used to treat this disease (Table 2).

Biological Consideration in Ovarian Cancer	Pharmacological Consideration of IP Therapy
Majority of women present with metastases to intra-abdominal lymph nodes and peritoneal surfaces	Delivery of chemotherapy directly into abdominal cavity with a high degree of safety
Susceptible to cytoreductive surgery, leaving small volume residual disease behind	Small tumor volumes allow delivery of high concentrations of drug to tumor surface and penetration to a depth of 0.5-1.0 mm
The region at risk (the peritoneum) represents a large surface area of highly vascularized tissue	Promotes absorption of drug to systemic circulation and delivery of drug via tumor vasculature
Responsive to platinum and taxane therapy	IP:IV pharmacological advantage of 20 for platinum and 1,000 for taxanes

Abbreviations: IP, intraperitoneal; IV, intravenous.

Initially, small phase I and II clinical trials confirmed the feasibility of this approach and observed that higher concentrations of drug could be achieved in the peritoneal space with IP than with IV therapy (142,143). In addition, they noted absorption across the peritoneum was sufficient to achieve clinically active systemic concentrations of the drug (143). Lastly, they observed clinical benefit in terms of reduction of tumor bulk and ascites (143). This led to a period of approximately 10 years during which various drugs were administered intraperitoneally in women with recurrent, small volume disease in a series of phase II trials. These trials were important in defining the efficacy as well as toxicity of IP therapy in this setting. However, controversy remained over what role, if any, IP therapy had as part of first-line treatment of epithelial ovarian cancer. Three randomized trials performed by the US cooperative groups during the past 10 years provide the most extensive data on the use of IP chemotherapy in the first-line setting.

DATA FROM CLINICAL TRIALS

In the Southwest Oncology Group (SWOG) 8501/ Gynecologic Oncology Group (GOG) 104 trial, Alberts et al compared IP cisplatin and intravenous (IV) cyclophosphamide to IV cisplatin and IV cyclophosphamide in women with stage III epithelial ovarian cancer after exploratory laparotomy and removal of all tumor masses larger than 2 cm (144). Six hundred fifty-four patients were randomly assigned and 546 were eligible for the study. The patients received six cycles of IV cyclophosphamide 600 mg/m² plus either IP cisplatin 100 mg/m² or IV cisplatin 100 mg/m² at 3-week intervals. At the completion of therapy, patients with a complete clinical response underwent a second-look laparotomy to determine pathologic response. During accrual, and without knowledge of the therapeutic results, the sample size was increased in order to stratify response according to size of residual tumor after surgery. This was done because of the hypothesis that patients with the smallest residual tumor — ≤ 0.5 cm in greatest dimension — would be the group most likely to benefit from IP chemotherapy. In both the IP and IV groups, 58% of patients received all six cycles of cisplatin chemotherapy. Two hundred ninety-seven patients underwent second-look laparotomy. The rate of complete pathologic response was 47% in the IP group and 36% in the IV group. Statistical comparison was not performed due to the small percentage of patients that underwent second-look laparotomy. All eligible patients were included in survival analysis, regardless of whether they completed their assigned treatment or not. The median survival was 41 months in the IV group and 49 months in the IP group. The hazard ratio for risk of death in the IP group, as compared with the IV group, was 0.76 (95% CI, 0.61 to 0.96; P = .02). The effect of the treatment, IV or IP, was not influenced by the extent of residual disease. Two treatment related deaths occurred in the IP group and none occurred in the IV group. Granulocytopenia and leukopenia ≥ grade 3 was significantly higher in the IP group as was abdominal pain ≥ grade 2 and transient dyspnea. Tinnitus, hearing loss, and grade 2 or 3 neuromuscular toxic effects at the end of treatment were significantly higher in the IV group. This study was published in 1996. At this time it was also shown that IV paclitaxel and cisplatin was superior to IV cyclophosphamide and cisplatin.

Therefore, interest was turned to combining IV paclitaxel and IP cisplatin. In the GOG 114/SWOG 9227 trial, Markman et al (145) compared a control arm of IV paclitaxel and cisplatin for six cycles with an experimental arm of two doses of high-dose carboplatin followed by IV paclitaxel and IP cisplatin for six cycles in patients with optimally debulked (largest residual tumor nodule ≤ 1 cm in maximum diameter) stage III epithelial ovarian cancer. Initially, a third arm consisting of IV cyclophosphamide and cisplatin was included, but this was discontinued due to evidence of the superiority of IV paclitaxel over cyclophosphamide. Five hundred twenty-three patients were enrolled and 462 were eligible. Six point eight percent of patients randomly assigned to the IP arm received no IP therapy and 18.3% received two or fewer cycles. Two patients from each group died from chemotherapy-related causes. Grade 4 neutropenia, grade 3 to 4 thrombocytopenia, grade 3 to 4 gastrointestinal and metabolic toxicity were all higher in the IP arm. Progression-free survival was longer in the IP arm, with median time to tumor recurrence of 27.9 months compared with 22.2 months in the IV arm. The relative risk estimate in the IP arm compared with the IV arm was 0.78 (90% CI, 0.66 to 0.94). Overall survival was also longer in the IP arm, 63.2 months versus 52.2 months ($P = .05$). The estimated relative risk for death of a patient treated on the IP arm compared with the IV arm was 0.81 (90% CI, 0.65 to 1.00). The results of this trial coupled with promising results of a phase II trial exploring the combined use of IP cisplatin and IP/IV paclitaxel (146) led to the GOG 172 trial, which compared the standard arm of IV paclitaxel over 24 hours followed by IV cisplatin on day 2 to IV paclitaxel over 24 hours following by IP cisplatin on day 2 and IP paclitaxel on day 8 in women with stage III ovarian carcinoma with largest residual mass less than or equal to 1.0 cm (147). Four hundred twenty-nine patients were randomly assigned and 415 were eligible. Ninety percent of patients in the IV group received six cycles of chemotherapy and 83% received six cycles of the assigned treatment. Eighty-three percent of patients in the IP group received six cycles of chemotherapy and 42% received six cycles of the assigned IP treatment. The primary reason for discontinuing IP therapy was catheter-related complications. There were significantly more patients in the IP group with severe (grade 3 or 4) fatigue, pain, and hematologic,

gastrointestinal, metabolic, and neurologic toxicity. The median progression-free survival was 23.8 months in the IP group versus 18.3 months in the IV group ($P = .05$). The median overall survival was 65.6 months in the IP group versus 49.7 months in the IV group ($P = .03$). Second-look laparotomy was optional. Complete pathologic response was noted in 57% of the IP group (46 of 81 patients) and 41% of the IV group (35 of 85 patients). Although fewer than one half of patients in the IP group received six cycles of IP therapy, the group had superior survival to the IV group. The results of GOG 172, combined with the consistent results in the two previous trials, led the National Cancer Institute to issue a clinical announcement in January 2006, recommending that women with stage III ovarian cancer that undergo optimal surgical cytoreduction be considered for IP chemotherapy (148). The clinical announcement mentions that a significant improvement in overall survival is associated with IP chemotherapy with an increase in toxicity, although this is short-term and manageable.

STRENGTHS OF THE DATA

The data confirm biologic and pharmacologic hypotheses. The peritoneal route of spread for ovarian cancer coupled with the pharmacologic advantage for platinum and taxanes administered via the IP route suggest that IP administration should result in superior therapeutic outcomes compared with exclusively IV administered drugs. The data are consistent across studies. In all three trials, the IP arm outperformed the IV only arm in terms of progression-free and overall survival. IP therapy resulted in a 20% reduction in risk of recurrence and a 20% to 25% reduction in the risk of death (Table 2).

Author	Trial	Sample Size	Treatment		PFS (IP/IV v IV)			OS (IP/IV v IV)		
			IV	IP	Median (months)	P	HR	Median (months)	P	HR
Alberts et al ⁴	SWOG 8501/GOG 104	267	Cyclophosphamide 600 mg/m ² IV q 21 days × 6 cycles	Cisplatin 100 mg/m ² IP q 21 days × 6 cycles	NS	NS	NS	49		
		279	Cyclophosphamide 600 mg/m ² IV + cisplatin 100 mg/m ² IV q 21 days × 6 cycles	None	NS	NS	NS	41	.02	.76
Markman et al ⁶	GOG 114/SWOG 9227	235	Carboplatin AUC 9 IV × 2 cycles prior to any IP treatment; paclitaxel 135 mg/m ² IV over 24 hours day 1 q 21 days × 6 cycles	Cisplatin 100 mg/m ² IP day 2 q 21 days × 6 cycles	27.9			63.2		
		227	Paclitaxel 135 mg/m ² IV over 24 hours day 1; cisplatin 75 mg/m ² IV day 2 q 21 days × 6 cycles	None	22.2	.01	.78	62.2	.05	.81
Armstrong et al ⁷	GOG 172	205	Paclitaxel 135 mg/m ² IV over 24 hours day 1	Cisplatin 100 mg/m ² IP day 2 q 21 days × 6 cycles; paclitaxel 60 mg/m ² IP day 8	23.8			65.6		
		210	Paclitaxel 135 mg/m ² IV over 24 hours day 1; cisplatin 75 mg/m ² IV day 2	None	18.3	.05	.80	49.7	.03	.75

Abbreviations: IP, intraperitoneal; IV, intravenous; PFS, progression-free survival; OS, overall survival; HR, hazard ratio; SWOG, Southwest Oncology Group; GOG, Gynecologic Oncology Group; NS, not stated; q, every; AUC, area under the curve.

These results are clinically meaningful. The magnitude of improvement in median survival observed in these three trials ranges from 8 to 16 months. These differences are both statistically and clinically meaningful. These results are supported in a larger context. A recently published meta-analysis that included 38,440 women who participated in 198 trials over a 35-year period reported a 22% reduction in death hazard ratio for women who received IP therapy in the subset of 12 trials comparing an IP versus non-IP regimen (149).

WEAKNESS OF THE DATA

The trials were not pure tests of IP therapy. No trial evaluated chemotherapy administered exclusively by the IP route. Rather, each trial compared a combined IP/IV regimen with an IV regimen. The control arms did not represent the standard of care. Although the control arms represented the standard of care at the time each trial was designed, the standard of care in clinical practice changed during the course of each of these studies. This led to skepticism by some that the trial results were not relevant to clinical practice and, therefore, should not lead to a change in patient management. The use of the cisplatin plus paclitaxel combination instead of carboplatin plus paclitaxel in GOG 172 could have inflated the benefit of the IP/IV arm. Although a prior GOG

study (GOG 158) demonstrated no statistically significant difference between these regimens, there was a trend for improved progression-free and overall survival for the carboplatin plus paclitaxel arm (127,150). Therefore, the superiority of IP/IV therapy has not been firmly established and the carboplatin IV plus paclitaxel IV combination remains a viable option in 2007 for women with optimally debulked epithelial ovarian cancer (150,151). IP therapy could not be administered as planned due to intolerance and toxicities. In these three trials, only 42% to 71% of women could receive all six cycles of IP/IV therapy as planned. The most common reasons for discontinuation of IP treatment were catheter-related complications, pain, fatigue, myelosuppression, gastrointestinal or metabolic toxicities. Substantial concerns about quality of life, technical difficulties associated with IP administration, and lack of reimbursement for the additional treatment time involved in delivering IP therapy continue to limit the adoption of this as standard of care. These are considered to be substantial contributing causes to the lack of more widespread adoption of IP therapy in the community.

IMMUNOTHERAPY OF OVARIAN CANCER

Although the cancer cell remains the main target of oncologic therapy, it is becoming progressively clear that the tumor microenvironment provides critical support to tumor growth and therefore opportunities for therapy. Inhibition of tumor angiogenesis is an obvious example of effective biological therapy that has produced clinical results. Importantly, complex mechanisms regulating immune response and inflammation interface with angiogenesis at the tumor microenvironment, and their balance can greatly affect the fate of tumors. The overall balance of tumor inflammatory mechanisms is polarized to promote angiogenesis, tumor cell survival and immune escape, all contributing to tumor growth. However, it is becoming clear that many patients with gynecologic malignancies mount a spontaneous antitumor immune response. Although ineffective to reject tumor, this can be potentially harnessed therapeutically. The use of immunomodulatory therapy is predicated on the notion that gynaecologic cancers are potentially immunogenic tumors, i.e., they

can be recognized and attacked by cell-based immune mechanisms. Cervical and lower genital tract cancers induced by human papillomavirus (HPV) are the prototype of potentially immunogenic tumors that can elicit a spontaneous immune response. HPV xenoantigens expressed by tumor cells are readily recognized by the immune system. Cell-mediated immune responses are important in controlling HPV infections as well as HPV-associated neoplasms (152). The prevalence of HPV-related diseases is increased in patients with impaired cell-mediated immunity, including transplant recipients (153) and HIV-infected patients (154,155). Infiltrating CD4⁺ (T helper cells) and CD8⁺ (cytotoxic) T cells have been observed in spontaneously regressing warts (156), and warts often disappear in patients who are on immunosuppressive therapy when treatment is discontinued (157). In addition, animals immunized with viral proteins are protected from HPV infection or the development of neoplasia and experience regression of existing lesions (158,159). Nevertheless, patients with invasive cervical cancer exhibit exhausted and tolerized T cells that recognize antigen in vitro but are unable to reject tumors in vivo (160,161). The emergence of immunomodulatory therapies revives opportunities to activate and invigorate such T-cell immunity and warrants clinical testing. Although tumor-associated antigens have not undergone rigorous scrutiny in other gynaecologic malignancies (162), similar mechanisms of spontaneous antitumor immune response have been convincingly demonstrated. Tumor-reactive T cells and antibodies have been detected in peripheral blood of patients with advanced stage ovarian cancer at diagnosis (163,164), while oligoclonal tumor reactive T cells have been isolated from tumors or ascites (165-173). Importantly, the detection of intratumoral or intraepithelial tumor infiltrating lymphocytes (TIL), i.e., T cells infiltrating tumor islets predicts significantly improved progression survival and overall survival in ovarian cancer. It has been reported in an Italian cohort that patients whose tumors had intraepithelial T cells experienced 3.8-fold longer median progression-free survival and 2.8-fold longer overall survival as compared to patients whose tumors lacked intraepithelial T cells. Remarkably, survival rate at 5 years was 38% in patients whose tumors had intraepithelial T cells (n = 102) and 4.5% in patients lacking them (n = 72). The impact of intraepithelial T cells was

confirmed by multiple independent studies on ethnically diverse populations (174-180). Similar observations were made in endometrial cancer (181-183) and other solid tumors (184). Retrospective studies showing that the incidence of many non-virally induced solid tumor types is in fact 4- to 30-fold increased in immunosuppressed transplant recipients (185-189) provide evidence that immune recognition is probably a universal mechanism in tumors.

CHEMOTHERAPY AS AN IMMUNE MODULATOR

Although it has been traditionally thought that chemotherapy antagonizes immune mechanisms altogether, recent evidence has challenged this view. Indeed, agents such as cyclophosphamide, doxorubicin and paclitaxel increase the number and function of antigen-specific T cells and thus may enhance cancer immunity (190). It is becoming progressively clear that conventional chemotherapy has important “off-target” immunologic effects and, in fact, may depend on activation of immune mechanisms to achieve its full efficacy. In mouse models of solid tumors, increased tumor inflammation following administration of chemotherapy predicts better prognosis (191), while tumors grown in immunodeficient mice fail to respond to chemotherapy (192), clearly highlighting a role for the immune system in cancer clearance in the context of cytotoxic therapy. Similar events may occur in humans; tumor-infiltrating lymphocytes predicted complete pathologic response in breast cancer patients after neoadjuvant chemotherapy (193). Furthermore, neoadjuvant taxol therapy was found to increase TIL (194). Interestingly, breast cancer patients bearing a loss-of function Asp299Gly polymorphism of the Toll-like receptor (TLR) 4 receptor exhibit a higher risk of relapse after treatment with chemotherapy and radiation therapy (195). The immunomodulatory effects of chemotherapy can be broadly grouped in three mechanisms: (a) induction of immunogenic cancer cell death, which facilitates tumor antigen presentation (in situ vaccination); (b) direct activation of antigen presenting or effector mechanisms; and (c) suppression of immune inhibitory cells, thereby releasing regulatory breaks on antitumor immune response (Fig. 4). These mechanisms are quite complex and our understanding is still in its infancy, but effects appear to be

dependent on drug type, dose and schedule, as well as the immune cell type.

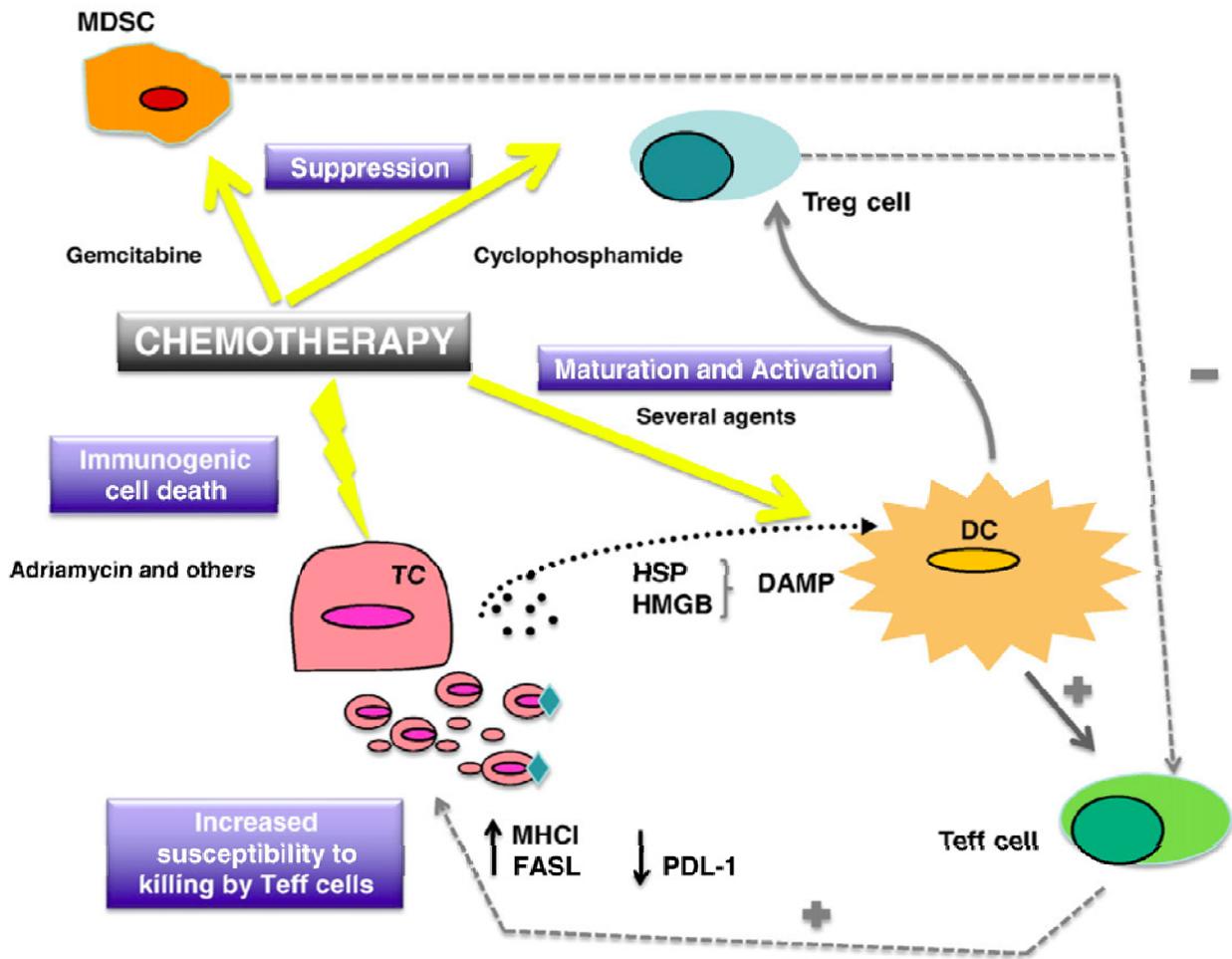


Figure 4. Immunomodulation by chemotherapy (schematic representation)

NON-SPECIFIC IMMUNE ACTIVATION

Multifaceted, pleiotropic immune activation can be achieved with cytokines and Toll-like receptor agonist therapy and is suitable for combination with immunomodulatory chemotherapy.

INTERFERONS

Interferons were first described as antiviral cytokines, but have since been shown to be secreted in response to a vast number of stimulatory factors other than viruses. They are divided into two broad

categories: type I and type II interferons. Type I interferons are subdivided into two classes, known as alpha and beta. Interestingly, 12 forms of IFN- α have been identified, while only one form of IFN- β has been isolated. Signalling through their corresponding receptors on target cells is mediated by a series of Jak/STAT proteins and results in several antiviral activities. Additionally, they have potent effects on cell proliferation. Mouse models have demonstrated that gene therapy with IFN- β can greatly enhance tumor cell death in the context of several different malignancies (198). Many clinical trials have demonstrated the efficacy of type I interferon therapy in the treatment of hematologic malignancies (199-201), melanoma (201-206) and renal cell carcinoma (207-209). Phase I/II clinical studies have examined the therapeutic value of type I IFNs in ovarian cancer. Intraperitoneal recombinant IFN- α alone or combined with cisplatin as salvage therapy for persistent ovarian cancer after primary chemotherapy has shown clinical efficacy in small volume disease (210,211), but there was no significant effect in a cohort of patients with recurrent, platinum-resistant disease (212). Although encouraging, these results did not support additional clinical development of type I interferon in ovarian cancer. One of the limitations of interferon therapy relates to the high intratumoral cytokine levels required to induce antitumor responses, which cannot be achieved without eliciting systemic toxicity and cannot be sustained owing to the short half-life of recombinant proteins. Cytokine gene therapy using recombinant viral vectors can achieve much higher and sustained cytokine levels at the tumor site than those resulting from systemic or regional administration of recombinant cytokine proteins without engendering systemic toxicity (213). A trial of intrapleural adenovirus delivering human IFN- β was recently completed at the University of Pennsylvania. Toxicity was minimal. One patient with recurrent, platinum-resistant low-grade ovarian carcinoma achieved complete objective and cytologic response of both pleural and intraperitoneal disease following a single intrapleural injection of adenovirus vector in this trial (214). Disease stability or objective responses were also observed in patients with malignant pleural mesothelioma enrolled in the study (215). These data present promising evidence that IFN- β can serve as a potent anticancer agent, and its use in combination with other forms of

chemo and immunotherapy certainly warrants further consideration. Structurally unrelated to type I interferons, IFN- γ is secreted by activated effector T cells and NK cells in response to target recognition. IFN- γ has been shown to have direct anti proliferative activity on ovarian cancer cells in vitro, which proved to be synergistic with cisplatin and doxorubicin (216-218). In vitro and in vivo, IFN- γ upregulates HLA class I and class II molecules and antigen presentation in ovarian tumor cells (219), a requisite for recognition by T cells. In fact, HLA class I expression by the tumor correlates with the intensity of T cell infiltration (220), a predictor of longer survival. Furthermore, IFN- γ has antiangiogenic effects (221). Encouraging results have been reported with recombinant human (rh)IFN- γ either as intraperitoneal monotherapy or in combinations in early phase trials (222-225). Theoretically, the effects are likely to be greatest in women who are also receiving chemotherapy because of IFN- γ 's non-specific immunomodulatory effects (226). Confirming expectations, a three-fold prolongation of progression-free survival was observed in a phase III multi-center study from Europe with subcutaneous administration of rhIFN-combined with MTD cisplatin and cyclophosphamide chemotherapy, with minimal added toxicity. However, in a subsequent randomized phase-III trial conducted in the United States, addition of subcutaneous rhIFN- γ to carboplatin and paclitaxel did not improve survival (227). Although one cannot exclude that racial and other demographic differences may account for opposite results, these data may indicate that the choice of chemotherapy drugs is in fact critical in combinatorial approaches with immune therapy. Indeed, whereas cyclophosphamide has potent immunomodulatory effects on many cell subsets including suppressing T regulatory (T_{reg}) cells, high dose steroids, which are necessarily given with paclitaxel to prevent acute hypersensitivity reactions, are immunosuppressive and induce T_{reg} in the setting of antigen presentation.

INTERLEUKINS

Interleukin-2 (IL-2) promotes expansion and enhances the cytotoxicity of effector immune cells (228). In addition, IL-2 can restore T-cell function following suppression by negative regulatory

receptors such as PD-1. IL-2 represents the most widely investigated cytokine for use in cancer therapy, having shown clinical efficacy in malignant melanoma and renal cell carcinoma (229,230), for which it is now FDA approved. Additionally, it has been used to enhance the efficacy of immunotherapy including vaccines and adoptive T-cell therapy (231). However, its use has several limitations. In monotherapy and in the context of adoptive immunotherapy, IL-2 is used at MTD, which induces a systemic inflammatory response, with significant morbidity including multiple organ toxicities, most significantly the heart, lungs, kidneys, and central nervous system. Other manifestation of IL-2 toxicity is capillary leak syndrome, resulting in a hypovolemic state and fluid accumulation in the extravascular space (232). Because ovarian cancer patients exhibit spontaneous antitumor immune response, IL-2 therapy may be a rational approach to activate pre-existing immunity or enhance immunomodulatory therapy. Intraperitoneal IL-2 was used in a phase I/II study in 41 patients with laparotomy-confirmed persistent or recurrent ovarian cancer. Weekly IL-2 infusion was relatively well tolerated and demonstrated evidence of long-term efficacy in a modest number of patients. Twenty percent of patients had a negative third look, i.e., exhibited pathologic evidence of complete response and no residual disease at repeat abdominal exploration (233). Rejecting pre-therapy T-cell activity, low expression of the CD3-zeta chain in peripheral blood T cells prior to therapy, a biomarker of T-cell functional suppression by tumor derived factors, predicted poor of response to IL-2 therapy (234). Importantly, IL-2 is essential for the peripheral homeostasis of CD4⁺CD25⁺Foxp3⁺ Treg cells, and it is now known that IL-2 is also an important activator of Treg suppressive activity in vivo (235). After IL-2 cessation, the number of Treg cells more efficiently dropped in patients who experienced a clinical response than in non responders (236). Together, these data indicate that patients with pre-existing tumor-reactive, functional T cells and low prevalence of Treg are those likely to benefit from IL-2 monotherapy. In another phase II study, 44 patients with EOC responding to primary chemotherapy were treated with subcutaneous low dose IL-2 and oral retinoic acid for 1 year and with intermittent schedules for up to 5 years. Patients experienced significantly improved progression-free and overall survival relative to 82

well-matched controls treated with standard therapy (237). Alternate cytokines that selectively support activation of effector cells without promoting T_{reg} cells may prove even more effective. IL-7, IL-15, IL-18 and IL-21 provide possible alternatives to IL-2; however, their function and clinical use are still under investigation. The function of IL-7 has not been completely appreciated until recently. It serves an essential role not only in lymphopoiesis but also in T-cell activity and maintenance and can promote antitumor immunity (238,239). A recent study using a mouse model of lung cancer examined the effects of IL-7 administration and found significant reduction in tumor burden, with a correlating increase in CD4⁺ and CD8⁺ T cells (240,241). IL-15 has similar functions to IL-2 in its effects of T cells, but also potentiates NK cell maturation and activity (242). IL-21 is a promising cytokine as it enhances the cytolytic activity of CD8⁺ T cells and NK cells but also modulates the activity of CD4⁺ T cells and B cells and suppresses T_{reg} cells (243). A recent phase II trial demonstrated that administration of IL-21 was associated with antitumor activity in patients with unresectable metastatic melanoma (244). IL-18 is a novel cytokine that has been shown to have very potent immunostimulatory effects, including induction of IFN- γ , TNF- α , IL-1 β , and GM-CSF, augmentation of NK cell cytotoxicity, activation of effector T cells, and promotion of T_{H1} responses, which are critical for tumor rejection. In a recent study, rhIL-18 was found to expand human effector T cells and reduce human T_{reg} in a mouse model transplanted with human peripheral blood lymphocytes (245). Clearly this biology points to a strong potential for the use of IL-18 in cancer immunotherapy. The immunostimulatory activity of IL-18 in vivo has been demonstrated in non-human primates (246) and humans (247). In phase I clinical evaluation, recombinant human (rh)IL-18 was safely administered as monotherapy to 28 patients with solid tumors, with minimal dose-limiting toxicities and two partial tumor responses (247). Toxicity has generally been mild to moderate even with repeat administration and a maximum tolerated dose has not been reached to date (248). IL-18 enhanced activation of peripheral blood CD8⁺ T cells, NK cells and monocytes and induced a transient increase in the frequency and expression level of Fas ligand (FasL) in peripheral blood CD8⁺ T cells and NK cells (248). The relatively minor toxicity of rhIL-18,

compared with other immunostimulatory cytokines that have undergone clinical development, is remarkable and renders IL-18 a well-suited drug for combinatorial approaches with chemotherapy. In mice with established ovarian carcinoma, administration of IL-18 alone was shown to have modest effects on tumor immunity, but when combined with pegylated liposomal doxorubicin chemotherapy, its effects were greatly enhanced. Clearly the use of IL-18 therapy with other immunogenic chemotherapy warrants further investigation. A phase I study is currently under way to test this hypothesis.

