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ANTI-TUMOR ACTIVITY OF CpG-ODN IN OVARIAN XENOGRAFT TUMORS

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ABSTRACT

Synthetic oligodeoxynucleotides expressing CpG motifs (CpG-ODN), Toll-like receptor 9 (TLR9) agonists, are able to induce innate/adaptive immune responses and can enhance the antitumor activity of DNA-damaging chemotherapy and radiation therapy in preclinical mouse models.

It was recently reported that peritumoral CpG-ODN treatment in preclinical models of ovarian cancer, activating TLR-9 expressing cells in tumor microenvironment, induces modulation of DNA repair genes and sensitizes cancer cells to DNA-damaging Cisplatin treatment.

In this thesis we investigated whether this treatment induces modulation of miRNAs in tumor cells and their relevance to chemotherapy response. Array analysis identified 20 differentially expressed miRNAs (16 down- and 4 up-regulated) in human IGROV-1 ovarian tumor cells from CpG-ODN-treated mice versus controls. Evaluation of the role of the 3 most differentially expressed miRNAs on sensitivity to Cisplatin of IGROV-1 cells revealed significant increased Cisplatin cytotoxicity upon ectopic expression of hsa-miR-302b (up-modulated in our array), but no increased effect upon reduced expression of hsa-miR-424 or hsa-miR-340 (down-modulated in our array). The impact of expression levels of all 20 differentially expressed miRNAs were associated with time to replase and overall survival probability in two data sets of ovarian cancer patients treated with platinum. It was found that hsa-miR-302b expression was significantly associated with time to relapse or overall survival in these patients. Use of bio-informatics tools identified 19 mRNAs potentially targeted by hsa-miR-302b, including HDAC4 gene, which has been reported to mediate Cisplatin sensitivity in ovarian cancer. Both HDAC4 mRNA and protein levels were significantly reduced in IGROV-1 cells overexpressing hsa-miR-302b. Altogether, these findings indicate that hsa-miR-302b acts as a ‘‘chemosensitizer’’ in human ovarian carcinoma cells and may represent a biomarker able to predict response to Cisplatin treatment. Moreover, the identification of miRNAs that improve sensitivity to chemotherapy provides the experimental underpinning for their possible future clinical use.

In the second part of this thesis we tested the efficacy of CpG-ODN in combination with other possible therapeutic agents in ovarian carcinoma ascites-bearing athymic mice, to mimic clinical treatment situations in advanced human ovarian disease.

Mice injected i.p. with IGROV-1 ovarian cancer cells were treated at different stages of ascites progression for 4 weeks with CpG-ODN alone or in combination with Bevacizumab, Polyinosinic:Polycytidylic acid (Poly(I):Poly(C)), Gefitinib, Cetuximab and Cisplatin.

In mice treated when ascitic fluid began to accumulate, CpG-ODN combined with Bevacizumab, Poly(I): Poly(C) or Gefitinib did not significantly increase Median Survival Times (MST), as compared with that using CpG-ODN alone, whereas MST in mice treated with CpG-ODN plus Cetuximab was significantly increased (>103 days for combination vs 62 days for CpG alone; P =

0.0008), with 4/8 mice alive at the end of the experiment. In mice showing evident and established ascites, evaluated with increase of abdominal volume and body weight (27.9 ± 0.8 g after vs 23 ± 1.1 g before tumor cell injection), treatment with Cisplatin in addition to CpG-ODN/Cetuximab led to significantly increased MST (105.5 days; P = 0.001), with all mice still alive at 85 days, over that using CpG ODN/Cetuximab (66 days), Cetuximab/Cisplatin (18.5 days), Cisplatin (23 days) or saline (16 days). At a very advanced stage of disease (body weight: 31.4 ± 0.9 g), when more than half of control mice had to be sacrificed 6 days after starting treatments, the triple-combination therapy still increased MST (45 days; P = 0.0089) vs controls.

These data indicated that CpG-ODN combination therapies that enhance the immune response in the tumor microenvironment and concomitantly target tumor cells are highly efficacious even in experimental advanced malignancies. Although differences in the distribution of TLR9 in mice and humans and the enrichment of this receptor on innate immune cells of athymic mice must be considered, our results indicate a promising strategy to treat ovarian cancer patients with bulky ascites.

INTRODUCTION

1. IMMUNE SYSTEM and TOLL-LIKE RECEPTORS

The immune system has the capabiliy to detect and eliminate pathogens through several mechanisms, and it may be broadly divided into innate and adaptive systems. For many years, investigations into the pathogenesis of immune diseases focused on the role of the B and T cells of the adaptive immune system. In recent years, it has become increasingly well accepted that the innate immune system plays an important role in triggering these adaptive immune responses.

Cells of the innate immune system, represented by natural killer (NK) cells, monocytes, and granulocytes, rapidly detect invading pathogens and tumors in a non-specific manner. The innate immune system will respond to and contain the invading pathogens and prevent their spread. The adaptive immune system, represented by cytolytic T cells (CTL), T helper cells (TH), and antibodies, is activated by presentation of antigen in a cognate fashion and will develop an antigen-specific response to eliminate the pathogen (1).

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 (2). 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 (3). The key characteristic of innate immune cells that enables them to identify and classify infection seems to be their repertoire of 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. The best understood family of PRRs are the Toll-like receptors (TLRs), of which 10 are known in humans (4). 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 (5, 6). TLRs are considered sensors for microbial infections or other ‘danger signals’, and are critical to the linkage between innate and adaptive immune responses (7). 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 microorganisms 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 (**Figure 1**) (8,9). 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 (10). Each family is attributed to a general class of PAMPs (Pathogen-Associated Molecular Pattern). TLRs 3, 7, 8 and 9 are located mainly in endosomes; double-stranded RNA are ligands for TLR3 (11), while TLRs 7 and 8 recognize single-stranded viral RNA (12), TLR9 recognizes unmethylated CpG sequences in DNA molecules. The other TLRs are located on the cell surface (13); TLRs 1, 2, 5, 6 and 10 respond to bacterial, fungal and viral PAMPs (14-16). Lipopolysaccharides are TLR4 ligands (17). 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 (18). One pathway involves the MyD88-independent production of type I interferons. The second uses MyD88 to activate nuclear factor-kappa B (NF-kB), 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 (19,4). Additionally, TLR triggering induces DC maturation, which leads to the up-regulation 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 (13,20,21). 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 (22,23).

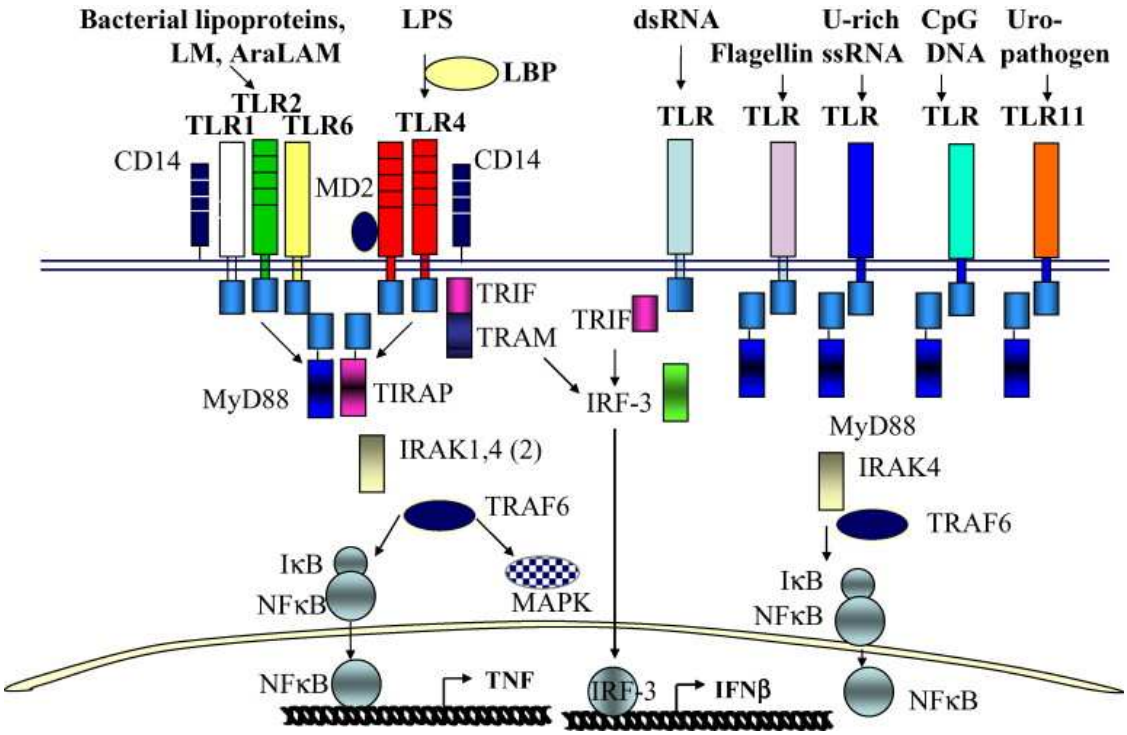

Figure 1. Microbial ligands and association with known TLRs and adaptor molecules. Schematic representation of the structure of TLRs and the major TLR ligands. Most TLRs form homodimers, while TLR2 associates with either TLR1 or TLR6. TLR signalling is mediated through adaptators such as MyD88, TIRAP, TRIF or TRAM.

Tumor immunotherapy has evolved since William Coley used crude bacterial extracts to treat cancer (24). 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 (24). 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 (25). These investigators suggested that the immunostimulatory effects of bacterial DNA were caused by the palindromic nature of the DNA sequences (26). In 1995, Krieg *et al.* (27) reported that the immunostimulatory effects of bacterial DNA were not caused by palindromes, but rather by 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 (26). 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 (28), although these studies could not be confirmed (29). Gene knock down and gain of function experiments finally identified TLR9 as the receptor conferring CpG reactivity by directly engaging bacterial DNA or synthetic CpG-ODN in a CpG motif-dependent manner (25,30-33). Research over the past 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 preferentially in memory B cells (34, 35) and plasmacytoid dendritic cells (pDC) (36-38), but is still a matter of discussion the expression in monocyte/macrophage cells (39-41). In murine, TLR9 is expressed on B cells, pDC, monocytes, macrophages, and dendritic cells (42,43). In non-activated 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 (44-46) (**Figure 2**).

It is well known that the TLR9 activation on pDCs induces secretion of type I interferon and increases expression levels of co-stimulatory molecules, such as CD80 and CD86; this is believed to initiate a range of secondary effects, including the secretion of cytokines/chemokines MCP-1, IP-10 and IL-12, the activation of NK cells and expansion of type 1 helper T cells and cytotoxic T lymphocytes (47;2).

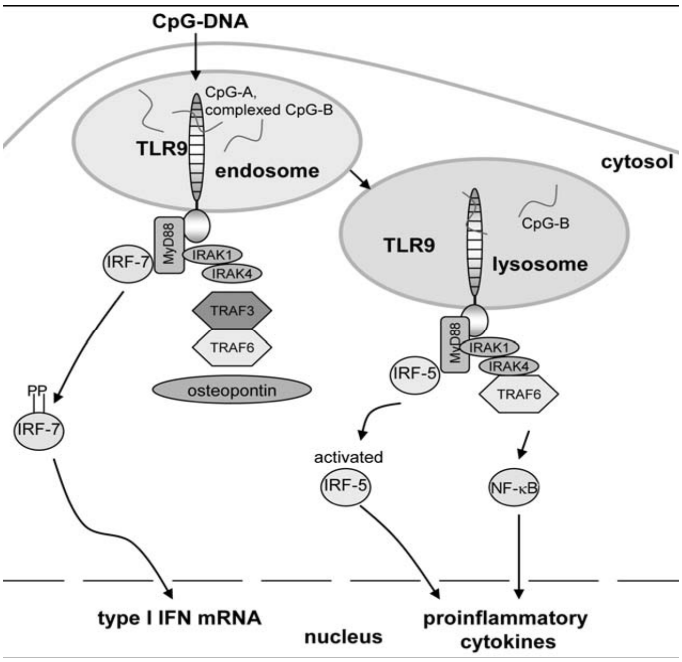

Figure 2. Activation of TLR9 induces secretion of proinflammatory cytokines and type I IFN dependent on ligand localization. CpG-A ODN or CpG-B ODN complexed to transfection agents reside in the endosome and initiate IRF-7 activation leading to type I interferon. In contrast, CpG-B ODN itself traffics to the lysosome and activates IRF-5 and NF-κB. 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 (48,49). 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 implied in trafficking (50,51), although a recent report has challenged this view. Accordingly, this report demonstrates that a tyrosine-based (YNEL) targeting motif in the cytoplasmic domain and the extracellular domain per se regulates TLR9 trafficking independent of the transmembrane domain (45). 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 usually feature of 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 non-responsiveness of TLR9 (together with TLR3, TLR7) accompanied by the disruption of TLR-unc93b interaction (52,53). To explore settings in which human and murine TLRs may play a role, it was found that TLR mRNAs is expressed in normal human and murine tissues and in cells

activated by microbial or inflammatory compounds. Indeed, TLR9 expression has been detected on intestinal epithelial cells, and an involvement in the maintenance of colonic homeostasis has been suggested (54,55). 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-κB) pathway, apical TLR9 activation prevents NF-κB activation by accumulation of NF-κB inhibitory protein I kappa B-alpha (IκB−α). Furthermore, apical TLR9 stimulation confers tolerance to subsequent TLR challenges, suggesting that apical exposure to luminal microbial DNA controls intestinal inflammation (55). This mechanism was demonstrated for pro-inflammatory bacterial product (or pathogen-associated molecular pattern) flagellin, that is a potent activator of intestinal epithelial pro-inflammatory gene expression. Flagellin is secreted by commensal and pathogenic bacteria and promotes inflammation only if it crosses intestinal epithelia and contacts their basolateral membranes, apical flagellin has no effect. TLR5 could activate proinflammatory gene expression in response to flagellin. Further, TLR5 is expressed on the basolateral, but not apical, surface of model epithelia, thus providing a mechanism by which microbes that invade or translocate flagellin, but not commensal bacteria, induce intestinal epithelia to orchestrate an inflammatory response (56).

TLR9 EXPRESSION ON TUMOR CELLS

TLR9 expression has also been reported in non-immune cells, including pulmonary epithelial and endothelial cells (57,58), keratinocytes (59), and intestinal epithelium (60,61).

Kundu et al. (62) have shown that immortalized prostate epithelial cells, expressing TLR9, exhibit enhanced proliferation when cultured in the presence of CpG-DNA. These stimulated cells were shown to be less susceptible to TNF-alpha induced apoptosis and to cell death.

Other authors, such as Ilvesaro et al. (63), provided evidence that TLR9 agonistic unmethylated CpG oligonucleotides (CpG-ODN) promote matrix metalloproteinase-13 (MMP-13) activity, resulting in enhanced migration of human prostate cancer cells expressing TLR9. CpG-ODN is well-known surrogate molecule for pathogens residing in genitourinary system, such as E. coli and some DNA viruses (HPV for example). These data show that pathogens frequently encountered in this milieu may enhance malignant transformation and boost cancer cell spreading. Moreover, it has been found that not only natural, but also synthetic CpG-ODN may function as vaccine adjuvants for infectious diseases as well as for cancer (64).

The role of TLRs expressed on tumor cells in the evasion of immune surveillance was elegantly demonstrated in animal experiments (65).

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 few studies have discussed the significance of TLR9 expression on tumor cells (66). It was demonstrated that TLR9 activation can lead to the proliferation of immortalized prostate cells (62), or to the promotion of matrix metalloproteinase (MMP)-13 activity, resulting in enhanced migration of human prostate cancer cells expressing TLR9 (67,63). These studies demonstrated how TLR9 agonists from pathogens encountered in the genitourinary system may enhance malignant transformation and boost cancer cell spreading through inflammation-dependent mechanisms (66,68). 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 (69,70). 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 (71). Basically, 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 (72,73). Unlike TLR4-mediated signaling, the TIR domain-containing adaptor protein/MyD88-adapter-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 (7,8). Several studies suggest that this molecule, TIR domain containing 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 (74-76). 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 (75,77). 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 up-regulate cytokine/chemokine gene expression (**Figure 3**) (75,78-80).

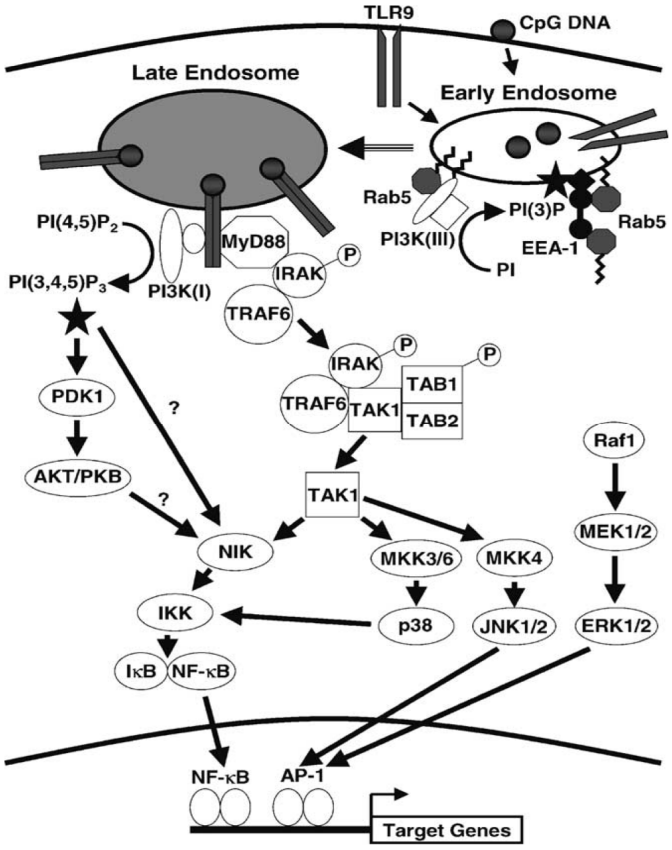

Figure 3. Class III PI3K (PI3K (III)), EEA1, and Rab5 mediate the trafficking and maturation of endosomes containing CpG DNA and TLR9, by which TLR9 transduces intracytoplasmic signal. The signal initiates with the recruitment of MyD88 to the TIR, which then activates IRAKTRAF6- TAK1 complex. This leads to the activation of both MAPKs (JNK1/2 and P38) and IKK complex, culminating upregulation of transcription factors including NF-kB 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 up-regulates STAT1 phosphorylation and IP-10 production through IFN-α/β receptor in an autocrine manner (81). Studies using chloroquine (CQ) or wortmannin (WM) showed that these agents could block CpG-DNA/TLR9 signaling but not LPS/TLR4 signaling (29,78,82). 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 (29,78). 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) (18), others find that DNA-PK KO mice and SCID mice respond normally to CpG-DNA (82,83). It was 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 (82). 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 (84-86). 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) (87,88). Ligand-induced association of TLR2 ICD and PI3K (I) was reported to activate the AKT/PKB-NF-kB pathway (89). CpG-DNA also induces phosphorylation of AKT/PKB thereby inhibiting apoptosis in DCs, an effect that is reversed by a PI3K inhibitor, LY294002 (90). 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-kB 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-kB 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 (27). 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 (**Figure 4**). B-Class ODN with one or more 6mer CpG motif with the general formula "purine-pyrimidine-C-G-pyrimidine-pyrimidine" (27) are strong stimulators of human B cell responses, and induce maturation of human pDCs. The 6mer motif 5′-GTCGTT-3′ represents the optimal human CpG motif (76), whereas 5′-GACGTT-3′ is the optimal murine CpG motif (27,91). The length, the 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 and are between 18 and 26 nucleotides in length (91), additional CpG motifs do not much further enhance activity. Chemical modifications of the backbone, the heterocyclic nucleobase or the sugar moiety further may enhance the activity of B-Class CpG-ODN. Phosphorothioate (PS) modifications of CpG-ODN stabilize them against nuclease degradation and enhance their activity by about 10 to 100 fold compared to phosphodiester (PO)-ODN that either have to be added repeatedly or to be combined with an uptake enhancer to result in similar activity (92,93).

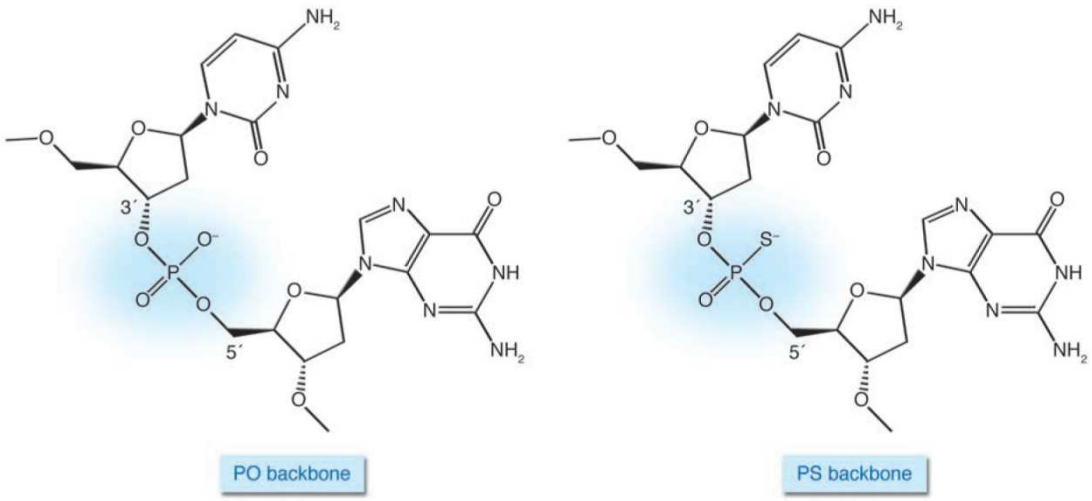

Figure 4. PS ODN differ from native phosphodiester (PO) DNA ODN only in the substitution of a sulfur for one of the non-bridging oxygen atoms. This change improves the in vivo stability of the ODN from a half-life of a few minutes to about two days for the PS ODN.

In contrast to the charged phosphodiester and phosphorothioate backbones, replacement with non charged backbones results in decreased immune stimulatory activity (94). CpG-ODN with 2′-Omethyl or 2′-O-methoxyethyl sugar modifications induce decreased immune stimulation (95,96), substitutions with a RNA derivative, locked nucleic acid (LNA) and even can eliminate the immune stimulatory effects of CpG-containing phosphorothioate ODN (97). In principle, any modification of cytosine at the CpG motifs is usually not well tolerated, while TLR9 appears to be more forgiving to modifications at the guanosine position (96,98).

A-Class CpG-ODN is defined by G runs with PS linkages at the 5′ and 3′ ends surrounding a phosphodiester palindromic CpG-containing sequence (99,100). Intermolecular tetrad and high molecular weight aggregates are formed via the G residues that enhance stability, increase endosomal uptake and ligand concentrations (96,101), 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 (102). 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 (99,100). The CpG C-Class has some sequence requirements similar to the B-Class and combines the modulatory 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 (102). 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-alpha production (102). 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 (103). 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. 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 (104).

