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***Antitumor activity of locoregional combined  
CpG-ODN therapy in experimental  
carcinoma models***

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# ***ABSTRACT***

Several studies in preclinical models have demonstrated the superior anti-tumor effect of CpG oligodeoxynucleotides (CpG-ODN) when administered repeatedly and locally rather than systemically (De Cesare M, et al. Clin Cancer Res 2008;14:5512-8; De Cesare M, et al. J Immunother 2010;33:8-15).

Based on those evidences, we evaluated aerosolized pulmonary delivery of CpG-ODN and its efficacy in treating established malignant lung lesions in two different murine tumors, the immunogenic N202.1A mammary carcinoma cells and the weakly immunogenic B16 melanoma cells. Upon reaching the bronchoalveolar space, aerosolized CpG-ODN activated a local immune response, as indicated by production of IL-12p40, IFN- $\gamma$  and IL-1 $\beta$  and by recruitment and maturation of DC cells in bronchoalveolar lavage fluid of mice. Treatment with aerosolized CpG-ODN induced an expansion of CD4<sup>+</sup> cells in lung and was more efficacious than systemic i.p. administration against experimental lung metastases of immunogenic N202.1A mammary carcinoma cells, whereas only i.p. delivery of CpG-ODN provided anti-tumor activity, which correlated with NK cell expansion in the lung, against lung metastases of the poorly immunogenic B16 melanoma. The inefficacy of aerosol therapy to induce NK expansion was related to the presence of immunosuppressive macrophages in B16 tumor-bearing lungs, as mice depleted of these cells by clodronate treatment responded to aerosol CpG-ODN through expansion of the NK cell population and significantly reduced numbers of lung metastases. Our results indicate that tumor immunogenicity and the tumor-induced immunosuppressive environment are both critical factors to the success of CpG therapy in the lung. These results indicated that aerosol delivery might be a valuable, practical approach to CpG-ODN therapy for lung tumors. Moreover, to mimic clinical treatment situations in advanced human ovarian disease, we tested the efficacy of CpG-ODN in combination with other therapeutic reagents in IGROV-1 ovarian carcinoma ascites-bearing athymic mice. Our results indicated that CpG-ODN and cetuximab combination therapy, enhancing the immune response in the tumor microenvironment and concomitantly targeting tumor cells, was 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 evidenced indicated a promising strategy to treat ovarian

cancer patients with bulky ascites. Despite an aggressive multimodal approach, more than 50% of patients with locally advanced SCCHN will relapse. The worse prognosis of those cancers must certainly be linked to the HNSCCs strong influence on the host immune system. We observed that the combination of cetuximab plus CpG-ODN led to a significant survival-time increase of IGROV-1 ovarian tumor ascites-bearing athymic mice, .as compared to single CpG-ODN or cetuximab treatments. Cetuximab is actually approved by FDA also to treat late-stage head and neck cancer. Based on those observations, we evaluated if the combined therapy with local and repeated CpG-ODN might improve the therapeutic efficacy of this monoclonal antibody. Our results indicated that the association of CpG-ODN was not able to significantly improve the antitumor effect of cetuximab administered as single agent.

# ***INTRODUCTION***

## **CLINICOPATHOLOGICAL FEATURES OF THE MAJOR TYPES OF LUNG CARCINOMAS**

Lung cancer (LC) is the second most common cancer in both men and women in Europe and in the United States. In particular, each year there are approximately 1,095,000 new cancer cases and 951,000 cancer-related deaths in men and 514,000 new cases and 427,000 deaths in women (Sant M. et al., *Eur. J Cancer* 2009;45:931-91) The main histological categories of lung cancer are non-small cells lung cancer (NSCLC), small-cell lung carcinoma (SCLC) and neuroendocrine tumours (NET) (Brennan P. et al., *Lancet Oncol.* 2010) NSCLC accounts for 85–90% of all LC (American Cancer Society; 2010) and includes three main types: squamous-cell carcinoma, adenocarcinoma, and large-cell carcinoma. The first two types represent about 80% of all LCs worldwide. Squamous cell carcinomas are also predominantly associated with a smoking history and tend to form large tumours in the center of the lung (Langer CJ et al., *J Clin Oncol* 2010). On the contrary, adenocarcinomas usually occur at the lung periphery. They can be subdivided into acinar, papillary, bronchiolo-alveolar carcinoma (BAC) and solid adenocarcinoma with mucin production. Mixed histologic patterns, however, are observed in the majority of cases. Adenocarcinoma is the most frequent type of lung cancer in non-smokers, however its incidence has been increasing in recent years also in smokers (Devesa SS et al., *Int J Cancer* 2005;117-294-9. Large-cell carcinomas are relatively rare (approximately 5% of LC) and show no evidence of squamous or glandular differentiation (Ginsberg MS. Et al., *Radiol Clin North Am* 2007;45:21-43). SCLC is the most aggressive lung tumour as a consequence of its high metastatic potential as compared to other forms of LC. The association with active smoking is evident since nearly all patients (over 95%) with SCLC are current or ex-smokers (Jackman DM, et al., *Lancet* 2005;366:1385–96). The lung is the second most common site of NET primary localization, following the gastro-enteric tract. The prognosis of lung NET is relatively more favourable compared to the previously described tumour types since NET

are characterized by a more indolent progression and lower proliferation rate (Modlin IM, et al., *Cancer* 2003;97:934–59; Gustafsson BI, et al., *Curr Opin Oncol* 2008;20:1–12).

### **Pharmaceutical management of lung cancer**

The present approaches to lung cancer treatment include: surgery, chemotherapy, radiation therapy, targeted therapy and immunotherapy. Over the past decade, cytotoxic agents including paclitaxel, docetaxel, gemcitabine, and vinorelbine have emerged to offer multiple therapeutic choices for patients with advanced NSCLC; however, these regimens provide only modest survival benefits as compared with cisplatin-based therapies. The standard treatment of limited-stage SCLC is concurrent cisplatin and etoposide with thoracic radiation therapy, which was shown to be beneficial compared with chemotherapy alone in 2 meta-analyses in the early 1990s. Treatment of extensive-stage disease is typically chemotherapy alone with a platinum compound plus etoposide. Topotecan is the only agent that is approved by the US Food and Drug Administration for use in relapsed SCLC; a related agent, irinotecan, also has activity in relapsed SCLC, with a response rate of 47% and a median survival of about 6 months, as observed in a small single-arm trial (Masuda N, et al., *J Clin Oncol* 1992;10:1225–9). Irinotecan has reasonably good tolerability, with low myelosuppression and controllable diarrhea. Topotecan has not been directly compared with irinotecan; however, irinotecan is another reasonable second-line treatment option in SCLC. Other agents that have some activity in relapsed SCLC but have not been extensively studied include cyclophosphamide/doxorubicin/vincristine, paclitaxel, docetaxel, gemcitabine, and vinorelbine (Jassem J, et al., *Eur J Cancer* 1993;29:1720–2; Masters GA, et al., *J Clin Oncol* 2003;21:1550–5; Smyth JF, et al., *Eur J Cancer* 1994;30A:1058–60; von Pawel J, et al., *J Clin Oncol* 1999;17:658; Yamamoto N, et al., *Anticancer Res* 2006;26:777–81).

### **Immunotherapy trials for the treatment of lung cancer**

In recent years, substantial progress in understanding of the mechanisms regulating immune responses in lung cancer has been made and new methods of immune therapy have been developed. A number of clinical trials have shown promise and a number of studies have demonstrated that a combined modality of cancer treatment can improve the outcome of

standard treatment. Since MUC1 is expressed on the cell surface of many common adenocarcinomas, including lung cancer, it was used in immune therapy. A phase I study, using a modified vaccinia virus (Ankara) expressing human MUC1, which also contains a coding sequence for human IL-2 (TG4010), revealed a safe toxicity profile and some clinical activity (Jassem J, et al., *Eur J Cancer* 1993;29:1720–2). In 2 phase I multicentre studies, including 4 patients with lung cancer (3 with NSCLC and 1 with SCLC), patients were immunized with a modified vaccinia virus expressing human MUC1. One of the lung cancer patients showed a marked decrease in the size of metastases. This antigen-specific immunotherapy was tolerated by all 13 patients without major side effects. The cytoplasmic domain of MUC1 (MUC1-CD), that induces tumorigenesis and resistance to DNA-damaging agents, was found to be associated with poor outcomes in patients with lung adenocarcinoma (Masters GA, et al., *J Clin Oncol* 2003;21:1550–5). More recently, the therapeutic vaccination with TG4010 and first-line chemotherapy was tested in a controlled phase II trial in 148 patients with advanced stage. Another novel approach of targeting MUC1 is the use of 86-base DNA aptamer (MA3) that binds to a peptide epitope of MUC1. In vitro experiments showed the specificity of MA3 for MUC1-positive tumors. An aptamer-doxorubicin complex was designed and was found capable to carrying the drug to the MUC1-positive tumor cells. Results of this study demonstrated that the drug intake, at the level of MUC1-negative cells, was significantly lower than in MUC1-positive cells (von Pawel J, et al., *J Clin Oncol* 1999;17:658). MUC1 has also been targeted in another trial of patients with NSCLC using the vaccine L-BLP25 (Stimuvax®) developed by EMD Serono Inc. and Merck. A multicentre study investigating the effect of vaccine in stage IIIB and IV NSCLC patients has initially demonstrated safety and a promising clinical effectiveness (Yamamoto N, et al., *Anticancer Res* 2006;26:777–81; Butts C, Murray N, et al., *J Clin Oncol* 2005;23:6674-6681). This vaccine is being tested in a phase III randomized, placebo-controlled trial in stage III NSCLC patients (Powell E, et al., *Expert Rev Respir Med* 2008;2:37-45). Another protein vaccination strategy aims at MAGE-3. First results, reporting the successful induction of humoral and cellular immune responses in patients with NSCLC following vaccination with MAGE-3 with and without adjuvant chemotherapy, were published in 2004 (Atanackovic D, et al., *J Immunol* 2004;172:3289-3296). A recent review of NSCLC vaccines suggested MAGE-A3 as one of the promising alternatives for adjuvant therapy in lung cancer (De Pas T, et al., *Crit Rev Oncol Hematol* 2012). In a phase



I study reported in 2011, vaccination of patients with a NY-ESO-derived peptide that includes multiple epitopes recognized by antibodies, CD4 and CD8 T, an increase in the titer of NY-ESO-1 antibodies was detected in nine out of ten patients. CD8 and CD4 T cells responded with distinct specificity in all patients. Two patients with lung cancer showed stable disease (Kakimi K, et al., *Int J Cancer* 2011;129:2836-2846). Two early-phase clinical trials using GM-CSF-secreting autologous tumor cells (GVAX) in patients with NSCLC have shown encouraging preliminary results. Salgia et al. (Salgia R, et al., *J Clin Oncol* 2003;21:624-630) reported the safety and feasibility of this approach in 33 advanced NSCLC patients with the most common toxicities limited to local injection site reactions and flu-like symptoms. A mixed response in one patient and long recurrence-free intervals in two other patients following isolated metastectomy were observed. In another phase I/II trial using the GVAX platform, autologous tumor cells were transduced with GM-CSF through an adenoviral vector (Ad-GM) and administered as a vaccine (Nemunaitis J, et al., *J Natl Cancer Inst* 2004;96:326-331). Seventy eight percent of patients developed antibody reactivity against allogeneic NSCLC cell lines. Three durable complete responses were observed. More recently, in a phase I/II trial on advanced-stage NSCLC, autologous tumor was mixed with an allogeneic GVAX vaccine. Although objective tumor responses were not seen, the evidence of vaccine-induced immune activation was demonstrated with minimal toxicity (Nemunaitis J, et al., *Cancer Gene Ther* 2006;13:555-562). A novel therapeutic approach, in patients with NSCLC, is the direct vaccination with messenger RNA (mRNA) encoding tumor antigens. This vaccine can induce immune response, consisting in antigen specific CD4+ and CD8+ T cells and B cells. Clinical data with promising results were obtained from a phase I/II trial (Fotin-Mleczek M, et al., *J Gene Med* 2012).

## **CLINICOPATHOLOGICAL FEATURES OF THE MAJOR TYPES OF OVARIAN CARCINOMAS**

During the 1990's, a number of advances were made in the histopathological classification of ovarian carcinomas (Seidman JD, et al., *Int J Gynecol Pathol* 2004;23:41-44). These include better recognition of patterns of metastatic carcinoma previously misinterpreted as

primary ovarian tumors, establishment of improved criteria for distinguishing invasive from non-invasive endometrioid and mucinous ovarian carcinomas, and interpretation of carcinosarcomas (malignant mixed mesodermal/Müllerian tumors) as carcinomas with areas of “sarcomatous” differentiation (or epithelial-mesenchymal transition), rather than sarcomas. In addition, primary peritoneal serous carcinomas and ovarian serous carcinomas are now considered essentially interchangeable for the purposes of diagnosis and treatment. These and perhaps other factors have altered the current histologic type and stage distribution of ovarian carcinomas compared to earlier case series. Seidman and colleagues recently analyzed the histologic type and stage distribution of 220 consecutive ovarian and peritoneal carcinomas. In Seidman’s series, nearly 70% of tumors were serous and fewer than 5% of these were confined to one or both ovaries (Stage 1) at diagnosis. Serous carcinomas typically display papillary or solid growth with slit-like spaces. Nuclear atypia is usually marked and mitotic activity abundant. Endometrioid adenocarcinomas account for 10–20% of ovarian carcinomas in most older reports, but in the more recent series of Seidman and colleagues, only 7% were endometrioid. These tumors have morphological features similar to their endometrial counterparts, showing varying quantities of overt gland formation, sometimes accompanied by squamous differentiation. In contrast to the serous carcinomas, over 50% of endometrioid adenocarcinomas are confined to the ovaries at diagnosis and nuclear atypia is usually less pronounced. Approximately 10% of ovarian carcinomas in the Seidman series showed clear cell differentiation. Several growth patterns (e.g., solid, papillary, tubulocystic) for clear cell carcinoma have been recognized. Although nearly one-third are Stage 1 at diagnosis, some studies have noted a relatively unfavorable prognosis of these tumors, even when corrected for tumor stage (Tammela J, et al., *Eur J Gynaecol Oncol* 1998;19:438–440; Sugiyama T, et al *Cancer* 2000;88:2584–2589). Finally, it is worth noting that mucinous carcinomas comprised fewer than 3% of primary ovarian carcinomas in the Seidman series and were almost always confined to the ovary at diagnosis. Mucinous adenocarcinomas also show overt gland formation, but in contrast to endometrioid adenocarcinomas, the tumor cell cytoplasm is mucin-rich. It should be kept in mind that many previous clinical and molecular analyses of mucinous adenocarcinomas were almost certainly compromised by inadvertent inclusion of metastatic adenocarcinoma to the ovaries (frequently from the gastrointestinal tract) misclassified as primary ovarian carcinomas (Hart WR. Et al., *Int J Gynecol Pathol* 2005;24:4–25). For many types of common adult solid

tumors, such as those of the colon, breast, and uterine cervix, the stages of neoplastic progression are fairly well defined and reflected by morphologically recognizable entities that represent a continuum including normal epithelium, preinvasive lesions, invasive carcinoma, and metastatic disease. In contrast, our understanding of the progression of ovarian carcinoma is incomplete, perhaps because we have only recently begun thinking about the different histological types of ovarian carcinomas as largely distinct disease entities.

### ***New concepts of ovarian tumor classification***

This “two pathway” scheme for ovarian endometrioid carcinoma pathogenesis is reminiscent of one previously proposed for ovarian serous carcinomas (Care A, et al., *Cancer Res* 2001;61(17):6532–6533; Smith-Sehdev AE, et al., *Am J Surg Pathol* 131 2003;27 (6):725–736). Low-grade serous carcinomas typically show micropapillary architecture, and often arise in association with recognizable precursors. The low grade serous carcinomas characteristically have mutations of KRAS or BRAF, but TP53 mutations are uncommon in these tumors. Mutations of KRAS or BRAF lead to constitutive activation of the MAPK (mitogen-activated protein kinase) signaling pathway. MAP kinases are serine/threonine-specific protein kinases that respond to extracellular stimuli (mitogens) and regulate various cellular activities, such as gene expression, mitosis, differentiation, and cell survival/apoptosis. Although the low-grade serous carcinomas tend to behave in a more indolent fashion than their high grade counterparts, some investigators have noted poor response of low-grade serous carcinomas to platinum-based therapeutic regimens. The great majority of serous carcinomas are high-grade and precursor lesions for these tumors remain poorly defined. Possible sites of origin include the ovarian surface epithelium, surface epithelial inclusion cysts, and the distal fallopian tube. High-grade serous carcinomas have a high prevalence of TP53 gene mutations, while mutations of KRAS or BRAF are rare. Collectively, studies of the type described above have led to the proposal of a new model for classifying ovarian carcinomas - in which the surface epithelial tumors can be divided into two broad categories designated Type I and Type II tumors based on their pattern of tumor progression and molecular genetic changes (Shih, IeM; Kurman, RJ. Et al., *Clin Cancer Res*

2005;11(20):7273–7279). Importantly, in this model, Type I and Type II refer to tumorigenic pathways and are not specific histopathologic diagnostic terms. Type I tumors include low-grade serous carcinoma, low-grade endometrioid carcinoma, mucinous carcinoma, and a subset of clear cell carcinomas, which develop in a stepwise fashion from well-recognized precursors, in most cases, borderline tumors. The borderline tumors, in turn, appear to develop from the ovarian surface epithelium or inclusion cysts in the case of serous and mucinous tumors and from endometriosis in the case of endometrioid and clear cell tumors. Most Type I tumors are slow growing as evidenced by the observation that they are generally large and often confined to the ovary at diagnosis. In contrast, the Type II tumors are high-grade and almost always have spread beyond the ovaries at presentation. Type II carcinomas include high-grade serous carcinoma, high-grade endometrioid carcinoma, undifferentiated carcinoma, probably some clear cell carcinomas, and malignant mixed mesodermal tumor (carcinosarcoma). Other than their association with endometriosis (in keeping with the Type I pathway), the clinicopathologic and molecular features allowing distinction of Type I from Type II clear cell carcinomas are yet to be defined. Type II carcinomas presumably evolve rapidly, disseminate early in their clinical course and are highly aggressive. In contrast to Type I tumors, Type II tumors are rarely associated with morphologically recognizable precursor lesions; however, Type II tumors may arise from “dysplasia” in inclusion cysts or serous intraepithelial carcinoma in the fallopian tubes (92-94). These precursor lesions may be difficult to recognize because they presumably undergo rapid transit from the occult lesion to a clinically diagnosed carcinoma. Type I and Type II tumors have very different molecular profiles. Chromosomal instability levels, as reflected by genome-wide changes in DNA copy number, are much higher in Type II tumors than in Type I tumors. Type I tumors often harbor somatic mutations of genes encoding protein kinases including KRAS, BRAF, PIK3CA and ERBB2, and other signaling molecules including CTNNB1 and PTEN. In contrast, Type II tumors generally lack these mutations but are characterized by a high frequency of TP53 mutations which are rare in Type I tumors. The division of ovarian cancer into two broad groups, Type I and Type II, continues to emphasize the heterogeneity of ovarian cancers, but also provides a morphological and molecular framework for future studies aimed at improving our understanding of ovarian cancer pathogenesis and developing more effective strategies for their early detection. Previous attempts to improve early diagnosis of ovarian cancer were based on the

assumption that ovarian cancer represents one disease when, from a pathogenetic view, it is at least two - with each having different implications for early detection. Current strategies are largely aimed at detecting low stage tumors. These strategies are most suitable for detecting tumors belonging to the Type I group, which often present as large tumor masses without dissemination at the time of diagnosis. A more useful endpoint for early detection of Type 2 ovarian carcinomas may be low tumor volume rather than low tumor stage. Importantly, it is well recognized that the most important prognostic indicator for Type 2 tumors is not stage at diagnosis, but the volume of residual disease following cytoreductive surgery (Bristow RE, et al., J Clin Oncol 2002;20(5):1248–1259). The published studies should prompt practicing pathologists to strongly consider the merits of a two-grade system for assigning tumor grade to ovarian carcinomas, rather than the traditional three-grade system used for most tumors. Indeed, two-tiered systems for grading serous carcinoma, the most common type of ovarian carcinoma, have already been proposed (10,11). Although uncertainties remain regarding the specific criteria that should be used to distinguish Type 1 from Type 2 ovarian carcinomas, careful analysis of both morphological and molecular features should help in determining which criteria are most appropriate.

### **Pharmaceutical management of ovarian cancer**

Currently, the standard therapy involves combining maximal cytoreductive surgery with chemotherapy that consists of a platinum agent and a taxane compound (Bristow RE, et al., J Clin Oncol 2002 20(5):1248–1259). The response rate of the standard regimen for ovarian cancer exceeds 80 %. However, more than 70 % of patients with advanced ovarian cancer experience recurrence within five years and eventually die because their cancer becomes resistant to platinum and taxane (Heintz AP, et al., Int J Gynaecol Obstet 95(Suppl 1):S161–S192). Most of these patients are subsequently treated with other agents, such as liposomal doxorubicin, gemcitabine, topotecan, or etoposide. The overall response rates to these other drugs, however, are only 10–25 %, with a relatively short duration (Agarwal R, et al Nat Rev Cancer 3:502–516). Therefore, novel treatment strategies are needed to improve outcomes for patients with advanced and recurrent ovarian cancer. Recent advances in studies about

the molecular biology of cancer and carcinogenesis have led to a variety of targeted agents, which affect tumor cells, tumor stroma, tumor vasculature, and cellular signaling mechanisms that are aberrant in tumor tissue. The majority of targeted agents are monoclonal antibodies and small-molecule protein-kinase inhibitors.

### **Immunotherapy of ovarian cancer**

Although the cancer cells remain the main objective of the research of new cancer therapies, it is gaining more and more the idea that the tumor microenvironment provides a fundamental support to the growth of the tumor and therefore also it could be an important target for anticancer therapy. A clear example of the influence of the tumor microenvironment is represented by the anti-angiogenic therapy, that has produced great clinical outcomes. Other mechanisms very important in the maintenance of the tumor microenvironment are the immune response and inflammation. In the tumor microenvironment the inflammatory process is directed to the maintenance of tumor cell survival, promotion of angiogenesis and evasion of the immune response. Tumors generally evade the immune response through several mechanisms including the negative modulation of MHC class I, the selection of cells that do not express tumor antigens , production of substances with immunosuppressive action and the induction of tolerance against tumor antigens . The tumor immunotherapy aims to enhance the immune response against tumors or to provide patients with anti-tumor effector cells or molecules, while also reducing the toxicity on normal cells, typical of cytotoxic therapies available today. This feature makes immunotherapy tumor therapeutic strategy of great interest especially for highly immunogenic tumors such as gynecologic tumors. The prototype of the immunogenic tumors can be identified in cervical cancers and in those of the lower genital tract induced by human papilloma virus (HPV), whose viral antigens expressed by the cancer cells are easily recognized by the immune system. Statistics show that the prevalence of HPV related cancers is higher in patients with a reduced ability to provide cellular immune responses , such as patients with HIV infection and patients undergoing a transplant (Laga, M., et al. Int J Cancer. 1992 50, 45-48). In clinic many cases of patients suffering from gynecologic cancers that have developed spontaneously antitumor immune response have been

documented. For example, in patients with advanced ovarian cancer at diagnosis, it was found the presence of antibodies and tumor-reactive T lymphocytes in the peripheral blood, while from the tumor masses and ascites oligoclonal reactive T cells were isolated (Hayashi, K., et al Gynecol. Oncol. 1999 74, 86-92). It has also been demonstrated that a very important factor for the prognosis of ovarian tumors is the presence of tumor infiltrating lymphocytes (TIL), in fact, the presence of TIL is predictive of a significant survival of patients with ovarian cancer. A study conducted in a cohort of Italian patients with EOC (epithelial ovarian cancer) showed that patients with tumor -enriched intraepithelial T cells showed a progression-free survival 3.8 times higher than in patients with detectable TIL. In the latter, the 5-year survival is assessed to 4.5% , while it was 38 % in patients whose tumors showed an infiltrate of T cells ( n = 102). This finding has been confirmed by other studies in different ethnic populations (Pujade-Lauraine, E., et al J. Clin. Oncol. 1996 14, 343-350). The presence of a spontaneous antitumor immune response in many patients with gynecologic cancers, although it is not sufficient to remove the tumor, is a starting point for the development and strengthening of immunomodulatory therapies alternative to traditional approaches . The immunotherapeutic strategies today in development include the active immunization with vaccines (for example anti-HPV vaccines), the administration of cytokines and adoptive immunotherapy with T cells. As regard active immunization , the main limitation of the development of vaccines against the EOC is the lack of tumor-specific antigens (except those resulting from infection of HPV). On the contrary it is becoming increasingly common, even in clinical trials, the use of cytokines and TLR agonists capable of inducing nonspecific immune activation to enhance the anti-tumor response.

## ***Enhancement of anti-tumor response***

### INTERFERONS

Interferons have been described initially as antiviral cytokines, but it was then shown that they are also secreted in response to a large number of immunostimulants factors. These molecules are divided into two broad categories: type I and type II. The first category is divided into two classes:  $\alpha$  and  $\beta$  . 12 forms of IFN-  $\alpha$  have been reported, and only one form of IFN-  $\beta$  . Interferons are known especially for their antiviral activity, but they also have an important effect on cell proliferation. Various clinical trials have demonstrated the efficacy

of type I interferons in the treatment of hematologic malignancies in melanoma and renal cell carcinoma (Colombo, N., et al. Crit Rev. Oncol Hematol. 2006 60, 159-179). Some phase I / II studies also examined the therapeutic efficacy of interferons in ovarian cancer. The intraperitoneal administration of recombinant IFN-  $\alpha$  as single agent or in combination with cisplatin in patients with ovarian carcinoma already treated with chemotherapy, was found to be effective in patients with a disease of small size, sensitive to treatment with platinum. This therapy revealed no efficacy for cancer patients resistant to platinum therapy . Although encouraging, these data were not sufficient to support the introduction of type I interferons in the treatment protocols of this type of cancer. One of the main limitations of the use of interferons is the high concentration of cytokine required to induce an antitumor response. Given the short half-life of recombinant proteins, high levels of cytokine can't be achieved without encountering a systemic toxicity. An alternative would be represented by gene therapy using viral vectors that allow to achieve and sustain high concentrations of cytokine at the level of the tumor site, without causing systemic toxicity. At the University of Pennsylvania has recently completed a gene therapy trial involving the production of IFN-  $\beta$  by a recombinant adenovirus. The toxicity recorded was minimal and it was observed in a patient with a low-grade ovarian carcinoma resistant to platinum therapy . These promising results demonstrate the potential ability of IFN -  $\beta$ , as an antitumor agent and its use in combination with other forms of chemotherapy and immunotherapy. IFN-  $\gamma$  is structurally different from the interferons of type I. It is produced by NK cells and activated T lymphocytes following the recognition of the target, and it is also able to convert pre- cytotoxic T cells in CTL . It also upregulate the expression of MHC class I and class II in ovarian cancer cells , unlike IFN-  $\alpha$  that acts only on the expression of MHC I molecules (Freedman, R. S., et al Clin Cancer Res.2000 6, 2268-2278). IFN-  $\gamma$  also increases the frequency of activation of T lymphocytes against malignant cells, and intensifies lymphocytic infiltration , an important predictor of survival. In vitro, this molecule showed a direct antiproliferative activity on ovarian cancer cells, which could synergize with the action of cisplatin and doxorubicin. Several trials conducted using (rh) recombinant human IFN-  $\gamma$  have reported encouraging results both in monotherapy in the treatment in combination with other pharmacological agents (Pujade-Lauraine, E., et al.J. Clin. Oncol. 1996 14, 343-350). In a multicenter phase III study conducted in Europe , there was a prolongation of progression-free survival 3 times higher in patients who had received rhIFN



-  $\gamma$  subcutaneously in combination with cisplatin MTD ( maximum tolerated dose ) and cyclophosphamide, compared to patients treated with conventional therapy. On the contrary, a study in the USA showed instead that the addition of rhIFN -  $\gamma$  to chemotherapy based on paclitaxel and carboplatin does not improve survival. These different results may be partially explained by demographic and racial differences between the two clinical trials. However , it would seem that the choice of chemotherapy drugs is a crucial factor in approaches combined with the immunological therapies.