TOLL-LIKE RECEPTOR AGONISTS

One of the most basic mechanisms for activation of the immune system is through the Toll-like receptors (TLRs). TLRs belong to the type I transmembrane receptor family. Their expression is ubiquitous, from epithelial to immune cells. The TLR family members are pattern recognition receptors that collectively recognize lipid, carbohydrate, peptide and nucleic acid structures that are broadly expressed by different groups of microorganisms. Some TLRs are expressed at the cell surface, whereas others are expressed on the membrane of endocytic vesicles or other intracellular organelles. There are at least 10 known TLRs in humans grouped in six major families, based on their phylogenetic background (249). Each family is attributed to a general class of PAMPs. TLRs 3, 7, 8 and 9 are located mainly in endosomes; double-stranded RNA are ligands for TLR3 (250), while TLRs 7 and 8 recognize single-stranded viral RNA (251). The other TLRs are located on the cell surface (252); TLRs 1, 2, 5, 6 and 10 respond to bacterial, fungal and viral PAMPs (253-255). Lipopolysaccharides are TLR4 ligands (256). TLR engagement alerts the immune system and leads to the activation of innate immune cells. Two major signaling pathways are generally activated in response to a TLR ligand (257). One pathway involves the MyD88-independent production of type I interferons. The second uses MyD88 to activate nuclear factor-kappa B (NF- κ B), JUN kinase (JNK) and p38, finally resulting in the production of proinflammatory cytokines such as TNF- α , IL-12 and IL-1 and induction of innate effector mechanisms (258,259). Additionally, TLR triggering

induces DC maturation, which leads to the upregulation of costimulatory molecules such as CD40, CD80 and CD86, and secretion of immune modulatory cytokines and chemokines. In addition, TLRs can directly stimulate the proliferation of CD4⁺ and CD8⁺ T cells as well as reverse the suppressive function of Treg cells (252,260,261). Adding TLR 3, 4, 7 or 9 ligands was shown to activate CD8⁺ cytotoxic T cells with increased IFN- γ production and promote a stimulatory cytokine milieu at the tumor microenvironment (262,263). Optimal antitumor immunity requires robust enhancement of the effector T-cell response induced by tumor antigenic peptides and control or elimination of T_{reg} suppressive function. Thus, the combination of peptide-based vaccines with TLR agonists, in particular a TLR8 agonist, may greatly improve the therapeutic potential of cancer vaccines. Several clinical trials have demonstrated that administration of agonists for TLRs 3,4,7 and 9 can enhance activity of cancer vaccines in the context of non-small cell lung cancer (264), non-Hodgkins lymphoma (265,266), glioblastoma (267), and superficial basal cell carcinoma (268). Multiple TLR agonists have also been explored in melanoma. TLR 7 or 9 agonists were used in combination with melanoma antigen vaccine in advanced melanoma (182,269,270). In addition, the TLR ligand Ribomunyl has been used in conjunction with a dendritic cell vaccine in a phase I/II trial, which reported a median survival of 10.5 months in patients with advanced melanoma (271). The use of TLR agonists in the clinic requires careful preclinical evaluation. For example, in the absence of specific cell-mediated antitumor immunity, non-specific activation of inflammation could in fact promote tumor growth rather than reducing it, because of the potent tumor-promoting effects of inflammation (272). Thus, combinations with active immunization or adoptive immunotherapy seem ideal, as these approaches greatly benefit from concomitant activation of innate immune response. If combination with chemotherapy is designed, it seems rational to combine TLR agonists with chemotherapy drugs that can activate cellular immune mechanisms. Finally, the choice of TLR agonists may matter. Whereas TLR 3 and 9 agonists induce apoptosis of TLR-expressing tumor cells (273) TLR4 agonists were shown to promote tumor cell survival, tumor growth and paclitaxel resistance in a proportion of ovarian cancer cells (274,275).

ACTIVATION OF CELLULAR IMMUNITY

Generation of a successful antitumor adaptive immune response requires first and foremost the primary signal provided by the binding of T-cell receptor to cognate tumor antigen. However, multiple secondary signals can activate or suppress this response. Characterization of these pathways in tumors and the development of specific agonistic or antagonistic antibodies or ligands have created new opportunities for powerful stimulation of antitumor immune response (Fig. 5).

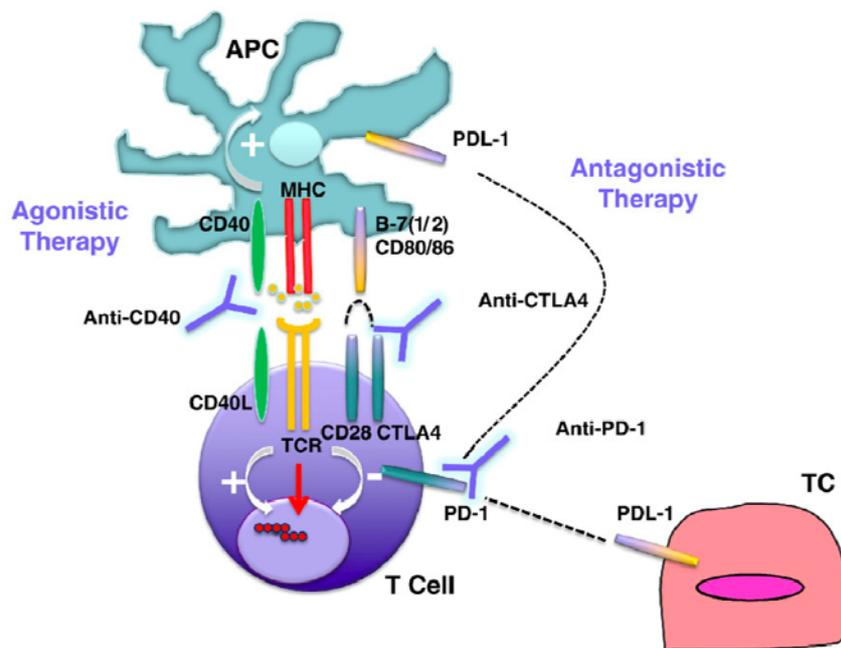


Figure 5. Activation of cellular immunity (schematic representation)

DC ACTIVATION VIA CD40

The CD40 receptor is a member of the TNF receptor family expressed by antigen-presenting cells and B cells. Its ligand, CD40L, is transiently upregulated on activated T cells, activated B cells and platelets; and under inflammatory conditions is also induced on monocytes and other innate immune cells. CD40 is a potent stimulator of antigen presenting cells and cellular immunity, and CD40/CD40L interaction is critical in the development of protective anti-tumor immunity. Mice deficient in CD40 fail to mount a protective anti-tumor immune response following vaccination. In addition, neutralizing anti-CD40L monoclonal antibody can abrogate the therapeutic value of potent tumor vaccines (276). Vice versa, a CD40 agonistic antibody was shown to be able to overcome

peripheral tolerance and generate antitumor immunity able to reject tumors (277). The main mechanism of immune stimulation by CD40 ligands is activation of DCs resulting in increased survival, upregulation of costimulatory molecules, and secretion of critical cytokines for T cell priming such as IL-12. This promotes antigen presentation, priming and cross-priming of CD4⁺ and CD8⁺ effector T cells (278). However, agonistic anti CD40 antibody alone can have adverse effects on antitumor immunity as in the mouse it can ultimately impair the development of tumor-specific T cells (279) or accelerate the deletion of tumor-specific cytotoxic lymphocytes in the absence of antigen vaccination (280). CD40 ligation could thus be best used in combinatorial approaches including vaccines and TLR agonists (278,281). Based on the immunomodulatory effects of select chemotherapy agents, the combination of CD40 ligands with chemotherapy is also a rational approach that warrants thorough investigation. For example, in mice with established solid tumors, the administration of gemcitabine with CD40L triggered potent antitumor immune response that eliminated tumor burden, and these mice became also resistant to repeated tumor challenge (282). Interestingly, the CD40 receptor is expressed on a variety of tumors including melanoma, lung, bladder and prostate cancers, but also cervix (283) and the majority of ovarian cancers (284-288). Because tumor cells also express the CD40L, it is likely that low-level constitutive engagement of CD40 facilitates malignant cell growth. However, transient potent activation of CD40 on carcinomas with ligand results in direct anti-proliferative effects and apoptosis. CD40 agonists promoted apoptosis and resulted in growth inhibition of ovarian carcinoma lines expressing CD40. CD40 ligation also induced NF- κ B activation and TNF- α , IL-6 and IL-8 production in most EOC cell lines (284,289). In vivo, administration of rhuCD40L inhibited the growth of several ovarian adenocarcinoma xenografts in severe combined immunodeficient mice through a direct effect causing apoptosis, fibrosis and tumor destruction. The antitumor effect of rhuCD40L was further increased by cisplatin (290). Interestingly, rIFN- γ enhanced expression of CD40 on tumor cells and the efficacy of on EOC cell lines (285). Thus, CD40 agonists can have direct cytotoxic effects on tumors, even in the absence of any additional immune responses and cells. Early clinical experience

with monoclonal IgG agonistic antibodies is encouraging. In a recent phase I study, patients with advanced solid tumors received single doses of CD40 agonistic antibody CP-870,893 intravenously. CP-870,893 was well tolerated; the most common adverse event being cytokine release syndrome including chills, rigors, and fever; 14% of all patients and 27% of melanoma patients had objective partial responses (291).

ACTIVATION OF T EFFECTOR CELLS VIA BLOCKADE OF INHIBITORY CHECKPOINTS

T-cell activation is triggered through the T-cell receptor by recognition of the cognate antigen complexed with MHC. T-cell activation is regulated by complex signals downstream of the diverse family of CD28 family immune receptors, which includes costimulatory (CD28 and ICOS) and inhibitory receptors (CTLA-4, PD-1 and BTLA). CD28 and CTLA-4 share the same ligands, B7-1 (CD80) and B7- 2 (CD86), whereas PD-1 interacts with PD ligand 1 (PD-L1), also named B7-H1, and PD-L2, also named B7-DC. Simultaneous recognition of the cognate MHC–peptide complex by the TCR (signal 1) and CD80 or CD86 by CD28 (signal 2) results in T-cell activation, proliferation, and differentiation, as well as effector cytokine production. PD-1 and CTLA-4 are induced on T cells following a TCR signal and result in cell cycle arrest and termination of T-cell activation. The importance of the PD-1 and CTLA-4 pathways in the physiologic regulation of T-cell activation is demonstrated by the autoimmune diseases occurring in CTLA-4 and PD-1 knockout mice (292) and further illustrated by the inflammatory side effects that can result from a therapeutic blockade of CTLA-4 in vivo, both in animal models and in humans (293-296). The use of blocking CTLA-4 or PD-1 mAbs can sustain the activation and proliferation of tumor-specific T cells, preventing anergy or exhaustion and thereby allowing the development of an effective tumor-specific immune response.

TOLL-LIKE RECEPTOR 9 AND ITS AGONISTS

The immune system has the ability to detect and eliminate pathogens through several mechanisms, and it may be broadly divided into innate and adaptive systems. Cells of the innate immune system, represented by natural killer (NK) cells, monocytes, and granulocytes, rapidly detect invading pathogens and tumors in a nonspecific manner. The innate immune system will respond to and contain the invading pathogens and prevent its spread. The adaptive immune system, represented by cytolytic T cells (CTL), T helper cells (T_H), and antibodies, is activated by presentation of antigen in a cognate fashion and will develop an antigen-specific response to eliminate the pathogen (297). To protect the host from succumbing to infections, the innate immune system, which is evolutionarily more ancient than adaptive immunity, must accomplish four fundamental tasks. First, it must rapidly detect any infectious agent, regardless of whether it is a virus, bacteria, fungus or parasite. Second, innate immune cells seem to rapidly categorize the type of invading infectious agent as to whether it is located extracellularly or intracellularly. Third, innate immune defences appropriate to the pathogen class are activated to either eradicate or at least temporarily contain the infection (298). Fourth, innate effectors have the ability to activate dendritic cells (DCs), which act as a bridge between the innate and adaptive immune responses, to express co-stimulatory molecules and effector cytokines. This will result in an enhanced ability to activate specific humoral and cellular immune responses (299). The key feature of innate immune cells that enables them to detect and categorize infection seems to be their repertoire of what have been termed pattern-recognition receptors (PRRs), which bind certain general types of molecules that are expressed across broad classes of pathogens, but which are absent or restricted in some way in vertebrates. As previously reported, the best understood family of PRRs is the Toll-like receptors (TLRs), of which 10 are known in humans (259). Toll-like receptors (TLRs) are a family of evolutionarily conserved pathogen recognition receptors; they are the mammalian homologues of *Drosophila* toll protein, and belong to the interleukin-1 receptor (IL-1R) superfamily (300,301). TLRs are considered sensors

for microbial infections or other ‘danger signals’, and are critical to the linkage between innate and adaptive immune responses (302). TLRs are part of the innate immune system, which recognizes pathogen-associated molecular patterns through germ-line encoded pattern-recognition receptors (PRRs). These receptors are present on different immune cells, and will recognize and bind certain molecules that are restricted to micro-organisms and absent from vertebrates, or expressed and not normally accessible to TLRs. The specificity of different TLRs is partially influenced by their structure and cellular location, which could be either intracellular or on the cell surface, depending on their specificity to intracellular or extracellular pathogens (303,304).

Tumor immunotherapy has evolved since William Coley used crude bacterial extracts to treat cancer (305). William Coley was a New York surgeon who injected bacteria into patients after observing that cancerous tumors could regress in the face of bacterial infection. His initial observations with this dangerous, but in some cases effective, therapy led to use of heat-killed *Serratia marcescens* and group A streptococci, now known as *Coley’s toxins*. Coley treated hundreds of patients over many years and reported that as many as 40% of patients achieved some level of clinical response (305). In the century that has followed, others have tried to duplicate his work, with less success. The reductionistic approach of subsequent research led to identification of the immunostimulatory effects of various bacterial components, including lipopolysaccharide. Indeed, lipopolysaccharide was thought for many years to be responsible for the antitumor effects of Coley’s toxins. It was not until the 1980s that a group of Japanese investigators identified bacterial DNA itself as a potent immunostimulatory fraction of prokaryotic cultures (306). These investigators suggested that the immunostimulatory effects of bacterial DNA were caused by the palindromic nature of the DNA sequences (307). In 1995, Krieg *et al.* (308) reported that the immunostimulatory effects of bacterial DNA were not caused by palindromes, but rather the presence of motifs containing unmethylated CG dinucleotides. The identification of this motif spawned a new field of research focused on identifying and characterizing the effects of so-called *CpG-containing oligonucleotides* (CpG ODNs) and the mechanisms through which they act. At the

time of the first report describing CpG-dependent immune stimulation by bacterial DNA, the receptor was not yet identified (307). An early study indicated that the catalytic subunit of the DNA-dependent protein-kinase (DNA-PKcs), involved in the repair of DNA double-strand breaks, is the mediator of CpG innate immune activation (309), although these studies could not be confirmed (310). Gene knock down and gain of function experiments finally identified TLR9 to be the receptor conferring CpG reactivity by directly engaging bacterial DNA or synthetic CpG ODN in a CpG motif-dependent manner (306,311-314). Research over the past 5 years suggests exploitation of these mechanisms holds significant promise for development of new cancer immunotherapies.

EXPRESSION AND LOCALIZATION OF TLR9

In humans, in bone marrow derived cells TLR9 is expressed only in memory B cells (315,316) and plasmacytoid dendritic cells (pDC) (317-319). Expression of TLR9 and responsiveness to CpG-DNA in other immune cells, such as human monocytederived dendritic cells and monocytes, has been reported, but is still a matter of debate (320,321). In contrast, murine TLR9 expression is not limited to B cells and pDC, but is also detected in monocytes, macrophages, and dendritic cells (322,323). In nonactivated immune cells TLR9 is expressed in the endoplasmic reticulum (ER). Upon cellular activation, TLR9 traffics to endosomal and lysosomal compartments, where it interacts with endocytosed CpG-DNA at acidic pH, a condition that is thought to be necessary for DNA recognition (324-326). (Figure 6)

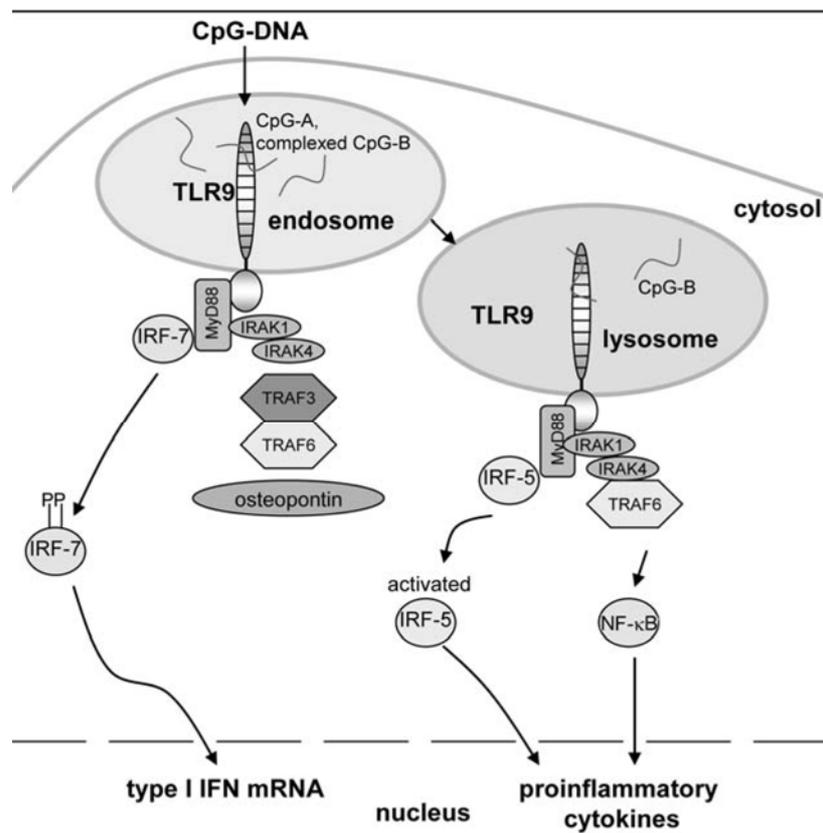


Figure 6. Spatiotemporal signaling of TLR9. Activation of TLR9 induces secretion of proinflammatory cytokines and type I IFN dependent on ligand localization. CpG-A ODN or CpG-B ODN complexed to transfection agents reside in the endosome and initiate IRF-7 activation leading to type I interferon. In contrast, CpG-B ODN itself traffics to the lysosome and activates IRF-5 and NF- κ B. In general, expression of proinflammatory cytokines is induced via IRAK1, IRAK4, TRAF6, and TRAF3. Type I IFN production in plasmacytoid dendritic cells is controlled by TRAF3, TRAF6, osteopontin, and IRF-7.

Compounds that interfere with endosomal acidification, such as the weak base chloroquine and bafilomycin A1, an inhibitor of the ATP-dependent acidification of endosomes, consequently, prevent CpG-DNA-driven TLR9 activation (327,328). The molecular basis for the retention of TLR9 in the endoplasmic reticulum (ER) in quiescent cells and the subsequent trafficking to the endosome upon cellular stimulation is unclear. Recently the membrane portion of TLR9 has been implicated in trafficking (329,330), although a recent report challenges this view. Accordingly, this report demonstrates that a tyrosine-based (YNEL) targeting motif in the cytoplasmic domain and the extracellular domain per se regulate TLR9 trafficking independent of the transmembrane domain (325). Despite these conflicting results on the trafficking-determining domain of TLR9, it is important to note that TLR9 trafficking to the endosome/lysosome does not seem to involve the Golgi apparatus, since the mature protein retains the sensitivity to the glycosidase Endo H, a feature

of usually ER-resident proteins. Which alternative route TLR9 uses to reach the endosomal/lysosomal compartment is currently unknown. The recently described ER resident protein unc93b may be involved in TLR9 trafficking since a dominant negative mutant of unc93b leads to nonresponsiveness of TLR9 (together with TLR3, TLR7) accompanied by the disruption of TLR-unc93b interaction (331,332). Recently TLR9 expression has also been detected on intestinal epithelial cells, and an involvement in the maintenance of colonic homeostasis has been suggested (333,334). Interestingly, on epithelial cells TLR9 is expressed on the apical and basolateral membrane, and TLR9 signaling varies in a site-specific manner. Whereas basolateral TLR9 stimulation leads to activation of the nuclear factor-kappa B (NF- κ B) pathway, apical TLR9 activation prevents NF- κ B activation by accumulation of NF- κ B inhibitory protein I kappa B-alpha (I κ B- α). Furthermore, apical TLR9 stimulation confers tolerance to subsequent TLR challenges, suggesting that apical exposure to luminal microbial DNA controls intestinal inflammation (334).

TLR9 EXPRESSION ON TUMOR CELLS

While numerous basic and clinical studies have investigated the immunostimulatory effects of TLR9 agonists on the innate and adaptive immune systems that could lead to the regression of tumors in vivo, only a few studies have discussed the significance of TLR9 expression on tumor cells (335). It was demonstrated that TLR9 activation can lead to the proliferation of immortalized prostate cells (336), or to the promotion of matrix metalloproteinase (MMP)-13 activity, resulting in enhanced migration of human prostate cancer cells expressing TLR9 (337,338). The aim of these studies was to demonstrate how TLR9 agonists from pathogens encountered in the genitourinary system may enhance malignant transformation and boost cancer cell spreading through inflammation-dependent mechanisms (335,339). On the other hand, other research showed mixed results regarding the direct effects of TLR9 agonists on tumor cells expressing TLR9. While some studies have shown that treatment of tumor cells expressing TLR9 in vitro with TLR9 agonist, at different doses, did not produce any effect on tumor growth, others have shown that the expression

of molecules, such as CD22, CD25, CD52, and HLA-DR might be enhanced on tumor cells, making them targets for different therapeutic approaches, such as the use of monoclonal antibodies (340,341). Others have reported that TLR9 signaling could enhance the metastatic potential of human lung cancer cells (95D) in nude mice, which might be related to the elevated proliferation and IL-10 secretion by the cells (342). Basically, we believe that the direct effect of TLR9 agonists on tumor cells needs to be further explored, and will depend, among other things, on the expression of TLR9.

CELLULAR SIGNALING MEDIATED BY TLR9

TLR9-mediated signaling proceeds through MyD88, an adaptor protein recruited to the TIR, which then activates the IRAK1-TRAF6-TAK1 pathway (343,344). Unlike TLR4-mediated signaling, the TIR domain-containing adaptor protein/MyD88-adaptor-like (TIRAP/MAL) is not involved in TLR9-mediated signaling. Recently, a novel adapter molecule associated with MyD88-independent as well as MyD88-dependent pathways was identified (303,304). Ongoing studies suggest that this molecule, TIR domaincontaining adapter inducing IFN- β (TRIF), is also involved in TLR9-mediated signaling. The TLR9 signaling cascade involves mitogen-activated protein kinases (MAPKs), such as p38, c-Jun NH2-terminal kinase (JNK), extracellular receptor kinase (ERK), and NF-kB-inducing kinase (NIK)-IKK-IkB pathways (348-350). The activation of ERK by CpG DNA contributes to the production of IL-10 by macrophages, but is not active in dendritic cells (DCs) or B cells (349,351). The signaling cascade culminates in the activation of several transcription factors including NF-kB, activating protein-1 (AP-1), CCAAT/enhancer binding protein (C/EBP), and cAMP-responsive element-binding protein (CREB), which directly upregulate cytokine/chemokine gene expression (see [Fig. 7](#)) (349,352-354).

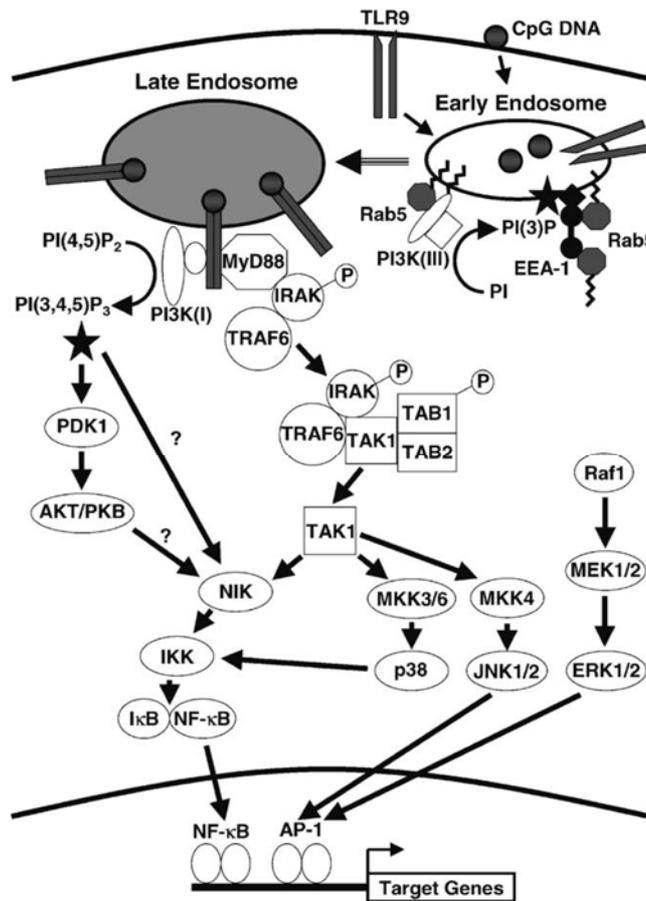


Figure 7. Scheme of CpG DNA/TLR9-mediated cellular signaling. Class III PI3K (PI3K (III)), EEA1, and Rab5 mediate the trafficking and maturation of endosomes containing CpG DNA and TLR9, by which TLR9 transduces intracytoplasmic signal. The signal initiates with the recruitment of MyD88 to the TIR, which then activates IRAK-TRAF6-TAK1 complex. This leads to the activation of both MAPKs (JNK1/2 and P38) and IKK complex, culminating upregulation of transcription factors including NF- κ B and AP-1. Raf1-MEK1/2-ERK1/2-AP-1 pathway is involved in CpG DNA-induced IL-10 production in macrophages. The alternative pathway mediated by class I PI3K (PI3K (I))-PDK1-AKT/PKB is also suggested to be involved in TLR9-mediated cellular activation.