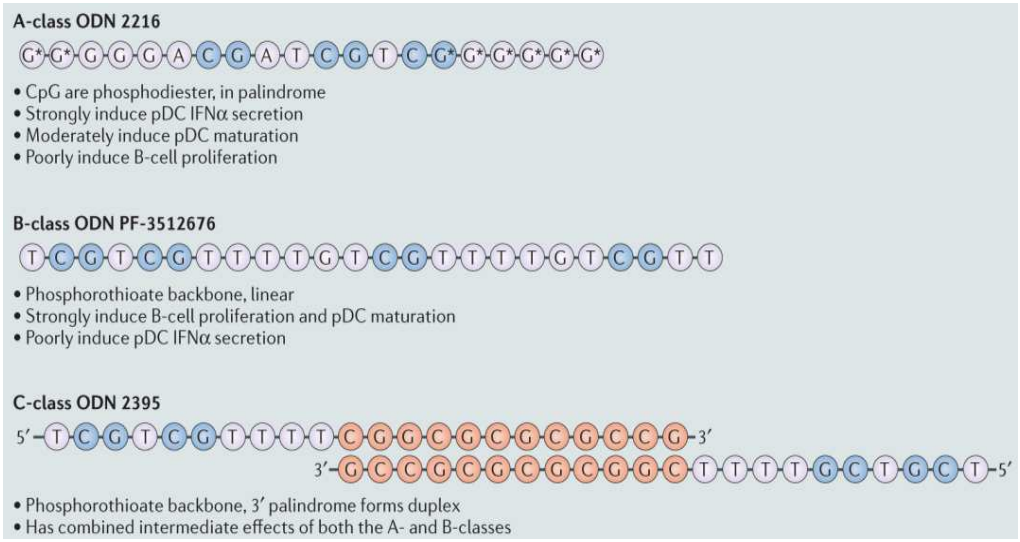

Figure 5. Three major classes of CpG-ODN that are structurally and phenotypically distinct have been described. Examples of each class are shown in the figure, using the ID numbers from the published reports (PF-3512676 formerly was also known as ODN 2006 and CpG 7909), together with the immune effects 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. 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 IFNα secretion. 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. The C-class CpG-ODN has immune properties intermediate between the A and B classes, inducing both B-cell activation and IFN-α secretion. 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 *TH1*-LIKE INNATE AND ADAPTIVE IMMUNITY

The immune effects of administering CpG-ODN to humans seem to result directly 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 (2). 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 CTLs and NK cells 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, followed by antigen-specific adaptive immunity (**Figure 6**). 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 co-stimulatory 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 (4). 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 (4). The pDCs activated through TLR9 become competent to induce effective $CD4^+$ and $CD8^+$ T cell responses (105-109). 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 (110). B cells are strongly costimulated if they bind specific antigen at the same time as TLR9 stimulation (**Figure 6**). 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.

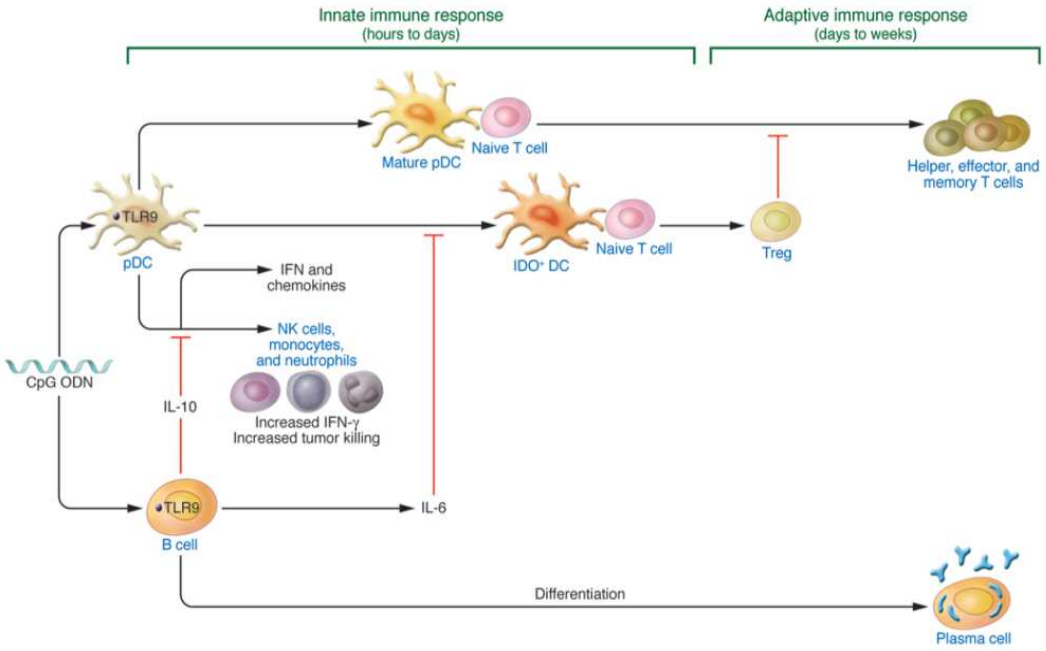

Figure 6. Among human immune cells, B cells and pDCs constitutively express TLR9. These cells endocytose DNA into an endosomal compartment where it binds to TLR9, forming a signaling complex. If the DNA contains unmethylated CpG motifs, TLR9 is stimulated, and the cell becomes activated. In pDCs, this 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 counter-regulatory 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 counter-regulatory pathways such as the induction of T_{regs} limit TLR9-induced immune activation, offering a potential for enhancing the therapeutic efficacy of TLR9 agonists by co-administration 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 phosphorothioate (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 (111,112). 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. 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 (43,113). Also 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 (114). 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 humans (**115**). Because the pharmacodynamics of subcutaneous CpG-ODN results from the local ODN concentration in the draining lymph nodes, they do not match the systemic pharmacokinetics.

Drug-like characteristics
• 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' compound[201]
Non-drug-like characteristics
• 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

Table 1. Characteristics of CpG oligodeoxynucleotides

PRECLINICAL STUDIES OF TLR9 AGONISTS

CpG-ODN has been tested in several mouse tumor models (116) 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 (116). Thus, there is some problem to extrapolate the positive effects seen in mouse models to humans.

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 (117-119). CpG-ODNs used as monotherapy could be effective in inducing regression in C3 model of cervical cancer, when it was injected subcutaneusly (120). However, the injection site was critical, since injection of CpG-ODN at distant sites was ineffective or less effective, in the treatment of other tumor models, compared with peritumoral or intratumoral injection. Mice with two bilateral C26 tumors rejected both tumors upon peritumoral injection of one tumor, indicating the development of a systemic immune response. Mice that rejected a tumor upon peritumoral CpG treatment remained tumor free and were protected against rechallenge with the same tumor cells, but not with the other tumor, demonstrating long term memory (121,122). Peritumoral administration of CpG-ODN was also effective in impeding the progression of tumors in BALB/c mice transgenic for the rat/neu transforming oncogene (123).

When CpG-ODN was combined with chemotherapy, it was more effective than chemotherapy alone (116). Mouse tumor models treated with CpG-ODN in combination with fluorouracil, topotecan (topoisomerase I inhibitor) (124), cyclophosphamide (125), or paclitaxel (126) 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
(127).

VACCINES

CpG-ODNs have also been used in vaccination studies as adjuvants, and have induced a good T_{H1}-type immune response (128,129). The efficiency of CpG-ODNs in inducing a T_{H1} based 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 (2,27,130,131). 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 (127). 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 MUCI transgenic mice (132). The immune response caused complete rejection of tumor cells in the prophylactic setting, while in the therapeutic setting, tumor burden was significantly reduced (132). 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 (133). Moreover, the use of CpG-ODN in mice as a vaccine adjuvant allowed to decrease the antigen dose by half, while maintaining the same level of antibody response, when compared with those mice receiving the full dose of antigen without the CpG-ODN adjuvant (134). 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 (135). The activity of CpG-ODN to induce humoral immune responses has also been confirmed in non-human primates and in humans (136-138).

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 (139-141). Agatolimod (CpG 7909) has been used in a phase I trial, to test its efficacy as monotherapy in previously treated NHL patients. Twenty-three patients were treated with 67 three weekly IV infusions of Agatolimod at doses ranging from 0.01 to 0.64

mg/kg/w. 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-1b), 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 previously treated NHL patients, with evidence for immunomodulatory effects primarily in the dosage range of 0.04–0.16 mg/kg (142,143).

A phase I trial was designed to investigate the safety, tolerability, and preliminary antitumor activity of Agatolimod in combination with monoclonal antibody.

A promising treatment of various B-cell lymphomas, as demonstrated in murine models, 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 (144).

Patients with relapsed/refractory $CD20^+$ B-cell NHL received Agatolimod through IV or SC routes, in combination with standard-dose Rituximab (145). 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/m^2/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 (146).

In another phase I trial, 1018 ISS, also this CpG-ODN, 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 (147). 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 $IFN^{-/-}$-inducible genes was observed 24 hours after the injection of 1018 ISS.

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 (148). 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 2'-5' 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 (149). 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 8 mg 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 (149).

MELANOMA

The limitations of immunotherapy for melanoma, like other cancers, arise 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 (150). 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 TLR9 activation with weekly SC administration of Agatolimod in melanoma patients (151,152). 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 (151,152). Clinical examination and laboratory safety assessments including hematology, blood chemistry, and baseline coagulation were performed weekly. 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 (151,152). 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 850 mg/ml 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 (153). CpG-ODNs have been used also in vaccination protocols as an adjuvant. A pilot trial was designed to study the immunogenicity of the analog peptide NY-ESO-1 157-165V, human leukocyte antigen (HLA)-A2 epitope, 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) (154). 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 (154). 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 (155). The enhanced T cell populations consisted primarily of effector memory cells, which in part secreted IFN-α 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 (155). 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 (156). 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 (156).

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 (124,125,157,158). 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 (159). 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 (160). 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 0.2 mg/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) (160), Erlotinib (Tarceva) (161) or Pemetrexed (Alimta) (162). Coley Pharmaceuticals (Wellesley, MA, USA) initiated two randomized, international, multicenter, phase III trials to assess the efficacy and safety of Agatolimod administered in combination with Paclitaxel/Carboplatin or Gemcitabine/Cisplatin chemotherapy as first-line treatment in patients with locally advanced or metastatic Non-Small-Cell Lung Cancer (NSCLC) (164,165). Over 800 patients were enrolled in these trials. These trials were stopped in 2007, after analysis of the phase III clinical trial results showed no evidence of any additional efficacy over standard chemotherapy alone.

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

2. 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 (166). 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 (166). 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 (167,168). Some investigators have suggested they may develop from the secondary Müllerian system, which includes paraovarian and paratubal cysts, the rete ovarii, endosalpingiosis, and endometriosis (169). 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 10**.

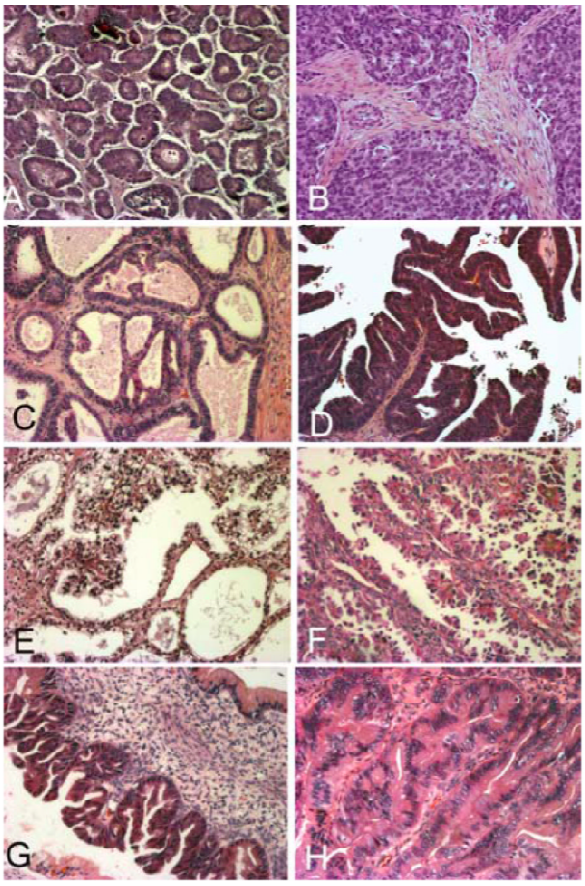

Figure 7. Pictures of the four most common histologic types of ovarian cancer, stained with hematoxylin and eosin. A, Ovarian serous carcinoma showing papillae formation. B, Ovarian serous carcinoma with predominant solid growth pattern. C, Ovarian endometrioid tumor of low malignant potential showing 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 (170). 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 (171,172). 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) (173). 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 (174), and two binary grading systems have been proposed for ovarian serous carcinomas, the most common type (175,176). 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 (177). Tumor grade is an important, albeit not sole factor, distinguishing Type I from Type II tumors.

PHARMACEUTICAL MANAGEMENT OF OVARIAN CANCER

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 (178). During the earliest days of the modern chemotherapeutic era, the newly identified alkylating agents were examined as therapeutic strategies in this malignancy (178,179). 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 (180). 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 (181,182). 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 (180,183). 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) (183-185). 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 (186-189). 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 (190-192). Cisplatin was initially revealed to produce objective responses in women with ovarian cancer, whose disease was shown to be resistant to alkylating agent therapy (191,192). 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 (193-196) 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 (193-197).

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, (198-200) but with several meta-analyses suggesting the superiority of a three-drug regimen of Cyclophosphamide, Doxorubicin and Cisplatin (201-203). 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 (204).

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 (205-209). 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) (207-210). 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 (207-210).

PLATINUM PLUS TAXANE-BASED PRIMARY CHEMOTHERAPY

In the late 1980s, Paclitaxel was demonstrated to be an active agent in platinum-resistant ovarian cancer (211-213). 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 (212). Of interest, similar to the initial experience with Cisplatin in ovarian cancer (188,189), the early experience with Paclitaxel suggested the drug was quite toxic (214,215), and its continued use was justified principally by the level of biological and clinical activity observed (211-213). 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 (211-213), Paclitaxel was quickly combined with Cisplatin in the front-line setting and directly com- pared with the 'standard of care' at this point in time, which was a platinum agent plus Cyclophosphamide (197,216,217). 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 (216,217), this outcome was not observed in all phase III evaluations (197,218). More recent evidence-based data have documented the equivalence of a Cisplatin plus Caclitaxel versus a Carboplatin plus Paclitaxel regimen employed as primary treatment of advanced ovarian cancer (**Table 3**) (207-210).

Table 3 Evidence-based platinum-taxane regimens employed as primary chemotherapy of advanced ovarian cancer

Cisplatin (75 mg/m^2) + paclitaxel (135 mg/m^2 over 24 h) q21d × six cycles[34,40]
Carboplatin (AUC 6–7.5) + paclitaxel (175 mg/m^2 over 3 h) q21d × six cycles[31-34]
Carboplatin (AUC 6) + docetaxel (75 mg/m^2) 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) (207-210,217,219), 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 (220). 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 (221). Key aspects of the biologic behaviour of ovarian cancer lend themselves particularly well to the pharmacology of drugs used to treat this disease (**Table 4**).

Table 4

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 platinums 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 (222,223). In addition, they noted absorption across the peritoneum was sufficient to achieve clinically active systemic concentrations of the drug (223). Lastly, they observed clinical benefit in terms of reduction of tumor bulk and ascites (223). 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 (224). 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/m^2 plus either IP Cisplatin 100 mg/m^2 or IV Cisplatin 100 mg/m^2 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, regard-less 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 (225) 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 (226) 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 (227). 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 o 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. Com- plete 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 cyto-reduction be considered for IP chemotherapy (228). 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. The data confirm biologic and pharmacologic hypotheses. The peritoneal route of spread for ovarian cancer coupled with the pharmacologic advantage for platinums 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 5**).

Table 5

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	.02	.76
		279	Cyclophosphamide 600 mg/m^2 IV + cisplatin 100 mg/m^2 IV q 21 days × 6 cycles	None	NS	NS	NS	41		
Markman et al[5]	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	.01	.78	63.2	.05	.81
		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			52.2		
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	.05	.80	65.6	.03	.75
		210	Paclitaxel 135 mg/m^2 IV over 24 hours day 1; cisplatin 75 mg/m^2 IV day 2	None	18.3			49.7		

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 (229). 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. 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 scepticism 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 (207,230). 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 (230,231). 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 (232). The prevalence of HPV-

related diseases is increased in patients with impaired cell-mediated immunity, including transplant recipients (233) and HIV-infected patients (234,235). Infiltrating $CD4^+$ (T helper cells) and $CD8^+$ (cytotoxic) T cells have been observed in spontaneously regressing warts (236), and warts often disappear in patients who are on immunosuppressive therapy when treatment is discontinued (237). In addition, animals immunized with viral proteins are protected from HPV infection or the development of neoplasia and experience regression of existing lesions (238,239). 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 (240,241). 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 (242), 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 (243,244), while oligoclonal tumor reactive T cells have been isolated from tumors or ascites (245-253). 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 (254-260). Similar observations were made in endometrial cancer (261-263) and other solid tumors (264). 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 (265-269) 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 (270). 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 (271), while tumors grown in immunodeficient mice fail to respond to chemotherapy (272), 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 (273). Furthermore, neoadjuvant taxol therapy was found to increase TIL (274). 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 (275). 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 (**Figure 11**). These mechanisms are quite complex and our understanding are still in its infancy, but effects appear to be dependent on drug type, dose and schedule, as well as the immune cell type.

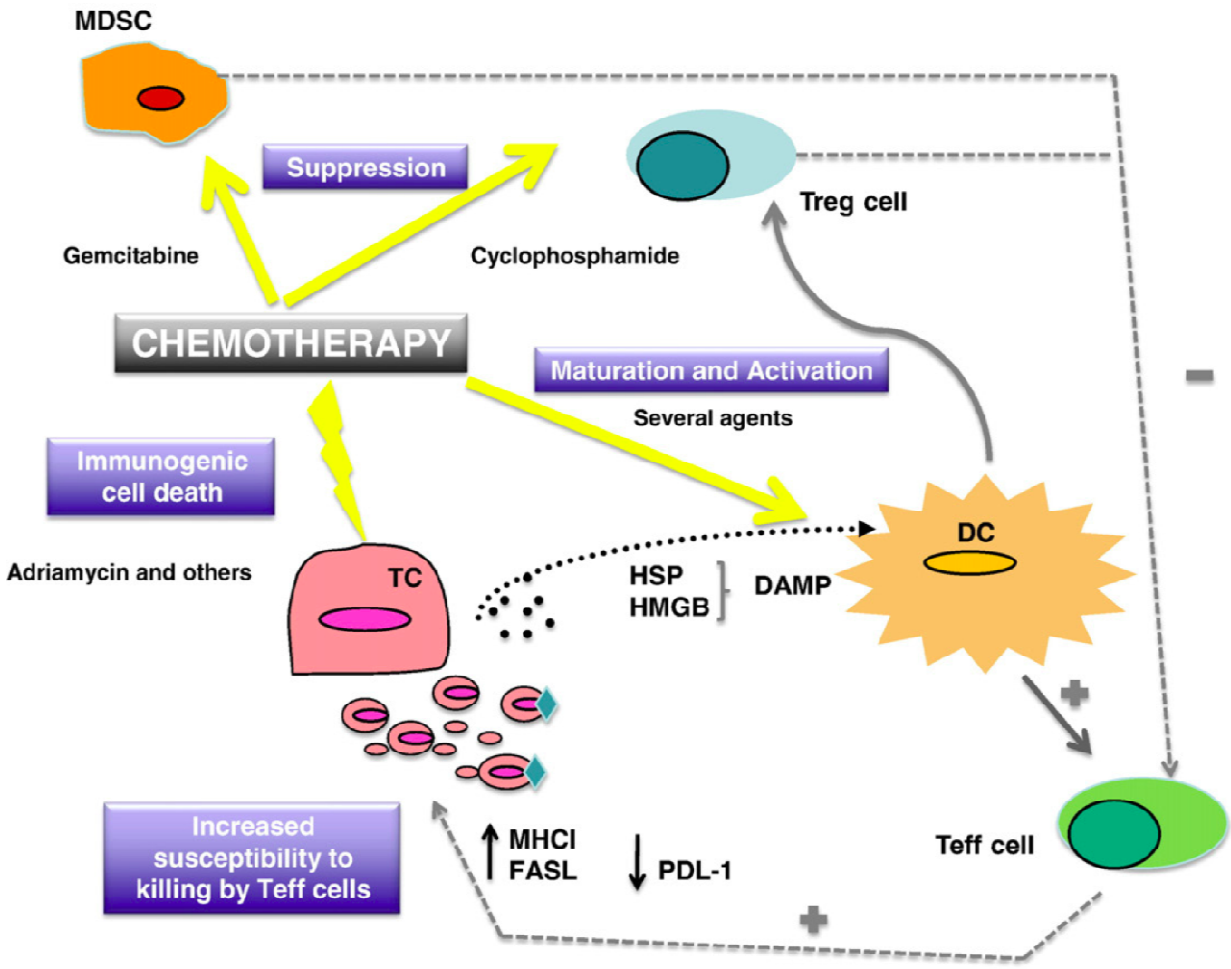

Figure 8. Immunomodulation by chemotherapy (schematic representation)

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 main 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 (276). Many clinical trials have demonstrated the effacacy of type I interferon therapy in the treatment of hematologic malignancies (277-279), melanoma (279-284) and renal cell carcinoma (285-287). 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 (288,289), but there was no significant effect in a cohort of patients with recurrent, platinum-resistant disease (290). 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 (291). 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 (292). Disease stability or objective responses were also observed in patients with malignant pleural mesothelioma enrolled in the study (293). 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 (294-296). In vitro and in vivo, IFN-γ upregulates HLA class I and class II molecules and antigen presentation in ovarian tumor cells (297), a requisite for recognition by T cells. In fact, HLA class I expression by the tumor correlates with the intensity of T cell infiltration (298), a predictor of longer survival. Furthermore, IFN-γ has antiangiogenic effects (299). Encouraging results have been reported with recombinant human (rh)IFN-γ either as intraperitoneal monotherapy or in combinations in early

phase trials (303-305). Theoretically, the effects are likely to be greatest in women who are also receiving chemotherapy because of IFN-γ's non-specific immunomodulatory effects (145). 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 (304). 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 Treg in the setting of antigen presentation.

INTERLEUKINS

Interleukin-2 (IL-2) promotes expansion and enhances the cytotoxicity of effector immune cells (305). 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 (306,307), 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 (308). 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 (309). 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 (310). 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 (311). 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 (312). After IL- 2 cessation, the number of Treg cells more efficiently dropped in patients who experienced a clinical response than in non responders (313). 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 (314). 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 lymphopoeisis but also in T-cell activity and maintenance and can promote antitumor immunity (315,316). 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 (317,318). IL-15 has similar functions to IL-2 in its effects of T cells, but also potentiates NK cell maturation and activity (319). 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 (320). A recent phase II trial demonstrated that administration of IL-21 was associated with antitumor activity in patients with unresectable metastatic melanoma (321). IL-18 is a novel cytokine that has been shown to have very potent immunostimulatory effects, including induction if IFN-□, TNF-□, IL-1□, 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 (322). 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 (323) and humans (324). 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 (324). Toxicity has generally been mild to

moderate even with repeat administration and a maximum tolerated dose has not been reached to date (325). 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 (325). 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 chemo-therapy. In mice with established ovarian carcinoma, administration of IL-18 alone was shown to have modest effects on anti-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). 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 (326), non-Hodgkins lymphoma (142,143), glioblastoma (327), and superficial basal cell carcinoma (328). 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 (262,329,330). 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 (331). 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 (332). 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 (333) TLR4 agonists were shown to promote tumor cell survival, tumor growth and Paclitaxel resistance in a proportion of ovarian cancer cells (334,335).

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 (**Figure 12**).