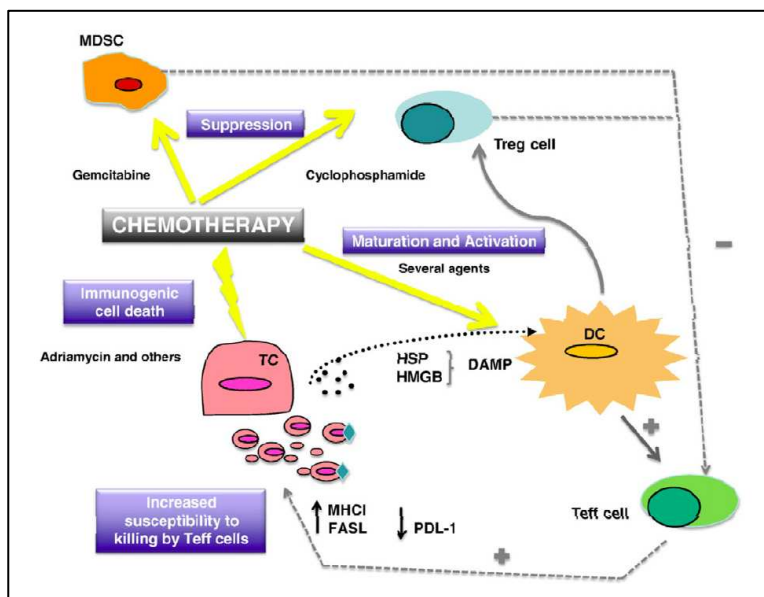
### INTERLEUKINS

Interleukin 2 (IL -2 ) promotes first clonal expansion and the cytotoxic activity of immune effector cells, and it is also able to restore the function of T cells following the suppression induced by negative regulators, as for example the activation of the receptor PD-1. This molecule is one of the main cytokines studied in the context of anticancer therapy , thanks to the important results obtained in the treatment of melanoma and renal cell carcinoma that led to its approval by the FDA. However, the use of IL- 2 in clinical trials shows many limitations. In monotherapy and in adoptive immunotherapy in fact, IL- 2 is used to the maximum tolerated dose (MTD) leads to serious manifestations of toxicity, including a systemic inflammatory response syndrome, capillary leak and multi organ toxicity that primarily affects the heart, kidneys, lungs and central nervous system. Because the tumor of the ovary is able to generate a response antitumor immune spontaneously, IL- 2 could be used in order to enhance pre -existing immunity. In a phase I / II study of 41 patients with recurrent ovarian cancer, IL-2 was administered every week by ip infusions . The treatment was well tolerated and demonstrated long-term efficacy in a small number of patients , for example 20% of the patients , after careful and repeated observations of the abdomen , showed a pathological complete response and no sign of residual disease . Interleukin- 2 is essential for the homeostasis of peripheral CD4 + CD25 + Treg cells FoxP3 +, and is also important for the suppressive activity of Treg in vivo. Following the completion of treatment with IL-2, the number of Treg cells is lowered more in patients who experienced a clinical response compared with non- responder . In light of these data, it is supposed that the patients with functional T lymphocytes, with a low prevalence of Treg lymphocytes, with a high immune reactivity towards the tumor, are those who derive greater benefit from monotherapy with IL- 2. Alternate the administration of different cytokines, that support

selectively the activation of effector cells without promoting the proliferation of Treg cells, may be an additional effective therapeutic strategy . Among the cytokines that could potentially replace IL-2 can be taken into account IL-7 , IL- 15 , IL- 18 , IL-21 , but their function and their clinical use is still under investigation. The function of IL-7 has never been fully appreciated until recently. It plays an essential role both in lymphopoiesis and in the activity of T cells and in the maintenance of anti-tumor immunity. A recent study on a mouse model of lung cancer examined the effects of administration of IL-7 and found a significant reduction in the spread of the tumor, correlated with an increase in CD4 + and CD8 + T cells (Andersson, A., et al J Immunol. 2009 182, 6951-6958). Functions similar to IL- 2 were also held by IL -15 . It is also able to enhance the maturation of NK cells and their activity . Interleukin 21 is a promising cytokine because it can promote the cytolytic activity of CD8 + lymphocytes and NK cells , to modulate the activity of CD4 + T cells and B lymphocytes and simultaneously reduce Treg cells . Interleukin 18 is a new cytokine that has been shown to have very powerful immunostimulatory effects , including the induction of IFN-  $\gamma$  , TNF -  $\alpha$  , IL- 1 $\beta$  and GM - CSF , the activation of effector T lymphocytes and the promotion of Th1 responses critical to tumor rejection . In a study conducted in a mouse model transplanted with human peripheral lymphocytes recent use of recombinant human IL- 18 allowed the expansion of effector T cells and the reduction of Treg cells (Carroll, R. G., *et al. PLoS. ONE.* 2008 3, e3289). In a phase I study, recombinant human IL-18 was administered as monotherapy in 28 patients with solid tumor with a very low toxicity, to the point that during the trial was never reached the maximum dose tolerated by the body. IL- 18 also increases the activation of NK , monocytes and CD8 + peripheral T lymphocytes and induces a transient increase in the frequency and in the levels of expression of FasL in CD8 + cells and NK cells. The lower toxicity compared to other cytokines makes IL- 18 a potential drug to be used in combination with chemotherapeutics approaches. In mice with advanced ovarian cancer , the administration of interleukin 18 showed modest antitumor effect on immunity, while in combination with chemotherapy its immunostimulating effects are significantly higher. The use of a therapy based on a the -18 in combination with other chemotherapeutic agents will be subjected to further studies. Currently a phase I trial is ongoing.

## **Chemotherapy as immunomodulator**

It has always been believed that chemotherapy contrasts immune mechanisms, however recent studies have modified this traditional view. In fact, drugs such as cyclophosphamide, doxorubicin and paclitaxel increase the number and function of antigen-specific T cells, thereby enhancing antitumor immunity. In immunocompetent animal models, increased tumor inflammation following the administration of chemotherapy predicts a better prognosis, while tumors growing in immunodeficient mice often do not respond to chemotherapy. These evidences clearly demonstrate an important role of the immune system in preventing the growth of a tumor which has already undergone cytotoxic therapy. Similar results have been observed in humans. For example, the presence of tumor-infiltrating lymphocytes is predictive of pathologic complete response in patients with metastatic breast cancer after neoadjuvant chemotherapy (Hornychova, H., *et al. Cancer Invest.* 2008 26, 1024-1031). The immunomodulatory effects of chemotherapy can be divided into three mechanisms: 1) induction of tumor cells, that facilitates the presentation of tumor antigens, 2) direct activation of effector cells and antigen presenting cells, and 3) suppression of immune inhibitory cells (Figure 1). These mechanisms are very complex and the current knowledge are still at an early stage. The effects of those mechanisms appear to depend on the type of chemotherapeutic drug administered, the dose and schedule of administration, as well as on the type of immune cells involved.



**Figure 1.** Schematic representation of immunomodulation mediated by chemotherapy

## **CLINICOPATHOLOGICAL FEATURES OF THE MAJOR TYPES OF HEAD AND NECK CARCINOMAS**

Head and neck cancer refers to a group of biologically similar cancers that start in the lip, oral cavity (mouth), nasal cavity (inside the nose), paranasal sinuses, pharynx and larynx. Ninety percent of head and neck cancers are squamous cell carcinomas (SCCHN) (<http://www.macmillan.org.uk/Cancerinformation/Cancertypes/Headneck/Aboutheadneckcancers/Typesofheadneckcancer.aspx>) originating from the mucosal lining (epithelium) of these regions. Head and neck cancers often spread to the lymph nodes of neck and this is often the first (and sometimes only) sign of the disease at the time of diagnosis. Head and neck cancer is strongly associated with certain environmental and lifestyle risk factors, including tobacco smoking, alcohol consumption, UV light, particular chemicals used in certain workplaces. Recent evidence pointing to a viral origin for some head and neck cancers are accumulating (Everett E. Head and Neck Cancer. 2007). Human papillomavirus (HPV), in particular HPV16, is a causal factor for some head and neck squamous cell carcinoma (HNSCC) (D'Souza, G The New England Journal of Medicine 2007). Approximately 15 to 25% of HNSCC contain genomic DNA from HPV (Kreimer, A. Cancer Epidemiology, Biomarkers & Prevention 2005) and the association varies based on the site of the tumor, In particular, HPV-positive oropharyngeal cancer, with highest distribution in the tonsils, where HPV DNA is found in (45 to 67%) of the cases (Perez-Ordoñez, B Journal of clinical pathology 2006) less often in the hypopharynx (13%–25%), and least often in the oral cavity (12%–18%) and larynx (3%–7%) (Paz, I. B Cancer 1997) Some experts estimate that while up to 50% of cancers of the tonsil may be infected with HPV, only 50% of these are likely to be caused by HPV (as opposed to the usual tobacco and alcohol causes). The presence of acid reflux disease (GERD – gastroesophageal reflux disease) or larynx reflux disease can also be a major risk factor. In the case of acid reflux disease, stomach acids flow up into the esophagus and damage its lining, making it more susceptible to throat cancer. Patients after hematopoietic stem cell transplantation (HSCT) are at a higher risk for oral squamous cell carcinoma. Post-HSCT oral cancer may have more aggressive behavior with poorer prognosis, when compared to oral cancer in non-HSCT patients. This effect is

supposed to be owing to the continuous lifelong immune suppression and chronic oral graft-versus-host disease.

## **Pharmaceutical management of head and neck cancer**

The use of surgery, radiation, and/or chemotherapy depends on tumor respectability and location, as well as whether an organ preservation approach is feasible (National Comprehensive Cancer Network. NCCN clinical practice guidelines in oncology: head and neck cancers. Vol. 2, 2008. [Accessed in Jan 30 2013]) The main treatment option for primary and secondary malignancy as well as recurrent disease is surgical therapy (Boehm A, et al., HNO 2010;58(8):762-9). The use of transoral laser assisted surgery followed by radiotherapy is a common practice in the treatment of early stage oropharyngeal, hypopharyngeal and supraglottic carcinomas. On the other hand early glottic carcinomas show excellent oncologic results after single modality treatment. Transoral laser surgery is the treatment of choice but radiotherapy is also a good alternative. In general, there are 3 main approaches to the initial treatment of locally advanced disease:

- 1) concurrent platinum-based chemoradiation, with surgery reserved for residual disease
- 2) surgery with neck dissection and reconstruction, followed by adjuvant radiation or chemoradiation, depending on the presence of adverse risk factors
- 3) induction chemotherapy followed by definitive chemoradiation and/or surgery

Approximately 60% of patients with HNSCC present at a locally advanced stage, in which combined modality therapy with curative intent is recommended (Shin DM, et al., Head Neck. 2011). Cisplatin remains the cornerstone of treatment in recurrent and metastatic HNSCC. Moreover, postoperative concurrent administration of high-dose cisplatin with radiotherapy is more efficacious than radiotherapy alone in patients with locally advanced HNSCC and does not cause an undue number of late complications (Bernier J, et al., N Eng J Med. 2004;350(19):1945-52). Data shows that radiation therapy combined with simultaneous 5-fluorouracil (5-FU), cisplatin, carboplatin, and mitomycin C as single drug or combinations of 5-FU with one of the other drugs results in a large survival advantage irrespective to the employed radiation schedule. If radiation therapy is used as single modality, hyperfractionation leads to a significant improvement of overall survival.

Accelerated radiation therapy alone, especially when given as split course radiation schedule or extremely accelerated treatments with decreased total dose, does not increase overall survival (Budach W, et al., BMC Cancer. 2006;6:28). The taxanes docetaxel and paclitaxel (Taxol®) are also active molecules in HNSCC therapy. Several phase II studies have indicated that adding a taxane improves responsiveness to 5-FU based induction chemotherapy. Results of a randomized phase III trial that compared induction chemotherapy using docetaxel and 5-FU together with 5-FU alone indicated that incorporation of a taxane substantially improves clinical response and survival in locally advanced head and neck cancer. However, paclitaxel may develop neurotoxicity and become problematic, particularly when used in combination with other neurotoxic agents such as cisplatin (Oncologist. 2005;10Suppl 3:11-9). Regarding to radiotherapy (RT), RT intensity modulated (IMRT) has increasingly been shown to be advantageous compared with traditional techniques such as conventional RT (2D) and conformation (3D), in that it provides a more homogeneous coverage of dose to the target volume and a decrease in the dose in the surrounding tissues. The highest dose is related to a better tumor control and better survival rates (Duprez F, et al., Radiother Oncol. 2009;93(3):563-9). Also hyperfractionated radiation therapy can be utilized in patients with HNSCC. However, this treatment option can develop reaction of different intensities in the mucosa, as oral mucositis, that causes significant pain, chewing and swallowing difficulties and is considered the most debilitating acute reaction during head and neck cancer treatment. The use of brachytherapy treatment in patients with HNSCC, that uses radiation sources in direct contact with the tissues to be irradiated, increases the risk to develop soft tissue necrosis, which may be defined as an ulcer located in the radiated tissue, without the presence of residual malignancy (Jham BC, et al., Otorhinolaryngol. 2006;72(5):704-8).

### **Immunotherapy of head and neck cancer**

Several immunotherapeutic strategies have been investigated in HNSCC patients, such as skewing the immune system by using monoclonal antibody or cytokines approaches. Several cell-based therapies also show potential as adjuvant treatment for HNSCC patients.

#### *PASSIVE IMMUNOTHERAPY:*

*Antibody approaches* For HNSCC patients, the present focus is on antibodies targeting the Epidermal Growth Factor Receptor (EGFR), since in 90% of HNSCCs the EGFR is overexpressed (Kalyankrishna S, et al., *J. Clin. Oncol.* 2006 24(17), 2666–2672; Grandis JR, et al., *Cancer Res.* 1003 53(15), 3579–3584). Triggering of the EGFR by EGF activates pathways leading to proliferation, survival and metastasis of HNSCC (Cassell A, et al., *Expert Opin. Invest. Drugs* 2010 19(6), 709–722). Cetuximab and panitumumab are clinically approved anti-EGFR antibodies used to treat HNSCC patients, characterized by similar working mechanisms. Cetuximab is a human–mouse chimeric monoclonal antibody, whereas panitumumab does not contain murine components. Binding of these monoclonal antibodies results in a downregulation of EGFR expression (Saltz L, et al., *N. Engl. J. Med.* 354(6), 567–578 (2006). In a randomized study with 424 patients, cetuximab in combination with radiotherapy was shown to increase overall survival of patients with locoregionally advanced HNSCC. Patients receiving radiotherapy alone had an overall survival of 29 months, whereas patients receiving radiotherapy in combination with cetuximab show an overall survival of 49 months. Instead of directly targeting tumor cells, the vascular system of the tumor can also be targeted, resulting in antitumor effects. Bevacizumab is a humanized VEGF antibody which has been theorized to increase the effectiveness of chemotherapeutics (Kundu SK, et al., *Nestor. Tumour Biol.* 33(3), 707–721 (2012). Bevacizumab is being tested in HNSCC patients in combination with chemotherapy (Seiwert TY, et al., *J. Clin. Oncol.* 26(10), 1732–1741 (2008); Lee NY, et al., *Lancet Oncol.* 13(2), 172–180 (2012).

#### *ACTIVE IMMUNOTHERAPY:*

The cytokine most investigated for immunotherapy is IL-2; several trials have been performed for HNSCC (Clayman GL, et al., *Ann. Otol. Rhinol. Laryngol.* 1992 101(11), 909–915); Dadian G, et al., *Eur. J. Cancer B Oral Oncol.* 1993 29B(1), 29–34); De Stefani A, et al., *J. Immunother. Emphasis Tumor Immunol.* 1996 19(2), 125–133); Mantovani G, et al., *Biotherapy* 1994 8(2), 91–98); Mantovani G, et al., *Cancer Immunol. Immunother.* 1998 47(3), 149–156); Recchia F, et al., *J. Immunother.* 2008 31(4), 413–419); Timar J, et al., *J.*

Clin. Oncol. 2005 23(15), 3421–3432). Low doses of IL-2 have been evaluated in a Phase III trial where patients were given either chemotherapy alone or in combination with low-dose IL-2. However, in this trial, no differences were found between the patient group receiving chemotherapy alone compared with the group receiving chemotherapy in combination with IL-2 (Mantovani G, et al., *Cancer Immunol. Immunother.* 1998 47(3), 149–156). Instead of a single recombinant cytokine, a mix of cytokines named IRX-2 has been also tested on HNSCC patients (Meneses A, et al., *Arch. Pathol. Lab. Med.* 1998 122(5), 447–454). It was injected intralymphatically and resulted well tolerated by HNSCC patients increasing their survival (Freeman SM, et al., *Am. J. Clin. Oncol.* 2011 34(2), 173–178); Hadden J, et al., *Int. Immunopharmacol.* 2003 3(8), 1073–1081); Barrera JL, et al., *Arch. Otolaryngol. Head Neck Surg.* 2000 126(3), 345–351). Berinstein *et al.* demonstrated increased numbers of TIL in patients following IRX-2 treatment. High lymphocyte immune infiltrate correlated with decreased tumor size and improved 5-year survival (Berinstein NL, et al., *Cancer Immunol. Immunother.* 2011 61(6), 771–782). Currently, the administration of recombinant IFN- $\alpha$ , IL-12 and GM-CSF are also being tested for use as adjuvant treatment in HNSCC patients (Schutt C, et al., *Int. Rev. Immunol.* 2012 31(1), 22–42); Rapidis AD, et al., *J. Oncol.* 2009, 346345). High immune infiltrate in HNSCC tumors resulted to be correlated with a relatively good prognosis (Watanabe Y, et al., *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* 2010 109(5), 744–752; Badoual C, et al., *Clin. Cancer Res.* 2006 12(2), 465–472; Wansom D, et al., *Laryngoscope* 2012 122(1), 121–127). In a study by Wansom *et al.*, increased levels of TIL were correlated with a good prognosis (Wansom D, et al., *Laryngoscope* 2012 122(1), 121–127). However, TIL levels were not found to be increased in HPV-positive tumors, compared with HPV-negative tumors. In other studies it has been found that patients with HPV-positive tumors have increased circulating CD8+ T cells compared with patients with HPV-negative tumors (Wansom D, et al. *Arch. Otolaryngol. Head Neck Surg.* 2010 136(12), 1267–1273; Turksma AW, et al., 2012 *Oral Dis.*). Furthermore, T cells specific for HPV16 have been found around the tumor and in the peripheral blood of patients with HPV16-positive head and neck tumors (Heusinkveld M, et al., *Int. J. Cancer* 2012 131(2), e74–e85). Increased immunogenicity of HPV-positive tumors may, in part, be explained by HPV-specific responses. Since persistent infection of high-risk HPV may ultimately lead to the formation of malignant tumors, use of HPV vaccinations might be considered to prevent the development of tumors. Prophylactic



vaccines have been developed and marketed in recent years for the prevention of genital warts and cancer of the cervix, penis and anus (Szarewski A. et al., *Expert Rev. Vaccines* 2012 11(6), 645–657; Goldstone SE, et al., *Expert Rev. Vaccines* 2012 11, 395–406). At present, young girls are vaccinated to prevent persistent HPV infections. In view of the increase in HPV-positive tumors in the head and neck region, it might also be worth the effort to vaccinate also young boys against high-risk HPV types. Another type of tumor in the head and neck region is nasopharyngeal carcinoma (NPC). Similar to HPV-positive tumors, a virus is associated with tumor genesis, in this case EBV. In the western world, the incidence of NPC is low; however, in south China and southeast Asia, the incidence is much higher. Nearly all undifferentiated NPC are associated with EBV infection (Chan AT. Nasopharyngeal carcinoma. *Ann. Oncol.* 21(Suppl. 7), 308–312 (2010). EBV antigens are attractive candidates as targets for immunotherapy since they are not expressed in healthy tissue. Viral antigens recognized by T cells include EBNA1, LMP-1 and LMP-2. In a Phase I study, autologous monocyte-derived DCs were pulsed with multiple LMP-2 peptides and injected into a lymph node. In nine of the 16 patients, LMP-2-specific T-cell responses were measured, resulting in two partial responses (Lin CL, et al., *Cancer Res.* 2002 62(23), 6952–6958). Clinical responses remained minimal, possibly because of the low expression of immunogenic virus proteins in the infected cells when the EBV is in its latent state. An appealing way to enhance immunotherapy in NPC patients is to reactivate the EBV virus, thereby enhancing immune recognition. Promising results were obtained in a pilot study where three patients with end-stage NPC received virus-activating drugs, which resulted in an increase of viral DNA levels in the circulation (Wildeman MA, et al., *Clin. Cancer Res.* 18(18), 5061–5070 (2012). The increase of viral DNA levels suggest the EBV is no longer latent and can therefore be more easily targeted.

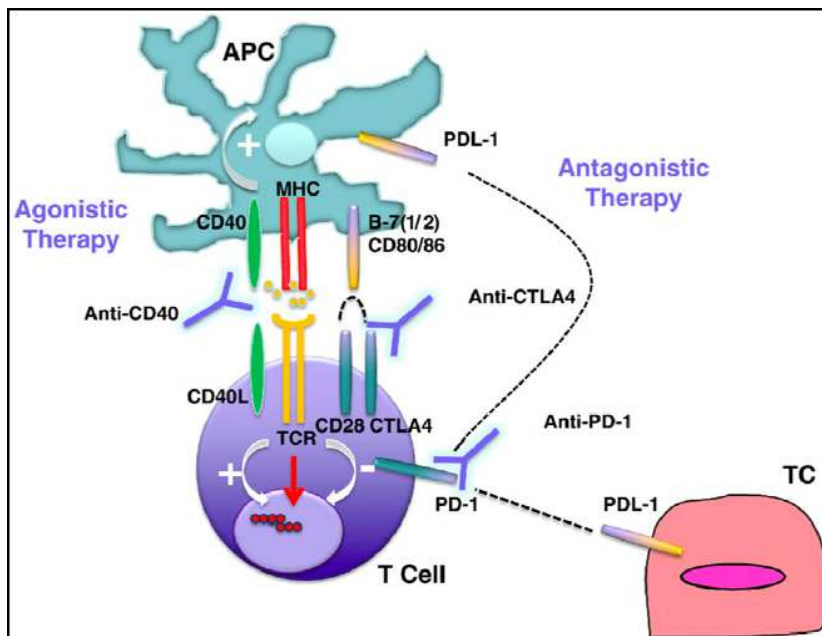
## **TOLL-LIKE RECEPTORS AND AGONISTS**

One of the most basic mechanisms for activation of the immune system is through the Toll-like receptors (TLRs). TLRs belong to the type I transmembrane receptor family. Their expression is ubiquitous, from epithelial to immune cells. The TLR family members are pattern recognition receptors that collectively recognize lipid, carbohydrate, peptide and nucleic acid structures that are broadly expressed by different groups of microorganisms. Some TLRs are expressed at the cell surface, whereas others are expressed on the membrane of endocytic vesicles or other intracellular organelles. There are at least 10 known TLRs in humans grouped in six major families, based on their phylogenetic background (Roach JC, et al., Proc Natl Acad Sci U S A 2005;102:9577–82). Each family is attributed to a general class of PAMPs. TLRs 3, 7, 8 and 9 are located mainly in endosomes; double-stranded RNA are ligands for TLR3 (Alexopoulou L, et al., Nature 2001;413:732–8), while TLRs 7 and 8 recognize single-stranded viral RNA (Heil F, et al., Science 2004;303:1526–9). The other TLRs are located on the cell surface (Peng G, et al., Science 2005;309:1380–4); TLRs 1, 2, 5, 6 and 10 respond to bacterial, fungal and viral PAMPs (Takeuchi O, et al., Int Immunol 2001;13:933–40; Takeuchi O, et al., J Immunol 2002;169:10–4; Hayashi F, et al., Nature 2001;410:1099–103). Lipopolysaccharides are TLR4 ligands (Yang H, et al., J Biol Chem 2000; 275:20861–6). 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 (Spaner DE, et al., Leukemia 2007;21:53–60). One pathway involves the MyD88-independent production of type I interferons. The second uses MyD88 to activate nuclear factor-kappa B (NF- $\kappa$ B), JUN kinase (JNK) and p38, finally resulting in the production of proinflammatory cytokines such as TNF- $\alpha$ , IL-12 and IL-1 and induction of innate effector mechanisms (Ausubel FM. Nat Immunol 2005;6:973–9; Iwasaki A, et al., Medzhitov Nat Immunol 2004;5:987–95). Additionally, TLR triggering induces DC maturation, which leads to the upregulation of costimulatory molecules such as CD40, CD80 and CD86, and secretion of immune modulatory cytokines and chemokines. In addition, TLRs can directly stimulate the proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells as well as reverse the suppressive function of Treg cells (Crellin NK, et al., J Immunol 2005;175:8051–9; Tabiasco J, et al., J Immunol 2006;177:8708–13). Adding TLR 3, 4, 7 or 9 ligands was shown to activate CD8<sup>+</sup> cytotoxic T cells with increased IFN- $\gamma$  production and to promote a stimulatory cytokine milieu at the tumor

microenvironment (Hamdy S, et al., *Vaccine* 2008;26: 5046–57; Ramakrishna V, et al., *J Transl Med* 2007;5:5). Optimal antitumor immunity requires robust enhancement of the effector T-cell response induced by tumor antigenic peptides and control or elimination of Treg 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 (Manegold C, et al., *J Clin Oncol* 2008;26:3979–86), non-Hodgkins lymphoma (Link BK, et al., *J Immunother* 2006;29:558–68.149; Leonard JP, et al., *Clin Cancer Res* 2007;13:6168–74), glioblastoma (Carpentier A, et al., *Br J Dermatol* 2003;149(Suppl 66):53–6) and superficial basal cell carcinoma (Stockfleth E, et al. *Br J Dermatol* 2003;149 (Suppl 66):53–6). 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. 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 (Lesimple T, et al., *Clin Cancer Res* 2006;12:7380–8). 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 (Vakkila J, et al., *Nat Rev Immunol* 2004;4:641–8). Thus, combinations with active immunization or adoptive immunotherapy seem ideal, as these approaches greatly benefit from concomitant activation of innate immune response. If combination with chemotherapy is designed, it seems rational to combine TLR agonists with chemotherapy drugs that can activate cellular immune mechanisms. Finally, the choice of TLR agonists may matter. Whereas TLR 3 and 9 agonists induce apoptosis of TLR-expressing tumor cells (273) TLR4 agonists were shown to promote tumor cell survival, tumor growth and paclitaxel resistance in a proportion of ovarian cancer cells (Kelly MG, et al., *Cancer Res* 2006;66:3859–68 Kim KH, et al., *J Transl Med* 2009;7:63).

## Activation of cellular immunity

Generation of a successful antitumor adaptive immune response requires first and foremost the primary signal provided by the binding of T-cell receptor to cognate tumor antigen. However, multiple secondary signals can activate or suppress this response. Characterization of these pathways in tumors and the development of specific agonistic or antagonistic antibodies or ligands have created new opportunities for powerful stimulation of antitumor immune response (Fig. 2).



**Figure 2.** 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 by a monoclonal antibody can abrogate the therapeutic value of potent tumor vaccines (Mackey MF, et al., *Cancer Res* 1997;57:2569–74). Vice versa, a CD40 agonistic antibody was shown to be able to overcome peripheral tolerance and generate antitumor immunity able to reject tumors (Diehl L, et al., *Nat Med* 1999;5:774–9). The main mechanism of immune stimulation by CD40 ligands is activation of DCs, resulting in increased survival, upregulation of costimulatory molecules, and secretion of critical cytokines for T cell priming such as IL-12. This promotes antigen presentation, priming and cross-priming of CD4<sup>+</sup> and CD8<sup>+</sup> effector T cells (Elgueta R, et al., *Immunol Rev* 2009;229:152–72). 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 (Bernier V, et al., *NatMed* 2007;13:354–60) or accelerate the deletion of tumor-specific cytotoxic lymphocytes in the absence of antigen vaccination (Kedl RM, et al., *Proc Natl Acad Sci U S A* 2001;98:10811–6). CD40 ligation could thus be best used in combinatorial approaches including vaccines and TLR agonists (Elgueta R, et al., *Immunol Rev* 2009;229:152–72; Scarlett UK, et al., *Cancer Res* 2009;69(18):7329–37). Based on the immunomodulatory effects of select chemotherapeutic 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 (Nowak AK, et al *J Immunol* 1999;162:4140). Interestingly, the CD40 receptor is expressed on a variety of tumors including melanoma, lung, bladder and prostate cancers, but also cervix (Altenburg A, et al., *J Immunol* 1999;162:4140–7) and the majority of ovarian cancers (Gallagher NJ, et al., *Mol Pathol* 2002;55:110–20; Melichar B, et al., *Gynecol Oncol* 2007;104:707–13; Hakkarainen T, et al., *Clin Cancer Res* 2003;9:619–24; Jiang E, et al., *Eur J Gynaecol Oncol* 2004;25:27–32). 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- $\kappa$ B activation and TNF- $\alpha$ , IL-6 and IL-8 production in most EOC cell lines (Toutirais O, et al., *Clin Exp Immunol* 2007;149:372–7). 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 (Ghamande S, et al., Cancer Res2001;61:7556–62). Interestingly, rIFN-g enhanced expression of CD40 on tumor cells and its efficacy on EOC cell lines (Melichar B, et al., Gynecol Oncol2007;104:707–13). 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 showed objective partial responses (Vonderheide RH, et al., J Clin Oncol 2007;25: 876–83).