In macrophages, CpG DNA also induces IFN- β production, which then upregulates STAT1 phosphorylation and IP-10 production through IFN- α/β receptor in an autocrine manner (355). Studies using chloroquine (CQ) or wortmannin (WM) showed that these agents could block CpG DNA/TLR9 signaling but not LPS/TLR4 signaling (310,352,356). Since cell surface binding and uptake of an ODN is not influenced by the presence of a CpG motif, endosomal maturation, which is the target of CQ, is believed to be an essential step in signaling (310,352). Taken together with the data on the subcellular distribution of CpG DNA described above, co-localization of CpG DNA with TLR9 in endosomal vesicles, and the accompanying maturation and movement of those vesicles, seems to be involved in signaling initiation. Although one group reported that the

suppression of CpG DNA signaling by WM reflected the inhibition of DNA-dependent protein kinase (DNA-PK) (257), others find that DNA-PK KO mice and SCID mice respond normally to CpG DNA (356,358). We observed that WM treatment led to a reduction in the size and number of endosomes containing both TLR9 and CpG ODN, suggesting that phosphatidylinositol 3 kinases (PI3K), which are also targets of WM, are involved in vesicular trafficking of CpG DNA (356). Indeed, Rab5-mediated recruitment of class III PI3K (PI3K (III)) leads to the production of PI(3)P in the endosomal membrane, which binds to the FYVE domain of early endosome antigen 1 (EEA1), recruiting it on to the membrane. The recruited EEA1 also associates with Rab5 and regulates homotypic fusion and trafficking of early endosomes (359-361). The PI(3,4, 5)P₃, product of class I PI3K (PI3K (I)), has been demonstrated to activate a signaling cascade consisting of 3-phosphoinositide-dependent kinase-1 (PDK1) and the protein kinase Akt/protein kinase B (AKT/PKB) (362,363). Ligand-induced association of TLR2 ICD and PI3K (I) was reported to activate the AKT/PKB-NF- κ B pathway (364). CpG DNA also induces phosphorylation of AKT/PKB thereby inhibiting apoptosis in DCs, an effect that is reversed by a PI3K inhibitor, LY294002 (365). However, recent data demonstrate that DN-p85a, which specifically blocks the function of PI3K (I), but neither DN-PDK1 nor DN-AKT/PKB, inhibits TLR9-mediated NF- κ B activation in HEK293 cells. This suggests that (1) PI3K(I) also regulate vesicular trafficking of CpG DNA and TLR9 and/or (2) another pathway mediated by PI3K(I) but not through the PDK1-AKT/PKB pathway is involved in TLR9-mediated NF- κ B activation in HEK293. PI3Ks and their second messengers therefore seem to play pivotal roles at distinct steps (i.e. vesicular trafficking for the association between CpG DNA and TLR9 and the signaling pathway directing AKT/PKB activation) in CpG DNA/TLR9-mediated cellular activation.

CLASSES OF SYNTHETIC CpG OLIGODEOXYNUCLEOTIDES

The immune stimulatory effects of CpG DNA are explained at least in part by differences inherent to genomic DNA of vertebrates and pathogens: vertebrate CpG dinucleotides are methylated and

their frequency is suppressed, while viral and bacterial CpG dinucleotides are non-methylated and occur with a much higher frequency (308). Synthetic CpG ODN can be generated containing specific CpG sequence motifs, sugar, base or backbone modifications as well as secondary and tertiary structures that all affect the immune modulatory effects of CpG ODN TLR9 ligands to different degrees (Fig. 1). B-Class ODN with 6mer CpG motifs with the general formula “purine-pyrimidine-C-G-pyrimidine-pyrimidine” (308) are strong stimulators of human B cell responses, and induce maturation of human pDCs and monocytes. The 6mer motif 5'-GTCGTT-3' represents the optimal human CpG motif (350), whereas 5'-GACGTT-3' is the optimal murine CpG motif (308,366). The length, number of CpG motifs, their spacing, position and the surrounding bases also determine the activity of B-Class ODN. The most potent ODN for activating human cells usually have three CpG motifs, additional CpG motifs do not much further enhance activity, and are between 18 and 26 nucleotides in length (366). Chemical modifications of the backbone, the heterocyclic nucleobase or the sugar moiety further enhance the activity of B-Class CpG ODN. Phosphorothioate modifications of CpG ODN stabilize them against nuclease degradation and enhance their activity by about 10 to 100 fold compared to phosphodiester ODN that either have to be added repeatedly or to be combined with an uptake enhancer to result in similar activity (367,368).

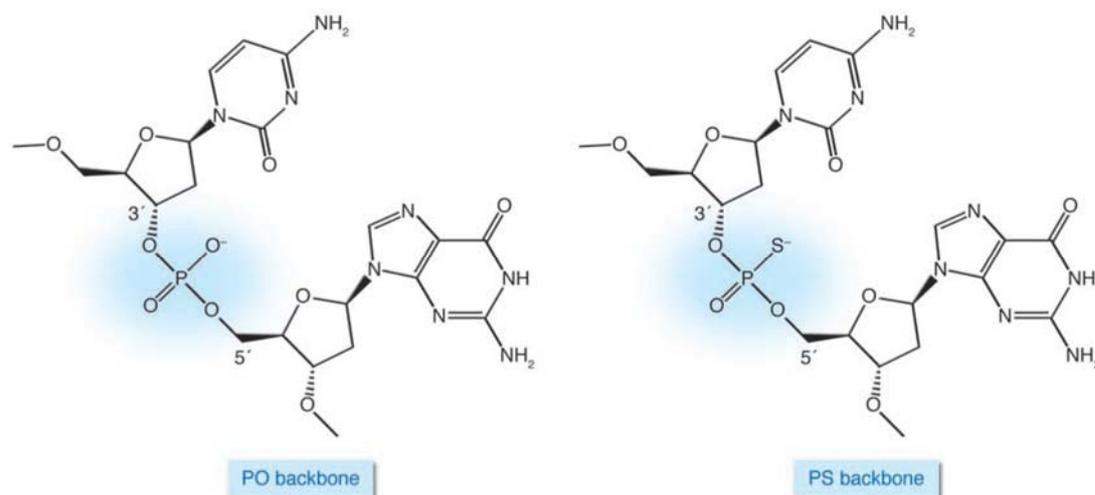


Figure 8. Backbones of native and modified DNA. PS ODN differ from native phosphodiester (PO) DNA ODN only in the substitution of a sulfur for one of the nonbridging oxygen atoms. This change improves the in vivo stability of the ODN from a half-life of a few minutes to about two days for the PS ODN.

In contrast to the charged phosphodiester and phosphorothioate backbones, replacement with non-charged backbones results in decreased immune stimulatory activity (369). CpG ODN with 2'-O-methyl or 2'-O-methoxyethyl sugar modifications induce decreased immune stimulation (370,371) and unpublished observations), substitutions with a RNA derivative, locked nucleic acid (LNA), even can eliminate the immune stimulatory effects of CpG-containing phosphorothioate ODN (372). In principle, any modification of cytosine at the CpG motifs is usually not well tolerated, but TLR9 appears to be more forgiving to modifications at the guanosine position (371,373). Another CpG ODN class is defined by G runs with PS linkages at the 5' and 3' ends surrounding a phosphodiester palindromic CpG-containing sequence (374,375). Intermolecular tetrad and high molecular weight aggregates are formed via the G residues that enhance stability, increase endosomal uptake and ligand concentrations (371,376), resulting in strong pDC IFN- α production by these CpG A-Class ODN. Albeit strong IFN- α and IFN- β stimulators, A Class CpG ODN are relatively weak in inducing other TLR9-dependent effects such as pDC maturation or B cell proliferation (377). Similar to the B-Class, the activity of A-Class ODN is influenced by length, modifications of the base, sugar or backbone. A-Class ODN require a chimeric backbone, the stimulatory effect is lost when the entire length of the backbone is PS modified (374,375). The CpG C-Class has some sequence requirements similar to the B-Class and combines the characteristics of the A- and B-Classes, stimulating strong B cell and pDC type I interferon production. C-Class ODN consist of a stimulatory hexameric CpG motif positioned at or near the 5' end and linked by a T spacer to a GC-rich palindromic sequence (377). The full immune activity requires physical linkage between the two domains, and a wide range of modifications that maintain the GC-rich palindrome are well tolerated, although destroying the palindrome abrogates IFN- α production (377). The stimulatory capacities of C-Class CpG ODN are similar to the A- and B Classes and depend on the length, base content and are influenced by chemical modifications. The formation of secondary and tertiary structures appears to control compartmental retention and intracellular distribution. The A- and C-Classes localize to different endolysosomal compartments

than the B-Class CpG ODN (378). The A- and C-Classes trigger IRF-7- mediated intracellular signaling pathways from early endosomes leading to strong IFN- α induction, whereas the B-Classes mainly stimulate NFkB-mediated signaling from late endosomes resulting in strong B cell activation. Palindromic sequences are involved in the formation of higher ordered structures and immediately affect stability, uptake characteristics and intracellular localization. Introducing a palindrome and increasing its length in a B-Class CpG ODN result in a stepwise increase of type I IFN production. Within the oligos tested, the strongest IFN- α induction is observed with CpG ODN having the longest palindrome. It is also possible to combine the 3' GC-rich palindrome of C-Class ODN with a non-GC-rich 5' palindrome. Such double palindromic or P-Class CpG ODN do not only form hairpins at their GC-rich 3' ends, but also form concatamers due to the presence of the 5' palindrome. These highly ordered structures appear to be responsible for the strongest type I IFN induction observed with CpG ODN. Similar to the A-Classes, P-Class ODN may enter early endolysosomal compartments preferentially inducing the IRF7 signaling pathway (379).

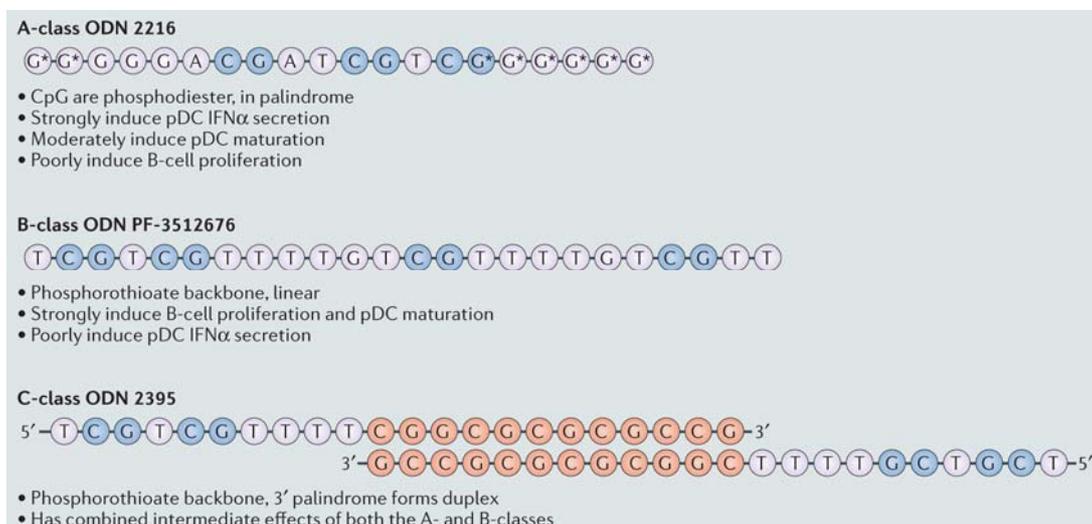


Figure 9. Three major classes of CpG ODN that are structurally and phenotypically distinct have been described. Examples of each class are shown in the figure, using the ID numbers from the published reports (PF-3512676 formerly was also known as ODN 2006 and CPG 7909), together with the immune effects and structural characteristics that are specific to the class. The A-class CpG ODN (also referred to as type D) are potent inducers of interferon- α (IFN- α) secretion (from plasmacytoid dendritic cells), but only weakly stimulate B cells. The structures of A-class ODN include poly-G motifs (three or more consecutive guanines) at the 5' and/or 3' ends that are capable of forming very stable but complex higher-ordered structures known as G-tetrads, and a central phosphodiester region containing one or more CpG motifs in a self complementary palindrome. These motifs cause A-class ODN to self-assemble into nanoparticles 193. B-class ODN (also referred to as type K) have a completely phosphorothioate backbone, do not typically form higher-ordered structures, and are strong B-cell stimulators but weaker inducers of IFN α secretion¹⁰. However, if B-class CpG ODN are artificially forced into higher-ordered structures on beads or microparticles, in dendrimers or with cationic lipid transfection, they exert the same immune profile as the A-class CpG ODN, thereby linking the formation

of higher-ordered structures to biological activity^{45,193–195}. The C-class CpG ODN have immune properties intermediate between the A and B classes, inducing both B-cell activation and IFN- α secretion^{43,196,197}. These properties seem to result from the unique structure of these ODN, with one or more 5' CpG motifs, and a 3' palindrome, which is thought to allow duplex formation within the endosomal environment.

CpG ODN STIMULATE T_H1-LIKE INNATE AND ADAPTIVE IMMUNITY

The immune effects of administering CpG ODN to humans seem to result directly and indirectly from activation of the immune cells that constitutively express TLR9, B cells, and pDCs. CpG ODN require no delivery system in vitro or in vivo — they can simply be administered in saline and are spontaneously taken up by most immune cells, in particular B cells and DCs (ODN uptake is not restricted to TLR9-expressing cells). ODN uptake by lymphocytes is energy and temperature dependent and greatly increased by cell activation; it also seems to be receptor mediated, although the specific receptors remain largely obscure (298).

Immune responses can be broadly divided into two types: T_{H1} and T_{H2}. T_{H1} immune activation is optimized for fighting intracellular infections such as viruses and involves the activation of NK cells and CTLs that can lyse infected cells. This type of immune activation is the most highly desired for cancer therapy, as the same defenses can be directed to kill tumor cells. In contrast, T_{H2} immune responses are directed more at the secretion of specific antibodies and are relatively less important for tumor therapy. One of the most notable features of TLR9 activation is the remarkably strong T_{H1} responses that are triggered. The immune response to infection or TLR stimulation occurs in two phases: the first to be activated is antigen-nonspecific innate immunity, which is followed by antigen-specific adaptive immunity (Figure 10). TLR9 stimulation with any class of CpG ODN activates innate immunity with a predominantly T_{H1} pattern of cytokine and chemokine secretion by B cells and pDCs (and by other immune cells that are activated secondarily). In response to TLR9 stimulation, B cells and pDCs also express increased levels of costimulatory molecules (such as CD80 and CD86), TNF-related apoptosis-inducing ligand (TRAIL), which can

induce tumor cell death, and CC chemokine receptor 7 (CCR7), activation of which causes cell trafficking to the T cell zone of the lymph nodes, and show increased resistance to apoptosis (259). Together, these innate immune effects of TLR9 activation can promote tumor regression either directly, through the antitumor activity of factors such as IFN- α and TRAIL, or indirectly, through the activation of NK cell-mediated tumor killing (298). TLR9-mediated innate immune activation and pDC and B cell maturation are followed by the generation of antigen-specific antibody and T cell immune responses (259). The pDCs activated through TLR9 become competent to induce effective CD4⁺ and CD8⁺ T cell responses (380-384). Both A-class and B-class CpG ODN increase the ability of pDCs to induce antigen-specific CD8⁺ T cells with a memory phenotype; the B-class CpG ODN also increase the frequency of CD8⁺ T cells with a naive phenotype (385). B cells are strongly costimulated if they bind specific antigen at the same time as TLR9 stimulation (Figure 10) (14). This selectively enhances the development of antigen specific antibodies, suggesting that CpG ODN might be useful as vaccine adjuvants, especially for the induction of strong T_{H1}-biased immunity.

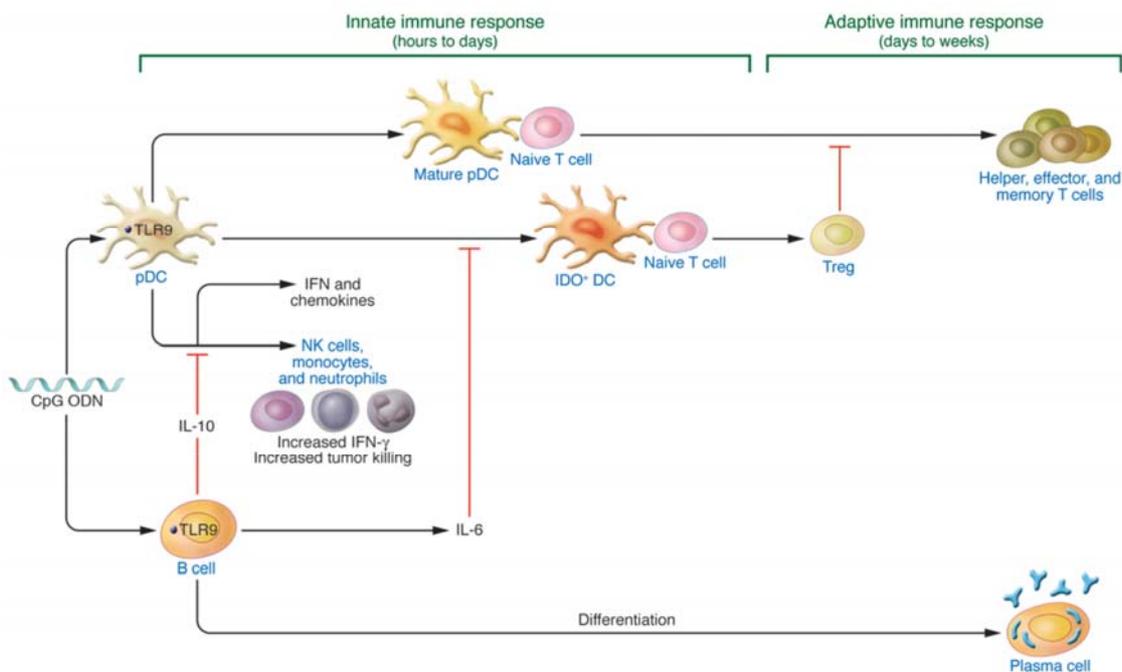


Figure 10. Activation of innate and adaptive immunity by TLR9 activation. Among human immune cells, only B cells and pDCs constitutively express TLR9. These cells endocytose DNA into an endosomal compartment where it binds to TLR9, forming a signaling complex. If the DNA contains unmethylated CpG motifs, TLR9 is stimulated, and the cell becomes activated. In pDCs, this results in type I IFN secretion, which activates NK cells, monocytes, and other APCs, and in the pDC maturation into a more effective APC able to activate naive T cells. Opposing these immuneboosting

effects, pDCs activated through TLR9 also mediate immune-suppressive effects through counterregulatory factors such as indoleamine 2,3-dioxygenase and the generation of T_{regs}. In B cells, TLR9 stimulation results in the secretion of proinflammatory cytokines, such as IL-6, and in the release of immune regulatory cytokines that might limit the intensity of the inflammatory response, such as IL-10. TLR9 activation of B cells confers a greatly increased sensitivity to antigen stimulation and enhances their differentiation into antibody-secreting plasma cells. On balance, these immune effects of CpG DNA generally promote strong T_{H1} CD4⁺ and CD8⁺ T cell responses. However, the concurrent activation of counterregulatory pathways such as the induction of Tregs limit TLR9-induced immune activation, offering a potential for enhancing the therapeutic efficacy of TLR9 agonists by coadministration of antagonists of one or more of these inhibitory pathways.

DRUG-LIKE PROPERTIES OF SYNTHETIC CpG-ODN

Some of the characteristics of synthetic ODN are quite attractive for drug development, whereas others are less favourable. The technology for commercial-scale (multi-kilogram) ODN synthesis and purification, carried out according to Good Manufacturing Practices, has been well developed during the past decade of antisense and aptamer drug development. Antisense and aptamer oligonucleotide drugs have been approved by the US FDA, establishing a regulatory pathway for this general class of drugs. The absorption, distribution, metabolism and elimination (ADME) properties of synthetic PS-ODN with and without CpG motifs have been well characterized and reported in the extensive literature on antisense ODN, which has shown these characteristics to be essentially sequence-independent (386,387). ODNs given subcutaneously are slowly absorbed from injection sites (with the highest concentration in the draining lymph nodes for the first several days after injection), and then enter the systemic circulation, where they demonstrate high-capacity, low-affinity binding to plasma proteins, principally albumin. ODN are rapidly cleared into tissues, especially the liver, kidneys and spleen, but do not seem to cross the blood–brain or blood–testes barriers. Catabolism of ODN typically occurs by exonuclease digestion and base clipping, primarily at the 3' end, resulting in natural DNA bases and thiophosphate metabolites that are excreted in the urine. The immune effects of CpG ODN administration through different routes result from their ADME characteristics. For example, subcutaneous administration of CPG 7909 (Coley), which results in high levels of the compound in the draining lymph node (which would contain a relatively

high concentration of TLR9- expressing cells), induces high levels of serum cytokines and chemokines (388). On the other hand, even relatively high-dose intravenous administration of CPG 7909, which is rapidly diluted in the blood and is approximately 95% protein bound, fails to induce measurable serum cytokine responses in humans⁵⁹. Because the pharmacodynamics of subcutaneous CpG ODN result from the local ODN concentration in the draining lymph nodes, they do not match the systemic pharmacokinetics.

<p>Drug-like characteristics</p> <ul style="list-style-type: none"> • Excellent aqueous solubility • Spontaneous intracellular uptake by certain immune cells (including especially those that express Toll-like receptor 9 (TLR9)) • Relatively simple solid-phase Good Manufacturing Practice synthesis (multi-kilogram scale) and chromatographic purification • Comparatively well-understood chemistry enables diverse studies of structure–activity relationships • Metabolites are mostly normal components of DNA, not novel small molecules • Range of backbones available for modulating compound stability for different applications • Can be administered through virtually any drug route (including oral) • Dose exposure required for immune stimulation is ~0.1–1% of that required for antisense applications • Excellent stability in aqueous solutions at physiologic pH, even at room temperature • Well-developed highly analytic methods for Chemistry, Manufacturing and Controls (liquid chromatography–mass spectrometry is state of the art) • Very sensitive methods available for detection of ‘cold’ compound²⁰¹ <p>Non-drug-like characteristics</p> <ul style="list-style-type: none"> • Medium size: molecular mass ~6,000-8,000 Da (length typically 18-25 bases) • Highly charged polyanions • Phosphorothioate and some other backbones are chiral • Poor stability of purines in acid solution • Cleaved by nucleases in serum or cell extracts (phosphorothioate backbone is relatively nuclease resistant) • Highly protein bound • Non-uniform organ distribution; highest tissue levels in kidney, liver and spleen after systemic delivery • Pharmacokinetics do not match pharmacodynamics after subcutaneous delivery • Sequence-independent effects, including concentration-dependent activation of complement proteins and prolongation of partial thrombin time
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Table 3. Characteristics of CpG oligodeoxynucleotides

PRECLINICAL (ANIMAL) STUDIES OF TLR9 AGONISTS

Cellular expression of TLR9 varies between humans and mice. TLR9 expression in mice is broader, and includes monocytes and macrophages. Thus, it is difficult to extrapolate the positive effects

seen in mouse models to humans. Nevertheless, some of the ground-breaking work on CpGs was done in animals (259). In studies with TLR9 knock-out mice, TLR9 was found to be the receptor for CpG ODNs, and proved that CpG ODN exerted its effect through the activation of TLR9 (323,389). CpG ODN has been tested in several mouse tumor models (390) and has shown moderate success in inducing rejection of established tumors when used alone. On the other hand, CpG ODN induced the rejection of larger tumors when it was combined with other antitumor treatments, such as radiation and monoclonal antibodies (390).

MONOTHERAPY

The effects of CpG ODN monotherapy can vary widely, depending on the tumor type. Moreover, its mechanism of action varies depending on several factors, such as MHC expression of the tumor, the susceptibility of the tumor to several immune effectors such as NK cells, T cells, or even TLR9 expression on the tumor cells (391-393). While using CpG ODNs as monotherapy could be effective in inducing regression in some tumors, such as the C3 model of cervical cancer (394), it is ineffective or less effective in the treatment of other tumors when given by systemic injection, compared with peritumoral or intratumoral injection (395,396). Peritumoral administration of CpG ODN was effective in impeding the progression of tumors in BALB/c mice transgenic for the rat neu transforming oncogene (397).

CHEMOTHERAPY

When CpG ODN was combined with chemotherapy, it was more effective than chemotherapy alone (390). Mouse tumormodels treated with CpG ODN in combination with fluorouracil,[106] topotecan (topoisomerase I inhibitor) (399), cyclophosphamide (400), or paclitaxel (401) showed substantial improvements in survival. The increased efficacy of these combinations in mouse models led to several clinical trials, where CpG ODN (agatolimod) was used in combination with standard taxane/platinum chemotherapy in phase II and III trials in patients with non-small cell lung

cancer (NSCLC). CpG ODN was also combined effectively with chemotherapy (fluorouracil plus leucovorin or irinotecan) and DC-based immunotherapy in the C26 mouse model of colon carcinoma (402).

