



## Immuno-chemotherapy reduces recurrence of malignant pleural mesothelioma: an experimental setting<sup>☆</sup>

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### Abstract

**Objective:** To assess the effect of immuno-chemotherapy on the extent of local tumour recurrence in an established rat model of malignant pleural mesothelioma (MPM). **Methods:** Six days after subpleural inoculation of a syngeneic MPM cell line Interleukin-45 (IL-45), left-sided pneumonectomy and resection of the tumour nodule was performed. Animals were randomised into four treatment groups for intrapleural therapy: control ( $n = 6$ ), 500  $\mu\text{g}$  cytosine phosphate guanosine oligodeoxynucleotide (CpG-ODN) ( $n = 6$ ), cisplatin-fibrin ( $n = 6$ ), cisplatin-fibrin + 500  $\mu\text{g}$  CpG ( $n = 6$ ). Six days later the volume of tumour recurrence was assessed, which was the primary endpoint. Secondary endpoints were quantification of the ratio host/tumour cells in the local recurrence and cytokine expression profile in the tumour tissue by real time quantitative PCR (qPCR). T lymphocyte subpopulations in the tumour recurrence tissue were evaluated by immunohistochemistry. Treatment-related toxicity was monitored by measuring blood chemistry and complete blood count. **Results:** The volume of tumour recurrence was significantly reduced from 610  $\text{mm}^3$  in the control group to 11.7  $\text{mm}^3$  in the cisplatin-fibrin group ( $p = 0.004$ ) and to 21.8  $\text{mm}^3$  in the cisplatin-fibrin + CpG group ( $p = 0.004$ ). Pro-inflammatory cytokines (Interferon- $\gamma$  (IFN- $\gamma$ ), Interleukin-6 (IL-6), Interleukin-12 (IL-12)) were increased after treatment with cisplatin-fibrin + CpG in comparison to cisplatin-fibrin alone but differences were not statistically significant. We found a higher ratio of host/tumour cells in the cisplatin-fibrin + CpG group (45/55%) compared to the cisplatin-fibrin group (27/73%). In comparison to the control group, animals treated with cisplatin-fibrin + CpG showed a higher number of CD8+ T-cells in the tumour tissue. No significant treatment-related toxicity was observed. **Conclusions:** Adjuvant treatment with chemotherapy or immuno-chemotherapy leads to significant reduction of mesothelioma recurrence after surgery in this rat MPM model. Immuno-chemotherapy resulted in an increased recruitment of inflammatory cells to the site of tumorigenesis and elicited higher level of tumour growth inhibiting cytokines.

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**Keywords:** Mesothelioma; Immunotherapy; Cisplatin; Intrapleural; Toll-like receptor; CpG-ODN

### 1. Introduction

Asbestos is a known carcinogen. Its use has been banned from the European Union since 2005 [1]. Due to the time lag between asbestos exposure and cancer development, initial analysis predicted that malignant pleural mesothelioma (MPM) related deaths will continue to increase and peak in the year 2020 [1]. Recent analysis suggests that the increase may be levelling off and the peak might be anticipated earlier than predicted, around 2015 [2].

In some villages in Turkey asbestosis is present in the environment, this results in a rate of mesothelioma-related mortality more than 100 fold higher than in control villages. The clinical course of mesothelioma disease is characterised by locally aggressive growth of the tumour. Multimodality treatment including chemotherapy, surgery and radiotherapy can offer a long-term survival [3,4].

However, despite this aggressive approach, local recurrence still remains the main problem of this type of tumour [4]. Hence, new therapeutic strategies are required. Intrapleural chemotherapy provides the advantage to achieve high local concentration while reducing systemic side effects and has been used in clinical and experimental settings [5–9].