## *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 autoimmune diseases occurring in CTLA-4 and PD-1 knockout mice (Chen ML, et al., Proc NatlAcad Sci U S A 2005;102:419–24) 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. The use of blocking CTLA-4 or PD-1 mAbs can sustain the activation and proliferation of tumor-specific T cells, preventing anergy or exhaustion and thereby allowing the development of an effective tumor specific immune response.

## **TOLL-LIKE RECEPTOR 9 AND ITS AGONISTS**

The immune system has the ability to detect and eliminate pathogens through several mechanisms, and it may be broadly divided into innate and adaptive systems. Cells of the innate immune system, represented by natural killer (NK) cells, monocytes, and granulocytes, rapidly detect invading pathogens and tumors in a nonspecific manner. The innate immune system will respond to and contain the invading pathogens and prevent its spread. The adaptive immune system, represented by cytolytic T cells (CTL), T helper cells (TH), and antibodies, is activated by presentation of antigen in a cognate fashion and will develop an antigen-specific response to eliminate the pathogen. 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 (Krieg AM..Nat Rev Drug Discov 2006.5(6):47-84). 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 (Murad YM, et al., Biodrugs 2009. 23(6):361-375). The key feature of innate immune cells that enables them to detect and categorize 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 is the Toll-like receptors (TLRs), of which 10

are known in humans (Iwasaki A, et al., Medzhitov NatImmunol 2004;5:987–95). 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 (Takeda K, et al., Annu Rev Immunol 2003; 21: 335-76; Medzhitov R, et al., Nature 1997; 388 (6640): 394-7). TLRs are considered sensors for microbial infections or other ‘danger signals’, and are critical to the linkage between innate and adaptive immune responses (Akira S, et al., Nat Immunol 2001 08; 2 (8): 675-80). These receptors are present on different immune cells and recognize and bind certain molecules that are restricted to micro-organisms and absent from vertebrates, or expressed and not normally accessible to TLRs. The specificity of different TLRs is partially influenced by their structure and cellular location, which could be either intracellular or on the cell surface, depending on their specificity to intracellular or extracellular pathogens (Akira S, et al., Nat Rev Immunol 2004 07; 4 (7): 499-511; Akira S. Curr Top MicrobiolImmunol 2006; 311: 1-16). Tumor immunotherapy has evolved since William Coley used crude bacterial extracts to treat cancer (Wiemann B, et al., Pharmacol Ther 1994; 64 (3): 529-64). 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. 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 (Mashiba H, et al., Jpn J Med Sci Biol 1988, 41:197-202). These investigators suggested that the immunostimulatory effects of bacterial DNA were caused by the palindromic nature of the DNA sequences (Yamamoto S, et al., Microbiol Immunol 1992, 36:983-997). In 1995, Krieg *et al.* (Krieg AM, et al., Nature 1995, 374:546-549) 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

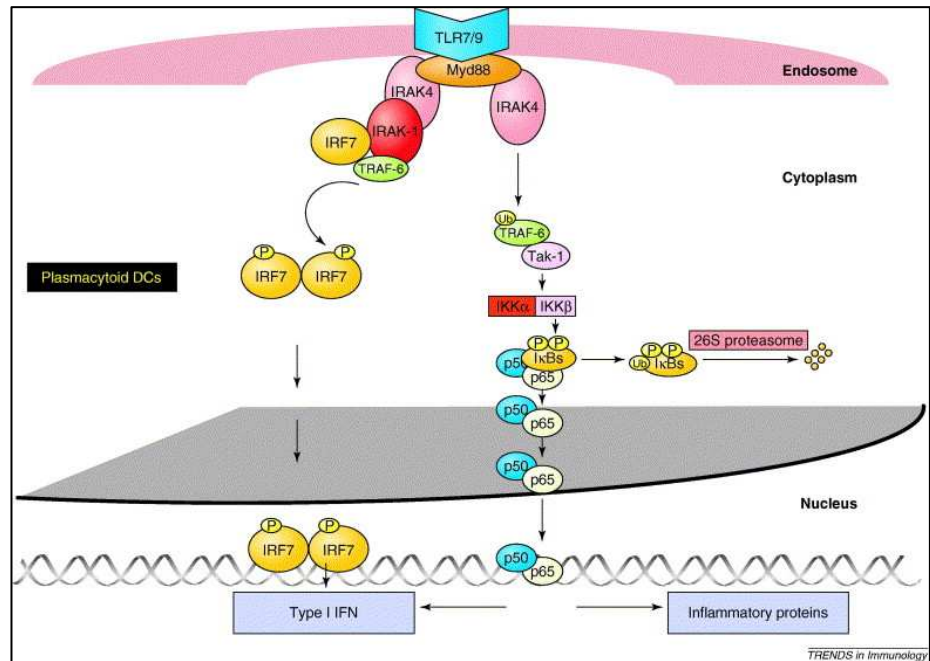


spawned a new field of research focused on identifying and characterizing the effects of so-called *CpG-containing oligonucleotides* (CpG ODNs) and the mechanisms through which they act. At the time of the first report describing CpG-dependent immune stimulation by bacterial DNA, the receptor was not yet identified (Yamamoto S, et al., *Microbiol Immunol* 1992, 36:983-997). 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 (Krieg AM, et al., *Nat Med* 2003, 9:831-835), although these studies could not be confirmed (Yi AK, et al., *J Immunol* 1998, 160:4755-4761). Gene knock down and gain of function experiments finally identified TLR9 to be the receptor conferring CpG reactivity by directly engaging bacterial DNA or synthetic CpG ODN in a CpG motif-dependent manner (Bourke E, et al., *Blood* 2003, 102:956-963). Research over the past 5 years suggests exploitation of these mechanisms holds significant promise for development of new cancer immunotherapies.

### **Expression and localization of TLR9**

In humans, in bone marrow derived cells TLR9 is expressed only in memory B cells (Bernasconi NL, et al., *Blood* 2003, 101:4500-4504; Bourke E, et al., *Blood* 2003, 102:956-963) and plasmacytoid dendritic cells (pDC) (Hornung V, et al., *J Immunol* 2002, 168:4531-4537; Kadowaki N, et al., *J Exp Med* 2001, 194: 863-869; Krug A, et al., *Eur J Immunol* 2001, 31:3026-3037). Expression of TLR9 and responsiveness to CpG-DNA in other immune cells, such as human monocytederived dendritic cells and monocytes, has been reported, but is still a matter of debate (Housse V, et al., *J leukoc Biol* 2006, 80:1328-1336; Saikh KU, et al., *J Immunol* 2004, 173: 7426-7434). In contrast, murine TLR9 expression is not limited to B cells and pDC, but is also detected in monocytes, macrophages, and dendritic cells (Edwards AD, et al., *Eur J Immunol* 2003, 33: 827-833; Hemmi H, et al., *Nature* 2000, 408:740-745). 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 (Latz E, et al., *Nat Immunol* 2004, 5:190-198; Leifer CA, et al., *J Immunol* 2004, 173:1179-1183; Rutz M, et al., *Eur J Immunol* 2004, 34:2541-2550). (Fig.3)

**Figure 3.** Spatiotemporal signaling of TLR9. Activation of TLR9 induces secretion of proinflammatory cytokines and type I IFN dependent on ligand localization. CpG-A ODN or CpG-B ODN complexed to transfection agents reside in the endosome and initiate IRF-7 activation leading to type I interferon. In contrast, CpG-B ODN itself traffics to the



lysosome and activates IRF-5 and NF-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.

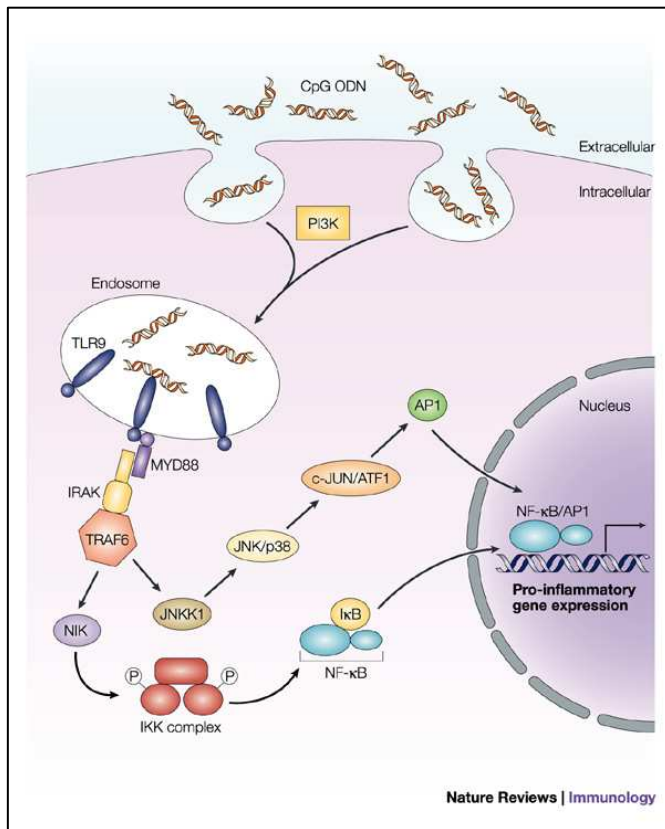
Compounds that interfere with endosomal acidification, such as the weak base chloroquine and bafilomycin A1, an inhibitor of the ATP-dependent acidification of endosomes, consequently, prevent CpG-DNA-driven TLR9 activation (Hacker H, et al., *Nature* 2006, 439:204-207; Yi AK, et al., *Int Immunol* 1999, 11:2015-2024). 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 (Barton GM, et al., *Nat Immunol* 2006, 7:49-56; Kajita E, et al., *Biochem Biophys Res Commun* 2006, 343:578-584), although a recent report challenges this view. Accordingly, this report demonstrates that a tyrosine-based (YNEL) targeting motif in the cytoplasmic domain and the extracellular domain per se regulate TLR9 trafficking independent of the transmembrane domain (Leifer CA, et al., *J Immunol* 2004, 173:1179-1183). Despite these conflicting results on the trafficking-determining domain of TLR9, it is important to note that TLR9 trafficking to the

endosome/lysosome does not seem to involve the Golgi apparatus, since the mature protein retains the sensitivity to the glycosidase Endo H, a feature of usually ER-resident proteins. Which alternative route TLR9 uses to reach the endosomal/lysosomal compartment is currently unknown. The recently described ER resident protein unc93b may be involved in TLR9 trafficking since a dominant negative mutant of unc93b leads to nonresponsiveness of TLR9 (together with TLR3, TLR7) accompanied by the disruption of TLR-unc93b interaction (Brinkmann MM, et al JCell Biol 2007, 177:265-275; Tabeta K, et al., Nat Immunol 2006, 7:156-164). Recently TLR9 expression has also been detected on intestinal epithelial cells, and an involvement in the maintenance of colonic homeostasis has been suggested (Ewaschuk JB, et al., Infect Immunol 2007, 75: 2572-2579; Lee J, et al., Nat Cell Biol 2006, 8:1327-1336). 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- $\kappa$ B) pathway, apical TLR9 activation prevents NF- $\kappa$ B activation by accumulation of NF- $\kappa$ B inhibitory protein I kappa B-alpha (I $\kappa$ B- $\alpha$ ). Furthermore, apical TLR9 stimulation confers tolerance to subsequent TLR challenges, suggesting that apical exposure to luminal microbial DNA controls intestinal inflammation.

### **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. Unlike TLR4-mediated signaling, the TIR domain-containing adaptor protein/MyD88-adaptor-like (TIRAP/MAL) is not involved in TLR9-mediated signaling. Recently, a novel adapter molecule associated with MyD88-independent as well as MyD88-dependent pathways was identified. Ongoing studies suggest that this molecule, TIR domaincontaining adapter inducing IFN- $\beta$  (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- $\kappa$ B-inducing kinase (NIK)-IKK-I $\kappa$ B pathways (Hacker H, et al., EMBO J 1999;18:6973-82; Yi AK, et al., J Immunol 2002;168:4711-20; Hartmann G, et al., J Immunol 2000;164:944-53). 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. The signaling cascade culminates in the activation of several transcription factors including NF- $\kappa$ B, activating protein-1 (AP-1), CCAAT/enhancer binding protein (C/EBP), and cAMP-responsive element-binding protein (CREB), which directly upregulate cytokine/chemokine gene expression (Fig.4).



**Figure 4.** Scheme of CpG DNA/TLR9-mediated cellular signaling. Class III phosphatidylinositol 3-kinase (PI3K) facilitates the internalization of CpG oligodeoxynucleotides (ODNs) into endosomal vesicles that contain Toll-like receptor 9 (TLR9). The interaction between CpG DNA and TLR9 transduces an intracytoplasmic activation signal. The signal initiates with the recruitment of myeloid differentiation primary response gene 88 (MYD88) to the Toll-interleukin-1 receptor (TIR) domain of TLR9, followed by activation of the IRAK-TRAF6 complex. This leads to the activation of both the mitogen-activated protein kinase (MAPK: JNK1/2 and p38) and inhibitor of nuclear factor- $\kappa$ B (NF- $\kappa$ B) kinase (IKK) complexes, culminating in the upregulation of transcription factors, including NF- $\kappa$ B and activating protein 1 (AP1). ATF1, activating transcription factor 1; IRAK, IL-1 receptor-activated kinase; JNKK1, c-JUN N-terminal kinase (JNK) kinase 1; NIK, NF- $\kappa$ B-inducing kinase; TRAF6, tumour-necrosis factor receptor-associated factor 6

activated kinase; JNKK1, c-JUN N-terminal kinase (JNK) kinase 1; NIK, NF- $\kappa$ B-inducing kinase; TRAF6, tumour-necrosis factor receptor-associated factor 6

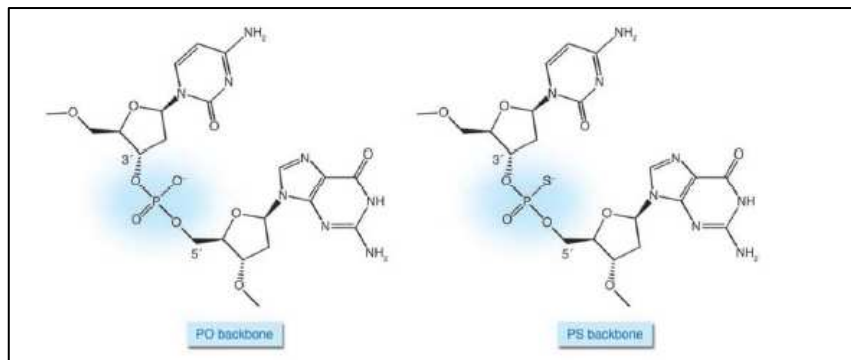
In mouse macrophages, CpG DNA also induces IFN- $\beta$  production, which then upregulates STAT1 phosphorylation and IP-10 production through IFN- $\alpha/\beta$  receptor in an autocrine manner (Hoshino K, et al., *Int Immunol* 2002;14:1225– 31). Studies using chloroquine (CQ) or wortmannin (WM) showed that these agents could block CpG DNA/TLR9 signaling but not LPS/TLR4 signaling (Ishii KJ, et al., *J Exp Med* 2002;196:269–74). 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. 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), others find that DNA-PK KO mice and SCID mice respond normally to CpG DNA (Ishii KJ, et al., *J Exp Med* 2002;196:269–74). We observed that WM treatment led to a reduction in the size and number of endosomes containing both TLR9 and CpG ODN, suggesting that phosphatidylinositol 3 kinases (PI3K), which are also targets of WM, are involved in vesicular trafficking of CpG DNA. 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 (Backer JM. *Mol Cell Biol Res Commun* 2000;3:193–204; Siddhanta U, et al., *J Cell Biol* 1998;143:1647–59; Vieira OV, et al., *Biochem J* 2002;366:689–704). The PI(3,4, 5)P<sub>3</sub>, 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). Ligand-induced association of TLR2 ICD and PI3K (I) was reported to activate the AKT/PKB-NF- $\kappa$ B pathway. CpG DNA also induces phosphorylation of AKT/PKB thereby inhibiting apoptosis in DCs, an effect that is reversed by a PI3K inhibitor, LY294002. 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- $\kappa$ B activation in HEK293 cells. This suggests that (1) PI3K(I) also regulate vesicular trafficking of CpG DNA and TLR9 and/or (2) another pathway mediated by PI3K(I) but not through the PDK1- AKT/PKB pathway is involved in TLR9-mediated NF- $\kappa$ B activation in HEK293. PI3Ks and their second messengers therefore seem to play pivotal roles at distinct steps (i.e. vesicular trafficking for the association between CpG DNA and TLR9 and the signaling pathway directing AKT/PKB activation) in CpG DNA/TLR9-mediated cellular activation.

## Classes of synthetic CpG oligodeoxynucleotides

The immune stimulatory effects of CpG DNA are explained at least in part by differences inherent to genomic DNA of vertebrates and pathogens: vertebrate CpG dinucleotides are methylated and their frequency is suppressed, while viral and bacterial CpG dinucleotides are non-methylated and occur with a much higher frequency. Synthetic CpG ODN can be generated containing specific CpG sequence motifs, sugar, base or backbone modifications as well as secondary and tertiary structures that all affect the immune modulatory effects of CpG ODN TLR9 ligands to different degrees (Fig. 5). B-Class ODN with 6mer CpG motifs with the general formula “purine pyrimidine- C-G-pyrimidine-pyrimidine” are strong stimulators of human B cell responses, and induce maturation of human pDCs and monocytes. The 6mer motif 5'-GTCGTT-3' represents the optimal human CpG motif, whereas 5'-GACGTT-3' is the optimal murine CpG motif (G. Hartmann, et al., J. Immunol. 164 (2000) 1617–1624). The length, number of CpG motifs, their spacing, position and the surrounding bases also determine the activity of B-Class ODN. The most potent ODN for activating human cells usually have three CpG motifs, additional CpG motifs do not much further enhance activity, and are between 18 and 26 nucleotides in length. Chemical modifications of the backbone, the heterocyclic nucleobase or the sugar moiety further enhance the activity of B-Class CpG ODN. Phosphorothioate modifications of CpG ODN stabilize them against nuclease degradation and enhance their activity by about 10 to 100 fold compared to phosphodiester ODN that either have to be added repeatedly or to be combined with an uptake enhancer to result in similar activity (D.P. Sester, et al., J. Immunol. 165 (2000) 4165–4173; K. Yasuda, et al., P. J. Immunol. 174 (2005) 6129–6136).

**Figure 5.** Backbones of native and modified DNA. PS ODN differ from native phosphodiester (PO) DNA ODN only in the substitution of a sulfur for one of the nonbridging oxygen atoms. This change improves the in vivo stability of the ODN from a half-

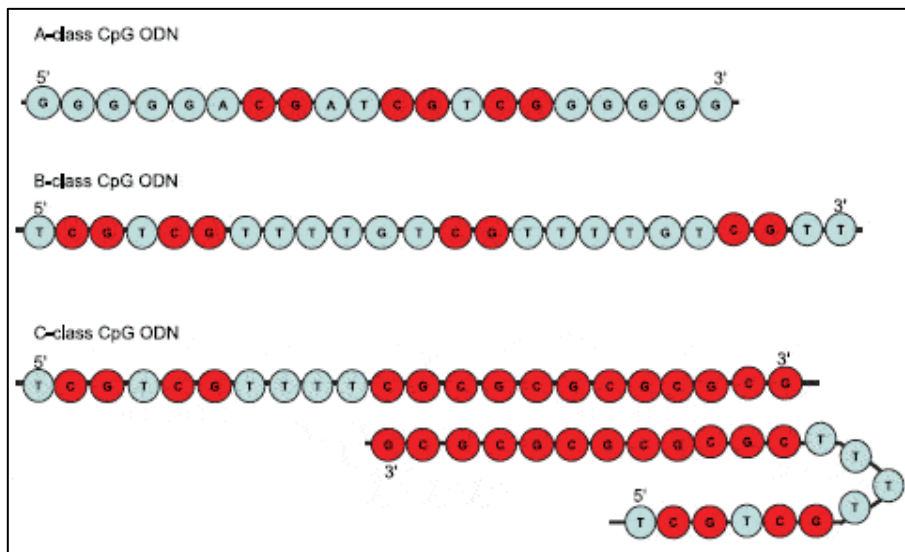


life of a few minutes to about two days for the PS ODN.

In contrast to the charged phosphodiester and phosphorothioate backbones, replacement with noncharged backbones results in decreased immune stimulatory activity. CpG ODN with 2'-O-methyl or 2'-O-methoxyethyl sugar modifications induce decreased immune stimulation (E. Uhlmann, J. Vollmer, et al., *Curr. Opin. Drug Discov. Dev.* 6 (2003) 204–217.) and unpublished observations), substitutions with a RNA derivative, locked nucleic acid (LNA), even can eliminate the immune stimulatory effects of CpG-containing phosphorothioate ODN (J. Vollmer, et al., *Oligonucleotides* 14 (2004) 23–31). In principle, any modification of cytosine at the CpG motifs is usually not well tolerated, but TLR9 appears to be more forgiving to modifications at the guanosine position (J. Vollmer, et al., *J. Leukoc. Biol.* 76 (2004) 1–9). Another CpG ODN class is defined by G runs with PS linkages at the 5' and 3' ends surrounding a phosphodiester palindromic CpG-containing sequence (A. Krug, et al., *J. Immunol.* 31 (2001) 2154–2163). Intermolecular tetrad and high molecular weight aggregates are formed via the G residues that enhance stability, increase endosomal uptake and ligand concentrations (J.D. Marshall, et al., *J. Leukoc. Biol.* 73 (2003) 781–792), resulting in strong pDC IFN- $\alpha$  production by these CpG A-Class ODN. Albeit strong IFN- $\alpha$  and IFN- $\beta$  stimulators, A Class CpG ODN are relatively weak in inducing other TLR9-dependent effects such as pDC maturation or B cell proliferation. 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. The CpG C-Class has some sequence requirements similar to the B-Class and combines the characteristics of the A- and B-Classes, stimulating strong B cell and pDC type I interferon production. C-Class ODN consist of a stimulatory hexameric CpG motif positioned at or near the 5' end and linked by a T spacer to a GC-rich palindromic sequence (J. Vollmer, et al., *Eur. J. Immunol.* 34 (2004) 251–262). 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. The stimulatory capacities of C-Class CpG ODN are similar to the A- and B Classes and depend on the length, base content and are influenced by chemical modifications. The formation of secondary and tertiary structures appears to control

compartmental retention and intracellular distribution. The A- and C-Classes localize to different endolysosomal compartments than the B-Class CpG ODN (K. Honda, et al., Nature434 (2005) 1035–1040). The A- and C-Classes trigger IRF-7- mediated intracellular signaling pathways from early endosomes leading to strong IFN- $\alpha$  induction, whereas the BClasses mainly stimulate NFkB-mediated signaling from late endosomes resulting in strong B cell activation. Palindromic sequences are involved in the formation of higher ordered structures and immediately affect stability, uptake characteristics and intracellular localization. Introducing a palindrome and increasing its length in a B-Class CpG ODN result in a stepwise increase of type I IFN production. Within the oligos tested, the strongest IFN- $\alpha$  induction is observed with CpG ODN having the longest palindrome. It is also possible to combine the 3' GC-rich palindrome of C-Class ODN with a non-GC-rich 5' palindrome. Such double palindromic or P-Class CpG ODN do not only form hairpins at their GC-rich 3' ends, but also form concatamers due to the presence of the 5' palindrome. These highly ordered structures appear to be responsible for the strongest type I IFN induction observed with CpG ODN. Similar to the A-Classes, P-Class ODN may enter early endolysosomal compartments preferentially inducing the IRF7 signaling pathway (U. Wille-Reece, et al., BJ. Exp. Med. 203 (2006) 1249–1258).

**Figure 6.** 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- $\alpha$  (IFN-) secretion (from plasmacytoid dendritic cells), but only weakly stimulate B cells. The structures of A-class ODN include poly-G motifs (three or more consecutive guanines) at the 5' and/or 3'

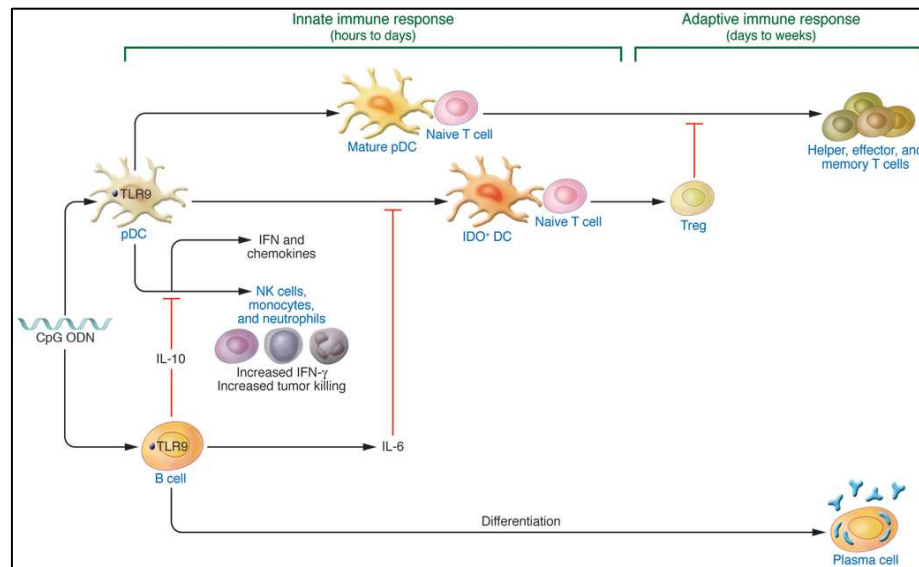


ends that are capable of forming very stable but complex higher-ordered structures known as G-tetrads, and a central phosphodiester region containing one or more CpG motifs in a self complementary palindrome. These motifs cause A-class ODN to self-assemble into nanoparticles 193. B-class ODN (also referred to as type K) have a completely phosphorothioate backbone, do not typically form higher-ordered structures, and are strong B-cell stimulators but weaker inducers of IFN $\alpha$  secretion<sup>10</sup>. 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 have immune properties intermediate between the A and B classes, inducing both B-cell activation and IFN- $\alpha$  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 and indirectly from activation of the immune cells that constitutively express TLR9, B cells, and pDCs. CpG-ODN require no delivery system *in vitro* or *in vivo*, they can simply be administered in saline and are spontaneously taken up by most immune cells, in particular B cells and DCs (ODN uptake is not restricted to TLR9-expressing cells). ODN uptake by lymphocytes is energy and temperature dependent and greatly increased by cell activation; it also seems to be receptor mediated, although the specific receptors remain largely obscure. Immune responses can be broadly divided into two types: Th1 and Th2. Th1 immune activation is optimized for fighting intracellular infections such as viruses and involves the activation of NK cells and CTLs that can lyse infected cells. This type of immune activation is the most highly desired for cancer therapy, as the same defenses can be directed to kill tumor cells. In contrast, Th2 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 Th1 responses that are triggered. The immune response to infection or TLR stimulation occurs in two phases: the first to be activated is antigen-nonspecific innate immunity, which is followed by antigen-specific adaptive immunity (Fig.7). TLR9 stimulation with any class of CpG ODN activates innate immunity with a predominantly Th1 pattern of cytokine and chemokine secretion by B cells and pDCs (and by other immune cells that are activated secondarily). In response to TLR9

stimulation, B cells and pDCs also express increased levels of costimulatory molecules (such as CD80 and CD86), TNF-related apoptosis-inducing ligand (TRAIL), which can induce tumor cell death, and CC chemokine receptor 7 (CCR7), activation of which causes cell trafficking to the T cell zone of the lymph nodes, and show increased resistance to apoptosis (259). Together, these innate immune effects of TLR9 activation can promote tumor regression either directly, through the antitumor activity of factors such as IFN- $\alpha$  and TRAIL, or indirectly, through the activation of NK cell-mediated tumor killing. 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. The pDCs activated through TLR9 become competent to induce effective CD4+ and CD8+ T cell responses. 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 (Rothenfusser, S., et al. 2004. Blood. 103:2162–2169). B cells are strongly costimulated if they bind specific antigen at the same time as TLR9 stimulation (Fig. 7). 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 TH1-biased immunity.