VACCINES

CpG ODNs have also been used in vaccination studies as adjuvants, and have induced a good T_{H1}-type immune response (403,404). The efficiency of CpG ODNs in inducing a T_{H1} biased response is thought to be due to synergy between TLR9 and the B-cell receptor, which results in antigen-specific B-cell stimulation, inhibition of B-cell apoptosis, enhanced IgG class switching and DC maturation and differentiation (298,308,405,406). The co-injection of antigen-pulsed, mature DCs and CpG ODNs with a peritumoral injection of CpG ODNs elicited a CD8⁺ T-cell response resulting in tumor rejection and long-term protection in the C26 model of colon carcinoma (402). Moreover, in a preclinical model of colon cancer, a vaccine combining CpG ODN with GM-CSF and class I and class II restricted mucin (MUC) 1 peptides was successful in breaking MUC1 self-tolerance, and in eliciting a robust antitumor response in MUC1 transgenic mice (407). The immune response caused complete rejection of tumor cells in the prophylactic setting, while in the therapeutic setting, tumor burden was significantly reduced (407). When a DC-tumor cell fusion vaccine was used in mice, along with the TLR9 agonist ODN 1826 and the TLR3 agonist PolyICLC, a synergistic effect was shown, which was enough to achieve tumor rejection that could not be achieved by the vaccine alone. This effect was shown to be mediated by IL-12 (408). Moreover, the use of CpG ODN in mice as a vaccine adjuvant allowed for decreasing the antigen dose by half, while maintaining the same level of antibody response, when compared with those receiving the full dose of antigen without the CpG ODN adjuvant (409). Also, when CpG ODN was used with the recombinant hepatitis B virus surface antigen vaccine in mice, the titers of antibodies against hepatitis B surface antigen (HbsAg; anti-Hbs) were 5-fold higher than in mice immunized with HbsAg and the standard adjuvant, aluminum hydroxide (410). The activity of CpG ODN to

induce humoral immune responses has also been confirmed in non-human primates and in humans (411-413).

CpG-ODNs IN CANCER CLINICAL TRIALS

NON-HODGKIN'S LYMPHOMA

Non-Hodgkin's lymphoma (NHL) normally responds to immune-modulating treatments, such as IFN, IL-2, and monoclonal antibodies (414-416). Agatolimod has been used in a phase I trial, to test its efficacy as monotherapy in patients with previously treated NHL. Patients were divided into seven cohorts (three patients each), and received three weekly IV infusions of agatolimod at one of six dose levels (0.01, 0.04, 0.08, 0.16, 0.32, and 0.64 mg/kg). Patients were evaluated for several immunologic parameters and clinical endpoints before, during, and after treatment with agatolimod. These included a blood count, urinalysis, serum chemistries (including glucose, renal, and hepatic profiles), coagulation proteins (including prothrombin time, activated partial thromboplastin time, and fibrinogen levels), ECG recordings, and immunologic assays (including erythrocyte sedimentation rate, antinuclear antibodies, anti-double-stranded DNA, C3, C4, and CH50 activity). Tumor measurements were obtained by CT. Twenty-three patients completed therapy, and the treatment was well tolerated with infrequent transient grade 1 and 2 adverse events, including hyperglycemia, nausea, chills/rigors, hypotension, and fever. Serious adverse hematologic events, observed more than once, included anemia (n = 2), thrombocytopenia (n = 4), and neutropenia (n = 2), and were largely judged to be related to disease progression. Beginning day 2, there was an increase in the absolute numbers of NK cells and the mean ratio of NK cell concentrations when compared with pretreatment levels was 1.44 (95% CI 0.94, 1.94) on day 2 and was 1.53 (95%CI 1.14, 1.91) on day 42. NK activity also increased in patients, along with antibody-dependent cellular cytotoxic activity, which increased in select cohorts. There were no biologically significant changes

in the levels of serum cytokines (IL-12, IL-18, TNF- α), chemokines (IP-10, MCP-1, MIP-1 α), or markers of immune activation (IgM, IgG, C-reactive protein) at any of the dose levels tested; serum IL-6 levels rose transiently after the first injection, then returned to baseline within 48 hours. In general, immunomodulatory effects of agatolimod were greater at lower rather than at higher dose levels. No clinical responses were documented at day 42. A partial radiographic response was observed in two patients at 3 months, without further NHL therapy. This study concluded that agatolimod can be given safely to patients with previously treated NHL over a range of doses, with evidence for immunomodulatory effects primarily in the dosage range of 0.04–0.16 mg/kg (265,417). In a murine lymphoma model, there was clear synergy between CpG ODN and antitumor monoclonal antibody therapy when used in combination (418). A promising treatment of various B-cell lymphomas is the combination of CpG ODN with rituximab, an antibody against CD20, a cell surface marker that is widely expressed on B cells. CpG ODN was found to enhance the expression of CD20, the target antigen for rituximab, on various types of B-cell lymphoma (419). A phase I trial was designed to investigate the safety, tolerability, and preliminary antitumor activity of agatolimod in combination with rituximab. Patients with relapsed/refractory CD20⁺ B-cell NHL received agatolimod through IV or SC routes, in combination with standard-dose rituximab (226). Patients with relapsed or refractory NHL, who were candidates for rituximab as a single agent, were enrolled in one of three cohorts. All patients received IV rituximab 375mg/m²/week for 4 weeks followed by agatolimod weekly for 4 weeks administered SC (0.01, 0.04, 0.08, or 0.16mg/kg; cohort 1; n = 19) or IV (0.04, 0.16, 0.32, or 0.48mg/kg; cohort 2; n = 19). Cohort 3 (n = 12) received agatolimod 0.24mg/kg administered SC weekly for 20 weeks. Patients were monitored for toxicity and tumor response. The combination of agatolimod with rituximab was well tolerated, and across all groups, 38 of 50 patients had one or more adverse events. The most frequent adverse events were mild or moderate flu-like symptoms (e.g. fever, fatigue, headache), and local injection-site reactions, including erythema, pain, and edema. Grade 3/4 adverse events, which included lymphopenia, neutropenia, diarrhea, and dehydration, rarely

occurred in more than one patient or at >1 dose level. Among patients enrolled in the 4-week dosing cohorts, 4 of 19 (21%) in the IV arm and 2 of 19 (10.5%) in the SC arm had a complete response (CR) or partial response (PR), and there were 11 (57.9%) and 10 (52.6%) patients, respectively, with stable disease as best response. A total of 6 of 12 (50%) patients in cohort 3 had a CR or PR, and there were three (25%) patients with stable disease. Cytokine and chemokine measurements demonstrated biological activity in cohort 3. It was concluded from this study that agatolimod can be given safely in combination with rituximab to NHL patients by both the IV and SC routes, without apparent exacerbation of rituximab-related infusion toxicity (266). In another phase I trial, 1018 ISS in combination with rituximab was used to treat relapsed NHL patients. Twenty patients were treated with four weekly rituximab infusions and 1018 ISS was administered SC once a week for 4 weeks, starting after the second dose of rituximab (420). Patients were assigned to one of four doses of 1018 ISS (0.01, 0.05, 0.2, or 0.5mg/kg). As expected, 50% of patients had infusion reactions associated with the initial dose of rituximab; there was no exacerbation of rituximab toxicity after initiation of therapy with 1018 ISS. Nineteen patients were evaluable for clinical responses. Six patients showed objective responses (one unconfirmed CR, five PR) for an overall response rate of 32%. Additionally, 13 patients had stable disease after therapy. Median progression-free survival in responding patients was 12 months (range 5–23.5 months). Four patients remained alive without progression at a median of 10 months follow-up (range 3.2–23.4 months). Quantitative PCR analysis was done, to evaluate changes in mRNA expression in a panel of IFN inducible genes, on PBMCs isolated before and 24 hours after the second and fourth doses of 1018 ISS. There was no evidence of gene induction in vivo with the 0.01mg/kg dose, but in the three higher-dose groups a dose-related increase in the induction of several IFN α / β -inducible genes was observed 24 hours after the injection of 1018 ISS. A phase I/II study evaluating the effects of intratumoral injection of agatolimod combined with local radiation in recurrent low-grade lymphomas is underway. Patients will receive low-dose radiotherapy to a single tumor site on days 1 and 2 (2 Gy each day). CpG ODN injections will be administered into the same tumor site within

the 24 hours before and the 24 hours after the radiation, and on days 8 and 15. Weekly doses of CpG ODN will be then administered SC in the region of previous injections for six additional doses. The estimated enrollment in this trial is 30 patients (421).

RENAL CELL CARCINOMA

In a phase I, multicenter, dose-escalation trial, the effect of weekly SC doses of agatolimod (0.08 to 0.81 mg/kg) was evaluated for 24 weeks or until disease progression in patients with advanced renal cell carcinoma (422). Thirty-one patients were enrolled; 18 males and 13 females, aged 35–79 years. One patient had a durable PR (8 months), nine had stable disease and 17 patients progressed despite treatment with agatolimod. Four patients continued to receive treatment. Median time to progression was 112 days. No drug-related serious adverse events were reported, and agatolimod was well tolerated up to weekly doses of 0.54 mg/kg. Pro-inflammatory or cytokine effects (erythematous injection-site reactions, chills, myalgias, arthralgias, and fatigue) were dose-related and reversible. Biologic responses were consistent with the mechanism of action of CpG TLR9 and the most consistent effects observed were increased levels of plasma IP-10 and 205 oligoadenylate synthetase (OAS). This study concluded that agatolimod can be safely administered at doses up to 0.54 mg/kg weekly. In another trial involving patients with progressive metastatic renal cell carcinoma, patients were vaccinated with autologous tumor cells (ATC) derived from the primary tumor or metastases (423). Vaccines consisted of irradiated ATC, agatolimod, and GM-CSF. The first three induction vaccinations were given weekly followed by SC administration of IFN- α (6 MIU, three times weekly) and agatolimod 8mg bi-weekly. Tumor evaluation was performed after 3 months. In case of a remission or stable disease, patients continued with 3-monthly vaccinations and treatment with SC agatolimod and IFN- α . Blood was collected for immunomonitoring and delayed-type hypersensitivity responses (DTH) against ATC were measured before and after vaccination. The treatment was well tolerated. Twelve patients were included and treated according to the protocol. Three patients (25%) achieved a PR (durations 6, 4+, 4+ months) and two patients

(17%) remained stable. Adverse effects experienced by some patients included flu-like symptoms, fever, fatigue, and erythema, and induration at the vaccination site. A DTH response (>10mm) was observed after vaccination in all patients, suggestive of a specific antitumor response (423). Idera has tested their TLR9 agonist IMO 2055 (IMOXINE) in a phase I trial for safety (424). This compound is being evaluated at two dose levels in a phase II trial for metastatic renal cell carcinoma. This trial was designed for treatment-naïve patients and patients who had received one prior therapy. Stage A of the trial has been closed for further enrollment as of 2 June, 2009 (425).

MELANOMA

The limitations of immunotherapy for melanoma, like other cancers, stem from tumor-induced mechanisms of immune evasion that render the host tolerant of tumor antigens. For example, melanoma inhibits the maturation of APCs, preventing full T-cell activation and down-regulating the effector antitumor immune response (426). Of the new immunotherapies targeting critical regulatory elements of the immune system that may overcome tolerance, CpG ODNs have been used in melanoma treatment protocols either as monotherapy or in combination with other treatments. An open-label, multicenter, phase II clinical trial was carried out to assess the clinical and immunologic effects of chronic TLR9 activation with weekly SC administration of agatolimod in melanoma patients (427,428). Twenty patients from six centers, with histologically confirmed non-ocular unresectable clinical stage IIIb/c or IV melanoma, were enrolled. Patients received treatment with agatolimod 6mg weekly by SC injection for 24 weeks or until disease progression and clinical and immunologic activity as well as safety were evaluated (427,428). Clinical examination and laboratory safety assessments including hematology, blood chemistry, and baseline coagulation were performed weekly. Antinuclear antibodies, anti-double-stranded DNA (dsDNA) and antithyroid antibodies (Ab), and rheumatoid factor levels were determined every 8 weeks. Complete tumor imaging (CT scan or MRI of chest, abdomen/pelvis, and brain) was performed according to Response Evaluation Criteria in Solid Tumors. Laboratory and clinical

adverse events were limited, transient, and did not result in any withdrawals. Two patients experienced a confirmed partial response and three patients achieved stable disease. Immunologic measurements revealed a moderate but consistent increase in the proportions of CD86⁺ blood pDCs, and an elevation of the mean fluorescence intensity for HLA-DR on blood pDCs, both features indicating pDC activation. Serum levels of 20,50-oligoadenylate, a surrogate marker of type I IFN production, which remains elevated in serum for more than 1 week after induction, indirectly confirming sustained induction of type I IFN expression. Also, agatolimod induced a decrease in CD56⁺CD16⁺ NK cell numbers, presumably reflecting NK cell recruitment into tissues. Stimulation of NK cell cytotoxicity (NKC), however, was less consistent with some patients showing an increase and others showing a decrease in NKC; a sustained increase in NKC was associated with clinical benefit. The authors concluded that TLR9-targeted therapy can stimulate innate immune responses in cancer patients and enabled the identification of biomarkers that may be associated with TLR9-induced tumor regression (427,428). In another randomized phase II trial, 184 patients with the diagnosis of metastatic melanoma were enrolled in 48 sites. Patients were randomized into four arms: agatolimod 10 or 40 mg, agatolimod 40 mg in combination with DTIC (dacarbazine), or DTIC alone. DTIC 850mg/m² was administered IV every 21 days, and agatolimod was administered by weekly SC injection into multiple sites. Treatment was continued until disease progression. A preliminary response assessment in 92 patients showed four PRs in the combination arm compared with two PRs in the DTIC arm, one PR in the agatolimod 10 mg arm and no responses with agatolimod 40 mg. Fifty-seven patients had disease progression at or before the ninth week (third cycle). The authors concluded that a combination of DTIC with agatolimod may give a better response than DTIC alone in patients with metastatic melanoma (429). CpG ODNs have been used also in immunotherapy protocols as an adjuvant. A pilot trial was designed to study the immunogenicity of the analog peptide NY-ESO-1 157-165V in combination with agatolimod and montanide ISA 720 in eight patients with stage III/IV NY-ESO-1-expressing melanoma. Patients were immunized with montanide and agatolimod (arm 1, three patients); montanide and

peptide NY-ESO-1 157-165V (arm 2, two patients); or with montanide, agatolimod, and peptide NY-ESO-1 157-165V (arm 3, three patients) (430). Data from this study showed that the peptide vaccine, in combination with agatolimod and montanide promoted the expansion of NY-ESO-1-specific CD8⁺ T cells in patients with advanced cancer. The data also suggest that the presence of tumor-induced NY-ESO-1-specific T cells of well defined clonotypes is critical for the expansion of tumor-reactive NY-ESO-1-specific CD8⁺ T cells after peptide-based vaccine strategies (430). In a phase I trial conducted at the Ludwig Institute for Cancer Research (Lausanne, Switzerland), eight HLA-A2⁺ melanoma patients received four monthly vaccinations of low-dose agatolimod mixed with melanoma antigen A (Melan-A, identical to MART-1) analog peptide and incomplete Freund's adjuvant. All patients exhibited rapid and strong antigen-specific T-cell responses; the frequency of Melan-A-specific T cells reached over 3% of circulating CD8⁺ T cells. This was one order of magnitude higher than the frequency seen in eight control patients treated similarly but without agatolimod and one to three orders of magnitude higher than that seen in previous studies with synthetic vaccines (431). The enhanced T cell populations consisted primarily of effector memory cells, which in part secreted IFN- γ and expressed granzyme B and perforin *ex vivo*. *In vitro*, T-cell clones recognized and killed melanoma cells in an antigen-specific manner. The authors concluded that agatolimod is an efficient vaccine adjuvant that promotes strong antigen-specific CD8⁺ T-cell responses in humans (431). Finally, a phase I study investigated the safety, serum cytokine levels, cellular immune responses, and clinical activity of intralesional agatolimod in patients with basal cell carcinoma (BCC) or cutaneous or subcutaneous melanoma metastases (432). Five patients with BCC and five patients with melanoma and cutaneous and subcutaneous metastases received treatment with escalating doses of agatolimod (up to 10 mg) injected intralesionally every 14 days. Local tumor regressions were observed in patients with BCC (one complete regression, four partial regressions) and metastatic melanoma (one complete regression). After treatment with agatolimod, IL-6 was increased in all patients, IFN- γ IP-10 in eight of ten patients, interleukin 12p40 in seven of ten patients, and TNF- α levels in six of ten patients (432).

NON-SMALL CELL LUNG CANCER

The combination of a TLR9 agonist and chemotherapy has been shown to improve survival over chemotherapy alone in several mouse tumor models, suggesting a possible therapeutic synergy between these two approaches (398-400,433). It was also shown that the immunomodulatory oligonucleotide had potent antitumor effects as monotherapy and in combination with conventional chemotherapeutic agents, and may act directly on NSCLC cells via TLR9 (434). A randomized phase II study was carried out to assess the antitumor activity and safety of the combination of agatolimod with taxane plus platinum chemotherapy in chemotherapy-naïve patients with stage IIIB to IV NSCLC (435). In this trial, 112 patients with stage IIIB/IV NSCLC were enrolled, and they received four to six 3-week cycles of chemotherapy alone or in combination with agatolimod 0.2mg/kg, administered SC. The response rate improved from 19% in patients receiving chemotherapy alone to 37% in patients receiving chemotherapy plus agatolimod. The median survival was 6.8 versus 12.8 months, and the 1-year survival 33% versus 50% in patients receiving chemotherapy alone versus chemotherapy plus agatolimod. The authors concluded that a TLR9-activating ODN may enhance the clinical activity of chemotherapy in the treatment of NSCLC. Pfizer has also disclosed its intention to investigate agatolimod for use in breast cancer patients, and to initiate three randomized phase II clinical studies of agatolimod in advanced NSCLC. Each study will combine agatolimod with either bevacizumab (Avastin) (436), erlotinib (Tarceva) (437) or pemetrexed (Alimta) (438). Coley Pharmaceuticals (Wellesley, MA, USA) initiated two randomized, international, multicenter, phase III trials to compare agatolimod in combination with platinum-based chemotherapy (paclitaxel plus carboplatin or gemcitabine plus cisplatin) with chemotherapy alone for the treatment of chemotherapy-naïve patients with locally advanced or metastatic NSCLC (439,440). Over 800 patients were enrolled in these trials. These trials were discontinued in 2007, after analysis of the phase III clinical trial results showed no evidence of any additional efficacy over standard chemotherapy alone. In December 2007, Idera initiated a phase I

trial to determine the recommended dosage of IMO 2055 in combination with erlotinib and bevacizumab in patients with NSCLC who had progressed on first-line chemotherapy (441).

Approach	Disease	CpG ODN	Clinical trial phase	References
Monotherapy	Renal cell carcinoma	Agatolimod (CpG 7909, PF 3512676)	I	131
Vaccine	Renal cell carcinoma	Agatolimod + irradiated autologous tumor cells + GM-CSF	I	132
Monotherapy	Melanoma	Agatolimod	II	136,137
Combination therapy	Melanoma	Agatolimod + dacarbazine (DTIC)	II	138
Combination therapy with vaccine	Melanoma	Agatolimod + montanide ISA 720+ analog peptide NY-ESO-1	I	139
Combination therapy with vaccine	Melanoma	Agatolimod + melanoma antigen A (Melan-A, identical to MART-1) analog peptide and incomplete Freund's adjuvant	I	140
Combination therapy with vaccine	Melanoma	Agatolimod + MAGEA3	I	141
Monotherapy	NHL	Agatolimod	I	81,124
Combination therapy	NHL	ODN 1018 + rituximab	I	129
Combination therapy	NHL	Agatolimod + rituximab	I	127,128
Combination therapy	NSCLC	IMO 2055 + bevacizumab (Avastin [®]) + erlotinib (Tarceva [®]) or pemetrexed (Alimta [®])	II	142
Combination therapy	NSCLC	Agatolimod + taxane + platinum chemotherapy	II	143
Combination therapy	NSCLC	Agatolimod + paclitaxel + carboplatin or gemcitabine + cisplatin	III	144,145

GM-CSF = granulocyte-macrophage colony-stimulating factor; **NHL** = non-Hodgkin's lymphoma; **NSCLC** = non-small cell lung cancer.

Table 4. A summary of some clinical trials using CpG oligodeoxynucleotides (ODNs) for the treatment of patients with cancer.

AIM OF THE THESIS

It was recently reported the critical role of the administration route in the treatment of human ovarian cancer xenografts in nude mice, with intraperitoneal (i.p.) injection of CpG-ODN leading to an impressive increase in survival time and in tumor-free rate as compared to the slight effect of treatment administered intravenously or subcutaneously. However, it should be noted that these results were observed in mice before the appearance of ascites and therefore with a relatively low tumor burden. Indeed, ascites formation is a major cause of morbidity and mortality in advanced ovarian cancer patients. In these patients, in whom the metastatic spread of tumor cells outside the peritoneum is uncommon, the tumor cell deposits in the peritoneal surface may prevent adsorption of i.p. fluid by mechanical obstruction, inducing ascites. The treatment of ovarian cancer ascites is characterized by different palliative therapeutic options which are often of limited efficacy. There are several reasons for giving immunotherapy locoregional in stead of systemic. In a lot of animal models it is shown that locoregional immune therapy is far more effective than systemic treatment because the concentration of drugs at the site of the tumor is very high after local application compared to intravenous (i.v.) infusion or subcutaneous (s.c.) administration and another advantage of locoregional administration compared with systemic administration is a lower toxicity. Ovarian cancer, which growth is mostly confined to peritoneal, is one of the very few tumor types suitable for an unproblematic delivery of CpG-ODN directly into the tumor bed, but with selective routes of CpG-ODN administration it can be possible to increase the local concentration of CpG-ODN within the microenvironment of tumors with other localizations.

The aims of this project are to:

Evaluate the antitumor activity of repeated loco-regional CpG-ODN administrations alone or in combination with other chemotherapeutic agents to treatment of experimental ovarian carcinomas and define the molecular mechanism(s) underlying CpG-ODN antitumor activity.

MATERIALS AND METHODS

Mice

All experiments were carried out using 8- to 12-week-old female Swiss nude mice (Charles River, Calco, Italy). Mice were maintained in laminar flow rooms at constant temperature and humidity, with food and water given ad libitum. Experiments were approved by the Ethics Committee for Animal Experimentation of the Fondazione IRCCS Istituto Nazionale Tumori of Milan according to institutional guidelines.

Oligodeoxynucleotides, drugs and antibodies

Purified, phosphorothioated ODN1826 (5'-TCCATGACGTTTCCTGACGTT-3') containing CpG motifs and the control, non-CpG-ODN 2137, were synthesized by Coley-Pharmaceutical Group (Ottawa, Canada). Phosphorothioate modification was used to reduce susceptibility of the ODN to DNase digestion, thereby significantly prolonging its *in vivo* half-life. Cisplatin (Platinex®), purchased from Bristol-Myers-Squibb, was dissolved in saline and administered i.p. at 4mg/Kg of body weight. Bevacizumab (Avastin®) was purchased from Genentech/Roche, Poly(I)Poly(C) was purchased from GE Healthcare, Cetuximab (Erbix®) was purchased from Merck. Anti-RAD51 (MS-988-P0) and anti-SIRT1 (S5322) antibodies were purchased from Thermo Fisher Scientific Inc. (Fremont, CA) and Sigma (St. Louis, MO), respectively.

Cell culture

The ovarian cancer cell line IGROV-1 (gift from Dr J Benard, Institute Gustave Roussy, Villejuif, France) was routinely maintained in RPMI medium 1640 supplemented with 10% FCS (Sigma) and 2 mM glutamine (Cambrex, East Rutherford, NJ). Cells were maintained at 37°C in a 5% CO₂ in air atmosphere.

Bio-Plex assay

Bio-Plex mouse assays (Bio-Rad Laboratories, Hercules, CA) for simultaneous quantitation of angiogenic factors, i.e., vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF), or of cytokines, i.e., interleukin (IL)-1, -6, -10, keratinocyte chemokine (KC), granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor (TNF) and interferon-gamma (IFN- γ) were used according to the manufacturer's instructions. (Briefly, mouse ascites-fluids were incubated on properly 96-well plate with polystyrene-beads coated with cytokine- and angiogenic factor- specific antibodies, and then exposed to detection antibodies prior incubation with Streptavidin-PE (phycoerythrin). Capture lysates were analyzed on the Bio-Plex™ 200 system (Bio-Rad). Samples were tested in duplicate.

***In vivo* studies (I)**

IGROV-1 human ovarian carcinoma cells were adapted to growth i.p. and maintained by serial i.p. passages of ascitic cells into healthy mice as described (442,443). For *in vivo* experiments, mice were injected i.p. with 2.5×10^6 ascitic cells in 0.2 ml of saline and treated starting 10-11 days later, when mice showed evident and established ascites, with CpG-ODN delivered i.p. at a dose of 20 $\mu\text{g}/\text{mouse}$ in different administration schedules.

For antitumor activity studies, mice bearing established ascites were selected from larger groups of mice injected i.p. 10 days before with IGROV-1 cells, and randomly divided into treated and control groups. Experimental groups (8-10 mice) were inspected daily and weighed three times weekly. Mice were individually killed by cervical dislocation prior to impending death, recognized as loss of reactivity to stimuli in mice with or without ascites. The ascitic fluid was withdrawn and measured. A careful necropsy was performed to observe tumor spread in the abdominal cavity. Solid i.p. masses were gently detached from organs, removed and weighed. Day of sacrifice was considered day of death, and the median day of death (median survival time; MST) was calculated for each group. Antitumor activity was assessed as T/C%, i.e., the ratio of MST in treated mice to

that of control mice x 100.

To evaluate the *in vivo* effect of CpG-ODN treatment in the anti-tumor activity of cisplatin, athymic mice were injected i.p. with 2.5×10^6 ascitic cells in 0.2 ml of saline and treated i.p. 8 days later, when ascitic fluid begins to accumulate, with CpG-ODN (20 μ g/mouse daily for 4 weeks), cisplatin (3 mg/Kg, once per week for 4 weeks) or both. Experimental groups (8-10 mice) were inspected daily and weighed 3 times weekly. Mice were sacrificed prior to impending death, recognized as loss of reactivity to stimuli. Day of sacrifice was considered day of death, and the median day of death (median survival time; MST) was calculated for each group. Antitumor activity was assessed as T/C%, i.e., the ratio of MST in treated mice to that of control mice x 100.

For cellular studies, ascites-bearing mice were sacrificed by cervical dislocation at several time points after treatment with saline or CpG-ODN. Ascitic fluid was collected using a heparinized syringe and the volume recorded. The fluid was transferred to a centrifuge tube maintained on ice. After centrifugation, supernatant was removed and stored at -80°C for analyses of cytokines and angiogenic factors (see Bio-Plex Assay). The pellet was suspended in 0.17 M ammonium chloride for 10 min at 4°C to lyse red blood cells. After washing once in saline, live cells were counted using the trypan blue exclusion assay.

To evaluate the role of NK and monocytes/macrophages in CpG-ODN-induced effects on total cell number in the peritoneal fluid and on cytokine production, ascites-bearing mice (3 mice/group) were previously i.p. inoculated with 200 μ l of HBSS containing 100 μ g of anti-NK1.1 (hybridoma PK136; ATCC, Rockville, MD, USA), or with 400 μ l of clodronate-loaded liposomes prepared as described (13) to deplete macrophages or with control liposomes. Depletion of NK cells and monocytes/macrophages was verified by staining peritoneal cells with FITC-conjugated anti-NK1.1 antibody (Pharmingen BD) and with PE-anti-mouse F4/80 (Caltag Laboratories, Bangkok, Thailand) and FITC-anti-mouse CD11b (BD Pharmingen), respectively.

Histology

Lymph node and spleen tissue collected from 6 tumor bearing mice treated 5 times/week for 4 weeks with CpG-ODN and from 2 saline-treated control mice were fixed in 10% neutral buffered formalin, embedded in paraffin, cut in 5 μm -thick sections and stained with hematoxylin and eosin. Slides were examined using a Nikon Eclipse 80i microscope fitted with a DS5000 digital camera (Tokyo Japan).