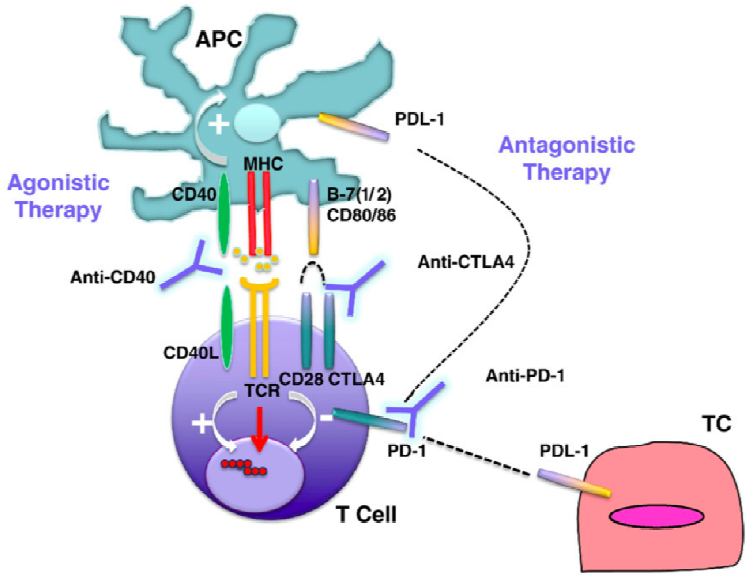

Figure 9. Activation of cellular immunity (schematic representation)

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 (336). Vice versa, a CD40 agonistic antibody was shown to be able to overcome peripheral tolerance and generate antitumor immunity able to reject tumors (337). The main mechanism of immune stimulation by CD40 ligands is activation of DCs resulting in increased survival, up-regulation 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 (338). 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 (339) or accelerate the deletion of tumor-specfic cytotoxic lymphocytes in the absence of antigen vaccination (340). CD40 ligation could thus be best used in combinatorial approaches including vaccines and TLR agonists (338,341). 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 (342). Interestingly, the CD40 receptor is expressed on a variety of tumors including melanoma, lung, bladder and prostate cancers, but also cervix (343) and the majority of ovarian cancers (344-348). 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-kB activation and TNF-α, IL-6 and IL-8 production in most EOC cell lines (344,349). 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 (350). Interestingly, rIFN-g enhanced expression of CD40 on tumor cells and the efficacy of on EOC cell lines (345). 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 (351).

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 (352) 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 (353-356). 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

3. miRNAs: NEW PLAYERS IN CANCER BIOLOGY

After the initial discovery in 1993, when a small RNA encoded by the *lin-4* locus was associated with the developmental timing of the nematode *Caenorhabditis elegans* by modulating the protein lin-14 (357), the miRNA field had undergone a long period of silence. It took several more years to appreciate that these small (19-22 nt) RNA molecules are expressed in several organisms, including *Homo sapiens*, are highly conserved across different species, highly specific for tissue and developmental stage, and that they play crucial functions in the regulation of important processes, such as development, proliferation, differentiation, apoptosis and stress response (**Figure 13**).

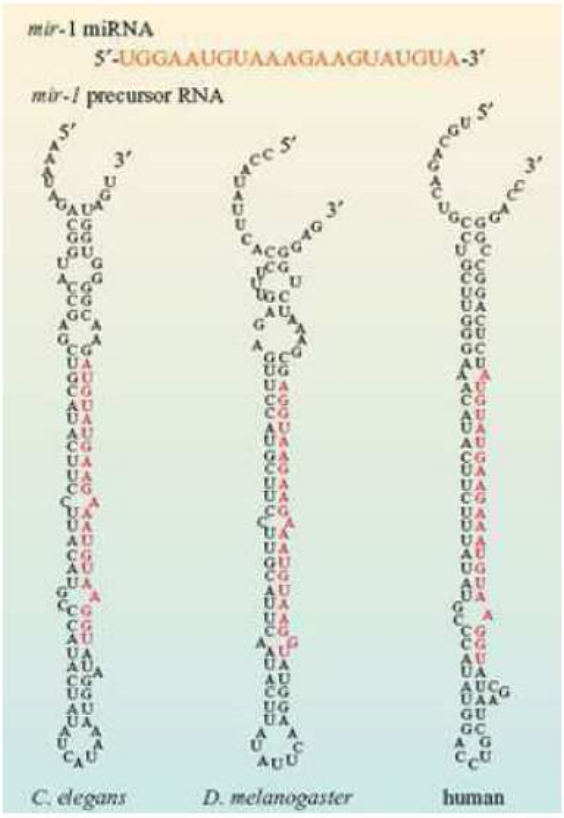

Figure 10. miRNAs are small RNA molecules, about 19-25 nucleotides long, that are highly conserved in the genomes of different species. The mature form (in red) derives from a partially double stranded precursor characterised by a hairpin structure.

In the last few years, miRNAs have taken their place in the complex circuitry of cell biology, revealing themselves as key regulators of gene expression. miRNA genes represent approximately 1% of the genome of different species, and each can bind to and regulate hundreds of different conserved or non-conserved targets: it has been estimated that about 30% of an organism's genes are regulated by at least one miRNA (358).

miRNAs are transcribed for the most part by RNA Polymerase II as long primary transcripts characterised by hairpin structures (pri-miRNAs), which are processed in the nucleus by the RNAse III Drosha into 70-100 nt long pre-miRNAs. These precursor molecules are exported by an Exportin 5-mediated mechanism to the cytoplasm, where an additional step mediated by the RNAse III Dicer generates a dsRNA of approximately 22 nucleotides, named miRNA/miRNA*. The mature single stranded miRNA product is then incorporated into the complex known as miRNP (*miRNA-containing ribonucleoprotein complex*), miRgonaute or miRISC (*miRNA-containing RNA-induced silencing complex*), whereas the other strand is usually subjected to degradation. In this complex, the mature miRNA is able to regulate gene expression at post-transcriptional level, binding through partial sequence complementarily for the most part to the 3'UTR of target mRNAs, and leading at the same time to some degree of mRNA degradation and/or translation inhibition (359*)* (**Figure 14**).

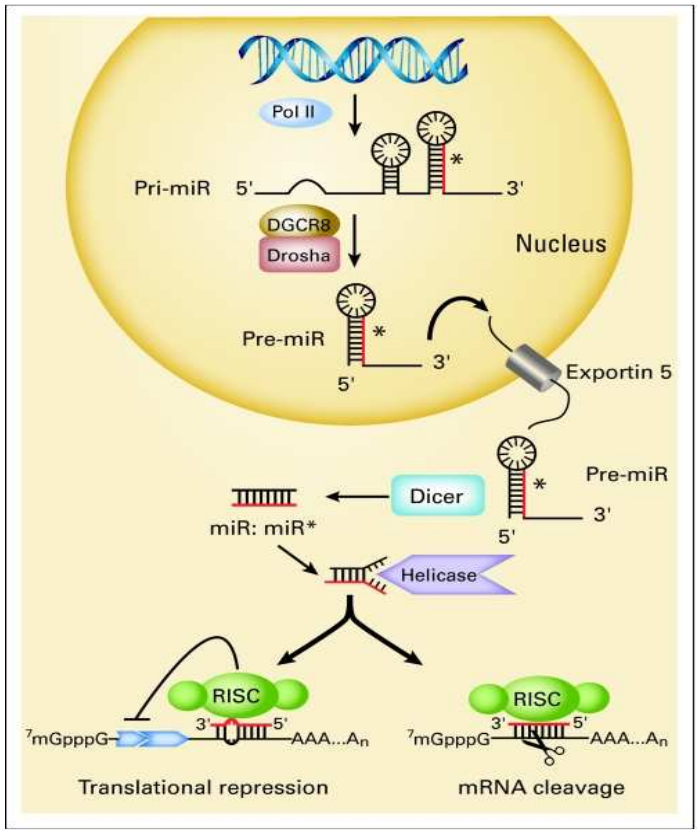

Figure 11. Biogenesis, processing, and maturation of miRNAs. miRNAs are transcribed mainly by RNA polymerase II as long primary transcripts characterised by hairpin structures (primiRNAs) and processed in the nucleus by RNAse III Drosha in a 70-nucleotide-long pre-miRNA. This precursor molecule is exported by the Exportin 5 to the cytoplasm, where RNAse III Dicer generates a dsRNA of approximately 22 nucleotides, named miRNA/miRNA*. The mature miRNA product is then incorporated in the complex known as miRISC, whereas the other strand is usually subjected to degradation. As part of this complex, the mature miRNA is able to regulate gene expression binding through partial homology the 3'UTR of target mRNAs and leading to mRNA degradation in case of perfect matching or translation inhibition when there is partial complementarity. RISC, RNA-induced silencing complex.

Croce at al. identified putative tumour suppressors at chromosome 13q14 that were involved in the pathogenesis of Chronic Lymphocytic Leukaemia (CLL), the most common human leukaemia in the Western world. Deletions of chromosome 13 at band q14 are detected by cytogenetic studies in approximately 50% of CLL, while loss of heterozygosity (LOH) studies identified deletions at 13q14 in approximately 70% of CLLs. They took advantage of chromosome translocations and small deletions to define a minimal critical region of deletion. However, they found that this critical region on 13q14 does not contain the expected protein coding tumour suppressor gene. Instead two non-protein coding miRNA genes, *miR-15a* and *miR-16-1*, that are expressed in the same polycistronic RNA were detected. This result indicated that the deletion of chromosome 13q14 caused the loss of these two miRNAs, providing the first evidence that miRNAs could be involved in the pathogenesis of human cancer (360). Study of a large series of primary CLL showed knock down or knock out of *miR-15a* and *miR-16-1* in approximately 69% of CLL. Since such alteration is present in most indolent CLL, they speculated that loss of *miR-15a* and *miR-16-1* could be the initiating or a very early event in the pathogenesis of the indolent form of this disease (360). Immediately after these initial observations, Croce et al. mapped all the known miRNA genes and found that many of them are located in regions of the genome involved in chromosomal alterations, such as deletion or amplification, in many different human tumors. In several instances the presumed tumor suppressor genes or oncogenes, respectively, had failed to be discovered after many years of investigation (361). A rapidly increasing body of experimental evidence has subsequently demonstrated that this was not just a random association, but that miRNAs can have a causal role in tumourigenesis, acting as oncogenes or tumor suppressor genes depending on the target molecules they regulate and on the cellular context (**Figure 15**).

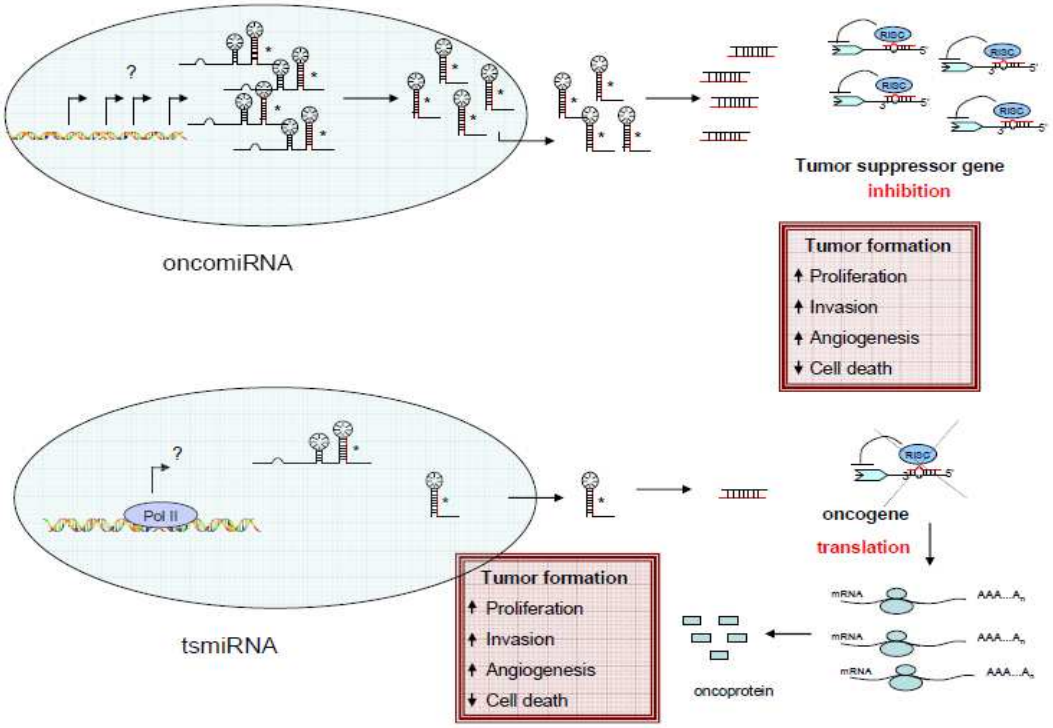

Figure 12. miRNAs as oncogenes or tumour suppressor genes. miRNAs can have oncogenic effects (oncomiRNA) when they target tumour suppressor genes. When an oncomiRNA is overexpressed, for example because the encoding gene is located in an amplified region of the genome, this will lead to down-regulation of the targets and to tumour formation (upper panel). Conversely, a miRNA can be characterised by tumour suppressor properties if the main target in that specific cellular context is an oncogene; in this case, if the miRNA expression is lost, for example because the encoding gene is located in a deleted region of the genome, the resulting effect will be tumourigenic (lower panel). In summary, what usually happens in a tumour is the overexpression of an oncogenic miRNA, and/or the loss of a miRNA with oncosuppressive properties.

Examples of tumor suppressor miRNAs are *miR-15* and *miR-16-1* or *let*-7a, able to target respectively oncogenes as BCL2 (362) and RAS (363), HMGA2 (363,364) and MYC itself (365), whereas a well known oncomiRNA is *miR-21*, overexpressed in several human tumors and able to induce proliferation, invasion and metastasis by repressing the expression of oncosuppressor molecules, as TPM1 (366) and PDCD4 (367). Alterations in miRNAs expression are not isolated but rather appear to be the rule in human cancer. After the early studies indicating the role of miRNA genes in the pathogenesis of human cancer, different platforms have been developed to assess the global expression of miRNA genes in normal and diseased tissues, and profiling studies have been carried out to assess miRNA dysregulation in human cancer. This was an attempt to establish whether miRNA profiling could be used for tumor classification, diagnosis and prognosis.

miRNA PROFILING IN CANCER DIAGNOSIS AND PROGNOSIS

Profiling of different cell types and tissues indicated that the pattern of miRNA expression is cell type and tissue specific, suggesting that the program regulating expression of miRNAs is exquisitely cell type dependent, and tightly associated with cellular differentiation and development. Some of the most important miRNAs which are aberrantly expressed in tumors are listed in **Table 6**.

Table 6

Tumour type	up-regulated miRNA	down-regulated miRNA	target
CLL		*miR-29, miR-181*	TCL1
	miR-155		
		miR-15a, miR-16-1	BCL2
AML		*miR-29*	MCL1
			DNMT
Lymphoma	*miR-155*		
	miR-17-92		PTEN, BIM,E2F1
	miR-106b-25		E2F1
MM	*miR-21*		
	miR-19a, miR-19b		SOCS1
Breast Cancer	*miR-21*		PTEN, PDCD4, TPM1
		miR-125b	HER2, HER3
		miR-205	HER3
		miR-10b (associated with metastasis)	HOXD10
	miR-373		
		miR-200	ZEB
Lung Cancer		*let-7*	RAS, HMGA2, C-MYC
	miR-155		
HCC		*miR-122a*	Cyclin G1
	miR-221		p27
		miR-34a	MET

Table 6. miRNAs aberrantly expressed in tumours. CLL, chronic lymphocytic leukemia; AML, acute myelocytic leukemia; MM, multiple myeloma; HCC, hepatocellular carcinoma.

The possible use of miRNA as biomarkers of diagnosis and prognosis is also strengthened by the relative stability of these small molecules, which enables them to be extracted and visualized not only from fresh or frozen samples, but also from archival paraffin embedded tissues (368), for which larger numbers of samples from diagnostic archives and more clinic-pathological follow up data are generally available. Moreover, it has been recently shown that miRNAs can also be detected in biological fluids, as blood (369,370,371), or in circulating exosomes (372). Notably, the expression profile of circulating miRNAs from cancer patients in comparison with healthy subjects often reflects the pattern observed in the tumor versus normal tissue (370). These reports provide

evidence of a possible future use of circulating miRNAs as useful biomarkers for less invasive diagnostics.

miRNA IN OVARIAN CANCER

The first report of a putative involvement of miRNAs in the biology of human ovarian cancer was the genomic study performed by Zhang *et al*., who used an array comparative genomic hybridisation (aCGH) approach to identify miRNA loci gained/lost in ovarian cancer, breast cancer and melanoma (373). After this initial evidence, several groups have investigated the role of miRNAs in the pathogenesis of ovarian cancer, either as biomarkers, potential research tools or targets for specific therapies. miRNA *let-7i* was recently found to be a tumor suppressor significantly down-regulated in platinum resistant ovarian tumors, and *let-7i* gain-of-function restored drug sensitivity of chemoresistant ovarian cancer cells, thus representing a candidate biomarker and therapeutic target (374). An oncosuppressive role for *miR-15/-16* has been described also in ovarian cancer, where these two miRNA regulate the expression of the oncogenic protein Bmi1 (375). In another study, 27 miRNAs significantly associated with chemotherapy response, showing that (similar to DNA methylation) miRNAs represent possible prognostic and diagnostic biomarkers for ovarian cancer (376). *miR-214* has been reported to target PTEN thus contributing to cisplatin resistance (377). Interestingly, levels of *Dicer* and *Drosha* mRNA in ovarian-cancer cells have been recently associated with outcomes in patients with ovarian cancer (378).

miRNAs/ANTI-miRNAs IN CANCER TREATMENT

The evidence collected to demonstrate that miRNAs may represent valid diagnostic, prognostic and predictive markers in cancer. Indeed, aberrant miRNA expression correlates with specific bio-pathological features, disease outcome and response to specific therapies in different tumor types. Considering the importance of miRNAs in development, progression and treatment of cancer, the potential usefulness of a miRNA-based therapy in cancer is now being exploited, with the attempt to modulate their expression, reintroducing miRNAs lost in cancer, or inhibiting oncogenic miRNAs by using anti-miR oligonucleotides (**Figure 16**).

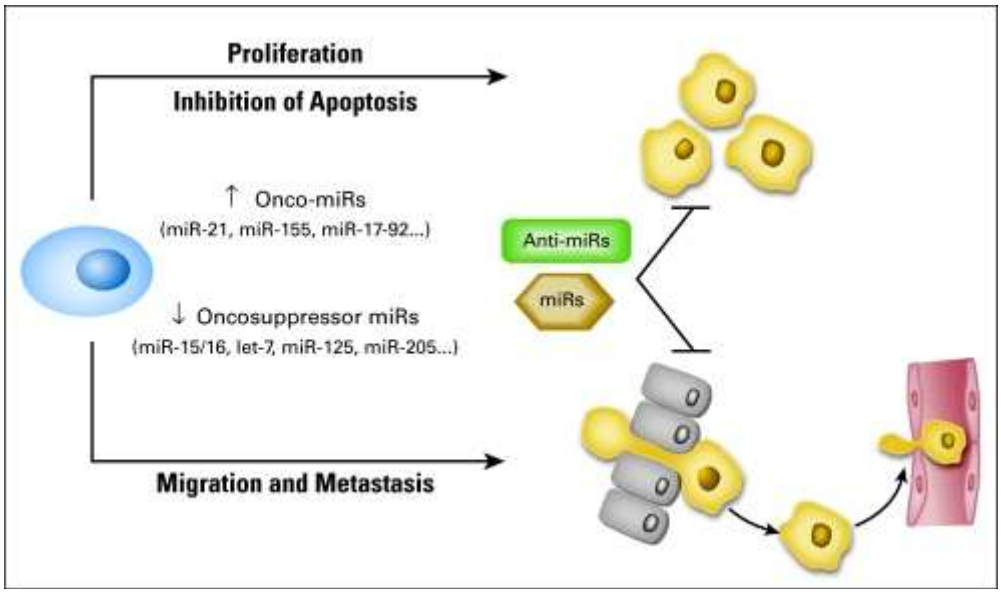

Figure 13. miRNAs as therapeutic tools. The reintroduction by transfection of synthetic miRNAs lost during cancer development or progression or the inhibition of oncogenic miRs by using anti-miRNA oligonucleotides could help counteract tumour proliferation, extended survival, and the acquisition of a metastatic potential, thus representing potential therapeutic tools.

For example, transfection of *miR-15a/16-1* induces apoptosis in leukaemic MEG01 cells and inhibits tumour growth *in vivo* in a xenograft model (379), while the inhibition of *miR-21* with antisense oligonucleotides generates a pro-apoptotic and antiproliferative response *in vitro* in different cellular models, and reduces tumor development and metastatic potential *in vivo* (380).
Moreover, miRNAs involved in specific networks, as the apoptotic, proliferation or receptor-driven pathways, could likely influence the response to targeted therapies or to chemotherapy: inhibition of *miR-21* and *miR-200b* enhances sensitivity to gemcitabine in cholangiocytes, probably by modulation of CLOCK, PTEN and PTPN12 (381). Beside targeted therapies and chemotherapy, miRNAs could also alter the sensitivity to radiotherapy, as recently reported by Slack's group (382): in lung cancer cells, *let-7* family of miRNAs can suppress the resistance to anticancer radiation therapy, probably through RAS regulation. Evidence described to date represents the experimental basis for the use of miRNAs as both targets and tools in anti-cancer therapy, but there are at least two primary issues to address to translate these fundamental research advances into medical practice: the development of engineered animal models to study cancer-associated miRNAs, and the improvement of the efficiency of miRNA/anti-miRNA delivery *in vivo*. Towards this aim, modified miRNA molecules with longer half-lives and efficiency have been developed, such as anti-miR oligonucleotides (AMOs) (383), locked nucleic acid (LNA)-modified oligonucleotides (384), and cholesterol-conjugated antagomirs (385). Interestingly, Ebert and colleagues (386) have recently described a new approach to inhibit miRNAs function: synthetic

mRNAs containing multiple binding site for a specific miRNA, called miRNA sponges, are able to mop up the miRNA preventing its association with endogenous targets.

To improve the *in vivo* delivery of either miRNAs or anti-miRNAs, the methods that have been tested in pre-clinical studies over the last decades for short-interfering RNAs (siRNA) or short heteroduplex RNA (shRNA) (387) could be applied also to miRNAs. Moreover, the advantage of miRNAs over siRNA/shRNA is their ability to affect multiple targets with a single hit, thus regulating a whole network of interacting molecules.

4. PREVIOUS RESULTS

The group where I conducted the thesis showed that intraperitoneally IGROV-1 tumor-bearing athymic mice treated weekly i.p. with CpG-ODN showed a significantly increased life span as compared to controls; however, all mice died with ascites (388). 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 μg/mouse) or saline, sacrificed in groups of 3 at different time points, and ascetic fluid removed for volume quantization 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 (**Figure 17A**). 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 (**Figure 17B**, P<0.001).

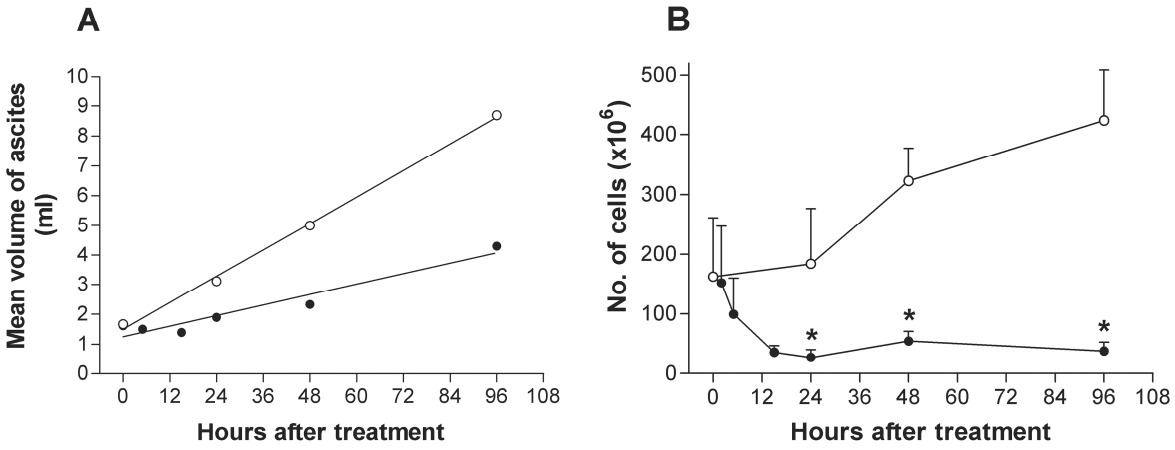

Figure 14. Effect of CpG-ODN treatment on ascites volume and cellularity. Ascites-bearing mice, treated i.p. with CpG-ODN or saline, were sacrificed at selected time points (3-6 mice/point), and ascitic fluid and cells collected. A) Ascites volume, plotted based on linear regression analysis and "best-fit" linear regression. Slopes of curves were compared using an unpaired *t*-test (p< 0.001); B) Number of live cells (mean ± SD) (* P<0.001); Open symbols: control mice; closed symbols: CpG-ODN-treated mice.