**Figure 7.** Activation of innate and adaptive immunity through stimulation of TLR9 on pDCs and B lymphocytes. Generally, the effects of CpG DNA are to promote a strong cellular response of Th1 type that includes the activation of CD4 + T cells, CD8 + and B lymphocytes capable of producing antigen-specific antibodies. On the other hand the

same activation of TLR9 also induces the immunosuppressive pathways that involve the production of cytokines such as IL-10 or other factors such as IDO to finely regulate the inflammatory response.

### **Drug-like properties of synthetic CpG-ODN**

Some of the characteristics of synthetic ODN are quite attractive for drug development, whereas others are less favourable. The technology for commercial-scale (multi-kilogram) ODN synthesis and purification, carried out according to Good Manufacturing Practices, has been well developed during the past decade of antisense and aptamer drug development. Antisense and aptamer oligonucleotide drugs have been approved by the US FDA, establishing a regulatory pathway for this general class of drugs. The absorption, distribution, metabolism and elimination (ADME) properties of synthetic PS-ODN with and without CpG motifs have been well characterized and reported in the extensive literature on antisense ODN, which has shown these characteristics to be essentially sequence-independent (Geary, R. S. et al. *Drug Metab. Dispos.* 1997 25, 1272–1281; Levin, A. A., et al., *Antisense Drug Technology* 2001 201–267). ODNs given subcutaneously are slowly absorbed from injection sites (with the highest concentration in the draining lymph nodes for the first several days after injection), and then enter the systemic circulation, where they demonstrate high-capacity, low affinity binding to plasma proteins, principally albumin. ODN are rapidly cleared into tissues, especially the liver, kidneys and spleen, but do not seem to cross the blood–brain or blood–testes barriers. Catabolism of ODN typically occurs by exonuclease digestion and base clipping, primarily at the 3' end, resulting in natural DNA bases and thiophosphate metabolites that are excreted in the urine. The immune effects of CpG-ODN administration through different routes result from their ADME characteristics. For example, subcutaneous administration of CPG 7909 (Coley), which results in high levels of the compound in the draining lymph node (which would contain a relatively high concentration of TLR9- expressing cells), induces high levels of serum cytokines and chemokines (Krieg, A. M., et al., *J. Immunother.* 2004 27, 460–471). 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. Because the pharmacodynamics of subcutaneous CpG ODN result from the local ODN concentration in the draining lymph nodes, they do not match the systemic pharmacokinetics.

<p><b>Drug-like characteristics</b></p> <ul style="list-style-type: none"> <li>• Excellent aqueous solubility</li> <li>• Spontaneous intracellular uptake by certain immune cells (including especially those that express Toll-like receptor 9 (TLR9))</li> <li>• Relatively simple solid-phase Good Manufacturing Practice synthesis (multi-kilogram scale) and chromatographic purification</li> <li>• Comparatively well-understood chemistry enables diverse studies of structure-activity relationships</li> <li>• Metabolites are mostly normal components of DNA, not novel small molecules</li> <li>• Range of backbones available for modulating compound stability for different applications</li> <li>• Can be administered through virtually any drug route (including oral)</li> <li>• Dose exposure required for immune stimulation is ~0.1-1% of that required for antisense applications</li> <li>• Excellent stability in aqueous solutions at physiologic pH, even at room temperature</li> <li>• Well-developed highly analytic methods for Chemistry, Manufacturing and Controls (liquid chromatography-mass spectrometry is state of the art)</li> <li>• Very sensitive methods available for detection of "cold" compound<sup>91</sup></li> </ul> <p><b>Non-drug-like characteristics</b></p> <ul style="list-style-type: none"> <li>• Medium size: molecular mass ~6,000-8,000 Da (length typically 18-25 bases)</li> <li>• Highly charged polyanions</li> <li>• Phosphorothioate and some other backbones are chiral</li> <li>• Poor stability of purines in acid solution</li> <li>• Cleaved by nucleases in serum or cell extracts (phosphorothioate backbone is relatively nuclease resistant)</li> <li>• Highly protein bound</li> <li>• Non-uniform organ distribution: highest tissue levels in kidney, liver and spleen after systemic delivery</li> <li>• Pharmacokinetics do not match pharmacodynamics after subcutaneous delivery</li> <li>• Sequence-independent effects, including concentration-dependent activation of complement proteins and prolongation of partial thrombin time</li> </ul>
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**Table 1.** Characteristics of CpG oligodeoxynucleotides

### ***Preclinical (animal) studies of TLR9 agonists***

As previously reported, cellular expression of TLR9 varies between humans and mice. TLR9 expression in mice is broader, and includes monocytes and macrophages. Thus, it is difficult to extrapolate the positive effects seen in mouse models to humans. Nevertheless, some of the ground-breaking work on CpGs was done in animals. 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. CpG ODN has been tested in several mouse tumor models (Krieg AM. *Curr Oncol Rep* 2004 Mar; 6 (2): 88-95) 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.

### *MONOTHERAPY*

The effects of CpG ODN monotherapy can vary widely, depending on the tumor type. Moreover, its mechanism of action varies depending on several factors, such as MHC expression of the tumor, the susceptibility of the tumor to several immune effectors such as NK cells, T cells, or even TLR9 expression on the tumor cells (Ballas ZK, et al., *J Immunol* 2001 Nov 1; 167 (9): 4878-86; Carpentier AF, et al., *Cancer Res* 1999 Nov 1; 59 (21): 5429-32; Lonsdorf AS, et al., *J Immunol* 2003 Oct 15; 171 (8): 3941-6). While using CpG-ODNs as monotherapy could be effective in inducing regression in some tumors, such as the C3 model of cervical cancer (Baines J, et al., *Celis Clin Cancer Res* 2003 Jul; 9 (7): 2693-700), it is ineffective or less effective in the treatment of other tumors when given by systemic injection, compared with peritumoral or intratumoral injection (Heckelsmiller K, et al., *J Immunol* 2002 Oct 1; 169 (7): 3892-9; Kawarada Y, et al., *J Immunol* 2001 Nov 1; 167 (9): 5247-53). Peritumoral administration of CpG-ODN was effective in impeding the progression of tumors in BALB/c mice transgenic for the rat neu transforming oncogene (Mastini C, et al., *Curr Cancer Drug Targets* 2008 May; 8 (3): 230-42).

### *CHEMOTHERAPY*

When CpG-ODN was combined with chemotherapy, it was more effective than chemotherapy alone. Mouse tumor models treated with CpG-ODN in combination with fluorouracil, topotecan (topoisomerase I inhibitor), cyclophosphamide (Weigel BJ, et al., *Clin Cancer Res* 2003 Aug 1; 9 (8): 3105-14), or paclitaxel (Weeratna RD, et al., *ASCO Meeting Abstracts* 2004 Jul 15; 22 (14 Suppl.): 7346) 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 (Bourquin C, et al., *Int J Cancer* 2006 Jun 1; 118 (11): 2790-5).

## VACCINES

CpG-ODNs have also been used in vaccination studies as adjuvants and have induced a good TH1- type immune response (Kim SK, et al., *Vaccine* 1999 Nov 12; 18 (7-8): 597-603; Chu RS, et al., *J Exp Med* 1997 Nov 17; 186 (10): 1623-31). The efficiency of CpG-ODNs in inducing a TH1 biased response is thought to be due to synergy between TLR9 and the B-cell receptor, which results in antigen specific B-cell stimulation, inhibition of B-cell apoptosis, enhanced IgG class switching and DC maturation and differentiation (Yi AK, et al., *J Immunol* 1998 Jun 15; 160 125898-906; He B, et al., *J Immunol* 2004 Oct 1; 173 (7): 4479-91). 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. Moreover, in a preclinical model of colon cancer, a vaccine combining CpG-ODN with GM-CSF and class I and class II restricted mucin (MUC) 1 peptides was successful in breaking MUC1 self tolerance, and in eliciting a robust antitumor response in MUC1 transgenic mice (Mukherjee P, et al., *Vaccine* 2007 Feb 19; 25 (9):1607-18). The immune response caused complete rejection of tumor cells in the prophylactic setting, while in the therapeutic setting, tumor burden was significantly reduced. When a DC-tumor cell fusion vaccine was used in mice, along with the TLR9 agonist ODN 1826 and the TLR3 agonist Poly(I:C), 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 (Zheng R, et al., *Cancer Res* 2008 Jun 1; 68 (11): 4045-9). Moreover, the use of CpG-ODN in mice as a vaccine adjuvant allowed for decreasing the antigen dose by half, while maintaining the same level of antibody response, when compared with those receiving the full dose of antigen without the CpG-ODN adjuvant. 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 (Davis HL, et al., *J Immunol* 1998 Jan 15; 160 (2): 870-6). The activity of CpG ODN to induce humoral immune responses has also been confirmed in non-human primates and in humans (Halperin SA, et al., *Vaccine* 2003 Jun 2; 21 (19-20): 2461-7; Verthelyi D, et al., *J Immunol* 2002 Feb 15; 168 (4): 1659-63; Davis HL, et al., *Vaccine* 2000 Mar 17; 18 (18): 1920-4).

## **TLR9 expression on tumor cells**

While numerous basic and clinical studies have investigated the immunostimulatory effects of TLR9 agonists on the innate and adaptive immune systems that could lead to the regression of tumors in vivo, only a few studies have discussed the significance of TLR9 expression on tumor cells (Zeromski J, et al., *Cancer Microenviron* 2008 Dec; 1 (1): 37-42). It was demonstrated that TLR9 activation can lead to the proliferation of immortalized prostate cells (Kundu SD, et al., *Prostate* 2008 Feb 1; 68 (2): 223-9), or to the promotion of matrix metalloproteinase (MMP)-13 activity, resulting in enhanced migration of human prostate cancer cells expressing TLR9 (Merrell MA, et al., *Prostate* 2007 May 15; 67 (7): 774-81; Coussens LM, et al., *Blood* 2000 Feb 1; 95 (3): 999-1006). The aim of these studies was to demonstrate how TLR9 agonists from pathogens encountered in the genitourinary system may enhance malignant transformation and boost cancer cell spreading through inflammation-dependent mechanisms. 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 (Jahrsdorfer B, et al., *Pathol Oncol Res.* Epub 2009 Mar 25). Others have reported that TLR9 signaling could enhance the metastatic potential of human lung cancer cells (95D) in nude mice, which might be related to the elevated proliferation and IL-10 secretion by the cells (342). Basically, we believe that the direct effect of TLR9 agonists on tumor cells needs to be further explored, and might depend also on the expression of TLR9.

## **CpG-ODN FOR CANCER TREATMENT**

Several TLR9 agonists are undergoing clinical testing in a range of tumors, including non-small cell lung carcinoma (NSCLC), basal cell carcinoma, metastatic melanoma, NHL, and cutaneous T cell lymphoma (Kim et al. 2004 ; Leonard et al. 2007 ; Manegold et al. 2005 ; Hofmann et al. 2008 ; Pashenkov et al. 2006 ). Currently, TLR9 agonist treatment has been associated with immune reactions and some evidence of antitumor activity, though complete responses have been

rare (Kim et al. 2004 ; Leonard et al. 2007 ; Manegold et al. 2005 ; Hofmann et al. 2008 ; Pashenkov et al. 2006).

### **Intra-or perilesional injection of CpG-ODN:**

Several clinical studies using CpG ODN TLR9 agonist as single agent have been completed and shown evidence of antitumor activity. Immunotherapies are often tested in the setting of melanoma and other skin cancers because they tend to be highly immunogenic. One approach to melanoma monotherapy that has been investigated in humans with PF- 3512676 is local therapy with intra- or perilesional injection. In a phase 1 trial of low dose intra- or perilesional injection of PF- 3512676 there was 1 local regression among 5 patients with metastatic melanoma and 1 complete response (CR) and 4 partial responses (PRs) among 5 patients with basal cell carcinoma has been observed (Hofmann et al., 2008). In a different trial involving 24 patients with clinical stage I to stage III melanoma, surgical resection of the primary tumor was followed by randomization to receive either saline or 8mg PF-3512676 intradermally at the excision site, followed 1 week later by a sentinel lymph node procedure (Molenkamp et al., 2007). PF-3512676 injection induced the release of inflammatory cytokines, decreased the number of regulatory T cells, and induced tumor-specific CD8+ T cells in sentinel lymph nodes of these patients (Molenkamp et al., 2007; Molenkamp et al., 2008; Sluijter et al., 2010). Furthermore, both myeloid and pDCs were activated (Molenkamp et al., 2007), and the degree of pDC activation correlated with the magnitude of the CD8+ T cell response (Molenkamp et al., 2008). Intratumoral monotherapy with TLR9 agonists has also been evaluated in patients with recurrent glioblastoma (GBM). A minor response was observed in 2 of 24 patients with recurrent GBM receiving intratumoral CpG-28 in a phase 1 dose-escalation trial (Carpentier et al., 2006). A phase 2 trial of this oligo CpG-28 in 34 patients with recurrent GBM at the highest dose reached in the phase 1, 20 mg, had a 6-month progression-free survival rate of 19%, which did not meet the goal of the study (Carpentier et al., 2010). The overall survival rate was 24% at 1 year and 15% at 2 years, which are longer than typical for GBM but could reflect patient selection in this small non-randomized trial. Important evidences of clinical benefit from CpG ODN therapy was seen in a phase 1/2 clinical trial in refractory non-Hodgkin's lymphoma (NHL) patients in which



intratumoral injection of the CpG ODN PF-3512676 was accompanied by local radiation to the injected tumor (Brody et al., 2010). In this clinical setting radiation therapy alone can cause local responses, but not systemic regression. Of 15 treated patients, there was 1 CR, 3 PRs, and several other patients with stable regressing disease (Brody et al., 2010).

### **Systemic therapy with CpG-ODN:**

A second approach to immunotherapy of melanoma has been systemic therapy with SC injection. In a phase 2 study in 20 patients with metastatic melanoma treated with SC PF- 3512676, 2 patients (10%) had a PR, and 3 patients (15%) had stable disease (Pashenkov et al., 2006). These patients with possible clinical benefit from PF-3512676 therapy tended to have increased levels of NK cell activity compared to the patients with progressive disease. In these studies the PF-3512676 monotherapy was generally well tolerated and was associated with some antitumor activity. In a second phase 2 clinical trial of SC PF-3512676 in 184 patients with advanced melanoma with or without dacarbazine chemotherapy (Weber et al., 2009), there was no statistically significant benefit from CpG therapy overall. Nevertheless, a single patient in one of the monotherapy arms had a prolonged course during therapy with apparently slow progression of the original tumor masses. On surgical excision this was found to be necrotic tissue with inflammatory cells and no remaining detectable tumor (Stoeter et al., 2008). In a phase 1/2 dose-escalation trial, 39 patients with stage IV renal cell carcinoma (RCC) received weekly PF-3512676 by SC injection at doses ranging from 0.08 mg/kg up to a high dose of 0.81 mg/kg for up to 24 weeks (Thompson et al., 2009). The maximal tolerated dose was not reached, but the side effects of treatment were tolerable, and the efficacy of CpG therapy in this setting was modest with 2 PR, one of which occurred at a weekly dose of 0.16 mg/kg and the other at a dose of 0.54 mg/kg (Thompson et al., 2009). In a phase 1 study of SC PF-3512676 monotherapy in patients (N= 28) with refractory relapsed cutaneous T cell lymphoma, 7 patients (25%) achieved an objective response (2 CRs, 5 PRs) (Kim et al., 2010). Patients typically experienced mild to moderate injection-site reactions (erythema, induration, edema, inflammation, and pain) and flu-like symptoms (fatigue, rigors, fever, and arthralgia) (Kim et al., 2010). PF- 3512676 has also been studied in a phase 1 trial in patients with (NHL). In patients (N= 23) receiving PF-3512676

(0.01 to 0.64 mg/kg) intravenously (IV) up to 3 times per week, NK cell numbers and activation were enhanced in most subjects and most side effects were again mild to moderate and transient (Link et al., 2006). Several phase 1 clinical trials have investigated the combination of a CpG ODN with the anti-tumor antibody rituximab in patients with relapsed or refractory NHL. In a dose-escalation study, 12 out of 50 NHL patients (24%) receiving PF-3512676 weekly for 4 weeks IV or SC in combination with rituximab had an objective response (including 5 CRs and 7 PRs) (Link et al., 2006; Leonard et al., 2007). In a separate dose-escalation phase 1 study, 20 patients with relapsed NHL were treated with the SC administration of weekly CpG ODN 1018 ISS following rituximab therapy (Friedberg et al., 2005). Dose-dependent induction of type 1 IFN-inducible genes was seen. In a phase 2 clinical trial performed by the same group, 1018 ISS was administered at a dose of 0.2 mg/kg weekly SC for 4 weeks in combination with rituximab to 23 patients with relapsed/refractory follicular lymphoma (Friedberg et al., 2009). Almost half of the patients showed clinical responses, and many patients also showed evidence of immune response, including infiltration of the tumors with CD8+ T cells and macrophages (Friedberg et al., 2009).

## ***AIM OF THE THESIS***

TLRs recognize several conserved pathogen-associated molecular patterns, allowing TLRs to detect microbial pathogens. TLR agonists represent a novel approach to stimulating an effective antitumor response because they are uniquely able to stimulate both innate and adaptive immune arms. In particular, synthetic CpG-ODN are agonists of TLR9, which is expressed on cells of the immune system and on endothelial cells, fibroblasts, and epithelial cells. CpG-ODN have demonstrated antitumor activity in different animal models and in patients with malignant melanoma, renal carcinoma and recurrent or refractory lymphoma. However, although both preclinical and early clinical trials suggest the value of CpG-ODN as a component of a variety of approaches to cancer therapy, clinical development of this recently discovered novel class of immunostimulatory agents is in the incipient stage and much remains unknown about the optimal approaches to their use. In fact, most past and ongoing clinical trials involve CpG-ODN administered subcutaneously (s.c.), since this route has been reported to effectively activate adaptive immunity, but, the immune modulating activities of CpG-ODN are not restricted to the

instruction and reprogramming of adaptive immunity, since they also involve activation and amplification of the innate effector cell response. Unlike cells of the adaptive immune response which can reach the antigen wherever they are activated, those of the innate immune must be activated locally. The superior antitumor effect observed in mice bearing intraperitoneal human ovarian carcinomas by CpG-ODN given intraperitoneally (i.p.) as compared to s.c. or intravenous (i.v.) routes of delivery points to the importance of innate effector cell activation at the site of tumor growth. Moreover, the significantly greater antitumor effect after daily instead of weekly i.p.CpG-ODN delivery in mice bearing advanced tumors (ascites) further supports the relevant role of activated innate immune system cells, which need frequent replenishment since they generally disappear within hours to days after quickly mediating their effector functions. The findings that repeated locoregional activation of innate immunity cells plays a relevant role in the therapy of experimental ovarian cancer has suggested the promise of locoregional CpG-ODN administration in an ovarian cancer phase I clinical trial. Indeed, ovarian cancer, in which growth is mostly confined to the peritoneal cavity, is one of the very few tumor types suitable for a simple CpG-ODN delivery directly into the tumor bed. Nevertheless, use of appropriate routes of CpG-ODN administration is likely to enable increased local concentrations of CpG-ODN in the microenvironment of tumors with other localizations.

The aims of this project are to:

- 1) Evaluate the antitumor activity of repeated locoregional aerosol CpG-ODN administrations in experimental lung carcinoma models**
- 2) Evaluate the antitumor activity of repeated locoregional CpG-ODN administrations combined with other anti-tumor therapies in experimental ovarian carcinoma models**
- 3) Evaluate the antitumor activity of repeated locoregional CpG-ODN administrations combined with other anti-tumor therapies in experimental head-and neck carcinom models**

# ***MATERIAL AND METHODS***

## **CELL LINES**

N202.1A cells, derived from a spontaneous mammary carcinoma in an FVB-neuN transgenic mouse (25), B16 mouse melanoma cells and Mouse leukemic monocyte/macrophage RAW 264.7 cells (American Type Culture Collection) were routinely maintained at 37°C in a 5% CO<sub>2</sub> atmosphere in Roswell Park Memorial Institute medium 1640 (RPMI, EuroClone) and Dulbecco's Modified Eagle's Medium (DMEM, Sigma) respectively, supplemented with 10% Fetal Calf Serum (FCS, Sigma) and 2 mM glutamine (Cambrex, East Rutherford, NJ, USA). For in vitro experiments, human IGROV-1 ovarian tumor cells (gift from Dr. J. Benard, Institute Gustave Roussy, Villejuif, France) were cultured in RPMI medium 1640 supplemented with 10% FCS and 2 mM glutamine. Established human squamous cell carcinoma lines FaDu and Cal27 (purchased from ATCC, Rockville, MD) were maintained in EMEM or DMEM supplemented with 10% FCS and 2 mM glutamine. All cultures were maintained at 37°C in a 5% CO<sub>2</sub> humidified environment.

## **MICE**

All experiments were carried out using 8- to 12-week-old female FVB, C57BL/6, Swiss nude (athymic) and SCID mice (Charles River, Calco, Italy) maintained in laminar-flow rooms at constant temperature and humidity, with food and water given ad libitum. Experiments were approved by the Ethics Committee for Animal Experimentation of the Fondazione IRCCS Istituto Nazionale dei Tumori of Milan according to institutional guidelines.

## **OLIGODEOXYNUCLEOTIDES, DRUGS AND ANTIBODIES**

Purified, phosphorothioated ODN1826 (5'-TCCATGA CGTTCCTGACGTT-3') 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. The following drugs were used: Bevacizumab (Roche, Basel, Switzerland); Poly(I)Poly(C) (Amersham Biosciences, Piscataway, NJ, USA); Cetuximab (ErbixW, Merck Serono, Darmstadt, Germany); Gefitinib (LC Laboratories, Woburn, MA, USA); and Cisplatin (Teva Italia, Milan, Italy). 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  $-20^{\circ}\text{C}$  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  $\mu\text{l}$  of sterile saline at the indicated concentrations just before administration. Sulforhodamine B (SRB) was purchased from Sigma-Aldrich. Anti-MICA, -MICB, -ULBP1, -ULBP2, ULBP4, -CD112, -CD155, -ICAM-1, and HLA-E antibodies were purchased from R&D Systems (Minneapolis, MN, USA). Anti-mouse Alexa Fluor 448- conjugated reagent was purchased from Invitrogen.  $^{51}\text{Cr}$  was purchased from PerkinElmer (Waltham, Massachusetts).

## IN VIVO STUDIES

Aerosol administration was performed using a mouse whole-body exposure system consisting of an AeronebVR Lab Micropump Nebulizer that generates aerosol into a plastic box with a sealed top and a wire netting floor that contains up to 10 mice (EMMS Unit 32 Bordon, Hants, UK). CpG-ODN was dissolved in 5 ml saline and placed in the nebulizer unit. The aerosolized solution was introduced into the box via a accordion tube at a short distance from the nebulizer at one end and discharged at the other end. Mice were exposed to aerosol for 15 min, with the 5-ml volume of liquid in the nebulizer nearly consumed in 10 min. Control mice received aerosolized saline. Intraperitoneal (i.p.) administration was performed as described (De Cesare M. *Et al J Immunother* 2010;33:8–1) using 20 $\mu\text{g}$  CpG-ODN in 200  $\mu\text{l}$  saline/mouse. FVB or C57BL/6 mice were injected intravenously (i.v.) with  $3 \times 10^5$  N202.1A carcinoma cells or  $5 \times 10^5$  B16 melanoma cells, respectively, and treated 72 hr later with aerosol (1.5 or 2.5 mg) or i.p. CpG-ODN administered at 72- to 96-hr intervals for 3 weeks. Control mice were injected with tumor cells only. All mice were anesthetized and euthanized 5 weeks after tumor injection, and macroscopic lung metastases were counted. In each experiment, mice were weighed twice weekly.

Clodronate-loaded liposomes or control PBS-liposomes were prepared as described (VanRooijen N. et al., J Immunol Methods 1994;174:83–93) and injected (100µl) intratracheally (i.t.) into anesthetized mice 96 hr after i.v. injection of  $5 \times 10^5$  B16 melanoma cells. After 24 hr (120 hr after tumor injection), mice were treated with aerosol or i.p. CpG-ODN 4 days/week for 2 or 4 weeks in experiments to evaluate lung infiltrate or protection from tumor growth, respectively 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 (22). Mice were injected i.p. with  $2.5 \times 10^6$  ascitic cells in 200µl 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  $27.9 \pm 0.84$  g,  $31.4 \pm 0.9$  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  $\times 100$  (T/C%). Cal-27 human head-and neck carcinoma cells were grown s.c and maintained by serial s.c. passages into healthy mice. To evaluate the CpG-ODN in association with cetuximab, mice have been injected s.c. in the right flank with human CAL-27 cells; when mice showed established tumors, they have been randomly divided into treated and control groups and animals have been treated with CpG-ODN i.p. (20 µg/mouse, 5 days/week) or CpG-ODN delivered using an Alzet osmotic pump (inserted s.c. in the proximity of tumor), cetuximab (1 mg/mouse administered i.v.) or combinations. FaDu tumor xenografts were established using ~50 mg nonnecrotic FaDu tumors pieces in 8–12-week-old female athymic mice. To evaluate the efficacy of cetuximab, mice bearing an established tumor were randomly divided into treated and

control groups and animals have been treated with treated with cetuximab (1 mg/mouse administered i.v.).

## SULFORHODAMINE B COLORIMETRIC ASSAY

To analyze in vitro the proliferation of selected cell lines, 5000 cells are seeded in 96-well plate to the bottom plate (Costar, Corning Incorporated, Corning, NY), in 6 replicates of 24 hours before testing the sulforhodamine B (SRB). The detection of cell growth is determined every 24 hours for 5 consecutive days. At the appointed time, the cells are fixed by adding 50 $\mu$ l/well of trichloroacetic acid (TCA) to 50% cold and the plates are incubated for 1 hour at 4 ° C. The fixed cells are stained with 50 $\mu$ l/well of SRB diluted to 0.4% in 1% acetic acid for 30 minutes at room temperature and after suction and evaporation of the dye, the cells are solubilized for 5 minutes stirring at room temperature in 100 l / well of 10 mM unbuffered Tris pH 10.5. It is estimated by reading the optical density in a spectrophotometer at 490 nm sample.

## LUNG ENZIMATIC DIGESTION

To evaluate cellular lung infiltrates, lungs from mice injected with tumor cells and treated 3 times at 24-hr intervals with TLR3 agonist (1.5 mg) or saline aerosol were digested in DMEM medium containing collagenase (300 U/ml) and hyaluronidase (100 U/ml) (Stemcell Technologies) for 1 hr at 37°C. Cell suspensions were filtered through 70- $\mu$ m cell strainers and, after lysis of red blood cells, analyzed for immune cell subtypes by flow cytometry.

## IMMUNOFLUORESCENCE ANALYSIS

Lung tissue was collected 4 weeks after i.v. injection of 5 $\times$ 10<sup>5</sup> B16 melanoma cells in mice treated or not with aerosolized TLR3 agonist (1.5 mg) and double-stained for CD68 (green) and arginase (red). Nuclear staining (blue) was performed using DAPI.

## ANNEXIN-V FITC ASSAY

Cells were incubated with for 48 h with TLR3 agonist (50 µg/ml) and then were incubated with FITC Annexin V in a buffer containing propidium iodide (PI) and analyzed by flow cytometry. Untreated cells were primarily FITC Annexin V and PI negative, indicating that they were viable and not undergoing apoptosis. After a 48 hour treatment there were primarily two populations of cells: cells that were viable and not undergoing apoptosis (FITC Annexin V and PI negative) and cells undergoing apoptosis (FITC Annexin V positive and PI negative).

## FLOW CYTOMETRY

For flow cytometry, cell suspensions obtained from digested lung were surface-stained for 30 min at 4°C with the following directly conjugated Abs: CD45APCeFluor780; CD11bPE, CD11bPECy5; CD11cPE-Cy7; F480PerCPCy5.5. Purified rat anti-mouse CD16/CD32 mAb was used to prevent nonspecific binding of Abs to mouse Fc receptors. Cells were examined using a FACSCanto flow cytometer and the data were analyzed using FlowJo software. All analyses were performed gating on CD45+ cells after dead cell and doublet exclusion to detect immune infiltrate. 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, 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. 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 100µCi <sup>51</sup>Cr 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:  $100 \times (\text{experimental cpm} - \text{spontaneous cpm}) / (\text{maximum cpm} - \text{spontaneous cpm})$ .