Flow cytometry

Twelve days after tumor cell injection, ascitic cells collected as above from mice (3 mice/group, 2 independent experiments) 24 h after treatment with saline, CpG-ODN or control ODN were incubated for 30 min at 4°C with 10 $\mu\text{g}/\text{ml}$ chimeric human IgG1 MOv19 antibody (kindly provided by Silvia Miotti, INT), which recognizes a folate-binding protein in ovarian tumor cells (14), or chimeric human IgG1 anti-CD20, Rituximab (Roche), as isotype control, followed by incubation with a polyclonal goat anti-human FITC antibody (KPL, Gaithersburg, MD, USA), to evaluate the percentage of tumor cells.

Ascitic cells from saline- or CpG-ODN treated mice (4 mice/group) were directly stained with FITC or phycoerythrin-labeled (FITC) antibodies directed against CD45 (Miltenyi Biotec.), NK1.1 (BD Biosciences), GR-1 (Southern Biotech.), F480 (Caltag Lab.), CD19 (BD Biosciences), CD11c (BD Biosciences) and PDCA-1 (Miltenyi Biotec.) at 4°C for 25 min to evaluate the composition of the immune cellular infiltrate. The percentage of different cell populations was analyzed by gating on CD45⁺ cells. Maturation of plasmacytoid dendritic cells was analyzed by staining ascitic cells with PE-coniugated anti-CD80 and anti-CD86 (BD Biosciences) antibodies. Interferon alpha production was evaluated by intracellular staining with FITC-conjugated anti IFN- α (PBL Biomedical Lab.) after *in vitro* stimulation of ascites cells for 4 h with **phorbol** 12-myristate 13-acetate (PMA) (50ng/ml) and ionomycin (500 ng/ml). Analysis of activation markers and IFN- α production was performed on PDCA-1⁺ gated cells.

All samples were analyzed by gating on live cells using FACSCalibur and CellQuest software (BD Biosciences).

***In vivo* experiments (II)**

Mice were treated i.p. with CpG-ODN (20 µg/mouse, 5 days/week for 4 weeks), cis-platinum (DDP, 3 mg/Kg i.p., once per week for 4 weeks), bevacizumab (5mg/Kg at 3-4 day intervals), or Poly(I):Poly(C) (20 µg/mouse at 2-3 day intervals), cetuximab (1 mg/mouse at 3-4 day intervals) or their combinations. Control mice received saline. Single agents were also included in the experiments. Control mice received saline. Mice were individually killed by cervical dislocation prior to impending death, recognized as loss of reactivity to stimuli in mice with or without ascites. Day of sacrifice was considered day of death, and the median day of death (median survival time; MST) was calculated for each group. Antitumor activity was assessed as T/C%, i.e., the ratio of MST in treated mice to that of control mice x 100.

***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 11-12 days later, when mice showed evident and established ascites, with CpG-ODN delivered i.p. at a dose of 20 µg/mouse daily for 3 days. Control mice received saline. Ascites-bearing mice were sacrificed by cervical dislocation at 24 h 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 µM of CpG-ODN in complete culture medium for 24 h. At the end of treatment, cells were collected and RNA extracted.

To evaluate the effect of ascitic fluid on IGROV-1 *in vitro*, 1×10^6 IGROV-1 were seeded in 6-well plates and incubated for 24 h with ascitic fluid from saline- or CpG-ODN-treated mice diluted in

culture medium without FCS (ratio medium:ascites, 1:1). At the end of treatment, cells were collected and RNA extracted.

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 $10 \mu\text{M}$ of CpG-ODN in complete culture medium for 24 h. At the end of treatment, cells were collected and RNA extracted.

To define whether local treatment at the tumor site is critical to down-regulate DNA repair genes, mice were injected i.p. with IGROV-1 tumor cells as described above and treated i.p. or subcutaneously (s.c.) with CpG-ODN at a dose of $20 \mu\text{g}/\text{mouse}$ daily for 3 days. At 24 h 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.

RNA isolation and expression profiling

Solid i.p. tumor masses were pulverized using a Mikrodismembrator (Braun Biotech International, Germany). Total RNA was extracted with the Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and DNA-digested with DNase using the RNeasy kit (Qiagen, Valencia, CA). After each extraction, a small fraction of RNA was used for quality and yield assessment. RNA total concentration and purity were determined by UV spectrometry. Total RNA electrophoretic profile was analyzed by the Agilent RNA 6000 NanoLabChip kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) using software provided by the manufacturer for determination of RIN (RNA integrity number). Total RNA (500 ng) was reverse-transcribed, labeled with biotin and amplified overnight (14 h) using the Illumina RNA TotalPrep Amplification kit (Ambion) according to the manufacturer's protocol. Biotinylated cRNA samples were hybridized to Bead Chip Human HT12_v3 (Illumina, Inc., San Diego, CA) at 58°C overnight (18 h). Array chips were washed with manufacturer's E1BC solution, stained with $1 \mu\text{g}/\text{ml}$ Cy3-streptavidin (Amersham Biosciences) and scanned with Illumina BeadArray Reader. Primary data

were collected using the supplied scanner software and the following analyses were performed using BeadStudio Version 3 software. Intensity values of each hybridization were quality-checked and the dataset was normalized using the quantile algorithm. Analyses were performed using BrB ArrayTools_v3.8.0, developed by Simon, R. and Lam, AP.

The microarray dataset and sample information are deposited in the Gene Expression Omnibus database (experiment number GSEXXX), according to MIAME (minimum information about a microarray experiment) guidelines.

In silico analysis

Five publicly available microarray datasets, on the gene expression modulation induced by CpG-ODN (4 datasets) or by imiquimod (1 dataset), were identified and selected for analysis. Gene expression analyses were performed on different platforms (in-house-assembled oligonucleotide, Affymetrix, Illumina and Agilent microarray). Whole-genome raw data were retrieved from the Gene Expression Omnibus [GEO] microarray data repository at <http://www.ncbi.nlm.nih.gov/geo/> (GSE11202 (444), GSE18203 (445), and GSE20032 (446)) and from ArrayExpress database at <http://www.ebi.ac.uk/microarray-as/ae/> (E-TABM-823(447)). Datasets were annotated based on the most recent gene annotation available, filtered and normalized using BrB ArrayTools_v3.8.0 (448).

A list of genes related to the DNA repair was retrieved from Gene Ontology (<http://www.geneontology.org>) using the AmiGO web application (449). To identify the genes varying over the time of CpG-ODN treatment, regression analysis of time-course expression data was carried out by the Time Course Analysis plug-in available in BrB ArrayTools_v3.8.0, whereas genes differentially expressed among CpG-ODN- or imiquimod-treated and untreated mice were identified through the Class Comparison option using the random variance model.

Our CpG-ODN microarray analysis identified a signature of 27 genes [false discovery rate (FDR) <0.01 and a fold-change filter > 1.5] used to test an ovarian cancer dataset retrieved from

<http://data.cgt.duke.edu/oncogene.php> and a breast cancer retrieved from <http://www.rii.com/publications/2002/nejm.html>, offering a complete clinical annotation in patients treated with DNA-damaging drugs. Univariate Kaplan-Meier survival and Cox's regression analysis was performed with log-rank test using WinSTAT for Excel (version 2009.1; R. Fitch software). Supplementary Table 2 lists the published datasets used for *in silico* analyses.

Ingenuity pathway analysis (IPA)

Pathways and networks significantly regulated in gene expression after CpG-ODN treatment were examined using the Core Analysis function included in IPA (Ingenuity System, Inc.). HUGO gene identifiers were mapped to networks available in the Ingenuity database and ranked by score for the likelihood that genes found together in a network was random.

MTT assay

IGROV-1 ovarian cancer cells in the log phase of growth were harvested, seeded into 96-well plates (3000 cells/well), and incubated for 48 h with ascitic fluid from ovarian tumor-bearing mice treated daily for 3 days with CpG-ODN or saline and then exposed to cisplatin (3 μ M) for 1 h. At the end of incubation with cisplatin, cells were washed and complete medium was added to the wells. After 72 h, 20 μ l of MTT (5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma) solution in PBS was added to each well for an additional 4 h. Supernatants in the well were carefully aspirated after centrifugation and replaced with 100 μ l DMSO. Optical density (OD) was evaluated by spectrophotometry at 550 nm with an ELISA microplate reader (Bio-Rad Lab, Inc., Hercules, CA). Samples without cisplatin were assigned the value of 100%.

Western blot

Pulverized tumors were lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100 (Sigma), 10% (vol/vol) glycerol, 2 mM Na-orthovanadate, 10 mM leupeptin, 10

mM aprotinin, 1 mM phenylmethylsulfonyl-fluoride, 100 mM Na-fluoride, and 10 mM Na-pyrophosphate for 30 min at 4°C. Insoluble material was removed by 10 min of centrifugation at $15,500 \times g$ at 4°C. Protein concentrations were determined using the Coomassie technique. Equal amounts of total lysates were loaded and separated on 10% precast NuPage SDS-Bis-Tris gel (Invitrogen) and transferred to PVDF membranes (Millipore, Billerica, MA). Total lysate (60 μ g) was loaded on the same gel. Western blots were performed with the indicated antibodies and binding was detected with peroxidase-conjugated secondary antibodies and chemiluminescence ECL (GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions.

Statistical analysis

To directly compare ascites volumes of saline- and CpG-treated mice, best-fit linear regression analysis was determined for volume data. Slopes of these lines were compared using an unpaired *t*-test. Percent survivorship over time was estimated by the Kaplan-Meier product limit method and compared with the log-rank test. Differences between groups in the percentage of cellular infiltrate populations, angiogenic factors and cytokines levels were analyzed by unpaired Student's *t*-test. Analyses were done with Graph Pad Prism (GraphPad Software, Inc., San Diego) and differences at $P < 0.05$ were considered significant.

RESULTS

It was recently reported that athymic mice bearing a relatively low IGROV-1 tumor burden and repeatedly treated weekly i.p. with CpG-ODN showed a significantly increased life span as compared to controls; however, all mice died with ascites (450). Interestingly, treatment of mice when they had developed ascites showed a reduced body weight (BW) the day after CpG-ODN administration. To further investigate this observation, IGROV-1-bearing mice with established ascites, i.e., increased abdominal volume and BW, were treated i.p. with a single injection of CpG-ODN (20 μ g/mouse) or saline, sacrificed in groups of 3 at different time points, and ascitic fluid removed for volume quantitation and cell collection. Ascitic volumes continued to increase in control mice, whereas the volumes in treated mice declined shortly after treatment and accumulated thereafter at a slower rate than in controls (Fig. 11A). At 96 h after CpG-ODN injection, the mean volume was increased up to 5-fold in controls and 2.5-fold in treated mice (from 1.7 ml to 8.7 ml and 4.3 ml, respectively). Total number of live peritoneal cells collected from the ascitic fluid was rapidly and dramatically reduced in treated mice compared to control mice, with the reduction still detectable at 96 h after CpG-ODN treatment (Fig. 11B, $P < 0.001$).

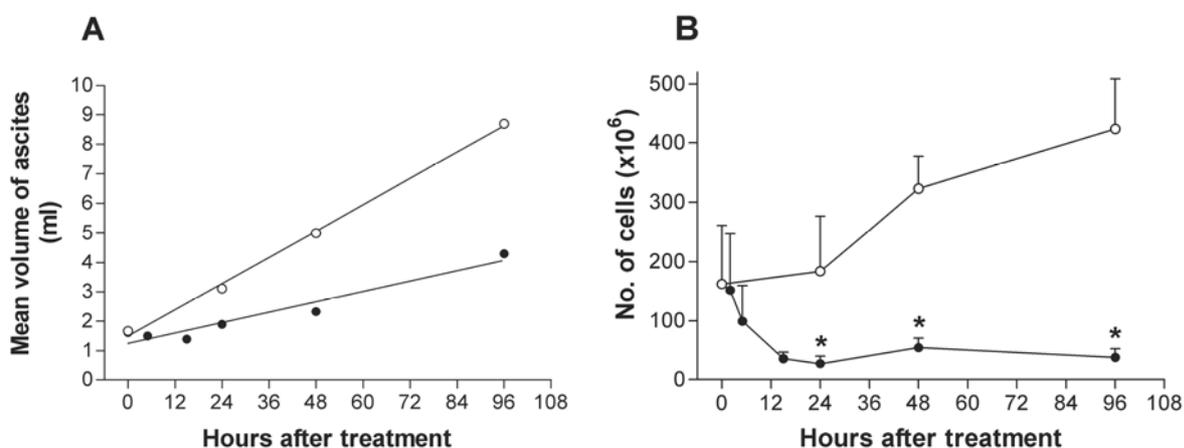


Figure 11. Effect of CpG-ODN treatment on ascites volume and cellularity. Ascites-bearing mice, treated i.p. with CpG-ODN or saline, were sacrificed at selected time points (3-6 mice/point), and ascitic fluid and cells collected. A) Ascites volume, plotted based on linear regression analysis and "best-fit" linear regression. Slopes of curves were

compared using an unpaired *t*-test ($p < 0.001$); B) Number of live cells (mean \pm SD) (* $P < 0.001$); Open symbols: control mice; closed symbols: CpG-ODN-treated mice.

Staining of peritoneal cells with antibody MOv19, which recognizes folate-binding protein expressed by IGROV-1 cells, revealed a markedly reduced percentage of tumor cells after CpG-ODN treatment (from 69.9 ± 3.5 of controls to 5.16 ± 2.0 of treated-mice), while no reduction in percentage of tumor cells was observed after non-CpG-ODN treatment (60.6 ± 9.97) (Fig. 12).

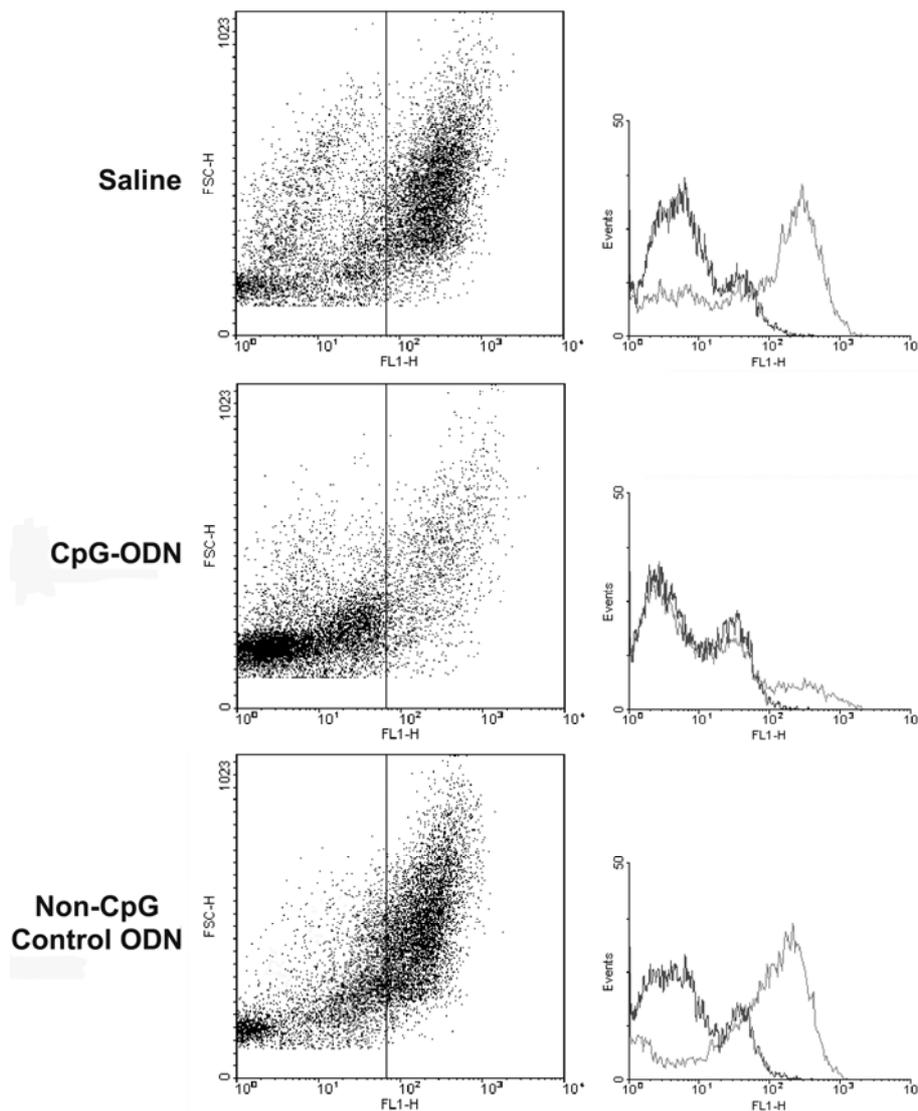


Figure 12. Effect of CpG-ODN treatment on cellularity. Percentage of MOv19-positive cells detected by FACS analysis 24 h after saline, CpG-ODN or non-CpG-ODN treatment (data from a representative experiment). Cells were stained with anti-MOV19 (gray line), an antibody which recognizes folate-binding protein in ovarian tumor cell, or with anti-CD20 Rituximab antibody as isotype control (black line).

Immune cell populations were analyzed by gating on live CD45⁺ cells. CpG-ODN treatment did not induced significant differences in the percentage of ascites fluid prominent immune populations such as neutrophils granulocytes [GR1⁺ cells: 76.8 ± 2.8 % (mean ± SD) in CpG-ODN-treated mice *versus* 62.7 ± 5.6 in saline-treated mice], NK cells (NK1.1⁺ cells: 9.9 ± 3.0 *versus* 12.1 ± 7.9), macrophages (F480⁺ cells: 14.1 ± 2.0 *versus* 16.5 ± 7.8) and plasmacytoid dendritic cells (PDCA-1⁺CD11c^{low} cells: 13.2 ± 1.8 *versus* 16.2 ± 3.6). Moreover, CpG-ODN treatment did not modify the expression of maturation markers (CD80 and CD86) on plasmacytoid dendritic cells, which might be directly activated by CpG-ODN through TLR9 triggering, nor modulate their production of IFN- α . The drastic reduction in total cell number in the peritoneal fluid after CpG-ODN treatment was not related to increased adhesion properties of tumor cells to peritoneal wall, since histological analyses of parietal mesothelial peritoneal tissue under abdominal trasversal muscle from control and CpG-ODN-treated mice revealed no substantial differences in the number of adhered tumor cells.

Based on the decreased tumor cell number in the peritoneal fluid and on the reduced ascitic volume after CpG-ODN injection, we investigated the effects of CpG-ODN administered using different schedules on survival times of ascites-bearing mice. In two separate experiments, mice were i.p. treated with CpG-ODN or saline every 7th day for 4 weeks. Saline-treated mice became moribund for tumor burden and were euthanized between days 18 and 24 after tumor cell injection (MST of 19.5 and 20.5 days, in the two experiments, respectively), whereas CpG-ODN-treated mice were euthanized between days 21 and 130 (MST of 21 and 22.5 days and a T/C% = 108 and 110 in the two respective experiments) (Fig. 13A). Despite the marginal increase in MST, 2 mice in each of the two experiments survived much longer than control mice, i.e., they were sacrificed 28, 38, 42 and 130 days after cell injection. When ascites-bearing mice were treated with CpG-ODN according to a more frequent schedule, i.e., 5 times/week for 3 or 4 weeks, survival was significantly increased compared with saline-treated groups (MST of 21 and 20.5 days for control mice in the two experiments, and 30.5 and 34 days for CpG-ODN-treated mice; T/C% of 145 and

166 for 3 or 4 weeks of treatment, $P=0.0023$ and $=0.0014$, respectively) (Fig. 13B). Note that 2 mice/experiment were sacrificed at days 51, 51, 55 and 97 and ascites did not regrow in the treated mice even in the long term survivors. To compare the antitumor effect with a clinical cytotoxic drug, ascites-bearing mice were treated i.p. with the maximum tolerated dose of cisplatin (4mg/Kg of body weight, once, a week for 3 weeks). Only a small effect on survival was achieved (MST of 20 days for control mice and 23 days for cisplatin-treated mice; T/C% of 115). Figure 13C shows that the daily CpG-ODN regimen controls ascites formation and thus prevents BW increases.

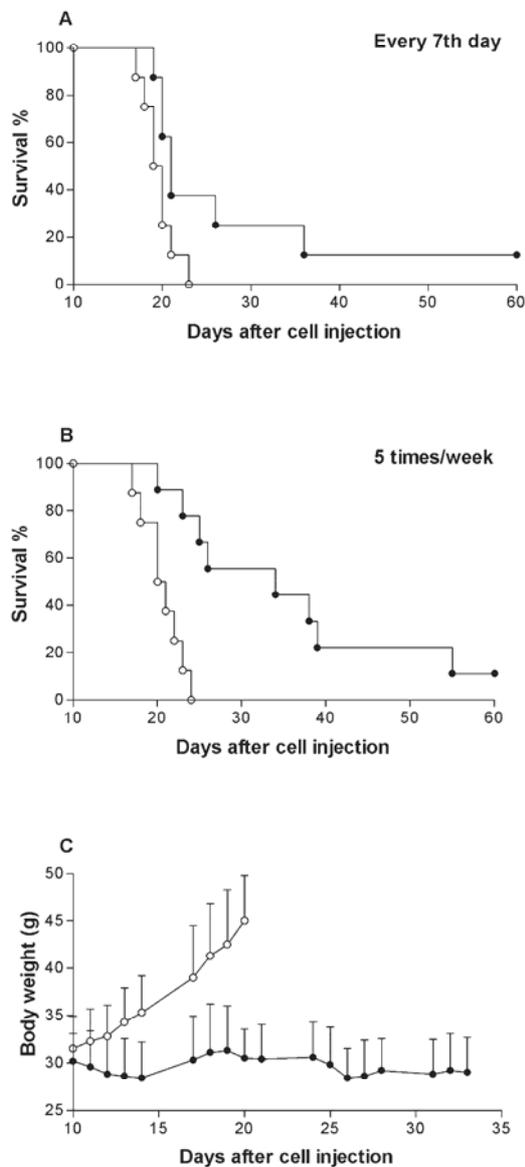


Figure 13. Effect of CpG-ODN treatment on survival and body weight. Kaplan-Meier plot of the percentage of survivors over time among IGROV-1 ascites-bearing mice treated with CpG-ODN (20 μ g/mouse) or saline for 4 weeks every 7th day (A) or 5 times/week (B); C) Body weight (mean \pm SEM) of experimental groups reported in panel B. Open symbols: control mice; closed symbols: CpG-ODN-treated mice.

When animals were sacrificed, ascitic fluid was withdrawn and measured, and solid i.p. masses removed and weighed. All control mice, sacrificed by day 24, showed large amounts of hemorrhagic fluid in the abdominal cavity and a large tumor mass that included the liver, stomach, duodenum and spleen without invading these organs. Moreover, multifocal, small nodules were widely scattered on the abdominal wall and a thin layer of tumor cells was present on diaphragm (mean mass of solid tumor material: 2 ± 0.9 g). By comparison, treated mice sacrificed at an advanced time (days 28 to 90) showed significantly reduced ascitic fluid amounts ($P = 0.0005$) in the peritoneal cavity and larger solid tumor masses that included nearly all of the bowel (4.5 ± 2.8 g; $P = 0.022$). The five consecutive CpG-ODN weekly deliveries were well-tolerated in terms of mouse status and BW, but histological examination revealed a disorganization of lymphoid organs (spleen and lymph nodes); in particular, 4 out of 6 analyzed mouse spleens showed some enlargement of the B-cell zone (Fig. 14A) and 2 spleens revealed a complete alteration of the follicular structure (Fig. 14B); no alterations were observed in saline-treated mice.

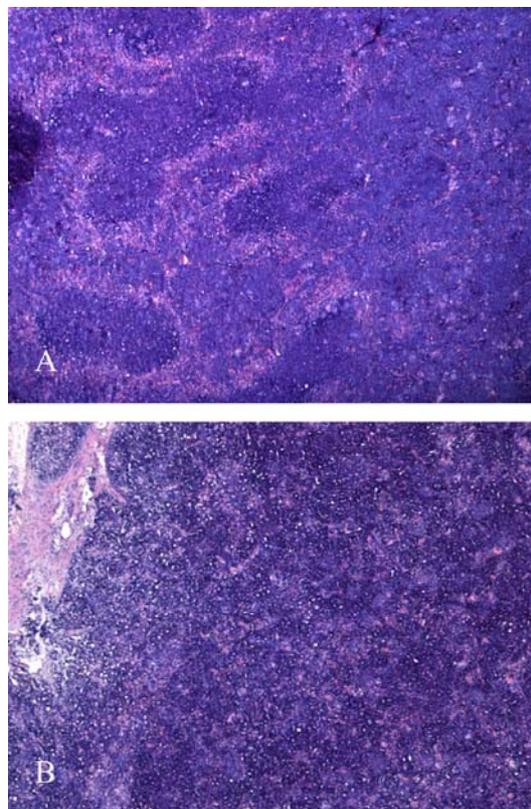


Figure 14. Effect of CpG-ODN -treatments on spleen morphology. Histological sections of spleen of mice treated 5 times/week for 4 weeks with CpG-ODN showed in some animals an enlargement of B-cell zone (A) or, in other, a complete alteration of follicular structure (B).

In light of a reported direct effect of CpG-ODN on tumor cell death (451), we tested IGROV-1 cells for expression of TLR9 and for their response to CpG-ODN. Low-level expression of TLR9 was detected both at the RNA (by reverse transcription-PCR) and protein (by FACScan analysis) levels, but neither viability nor proliferation was modified after CpG treatment at doses up to 10 µg/ml, and levels of proinflammatory mediators (IL-8, TNF-α, and IL-1β in the supernatant of CpG-ODN-treated IGROV-1 cells were comparable to those of control cells.

Markedly elevated peritoneal VEGF levels can be present in malignant ascites of ovarian cancer patients (452), and blocking of VEGF has been reported to strongly reduce ascites volume (453). Bio-Plex assay of VEGF or other angiogenic mediators, such as PDGF and bFGF, in the ascitic fluid of IGROV-1-bearing mice sacrificed 24, 48 and 96h after CpG-ODN administration (3 mice/group) revealed similar high, increasing VEGF levels in control mice and CpG-ODN-treated mice over time (Fig. 15A). Levels of PDGF and bFGF in ascitic fluid were also not significantly modified by CpG-ODN treatment.

The peritoneum is now recognized as a dynamic cellular membrane with important functions, including secretion of cytokines and growth factors such as IL-1, IL-6, KC, GM-CSF, and TNF. Moreover, the activation of different immune cells in the peritoneal cavity by CpG-ODN may trigger release of other cytokines such as IL-10, IFN-γ or IL-12 in the peritoneal fluid. Bio-Plex assay of cytokines and growth factors in peritoneal fluid 24 h after CpG-ODN injection (3 mice/group) indicated increased levels of IL-6, IL-10, IL-12 and IFN-γ over those in control mice but reduced levels of all of these cytokines in peritoneal fluids 48-96 h after CpG-ODN treatment similar to levels in control mice (Fig. 15B). Figure 15C shows the overall fold-changes in levels produced by CpG-ODN treatment. No significant differences were observed at the times analyzed (24, 48 and 96 h) in the amount of KC, probably related to the CpG-ODN-induced very rapid release of this cytokine (454), and only low levels of GM-CSF and TNF in saline- and CpG-ODN-treated mice were detected.