It was also recently 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) (**Figure 18A**). 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) (**Figure 18B**).

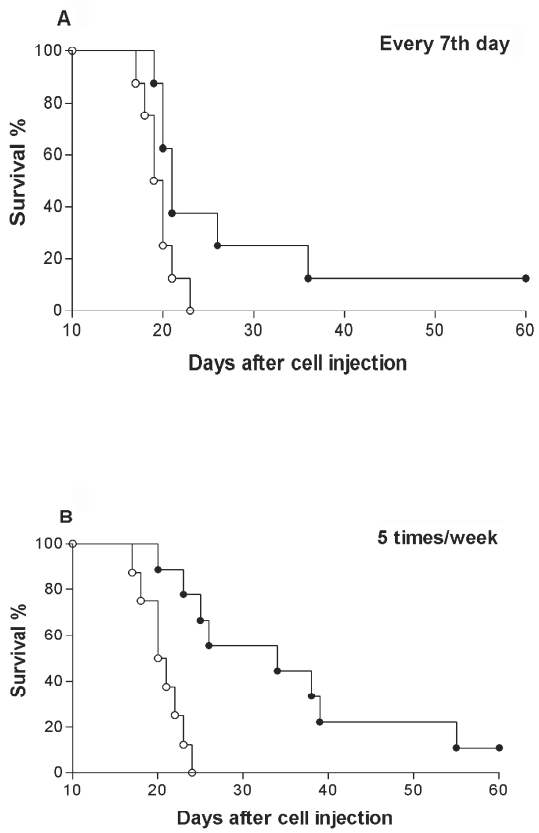

Figure 15. Effect of CpG-ODN treatment on survival and body weight. Kaplan-Meier plot of the percentage of survivors over time among IGROV-1 ascites-bearing mice treated with CpG-ODN (20 μg/mouse) or saline for 4 week severy 7th day (A) or 5 times/week (B). Open symbols: control mice; closed symbols: CpG-ODN-treated mice.

In light of a reported direct effect of CpG-ODN on tumor cell death (389), IGROV-1 cells were tested 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 pro-inflammatory 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 (390), and blocking of VEGF has been reported to strongly reduce ascites volume (391).
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 (**Figure 19**).

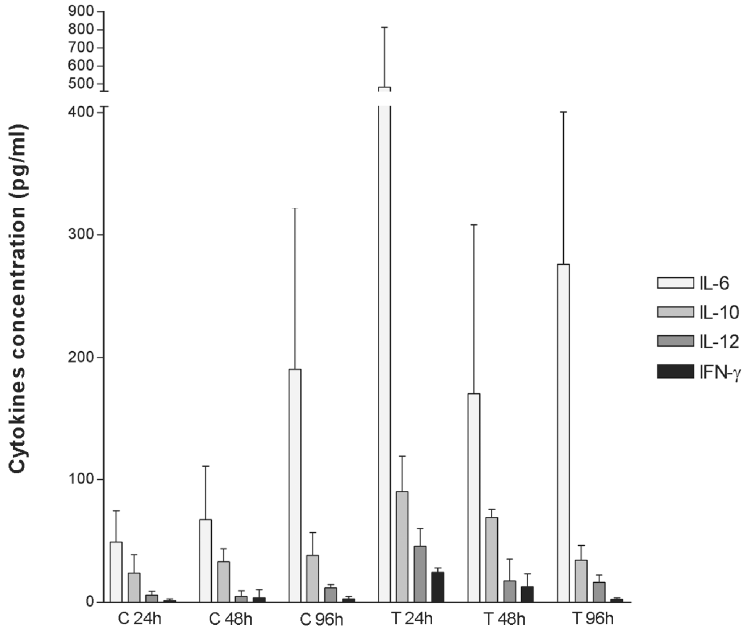

Figure 16. Effect of CpG-ODN treatments on ascitic fluid concentrations of angiogenic factors and cytokines Ascites-bearing mice were treated i.p. with CpG-ODN (T) or saline (C). At selected time points, CpG-ODN- and saline-treated mice (3 mice/point) were sacrificed, ascites fluids were recovered, and cytokine and angiogenic factor concentrations were determined by Bio-Plex assay.

CpG-ODN and DNA REPAIR

It was recently demonstrated 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.

In silico analysis were conducted on tumor and immune cells from mice treated or not i.p. with CpG-ODN.

In immune spleen cells from a list of 209 genes retrieved according to the "DNA repair" term from GeneOntology (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 20**).

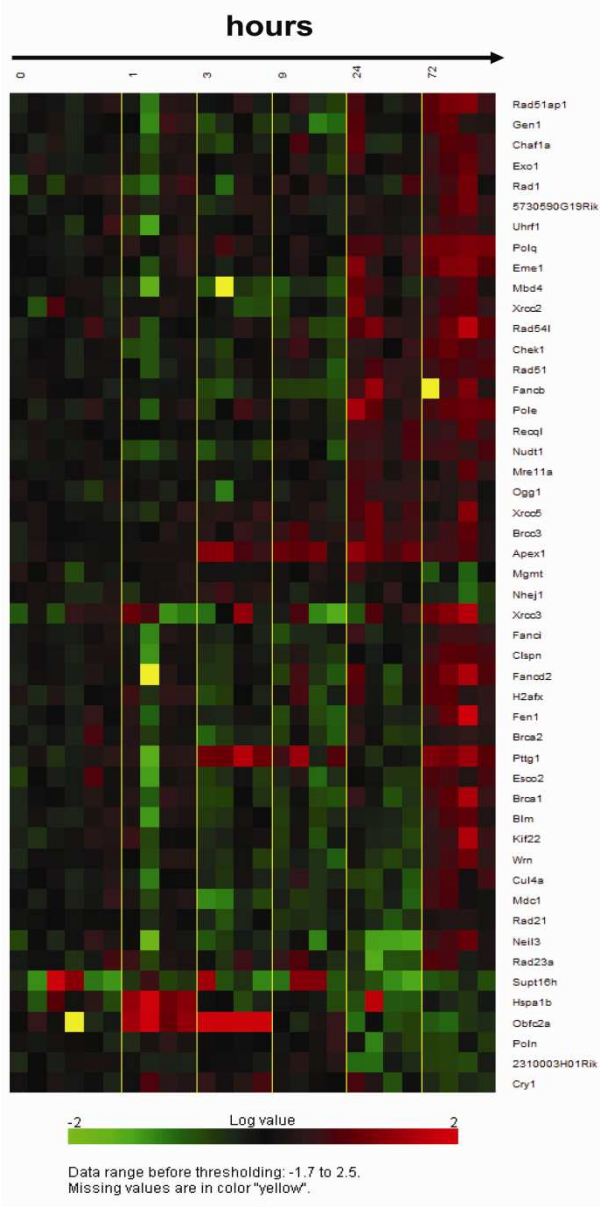

Figure 17. Microarray analysis of DNA repair pathway genes in spleen cells from CpG-ODN-treated mice. Mice were treated i.p. with CpG-ODN. RNA was extracted from spleen cells 1, 3, 9, 24 and 72 h after treatment and analyzed in-house-assembled oligonucleotide microarray platform. Of 209 genes involved 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 initialdownregulation, increased expression, and final downregulation, respectively. Each column represents a sample and each row, a gene.

In human IGROV-1 ovarian carcinoma cells 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**).

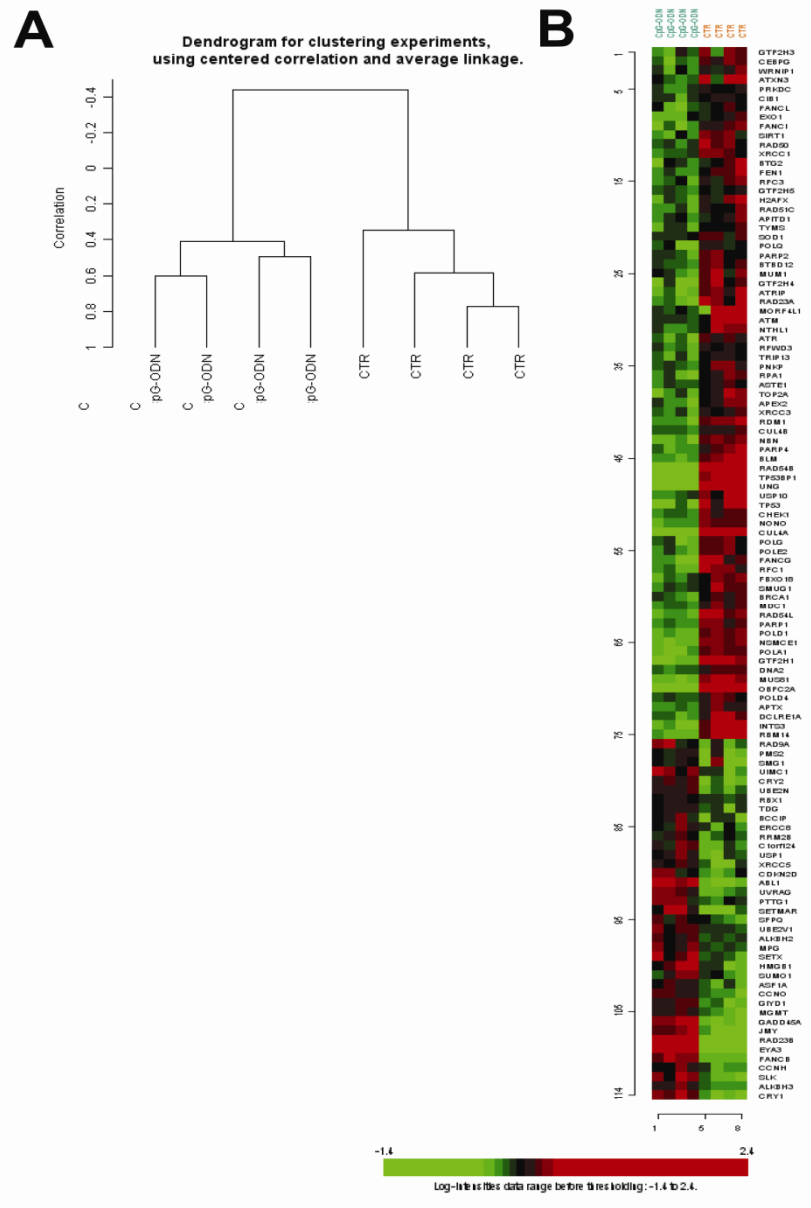

Figure 18. Microarray analysis of DNA repair pathway genes in IGROV-1 ovarian tumors from CpG-ODN-treated athymic mice. IGROV-1-bearing mice with established ascites, i.e., increased abdominal volume and body weight, were treated i.p. daily for 3 days with CpG-ODN or saline (control group) and sacrificed 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-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.

Thus, microarray analyses indicate that locally administered TLR9 agonists regulate DNA repair genes 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 (392), and SIRT1, whose activity promotes homologous recombination (393). RAD51 and SIRT1 proteins decreased their expression in treated mice as compared to controls (**Figure 22**).

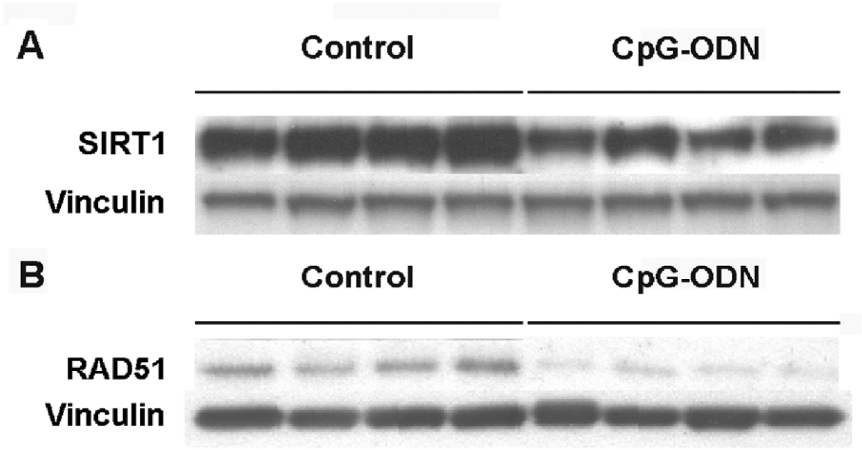

Figure 19. Western blot analysis of DNA repair proteins in IGROV-1 tumor cells adhering to the peritoneal wall after i.p. injection of CpG-ODN. Protein expression level of SIRT-1 (A), Rad51 (B) in IGROV-1 ovarian cancer cells from athymic mice treated daily for 3 days with CpG-ODN or saline (4 mice/group). Vinculin was used to normalize protein loading per lane.

To evaluate whether CpG-ODN-induced DNA repair gene modulations, observed in IGROV-1 microarray analysis, were relevant to increase the cell sensitivity to DNA damages, among the genes found differentially modulated between the CpG-ODN-treated and untreated IGROV-1 tumors, a set of 27 gene with a level of FDR less than 0.01 and a fold difference of more than 1.5 was selected (**Figure 23**) and the average expression of both CpG-ODN-treated and untreated tumors was calculated for each gene.

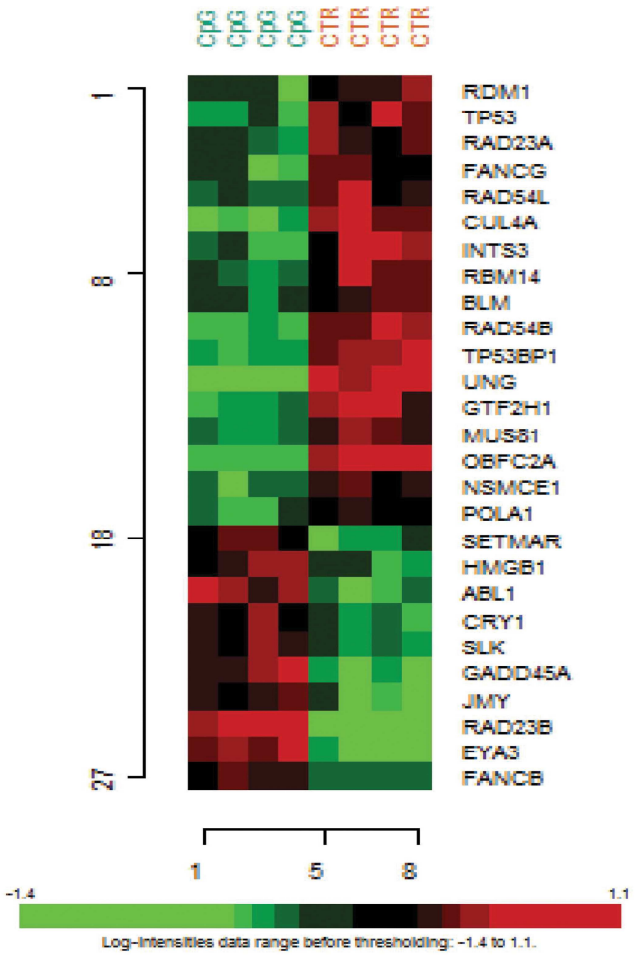

Figure 23. Heat-map of the 27 modulated genes in the DNA repair pathway obtained in IGROV-1 ovarian tumors from CpG-ODN-treated athymic mice.

The effect of CpG-ODN treatment on the antitumor activity of Cisplatin was evaluated to test for correlation between DNA repair gene down-modulation and sensitivity to DNA-damage-inducing drugs.

IGROV-1 ovarian tumor–bearing athymic mice were used in these experiments, as IGROV-1 cells are sensitive to Cisplatin (394) and as CpG-ODN in this model has been shown to prolong survival of mice with bulky disease, inducing an activation of different effector cells and cytokines of innate immunity at the site of tumor growth (388, 395). Mice were treated i.p. with CpG-ODN, Cisplatin, or both 8 days after tumor cell injection, when ascites start to form. Analysis of the effect of the combined treatment revealed a significant ($P < 0.0001$) increase in life span compared with the use of either reagent alone (**Figure 24**).

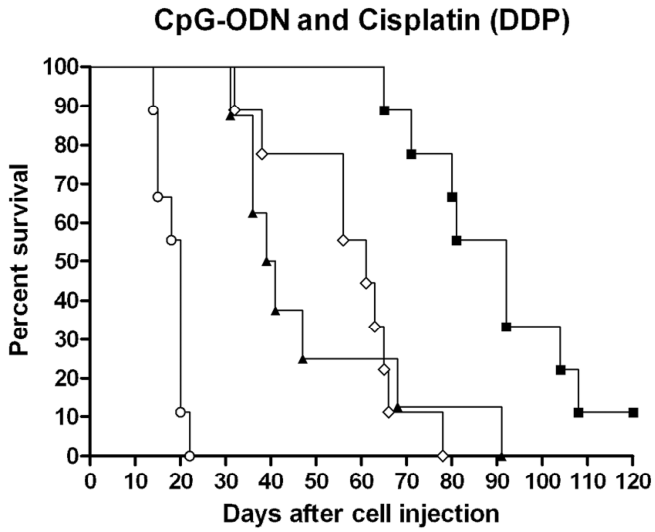

Figure 24. Kaplan-Meier plot of percent survivors over time among IGROV-1 ovarian tumor-bearing athymic mice. Mice were treated i.p., starting from 8 days after tumor cell injection, with CpG-ODN (20 μg/mouse, 5 days/week for 4 weeks), cis-platinum (DDP, 3 mg/Kg i.p., once per week for 4 weeks) or both. Control mice received saline. Saline-treated mice (○); CpG-ODN-treated mice (◊), cisplatin-treated mice (▲); CpG-ODN plus cisplatin-treated mice (■). Experimental groups consisted of 8-10 mice group.

It should be noted that the modulation of DNA repair genes in human ovarian IGROV-1 tumors and the increase in the antitumor effect of Cisplatin and CpG-ODN against IGROV-1 human ovarian tumor xenografts in mice were observed in mice injected with a CpG-ODN sequence specific for murine TLR9, making unlikely the possibility that the modulation was related to a direct interaction of CpG-ODN with tumor cells, as different DNA motifs are required for stimulation of mouse and human cells by CpG-ODN (76, 396).

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.

These findings provide the first evidence that the tumor microenvironment can sensitize cancer cells to DNA-damaging chemotherapy, thereby expanding the benefits of CpG-ODN therapy beyond induction of a strong immune response, underscoring the need for further investigation of the mechanisms and of the synergistic effect of CpG-ODN in combination with DNA-damaging drugs in cancer treatment.

MATERIALS AND METHODS

CELLS

Human IGROV-1 ovarian tumor cells (gift from Dr. J. Benard, Institute Gustave Roussy, Villejuif, France) were adapted to growth i.p. and maintained by serial i.p. passage of ascitic cells into healthy mice as described (397,398). Every 6 months, cells were authenticated by morphologic inspection and by FACS analysis for the presence of specific markers. For in vitro experiments, IGROV-1 cells were maintained in RPMI medium 1640 supplemented with 10% FCS (Sigma) and 2 mM glutamine (Cambrex, East Rutherford, NJ, USA) at 37uC in a 5% CO2 air atmosphere. Mouse leukemic monocyte/macrophage RAW 264.7 cells (American Type Culture Collection) were cultured in DMEM (Sigma) supplemented with 10% FCS (Sigma) and 2 mM glutamine (Cambrex).

MICE

Eight- to 12-week-old female Swiss nude (athymic) mice (Charles River, Calco, Italy) were maintained in laminarflow 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.

DRUGS AND ANTIBODIES

Purified phosphorothioated ODN1826 (59-TCCATGACGTTCCTGACGTT-39) containing CpG motifs was synthesized by TriLink Biotechnologies (San Diego, CA, USA). Phosphorothioate modification was used to reduce susceptibility of the ODN to DNase digestion, thereby significantly prolonging its in vivo half-life. Cisplatin was purchased from Teva Italia (Milan, Italy). Anti-HDAC4 (D15C3), anti-p21 (sc-397) and anti-GAPDH (GAPDH-71.1) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA), Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Sigma (St. Louis, MO, USA), respectively.

The following drugs were used: Bevacizumab (Roche, Basel, Switzerland); Poly(I)Poly(C) (Amersham Biosciences, Piscataway, NJ, USA); Cetuximab (ErbituxW, Merck Serono, Darmstadt, Germany); Gefitinib (LC Laboratories, Woburn, MA, USA). Lyophilized ODN1826 and Poly(I):Poly(C) were dissolved in sterile water at a concentration of 10 mg/ml and 2 mg/ml, respectively, and stored at −20°C until use. Gefitinib was dissolved in DMSO (10% v/v final

concentration) and diluted in carboxymethylcellulose (0.25% w/v) to a final concentration of 10 mg/ml. Bevacizumab, Cetuximab and Cisplatin (purchased in their commercial formulation) were diluted in 200 μl of sterile saline at the indicated concentrations just before administration.

miRNA EXTRACTION FROM TUMOR SAMPLES

miRNAs were extracted from the IGROV-1 xenograft tumors used for gene expression analysis or from a replica of the in vivo experiment (397). Briefly, solid i.p. masses were mechanically disrupted and homogenized in the presence of QIAzol Lysis reagent (Qiagen, Valencia, CA, USA) using a Mikrodismembrator (Braun Biotech International, Melsungen, Germany). RNA was extracted using the miRNeasy Mini kit (Qiagen) according to the manufacturer's instructions. RNA concentrations were measured with the NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), while RNA quality was assessed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA USA) using the RNA 6000 Nano kit (Agilent). Samples included in the present analysis had a RIN (RNA Integrity Number) score > 7 and a 28S:18S rRNA ratio ~ 2:1.

miRNA EXPRESSION PROFILING

Mature miRNAs were detected with the Illumina Human_v2 MicroRNA expression profiling kit, based on the DASL (cDNAmediated Annealing, Selection, Extension, and Ligation) assay, according to the manufacturer's instructions (Illumina Inc., San Diego, CA, USA). Briefly, 600 ng/sample total RNA was converted to cDNA followed by annealing of a miRNA-specific oligonucleotide pool consisting of: i) a universal PCR priming site at the 59 end; ii) an address sequence complementary to a capture sequence on the BeadArray; and iii) a miRNA-specific sequence at the 39 end. After PCR amplification and fluorescent labeling, probes were hybridized on Illumina miRNA BeadChips, washed, and fluorescent signals were detected by the Illumina BeadArrayTM Reader. Data were collected using BeadStudio V3.0 software. Raw and normalized data are available on the Gene Expression Omnibus website with accession numbers GSE41783 and GSE23441 for miRNA and gene expression profiling, respectively.

REAL-TIME QUANTITATIVE PCR (RT-qPCR)

RT-qPCR microRNA assays specific for hsa-miR-18a, hsamiR- 18b, hsa-miR-140-5p, hsa-miR-101, hsa-miR-556-3p, hsamiR-424, hsa-miR-136, hsa-miR-340, hsa-miR-302b were purchased from Exiqon (Vedbaek, Denmark). RT-qPCR was performed using the miRCURY LNA Universal RT microRNA PCR system (Exiqon) according to the manufacturer's instructions. Total RNA (20 ng) was polyadenylated and reverse-transcribed at 42uC (60 min), followed by heat-inactivation at 85uC (5 min) using a poly-T primer containing a 59 universal tag. The resulting cDNA was diluted 80-fold and 8 ml used in 20-ml PCR amplification reactions at 95uC for 10 min, 40 cycles of 95uC for 10 sec, and 60uC for 60 sec. Results were normalized with snord48 (Assay ID:203903). P-values were calculated using two-tailed Student's t-test.