## PHAGOCYTOSIS ASSAY

Macrophage antibody-dependent cell-mediated phagocytosis (ADCP) was assessed by flow cytometry (Correale P. Et al Int J Cancer 2012, 130(7):1577–1589. 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<sup>2+</sup>- and Mg<sup>2+</sup>-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.

## STATISTICAL ANALYSIS

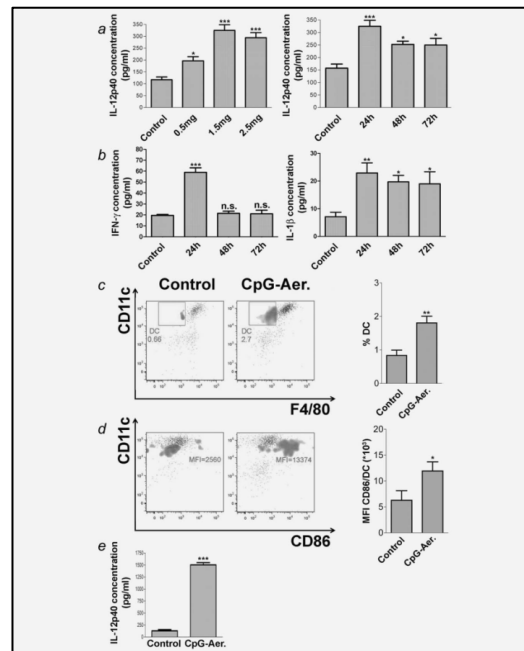
Analyses were performed using GraphPad Prism 5 (Graph- Pad Software). For studies comparing differences between two groups, the unpaired Student's t test was used. For differences between more than two groups, statistical significance was determined using one-way Anova test, followed by a Dunnett's or Tukey's post-test for comparison between groups.

Percent survivorship was estimated by the Kaplan-Meier product limit method and compared with the log-rank test.

# RESULTS

## ANTITUMOR ACTIVITY OF REPEATED LOCOREGIONAL CpG-ODN ADMINISTRATIONS IN EXPERIMENTAL LUNG CARCINOMAS

The efficacy of aerosolized CpG-ODN in reaching the lung and inducing an immune response was evaluated in BAL fluid 24, 48 and 72 hr after aerosol treatment with 5 ml of saline containing 0.5, 1.5 or 2.5 mg of oligonucleotide 1826 (5-10 FVB mice in the same aerosol box). Pesce and colleagues showed that among cytokines misured by ELISA-based multiplex analysis produced in lung after intranasal CpG administration, IL-12 was the most increased; based on those results, we evaluated the IL-12 production, a marker of the immunological response caused by CpG-ODN areosolized administration. IL-12p40 production was threefold higher in mice treated with 1.5 or 2.5 mg of CpG-ODN versus saline-treated control mice and remained high even after 48 and 72 hr, while no significant increase in IL-12p40 was detected with the dose of 0.5 mg (Fig. 8a).

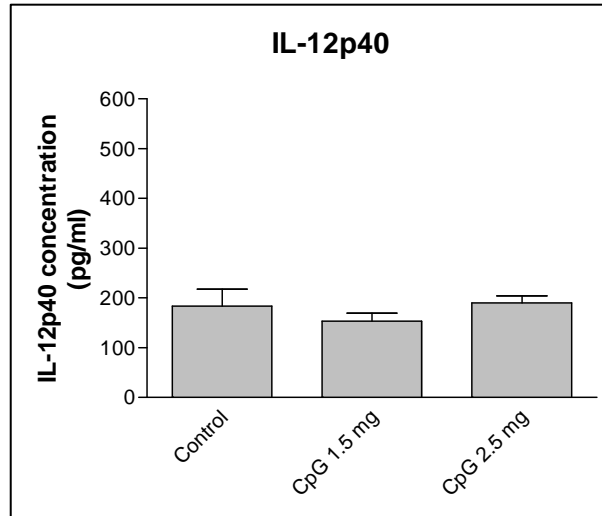


**Figure 8.** Immune effects of aerosolized CpG-ODN in BAL fluid. (a) IL-12p40 levels evaluated by ELISA in BAL of mice at 24 hr after aerosol with CpG-ODN at different concentrations (left) and at different times after a single

treatment with 1.5 mg of CpG-ODN (right). (b) IFN- $\gamma$  and IL-1 $\beta$  levels evaluated by proteomic analysis in BAL collected 24, 48 and 72 hr after a single CpG-ODN aerosolization (1.5 mg). All values (pg/ml) are expressed as mean $\pm$ SE (5–6 mice/group). \*\*\* $p$ <0.001, \*\* $p$ <0.01, \* $p$ <0.05 versus control, by one-way ANOVA followed by Dunnet's post-test. (c) A representative flow cytometric analysis of DC (identified as CD11c<sup>+</sup>F4/80<sup>-</sup> cells among CD45<sup>+</sup> cells after lymphocyte exclusion) in BAL after 4 CpG-ODN (1.5 mg) or saline aerosol treatments at intervals of 72–96 hr (left). Histogram on the right shows the frequency of DC in 8–10 mice per group. \*\* $p$ <0.01, \* $p$ <0.05 versus control, by Student's  $t$  test. (d) A representative flow cytometric analysis of CD86 expression level, represented as mean fluorescence intensity (MFI) performed in the DC gate (as described in Panel c) (left). Histogram on the right shows the CD86 MFI in the DC gate in 8–10 mice per group. \*\* $p$ <0.01, \* $p$ <0.05 versus control, by Student's  $t$  test. (e) IL-12p40 levels in BAL collected at 24 hr after the last of four treatments at 72–96 hr intervals with aerosolized CpG-ODN (1.5 mg). Values (pg/ml) are expressed as mean $\pm$ SE (5–6 mice/group). \*\*\* $p$ <0.001 versus control, by Student's  $t$  test.

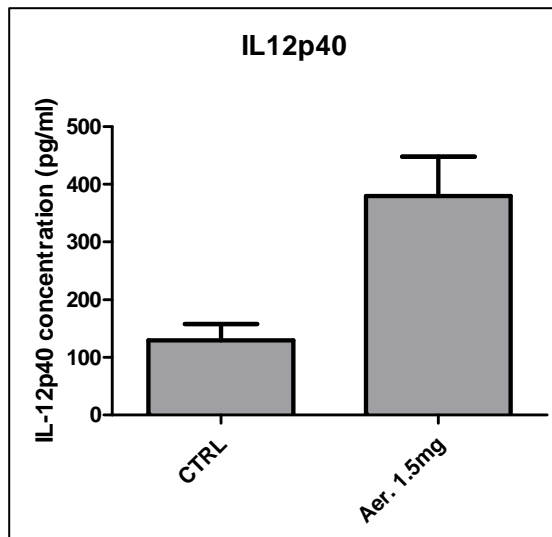
Proteomic analysis of a panel of pro-inflammatory cytokines at 24, 48 and 72 hr after aerosol administration at the selected dose of 1.5 mg CpG-ODN also revealed a significant increase in IL-1 $\beta$  and IFN- $\gamma$  (Fig. 8b) but no significant modulation of IL-2, IL-4, IL-5, IL12p70, TNF- $\alpha$  or IL-6 in the BAL fluids. After having evaluated the efficacy of aerosolized CpG-ODN in reaching the lung and inducing an immune response, we performed an analysis of DC cells (CD45<sup>+</sup>CD11c<sup>+</sup>F4/80<sup>-</sup>), which are selectively recruited by i.n.-delivered CpG in the bronchoalveolar space (Pesce I. et al., *J Innate Immun* 2010). The results of these experiments revealed no significant changes in the percentage of these cells at 24, 48 and 72 hr in BAL fluid recovered from mice treated once with aerosolized CpG-ODN at 0.5, 1.5 or 2.5 mg/5 ml saline, while repeated treatments with 1.5 mg CpG-ODN at intervals of 72–96 hr for 2 weeks induced a significant increase in the percentage of DC population in BAL fluid as compared to control mice (Fig. 8c) and enhanced maturation of these cells, as indicated by the significant increase in expression levels of the activation marker CD86 (Fig. 8d). This treatment schedule also induced high production of IL-12p40 in BAL fluid (Fig. 8e).

Moreover, our results indicated that the IL12p40 production after aerosolized CpG-ODN treatment could be locally restricted to the lung, by the fact that no modulation of IL12p40 concentration was observed in sera of mice treated at the different doses of CpG-ODN as compared to that in control mice (Fig. 9).



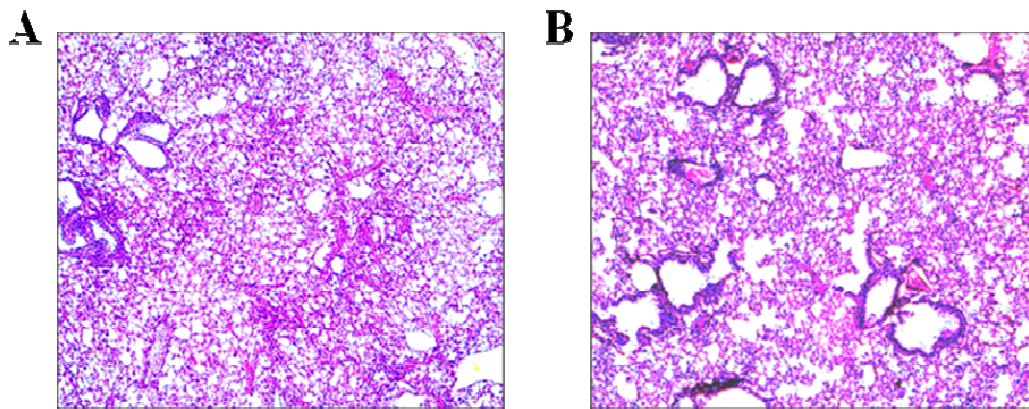
**Figure 9.** Immune effects of aerosolized CpG-ODN in mouse sera. L-12p40 levels were evaluated by ELISA in sera of mice at 24 hr after aerosol with CpG-ODN at different concentrations of CpG-ODN. Values (pg/ml) are expressed as mean $\pm$ SE (5–6 mice/group). \*\*\* $p$ <0.001 versus control, by Student's  $t$  test.

With the aim to study the effect of aerosolized CpG-ODN in other strain of mice, we evaluated the IL12p40 modulation also in C57BL/6 mice: we observed a similar production of IL-12p40 also in experiments using this mice model. (Fig. 10).



**Figure 10.** Immune effects of aerosolized CpG-ODN in BAL fluid. L-12p40 levels were evaluated by ELISA in BAL fluid of mice at 24 hr after aerosol with CpG-ODN at concentrations of 1.5mg of CpG-ODN. Values (pg/ml) are expressed as mean $\pm$ SE (5–6 mice/group). \*\*\* $p$ <0.001 versus control, by Student's  $t$  test.

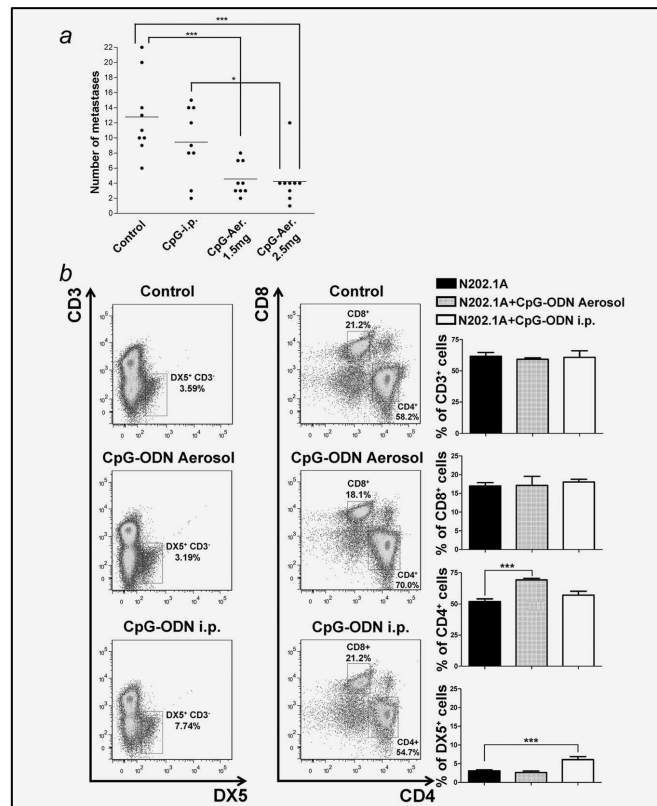
We therefore intended to check if this repeated CpG-ODN administration could generate toxic effects on our mice tissues. Unlike i.n.-administered CpG-ODN, which induces lung tissue inflammation associated with weight loss in rodents (Campbell JD. et al., J Clin Invest 2009; Knuefermann P. et al. *Respir Res* 2007; Tasaka S. et al., *Respir Res* 2009), prolonged repeated treatments at intervals of 72-96 hr with 1.5 mg CpG-ODN aerosol for 3 weeks were well tolerated. No effects on body weight and no histological changes in the structure of lungs, as indicated by histopathological examination of hematoxylin and eosin-stained sections of lung tissue, were observed in mice exposed to CpG-ODN aerosolization (Fig. 11).



**Figure 11.** Effect of CpG-ODN -treatments on mice lung morphology. Histopathological hematoxylin and eosin-stained sections of lung tissue of mice treated (B) or not (A) with prolonged repeated treatments at intervals of 72-96 hr of 1.5 mg CpG-ODN aerosol for 3 weeks.

Together, the results indicate that aerosolized CpG-ODN can reach the bronchoalveolar space in the lung and locally activate an innate immune response without apparent signs of toxicity. We decided to evaluate the effect of CpG-ODN treatment administered by aerosol firstly in mammary N202.1A tumor model. This tumor cell line is a mammary carcinoma clone derived from a HER-2/neu transgenic mouse of FVB background (N#202 transgenic line). N202.1A cells express high levels of surface HER-2/neu and their expression of major histocompatibility complex class I glycoproteins (H-2<sup>q</sup>) is low. The efficacy of CpG-ODN aerosolization versus i.p. administration in controlling the growth of experimental lung metastases was evaluated in mice bearing the immunogenic N202.1A tumor, a mammary carcinoma overexpressing the rat neu

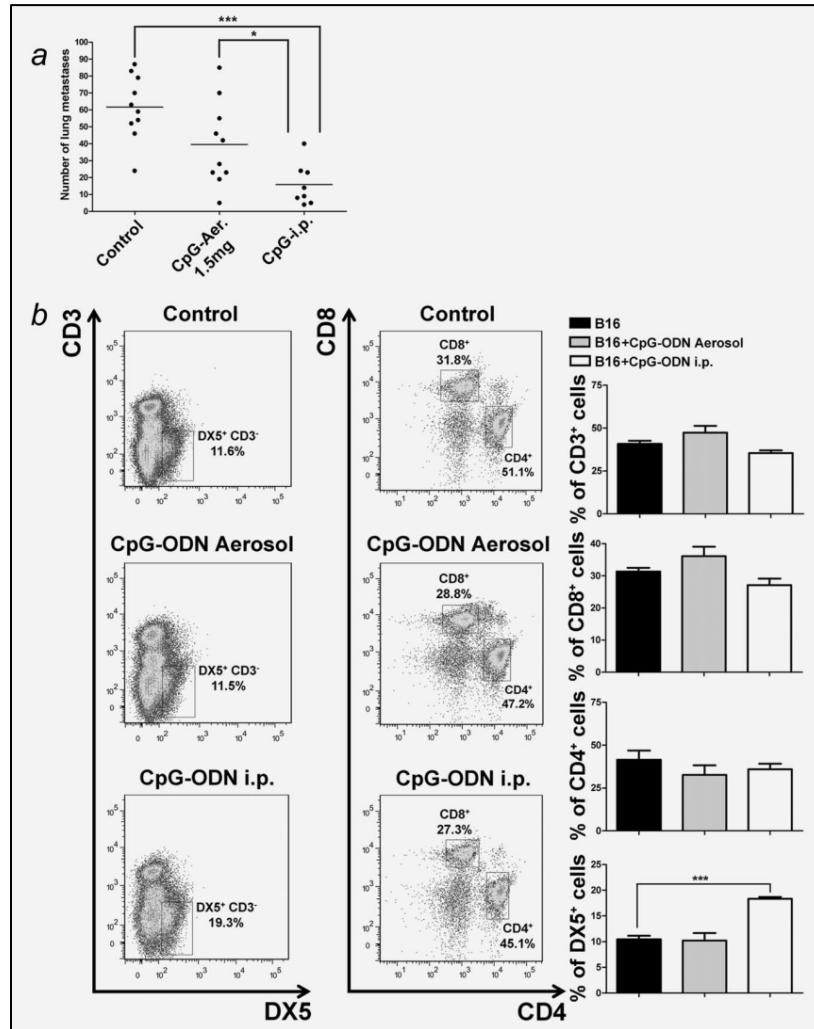
oncogene. FVB mice were injected i.v. with  $3 \times 10^5$  N202.1A cells and, 72 hr later, treated at 72–96 hr intervals for 3 weeks with aerosolized 5 ml saline containing 1.5 or 2.5 mg CpG-ODN (to treat up to 10 mice in the same aerosol box) or with 20  $\mu$ g/mouse CpG-ODN administered i.p. (in 200  $\mu$ l saline). At 5 weeks after treatment, the number of lung colonies was significantly lower in mice treated with 1.5 or 2.5 mg CpG-ODN aerosolization ( $p < 0.001$  1.5 mg CpG-ODN vs. control;  $p < 0.05$  2.5 mg CpG-ODN vs. i.p.), but not in mice treated i.p. with CpG-ODN, as compared to controls (Fig. 12a).



**Figure 12.** Effect of aerosol or intraperitoneal administration of CpG-ODN on N202.1A experimental lung metastases. (a) Number of macroscopic lung metastases at 5 weeks after i.v. injection of N202.1A mammary carcinoma cells in mice untreated or treated with CpG-ODN i.p. or by aerosol (9 mice/group). \* $p < 0.05$ ; \*\*\* $p < 0.001$  by one-way ANOVA followed by Tukey’s post-test. (b) A representative flow cytometric analysis of immune infiltrate of lungs of mice at 2 weeks after i.v. injection with N202.1A tumor cells untreated or treated with CpG-ODN aerosol or i.p. NK cells were identified as DX5+CD3-, while CD4+ and CD8+ T cells were identified in CD3+ gated cells (among CD45+ FSClowSSClow-gated cells). Histograms show the frequency of different populations in 5 mice per group (mean±6SE). \*\*\* $p < 0.001$  by oneway ANOVA followed by Dunnet’s post-test.

Moreover, each i.p. CpG-ODN injection, but not aerosolized CpG-ODN, induced transient weight loss (about 1 g) in mice. Our results indicated that local treatment with aerosolized CpG-

ODN is more efficient in reducing tumor lung colonies respect to systemic treatment, indicating a superior antitumor activity of the locoregional treatment. We checked if this different protective effect obtained from the CpG-ODN i.p. or aerosolized administration could be due to a different immunologic population stimulation. To compare the immune effector cells infiltration induced in tumors by the TLR agonist through the two administration routes, the experiment above was repeated and lung infiltration of CD45+ cells was evaluated by flow cytometry after enzymatic digestion of lung tissue at day 15 after tumor cell injection. Analysis of the FCS<sub>low</sub>SSC<sub>low</sub> fraction (Fig. 12b) revealed a significantly increased percentage of CD3+CD4+ T cells but no modulation of NK cells (DX5+CD3-) in the lung of mice receiving aerosolized CpG-ODN as compared to untreated N202.1A tumor-bearing mice, while i.p. administration induced a significant increase of NK cells but did not modulate the percentage of T cells (CD3+CD4+ or CD3+CD8+). Thus, locally administered CpG-ODN was more effective in promoting an expansion of CD4+ T cells in lungs bearing the immunogenic N202.1A tumor, whereas i.p. administration preferentially expanded NK cells. As most primary lung tumors and frequently lung metastases derived from other tumor histotypes in humans are only weakly immunogenic, we compared the efficacy of the two administration routes also against B16 murine melanoma, in which is reported that immunological protection is mediated primarily by NK cells (Sfondrini L. et al., *Cancer Immunol Immunother* 2004; Glasner A. et al., *J Immunol* 2012; Zheng S. et al., *Oncol Lett* 2012) Mice were injected i.v. with  $5 \times 10^5$  B16 cells and treated with 1.5 mg aerosolized or i.p.-administered CpG-ODN (20  $\mu$ g/mouse) at 72–96 hr intervals for 3 weeks, beginning 72 hr after tumor cell injection. A third group of mice was left untreated as a control for lung tumor colonization. Our results indicated that in contrast with the N202 tumor model, B16 tumor bearing mice treated with CpG-ODN aerosol showed no significant reduction in the number of lung metastases at 5 weeks, while i.p. CpG-ODN administration induced significant protection ( $p < 0.0001$  CpG-ODN i.p. vs. control) (Fig. 13a).

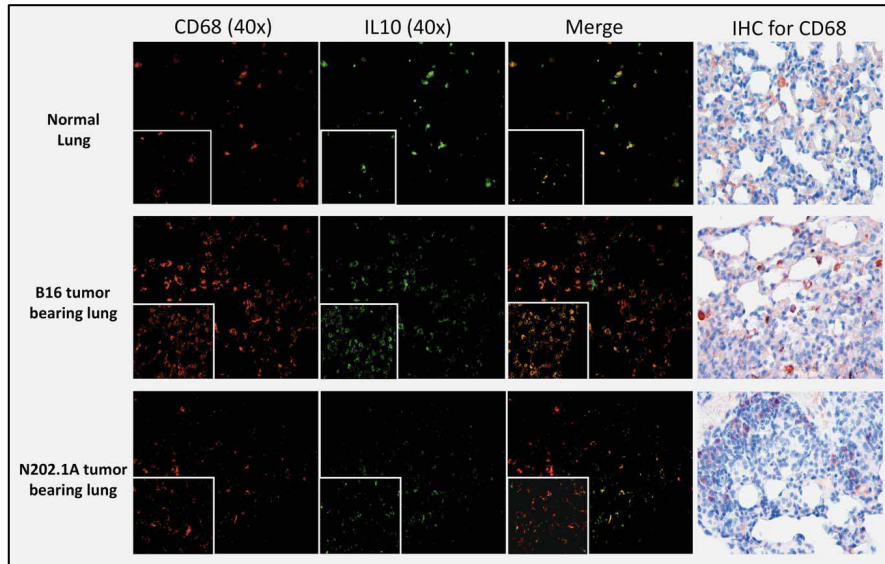


**Figure 13.** Effect of aerosol or intraperitoneal administration of CpG-ODN against B16 experimental lung metastases. (a) Number of macroscopic lung metastases at 5 weeks after i.v. injection of B16 melanoma cells in mice untreated (10 mice) or treated with CpG-ODN aerosol (10 mice) or i.p. (8 mice). \* $p < 0.05$ ; \*\*\* $p < 0.001$  by one-way ANOVA followed by Tukey's post-test. (b) A representative flow cytometric analysis of immune infiltrate of lungs of mice at 2 weeks after i.v. injection with B16 tumor cells untreated or treated with CpG-ODN aerosol or i.p. NK cells were identified as DX5<sup>+</sup>CD3<sup>-</sup>, while CD4<sup>+</sup> and CD8<sup>+</sup> T cells were identified in CD3<sup>+</sup> gated cells (among CD45<sup>+</sup> FSC<sup>low</sup>SSC<sup>low</sup>- gated cells). Histograms on the right show the frequency of different populations in 5 mice per group (mean $\pm$ 6SE). \*\*\* $p < 0.001$  by one-way ANOVA followed by Dunnett's post-test.

By the fact that in previous N202.1A tumor model we found that local aerosolized CpG-ODN therapy expanded a different immunological population compared to systemic CpG-ODN administration, we checked if results obtained also in B16 melanoma model could depend on



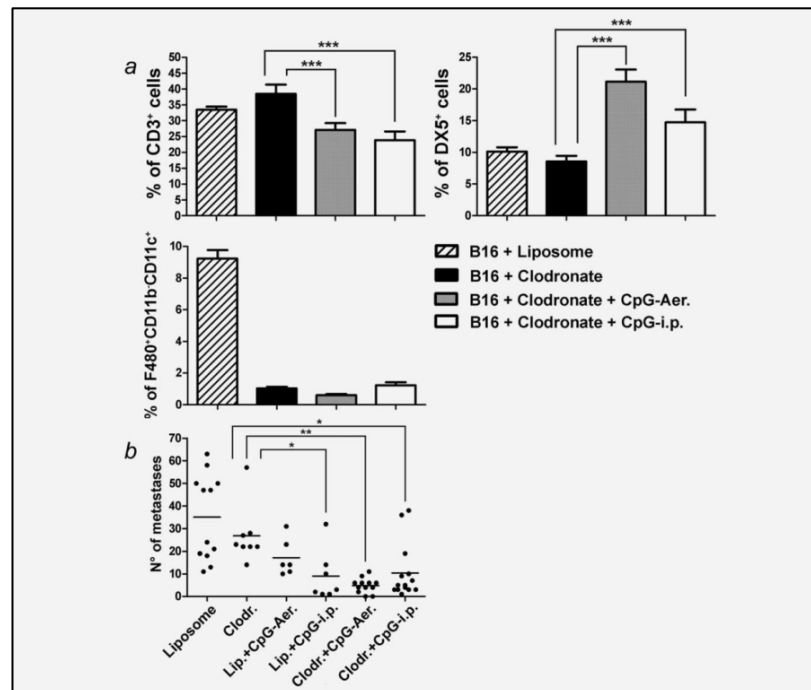
different stimulation of the immune population cells. We performed flow cytometric analysis of CD45<sup>+</sup> immune effector cells in treated or untreated mice after enzymatic digestion of lung tissues at day 15 after tumor injection and we noted that neither aerosol nor i.p. administration expanded CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> T cells in this tumor model, whereas i.p., but not aerosol CpG-ODN, treatment induced a strong increase in the percentage of NK cells (Fig. 13b). The increased percentage of NK cells in the lung induced by systemic treatment did not appear to reflect specific tumor-induced recruitment, but rather an increased number of CpG-induced circulating NK cells, because a similar expansion of DX5<sup>+</sup>CD3<sup>-</sup> cells was detected in lung as well as in spleen and blood of tumor-free mice after i.p. injection of CpG-ODN (23.7%±61.7% in i.p.-treated vs. 12.5%±60.9% in untreated mice in the lung; 4.8%±60.2% and 5.3%±60.5% in i.p.-treated vs. 3.4%±60.1% and 2.7%±60.3% in untreated mice in the spleen and blood, respectively; 4 mice/group). We hypothesized that the inability of aerosol CpG-ODN to induce effective immune activation in B16 tumor-bearing lungs could depend on a strong immunosuppressive activity possibly exerted by alveolar macrophages, which characterize this tumor microenvironment. It's reported in literature, in fact, that with progressive tumor growth, resident pulmonary alveolar macrophages, which are the most abundant inflammatory cells in the lungs, frequently shift their polarization to the M2 phenotype and exert suppressive activity on T and NK cells (Young MR. et al., *J Leukoc Biol* 1987; Jessup JM. et al., *Cell Immunol* 1985; Young MR. et al., *J Natl Cancer Inst* 1986; Bilyk N. et al., *Immunology* 1995). Therefore, we performed a double immunofluorescence to detect the presence of macrophages (CD68) secreting IL-10, a marker of the M2 phenotype, in lungs of mice injected i.v. with B16 or N202.1A tumor cells: our results revealed the presence of a high number of CD68<sup>+</sup> cells secreting IL-10 in B16 tumor-bearing mice, whereas only low number of CD68/IL-10 double-positive cells was detected in the lungs of N202.1A tumor-bearing mice (Fig. 14).



**Figure 14.** Analysis of IL-10-secreting macrophages in tumor-bearing lungs. Immunofluorescence analysis of sections of lung tissue collected 4 weeks after i.v. injection of B16 melanoma cells or N202.1A mammary carcinoma cells and double-stained for CD68 (red) and IL-10 (green). Representative images show single and double (merged) staining of formalin-fixed, paraffin-embedded samples. Immunohistochemical staining shows CD68+ cells populating alveolar spaces of normal lungs and lungs with either B16 or N202.1A tumor metastases. Original magnification 3400, 3200 inset magnification.