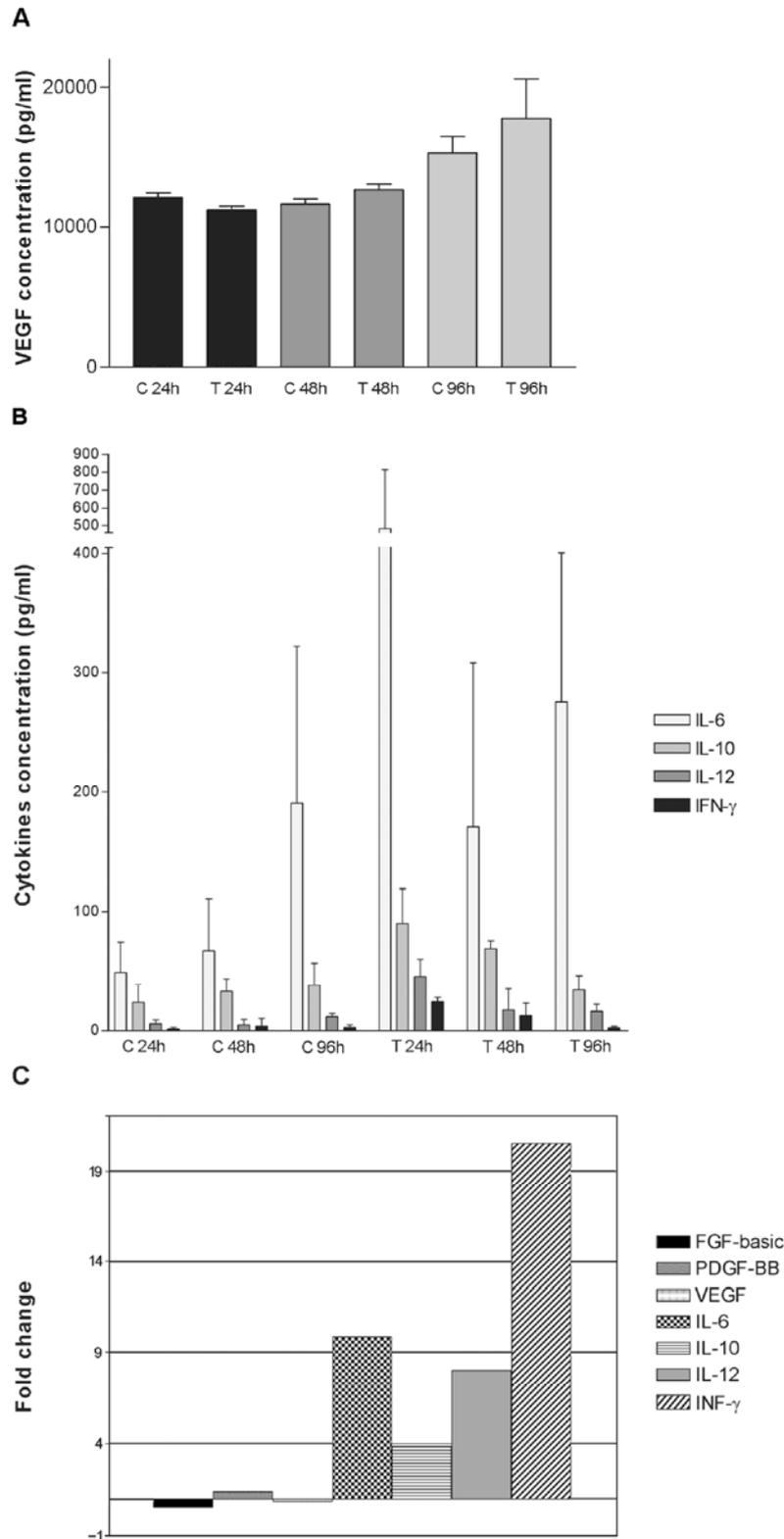


Figure 15. Effect of CpG-ODN treatments on ascitic fluid concentrations of angiogenic factors and cytokines Ascites-bearing mice were treated i.p. with CpG-ODN (T) or saline (C). At selected time points, CpG-ODN- and saline-treated mice (3 mice/point) were sacrificed, ascites fluids were recovered, and cytokine and angiogenic factor concentrations were determined by Bio-Plex assay. A) VEGF and B) IL-6, IL-10, IL-12 and IFN- γ concentrations (mean \pm SD); C) Fold-change in concentrations of angiogenic factors VEGF, PDGF and bFGF and of cytokines IL-6, IL-10, IL-12, and IFN- γ , comparing saline- and CpG-ODN-treated samples at 24 h after treatment.

Depletion experiments to investigate the role of NK cells and monocytes/macrophages and of cytokines produced by these immune cell populations on the CpG-ODN-induced reduction of ascites tumor cells and on CpG-ODN-induced ascites cytokine production revealed a significant decrease in IFN- γ in NK-depleted mice vs. non-depleted mice ($p < 0.01$) and of IL-6 and IL-12 in monocyte/macrophage-depleted vs. non-depleted mice ($p < 0.05$) in peritoneal ascitic fluid 24 h after CpG-ODN treatment; however, the absence of NK or monocytes/macrophages only slightly influenced the CpG-ODN-induced reduction of ascites tumor cells (79% reduction in NK-depleted mice vs. 86% reduction in non-depleted mice; 82% reduction in monocyte/macrophage-depleted mice vs. 89 % reduction in non-depleted mice).

Thus, a therapeutic effect on bulky disease appears to require locoregional treatment and also frequent multiple administrations. However daily i.p. administration of CpG-ODN induced a significant increase of survival-time but no cure of a single mouse (Fig. 13 A and B), therefore we screened the effectiveness of CpG-ODN in combination with different agents, including:

- 1) MAb bevacizumab, which targets the vascular endothelial growth factor (VEGF). VEGF is reportedly overexpressed in ovarian cancer (455-457), and VEGF-regulated angiogenesis is an important component of ovarian cancer growth (458,459);
- 2) the Poly(I):Poly(C) TLR3 agonist, which reportedly induces a synergistic effect when combined with TLR9 ligand by mediating an enhanced activation of innate immunity (460);
- 3) the cytotoxic chemotherapeutic drugs cis-platinum, which is currently used in ovarian cancer patients (461).

Mice were treated i.p. with CpG-ODN, Bevacizumab, Poly(I):Poly(C), cisplatin or their combinations at 8 days after tumor cell injection, when ascites starts to form.

Our results indicate that the combination of repeated i.p. CpG-ODN treatments plus bevacizumab (Fig. 16A) and Poly(I):Poly(C) (Fig. 16B) do not significantly increase median survival time,

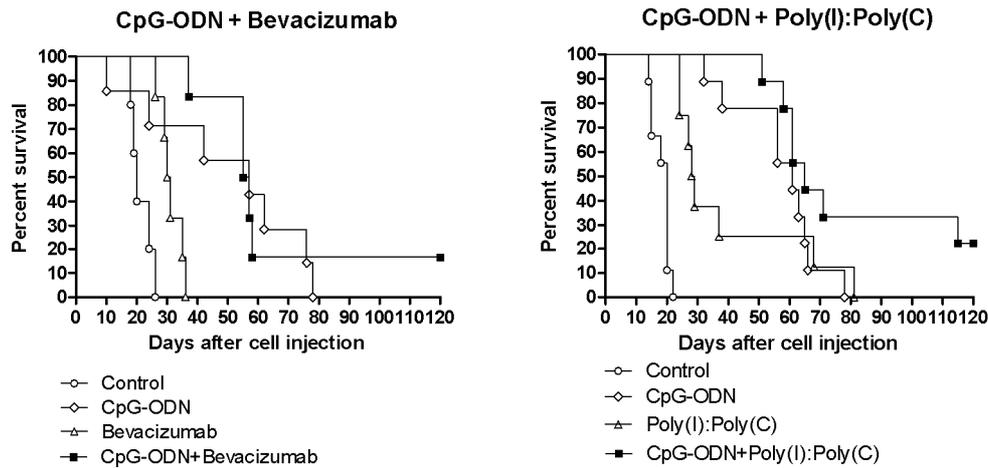


Figure 16. Kaplan-Meier plot of percent survivors over time among IGROV-1 ovarian tumor-bearing mice treated i.p. starting from day-8 with CpG-ODN (20 µg/mouse, 5 days/week) in combination with: bevacizumab (5mg/Kg at 3-4 day intervals) or Poly(I):Poly(C) (20 µg/mouse at 2-3 day intervals). Single agents were also included in the experiments. Control mice received saline.

The absence of synergy between the two immune modulators might be related to the reason that the massive activation of innate cells induced by repeated CpG-ODN stimulation can not be increased by the TLR3 agonist. Also the combination of repeated i.p. CpG-ODN treatments plus bevacizumab, did not enhanced the effect of CpG-ODN treatment alone. The little therapeutic benefit versus control mice observed in mice treated with the anti-VEGF antibody as a single treatment, might is related mainly to bevacizumab effects on controlling ascites formation, since in bevacizumab-treated mice ascites production was partially inhibited.

However, when we evaluated the effect of CpG-ODN treatment on the anti-tumor activity of cisplatin, a DNA cross-linking agent (462), the analysis of the effect of the combined treatment revealed a significant ($p < 0.0001$) increase in lifespan compared to the use of either reagent alone (T/C values: 200% for cisplatin, 305% for CpG-ODN, 460% for cisplatin plus CpG-ODN) (Fig. 17)

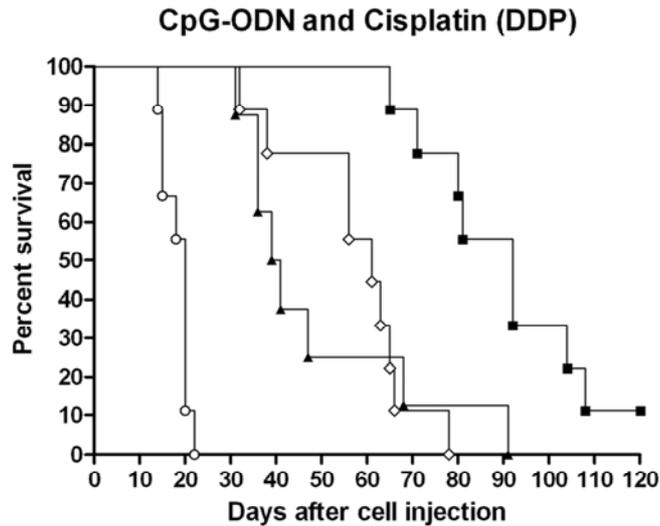


Figure 17. Kaplan-Meier plot of percent survivors over time among IGROV-1 ovarian tumor-bearing athymic mice. Mice were treated i.p., starting from 8 days after tumor cell injection, with CpG-ODN (20 μ g/mouse, 5 days/week for 4 weeks), cis-platinum (DDP, 3 mg/Kg i.p., once per week for 4 weeks) or both. Control mice received saline. Saline-treated mice (\circ); CpG-ODN-treated mice (\diamond); cisplatin-treated mice (\blacktriangle); CpG-ODN plus cisplatin-treated mice (\blacksquare). Experimental groups consisted of 8-10 mice group.

Different chemotherapeutic drugs have been reported to improve the efficacy of CpG-ODN in mouse tumor models (463,464). 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 (465). In immunocompromised athymic mice, the mechanisms underlying the improved anti-tumor effect when CpG-ODN was combined with gimatecan (466) or topotecan (499,400) have to be different from those in immunocompetent mice and remain unclear. It is noteworthy that, although these two 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 (467,468) and non-immunogenic tumors (469). For non-immunogenic tumors, the mechanism remains undefined since the rationale for even exploring the combination of CpG-ODN with radiation, i.e., that dendritic cells (DC) 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 appears 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 gamma-radiation (470).

Based on these disparate observations, we hypothesized that CpG-ODN modulate genes involved in DNA repair, increasing their expression in TLR9-expressing immune cells, but down-regulating 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 (471), and assessed the anti-tumor effect of CpG-ODN associated with DNA-damaging chemotherapy in this mouse model.

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 (accession number GSE11202) in immune spleen cells from mice treated i.p. with CpG-ODN (444). Spleen cells were chosen for this analysis based on previous studies establishing that the spleen accurately reflects the breadth of immunity induced by CpG-ODN *in vivo* (356,472-476). mRNA expression levels 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 (www.geneontology.org, GO:0006281mouse), 189 were present in the GSE11202 and 49 genes were found to be significantly modulated (FDR<0.05) during the course of CpG-ODN treatment, 43 of which were up-regulated (Figure 18).

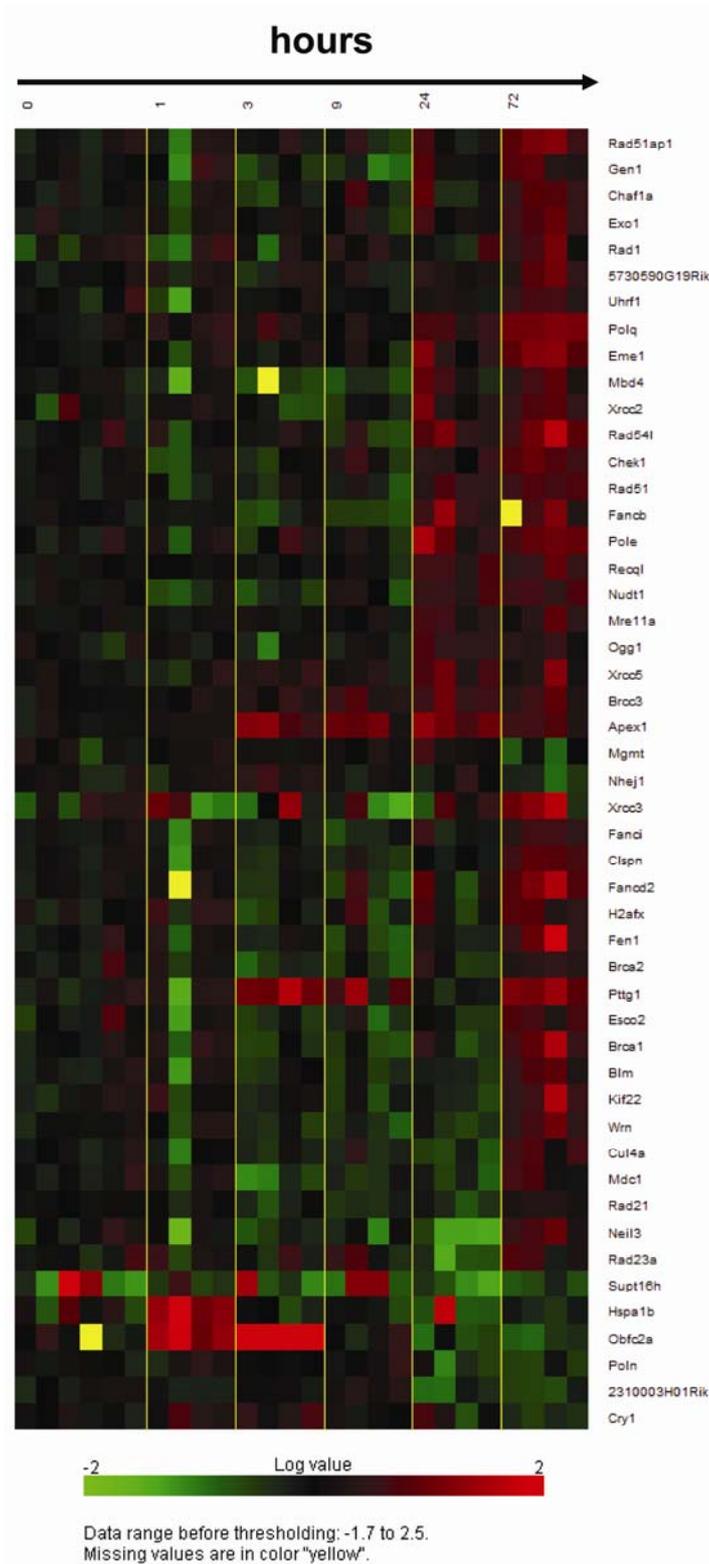


Figure 18. Microarray analysis of DNA repair pathway genes in spleen cells from CpG-ODN-treated mice. Mice were treated i.p. with CpG-ODN. RNA was extracted from spleen cells 1, 3, 9, 24 and 72 h after treatment and analyzed in-house-assembled oligonucleotide microarray platform. Of 209 genes involved in the DNA repair pathway (GO:0006281mouse), 189 were present in the GSE11202 dataset and 49 of these genes showed significant modulation (FDR<0.05) compared to that in untreated control mice (0 h). Color coding for each gene is normalized to the mean of the arrays for untreated controls (0 h). Black represents no change compared to controls; green and red represent down- and up-regulation with respect to the first time point, respectively. Changes from green to red to green indicate initial downregulation, increased expression, and final downregulation, respectively. Each column represents a sample and each row, a gene.

Accordingly, analysis of a published gene expression dataset (accession number: E-TABM-823) in immune mucosal lung tissue of mice 48 h after intranasal administration of CpG-ODN, when CpG-ODN induced recruitment of natural killer (NK) and DC into the bronchoalveolar spaces peaked (447), identified 29 genes in the DNA repair pathway that were significantly modulated by CpG-ODN treatment (FDR<0.05), 21 of which were up-regulated (Figure 19). Noteworthy, 13 of the 29 modulated genes were shared with those modulated in immune spleen cells. Thus, the presence of microbial DNA appears to induce up-regulation of genes involved in DNA repair in immune cells.

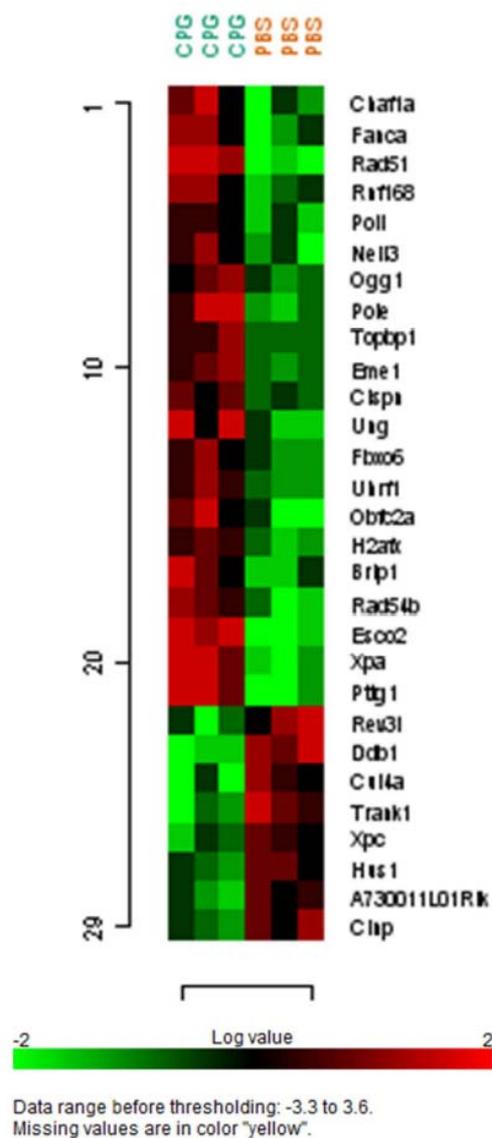


Figure 19. Microarray analysis of DNA repair pathway genes in lung mucosa from mice treated with CpG-ODN intranasally. RNA extracted from lung mucosa 48 h after CpG-ODN or saline treatment was analyzed on Agilent 44K whole-mouse genome microarray. Of 209 genes involved in the DNA repair pathway (GO:0006281mouse), 193 were detected in the E-TABM-823 dataset and 29 of these showed significant modulation (FDR<0.05). Each row represents the average expression kinetics of a gene.

To test whether CpG-ODN similarly affects non-immune TLR9-negative normal cells, we conducted expression analysis of DNA repair genes using a whole-mouse genome microarray dataset (accession number: E-TABM-506) obtained from murine quadriceps muscle injected with CpG-ODN in experiments to evaluate this bio-drug as a vaccine adjuvant (477); only 2 genes, NBR and FAM175, for which the specific function of the encoded proteins is unknown, were modulated (FDR <0.05). Thus, CpG-ODN does not induce significant modulation of DNA repair genes in non-immune normal cells.

The potential effect of CpG-ODN in tumor cells was evaluated based on the expression of the corresponding GO:0006281 mouse genes in a dataset obtained from MC38 murine colon carcinoma cells in mice injected intratumorally with CpG-ODN (445) (accession number: GSE18203). 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 down-regulation of DNA repair genes in tumor cells (40 of 50 modulated genes were down-regulated) (Figure 20).

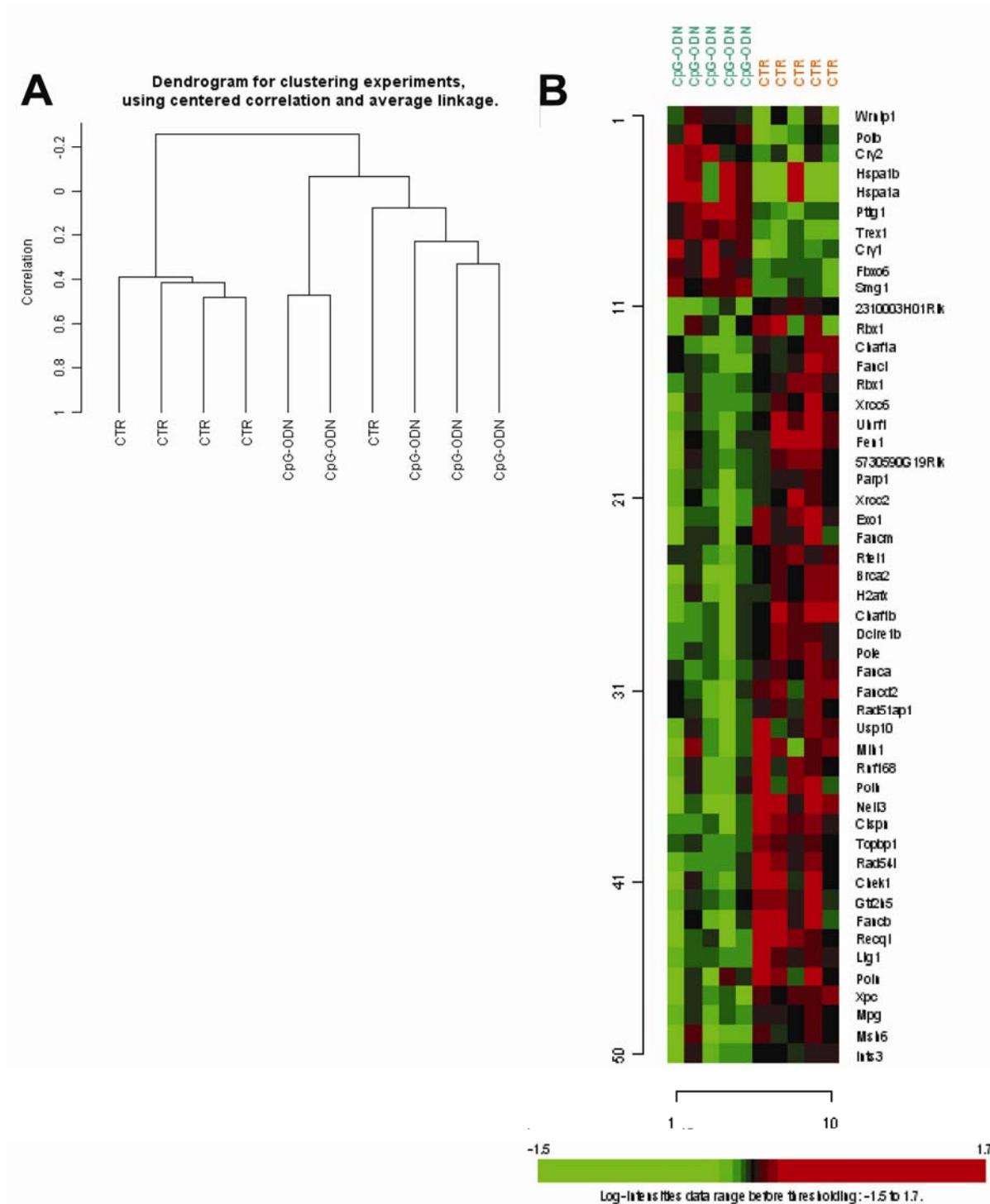


Figure 20. Microarray analysis of DNA repair pathway genes in MC38 murine colon tumors from CpG-ODN-treated mice. Mice bearing subcutaneous MC38 tumors were injected intratumorally with CpG-ODN or saline (control group). RNA extracted from tumors 6 h after treatment was analyzed on Affimetrix Platform using Affimetrix Mouse gene 1.0 ST chips; 201 genes in the DNA repair pathway (GO:0006281mouse) were detected in the Affimetrix 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 up-modulated (threshold $p < 0.05$), in CpG-ODN-treated mice (red: up-regulated genes; green: down-modulated genes). Each column represents a sample and each row, a gene.

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 at 3 days after evidence of ascites. At 24 h after the final treatment, tumors adhering to omentum were collected, and RNA extracted from the tumors cells was analyzed for gene expression profile. Among the 232 genes belonging to GO:0006281human, 227 genes available in our microarray platform clustered tumors according to saline or CpG-ODN treatment (Figure 21A) (accession number GSE23441), 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 down-regulated) (Figure 21B).