BIOINFORMATICS ANALYSIS

Analyses were performed using BRB-Array Tools v4.0 stable release developed by Dr. Richard Simon (NCI) and the BRBArray Tools development team (EMMES Corp.) and the R package (http://www.bioconductor.org/). The same data-processing was used in both miRNA and gene expression profiling to improve data integration. Quantile normalization was used to correct experimental distortions. A detection threshold of p,0.05 was set for each gene and miRNA. Probes detected in less than 50% of the samples were eliminated from the analysis. Genes and miRNAs differentially expressed were identified using a randomvariance t-test, which allows computation of a t-test statistic for each detected miRNA and genes between the classes of samples under investigation without assuming that all miRNAs have the same variance (399). To limit the number of false-positive findings, miRNAs and genes were considered statistically significant at a false-discovery rate (FDR) < 0.1. To identify the most likely targets, mRNA and miRNA expression data were integrated using the MAGIA web tool (400). A parametric linear correlation measure (Pearson's correlation coefficient, recommended for normally distributed data and a sample size > 5) was used to assess the degree of anti-correlation between miRNA and gene expression data.

IN SILICO BIOINFORMATICS ANALYSES

Two publicly available datasets GSE27290 (401) and GSE25204 (402) reporting miRNA expression and clinical annotated data were downloaded from the Gene Expression Omnibus (GEO) database. The former dataset consists of 62 diagnosed patients with stage III or IV serous ovarian cancer profiled on a precommercial version of miRNA chips (GPL7341) designed on miRBase 9.1. Raw array data were processed using GeneSpring software (Agilent) and quantile-normalized. The latter dataset reports profiling of 85 stage III or IV epithelial ovarian cancers, divided into a training set (55 cases) and test set (30 cases), profiled with Illumina human_v2 MicroRNA chips. Raw data were processed and quantile-normalized using BeadStudio V3.0 software. Non-biological experimental variations between training and test sets were adjusted using ComBat (403).

miRNA TRANSFECTION AND CELL VIABILITY ANALYSIS

IGROV-1 cells seeded in 6-well plates at 26105 cells/well were transfected with miRCURY LNA inhibitors of hsa-miR-424 or hsa-miR-340 or negative control A (Exiqon; final concentration, 100 nmol/L) using SiPort Neo-FX (Ambion) according to the manufacturer's instructions, or with hsa-miR-302b precursor or negative control #1 pre-miR (Ambion; final concentration, 50 nmol/l). Transfections were verified by qRT-PCR as described above. Cell viability after cisplatin treatment was assessed by propidium iodide staining and flow cytometry (397).

CELL GROWTH ASSAY

IGROV-1 cells were transfected with 50 nmol/l pre-hsa-miR-302b or scrambled oligonucleotide using SiPort Neo-FX transfection reagent according to the manufacturer's protocol (Ambion) and seeded in a 96-well plate at a density of 10^3, $1.5x10^3$, and $2x10^3$ cells/well. After 72 h of culture, cells were fixed with 10% trichloroacetic acid for 1 h at 4uC, washed 5 times with distilled and de-ionized water, air-dried, and incubated with 100 ml sulforodamine (SRB) 0.4% (w/v) for 30 min. Cells were then washed 4 times with 1% acetic acid, air-dried, and 10 mM Tris solution (pH 10.5) added to dissolve the bound dye. Cell growth was assessed based on optical density (OD) at 550 nm using an ELISA microplate reader (Bio-Rad Lab, Inc., Hercules, CA, USA).

IMMUNOBLOTTING

Transfected cells 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 4uC. Insoluble material was removed by 10-min centrifugation at 15,500 6 g at 4uC. Protein concentrations were determined using the Coomassie technique. Equal amounts of total lysates (20 mg) were loaded and separated on 10% precast NuPage SDS Bis-Tris gels (Invitrogen) and transferred to PVDF membranes (Millipore, Billerica, MA, USA). 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.

PLASMID CONSTRUCTION

For luciferase reporter experiments, a 1017-bp region of the HDAC4 39 untranslated region including the binding site for hsamiR- 302b was amplified from IGROV-1 cells. The PCR product was digested with XbaI and cloned into the reporter plasmid pGL3 control (Promega, Madison, WI, USA) downstream of the luciferase gene. Mutations into the hsa-miR-302b binding site of the HDAC4-39UTR were introduced using Quik-Change II Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA).

Primers for plasmid construction were:

HDAC4-wt-Fw: 5'-AATTTCTAGAGGGGGACTTAATTCTAATCTCATT-3'.

HDAC4-wt-Rw: 5'-AATTTCTAGATTTTGTGTCAGACCATTACGAA-3'.

HDAC4-Mut-Fw:5'GCACTGGCTGGGAGTCAGCAAGCGCCGCGGGTATATCCCTTTGACGGAAACCCTG-3'.

HDAC4-Mut-Rw: 5'-CAGGGTTTCCCTCAAAGGGATATACCCGCGGCGCTTGCTGACTCCCAGCCAGTGC-3'.

LUCIFERASE ASSAYS FOR TARGET AND PROMOTER IDENTIFICATION

pGL3 reporter vector (200 ng) containing the hsa-miR-302b binding site, 40 ng of the phRL-SV40 control vector (Promega), and 50 nmol/l miRNA precursors or scrambled sequence miRNA control (Ambion Inc, Austin, TX. USA) were co-transfected into IGROV 1 cells in 48-well plates. Cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Firefly luciferase activity was measured with a Dual Luciferase Assay Kit (Promega) 48 h after transfection and normalized with a Renilla luciferase reference plasmid. Reporter assays were carried out in quadruplicate. Data (mean6S.E.M.) were analyzed using unpaired Student's t-test.

ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY (ADCC) ASSAY

IGROV-1 cells were treated or not (controls) with Cetuximab (5 μg/ml for 72 h) and labeled with 100 μCi ^{51}Cr (PerkinElmer, Waltham, Massachusetts) for 1 h at 37°C. After 3 washes with PBS-5% FCS, cells were co-incubated for 4 h at 37°C with PBMC from 12 healthy donors (effector:target ratio 50:1) in 200 μl RPMI 1640 complete medium in triplicate 96-well U-bottomed plates in the presence of saturating concentrations of Cetuximab (10 μg/ml). Radioactivity of the supernatant (80 μl) was measured with a Trilux Beta Scintillation Counter (PerkinElmer).
Percent specific lysis was calculated as: 100 × (experimental cpm - spontaneous cpm)/ (maximum cpm - spontaneous cpm).

FLOW CYTOMETRY

IGROV-1 cells were exposed to Cetuximab (5 μg/m) for 72 h or left untreated, collected and incubated for 30 min at 4°C with anti-MICA, -MICB, -ULBP1, -ULBP2, ULBP4, -CD112, -CD155, -ICAM-1, and HLA-E antibodies (R&D Systems; Minneapolis, MN. USA), followed by incubation with anti-mouse Alexa Fluor 448- conjugated reagent (Invitrogen). Samples were analyzed by gating on live cells using FACSCanto II system (Becton-Dickinson, San Jose, CA) and BD FACSDiva™ software (Becton-Dickinson). EGFR expression levels on IGROV-1 cells were determined after incubation for 30 min at 4°C with Cetuximab (10 μg/ml), followed by incubation with anti mouse Alexa Fluor 448-conjugated antibody (Invitrogen).

PHAGOCYTOSIS ASSAY

Macrophage antibody-dependent cell-mediated phagocytosis (ADCP) was assessed by flow cytometry (404). Murine RAW264.7 effector cells were labeled with PKH26 (Red Fluorescent Cell Linker Mini Kit), while IGROV-1 target cells were labeled with PKH67 (Green Fluorescent Cell Linker Mini Kit) according to the manufacturer's instructions (Sigma). IGROV-1 cells were then seeded in tissue culture flasks and exposed to Cetuximab (5 μg/ml for 72 h) or left untreated. At the end of treatment, target and effector cells were mixed at E:T ratio of 3:1 in complete medium and incubated for 12 h at 37°C in overload conditions of monoclonal antibody (10 μg/ml). Cells were collected, washed, resuspended in cold Ca^{2+} - and Mg^{2+} -free Dulbecco's PBS and analyzed by flow cytometry (FACSCanto II, Becton- Dickinson). Phagocytosis of IGROV-1 cells by RAW264.7 cells was evaluated in triplicate as percentage and intensity of macrophages positive for green fluorescence in at least three separate experiments.

THERAPY STUDIES

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 (2). Mice were injected i.p. with 2.5 × 106 ascitic cells in 0.2 ml of saline and treated 7 days later, when ascitic fluid began to accumulate, with CpG-ODN i.p. daily for 4 weeks (20 μg/ mouse) in combination with: Bevacizumab (5 mg/kg i.p. at 3–4 day intervals); Poly(I):Poly(C) (20 μg/mouse i.p. at 2–3 day intervals); Gefitinib (100 mg/kg per os, 5 days/ week); or Cetuximab (1 mg/mouse i.p. at 3–4 day intervals). Single agents were also included and control mice received saline. In other experiments, mice with evident and established ascites were selected on the basis of a similar body weight (mean 27.9 ± 0.84 g, 31.4 ± 0.9 g, first and second experiment, respectively) from large groups of mice injected i.p. 11–12 days before IGROV-1 cell injection and randomly divided into saline-treated (controls) and groups treated with CpG-ODN, Cetuximab (both with the schedules reported above) and Cisplatin (3 mg/Kg i.p., once weekly for 4 weeks) or their combinations. Experimental groups (5–12 mice/group) were inspected daily for ascites formation and weighed three times weekly. Mice were individually sacrificed by cervical dislocation prior to impending death. Day of sacrifice was considered day of death, and the median day of death (median survival time; MST) was calculated for each group. Anti-tumor activity was assessed as the ratio of MST in treated vs. control mice × 100 (T/C%).

STATISTICAL ANALYSIS

The clinical impact on overall survival (OS) and time to relapse (TTR) in GSE27290 and GSE25204, respectively, was assessed by the Kaplan-Meier method, and differences between curves were compared using a non-parametric (log-rank) test, with hazard ratios and 95% confidence intervals also computed. GraphPadPrism v5 (GraphPad software, La Jolla, CA, USA) was used for statistical analyses. Percent survivorship was estimated by the Kaplan-Meier product limit method and compared with the log-rank test.

RESULTS

1. miRNAs MEDIATE THE INCREASE OF SENSITIVITY TO CHEMOTHERAPY AFTER TREATMENT WITH CpG-ODN

The results shown in this thesis represent the continuation of the studies on the anti-tumor activity and mechanism of action of oligonucleotides containing CpG-ODN sequences, which are TLR9 agonists.

As previously reported, in laboratory where I conducted my thesis, Sommariva et al. showed in a mouse model of human IGROV-1 ovarian cancer that treatment with a CpG-ODN down-modulated DNA repair genes in the tumor cells. Human IGROV-1 cells cannot interact direct manner with CpG-ODN due to the oligonucleotide's species specificity and to the lack of TLR9 expression in this cell line, raising the possibility that peritumoral TLR9-expressing cells, such as innate immune cells, and/or endothelial cells, fibroblasts and epithelial cells, induce down-regulation of DNA repair genes in tumor cells through a direct cell-cell interaction and/or by secreting soluble factors.

Based on these findings, one could assume that, as reported in **Figure 21**, CpG-ODN induced down-modulation of DNA repair genes in tumor cells might represent a physiologic phenomenon that occurs locally in the presence of an infectious event (**Figure 25**). When was detected an infectious agent via endosomal TLRs, immune cells might induce modulation of DNA repair genes in infected (or transformed) cells to facilitate their death (405).

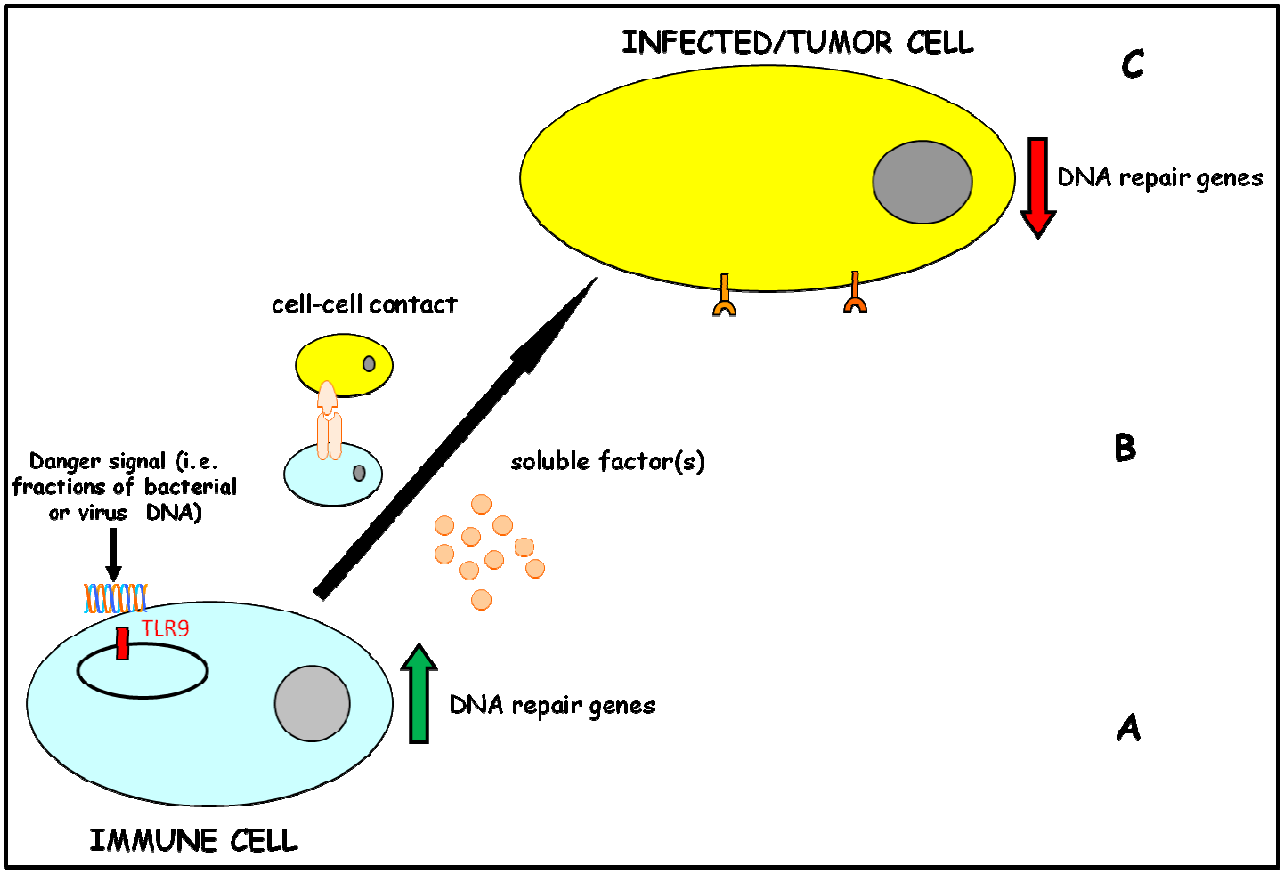

Figure 25. Hypothesized mechanisms of action of TLR9-expressing cells in modulating DNA repair genes during infections. (A) TLR9-positive cells upon detecting an infectious agent regulate DNA repair genes to decrease their susceptibility to proapoptotic signals and (B) induce modulation of DNA repair genes in infected cells (C) to facilitate their death.

Among the most relevant regulators of gene expression, microRNAs have been recently described as crucial players in most physiological and pathological conditions.

More than 1,200 human miRNAs have been identified and validated to date (www.mirbase.org). miRNAs can regulate about one-third of the human genome, with involvement in development and progression of many diseases (406–409), indeed several experimental and clinical findings have implicated miRNAs in the response to chemotherapy (409), demonstrating a role for miRNAs in modulation of genes involved in DNA repair (410,411).

Presumably, miRNAs evolved to allow organisms and cells to effectively deal with stress (412). Identification of miRNAs that are used ‘‘physiologically’’ to modulate DNA repair genes may have therapeutic implications. To this aim, using our preclinical model IGROV-1, we analyzed the effect of CpG-ODN on modulation of miRNAs in tumor cells, the integration of miRNA with mRNA expression modulation induced by CpG-ODN, and the importance of the identified miRNAs for the response to chemotherapy.

2. miRNAs EXPRESSION IN IGROV-1 OVARIAN TUMORS FROM CpG-ODN-TREATED MICE

miRNA expression, using Illumina human miRNA_v2 array, was conducted with RNA extracted 24 hours after the final treatment from omentum-adherent tumors of human IGROV-1 ovarian carcinoma-bearing mice treated i.p. with CpG-ODN or saline beginning 3 days after evidence of ascites. Among the 1145 miRNAs represented on the Illumina chips, 567 mature miRNAs annotated on miRBase12.0, along with 150 putative miRNAs, were detected. Class comparison identified 23 miRNAs showing a FDR< 0.1 and a fold-change > 1.8 between CpG-ODN- and control-treated mice (**Figure 26**).

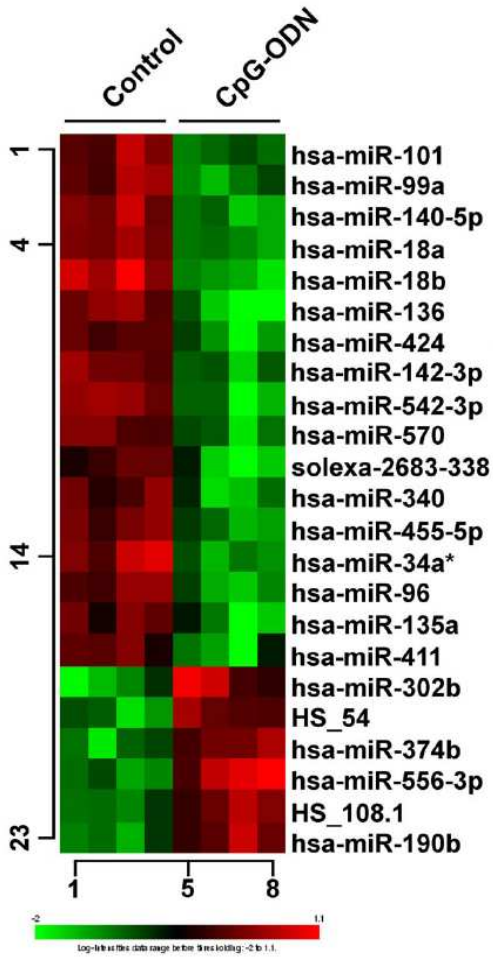

Figure 26. miRNA expression profiling in IGROV-1 ovarian tumors from CpG-ODN-treated athymic mice. Heat-map of 23 modulated miRNAs with FDR< 0.1 and fold change> 1.8 in CpG-ODN versus saline-treated mice. Among the 20 miRNAs belonging to miRBase12.0, 16 were down- and 4 up-modulated in CpG-ODN-treated mice (red: up-regulated

miRNAs; green: down-modulated miRNAs). Columns and rows represent samples and miRNAs, respectively. doi:10.1371/journal.pone.0058849.g001

Among the 23 miRNAs, 20 (16 up-regulated in saline- and 4 in CpG-ODN-treated mice) were annotated on miRBase12.0. The other three miRNAs were putative miRNA sequences derived from deep-sequencing approaches and were excluded from further analysis due to the lack of information.

Results of microarray analyses were validate by quantitative Real-Time PCR (RT-qPCR) examining the expression of the 9 miRNAs on the RNA profiled in microarray analysis and on the RNA extracted from tumor samples obtained from a replica of the IGROV-1 tumor-bearing mice treated as above described. Two miRNAs, the hsa-miR-18a and hsa-miR-18b, were selected based on their reported role in the pathogenesis of ovarian cancer (413,414), and two, the hsa-miR-101 and has-miR-302b for their described involvement in DNA repair processes and sensitivity to chemotherapy (401). The remaining 5 miRNAs were randomly selected. While RT-qPCR using the RNA profiled in microarray analysis validated all 9 miRNAs (**Figure 27**), RT-qPCR using the RNA of the replica confirmed 6 of 9 miRNAs ($p<0.05$). A trend was observed for hsa-miR-18b and hsa-miR-101 but not for hsa-miR-136 (**Figure 28**).

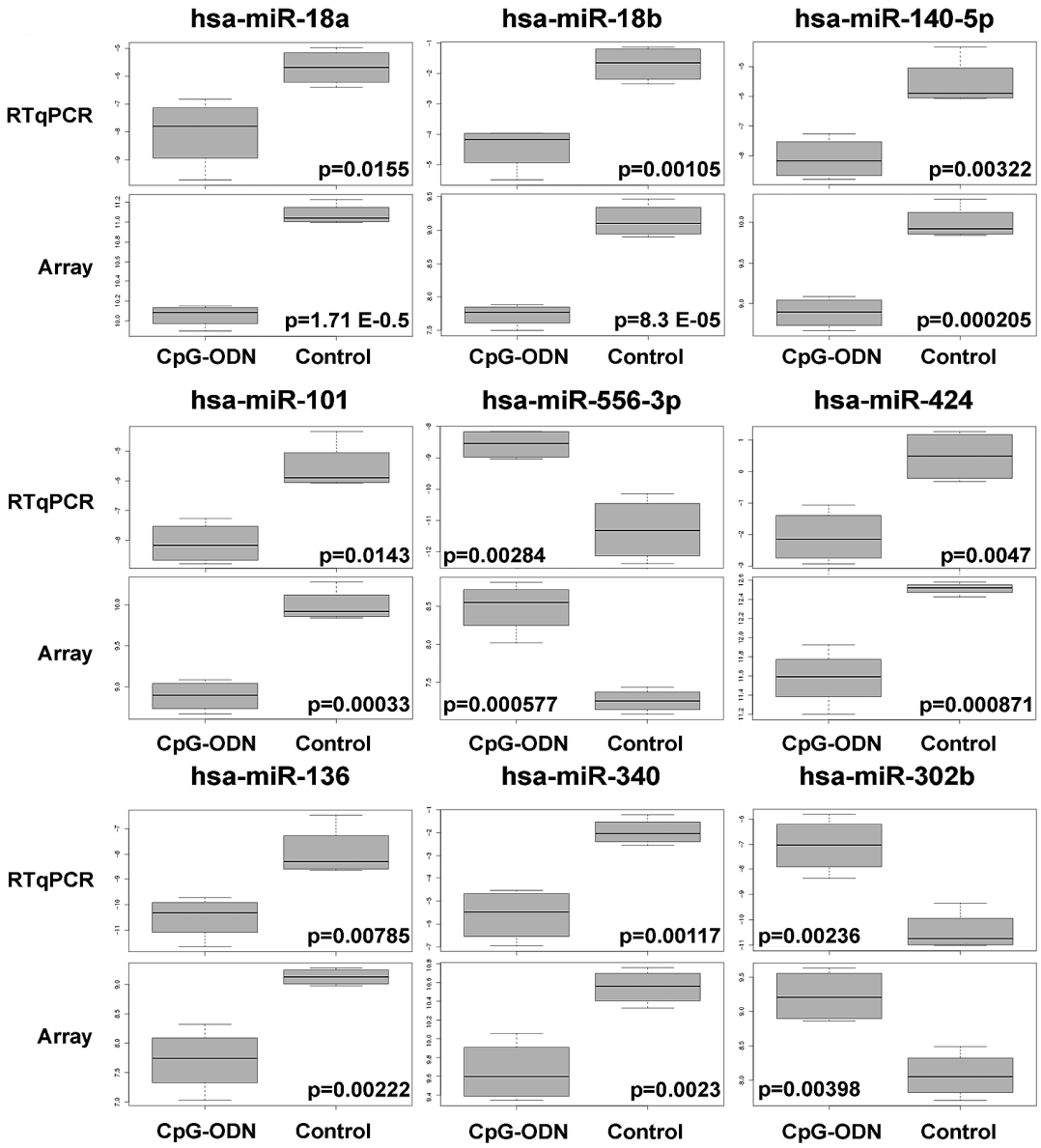

Figure 27. qRT-PCR validation of CpG-ODN miRNA profile. Comparison of hsa-miR-18a, hsa-miR-18b, hsa-miR-140-5p, hsa-miR-101, hsa-miR-556-3p, hsa-miR-424, hsa-miR-136, hsa-miR-340, hsa-miR-302b expression obtained by miRNA expression profile and qRT-PCR on tumors collected from human IGROV-1 ovarian tumor-bearing mice treated daily i.p. with CpG-ODN or saline (control group). *P* values of differential expression between control and CpG-ODN-treated IGROV-1 xenografts are reported. qRT-PCR data are plotted as -ΔCt and array data are plotted as $\log_2$ (expression).