Thus, the inability of aerosol CpG-ODN to induce effective immune activation in B16 tumor-bearing lungs might be due to a strong immunosuppressive activity established by alveolar macrophages in this tumor microenvironment. As a consequence, we performed experiments to deplete those immunosuppressive macrophages, in order to allow NK expansion induced by aerosol CpG-ODN in this tumor model. To deplete alveolar macrophages we treated animals with clodronate encapsulated liposomes. Liposomes are artificially prepared lipid vesicles, consisting of concentric phospholipid bilayers entrapping aqueous compartments. They can be used to encapsulate strongly hydrophilic molecules solved in aqueous solutions, such as clodronate, a non-toxic bisphosphonate, developed for human application. Freely solved clodronate will not cross liposomal or cellular phospholipid membranes. After injection, liposomes, used as Trojan horses in this case, will be ingested and digested by macrophages followed by intracellular release and accumulation of clodronate. At a certain intracellular concentration, clodronate induces apoptosis of the macrophage. We first determined empirically the effect of a single i.t. injection of clodronate encapsulated liposomes: results obtained

indicated that only a single i.t. injection greatly reduced the percentage of alveolar macrophages (% of CD45+CD11c+CD11b- in digested lungs:  $2.66 \pm 1.3$  in Cl<sub>2</sub>MDP liposome-treated versus  $11.17 \pm 2.3$  in liposometreated mice evaluated 72 hr after injection). Secondly, consistent with previous data (Tasaka S. et al., Respir Res. 2009; 10:84.), we tested the effect of macrophage depletion on aerosol CpG-ODN-induced NK cell expansion in four groups of mice (5 mice/group) injected i.v. with  $5 \times 10^5$  B16 cells; after 96 hr from the injection, we treated animals i.t. with 100 ml of Cl<sub>2</sub>MDP-liposome (three groups) or 100 ml of control liposome (the fourth group). After 24 hr, two groups of mice given Cl<sub>2</sub>MDP liposomes started the treatment with aerosol (1.5 mg) or i.p. (20  $\mu$ g) CpG-ODN for 4 days/week for 2 weeks, while the third and fourth groups treated with Cl<sub>2</sub>MDP liposome and control liposome, respectively, received no CpG-ODN. We then checked the effect of the double treatment by analyzing the phenotype and composition of the immune cell infiltrate in the lung at 24 hr after the last CpG-ODN treatment (Fig. 15a): results indicated that in the absence of resident macrophages, after i.t. injection of clodronate encapsulated liposomes, aerosol CpG-ODN treatment did indeed induce significant NK cell expansion in lungs of B16 tumor-bearing mice.

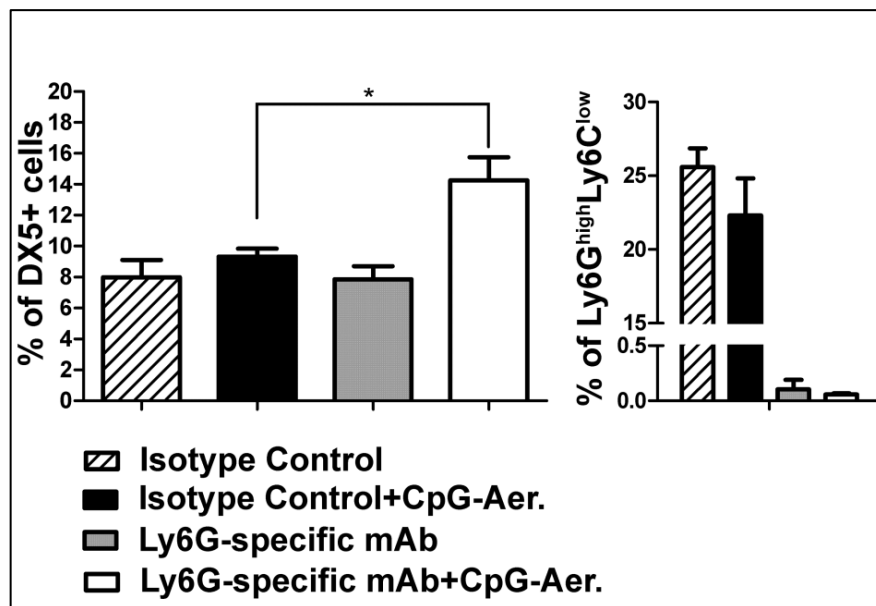


**Figure 15.** Effect of clodronate-induced macrophage depletion on aerosol or intraperitoneal CpG-ODN treatment of B16 lung metastases-bearing mice. (a) Frequency of CD3<sup>+</sup> T cells (among CD45<sup>+</sup> FSC<sub>low</sub>SSC<sub>low</sub>-gated cells), NK cells (DX5<sup>+</sup>CD3<sup>-</sup> among CD45<sup>+</sup> FSC<sub>low</sub>SSC<sub>low</sub>-gated cells) and alveolar macrophages (F4/80<sup>+</sup> CD11b<sup>-</sup> CD11c<sup>+</sup>

among CD45+ gated cells) evaluated by flow cytometric analysis of immune infiltrate of lungs of mice at 2 weeks after i.v. injection with B16 tumor cells and i.t. treatment 96 hr later with clodronate encapsulated liposomes or PBS-liposomes. At 24 hr after liposome treatment, two groups of clodronate-treated mice started treatment with CpGODN i.p. or aerosol. Histograms represent pooled data from 5 mice per group (mean±6SE). \*\*\*p<0.001 by one-way ANOVA followed by Dunnet's post-test. (b) Number of macroscopic lung metastases at 5 weeks after i.v. injection of B16 melanoma cells in mice injected i.t. with PBS-liposomes alone (12 mice), clodronate encapsulated liposomes alone (8 mice), PBS-liposomes followed by aerosol (6 mice) or i.p. (7 mice) CpG-ODN treatment, or clodronate encapsulated liposomes followed by aerosol (12 mice) or i.p. (14 mice) CpG-ODN treatment. \*p<0.05; \*\*\*p<0.001 by one-way ANOVA followed by Tukey's post-test.

Moreover, in these lungs the increased percentage of DX5+CD3- cells gated in the CD45+ population corresponded to a significant decrease of the percentage of CD3+ cells; on the other hand, mice treated with clodronate alone revealed a reduced percentage of alveolar macrophages without modification in the percentage of NK and CD3+ cells as compared to mice treated with control liposomes. Based on those encouraging results, we then determined whether the changes in cellular immune lung infiltrates induced by CpG-ODN aerosol in clodronate treated B16 tumor-bearing mice would lead to improved antitumor activity. Six groups of mice were injected i.v. with B16 cells and, after 96 hr, injected i.t. with Cl<sub>2</sub>MDP liposomes (three groups) or control liposome (three groups). Mice started treatment with CpG-ODN aerosol (one group Cl<sub>2</sub>MDP liposome-treated and one group liposome-treated) or i.p. (one group Cl<sub>2</sub>MDP liposome-treated and one group liposome-treated) at day 5 (120 hr) after tumor injection, in order to allow better depletion of resistant macrophage before CpG-ODN aerosol treatment. Treatment was continued for 4 days/week for 3 weeks. The remaining two groups of Cl<sub>2</sub>MDP liposome- and control liposome- treated mice received no CpG-ODN. Results indicated that resistant macrophage depletion was able to potentiate the local antitumor activity of aerosol CpG-ODN therapy: in macrophage-depleted mice, in fact, aerosolized oligonucleotide treatment induced a significant reduction in the number of lung metastases, as observed 5 weeks after tumor injection (Fig. 15b). On the other hand, macrophage depletion did not modify the efficacy of i.p. treatment. Moreover, clodronate-induced macrophage depletion per se did not reduce tumor growth, as the number of lung metastases in Cl<sub>2</sub>MDP liposome- versus control liposome-treated mice did not differ significantly. These data indicate that depletion of resident macrophages in tumor-bearing mice allows CpG aerosol therapy to stimulate a local immune response that confers significant protection from lung metastases. It is reported in literature that Ly6G granulocytic polymorphonuclear neutrophil subsets of myeloid-derived suppressor cells (PMN-

MDSC) are able to impair NK cell development specifically; it has also been shown that a cross-talk between tumor-associated macrophages and MDSCs determines a sustantion and an exacerbation of immune suppression in the tumor microenvironment (Ostrand-Rosenberg S. et al., Semin Cancer Biol 2012). Based on those observations, we tested whether MDSCs population was also involved in preventing NK cell expansion induced by CpG-ODN aerosol. We injected four groups of mice (5 mice/group with B16 cells as above and, 96 hr later, we treated animals every 72–96 hr with the Ly6G-specific mAb (1A8), which is reported to recognize and deplete Ly6G<sup>high</sup> neutrophilic populations, or with isotype control. At 24 hr after the first mAb treatment, two groups of mice started the treatment with aerosolized CpG-ODN (1.5 mg) for 4 days/ week for 2 weeks, while the other two groups of antibody treated mice received no CpG-ODN. Results demonstrated that 1A8 mAb treatment induced a strong depletion of granulocytic MDSC, identified as Ly6G<sup>high</sup>CD11b<sup>+</sup> cells not expressing or expressing low levels of the Ly6C marker, but in the lungs of these depleted mice, CpG-ODN aerosol therapy induced only a slight expansion of NK cells (Fig. 16).

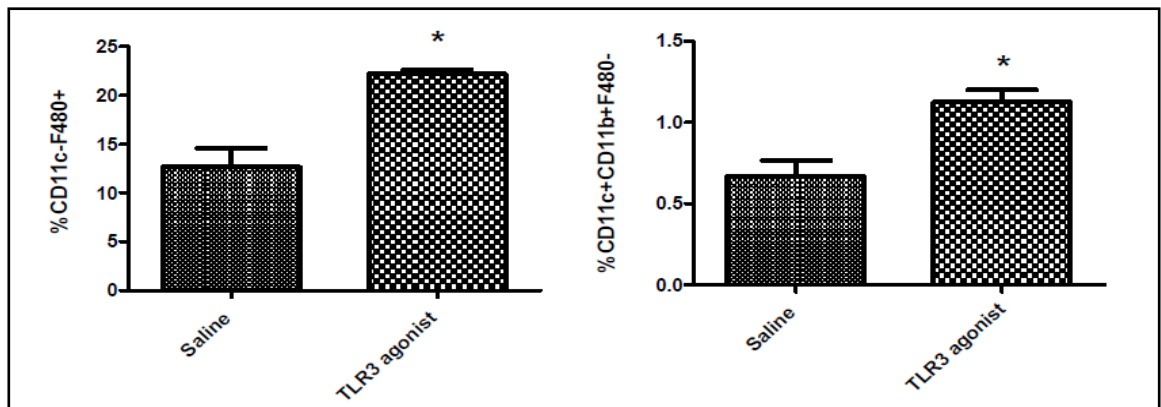


**Figure 16.** Effect of Ly6G<sup>+</sup> neutrophilic depletion on lung cellular infiltrates of B16 lung metastases-bearing mice treated with CpG-ODN aerosol

These evidences suggested that granulocytic MDSC do not play a major role in macrophage-induced immunosuppression.

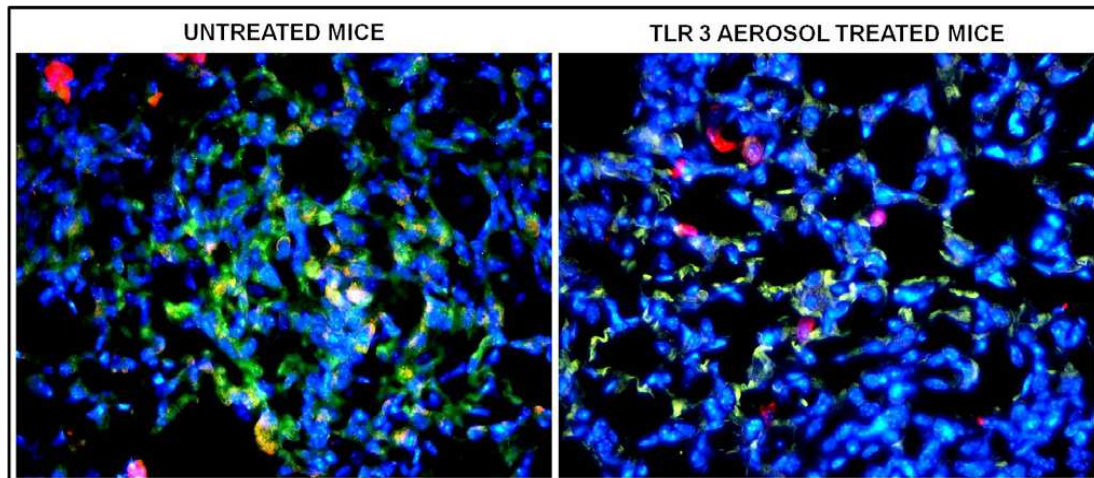
In a recent study in an experimental model has been shown that the TLR3 agonist Poly(I:C) can convert lung tumor-associated macrophages (TAM) from tumor supporters (M2) to those with tumoricidal properties (M1) (Lou Y. Et al., J Immunother 2011). The conversion is related to Poly(I:C) signaling through the TICAM-1/TRIF adaptor to induce expression of M1-related genes in TAM, unlike other TLR agonists, which usually activate a MyD88-dependent signaling pathway. TLR3 agonists are also reported to be able to trigger an innate immune response (Kline JN. Immunol Res 2007; 39:279– 86; Edwards L, et al., Eur J Immunol 2005; 35:273–81). Keeping those observations in mind, we proposed that aerosol-delivered TLR3 agonists might improve the CpG-ODN-induced local innate immune antitumor response even in the presence of an immunosuppressive tumor microenvironment. Moreover, TLR3 agonists might exert a direct cytotoxic effect on lung tumor cells expressing TLR3. In other recent studies it is demonstrated how a direct engagement of TLR3 agonists with TLR3-expressing tumor cell lines block proliferation and induce apoptosis (Garbuzenko OB. et al., Proc Natl Acad Sci USA 2010; Verschraegen CF. et al., Ann NY Acad Sci 2000), while in a clinical trial TLR3 agonist adjuvant treatment has revealed to be beneficial for patients with TLR3-overexpressing breast cancer. Lastly, TLR3 expression in patients with hepatocellular carcinoma was found to correlate with NK cell activation and with longer survival (vanRooijen N. et al., J Immunol Methods 1994). Together, the findings point to the potential of a combined treatment with aerosolized TLR-9 and -3 agonists in simultaneously blocking TAM-induced immunosuppression while activating a large number of innate immune subpopulations, thus providing a direct cytotoxic effect on TLR3-positive tumors. Based on those observations, we firstly evaluated the antitumor activity of aerosolized TLR9 agonist CpG-ODN plus TLR3 agonist Poly(I:C) on experimental lung carcinoma models and determine whether the two agonists synergize in their effects. We performed preliminary experiments to determine whether aerosolized TLR3 agonist reaches the bronchoalveolar space and recruits immune cells. We treated mice with 1.5 mg TLR3 agonist Poly(I:C) (diluted in 5 ml saline in the nebulizer unit to treat 4 mice in the same aerosol box) and control mice received saline. Our results revealed a significant increase of inflammatory macrophages and dendritic cells in immune infiltrates obtained after enzymatic digestion of lungs of mice treated with aereosolized Poly(I:C) as compared to immune infiltrates of saline-treated

mice (Fig.17). Those evidences were in agreement with the ones previously observed for TLR9 agonist CpG-ODN (Sorrentino R. Et al., J. Immunol 2010).



**Figure 17.** Recruitment of innate immune cells in lungs of athymic mice by aerosolized TLR3 agonist. Histograms show the percentage of inflammatory macrophages (CD11c-F4/80+ cells among CD45+ cells) and DC cells (CD11b+CD11c+F4/80- cells among CD45+ cells) in lung immune infiltrates of mice treated 3 times at 24-hr intervals with TLR3 agonist (1.5 mg) or saline aerosol (4 mice/group). Cell suspensions obtained after enzymatic digestion of lungs were analyzed by flow cytometry.\* $p < 0.05$ , Student's t-test.

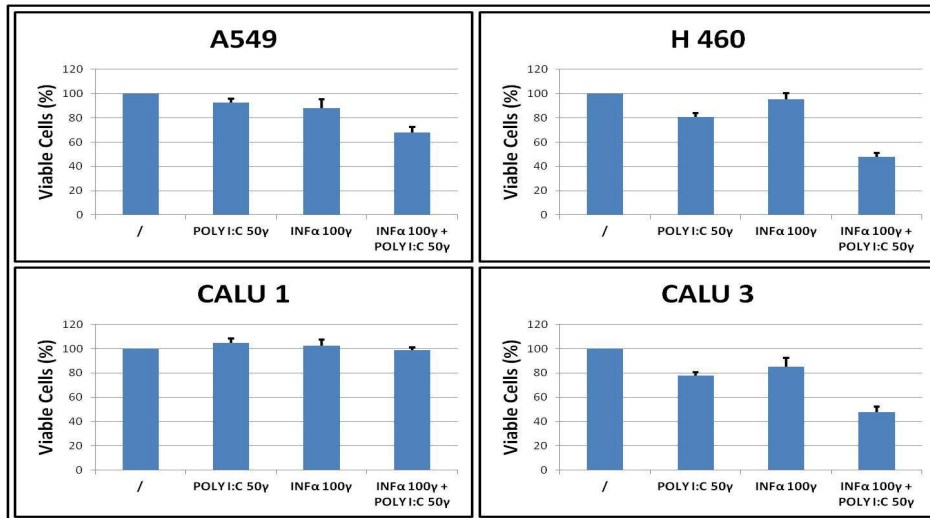
We then decided to examine the effect of TLR3 agonist aerosol treatment on M2 macrophages. Analysis of lung metastases from mice bearing B16 melanoma cells, which is demonstrated to promote a highly immunosuppressive microenvironment (Sfondrini L. et al., FASEB J. 2002;16:1749-1754), revealed that aerosol delivery of TLR3 agonist Poly(I:C) reduced the frequency of lung tumor-associated macrophages with M2 phenotype (Fig. 18).



**Figure 18.** Arginase-producing macrophages in lungs from B16 tumor-bearing mice treated with TLR3 agonist poly(I:C). Immunofluorescence analysis of sections of lung tissue collected 4 weeks after i.v. injection of  $5 \times 10^5$  B16 melanoma cells in mice treated or not with aerosolized TLR3 agonist (1.5 mg) and double-stained for CD68 (green) and arginase (red). Nuclear staining (blue) was performed using DAPI. Representative images show double (merged)-staining of formalin-fixed, paraffin-embedded samples; note the reduction of double-stained macrophages in lung of mice treated with aerosolized TLR3.

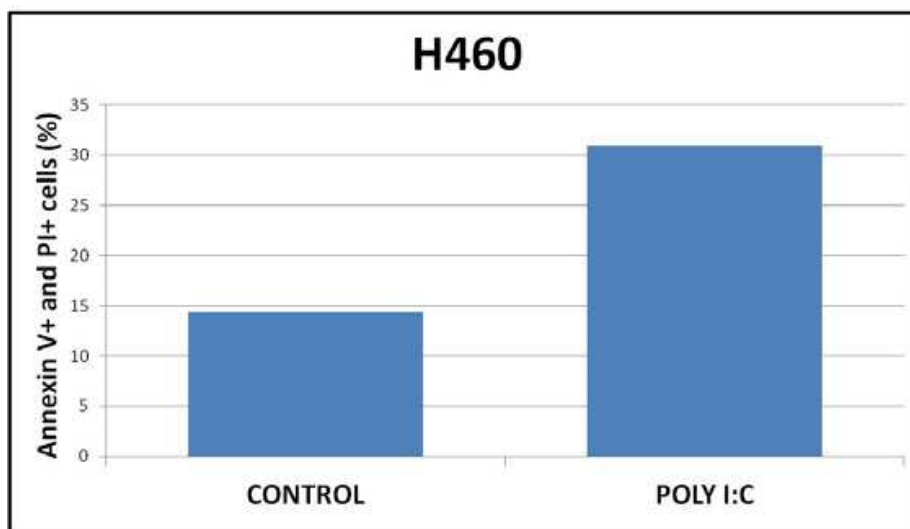
Since TLR3 agonists may have a direct inhibitory effect on TLR3-positive tumor cells that is independent of their activator effect on innate immune cells, we conducted preliminary experiments to evaluate TLR3 expression and responsiveness to TLR3 agonists of lung carcinoma cell lines. We tested the expression of TLR3 on four lung carcinoma cell lines, called Calu3, H460, A549 and Calu1, flow cytometry using anti-TLR3 monoclonal antibody (Fig. 19) revealed the receptor expression on all of four lung tumor cell lines tested. Moreover we analyze the effect of Poly(I:C) treatment on the viability of those cell lines: SRB assay showed a reduced cell viability induced by incubation for 48 h with TLR3 agonist Poly(I:C) of about 22% in Calu3 cells, 20% in H460 cells and 8% in A549 cells, while Calu1 cells remained insensitive, even when exposed for up to 72 h.





**Figure 19.** Effect of poly(I:C) and IFN $\alpha$  on viability of human lung cancer cell lines. A549, H460, CALU 1 and CALU 3 cells were incubated for 48h with Poly(I:C) (50  $\mu$ g/ml), IFN $\alpha$  (100  $\mu$ g/ml) or the combination and cell viability was evaluated with SRB test.

Finally, we performed an annexin V and PI staining of those carcinoma cell lines in order to understand if the reduced cell viability could be due to apoptosis: results indicated that Pol (I:C) treatment caused cell mortality through apoptosis cellular pathway (Fig.20).



**Figure 20.** Effect of poly(I:C) on apoptosis of H460 human lung cancer cell line. Cells were incubated for 48 h with TLR3 agonist (50  $\mu$ g/ml) and evaluated for apoptosis by flow cytometry (Annexin-VFITC assay). Data are representative of 3 independent experiments.

Studies of *in vivo* models of A549, H460, Calu1 and Calu3 are ongoing to evaluate the antitumor activity of the combined therapy of CpG-ODN and Poly(I:C).

## **COMBINATION OF CpG-ODN, MONOCLONAL ANTIBODY AND CHEMOTHERAPY AS HIGHLY EFFICACIOUS TREATMENT FOR ADVANCED OVARIAN XENOGRAFT TUMORS**

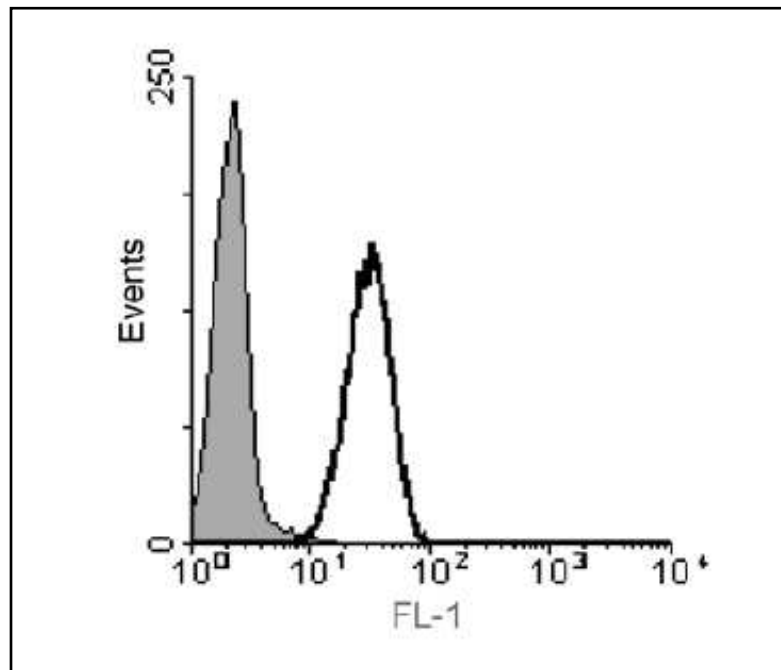
A therapeutic effect on bulky disease appears to require locoregional treatment and also frequent multiple administrations. Indeed, we had observed that a local, but not systemic, and a daily, but not weekly, stimulation of immune effector cells by targeted immunotherapy inhibited ascites production and significantly prolonged survival of athymic mice with bulky advanced-stage ovarian tumor disease. However, even this locoregional and repeated treatment was not able to cure animals (De Cesare M. et al., Clin Cancer Res 2008; De Cesare M. et al., J Immunother 2010). We then evaluated in this advanced-stage human ovarian tumor bearing mice, in which ascitic fluid formed 11 days after tumor cell injection and animals showed evident abdominal volume increase, if combination of CpG-ODN with other therapeutic agents could further increase benefits observed with targeted immunotherapy alone.

Therefore we screened the effectiveness of CpG-ODN in combination with different agents, including:

- 1) MAb bevacizumab, which targets the vascular endothelial growth factor (VEGF). VEGF is reportedly overexpressed in ovarian cancer (Dvorak HF et al., Am J Pathol 1995; Paley PJ. et al., Cancer 1997; Boocock CA. et al., J Natl Cancer Inst 1995), and VEGF-regulated angiogenesis is an important component of ovarian cancer growth (Hu L. et al., Clin Cancer Res 2005; Pourgholami MH. et al., Clin Cancer Res 2006);
- 2) the Poly(I:C) TLR3 agonist, which reportedly induces a synergistic effect when combined with TLR9 ligand by mediating an enhanced activation of innate immunity (Whitmore MM. et al., Cancer Res 2004);

- 3) MAb Cetuximab, which targets the ligand-binding domain of EGFR and is frequently overexpressed in ovarian cancer cells (Schilder RJ. Et al., Gynecol Oncol 2009);
- 4) Gefitinib, a tyrosine kinase inhibitor of EGFR.

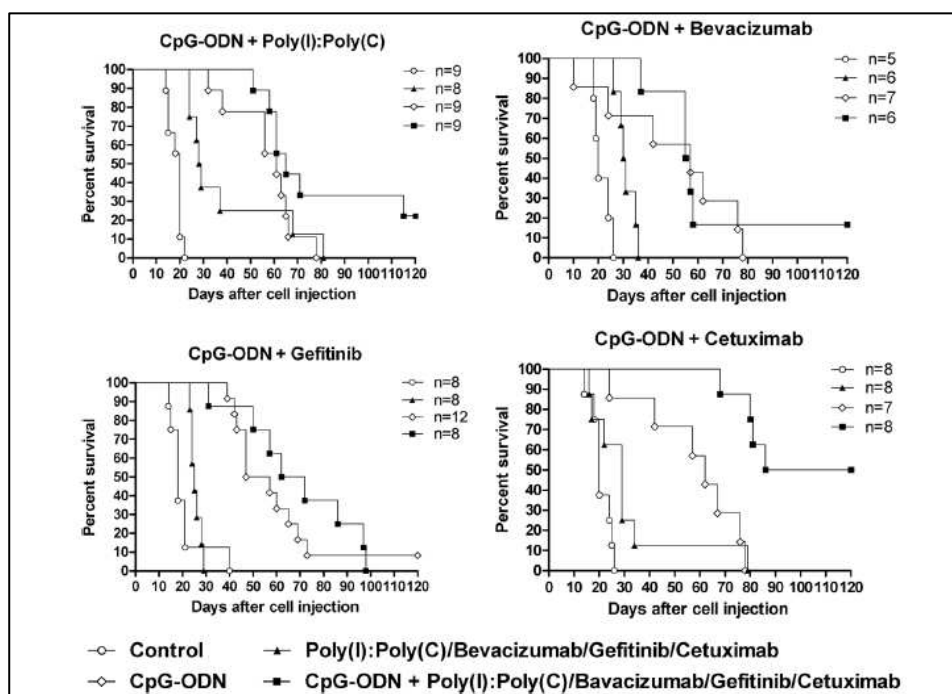
Before analyzing the therapeutic effect of the combination of those last two molecules, we firstly checked if our ovaric tumor model cell line IGROV-1 expressed EGFR, accordingly with literature data (Bijman MN. et al., Anticancer Drugs. 2009 Jul; 20(6):450-60). We performed cytofluorimetric analysis to confirm that IGROV-1 cells expressed EGFR (Fig. 21),



**Figure 21.** Flow cytometric analysis of IGROV-1 surface expression of EGFR. Cells were stained with cetuximab (black line, panel B), and with anti-CD20 rituximab antibody as isotype control (grey line).

To evaluate the efficacy of CpG-ODN in association with Poly(I:C), bevacizumab, gefitinib (Iressa) or cetuximab, mice were injected i.p. with  $2.5 \times 10^6$  IGROV-1 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.