Using our previously established rat mesothelioma model of tumour recurrence, we demonstrated a significant reduction of tumour recurrence by intrapleural chemotherapy with

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cisplatin loaded to the fibrin sealant Vivostat<sup>®</sup> [5]. Other groups have shown that intrapleural application of different immunomodulating therapies such as cytokines affected local tumour growth in MPM patients. Interleukin-2 (IL-2), for example, enhances the cytotoxic response of activated T-cells [8] and it has been proven to be superior to Interferon- $\alpha$  (IFN- $\alpha$ ) and Interferon- $\beta$  (IFN- $\beta$ ) in neoplastic pleural effusion and solid tumour comprised mesothelioma [8]. Lucchi et al. reported on the combination of IL-2 and chemotherapy in the multimodality treatment of malignant pleural mesothelioma patients [9].

One particularly promising approach is the stimulation of the innate immunity [6,7]. Toll-like receptors (TLRs) are pattern recognition receptors. They recognise conserved motifs of micro-organisms; their stimulation results in activation of the innate immune response, including release of cytokines, up-regulation of activation markers and of MHC molecules [6,7]. Currently, 13 TLRs (11 with known ligands) are described. TLRs differ by their cellular expression pattern, their recognition of conserved motifs and their ensuing activation they initiate. We focused our efforts on triggering TLR9: TLR9 binds preferentially unmethylated DNA motifs containing cytosine and guanosine, mainly present in bacteria and viruses. Synthetic TLR9 ligands containing unmethylated cytosine phosphate guanosine oligodeoxynucleotide (CpG-ODN) were generated and their value studied mainly for targeted immunomodulation. In fact, unmethylated CpG motifs are known to direct a strong immunostimulation of human B-lymphocytes, monocytes/macrophages, and dendritic cells. In addition, CpG induces the release of Th1-type cytokines such as Interleukin-12 (IL-12), Interleukin-6 (IL-6) and TNF- $\alpha$ , resulting in the activation of natural killer (NK) cells [8]. Chemotherapy and immunotherapy with CpG have synergistic effects [9] in several human and animal tumour models [10–14]. In a murine model of peritoneal mesothelioma, CpG resulted in a decrease of tumour growth and mortality when given intraperitoneally in combination with coramsine [10]. Currently there are no reports about CpG-based immunotherapy for malignant pleural mesothelioma.

In our rat mesothelioma recurrence model, we studied if CpG reduces the volume of tumour recurrence after surgical resection when applied intrapleurally either alone or in combination with chemotherapy. Furthermore we assessed the inflammatory response to this immunomodulating treatment at the tumour site.

## 2. Materials and methods

### 2.1. *In vitro* study

Prior to the *in vivo* study, we performed *in vitro* experiments in order to assess whether rat cells are susceptible to CpG treatment: peripheral blood mononuclear cells (PBMC) and the cells of our syngeneic rat mesothelioma cell line (IL-45) [15] were treated with different dosages of either 500 ng/ml or 5000 ng/ml of CpG and thereafter Interferon- $\gamma$  (IFN- $\gamma$ ), IL-6 and TNF- $\alpha$  were analysed in the supernatant by multiplex technology as described by Lagrelius et al. [16] by Cytolab ([www.cytolab.ch](http://www.cytolab.ch)).

### 2.2. *In vivo* study

The study protocol was approved by the local veterinary committee. Male Fisher rats weighing 260–300 g (Harlan, The Netherlands) were used. The animals were housed in the animal facility of the University Hospital of Zurich; after arrival, rats were acclimatised for 10 days prior to any experiments. They received humane care in accordance with the 'Guide for the Care and Use of Laboratory Animals' (National Institutes of Health publication no. 86-23, revised 1985).

### 2.3. Dose-escalating study to define the optimal dose of CpG

We initially performed a dose-escalating study in order to define the optimal dose based on the cytokine expression in the pleural tissue and considering treatment-related toxicity. The CpG28 (Microsynth AG, Balgach, Switzerland) has been previously evaluated to stimulate the rat-specific TLR9 [7]; its sequence is 5'-TAAACGTTATAACGTTATGACGTCAT-3'; phosphorothioate bonds were present at any position for stabilisation of the oligodeoxynucleotide. CpG were HPLC-purified followed by dialysis. Before using, CpG were resuspended in NaCl.