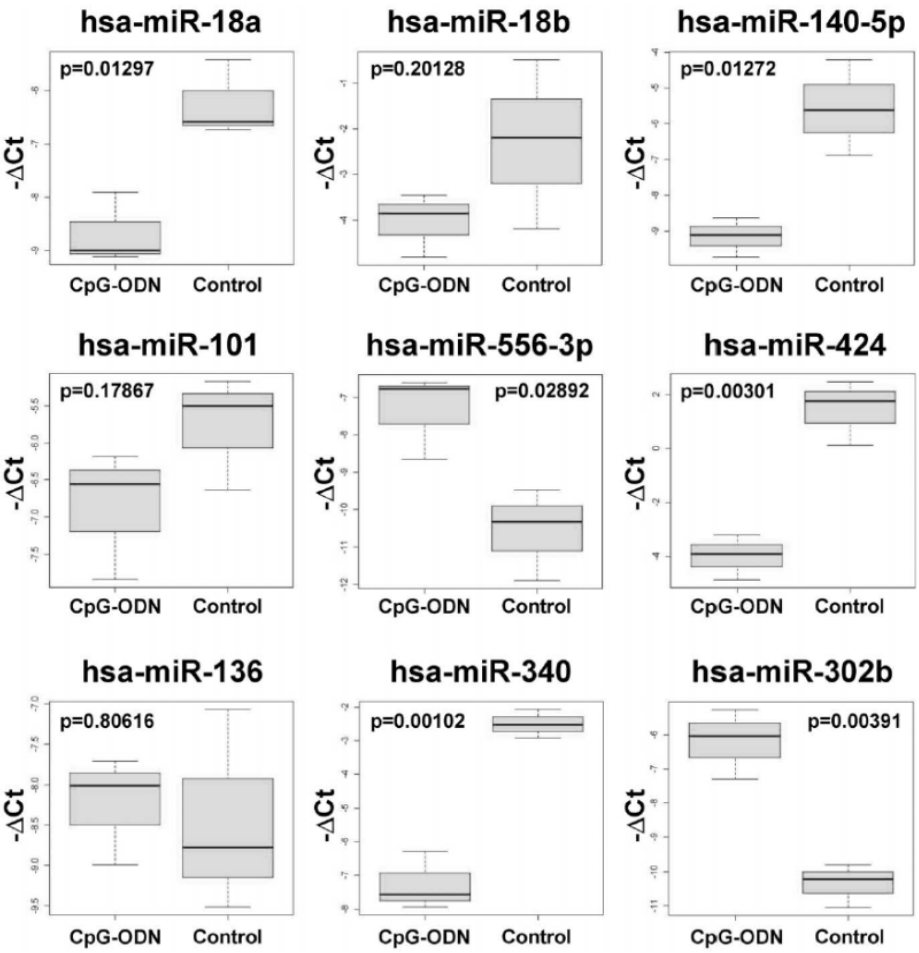

Figure 28. Independent biological validation of CpG-ODN miRNA profile. miRNA expression was assessed by RT-qPCR on IGROV-1 xenografts collected from a replica of a previous experiment (**397**). RT-qPCR data are plotted as 2-ΔCt. P-values were calculated using two-tailed Student's t-test.

3. UP-MODULATION OF hsa-miR-302b IN IGROV-1 CELLS ENHANCED CISPLATIN ACTIVITY

As previously reported, TLR9-expressing cells in the tumor microenvironment can sensitize cancer cells to DNA-damaging Cisplatin treatment by down-modulating DNA repair genes. To evaluate whether miRNAs modulated by CpG-ODN treatment are involved in the sensitivity to DNA damaging agents, the 3 most significantly differentially expressed miRNAs in tumor samples obtained from the replica of the in vivo experiment (hsa-miR-424, hsa-miR-340 and hsa-miR-302b) were examined. Hsa-miR-424 and hsa-miR-340 were found down-regulated by CpG-ODN treatment in our mRNA expression profile, while hsa-miR-302b was up-regulated. A gain- or loss-of-function phenotype was applied in order to mimic the up- or down-modulation observed in miRNA profiling (**see Figure 26**).

To down-regulate the expression of hsa-miR-340 and hsa-miR-424, IGROV-1 cells were transiently transfected with the respective LNA inhibitors or with a LNA negative control, whereas IGROV-1 cells were transfected with hsa-miR-302b precursor molecule or a scrambled oligonucleotide, as control, to increase expression of hsa-miR-302b. After 72 hours to transfection, cells were then treated with 50 mM of Cisplatin for 1 h. After 24 h the percentage of sub-G1 cells, an indicator of cell death, was analyzed by FACS.

Evaluation of the effect of hsa-miR-340 and hsa-miR-424 down-regulation on sensitivity to Cisplatin of IGROV-1 cells revealed no increase of cell death (**Figures 29A and 29B**).

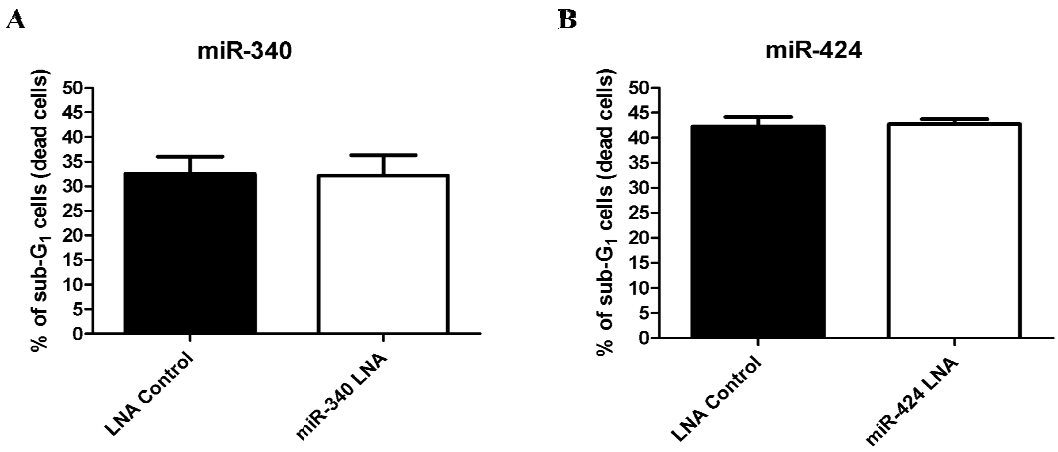

Figure 29. Down-regulation of expression of hsa-miR-340 (A) and hsa-miR-424 (B) not enhance Cisplatin sensitivity in IGROV-1. Hsa-miR-340 LNA/hsa-miR-424 LNA - and scrambled transfected cells after Cisplatin treatment not shown an increase of percent cell death. IGROV-1 cells were transfected with 50 nmol/l hsa-miR-340 LNA/hsa-miR-

424 LNA or LNA control, and 72 h later, exposed to cisplatin (50 mM) for 1 h. Cell viability was assessed 24 h after cisplatin treatment by propidium iodide staining and flow cytometry.

the increased expression of hsa-miR-302b significantly enhanced Cisplatin cytotoxicity, with an increase of cell death ranging from 26.5 to 43.9% as compared to negative scrambled-transfected cells ($p>0.0001$; **Figure 30A**), these experiments were repeated 6 time in independent manner. The transient transfection with hsa-miR-302b precursor molecule and control in IGROV-1 cells has not shown significant differences in cell growth (**Figure 30B**). This result excludes the possibility that hsa-miR-302b sensitized cancer cells to Cisplatin by stimulating cell proliferation.

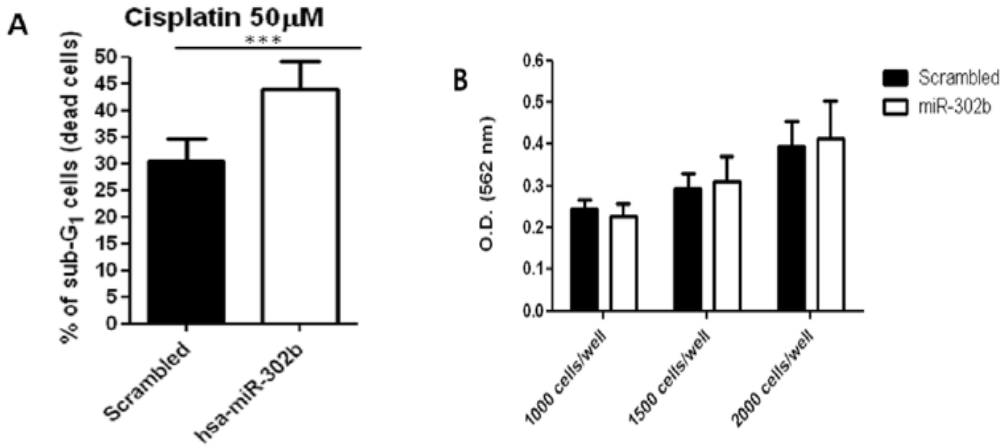

Figure 30. Forced expression of hsa-miR-302b increased Cisplatin sensitivity in IGROV-1 cells without affecting cell proliferation. (A) Percent cell death of hsa-miR-302b- and scrambled transfected cells after Cisplatin treatment. IGROV-1 cells were transfected with 50 nmol/l hsa-miR-302b precursor molecule or scrambled control, and 72 h later, exposed to Cisplatin (50 mM) for 1 h. Cell viability was assessed 24 h after Cisplatin treatment by propidium iodide staining and flow cytometry. Data represent mean 6 SEM of 6 independent experiments. ***p,0.0001 by paired t-test. (B) Evaluation of cell proliferation by SRB assay. Transfected cells were seeded in a 96-well plate at a density of 10^3, $1.5x10^3$, and $2x10^3$ cells/well. Cell growth was assessed by optical density (OD) determination 72 h after transfection. Data represent mean 6 SEM of 3 independent experiments

4. HDAC4 IS DIRECTLY TARGETED BY hsa-miR-302b IN IGROV-1 CELLS

Because miRNAs might play important regulatory roles modulating the expression of their predicted target genes, we searched for expression patterns regulated following CpG-ODN treatment by integrating the miRNA and mRNA expression profiles. Class comparison of whole gene expression, identified by Illumina HumanHT12_v3 beadchips using the same tumor extracted RNA assessed for miRNA profile, revealed 215 genes differentially expressed; 141 up-regulated in saline- and 74 in CpG-ODN treated mice (FDR< 0.1 and fold change> 1.8). We used freely available tool MAGIA (**400**) to identify functional miRNA-mRNA relationships. Specifically, the 20 miRBase-annotated miRNAs were altogether compared to the whole-expression profile dataset using the union of three prediction target algorithms (Pita, miRanda and TargetScan) available on MAGIA. The Pearson's correlation between each miRNA and its predicted target was then computed. Using Cytoscope the first 250 most significantly negatively correlated miRNA-mRNA interactions were identified, as a network. As shown in **Figure 31,** evidence of the concerted interplay of miRNAs regulated by CpG-ODN and their potential target mRNAs was observed for hsa-miR-302b and hsa-miR-374b, which are up-regulated in CpG-ODN-treated mice, and for 13 miRNAs up-regulated in saline-treated mice (hsa-miR-135a, hsa-miR-136, hsa-miR-340, hsa-miR-445-5p, hsa-miR-424, hsa-miR-96, hsa-miR-142-3p, hsa-miR-140-5p, hsa-miR-542-3p, hsa-miR-18a, hsa-miR-18b, hsa-miR-101, and hsa-miR-99a). The latter 13 form a highly interconnected cluster where different miRNAs exert their biological functions targeting the same genes.

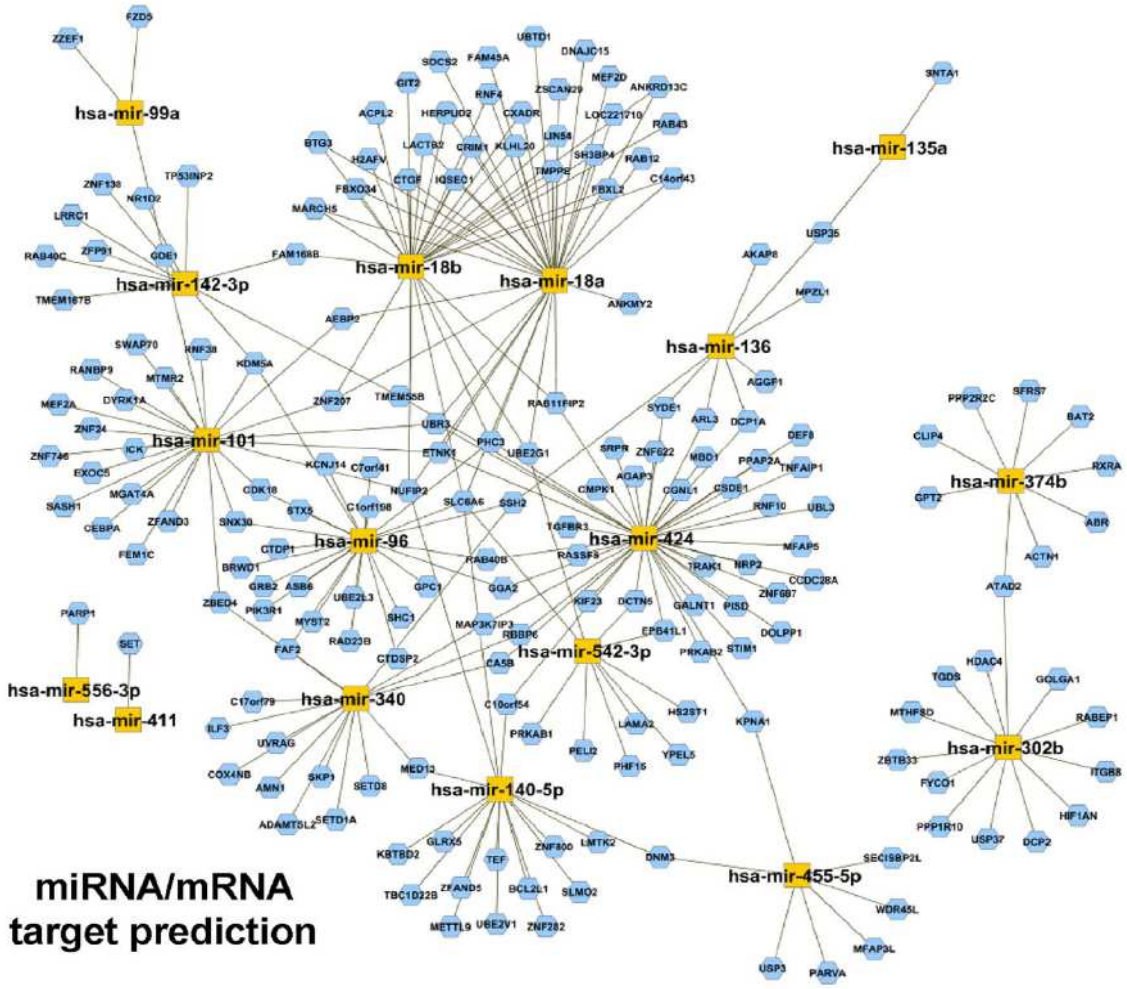

Figure 31. Computational integration of miRNA and gene expression profiles of tumor samples from CpG-ODN- and saline-treated mice. Network between 15 of 20 differentially expressed miRNAs and their anti-correlated target genes. The top 250 interactions were used to generate the network using the MAGIA tool.

Among the 19 genes potentially targeted by hsa-miR-302b as determined using MAGIA (q value< 0.1, **Table 7**), HDAC4, one of the top anti-correlated mRNAs, was evaluated as a potential molecular target of hsa-miR-302b associated with response to chemotherapy.

Table 7

Entrez ID	Gene Symbol	miR	Pearson correl	q value
2817	GPC1	hsa-mir-96	-0.989906	0.007708444
26052	DNM3	hsa-mir-455-5p	-0.985952	0.013812435
92370	ACPL2	hsa-mir-18b	-0.983891	0.013814912
7756	ZNF207	hsa-mir-18a	-0.982795	0.013814912
23328	SASH1	hsa-mir-101	-0.982131	0.013814912
9759	**HDAC4**	**hsa-mir-302b**	**-0.928993**	**0.024311363**
3908	LAMA2	hsa-mir-542-3p	-0.928964	0.024311363
87	ACTN1	hsa-mir-374b	-0.928388	0.024400169
1525	CXADR	hsa-mir-18a	-0.92831	0.024400169
401548	SNX30	hsa-mir-96	-0.92819	0.024407453
57695	USP37	hsa-mir-302b	-0.927045	0.024727786
58476	TP53INP2	hsa-mir-142-3p	-0.926939	0.024727786
51218	GLRX5	hsa-mir-140-5p	-0.925766	0.025173524
90441	ZNF622	hsa-mir-424	-0.925513	0.025216943

We focused on HDAC4, gene that belongs of the histone deacetylase family, since it encodes a protein that reportedly mediates Cisplatin sensitivity in ovarian cancer. Resistance to platinum chemotherapy continues to be a major complication in the treatment of ovarian cancer. HDAC4 was found over-expressed in clinically resistant cells and considerably potentiated Cisplatin response when it was silenced. Indeed, following knockdown of HDAC4, apoptotic response to platinum treatment in resistant cells was significantly enhanced (415). Forced hsa-miR-302b expression in IGROV-1 cells decreased HDAC4 mRNA and protein levels (**Figures 32 A and 32B**), supporting the interaction analysis data. Luciferase reported assay was than performed to determine if the down-modulation of HDAC4 after miR-302b over-expression was a consequence of a direct interaction between the miRNA and the mRNA of HDAC4. To this aim, the target site of miR-302b was identified within the HDAC4 3'UTR according to Target Scan database (**Figure 32C**) and the matching site was cloned into pGL3 reporter plasmid (Promega) downstream the luciferase gene, and the activity detected by a renilla/firefly dual-luciferase assay. When the reporter vector containing the HDAC4 3'UTR was co-transfected in IGROV-1 cells with miR-302b precursor or a scrambled oligonucleotide as control, enforced miR-302b over-expression was found to decrease luciferase activity as compared to scrambled transfected cells (~50% reduction, p=0.0088, **Figure**

32D), whereas mutated HDAC4-3'UTR escaped this inhibition (**Figure 32E**). These data indicate the direct effect of hsa-miR-302b on HDAC4 gene expression.

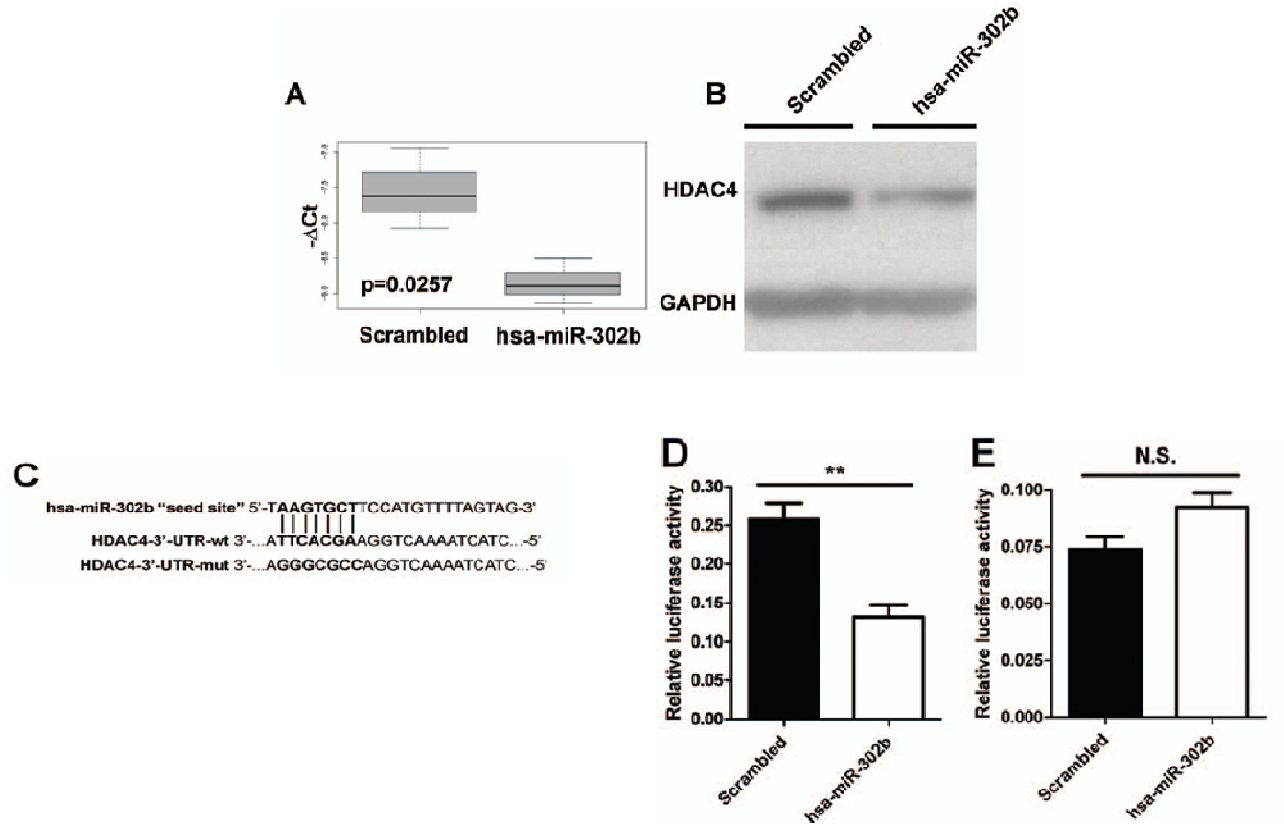

Figure 32. Targeting of HDAC4 in IGROV-1 cells by hsa-miR-302b. IGROV-1 cells were transfected with 50 nmol/l hsa-miR-302b or a scrambled oligonucleotide and RNA and proteins were collected after 72 h. HDAC4 mRNA levels were evaluated by RT-qPCR (A) and protein expression was evaluated by Western blot (B). GAPDH was used to normalize protein loading per lane. Data are representative of 6 independent experiments with superimposable results. (C) Schematic representation of the interaction between hsa-miR-302b and the binding site on the wild-type HDAC4-3'UTR and the mutated control. (D) Relative luciferase activity in IGROV-1 cells for HDAC4-3'UTR-wt co-transfected with reporter vector and with hsa-miR-302b precursor molecule or negative scrambled control for 48 h. (E) Relative luciferase activity in IGROV-1 cells for HDAC4-3'UTR-mut co-transfected with reporter vector and with hsa-miR-302b precursor molecule or negative scrambled control for 48 h.