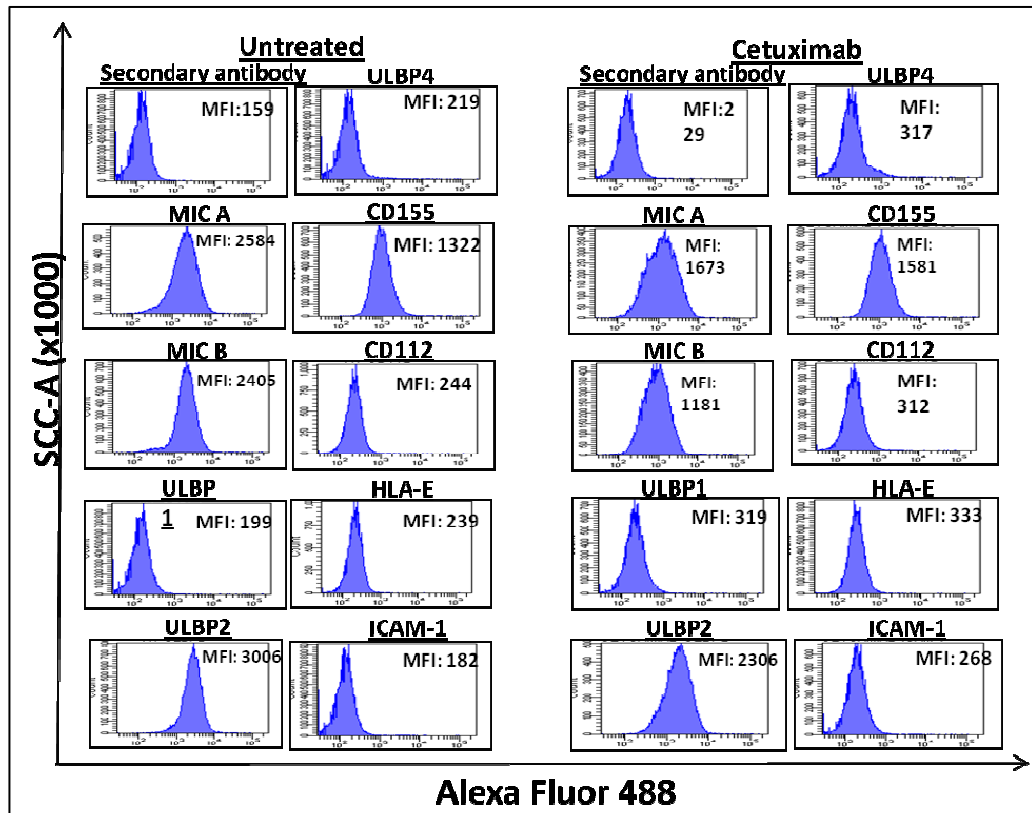


**Figure 22.** Kaplan-Meier plot of percent survival over time among IGROV-1 ovarian tumor-bearing mice. At 7 days after tumor cell injection, mice were treated i.p. with CpG-ODN (20  $\mu$ g/mouse, 5 days/week for 4 weeks) in combination with: Poly(I):C (20  $\mu$ g/mouse at 2- to 3-day intervals); 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.

As shown in figure 22, repeated i.p. CpG-ODN treatments plus Poly(I):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 end of the experiment (120 days). These results were not in agreement with those previously observed which demonstrated a clear synergy between the two immune modulators; this could be possibly due to the schedule of CpG-ODN administration. Indeed, daily CpG-ODN administration might induce massive innate cell activation hardly expandable by other immune modulators. Repeated i.p. CpG-ODN treatments plus anti-VEGF Bevacizumab (Fig.22) also did not enhance 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, 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 (Kobold S. et al., *Oncologist* 2009). 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,  $p = 0.4099$ ) (Fig.22). 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) (Fig.22), with 4 of 8 mice still alive at the end of the experiment. To note, even if IGROV-1 cells express EGFR, their growth has been showed to be independent from this receptor; as a consequence, treatment with Cetuximab alone was able to induce only a slight increase of mice lifespan compared to control mice. Different factors probably concur 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 the predominant immunological population is represented by NK cells and macrophages; also, those cells are reported to be much more biologically active when target cell's antigens have been bound by specific antibodies, exerting their cytotoxic activity through antibody-dependent cell cytotoxicity (ADCC). Additionally, as EGFR modulates a variety of downstream signaling pathways, such as NF- $\kappa$ B, PI3-K, MAPK, and PKC pathways (Zhang X. et al., *Int J Med Sci.* 2008 Jul 11; 5(4):209-17; Gadgeel SM et al., *Cancer.* 2009 May 15;115(10):2165-76), 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 and/or in phagocytosis (Kobold S. et al., *Oncologist* 2009).

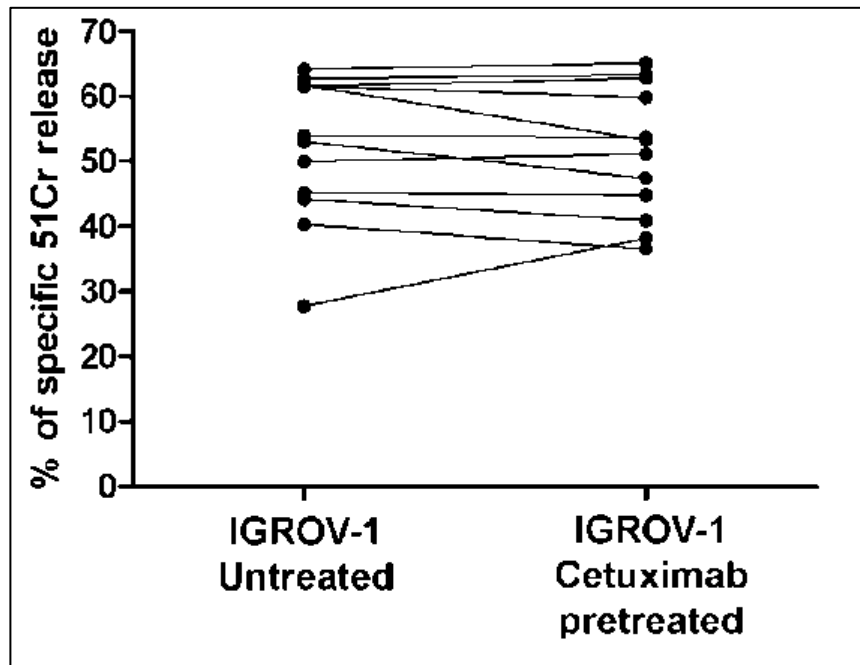
Because HER signaling in tumors regulates expression of MICA and MICB, key ligands that promote NK cell-mediated recognition and cytolysis (Benard J. et al., *Cancer Res* 1985), and because EGFR inhibitors enhance susceptibility to NK cell-mediated lysis by modulating expression of the NKG2D ligand ULBP-1 (Correale P. et al., *Int J Cancer* 2012; Kobold S. et al., *Oncologist* 2009; Roda JM, et al., *J Immunol* 2005, 175(3):1619–1627), 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. (Fig. 23).



**Figure 23.** Expression levels of molecules involved in NK-mediated cytotoxicity in IGROV-1 cancer cell line after cetuximab pretreatment. ( $p < 0.05$ )

Moreover, we performed a  $^{51}\text{Cr}$ -release ADCC assay using Cetuximab-pretreated or untreated IGROV-1 cell targets and PBMC from 12 healthy donors as effector cells. Tumor cells were pretreated with cetuximab for 72 hours, before their use as targets on ADCC assay, conducted with

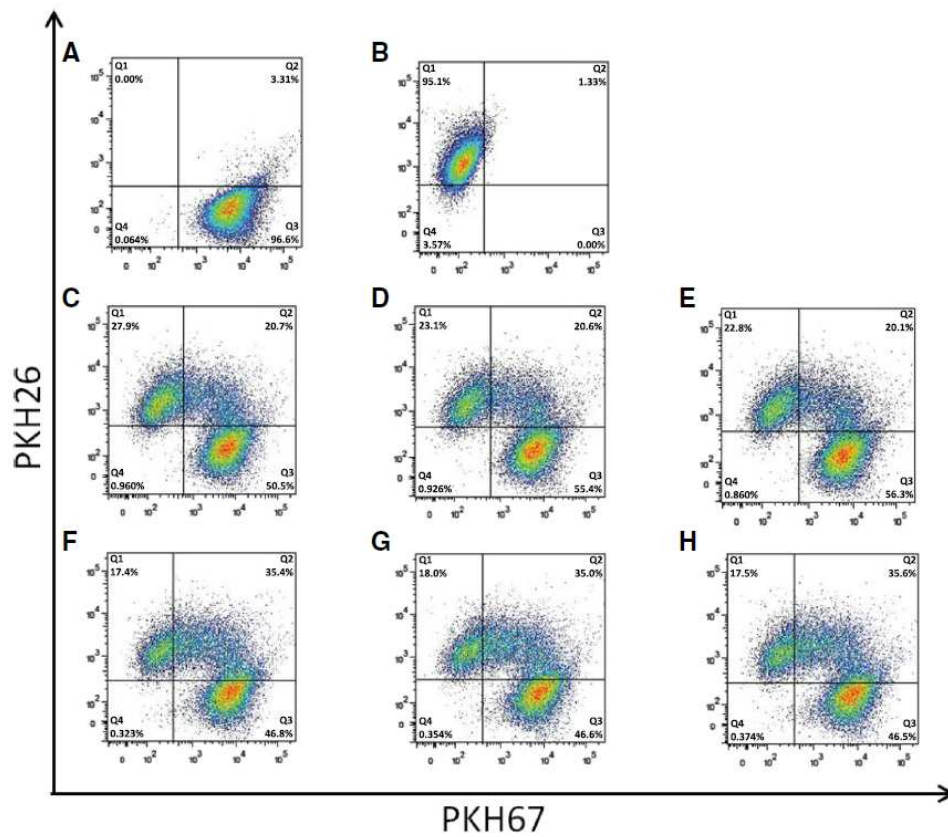
saturated concentration of cetuximab (10 $\mu$ g/ml) and using as effector cells PBMC from 12 healthy donors. Accordingly with FACS analysis, <sup>51</sup>Cr-release ADCC assay revealed no increase in death percentage in the pretreated tumor cells (Fig. 24).



**Figure 24.** 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.

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 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 5 $\mu$ g/ml). Also this experiment has been carried out in overload monoclonal

antibody condition (final concentration of cetuximab 10 $\mu$ g/ml). The results shown in the figure 25 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. These findings raise the possibility that the strong anti-tumor activity observed in the CpG-ODN/Cetuximab treatment might be due in part to increased susceptibility to phagocytosis of tumor cells induced by cetuximab.

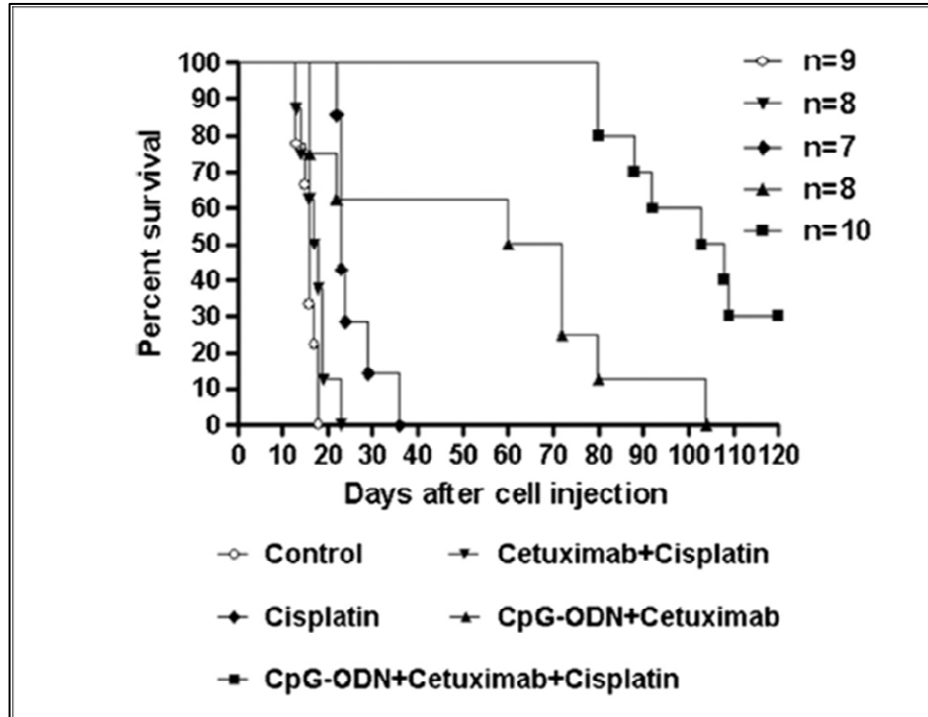


**Figure 25.** Effect of cetuximab pretreatment on phagocytosis of IGROV-1 cells. IGROV-1 target cells were stained green with PKH67 (A, right lower quadrant) and RAW264.7 effector cells were stained red with PKH26 (B, left upper quadrant). Tumor targets were pre-incubated for 72 h with 5  $\mu$ g/ml 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  $\mu$ g/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 in an 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, if the double combination of CpG-ODN and cetuximab added to another molecular agent could further increase its therapeutic effect. EGFR inhibitors are reported to interact with cisplatin (Ahsan A. et al., *Cancer Res* 2010; Sano D. et al., *Clin Cancer Res* 2011; Zhang Y. et al., *J Huazhong Univ Sci Technolog Med Sci* 2011; Weng Y. et al., *J Huazhong Univ Sci Technolog Med Sci* 2011), although their effect on sensitivity to this drug remains undefined; also, we recently reported the synergistic antitumor effect between CpG-ODN and cisplatin (Sommariva M, et al., *Cancer Res* 2011, 71:6382–6390). Keeping those evidences in mind, we selected mice for evident and established ascites from a large group of animals injected i.p. 11 days before with IGROV-1 cells (mean body weight  $\pm$  SEM  $27.9 \pm 0.84$  g vs  $23.00 \pm 1.08$  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 (Fig. 26).



**Figure 26.** Kaplan-Meier plot of percent survival over time in advanced-stage IGROV-1 ovarian tumor-bearing mice. 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  $\pm$  SEM  $27.89 \pm 0.84$  g vs  $23.00 \pm 1.08$  g before tumor cell injection) were treated with saline, cisplatin (3 mg/kg, once per week), CpG-ODN (20  $\mu$ g/ 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.

Together, results indicate that combinatorial therapies, enhancing immune response in the tumor microenvironment and concomitantly targeting tumor cells, are successful even in experimental advanced malignancies, and suggest, although the differences in the distribution of TLR9 in mice and human and the enrichment on innate immune cells in athymic cause for caution, a promising clinical strategy for treating ovarian patients with bulky malignant ascites.

# **ANTI-TUMOR ACTIVITY OF LOCOREGIONAL COMBINED CPG-ODN THERAPY IN EXPERIMENTAL HEAD-AND NECK CARCINOMA MODELS**

Despite an aggressive multimodal approach, more than 50% of patients with locally advanced SCCHN will relapse. The worse prognosis of these cancers must certainly be link to the fact that HNSCCs strongly influence the host immune system. Moreover, head and neck carcinomas, which are characterized by locoregional spread, are hardly accessible in the majority of patients, making them an attractive target for a local therapy. We observed that the combination of cetuximab plus CpG-ODN led to a significantly increased survival-time as compared to CpG-ODN or cetuximab alone in IGROV-1 ovarian tumor ascites-bearing athymic mice. Furthermore, Damiano et al., (Hu L, et al., Clin Cancer Res 2005, 11(22):8208–8212.) have shown that a synthetic agonist of TLR9 (IMO) impairs EGFR activity and its downstream signaling proteins; the authors have also demonstrated that the combination of IMO with cetuximab synergistically inhibits human colon cancer xenografts. Together, these findings provide the rationale to evaluate in experimental head and neck carcinoma models whether the combination of cetuximab, (approved by FDA to treat late-stage head and neck cancer), with a local CpG-ODN treatment might improve the therapeutic efficacy of the MAb. As head-and neck xenograft carcinoma model, we evaluated human CAL-27 tumor growth in athymic nude mice. This cell line expresses high levels of EGFR and is sensitive to cetuximab in nude mice xenograft models (Pourgholami MH, et al., Clin Cancer Res 2006, 12(6):1928–1935). Mice have been injected s.c. in the right flank with human CAL-27 cells and tumors have been constantly measured. Cal-27 tumors have demonstrated a significant proliferation without inducing animal suffering; therefore, we firstly utilized this xenograft model to evaluate the antitumor activity of CpG-ODN in head- and neck carcinomas. To treat animals locally with CpG-ODN we used Alzet Mini Osmotic Pumps: those devices are miniature, implantable pumps for research in mice, rats, and other laboratory animals. These minipumps deliver drugs, hormones, and other test agents at

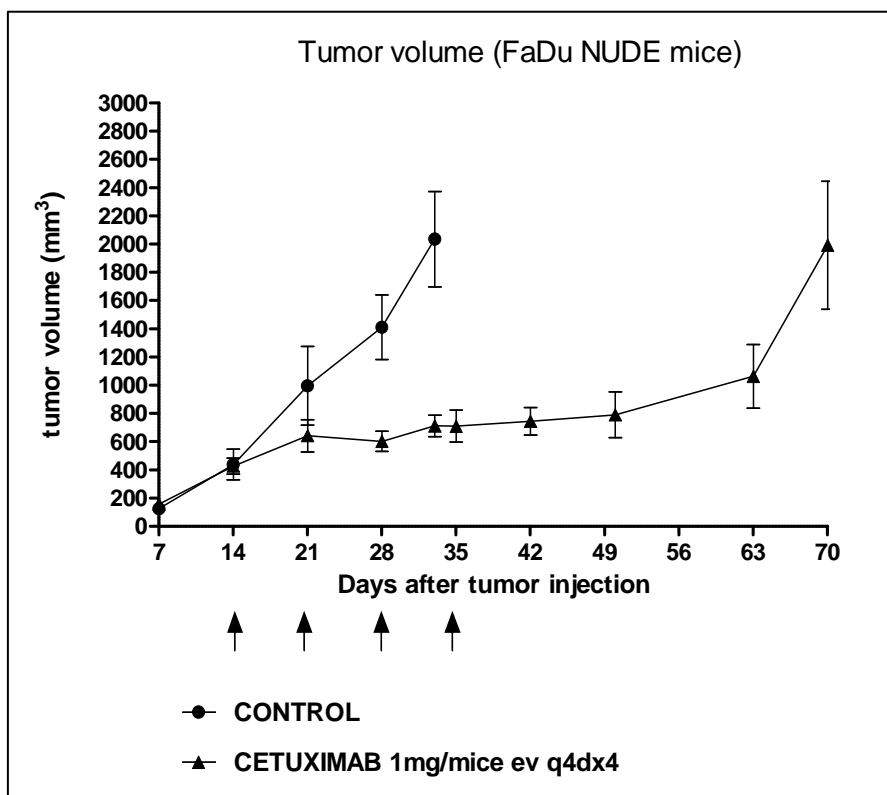
continuous and controlled rates, for durations ranging from one day to six weeks, without the need for external connections or frequent handling. Their unattended operation eliminates the need for repeated nighttime or weekend dosing by lab personnel (www.alzet.com). Both nude and SCID mice have been injected s.c. in the right flank with human CAL-27 cells; when mice showed established tumors, they have been randomly divided into treated and control groups and animals have been treated with CpG-ODN delivered using an Alzet osmotic pump (inserted s.c. in the proximity of tumor) or cetuximab (1mg/mouse administered i.v.). The Alzet pump, which has a pumping rate of 0.2 $\mu$ l/h ( $\pm$ 0.05 $\mu$ l/h), provides continuous infusion of CpG-ODN at about 20  $\mu$ g/day for 14 days. We treated animals also with CpG-ODN i.p. (20  $\mu$ g/mouse, 5 days/week), as we observed a therapeutic effect in previous ovarian IGROV-1 tumor model. Experimental groups (8-10 mice) have been inspected daily and weighed three times weekly. The effects on tumor growth has been evaluated by measuring the two perpendicular diameters of the tumor mass twice each week and calculating the tumor volume as  $\frac{1}{6} \times \text{length} \times \text{width}^2$ .

<i>TUMOR</i>	<i>ISOTOYPE</i>	<i>MICE STRAIN</i>	<i>MICE TUMOR STAGE</i>	<i>% OF TUMOR ERADICATION</i>
Cal-27	Oral Squamous Cell Carcinoma (tongue)	nude	early	100
Cal-28	Oral Squamous Cell Carcinoma (tongue)	nude	late	100
Cal-29	Oral Squamous Cell Carcinoma (tongue)	SCID	early	100
CaTC-1	Oral Squamous Cell Carcinoma	nude	early	100
FaDu	Oral Squamous Cell Carcinoma (pharynx)	nude	medium	/

**Tab. 2** Effect of cetuximab treatment on head and neck tumor models.

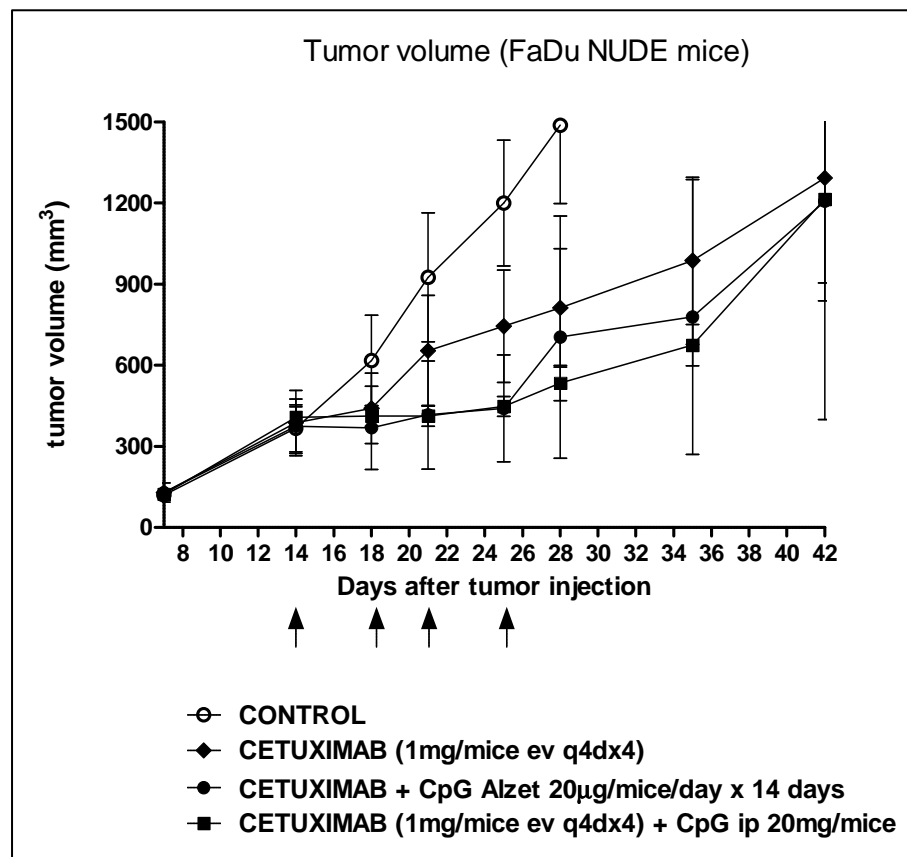
Our results indicated that treatment with cetuximab alone was able to completely eradicate Cal-27 tumors both in nude and in SCID mice model and that the combination of cetuximab and CpG-ODN delivered using an Alzet osmotic pump was not able to significantly enhance the antitumor effect the previous compound. Our experiments also indicated that treatment with CpG-ODN i.p alone or delivered using an Alzet osmotic pump was able to inhibit the growth of CAL-27 tumors, but did not to cure animals; infact, after the suspension of those treatments, tumors begun to grow again (Tab.2). Therefore, human CAL-27 tumor was found not suitable for testing the efficacy of the cetuximab plus CpG-ODN treatment, since cetuximab alone completely eradicated established tumors both in athymic and in SCID mice and also both in early and in an advanced tumor stage. Another experimental group (8-10 nude mice) has been injected s.c. in the right

flank also with human CaTC-1 tumor, another head and neck carcinoma delivered from patients. We tested the sensibility of this tumor firstly to cetuximab alone: when mice showed established tumors, they have been randomly divided into treated and control groups. Treated animals have been subjected to cetuximab administration (1mg/mouse administered i.v.); our results indicated that cetuximab alone was able to completely eradicate also this type of tumor. Based on those observations, we consequently evaluated the effect of cetuximab treatment on different type of head- and neck carcinoma. We firstly evaluated human FaDu tumor growth in athymic nude mice. This cell line has been demonstrated to show a moderate EGFR expression and lower in comparison to Cal27 and A431 cells (Dvorak HF, et al., Am J Pathol 1995, 146:1029–1039). Mice have been injected s.c. in the right flank with human FaDu cells and tumors have been constantly measured. FaDu tumors have demonstrated a significant proliferation without inducing animal suffering; therefore, we utilized this xenograft model to further evaluate the antitumor activity of CpG-ODN in head- and neck carcinomas.



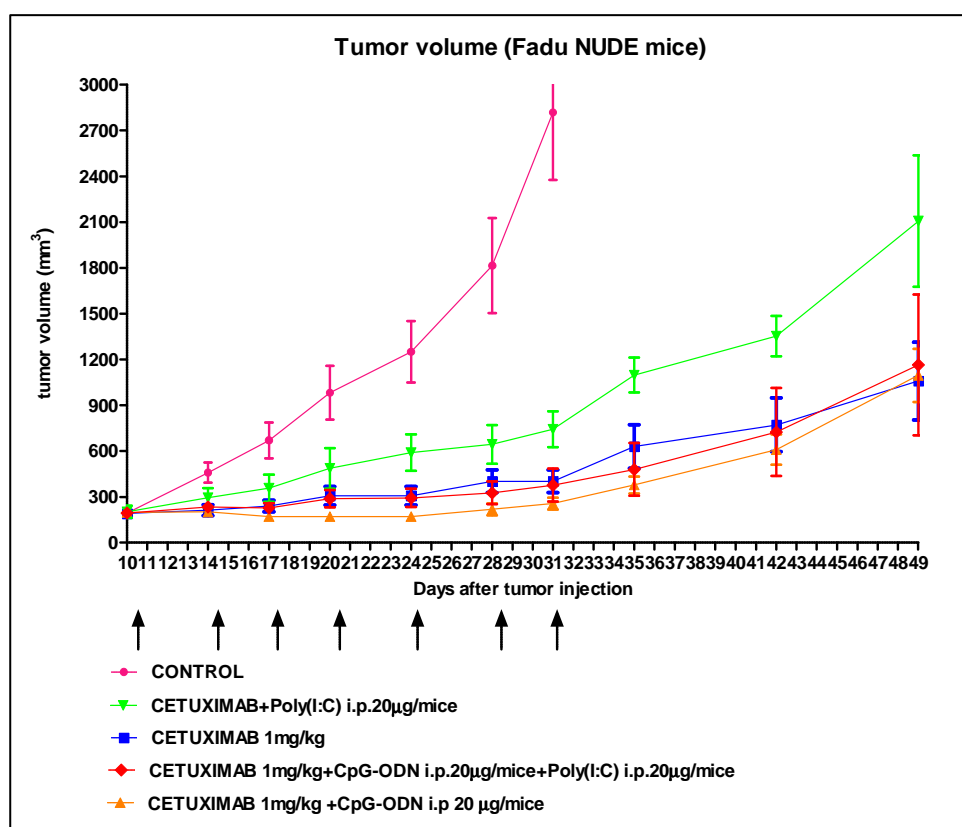
**Figure 27.** Mice bearing an established tumor ( ~ 100–200 mg, 7 days after implantation) were treated with cetuximab (1 mg/mouse administered i.v.).

FaDu tumor xenografts were established using ~50 mg nonnecrotic tumors pieces in 8–12-week-old female athymic mice. Mice bearing an established tumor (~100–200 mg, 7 days after implantation) were treated with cetuximab (1mg/mouse administered i.v.) (Fig.27). Results indicated that treatment with cetuximab alone was able to reduce tumor volume and to stabilize tumor growth; however, about 15 days after the suspension of the treatment, tumors began to grow again. Therefore, we considered this model suitable to evaluate the potentially improved therapeutic efficacy of local CpG-ODN treatment in combination with the monoclonal antibody cetuximab to reduce or completely inhibit human FaDu xenograft tumor growth and to speculate about possible *in vitro* molecular related mechanisms.