Under general anaesthesia, a suspension of 50  $\mu$ l containing three increasing different dosages of CpG was inoculated with a syringe (25 Gauge, 0.5  $\times$  16 mm) under the parietal pleura via a small left-sided thoracotomy in the fifth intercostal space according to the following experimental design: group 1 ( $n = 3$ ): control, untreated animals; group 2 ( $n = 3$ ): 100  $\mu$ g CpG; group 3 ( $n = 3$ ): 500  $\mu$ g CpG; group 4 ( $n = 3$ ): 1000  $\mu$ g CpG.

The rats were weighed before each surgical procedure and before any blood draw. Blood draws were obtained every other day to assess treatment-related toxicity by measuring the level of haemoglobin, haematocrit, leucocytes, platelets, urea, creatinine and transaminases. Six days after the instillation of CpG into the pleural space, the rats were euthanised and the chest wall was removed for evaluation of the cytokine expression profile, i.e., IL-12, IL-6 and IFN- $\gamma$ , in the pleural tissue. Notably, pleural samples from the CpG site of injection into tumour tissue and approximately 2 cm away from the injection sites were taken. The samples were snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Similarly, serum was analysed for its cytokine profile.

### 2.4. PCR assays

For quantitative PCR (qPCR), rat-specific primers for Interleukin-6, Interleukin-12, Interferon- $\gamma$ , Interferon- $\alpha$  and Interferon- $\beta$  were purchased from Qiagen AG (Hombrechtikon, Switzerland). Tumour specimens were processed for total RNA extraction using Qiagen RNeasy<sup>®</sup>. From the extracted RNA, reverse transcription was performed on 400–500 ng RNA (Qiagen QuantiTect<sup>®</sup> Reverse Transcription protocol). To investigate the quantitative expression of IL-6, IL-12 and IFN- $\gamma$ , cDNA were amplified by the SYBR-Green PCR assay using QuantiTect primer (Qiagen), and products were detected on a Prism 5700 detection system (SDS, ABI/Perkin-Elmer). We verified the specificity of the qPCR by

analysing the melting curve. GAPDH was used as house-keeping gene. Cytokine levels were expressed relative to the housekeeping genes by comparing (a) PCR cycle threshold between cDNA of cytokines and GAPDH ( $\Delta C$ ), and (b)  $\Delta C$  values between different  $\Delta C$  values and the highest  $\Delta C$  ( $\Delta\Delta C$ ).

### 2.5. Treatment strategy

We used a syngeneic mesothelioma tumour recurrence model as previously described [5]. Briefly, one million cells of a syngeneic mesothelioma cell line were injected into the subpleural space. Six days later, the tumour nodule which was grown meanwhile at the injection site was macroscopically completely resected and a left-sided pneumonectomy plus pleural abrasion was performed; this procedure was intended to imitate the treatment strategy of extrapleural pneumonectomy in humans. After resection, we applied the intrapleural treatment according to the following experimental design: (1) control, untreated animals (no adjuvant treatment); (2) cisplatin (100 mg/m<sup>2</sup>) loaded to fibrin; (3) 50  $\mu$ l of a CpG solution with a concentration of 10  $\mu$ g/ $\mu$ l at the site of the tumour resection; and (4) combined treatment of cisplatin loaded to fibrin and CpG (see above); the number in each group was six. The randomisation process was performed prior to the start of the experiment before inoculation of the tumour cells. For the treatment in groups 2 and 4, cisplatin was loaded to a fibrin-based carrier called Vivostat<sup>®</sup> as described previously [17]. CpG was injected subpleurally at the site of tumour nodule resection. Six days after treatment, the rats were euthanised and the chest wall dissected for analysis. Blood draws were taken daily to assess the possible treatment-related toxicity (see above). The primary endpoint was the volume of tumour recurrence. The length, width and thickness of the tumour nodule were accurately measured and the volume of the recurrence evaluated using the formula:  $V = 4/3\pi abc$  ( $a$ ,  $b$ ,  $c$  = semi-axes of the tumour).