Since IGROV-1 lack TLR9 expression, the effect of CpG-ODN on tumor cells is necessarily indirect, likely to be exerted on components of the immune system. An increasing body of evidence underlines how miRNAs can be released in body fluids, especially associated to microvescicles (MVs) as exosomes (416), probably as a mechanism of information exchange between different cell types and tissue districts in the organism (417). Moreover, since mature human and murine miR-302b have identical sequences, mature miRNA detected as increased in IGROV1 xenograft upon CpG-ODN treatment might be of murin origin. This observation raised the intriguing idea that CpG-ODN might act on TLR9 positive immune cells inducing up-regulation of miR-302b, which could be released in ascitic fluid associated to MVs, enter probably through endocytosis into IGROV1 cells, where it induces increased response to Cisplatin by HDAC4 modulation. To explore

this hypothesis we have analyzed precursor (pre-miR-302b) and mature (mm-miR-302b) forms of the microRNA both in spleens and tumors from CpG-ODN treated mice. A strong significant increase of murine pre-miRNA (p=6.92 E-05) upon treatment was observed in spleens, even though paralleled only by a moderate increase of mm-miRNA (p=0.0336), whereas human pre-miRNA in IGROV-1 was undetectable, despite the increase of mm-miRNA (**Figure 33**). As a positive control for human pre-miR-302b probe, human PBMC were treated with human CpG-ODN reveling increased levels of pre-miR-302b. The fact that human pre-miRNA seems undetectable in IGROV-1 whereas the murine form is induced in spleen upon CpG-ODN treatment supports the hypothesis revealed in tumors that the mature form has ectopic origin.

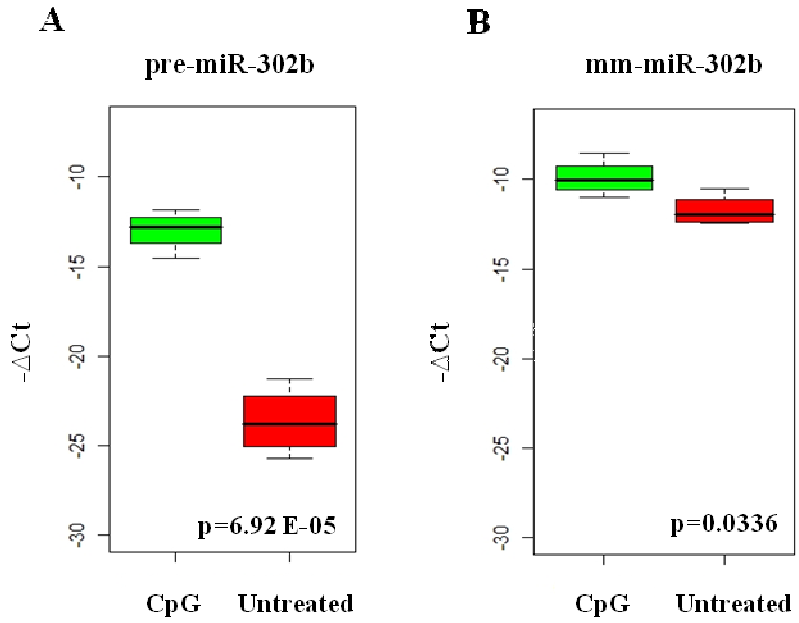

Figure 33. Analysis of precursor (pre-miR-302b) (A) and mature (mm-miR-302b) (B) forms of the miRNA in spleen from CpG-treated and untreated mice by RT-qPCR. A significant increase of murine pre-miRNA upon treatment was detected in spleens (p=6.92 E-05), while was observed a modest increase of mm-miRNA (p=0.0336). RT-qPCR data are plotted as 2-ΔCt. P-values were calculated using two-tailed Student's t-test.

5. CpG-ODN-MODULATED miRNAs AND OVARIAN CANCER PATIENTS' CLINICAL COURSE

The impact of expression levels of all 20 differentially expressed miRNAs, including those for which validation was not carried out on the clinical course of ovarian cancer patients undergoing chemotherapy, was evaluated in silico. The time to relapse (TTR) and overall survival (OS) with respect to each miRNA on two public datasets (GSE25204 and GSE27290) (402,403) were analyzed. Patients were stratified according to miRNA expression below (low expression) or above (high expression) the median expression value. In Bagnoli's dataset (403), Kaplan-Meier analysis showed that patients with low expression of hsa-miR-302b or with high expression of hsa-miR-340 had a shorter TTR (log-rank, P= 0.037; HR=1.75, 95% CI: 1.03–2.95 and P= 0.047; HR=1.7, 95% CI: 1.01–2.86, respectively) (**Figures 34A and 34B**). Median TTR was 11 and 25 months for low and high expression of hsa-miR-302b (**Figure 34A**), and 26 and 12 months for low and high expression of hsa-miR-340, respectively (**Figure 34B**). In Shih's dataset (401), only the expression of hsa-miR-302b was significantly associated to OS (log-rank, P = 0.034; HR= 2.02, 95%CI: 1.05–3.88), with a median OS of 33.7 and 101.2 months for low and high expression, respectively (**Figure 34C**). In both datasets, the impact of the other 18 miRNAs expression was not significantly associated to TTR or OS.

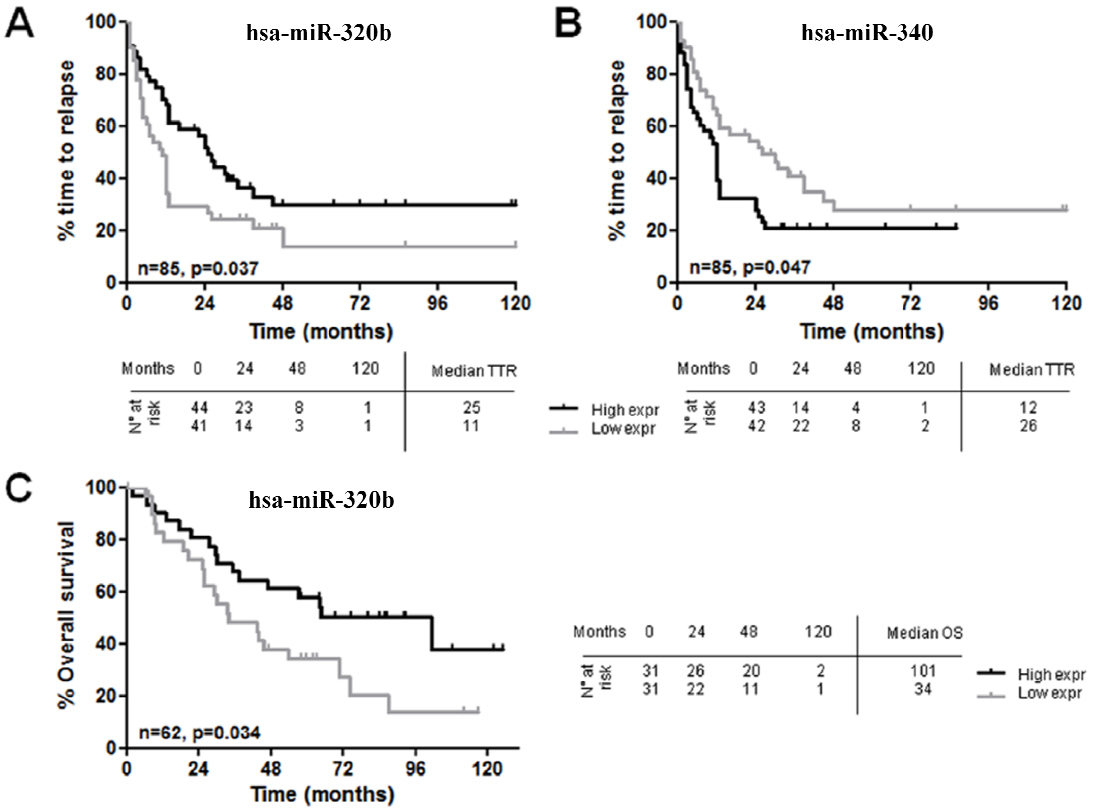

Figure 34. In silico evaluation of ovarian cancer patients' clinical course according to hsa-miR-302b and hsa-miR-340 expression levels. Kaplan-Meier survival curves of patients stratified according to hsa-miR-302b expression (A) and has-miR-340 expression (B) on GSE25204 and referred to TTR. (C) Kaplan-Meier survival curves for hsa-miR-302b expression on GSE27290 and referred to OS. Patients were dichotomized using median expression as threshold.

6. EFFICACY OF CpG-ODN IN COMBINATION WITH DIFFERENT AGENTS

TLR agonists, alerting the host to invading pathogens, are included in the National Cancer Institute list of immunotherapeutic agents with the highest potential to cure cancer in light of their immunostimulatory activity (418,168). However, while extensive preclinical and clinical data indicate the ability of TLR agonists to promote antitumor immunity *in vivo,* their low efficacy in some Phase III studies has delayed further development of several TLR agonists for cancer therapy (169). With the exception of Imiquimod, a synthetic TLR7 agonist topically applied to treat basal cell carcinoma, most clinical trials have involved subcutaneous (s.c.) administration of TLR agonists since this route was reported to effectively activate adaptive immunity (170).

Several immunoadjuvants, including RNA and DNA analogs, have been developed as anti-cancer drugs, one of which is Poly(I):Poly(C). This synthetic analog of double-strand RNA (dsRNA), displays antitumor functions and is currently used as an immune adjuvant in clinical trials. Poly(I):Poly(C) has been confirmed as an agonist of Toll-like receptor-3 (TLR-3) and retinoic acid-inducible gene I-like receptors (RLRs). Poly(I):Poly(C) initiates multiple signal pathways in different tumor cells by triggering TLR3, RIG-I, or MDA-5, including NF-κB and IRF3/7 pathways, and generates effectors, such as pro-inflammatory factors and type I IFN. Furthermore, Poly(I):Poly(C) directly induces apoptosis in tumor cells via intrinsic and extrinsic apoptotic pathways. An additional important effect in many types of cancers is the synergistic effect of Poly(I):Poly(C) in combination with other cytokines or chemotherapeutics (419).

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, in our preclinical model (**see Figures 18A and 18B**). Markedly elevated peritoneal VEGF levels can be present in malignant ascites of ovarian cancer patients (390), and blocking of VEGF has been reported to strongly reduce ascites volume (391). Bevacizumab is a recombinant humanized monoclonal IgG1 antibody that targets vascular endothelial growth factor (VEGF)-A, and is indicated in the treatment of metastatic colorectal cancer, non-small cell lung cancer, renal cell carcinoma, and glioblastoma multiforme (420-423). This antibody binds to and neutralizes all biologically active forms of VEGF-A, and then suppresses tumor growth and inhibits metastatic disease progression (424). The utility of VEGF antibodies in the treatment of ovarian carcinoma was initially explored in animal models, where

VEGF blockade was shown to inhibit ascites formation and slow tumor growth (425). In addition, VEGF-targeting agents are thought to enhance the effects of chemotherapy by normalization of primitive tumor vasculature, leading to decreased interstitial fluid pressure, increased tumor oxygenation, and enhanced delivery of cytotoxic drugs (426).

It was recently demonstrated that CpG-ODN treatment induces modulation of genes involved in DNA repair and sensitizes cancer cells to DNA-damaging Cisplatin treatment.

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 tumor cells, since several tumor entities are characterised by a substantial over-expression of EGFR (427,428). 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 (429,430). 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 (429). Using these pathways, EGFR is considered to modulate cell proliferation, differentiation as well as apoptosis but also DNA repair (431). 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 Gefitinib or the monoclonal antibody (mAB) Cetuximab (432,433). Gefitinib is a selective EGFR (ErbB1) tyrosine kinase inhibitor (434) and can also inhibit the growth of some ErbB2-overexpressing tumor cells (e.g. breast cancer) (435). Autophosphorylation of EGFR was prevented in various tumor cell lines and xenograft models by Gefitinib. It is speculated that upregulation of p27 via EGFR kinase inhibition results into inhibited cyclin-dependent kinase activity and arrest in the G1 cell cycle phase and it also inhibits tumor neoangiogenesis (436).

Cetuximab is a chimeric IgG_1 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 (437). Human ovarian carcinomas express EGFR to varying degrees (438) and the staining of IGROV-1 with Cetuximab revealed that this monoclonal antibody is able to bind the membrane surface of IGROV-1 (**Figure 35**).

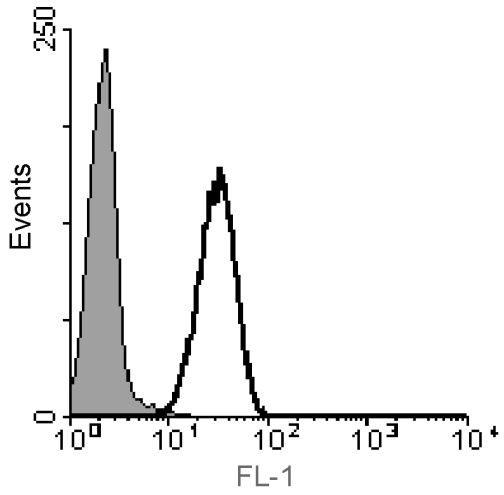

Figure 35. Flow cytometric analysis of IGROV-1 surface expression of EGFR. Cells were stained with cetuximab (black line, panel B), and with anti-CD20 rituximab antibody as isotype control (grey histogram).

Therefore it was screened the effectiveness of CpG-ODN in combination with:

1) MAb Cetuximab, which targets the ligand-binding domain of the epidermal growth factor receptor (EGFR) frequently expressed in ovarian cancer cells (438);

2) Gefitinib, a tyrosine kinase inhibitor of EGFR,

3) 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 (444);

4) MAb bevacizumab, which targets the vascular endothelial growth factor (VEGF). VEGF is reportedly overexpressed in ovarian cancer (439-441), and VEGF-regulated angiogenesis is an important component of ovarian cancer growth (442,443);

To evaluate the efficacy of CpG-ODN in association with Poly(I):Poly(C), Bevacizumab, Gefitinib (Iressa) or Cetuximab, mice were injected i.p. with 2.5 x 10^6 IGROV-1 (which express EGFR, **Figure 35**) cells in 0.2 ml of saline and treated starting 8 days later when mice showed an increase of body weight without an evident and established ascites.

Repeated i.p. CpG-ODN treatments plus Poly(I):Poly(C) was not able to induce a significant superior effect on Median Survival Times (MST) (65 days with Percent of Treated/Control (T/C%) of 325) compared with CpG-ODN treatment alone (61 days, T/C% 305), and only 2 of 9 mice from the combined treatment group showed long-term survival at the 120th day when the experiment was stopped (**Figure 36**). Those results were not in accordance to previous observations witch demonstrated a clear synergy between the two immune modulators (444); this could be possibly due

to the schedule of CpG-ODN administration. Indeed, daily CpG-ODN administration might induce considerable innate cell activation hardly expandable by other immune modulators.

As also shown in **Figure 36**, repeated i.p. CpG-ODN treatments plus anti-VEGF Bevacizumab also did not improve the effect of CpG-ODN treatment alone (MST 56 days for the combination vs 62 days for CpG-ODN alone). Of note, the therapeutic benefit versus control mice observed in mice treated with Bevacizumab alone, might be due, at least in part, to the ability of this monoclonal antibody effects to control ascites formation; infact, in mice treated with the anti-VEGF antibody as a single treatment, we observed an inhibition of ascites production. Those evidences are consistent with recent preclinical and clinical data and suggesting that targeting VEGF might suspend ascites production resulting from peritoneal metastasis (445).

The addition of the EGFR tyrosine kinase inhibitor Gefitinib (Iressa) to repeated i.p CpG-ODN treatment induced a slight but not significant increase in lifespan versus mice treated with CpG-ODN alone, (MST 67 days for the combination vs 52 days for CpG-ODN alone, p =0.4099). In contrast, a dramatic increase on survival was observed on mice treated with CpG-ODN plus Cetuximab versus those treated with CpG-ODN alone, (MST: 86 days combination, 29 days for Cetuximab alone; 62 days for CpG alone; P = 0.0008 combination versus CpG-ODN alone), with 4 of 8 mice still alive at the end of the experiment.

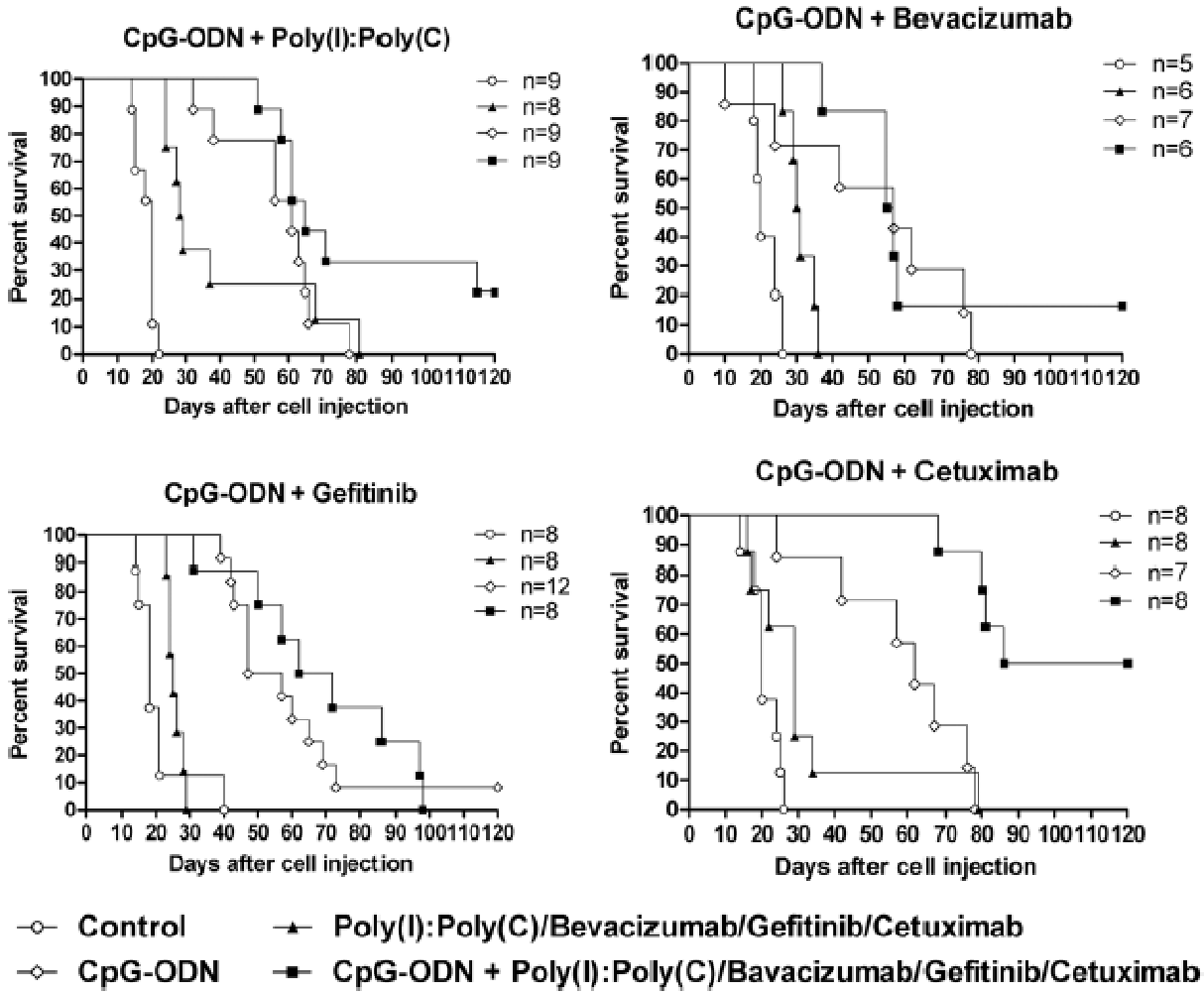

Figure 36. Kaplan-Meier plot of percent survival over time among IGROV-1 ovarian tumor-bearing mice. At 7 days after tumor cell injection, mice were treated i.p. with CpG-ODN (20 μg/mouse, 5 days/week for 4 weeks) in combination with: Poly(I):Poly(C) (20 μg/mouse at 2- to 3-day intervals); Bevacizumab (5 mg/Kg at 3- to 4-day intervals); Gefitinib (100 mg/mouse, 5 days/week) or Cetuximab (1 mg/mouse at 3- to 4-day intervals). Single agents were also tested. Control mice received saline. N = number mice/group.

Different factors probably agree for these impressive results. This synergistic effect is certainly due to the capacity of CpG-ODN to recruit and activate immune effectors cells at the site of tumor growth. Specifically, we performed our experiments with nude mice models, in which NK cells and macrophages are the predominant immunological population; also, those cells are reported to be much more biologically active when target cell's antigens have been bound by specific antibodies, exherting their cytotoxic activity through antibody–dependent cell cytotoxicity (ADCC).

To investigate whether treatment with Cetuximab increased the susceptibility of IGROV-1 cells to ADCC, cells were pre-treated with Cetuximab (5μg/ml) for 72 hours, before their use as targets on ADCC assay, conducted with saturated concentration of Cetuximab (10μg/ml) and using as effector

cells PBMC from 12 healthy donors (**Figure 37**). Additionally, as EGFR modulates a variety of downstream signaling pathways, such as NF-kB, PI3-K, MAPK, and PKC pathways (446,447), inhibition of these pathways by Cetuximab may lead to an increase susceptibility of tumor cells to the effector cells, such as NK cells, macrophages, neutrophils, involved in tumor eradication in nude mice. Therefore, those impressive results obtained in our mice tumor model in which the antibody alone had slight effect, might be also related to a Cetuximab-induced increase susceptibility of tumor cells to CpG-ODN-activated effector cells involved in ADCC (438,444).

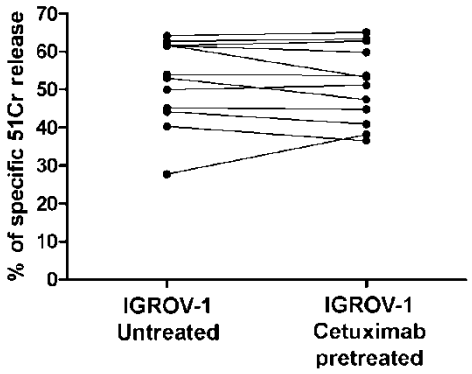

Figure 37. Comparison of ADCC activity untreated- and –cetuximab pretreated IGROV-1 cell line, using PBMC from 12 separate donors. IGROV-1 were the targets. Effector : target ratio was 50 : 1.

Not increased death in Cetuximab-pretreated tumor cells compared to untreated cells was observed. Because HER signaling in tumors regulates expression of MICA and MICB, key ligands that promote NK cell-mediated recognition and cytolysis (448), and because EGFR inhibitors enhance susceptibility to NK cell-mediated lysis by modulating expression of the NKG2D ligand ULBP-1 (448-450), we tested whether Cetuximab treatment of IGROV-1 cells modulates expression of molecules involved in NK-mediated lysis (MICA, MICB, ULBP1, ULBP2, ULBP4, CD112, CD155, ICAM-1 and HLA-E). FACS analysis of tumor cells pretreated with Cetuximab (5 µg/ml) for 72 h did not reveal any type of modulation but in some cases down-modulation of these receptors (**Figure 38**).