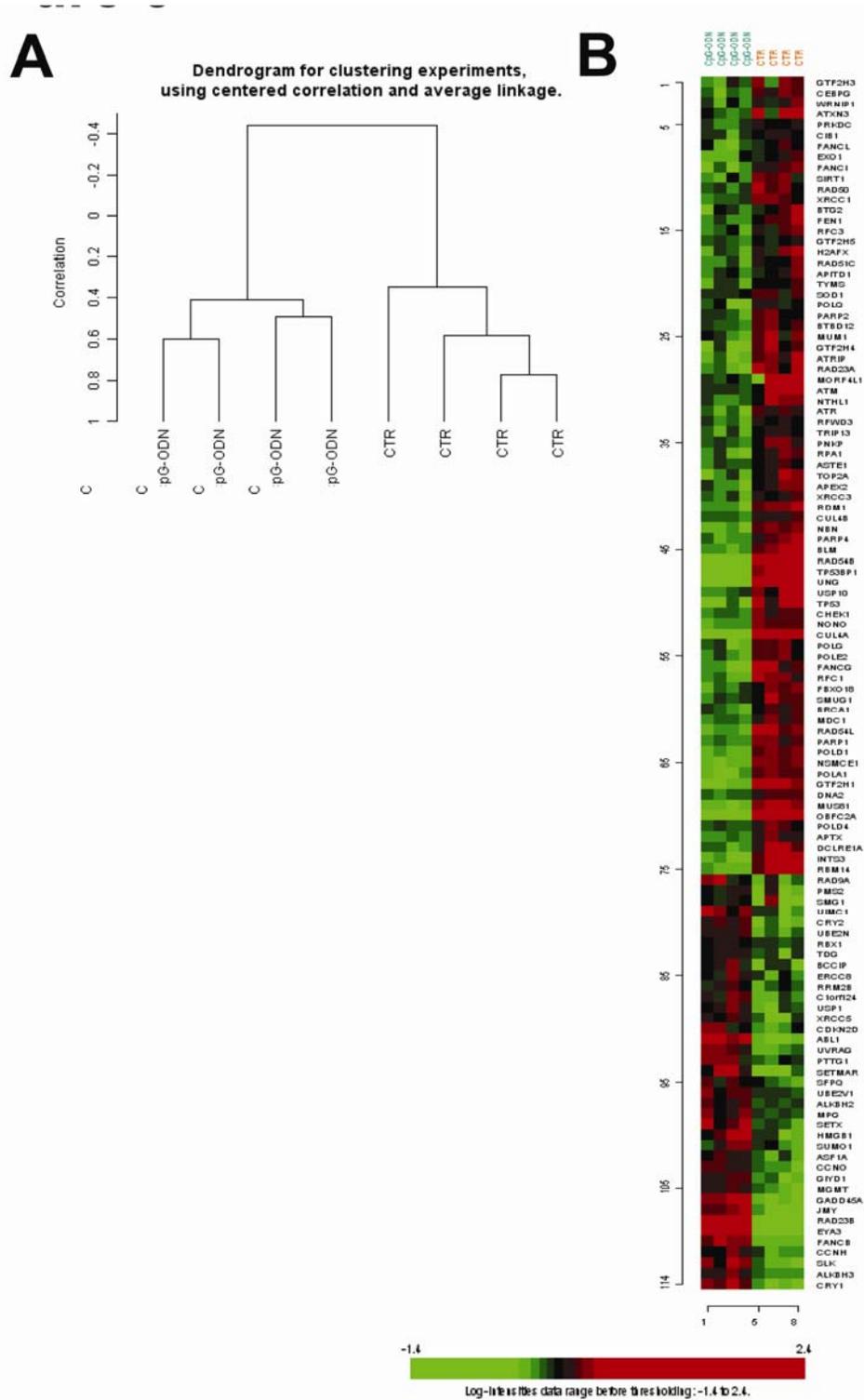


Figure 21. Microarray analysis of DNA repair pathway genes in IGROV-1 ovarian tumors from CpG-ODN-treated athymic mice. IGROV-1-bearing mice with established ascites, i.e., increased abdominal volume and body weight, were treated i.p. daily for 3 days with CpG-ODN or saline (control group) and sacrificed 24 h later. RNA, extracted from tumors was analyzed on Illumina human whole-genome beads chips; 227 genes in the DNA repair pathway (GO:0006281human) were detected in our microarray experiment (accession number GSE23441). (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 up-regulated (threshold $p < 0.05$), in CpG-ODN-treated mice; (red: up-regulated genes; green: down-modulated genes). Each column represents a sample and each row, a gene.

The power of CpG-ODN in modulating cancer cell DNA repair genes was confirmed by Ingenuity Pathway Analysis (IPA), since IPA of 1765 up- or down-modulated genes by CpG-ODN compared to controls revealed that the “Cell Death, Embryonic Development, DNA Replication, Recombination, and Repair pathway” was the most influenced (Figure 22).

Score	Focus Molecules	Top Functions
37	35	Cell Death, Embryonic Development, DNA Replication, Recombination, and Repair
34	34	Carbohydrate Metabolism, Small Molecule Biochemistry, Cellular Assembly and Organization
34	34	RNA Post-Transcriptional Modification, Cell Cycle, Humoral Immune Response
32	33	Cellular Function and Maintenance, Molecular Transport, RNA Trafficking
32	33	Genetic Disorder, Metabolic Disease, Cellular Assembly and Organization
32	33	Nucleic Acid Metabolism, Protein Synthesis, Small Molecule Biochemistry
30	32	Cancer, Gene Expression, DNA Replication, Recombination, and Repair
28	31	DNA Replication, Recombination, and Repair , Cellular Assembly and Organization, Cell Cycle
28	31	Nervous System Development and Function, Organ Morphology, Cell Morphology

Figure 22. Top nine networks (score>28; p<0.001) revealed by Ingenuity Pathway Analysis of 1765 genes modulated by CpG-ODN compared to controls.

Thus, microarray analyses indicate that locally administered TLR9 agonists regulate genes involved in DNA repair in tumor cells in the opposite way than in immune cells.

Results of microarray analyses were validated by examining on tumors from CpG-ODN-treated and control mice the expression of the gene products RAD51, a key protein in the homologous recombination DNA repair pathway (478), and SIRT1, whose activity promotes homologous recombination (479). Western blotting analysis of tumor cell lysates indicated decreased expression of RAD51, and SIRT1 proteins in treated mice as compared to controls (Figure 23).

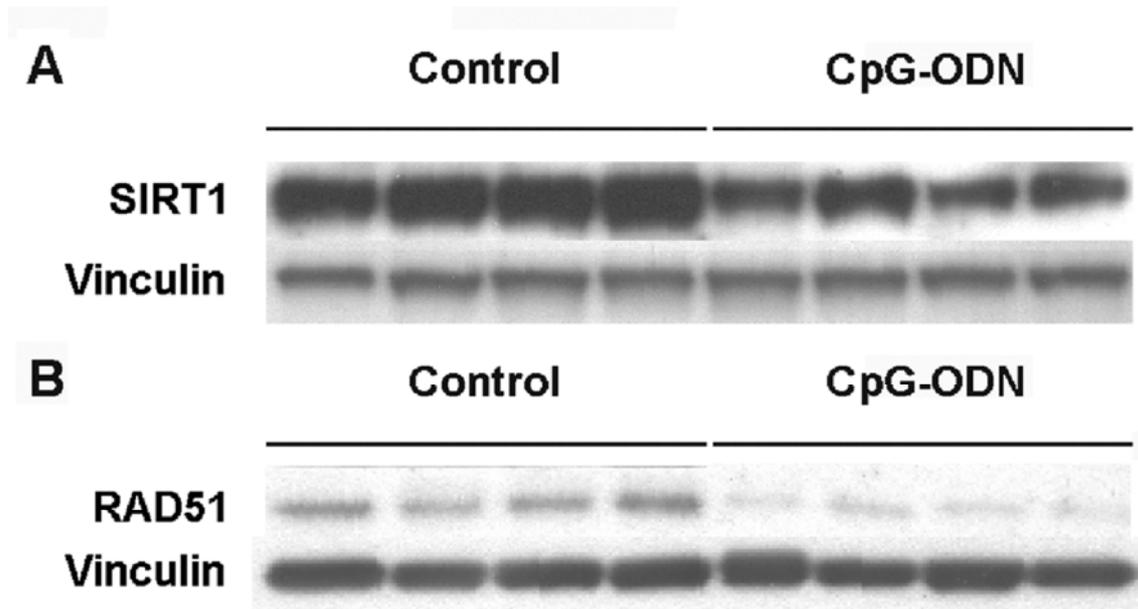


Figure 23. Western blot analysis of DNA repair proteins in IGROV-1 tumor cells adhering to the peritoneal wall after i.p. injection of CpG-ODN. Protein expression level of SIRT-1 (A), Rad51 (B) in IGROV-1 ovarian cancer cells from athymic mice treated daily for 3 days with CpG-ODN or saline (4 mice/group). Vinculin was used to normalize protein loading per lane.

To evaluate whether CpG-ODN induced DNA repair gene modulations, observed in IGROV1 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 $FDR < 0.01$ and a fold difference > 1.5 was selected (Figure 24) 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 dataset (<http://data.cgt.duke.edu/oncogene.php>) containing the gene expression data of ovarian cancer samples obtained at initial cytoreductive surgery from patients who then received platinum-based primary chemotherapy (480).

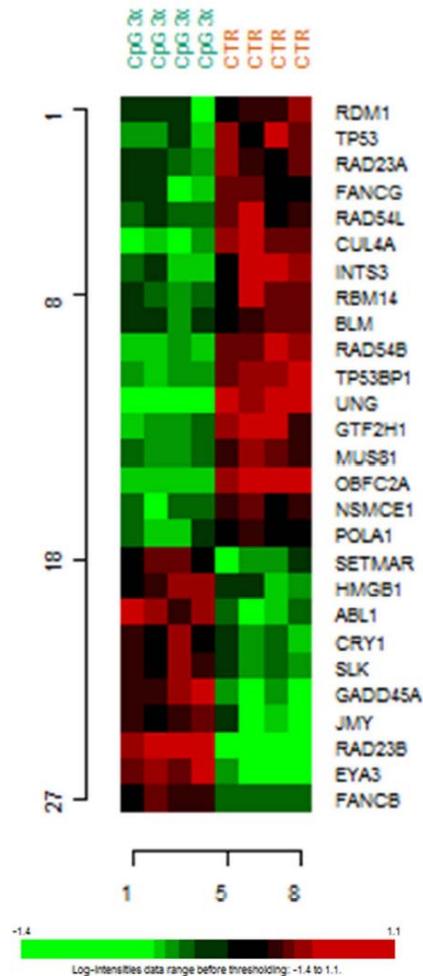


Figure 24. Heat-map of the 27 modulated genes in the DNA repair pathway obtained in IGROV-1 ovarian tumors from CpG-ODN-treated athymic mice. Genes were selected on the basis of FDR: <0.01, and a fold difference: >1.5 (red: up-regulated genes; green: down-modulated genes). Each column represents a sample and each row, a gene.

The Pearson correlation coefficient between expression values of the 27 genes and those available in the ovarian dataset 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”). As shown in Fig. 25 A, patients of the “CpG-like” group showed a significantly increased overall survival compared to the “CpG-untreated-like” group.

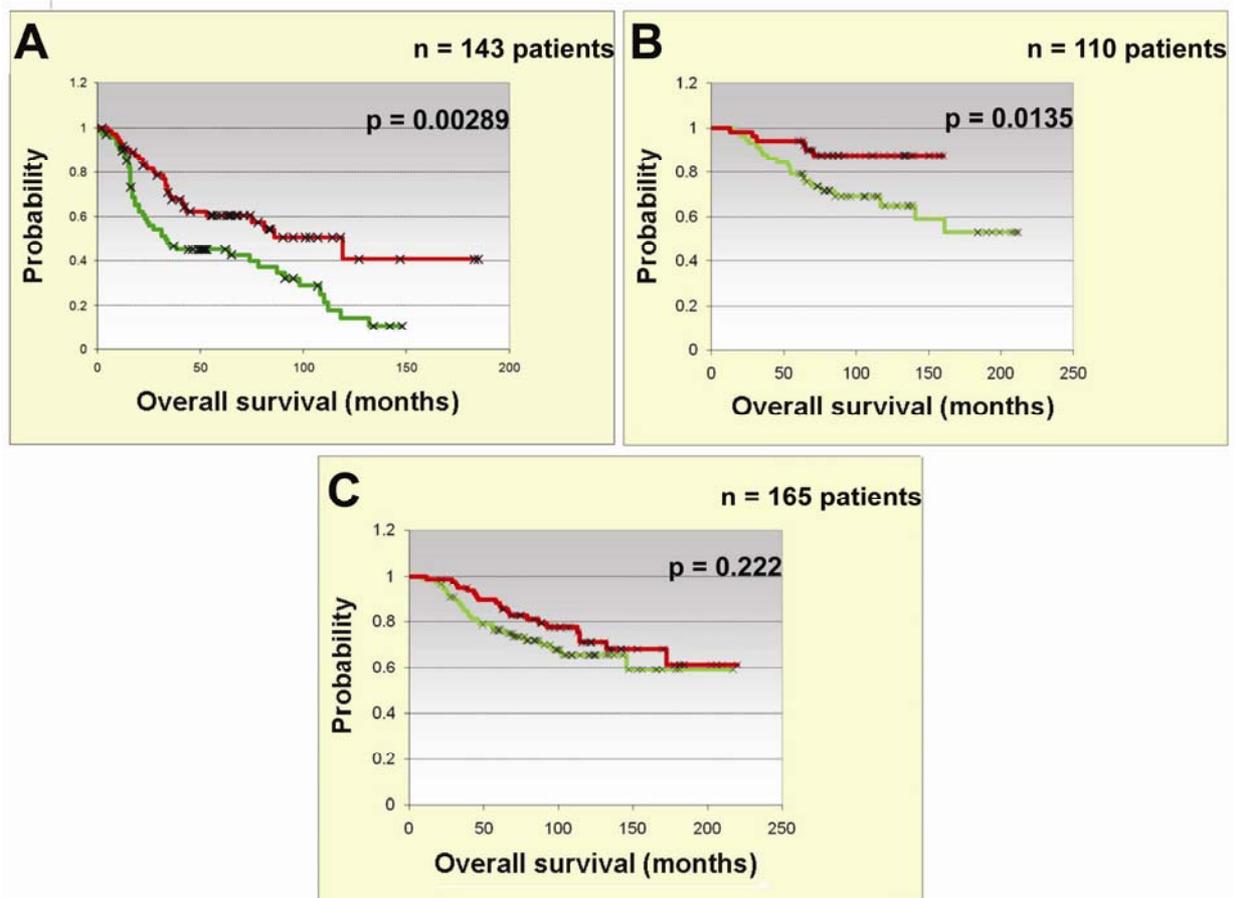


Figure 25. Association between DNA-repair genes modulated by CpG-ODN and survival in patients treated with chemotherapy in an adjuvant setting. Two datasets on whole-genome gene expression profiling of ovarian and breast cancer patients treated with chemotherapy were chosen (480,481). A Pearson correlation coefficient was calculated to assess the correlation between each tumor and the CpG-ODN signature. The correlation was determined based on expression values of the 27 genes found modulated, comparing the CpG-ODN treatment versus the control condition, and the corresponding genes available in each dataset. Tumors were split into two 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 ovarian cancer patients (panel A), breast cancer patients who received adjuvant systemic chemotherapy (panel B), and tumor patients who did not receive any systemic treatment (panel C). Patients with a greater correlation to the CpG-ODN compared to the untreated condition showed better outcome. Red curve: (CpG-ODN – CTRL) > average value; Green curve: (CpG-ODN – CTRL) < average value.

Cox's proportional hazard analysis confirmed above mentioned results (Hazard= 0.00131; p-value= 0.00578). A cross-validation procedure removing each single gene from the gene set and performing the Cox-analysis on the remaining gene set revealed that the prediction performance is not related to a particular gene, since in all analyses a p-value<0.05 was observed (Figure 26).

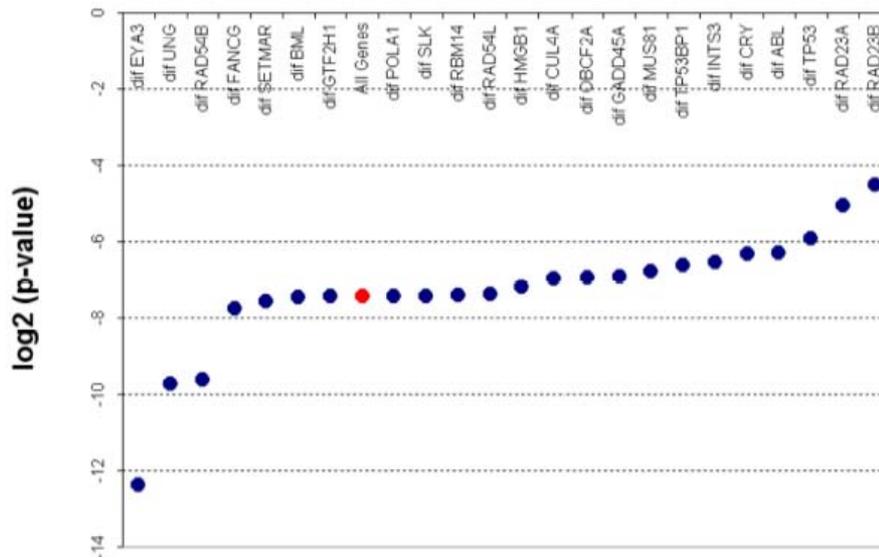


Figure 26. Plot of the p-values obtained by Cox hazard model comparing the entire gene set with the gene sets obtained removing each single gene.

When Kaplan-Meier survival analysis was performed on a breast cancer dataset of whole-genome expression of patients who received adjuvant DNA-damaging chemotherapy after surgery (<http://www.rii.com/publications/2002/nejm.html>) (481), patients classified as “CpG-like” showed a significantly increased overall survival compared to “CpG-untreated-like” patients (Fig. 25 B); on the contrary, no outcome difference was observed in the cohort of patients not treated with DNA damaging adjuvant therapy (Fig. 25 C). Altogether, these findings indicate that CpG-ODN modulate DNA repair gene expression that are relevant for cell sensitivity to DNA-damages.

It should be noted that the modulation of DNA repair genes in human ovarian IGROV-1 tumors and the increase in the anti-tumor effect of cisplatin and CpG-ODN against IGROV-1 human ovarian tumor xenografts in mice (IGROV-1 are sensitive to cisplatin (482), see Figure 17) 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 since different DNA motifs are required for stimulation of mouse and human cells by CpG-ODN (350,366). Nevertheless, we carried out a microarray analysis on mRNA extracted from IGROV-1 cells stimulated *in vitro* with murine CpG-ODN (accession number GSE23442); none of 16824 analyzed genes was found to be significantly modulated by CpG-ODN (no genes with FDR<0.1)

compared with IGROV-1 cells cultured in medium alone, excluding a direct action of CpG-ODN on tumor cells.

Our evidence of down-modulation in DNA repair genes in tumor cells in the analyses thus far involved the administration 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 (with FDR<0.1) as compared to tumors from saline-treated mice; thus, injection of CpG-ODN at the tumor site appears 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 down-regulation of DNA repair genes in tumor cells through a direct cell-cell interaction and/or by secreting soluble factors.

To evaluate the effect of soluble molecules, 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 and 24 h later, mRNA was extracted for microarray analysis. Genes involved in DNA repair clustered tumor cells according to treatment (Fig. 27A) and 25 genes were modulated (threshold $p < 0.05$) (Fig. 27B). Note that genes important in DNA repair such as PMS1 or REV1 were down-modulated.

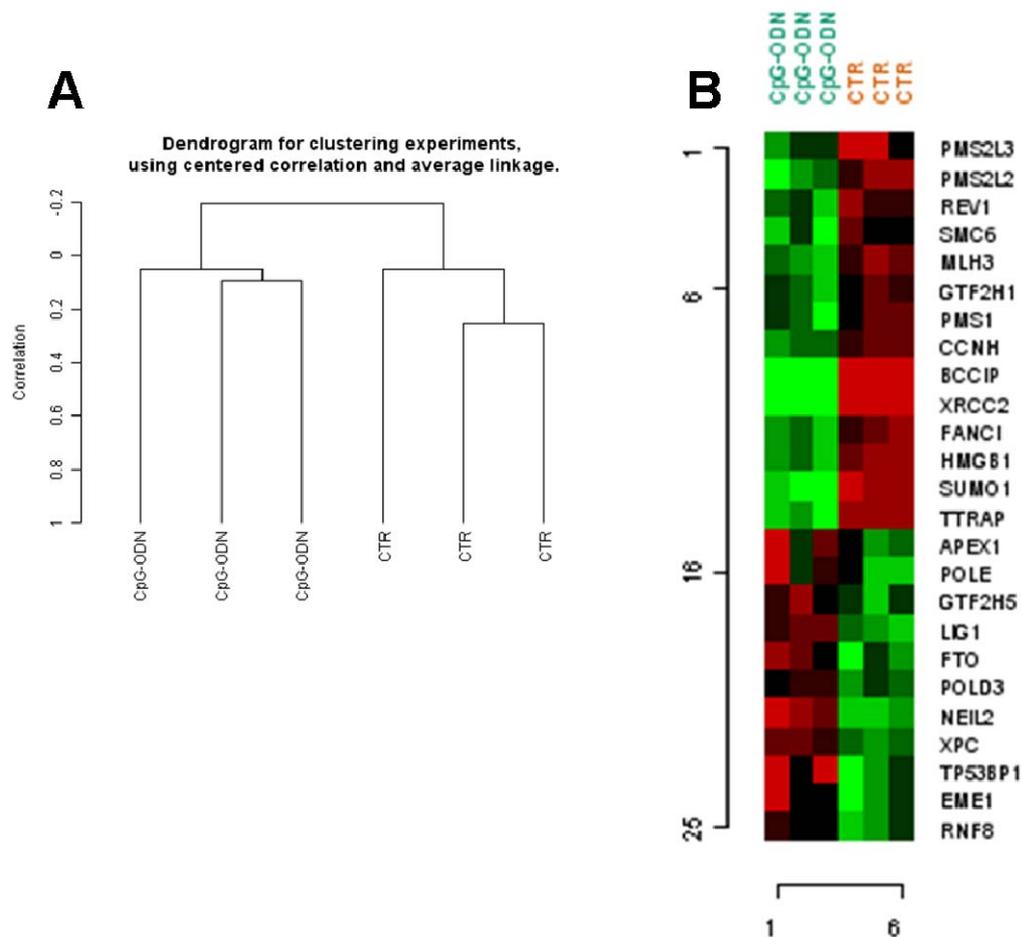


Figure 27. Microarray analysis of “DNA repair pathway” genes in IGROV-1 ovarian cancer cells pre-incubated *in vitro* with ascitic fluid from CpG-ODN treated mice IGROV-1 cells were cultured for 24 hours with ascitic fluid obtained from ovarian tumor bearing mice treated daily for 3 days with CpG-ODN or saline. RNA, extracted from IGROV-1 cells, was analyzed on Illumina human whole genome beads chips. Two hundred and forty genes belonging to the DNA repair pathway (GO:0006281) were found detectable in our microarray experiment. (A) Unsupervised hierarchical clustering of IGROV-1 cells according to the expression levels of DNA repair genes. (B) Heatmap of genes found modulated, 14 down and 11 up, considering a threshold of p-value <0.05, in CpG-ODN-treated mice; (red: up-regulated genes; green: down-modulated genes). Each column represents a sample and each row represents a gene.

Moreover, addition of ascitic fluid from *in vivo* CpG-ODN-treated mice to cultured IGROV-1 cells treated 48 h later with cisplatin enhanced cisplatin-mediated cytotoxicity of IGROV-1 cells compared to ascitic fluid of saline-treated tumor-bearing mice (Figure 28). Cell viability quantified by MTT assay after cisplatin (3 μ M, corresponding at LD₁₀) was 90.1 \pm 5.8% (mean \pm SEM) and 66.0 \pm 4.6% for cells pre-incubated with ascitic fluid from saline or CpG-ODN-treated mice respectively (p=0.0121).

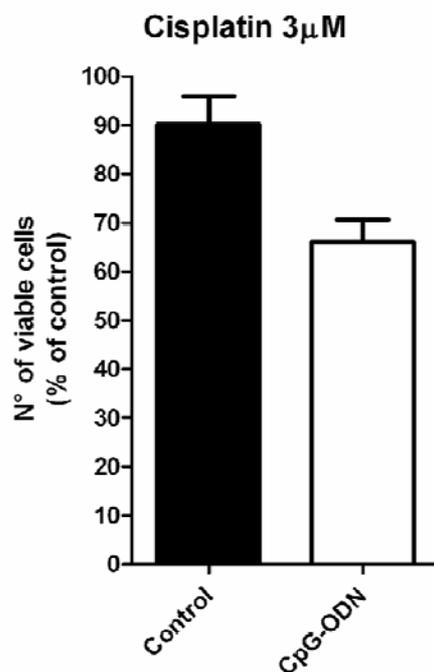


Figure 28. Ascitic fluid from CpG-ODN-treated mice sensitizes IGROV-1 cells to cisplatin. MTT assay of IGROV-1 ovarian cancer cells preincubated for 48 h with ascitic fluid from ovarian tumor-bearing mice treated daily for 3 days with CpG-ODN (open bar) or saline (closed bar) and exposed to cisplatin for 1 h. Cell viability of IGROV-1 cells cultured in the presence of ascites and cisplatin was calculated as percentage of cells treated only with ascites. Results are mean \pm SEM (n = 5-6 mice/ group).

Furthermore, to determine whether modulation of DNA repair genes in tumor cells is restricted to CpG-ODN induced TLR9 activation or occurs after activation with the another TLR agonist used in oncological therapy, the imiquimod, an immunomodulator that functions as agonist of TLR7 and/or TLR8 (483), a comprehensive *in silico* expression analysis of genes implicated in DNA repair (GO:0006281mouse) on mRNA extracted from spontaneous s.c. tumors from mice treated topically with imiquimod (446) (accession number: GSE20032) was performed. Imiquimod treatment was found to modulate DNA repair gene expression in tumors, which clustered according to treatment (treated vs. control) (Fig. 29A), 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 down-regulated) (Fig. 29B).

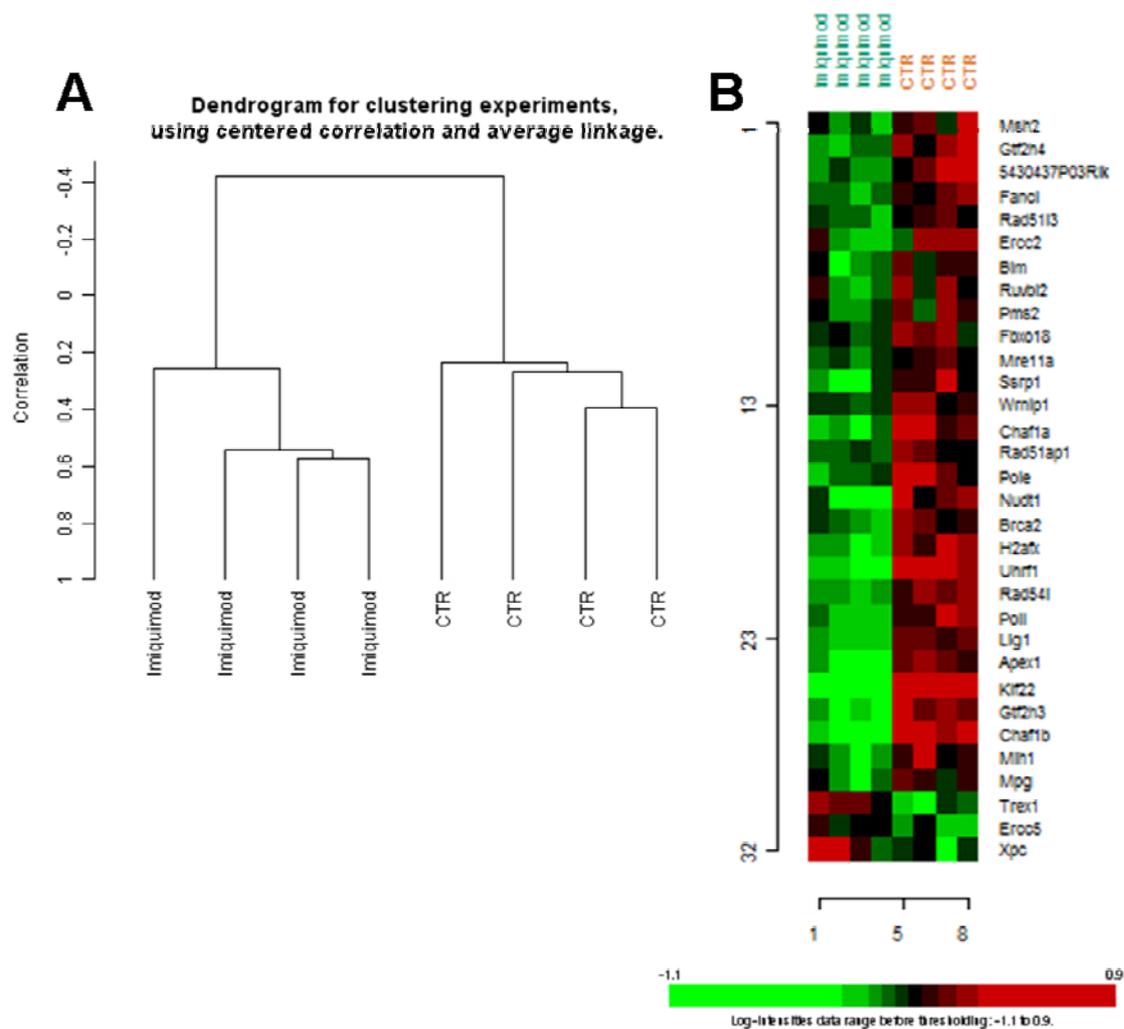


Figure 29. Microarray analysis of DNA repair pathway genes in tumors from mice treated with TLR7 agonist imiquimod. Mice bearing spontaneous tumors were treated topically with 5% imiquimod cream at the tumor sites for 3 consecutive days. RNA extracted from tumors and analyzed on Illumina mouse whole-genome bead chips revealed 144 genes in the DNA repair pathway (GO:0006281 mouse) present in the GSE20032 study. (A) Unsupervised hierarchical clustering of tumors according to expression levels of 144 DNA repair genes. (B) Heat-map of modulated genes, 29 down- and 3 up-modulated (threshold $p < 0.05$), in imiquimod-treated mice (red: up-regulated genes; green: down-modulated genes). Each column represents a sample and each row, a gene.