**Figure 28.** Mice bearing an established tumor ( ~ 100–200 mg, 7 days after implantation) were treated with cetuximab (1 mg/mouse administered i.v.). CpG-ODN (20µg/mouse administred i.p. or with Alzet Pumps)

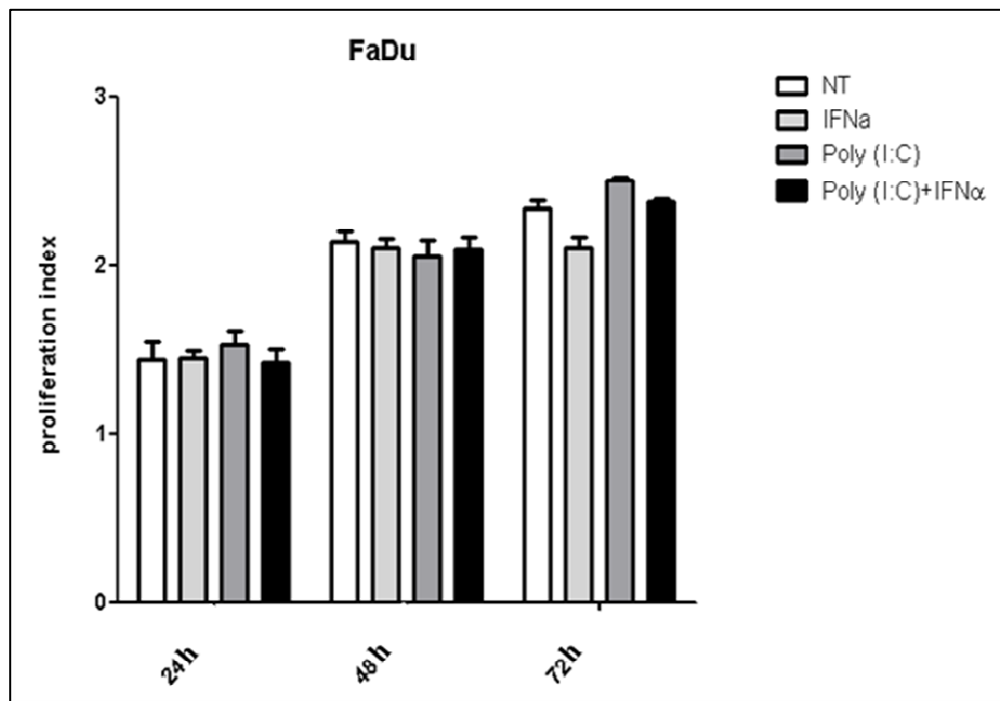
As expected, treatment with cetuximab alone was not able to eradicate tumor. However, the combinational therapy of cetuximab with CpG-ODN, delivered both with Alzet osmotic pumps and i.p, was able to slightly reduce FaDu tumor volume, even if not significantly, but did not completely eradicate tumor (Fig. 28). In order to improve the therapeutic effect of cetuximab combined with CpG-ODN, we decided to treat animals also with Poly(I:C) immunostimulant, a synthetic dsRNA TLR3 agonist, reported to elicit host immune responses and induce tumor cell apoptosis (Friedberg JW, et al., Br J Haematol 2009, 146(3):282–291)



**Figure 29.** Mice bearing an established tumor ( ~ 100–300 mg, 7 days after implantation) were treated with cetuximab (1 mg/mouse administered i.v.), CpG-ODN (20µg/mouse administered i.p. or with Alzet Pumps) and with Poly(I:C) (20µg/mouse administered i.p).

Moreover we tried to improve the effect of the therapy prolonging the number of treatments. As indicated by figure 29, treatment with cetuximab alone was able to reduce tumor volume and to

stabilize tumor growth; the combined therapy of cetuximab and CpG-ODN i.p. was able to better reduce tumor volume respect to cetuximab treatment alone, but not to determine a significant decrease or eradication of FaDu tumor. On the other hand, the combination of cetuximab and Poly(I:C) unexpectably was less effective than treatment with cetuximab alone and seemed also to determine a slight tumor growth, probably interfering with the inhibitory effect of the monoclonal antibody. The triple combination of cetuximab, CpG-ODN and Poly(I:C) was not able to induce an increased reduction of tumor growth, as respect to cetuximab treatment alone. In order to try to explain those unpected results, we decided to performed an *in vitro* proliferation test after treatment with Poly(I:C) treatment. We treated FaDu cells *in vitro* with Poly(I:C) and with IFN $\alpha$ , which is reported to potentiate Poly(I:C) effect in enhancing the TLR3 antiviral response (Tassari J et al., J Immunol. 2005 Apr 1;174(7):4289-94); treatments were performed as single agent or in combination.



**Figure 30.** FaDu cells were treated with Poly(I:C) (50  $\mu$ g/ml) or with IFN $\alpha$  (100  $\mu$ g/ml) or with the combination at different time point (24, 48 and 72 hours) and analyzed with SRB assay.



Our results indicated that FaDu cells are resistant to Poly(I:C) alone or to Poly(I:C) combined with IFN $\alpha$  treatments at all time points analyzed (Fig.30). In order to understand why in our head and neck tumor models (Cal27 and FaDu xenograft) CpG-ODN treatment was not able to determine a significant and consistent tumor growth inhibition in contrast to the effect observed in other tumor models, we are investigating if Cal-27 and FaDu cells could be able to evade immune response downmodulating TLR9 on mouse NK cells. Accordingly, it has been reported in literature that plasmacytoid dendritic cells (PDC), the major cell population responding to TLR9 agonists in humans, infiltrating head and neck cancer tissue are functionally impaired (Mimura K, et al., Int J Cancer 2011, 129(10):2408–2416.).

# ***DISCUSSION***

In recent years our concept of the non-specific nature of innate immunity has changed following the identification of a network of germline-encoded receptors that recognise with substantial specificity molecular motifs of microorganisms and many other cues produced during tissue injury. Stimulation of these innate sensors by their specific ligands triggers signalling pathways that result in the activation of innate effector mechanisms as well as the priming of naive lymphocytes for the type of response that must be induced (Montero Vega MT. *Allergol Immunopathol (Madr)*. 2008;36:164–175). Oligodeoxynucleotides (ODN) containing dinucleotides with unmethylated CpG motifs (CpG-ODN) are potent activators of both the innate and adaptive immune systems (Krieg AM. *Proc Am Thorac Soc*.2007 4:289–294; Abreu MT, et al., *J Immunol*.2005; 174:4453–4460.). Recognition of CpG-ODN is mediated by Toll-like receptor 9 (TLR9), a member of the TLR family, which is critically important in detecting microbial pathogens. TLRs, initially identified on cells of the immune system, are also expressed by non-professional immune cells such as endothelial cells, fibroblasts, and epithelial cells (Pratesi G, et al., *Cancer Research* 2005 Jul 15; 65 (14): 6388-93; Klinman DM. *Nat Rev Immunol*. 2004; 4:249–258). Both bone marrow and non-bone marrow-derived cells are thought to be involved in the response induced by TLR agonists. Successes in preclinical studies using CpG-ODN and early indications of its safe use in humans have led to considerable interest in the clinical development of these agents in the treatment of cancer patients (Lonsdorf AS, et al., *J Immunol* 2003 Oct 15; 171 (8): 3941-6; Krieg AM. *Proc Am Thorac Soc*.2007; 4:289–294; Krieg AM. *Curr Oncol Rep*. 2004; 6:88–95).

## ***Antitumor activity of repeated locoregional CpG-ODN administrations in experimental lung carcinomas***

With the aim to explore aerosol administration as a promising route for delivery of CpG-ODN to the murine lung, we performed experiments treating tumor bearing mice with aereosolized CpG-ODN. We previously demonstrated that alveolar macrophages respond to CpG-ODN with

production of IL-12p40 but not IL-12p70, in accordance to literature (Haynes A, et al., *PharmRes* 2005; 22:427–39.); we then performed experiments to evaluate production of same molecules also in murine BAL fluids. Results obtained in our experiments showed that aerosolized CpG-ODN reaches the bronchoalveolar space and can activate a local immune response, as indicated by the increased levels of IL-12p40, IFN- $\gamma$  and IL-1 $\beta$  and the recruitment and maturation of DC in BAL fluid. Results indicated also that there was no production of IL-12p70 in BAL fluids, whose immune cell population is characterized almost exclusively by alveolar macrophages. Moreover, our locoregional and repeated aerosolized CpG-ODN administration didn't show any sign of apparent toxicity usually evaluated as body weight loss and as histological changes in lung architecture. Those evidences were in contrast with the adverse effects reported in mice receiving CpG-ODN intranasal or intratumoral (Young MR, et al., *J NatlCancer Inst* 1986;76:745–50; Bilyk N, et al., *Immunology* 1995;86:231–7; de Haan A, et al., *Immunology* 1996;89:488–93.). We evaluated the antitumor activity of aerosol-delivered CpG-ODN in two murine tumor models with different characteristics and behavior: the mammary carcinoma N202.1A and the B16 melanoma. The first murine tumor model is reported to be highly immunogenic due to overexpression of the heterologous rat neu oncogene (Nanni P, et al., *Int J Cancer*2000; 87:186–94.), while the B16 melanoma is poorly immunogenic and reportedly expresses very low levels of MHC I molecules (Lollini P, et al., *Clin Exp Metastasis* 1990; 8:215.). These two tumors also differ in their sensitivity to immune effectors, with the neu-expressing mouse mammary tumor sensitive to cytotoxic activity of T but not NK cells (Reilly RT, et al., *Cancer Res* 2001; 61:880–3), while NK cells are required to counteract the growth of B16 melanomas (Sfondrini L, et al., *Cancer Immunol Immunother* 2004;53:697–704; Glasner A, et al *J Immunol*2012;188:2509–15; Zheng S, et al., *Oncol Lett* 2012;3:613–6.). Our experiments demonstrated that while N202.1A tumors in the lung induced a low level of CD68+ macrophages secreting IL-10, B16 melanoma cells strongly promoted the presence of double-stained CD68+IL-10+ cells, suggesting the potential of the latter model to induce an immunosuppressive microenvironment. These observations are in agreement with studies showing that alveolar macrophages in the presence of some growing tumors become immunosuppressive (Young MR, et al., *J LeukocBiol* 1987;42:682–8; Jessup JM, et al., *Cell Immunol* 1985;93:9–25; Young MR, et al., *J NatlCancer Inst* 1986;76:745–50.), developing, for

example, M2 tumor-associated macrophages, which play a role in the progression and prognosis of lung cancer (Zeni E, et al., *Eur Respir J*2007;30:627–32; Dai F, et al., *BMC Cancer*2010;10:220.). Depending on their relative importance, any or all of these differences might underlie the different results observed in the two models. In the immunogenic N202.1A model, we observed that aerosol locoregional therapy with CpG-ODN induced the preferential expansion of CD4+ cells and was more efficacious than i.p. administration in control lung metastases. Keeping those informations in mind, we conducted preliminary experiments which demonstrated a complete cure of N202.1A lung metastases in 5 of 10 mice treated with aerosol 15 mg CpG-ODN, without loss of body weight; those evidences suggest the promising possibility of increasing the dose of aerosolized CpG-ODN to achieve complete protection in this immunogenic model. Shifting on B16 melanoma mice tumor model, our experiments revealed that i.p. delivery of CpG-ODN stimulated a strong expansion of NK cells and induced significant protection against lung metastases, in contrast with results obtained in previous tumor model. On the other hand, in this melanoma model CpG aerosol treatment was not able to modify the number of NK cells in lung and did not confer significant protection. We supposed that this CpG resistance could be due to a less immunogenic microenvironment caused by the massive presence of alveolar macrophages. Keeping those observations in mind, we treated animals with liposome encapsulated clodronate, in order to deplete lung macrophages: results revealed that lung macrophages depletion allowed aerosolized CpG-ODN to expand NK cells and also was able to reduce the number of lung metastases. Aerosol inhalation to deliver Cl<sub>2</sub>MDP-liposomes to the lung has been recently reported (Kooguchi K, et al., *Infect Immun* 1998; 66:3164–9.), raising the possibility of aerosolized bisphosphonates in clinical use. We also depleted MDSC granulocytic component treating animals with anti-Ly6G antibody; experiments demonstrated that depletion of the Myeloid-derived suppressor cells (MDSCs) granulocytic component induced only a slight increase in NK cell expansion, in contrast with evidences obtained after lung macrophages depletion. Those results allowed us to exclude major involvement of this component in immunosuppression. Thus, other direct or indirect mechanisms, such as those induced by the production of suppressive cytokines as well as prostaglandin E, hydrogen peroxide and superoxide by alveolar macrophages that support the maintenance of the suppressive microenvironment (Young MR, et al., *J LeukocBiol* 1987;42:682–8.) might be involved in inhibition of CpG local activity. Several studies have reported the superior antitumor effect of

CpG administered directly rather than systemically into the tumor in both immunogenic or non-immunogenic tumor models, since CpG-ODN can activate both innate and adaptive immune responses at the tumor site (De Cesare M, et al., J Immunother 2010;33:8–15; De Cesare M, et al., Clin Cancer Res 2008;14:5512–8; Sommariva M, et al., Cancer Res 2011;71:6382–90; Lou Y, et al., J Immunother 2011;34:279–88.). Our results indicate that this is not true for tumors growing in the lung, where both tumor immunogenicity and the tumor-induced immunosuppressive environment are critical factors in the success of CpG therapy. Thus, different administration routes might be required for different tumors. Also, the composition of immune cells localized to the lung likely reflects the degree to which the immune system is subverted by the tumors, consistent with the reported prognostic value of characterization of immune infiltrate in the lung in non-small cell lung carcinoma and in lung adenocarcinoma (Al-Shibli KI, et al., Clin Cancer Res 2008; 14:5220–7; Zikos TA, et al., Cancer Immunol Immunother 2011;60:819–27; Sfondrini et al. Int. J. Cancer 2013:133, 383–394).

### **Combination of CpG-ODN, monoclonal antibody and chemotherapy as highly efficacious treatment for advanced ovarian xenograft tumors**

We had previously reported that a local, but not systemic, and a daily, but not weekly, stimulation of immune effector cells by targeted immunotherapy with CpG-ODN inhibits ascites production and significantly prolongs survival of athymic mice with bulky advanced-stage ovarian tumor disease. Although daily i.p. administration of CpG-ODN induced a significant increase of survival-time, this treatment did not determine the cure of a single mouse. To mimic clinical treatment situations in advanced human ovarian disease we tested the efficacy CpG-ODN in combination with other possible therapeutic reagents in ovarian carcinoma ascites-bearing athymic mice, with the aim to further increase benefits observed with targeted immunotherapy alone. After having checked citofluorimetrically that our ovaric tumor model cell line IGROV-1 expressed EGFR accordingly with literature data (Bijman MN. Et al., Anticancer Drugs. 2009 Jul;20(6):450-60), we evaluated the efficacy of treatment with CpG-ODN in association with Poly(I:C), bevacizumab, gefitinib (Iressa) or cetuximab in mice with early ascite tumor stage.

CpG-ODN combined with poly(I:C) bevacizumab, or gefitinib did not significantly increase MST as compared with that using CpG-ODN alone, whereas MST in mice treated with CpG-ODN plus cetuximab was significantly increased, with 4/8 mice alive at the end of the experiment. Results obtained with the combined treatment of CpG-ODN and Poly(I:C) were not in agreement with those previously observed, which demonstrated a clear synergy between the two immune modulators; this could be possibly due to the schedule of CpG-ODN administration. Indeed, daily CpG-ODN administration might induce massive innate cell activation hardly expandable by other immune modulators. The therapeutic benefit versus control mice observed in mice treated with bevacizumab, 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 (Kobold S. et al., Oncologist 2009). To note, even if IGROV-1 cells express EGFR their growth has been showed to be independent from this receptor; this evidence could explain why in our experiments treatment with cetuximab alone was able to induce only a slight increase of mice lifespan compared to control mice. Different factors probably concur for the impressive results obtained after the CpG-ODN/cetuximab double combination. 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 the predominant immunological population is represented by NK cells and macrophages; also, those cells are reported to be much more biologically active when target cell's antigens have been bound by specific antibodies, exerting their cytotoxic activity trough antibody-dependent cell cytotoxicity (ADCC). Additionally, as EGFR modulates a variety of downstream signaling pathways, such as NF-kB, PI3-K, MAPK, and PKC pathways (Zhang X. Et al., Int J Med Sci. 2008 Jul 11; 5(4):209-17; Gadgeel SM et al., Cancer. 2009 15;115(10):2165-76), 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 and/or in phagocytosis (Kobold S. et al.,

Oncologist 2009). HER signaling is reported to regulate expression of MICA and MICB in tumors; those molecules are known to be key ligands that promote NK cell-mediated recognition and cytotoxicity (Benard J. et al., *Cancer Res* 1985). Also, EGFR inhibitors are reported to be able to enhance susceptibility to NK cell-mediated lysis by modulating expression of the NKG2D ligand ULBP-1 (Correale P. et al., *Int J Cancer* 2012; Kobold S. et al., *Oncologist* 2009; Roda JM, et al., *J Immunol* 2005, 175(3):1619–1627). Based on those observations, we tested whether cetuximab treatment of IGROV-1 cells could be able to expression of molecules involved in NK-mediated lysis, as for example MICA, MICB, ULBP1, ULBP2, ULBP4, CD112, CD155, ICAM-1 and HLA-E. FACS analysis of IGROV-1 tumor cells pretreated with cetuximab did not reveal any type of modulation but in some cases down-modulation of these receptors. Moreover, we performed a <sup>51</sup>Cr-release ADCC assay using Cetuximab-pretreated or untreated IGROV-1 as cell targets and PBMC from 12 healthy donors as effector cells; <sup>51</sup>Cr-release ADCC assay revealed no increase in death percentage in the pretreated tumor cells, in accordance to evidences obtained from cytofluorimetric analysis previously reported. Another mechanism by which cetuximab is reported to employ its ADCC cytotoxic activity is inducing cell target phagocytosis (Correale P. et al., *International Journal of Cancer*, 2012). Keeping those observations in mind, we investigated if cetuximab treatment could make IGROV-1 cells more robustly phagocytosed by macrophages: cytofluorimetric analyses examining engulfment of PKH26-stained RAW 264.7 after have been co-cultured with PKH67-stained human IGROV-1 cells, pre-treated or not with cetuximab, revealed that cetuximab pre-exposure greatly increases macrophage-mediated phagocytosis of IGROV-1 ovarian tumor cells, and in particular that at 12 hours tumor cell incorporation was greater than 4 hours. These findings raise the possibility that the strong antitumor activity observed in the CpG-ODN/cetuximab treatment might be due in part to increased susceptibility to phagocytosis of tumor cells induced by cetuximab. It should be noted that these results were observed in mice before the appearance of ascites and therefore with a relatively low tumor burden. Indeed, ascites formation is a major cause of morbidity and mortality in advanced ovarian cancer patients. In these patients, in whom the metastatic spread of tumor cells outside the peritoneum is uncommon, the tumor cell deposits in the peritoneal surface may prevent adsorption of i.p. fluid by mechanical obstruction, inducing ascites (Jeon BH et al., *Cancer Res.* 2008; 68:1100–1109). Treatment of ovarian cancer ascites is characterized by different palliative therapeutic options, but in this advanced stage tumor is often much less

responsive to most anti-cancer therapies than limited disease. To this aim, we evaluated in advanced-stage human ovarian IGROV-1 bearing mouse, in which ascitic fluid formed 11 days after tumor cell injection and mice showed evident abdominal volume increase, if the double combination of CpG-ODN and cetuximab added to other molecular agents could further increase its therapeutic effect. EGFR inhibitors are reported to interact with cisplatin (Ahsan A. et al., *Cancer Res* 2010; Sano D. et al., *Clin Cancer Res* 2011; Zhang Y. et al., *J Huazhong Univ Sci Technolog Med Sci* 2011; Weng Y. et al., *J Huazhong Univ Sci Technolog Med Sci* 2011), although their effect on sensitivity to this drug remains undefined; also, we recently reported the synergistic antitumor effect between CpG-ODN and cisplatin (Sommariva M, et al., *Cancer Res* 2011, 71:6382–6390). Keeping those evidences in mind, we treated animals with the double combination of CpG-ODN and cisplatin and also with the triple combination of CpG-ODN, cetuximab and cisplatin. Results indicated that 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:  $31.4 \pm 0.9$  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. Together, results indicate that combinatorial therapies, enhancing immune response in the tumor microenvironment and concomitantly targeting tumor cells, are successful even in experimental advanced malignancies, and suggest, although the differences in the distribution of TLR9 in mice and human and the enrichment on innate immune cells in athymic cause for caution, a promising clinical strategy for treating ovarian patients with bulky malignant ascites. Preclinical studies in which treatment is initiated only after ascites is evident are rare and generally show a small effect on survival. Our results indicate that combination therapies to concomitantly enhance the immune response in the tumor microenvironment and target tumor cells can be effective even in advanced malignancies.



## **Anti-tumor activity of locoregional combined CpG-ODN therapy in experimental head-and neck carcinoma models**

HNSCCs are the sixth most common cancer in the world. Despite significant advances in the treatment modalities involving surgery, radiotherapy, and concomitant chemoradiotherapy, the 5-year survival rate remained below 50% for the past 30 years. More than 90% of squamous cell head- and neck carcinomas overexpress EGFR, often associated with poor clinical prognosis and outcome, and providing the rationale for developing therapies that target EGFR to treat this disease. Cetuximab, which binds to the EGF receptor, is approved by the US Food and Drug Administration as first-line treatment of locally or regionally advanced head and neck carcinomas in combination with radiotherapy. As a single agent, cetuximab is indicated for the treatment of patients with recurrent or metastatic head and neck carcinomas and for whom prior platinum-based therapy has failed (*Erbix® (cetuximab) Prescribing Information*. New York, NY, USA ImClone Systems Inc; 2008). Although it remains unclear whether the benefit of cetuximab is due to EGFR inhibition and downstream molecular effects on cell proliferation and apoptotic pathways or due to antibody-mediated immune responses, recent evidence has shown that MAbs mediate antibody-dependent cellular cytotoxicity and induce activation of cellular immunity, including that of NK and T cells, which contribute to clinical response. The worse prognosis of these cancers must certainly be link to the fact that HNSCCs strongly influence the host immune system (Turksma et al., *Immunotherapy* (2013) 5(1), 49–61). Based on those observations, we proposed to evaluate in experimental head and neck carcinoma models whether a local CpG-ODN treatment improves the therapeutic efficacy of cetuximab. As head-and neck xenograft carcinoma model, we firstly evaluated human CAL-27 tumor growth in athymic nude mice. This cell line expresses high levels of EGFR and is sensitive to cetuximab in nude mice xenograft models models (Pourgholami MH, et al., *Clin Cancer Res* 2006, 12(6):1928–1935). Cal-27 tumors have demonstrated a significant proliferation without inducing animal suffering; therefore, we utilized this xenograft model in our first experiments to evaluate the antitumor activity of CpG-ODN in head- and neck carcinomas. To treat animals locally with CpG-ODN we used Alzet Mini Osmotic Pumps: those devices are miniature, implantable pumps for research in mice, rats, and other laboratory animals. These minipumps deliver drugs, hormones, and other test agents at continuous and controlled rates, for durations ranging from one day to six weeks,

without the need for external connections or frequent handling. The Alzet pump, which has a pumping rate of 0.25  $\mu\text{l/h}$  ( $\pm 0.05$   $\mu\text{l/h}$ ), provided continuous infusion of CpG-ODN at about 20  $\mu\text{g/day}$  for 14 days, allowing a locoregional and repeated CpG-ODN therapy, which we reported to be superior to systemic administration in our IGROV-1 ovarian tumor model. We treated animals also with CpG-ODN i.p. (20  $\mu\text{g/mouse}$ , 5 days/week), as we observed a therapeutic effect in previous ovarian IGROV-1 tumor model. Our results indicated that treatment with cetuximab alone was able to completely eradicate Cal-27 tumors both in nude and in SCID mice model and that the combination of cetuximab and CpG-ODN delivered using an Alzet osmotic pump was not able to significantly enhance the antitumor effect the previous compound. Our experiments also indicated that treatment with CpG-ODN i.p. alone or delivered using an Alzet osmotic pump was able to inhibit the growth of CAL-27 tumors, but did not to cure animals; infact, after the suspension of those treatments, tumors begun to grow again. Since cetuximab alone completely eradicated established tumors both in athymic and in SCID mice and also both in early and in an advanced tumor stage, human CAL-27 tumor was found not suitable for testing the efficacy of the cetuximab plus CpG-ODN treatment. We then decided to test the possible efficacy of CpG-ODN therapy also in animals injected with human CaTC-1 tumor, another head and neck carcinoma. We tested the sensibility of this tumor firstly to cetuximab alone: our results indicated that cetuximab alone was able to completely eradicate also this type of tumor. Based on those observations, we consequently evaluated the effect of cetuximab as a single agent on different type of head- and neck carcinoma, with the subsequent intent to associate the possible efficacy of this antibody to local CpG-ODN administration. We performed preliminary experiments firstly to test the growth of human FaDu tumor cell line in athymic nude mice, which has been reported in literature to show a moderate EGFR expression and lower in comparison to Cal27 and A431 cells (Dvorak HF, et al., Am J Pathol 1995, 146:1029–1039). FaDu tumors have demonstrated a significant proliferation without inducing animal suffering; therefore, we utilized this xenograft model to further evaluate the sensitivity to cetuximab treatment. Treatment with cetuximab alone was able to reduce tumor volume and to stabilize tumor growth, but was not able to eradicate tumor. Infact, about 15 days after the suspension of the treatment, FaDu tumors begun to grow again. Therefore, this head and neck tumor model rised the possibility to test the effect association therapy of cetuximab and CpG-ODN, both in *in vivo* experiments and also in *in vitro* speculations about related molecular mechanisms. The combinational therapy of cetuximab

with CpG-ODN, delivered both with Alzet osmotic pumps and i.p, was able to reduce FaDu tumor volume, but did not completely eradicate tumor. With the aim to further improve the therapeutic effect of cetuximab combined with CpG-ODN, we decided to treat animals also with Poly(I:C) immunostimulant, a synthetic dsRNA TLR3 agonist and also to increase number of treatments. poly(I:C) TLR3 agonist is reported to elicit host immune responses and induce tumor cell apoptosis (Friedberg JW, et al., *Br J Haematol* 2009, 146(3):282–291). The combination of cetuximab and Poly(I:C) unexpectedly was less effective than treatment with cetuximab, probably interfering with the inhibitory effect of the monoclonal antibody. The triple combination of cetuximab, CpG-ODN and Poly(I:C) was not able to induce an increased reduction of tumor growth, as respect to cetuximab treatment alone. To try to explain those unexpected results, we decided to performed an *in vitro* proliferation test after treatment with Poly(I:C) treatment. We treated FaDu cells *in vitro* with poly(I:C) and with IFN $\alpha$ , which is reported to potentiate Poly(I:C) effect in enhancing the TLR3 antiviral response (Tassari J et al., *J Immunol*. 2005 Apr 1;174(7):4289-94); we then tested the effect of treatments administered as single agents or in combination. Our results indicated that FaDu cells are resistant to Poly(I:C) alone or to Poly(I:C) combined with IFN $\alpha$  treatments at all time points analyzed.

To conclude, our results obtained in mouse models of lung carcinoma suggest that evaluation of the patient lung immune status through analysis of BAL composition (Zikos TA, et al., *Cancer Immunol Immunother* 2011;60:819–27) might provide a non-invasive means of routinely sampling the immune environment of the lung to define the best immunotherapeutic strategy. Although differences in the distribution of TLR9 receptors in mice and humans as well as the enrichment of innate immune cells in athymic mice must be considered, our findings obtained from studies conducted in mouse IGROV-1 human ovarian tumor model point to a promising clinical strategy for treating ovarian cancer patients with bulky ascites. Thus, clinical trials of i.p. CpG-ODN treatment in association with cetuximab and cisplatin might now be contemplated in ovarian carcinoma patients with bulky disease. Finally, with the intent to understand why in our head and neck tumor models (Cal27 and FaDu xenograft) CpG-ODN treatment was not able to determine a significant and consistent tumor growth inhibition in contrast to the effect observed in other tumor models, we are investigating if Cal-27 and FaDu cells could be able to evade immune response downmodulating TLR9 on mouse NK cells. Accordingly, it has been reported

in literature that plasmacytoid dendritic cells (PDC), the major cell population responding to TLR9 agonists in humans, infiltrating head and neck cancer tissue are functionally impaired (Mimura K, et al., *Int J Cancer* 2011, 129(10):2408–2416.).

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