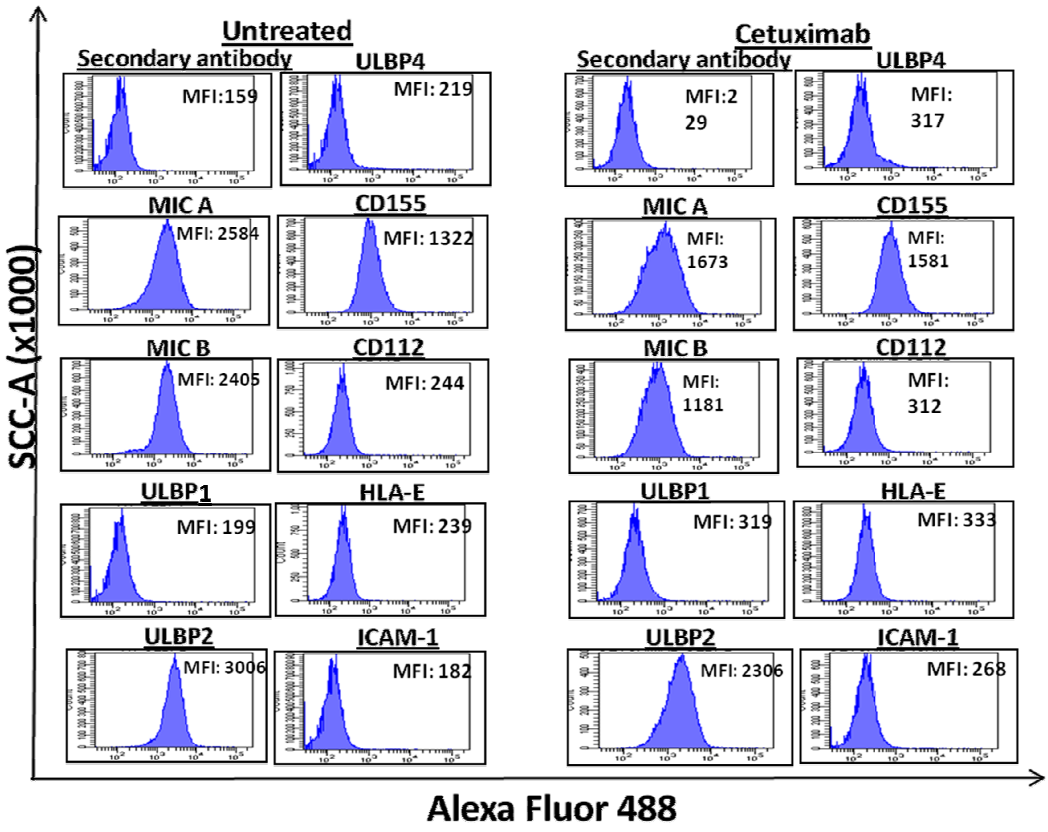

Figure 38. Expression levels of molecules involved in NK-mediated cytotoxicity in IGROV-1 cancer cell line after cetuximab pretreatment. ($p<0.05$)

We then investigated if Cetuximab treatment would make IGROV-1 cells more robustly phagocytosed by macrophages. To this aim we carried out cytofluorimetric analyses examining engulfment of PKH26-stained mouse leukemic monocyte/macrophage RAW 264.7 cells (red) that had been co-cultured for 4 and 12 with PKH67-stained human IGROV-1 cells (green) pre-treated or not with Cetuximab (final concentration 5µg/ml). Also this experiment has been carried out in overload monoclonal antibody condition (final concentration of Cetuximab 10µg/ml). The results shown in the **Figure 39** demonstrated that Cetuximab pre-exposure greatly increases macrophage-mediated phagocytosis of IGROV-1 ovarian tumor cells, as indicated by the significant increase of the percentage of double positive RAW 264.7-IGROV-1 in the Cetuximab pre-treatment group cells to untreated IGROV-1 group. In particular, at 12 hours tumor cell incorporation was greater than 4 hours.

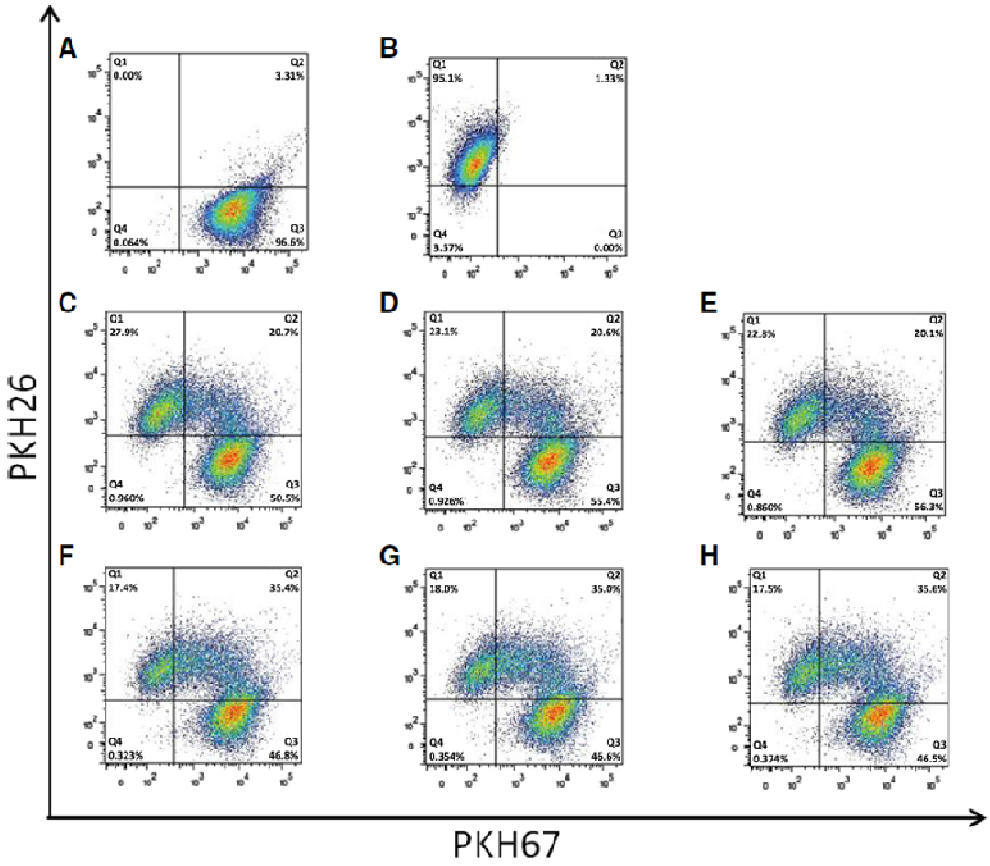

Figure 39. Effect of Cetuximab pre-treatment on phagocytosis of IGROV-1 cells. IGROV-1 target cells were stained green with PKH67 (A, right lower quadrant) and RAW264.7 effector cells were stained red with PKH26 (B, left upper quadrant). Tumor targets were pre-incubated for 72 h with 5 μg/ml Cetuximab (F,G,H) or left untreated (C,D,E). At the end of treatment, target and effector cells were mixed at effector/target (E/T) ratio of 3:1 in complete medium and incubated for an additional 12 h in overload conditions of monoclonal antibody (10 μg/ml). The percentage of double-positive cells present in the upper right quadrant (quadrant Q2) of the dot plots represents the percentage of RAW264.7 cells phagocytosing green-stained tumor cells. Data were obtained in triplicate and are representative of one of three experiments with similar results.

In vivo experiments reported above have been conducted in mice with an early tumor stage, starting treatment 8 days after tumor cell injection when mice showed an increase of body weight without an evident and established ascites. Unfortunately, advanced tumor disease in humans is often much less responsive than limited disease to most anti-cancer therapies. To this aim, we then evaluated if the double combination of CpG-ODN and Cetuximab added to another molecular agents could further increase it's therapeutic effect in advanced-stage human ovarian tumor bearing mouse, in which ascitic fluid formed 11 days after tumor cell injection and mice showed evident abdominal volume increase,

EGFR inhibitors are reported to interact with Cisplatin (451-454), although their effect on sensitivity to this drug remains undefined; also, we recently reported the synergistic antitumor effect between CpG-ODN and Cisplatin (397). Keeping those evidences in mind, we investigated the

therapeutic effect of the combination of CpG-ODN, Cetuximab and Cisplatin in mice selected for evident and established ascites from a large group of mice injected i.p. 11 days before with IGROV-1 cells (mean body weight ± SEM 27.9 ± 0.84 g vs 23.00 ± 1.08 g before tumor cell injection; increased body weight = 4.9 g). Mice were randomly divided into different groups and treated with saline, Cisplatin, CpG-ODN plus Cetuximab, CpG-ODN plus Cisplatin, Cetuximab plus Cisplatin, and CpG-ODN plus Cetuximab and Cisplatin. Saline-, Cisplatin-, or Cetuximab/Cisplatin-treated mice were euthanized on days 13 to 36 after tumor cell injection (MST 16, 23 and 18.5 days, respectively), CpG-ODN/Cetuximab-treated mice were euthanized between days 16–104 (MST 66 days; T/C% = 412.5), while 7 mice treated with the triple combination were euthanized on days 80–109, with 3 still alive at the end of experiment. Thus, survival was significantly increased (MST 105.5; T/C% 659.37; P = 0.001) compared with CpG-ODN/Cetuximab-treated mice (**Figure 40A**). Similar analysis in mice bearing even more advanced stage ascites (mean body weight ± SEM 31.4 ± 0.9 g vs 24.89 ± 0.68 g before tumor cell injection; increased body weight = 6.51 g) showed that the CpG-ODN/Cetuximab/Cisplatin combination still increased survival (MST 45;T/C% 250, P = 0.0089 vs controls) (**Figure 40B**). Note that 6 days after the start of treatment, 6 of 9 saline-treated mice became moribund and were sacrificed.

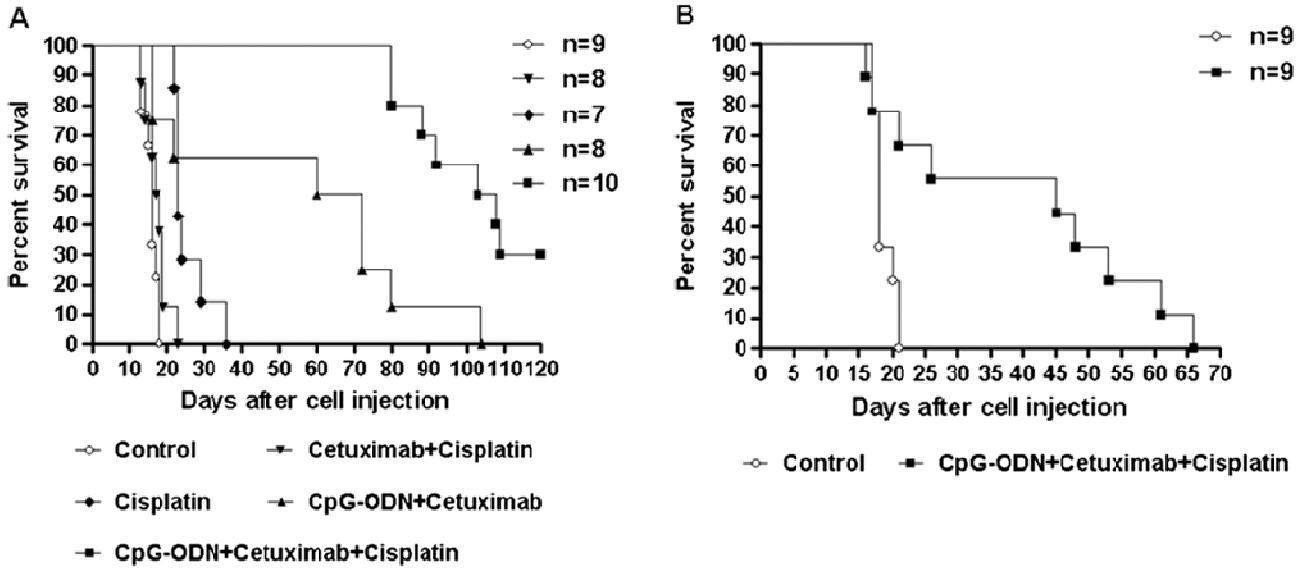

Figure 40. Kaplan-Meier plot of percent survival over time in advanced-stage IGROV-1 ovarian tumor-bearing mice. (A) Mice selected for the presence of evident and established ascites from a large group of mice injected i.p. 11 days before with IGROV-1 cells (mean body weight ± SEM 27.89 ± 0.84 g vs 23.00 ± 1.08 g before tumor cell injection) were treated with saline, Cisplatin (3 mg/kg, once per week), CpG-ODN (20 μg/ mouse, 5 days/week for 4 weeks) plus Cetuximab (1 mg/mouse at 3- to 4-day intervals), CpG-ODN plus Cisplatin, Cetuximab plus Cisplatin, and CpG-ODN plus Cetuximab and Cisplatin. (B) Mice selected for more advanced-stage disease (mean body weight ± SEM 31.4 ± 0.9 g vs 24.89 ± 0.68 g before tumor cell injection) were treated with saline or CpG-ODN plus Cetuximab and Cisplatin. N = number mice/group.

DISCUSSION

Oligodeoxynucleotides (ODN) containing dinucleotides with unmethylated CpG motifs (CpG-ODN) are strong activators of both the innate and adaptive immune systems (456,457). Recognition of CpG-ODN is promoted by Toll-like receptor 9 (TLR9), a member of the TLR family, which is necessary to detect microbial pathogens. TLRs, preferentially expressed by immune system cells, are also identified on non-professional immune cells such as endothelial cells, fibroblasts, and epithelial cells (158,458). 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 extensive interest in the clinical development of these agents in the treatment of cancer patients (118,456,459). Recently in the laboratory where I conducted my thesis it has been 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 (388). 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.

It was also demonstrated that TLR9 ligand CpG-ODN treatment induces down-modulation of DNA repair genes in tumor cells of both murine and human origins. Expression-level analysis of proteins, RAD51, a key protein in the homologous recombination DNA repair pathway (460), and SIRT1, whose activity promotes homologous recombination (461), in human tumor cells confirmed microarray results. These proteins are involved in homologous recombination and, consequently, are relevant for the repair of interstrand cross-links, which are the most cytotoxic lesions induced by Cisplatin. Accordingly, the combination of Cisplatin and CpG-ODN against IGROV-1 human ovarian tumor xenografts in athymic mice was found to induce a remarkable increase in life span compared with that using either reagent alone ($P < 0.0001$).

Down-modulation of DNA repair genes induced by CpG-ODN treatment in tumor cells and up-modulation in immune cells might represent a physiologic phenomenon that occurs locally in the presence of an infectious event. Thus, upon detecting the presence of an infectious agent via endosomal TLRs, immune cells might regulate DNA repair genes to decrease their susceptibility to possible pro-apoptotic signals during infections and, at the same time, directly and/or indirectly induce modulation of DNA repair genes in infected (or transformed) cells to facilitate their death.

The first aim of this thesis was to highlight the mechanisms used by the TLR9 ligand to increase the sensitivity to Cisplatin, inducing in this way also the increase of survival in our *in vivo* experimental models. It has been speculated that miRNA can collaborate in this mechanism.

Emerging evidence suggests that miRNAs play important roles in the regulation of immunological functions, including innate immune responses of macrophages and the development, differentiation, and function of T and B cells (462;463). miRNAs are a ubiquitous feature of all cells, and functional studies prompted by the growing number of miRNA targets identified have demonstrated the involvement of miRNAs in the regulation of cellular process (464).

Changes in miRNA expression induced by TLR ligand stimulation have been broadly investigated for their impact on development and function of innate immune cells, the primary expressors of TLRs (465).

In this thesis we show that *in vivo* treatment with CpG-ODN, the TLR9 agonist, also induces modulation of several miRNAs in tumor cells.

Our analysis of 3 miRNAs (hsa-miR-424, hsa-miR-340 and hsamiR-302b) for their significance to chemotherapy response showed that the enforced expression of hsa-miR-302b on IGROV-1 cells significantly enhanced Cisplatin cytotoxicity.

Members of the hsa-miR-302 cluster (hsa-miR-302a, hsa-miR-302b, has-miR-302c, has-miR-302d, and has-miR-367) regulate self renewal and pluripotency processes in human embryonic stem cells (hESCs) (466). Has-miR-302b is poorly expressed in gastric tumor and it could be considered a better marker of pluripotency. Has-miR-302 expression is positive correlated with induction of pluripotency (ips) genes, including OCT4 variants, in gastric adenocarcinoma (467). Recently bio-informatic analysis showed that EGFR might be a target of has-miR-302b. Has-miR-302b was frequently down regulated, whereas EGFR was up-regulated in 27 pairs of clinical HCC and non-tumors counterparts. Hsa-miR-302b suppression of HCC growth may due to targeting the EGFR/AKT2/CCND1 pathway (468). Consistently with our *in vitro* data, we found that hsa-miR-302b expression was significantly associated to TTR (time to relapse) or OS (overall survival) in two datasets of ovarian cancer patients treated with platinum-based therapy. These findings indicate that has-miR-302b enhanced chemosensibility of human ovarian carcinoma cells and may represent a biomarker able to predict response to Cisplatin treatment, leading to a more accurate selection of patients potentially responsive to a specific therapy. Moreover, the correlation between miRNA expression and response to specific therapies also suggests the potential usefulness of miRNAs as therapeutic adjuvants. Our study starts using an *in vivo* model to select a candidate miRNA, then validated *in vitro* as adjuvant tool and in human samples as predictive biomarker. The integration of miRNA and mRNA expression profiles upon CpG-ODN treatment revealed a broad concerted interplay of miRNAs with their predicted target mRNAs, suggesting a relevant role for miRNAs in CpG-ODN-induced expression of genes involved in different cellular pathways. Concerning genes involved in DNA repair, miRNA-mRNA interaction analysis identified HDAC4 as a gene

potentially targeted by hsa-miR-302b, as then validated by the decreased HDAC4 mRNA and protein levels upon enforced hsa-miR-302b expression in IGROV-1 cells. Inhibition of HDAC has been reported to induce hyperacetylation of core histones and consequent relaxation of chromatin structure; such an open chromatin configuration would be expected to enhance accessibility of genomic DNA to drugs targeting DNA (470;471). These data have led to clinical studies using HDAC inhibitors in combination with current DNA damaging agents, such as topoisomerase inhibitors, DNA synthesis inhibitors, DNA intercalators and agents that covalently modify DNA, as treatment of several types of cancer (471;472). However, whereas clinical studies have shown efficacy against human hematologic malignancies, results in solid tumor trials have been insufficient because of some HDAC inhibitor limitations such as cardiac toxicity (473;474). The observation that over-expression of miR-302b increased the sensitivity of ovarian tumor cells to Cisplatin, together with the reported tissue specificity of miRNAs (475), raises the possibility of using this miRNA to modulate DNA-damaging drug sensitivity and avoiding HDAC inhibitor toxicity.

A very recent study reports direct regulation of p21 protein by members of the miR-302 family activated following DNA damage in human embryonic stem cells (401), further suggesting that miR-302 can impact the response to DNA-damaging agents by modulating different target molecules.

Notably, the human IGROV-1 cells, model used for our project, are negative for TLR9 and do not respond to murine CpG-ODN, therefore the activity of CpG-ODN is not mediated by direct contact between the cells of the immune system and the tumor, instead is likely mediated by TLR9-positive cells in the tumor microenvironment directly and/or through soluble factors. Several studies indicating that miRNAs can also be transferred between cells, e.g., through exosomes (417), as a mechanism to interact and exchange information raising the intriguing possibility that the immune system responds to CpG-ODN treatment by boosting miRNA modulation and interaction with tumor cells.

Interestingly, our new preliminary data seem to support this hypothesis, since human pre-miR-302b is not even detectable in IGROV-1 xenograft tumors, whereas the murine precursor is significantly induced in spleens of CpG treated mice. Considering these encouraging results, we are now exploring the possibility that mature miR-302b is secreted by TLR9 positive immune cells and delivered to the tumor by a MV-mediated mechanism. Beside the understanding of the molecular mechanism behind the “gap” between activation of the immune system by CpG and biological effect on tumor cells, our future plan is also to exploit MVs to deliver miR-302b to the tumor, as a new adjuvant therapeutic tool.

The second propose of this thesis was to study the combination of CpG-ODN with different agents to improve therapy of advanced ovarian cancer. 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 avoid adsorption of i.p. fluid by mechanical obstruction, inducing ascites (476). 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 (477), are disappointing (478). 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 (478,479). Advance tumor diseases are generally difficult to treat, in animal models and in clinical studies, in which treatment is initiated only after ascites become evident, generally show a small effect on survival. 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, therefore we screened the effectiveness of CpG-ODN in combination with different agents, such as Bevacizumab, Poly(I):Poly(C), Gefitinib, Cetuximab and Cisplatin. Bevacizumab binds the vascular endothelial growth factor (VEGF) that is reportedly overexpressed in ovarian cancer (439-441), and VEGF-regulated angiogenesis is a key component of ovarian cancer growth (442,443). The Poly(I):Poly(C) TLR3 agonist induces a synergistic effect when combined with TLR9 ligand by mediating an enhanced activation of innate immunity (444). Cetuximab and Gefitinib target the epidermal growth factor receptor (EGFR) frequently expressed in ovarian cancer cells (438). As seen in the results, anti-VEGF Bevacizumab did not enhance the effect of CpG-ODN treatment alone on survival. Recent preclinical and clinical data (438) suggest that targeting VEGF might suspend ascites formation, indeed in mice treated with the anti-VEGF antibody as a single treatment, it was observed an inhibition of ascites production. Altought Bevacizumab is able to control ascites formation, the combination with CpG-ODN does not improve mice survival already induced by CpG-ODN alone. Also the combination with Poly(I):Poly(C) did not induced a significant superior effect on median survival times compared with CpG-ODN treatment alone. This result was not in accordance with observations which demonstrated a clear synergy between the two immune modulators (444). This could be possibly due to the schedule of CpG-ODN administration, daily CpG-ODN administration might induce considerable innate cell activation hardly expandable by other immune modulators. The addition of

the EGFR tyrosine kinase inhibitor Gefitinib (Iressa) to repeated i.p. CpG-ODN treatment induced a small but not significant increase in lifespan versus mice treated with CpG-ODN alone, but a strong increase of median survival time was observed when Cetuximab, a monoclonal antibody anti-EGFR, were administrated to the mice. IGROV-1 cells express EGFR, but their growth has been showed to be independent from this receptor; indeed treatment with Cetuximab alone was able to induce only a slight increase of mice lifespan compared to control mice. Since EGFR inhibitors interact with Cisplatin (449-452) and recently it was reported that CpG-ODN has a synergistic antitumor effect in combination with Cisplatin (453), we have also investigated the therapeutic effect of the combination of CpG-ODN, Cetuximab and Cisplatin in mice selected for evident and established ascites. The association of CpG-ODN plus Cetuximab and Cisplatin revealed a significant increase in lifespan compared to the use of either reagent alone. Note that 70% of control mice became moribund 6 days after the start of treatment. Since the nude mice models, used for performing our experiments, have NK cells and macrophages as predominant immunological population, we hypothesize that CpG-ODN recruit and activate immune effectors cells at the site of tumor growth through ADCC. An increased death in Cetuximab-pretreated tumor cells compared to untreated cells was not observed; indeed the increase of the susceptibility of IGROV-1 cells after treatment with Cetuximab is not mediated by antibody–dependent cell cytotoxicity. Therefore we suppose that Cetuximab treatment would make IGROV-1 cells more robustly phagocytosed by macrophages. Performing a phagocytosis assay we have demonstrated that the tumor cells after 12 hours were completely incorporated by macrophages, clarifying that Cetuximab active the innate immune system that kill tumor cells by phagocytosis. Together these results point to a promising clinical strategy for treatment of ovarian cancer patients with bulky ascites using TLR9 agonists as immune-modulator in combination with approved drugs. These findings may contribute also to understanding the implication of immune cells in tumor microenvironment, and the involvement of miRNAs as alternative mechanism in enhancing sensitivity to chemotherapy after immunostimulation. Indeed our preliminary data, suggesting that miRNAs might be exploited by the activated immune system to affect gene expression in tumor cells, namely modulating DNA repair genes, and thus increasing sensitivity to chemotherapy agents, strongly support the hypothesis of a possible future clinical use of miRNAs in the management of ovarian cancer.

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