In mammalian cells repair of DNA damage appears to be controlled also by the epidermal growth factor receptor (EGFR). This is considered to be of especial importance for tumour cells, since several tumour entities are characterised by a substantial over-expression of EGFR (484,485). This trans-membrane tyrosine kinase receptor, which belongs to the ErbB-family, is primarily located in cell membrane and is activated by ligands such as epidermal growth factor (EGF), amphiregulin, TGF- α but also by irradiation (486,487). Ligand binding leads to dimerization, which induces several down-stream signal cascades. The most prominent EGFR dependent signal cascades are the

Ras/Raf/MEK/ERK dependent MAPK cascade, the PI3K dependent AKT kinase cascade, the JAK/STAT and PKC dependent signalling (486). Using these pathways, EGFR is considered to modulate cell proliferation, differentiation as well as apoptosis but also DNA repair (488). The modulation of DNA repair is suggested especially to occur for radiation-induced DNA double-strand breaks (DSB). There was an increase in the number of residual DSBs as detected by the number of γ -H2AX foci measured 24 h after irradiation, when EGFR signalling was blocked either by tyrosine kinase inhibitor BIBX or the monoclonal antibody (mAB) Cetuximab (489,490). Cetuximab is a chimeric IgG₁ monoclonal antibody directed against the ligand-binding domain of the epidermal growth factor receptor. The proposed mechanisms for this monoclonal antibody include reducing tumor cell proliferation, angiogenesis, increasing apoptosis; cell cycle arrest and DNA repair capacity (491). Human ovarian carcinomas express EGFR to varying degrees (492) and the staining of IGROV.-1 with cetuximab revealed that this monoclonal antibody is able to bind the membrane surface of IGROV-1 (Fig. 30).

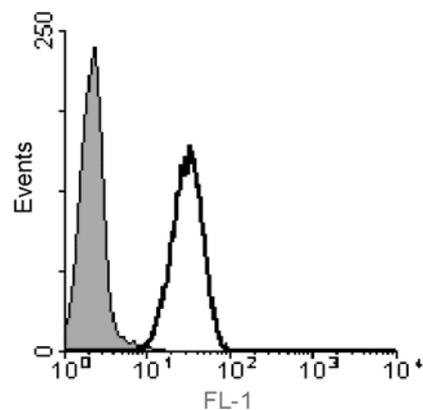


Figure 30. Flow cytometric analysis of IGROV-1 surface expression of EGFR. Cells were stained with cetuximab (black line, panel B), and with anti-CD20 rituximab antibody as isotype control (grey line).

We hypothesized that a combination Cetuximab-CpG-ODN could further improve the cisplatin cytotoxic activity. First we combined CpG-ODN with Cetuximab in IGROV-1 human ovarian carcinoma mouse model (Figure 31).

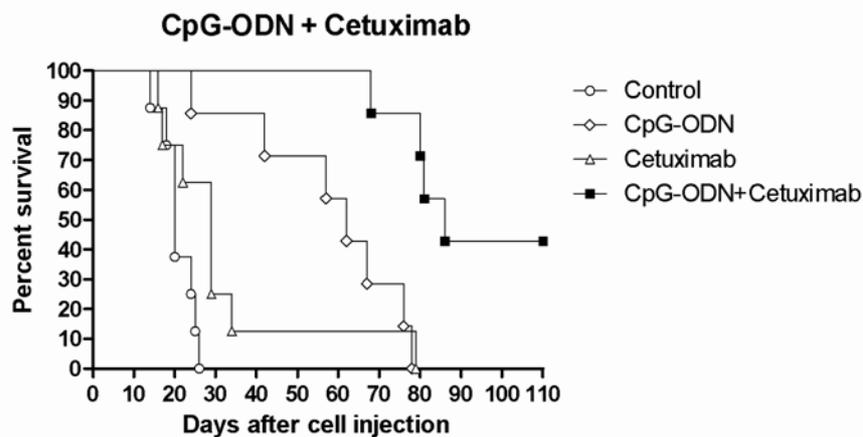


Figure 31. Kaplan-Meier plot of percent survivors over time among IGROV-1 ovarian tumor-bearing mice treated i.p. starting from day-8 with CpG-ODN (20 µg/mouse, 5 days/week) in combination with: cetuximab (1 mg/mouse at 3-4 day intervals). Single agents were also included in the experiment. Control mice received saline.

Mice treated with CpG-ODN plus cetuximab survived significantly longer than those treated with CpG-ODN or cetuximab alone. It is possible that the binding of this antibody to IGROV-1 cells can mediate ADCC by innate immune cells such as NK and granulocytes. Our results of the cetuximab experiments also suggest that the association of MAbs with CpG-ODN might allow the use of therapeutic MAbs to treat tumors that do not present amplification of the gene encoding the target of the MAb

Based on the results of the above described combination experiments and on the ability of CpG-ODN and Cetuximab in down-modulating DNA repair genes, we investigated the effects of CpG-ODN/Cetuximab in association with cis-platinum in an advanced human ovarian carcinoma model. Mice bearing established ascites, selected from larger groups of mice injected i.p. 11 days before with IGROV-1 cells, were randomly divided in different groups and treated with saline, cis-platinum, CpG-ODN plus cetuximab, CpG-ODN plus cis-platinum and CpG-ODN plus cetuximab and cis-platinum. Saline-, cis-platinum-, or cis-platinum/cetuximab-treated mice became moribund from tumor burden and were euthanized between days 13 and 36 after tumor injection (MST of 16, 23 and 18.5, respectively), whereas CpG-ODN/cetuximab-treated mice were euthanized between days 16 and 104 (MST of 66 and T/C%= 412.5). When ascites-bearing mice were treated with the

triple combination, CpG-ODN/cetuximab/cis-platinum, survival was significantly increased even compared with CpG-ODN/cetuximab treated groups (MST of 105.5, T/C% of 659.375; P=0.001 vs CpG-ODN/cetuximab treated mice) (Fig 32).

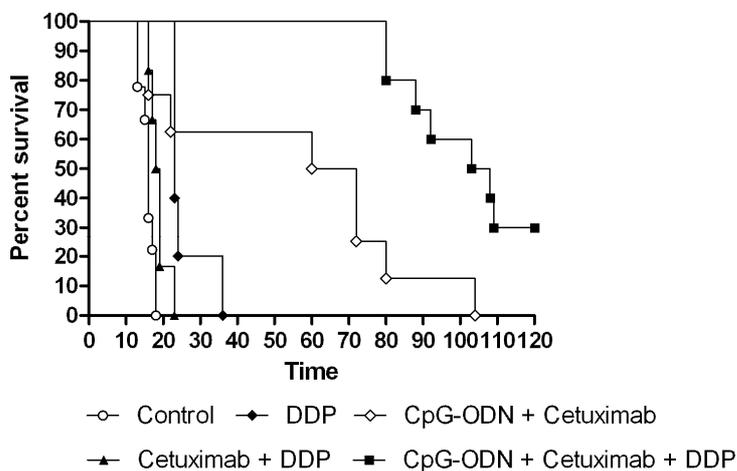


Figure 32. Kaplan-Meier plot of percent survivors over time among IGROV-1 ovarian tumor-bearing mice treated i.p. starting from day 11 with CpG-ODN (20 μ g/mouse, 5 days/week) in combination with cetuximab (1 mg/mouse at 3-4 day intervals), with cis-platinum alone (DDP, 3 mg/Kg i.p., once per week), with cetuximab with cis-platinum, or with CpG-ODN in combination with cetuximab and cis-platinum. Control mice received saline.

As described in Figure 32, mice treated with the combination of CpG-ODN/Cetuximab/Cisplatin were all still alive at 80th day. Therefore we can conclude that the association of these three drugs is effective in terms of survival in a mouse model of very advanced ovarian carcinoma.

DISCUSSION

In recent years our concept of the non-specific nature of innate immunity has changed following the identification of a network of germline-encoded receptors that recognise with substantial specificity molecular motifs of microorganisms and many other cues produced during tissue injury. Stimulation of these innate sensors by their specific ligands triggers signalling pathways that result in the activation of innate effector mechanisms as well as the priming of naive lymphocytes for the type of response that must be induced (493). Oligodeoxynucleotides (ODN) containing dinucleotides with unmethylated CpG motifs (CpG-ODN) are potent activators of both the innate and adaptive immune systems (494,495). Recognition of CpG-ODN is mediated by Toll-like receptor 9 (TLR9), a member of the TLR family, which is critically important in detecting microbial pathogens. TLRs, initially identified on cells of the immune system, are also expressed by non-professional immune cells such as endothelial cells, fibroblasts, and epithelial cells (433,496). Both bone marrow and non-bone marrow-derived cells are thought to be involved in the response induced by TLR agonists. Successes in preclinical studies using CpG-ODN and early indications of its safe use in humans have led to considerable interest in the clinical development of these agents in the treatment of cancer patients (392,494,497). It was recently reported the critical role of the administration route in the treatment of human ovarian cancer xenografts in nude mice, with intraperitoneal (i.p.) injection of CpG-ODN leading to an impressive increase in survival time and in tumor-free rate as compared to the slight effect of treatment administered intravenously or subcutaneously (450). However, it should be noted that these results were observed in mice before the appearance of ascites and therefore with a relatively low tumor burden. Indeed, ascites formation is a major cause of morbidity and mortality in advanced ovarian cancer patients⁹. In these patients, in whom the metastatic spread of tumor cells outside the peritoneum is uncommon, the tumor cell deposits in the peritoneal surface may prevent adsorption of i.p. fluid by mechanical

obstruction, inducing ascites (499). The treatment of ovarian cancer ascites is characterized by different palliative therapeutic options which are often of limited efficacy. Because tumor cell growth, even in advanced stages of ovarian cancer, is nearly always restricted to the peritoneal cavity, we tested whether repeated i.p. injections of CpG-ODN to recruit and activate innate effector cells throughout the abdominal cavity to the tumor site might control tumor cell growth and ascites formation in IGROV-1 ovarian tumor ascites-bearing mice. Here, we show that daily and multiple i.p. administration of CpG-ODN, but not weekly injections, significantly prolongs survival of mice with advanced-stage human ovarian tumors and established tumoral ascites as compared to control mice. Thus, bulky advanced-stage disease appears to require frequent challenges for a therapeutic effect. Because the athymic mouse model implies a relevant involvement of non-memory-endowed innate immune cells, which unlike adaptive immune cells, require repeated exposures to agonists to produce long-term activity, especially if the stimulating molecule has a very short half-life, the need for daily administrations of an immune modulator molecule such as CpG-ODN is not surprising. Moreover, CpG-ODN is known to induce short-time effects, upregulating mRNA within minutes and leading to short-term cytokine secretion and IgM production within hours (308,472). Several studies have suggested the efficacy of multiple and frequent administrations of CpG-ODN in providing long-term protection against bacterial infection (500), prion infection (501), cervical carcinoma (502) and mammary adenocarcinoma (503). While frequent administration of CpG-ODN might effectively combat infection and tumors, side effects due to immune overstimulation are possible. In our study, daily treatments were generally well-tolerated in terms of mouse status, but histological examination revealed a partial disorganization and enlarged B-cell zone in spleen of some animals, and a complete alteration of the spleen follicular structure in other mice, consistent with a previous report of malformed splenic lymphoid follicles after multiple administrations of CpG-ODN (504). CpG-ODN treatment was also found to inhibit ascites production. Indeed, even a single administration of a low-dose/low-volume CpG-ODN delivered i.p. to mice bearing an ascites volume roughly corresponding to 10% of their BW

(i.e., 2-3 ml over 25 g) rapidly reduced BW and ascites volumes, and multiple and frequent CpG-ODN administrations almost completely controlled ascites formation. The dramatic reduction in tumor cell number observed in treated mice, as well as the reversion in tumor cell/immune cell ratio in favor of immune cells, may contribute in restoring serial/functional drainage of ascitic fluid from the peritoneal cavity; however, the exact mechanism underlying ascites regression in our xenograft model remains unclear. Ovarian tumors have been reported to stimulate ascites by secreting VEGF, which enhances the permeability of preexisting microvessels lining the peritoneal cavity, and serves to improve tumor nutrient supply and waste removal for further expansion (505). The constitutive exposure of VEGF accumulated in ascitic fluid on the surrounding microvessels drives subsequent angiogenesis, which contributes to the long-term progressive growth of tumors. Several studies have supported a role for angiogenesis in ovarian cancer patients, and elevated VEGF levels in both serum and ascites have been associated with reduced survival (506,507). Clinical studies with agents targeting the VEGF pathway are ongoing or recently published and seem promising (508,509). In our study, levels of VEGF as well as of PDGF and bFGF in ascitic fluid increased as ascites developed, with levels higher for VEGF and PDGF than for bFGF; in no cases were the levels lowered by CpG-ODN treatment. Thus, the mechanisms responsible for CpG-ODN effects in controlling ascites formation do not appear to depend on angiogenic factors and instead might involve novel, as yet undiscovered pathways that await exploitation for therapeutic purposes. A previous report of a potent antitumor effect after i.p. but not i.v or s.c. CpG-ODN treatment in athymic mice implanted with human tumor ovarian cells in the peritoneal cavity (450) points to the importance of CpG-ODN treatment at the site of tumor growth. Advantages of i.p. administration of CpG-ODN may be related to the local activation of effector cells and might also include a lower clearance rate of CpG-ODN relative to plasma and a higher concentration of the drug in i.p. fluids relative to plasma (510). Various cells present in ascites fluid, including mesothelial and stromal cells as well as leukocytes, may be activated by i.p. injection of CpG-ODN. Increasing evidence suggests that the peritoneal mesothelium lining, comprised of cells uniquely located to regulate the

proliferation of submesothelial connective tissue and blood vessels, and able to produce of regulatory cytokines such as IL-1b, IL-6 and IL-8 (511,512), regulates important functions in controlling disease processes of the abdominal cavity (513,514). Moreover, mesothelial cells reportedly sense bacteria through TLR and secrete cytokines, chemokines and growth factors upon exposure to bacterial products (515). Such mesothelial cell-secreted molecules may control peritoneal tumor growth directly or by recruiting cells of innate immunity which, unlike cells of the adaptive immune response that can reach the antigen wherever they are activated, gather where the antigen induces their activation. The findings of high levels of cytokines such as IL-6, probably produced mostly by mesothelial cells, in peritoneal fluid of athymic mice treated with CpG-ODN suggest a central role for mesothelial cells in CpG-ODN-induced control of peritoneal tumor growth. Further studies are needed to define the specific contribution of these cells. The pleiotropic effects of CpG-ODN on different target cell populations raise the possibility of a direct or indirect activation even of bone marrow-derived innate immune cells, such as plasmacytoid dendritic cells, or NK cell and monocytes/macrophages, which can produce mixed effects on tumor growth (516). We previously observed that activated monocytes/macrophages accounted for at least some of the antitumor activity induced by CpG-ODN in early-stage human ovarian tumor xenografts (450). These cells are frequently the dominant population of leukocytes in the peritoneal fluid of patients with malignant ascites and, when activated, exert potent cytotoxic activity against neoplastic cells (517,518). However, our depletion experiments to investigate the role of a single immune cell subpopulation and its produced cytokines in the CpG-ODN-induced reduction of tumor cells in ascites revealed a strong reduction in the tumor cell number even in the absence of NK cells or monocytes/macrophages. Moreover, no differences in the percentage, maturation and activation of plasmacytoid dendritic cells, which directly respond to CpG-ODN through TLR9, were observed in ascitic fluid of CpG-ODN-treated mice. Thus, accordingly with the high degree of complexity of the processes that regulate the innate immune response and the cross-talk between the different signaling pathways, the antitumor activity of i.p. CpG-ODN treatment might not be related to a

specific cytokine or cell population but rather to the total repertoire of cytokines and cells accumulated in the peritoneal cavity. The clinical management of malignant ascites remains an unmet medical need because current treatments, which include diuretics, frequent large-volume paracentesis, i.p. or systemic chemotherapy, and a variety of other experimental strategies (519), are disappointing (520). None of these approaches has been established as standard therapy because of limited efficacy and the risk of severe side effects such as protein loss, bowel perforation and peritonitis (520,521). Although bulky tumor diseases are generally difficult to treat, either in patients and animal models, our data obtained in ascites-bearing athymic mice indicate that i.p. CpG-ODN treatments result in increased survival and inhibition of ascites formation, and suggest a relevant role for activation of cells and cytokines of innate immunity in the therapy of ovarian cancer patients with malignant ascites. Although daily i.p. administration of CpG-ODN induced a significant increase of survival-time, this treatment did not determine the cure of a single mouse, therefore we screened the effectiveness of CpG-ODN in combination with different agents, including such as bevacizumab, Poly(I):Poly(C) and cisplatin. Our results indicate that the combination of repeated i.p. CpG-ODN treatments plus bevacizumab and Poly(I):Poly(C) do not significantly increase median survival time, while the association of CpG-ODN and cisplatin revealed a significant increase in lifespan compared to the use of either reagent alone. Based on these results, we performed several experiments in order to gain insight into the molecular mechanisms by which CpG-ODN improves the therapeutic efficacy of cisplatin. Indeed, in the present study we show by microarray analyses that TLR9-ligand CpG-ODN treatment induces down-modulation of DNA repair genes in tumor cells of both murine and human origin. Expression level analysis of proteins, RAD51 and SIRT1, 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 lifespan compared to that using either

reagent alone ($p < 0.0001$). The combined data from our 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, 8 and 9, which reside intracellularly and recognize nucleic acids derived from the genome of viruses and bacteria (483). The findings that intratumoral delivery of CpG-ODN was critical in inducing DNA repair protein down-modulation in tumors and that CpG-ODN did not interact directly with the tumor cells to induce this down-modulations 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. Soluble molecules in ascitic fluid, presumably released by the above-mentioned cells via TLR9 stimulation, were found to be involved in the modulation of DNA repair genes, as indicated in IGROV-1 cells treated with ascitic fluid from CpG-ODN-treated mice of the down-modulation of genes important in DNA repair such as PMS1, encoding a protein reported to regulate cellular response to DNA damage by cisplatin (522), or REV1, encoding a DNA translesion polymerase able to confer resistance to a DNA-damaging agent and promote mutagenesis mainly through the interaction with other DNA translesion polymerases (523). Specifically, suppression of REV1 expression has been shown to reduce the rate at which human ovarian carcinoma cells acquire resistance to cisplatin (524). The lower number of modulated genes observed in that analysis compared with that in tumor cells from CpG-ODN-treated mice 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 (525). Based on our findings, this absence of CpG-induced enhanced chemosensitivity of tumor cells to DNA damage-inducing agents may rest in the subcutaneous

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 up-modulation of DNA repair genes in immune cells. Moreover, it is noteworthy that 19 of 49 genes modulated in spleen cells after CpG-ODN treatment, resulted modulated even in IGROV-1 tumor cells, but for almost of them the modulation was in an opposite way (14 up-regulated in spleen were down-regulated in tumor and 3 down-regulated in spleen were up-regulated in tumor). Despite numerous previous microarray analyses of immune cells after *in vitro* or *in vivo* stimulation with CpG-ODN (444-447,526), the up-regulation 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 pro-inflammatory genes (527), in light of the function of TLRs as innate immunity sensors of microbial products. CpG-ODN-induced down-modulation of DNA repair genes in tumor cells and up modulation in immune cells might represent a physiological phenomenon that occurs locally in the presence of an infective event. Thus, upon detecting the presence of an infectious agent via endosomal TLRs (Figure 33 BOX1, 1), immune cells might regulate DNA repair genes to decrease their susceptibility to possible pro-apoptotic signals during infections (Figure 33 BOX1, 2) and, at the same time, directly (by cell-cell interaction) and/or indirectly (by soluble factor/factors) (figure 33, BOX2) 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 down-modulation 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 expressing high levels of CpG-ODN-modulated DNA repair genes as compared to patients with low levels indicates the relevance of these genes in the tumor cell response to DNA-damaging drugs. In subjects who not received adjuvant chemotherapy, higher 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. Moreover, identification of a CpG-ODN-induced soluble molecule(s) involved in modulation of DNA repair genes could improve chemotherapeutic treatment even for tumors not amenable to CpG-ODN intratumoral administration.

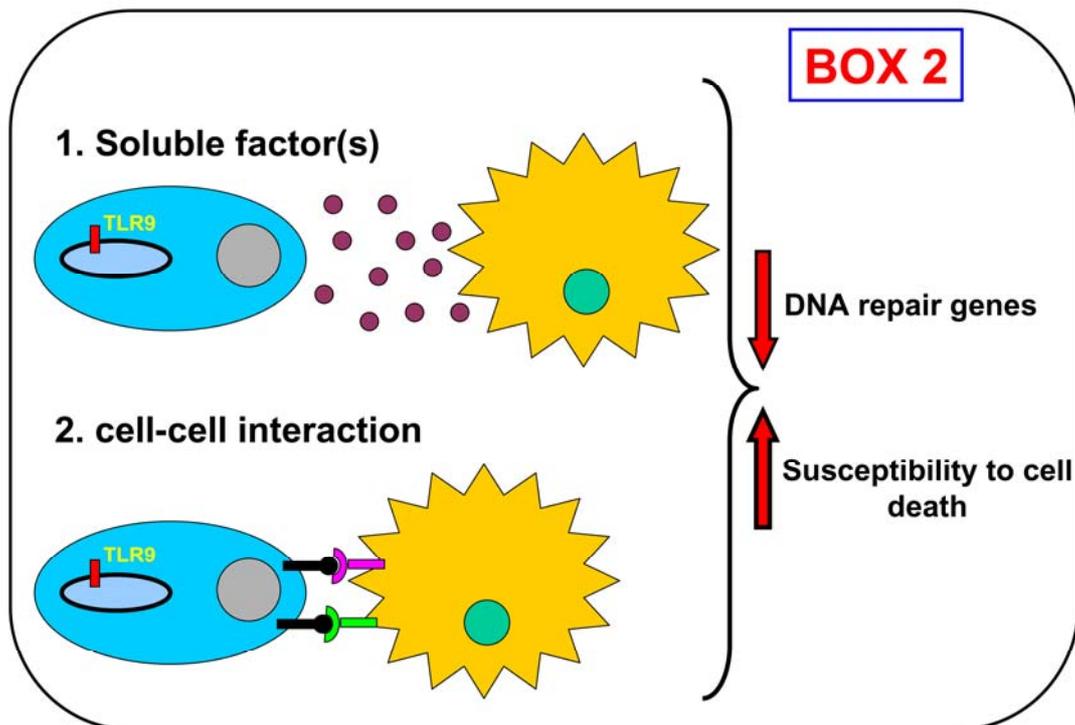
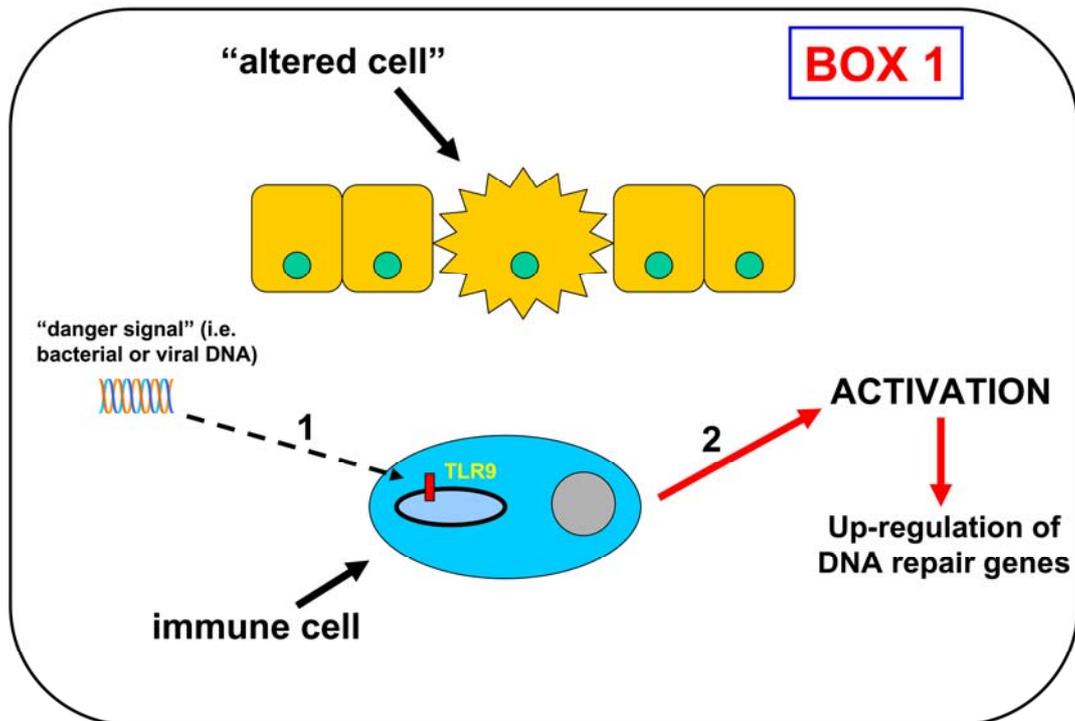


Figure 33. Possible mechanism of CpG-ODN ctivity in modulating DNA repair genes.

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