In order to determine the amount of tumour cells recurring we took advantage of the fact that the tumour cell line, H-45 cells are originally derived from a female rat [15] and are implanted in male rats. By comparing the levels of Y chromosome-gene sex determining region Y (SRY) versus the non-sex specific gene Rev3 allows determining the amount of host tissue in the whole specimen. Therefore, tumour samples were processed for DNA extraction using DNAeasy (Qiagen) and quantitative PCR was performed as described in the PCR section. Relative DNA levels were determined by comparing (a) PCR cycle threshold between SRY gene (Qiagen QuantiTect Cat. No. QT00459753) and Rev3 ( $\Delta C$ ), and (b)  $\Delta C$  values between different  $\Delta C$  values and the highest  $\Delta C$  ( $\Delta\Delta C$ ).

Secondary endpoints were the cytokine expression profile in the serum and in the tumour tissue as well as treatment-related toxicity (see above). Cytokine expression in the tumour tissue was evaluated by qPCR as described before. Cytokine levels were measured in the serum by Cytolab ([www.cytolab.ch](http://www.cytolab.ch)) using multiplex technology. The white blood cell subpopulations including T-cells, NK-cells, and macrophages were assessed by immunohistochemistry in biopsy specimens of tumour recurrence. For this purpose, we

constructed a tissue micro-array with biopsies of the tumour tissue at the time point of primary tumour resection and autopsy: sections (4.5  $\mu$ m) of tissue were transferred to an adhesive-coated slide system (Instrumedics, Hackensack, NJ, USA). Sections were manually stained after heat-induced epitope retrieval (3 min, 110 °C, citrate buffer, pH 6.0) using a standard multilink detection kit (Medi-Stain HRP DAB, mediateAG, Nunnigen, CH, Switzerland). The primary antibodies were purchased by Serotec AbD (MorphoSys AbD GmbH, Duesseldorf, Germany): monoclonal anti-rat CD3 (1F4; MCA772), anti-rat CD8 (OX-8; MCA48GA), anti-rat CD161 (10/78; MCA1427GA) and anti-rat CD68 (ED1; MCA341GA) diluted according to the manufacturer's protocol. The sections were assessed by two observers (A.S., L.A.). The number of positive stained cells were counted and a semi-quantitatively score was adopted as follows: 0 for 0–2 cells/core; 1 for 3–10 cells/core; 2 for 11–50 cells/core; and 3 for >50 cells/core.

The software package SPSS for Windows, version 15.0 (SPSS Inc., Chicago, Illinois) was used for the statistical analysis. Kruskal–Wallis test was performed to compare all groups;  $p$  values below 0.05 were considered significant. Mann–Whitney test was used for comparisons between single groups. Bonferroni correction was applied for multiple comparisons. A  $p$  value below 0.008 was then considered significant.

## 3. Results

### 3.1. In vitro study

The qPCR confirmed that TLR9 was expressed in peripheral blood mononuclear cells of Fisher rats and showed that cytokine expression increased in PBMC in response to CpG28: in particular, the higher the dosage of CpG injected, the higher expression level of cytokines found (data not shown).

### 3.2. Dose-escalating study to define the optimal dose of CpG

All animals were fine irrespective of the dose applied. In particular there was neither a significant difference in the weight nor differences in the haematological, renal or hepatic parameters measured in the blood during the observation period. qPCR for detection of different cytokines in the pleural samples taken at the site of CpG injection and far from the site of injection, revealed increased levels of pro-inflammatory cytokines (IL-6, IL-12 and IFN- $\gamma$ ) of the treated groups in comparison to the control group (Table 1). Similar changes of IFN- $\alpha$  and IFN- $\beta$  expression were not detected. We did not see any increase of local cytokine expression in the pleural tissue when the dosage of CpG instilled was increased from 500 to 1000  $\mu$ g. In the serum, TNF- $\alpha$  peaked the first day after treatment start, which was more pronounced after application of the higher dosages of CpG (Table 2); however, that was not the case for IFN- $\gamma$  and IL-6 expression in the serum. Based on these results, a dosage of 500  $\mu$ g CpG was chosen for further investigation in our rat recurrence model.

Table 1

Dose-escalating pilot study: IFN- $\gamma$  and IL-6 expression levels in the pleural tissue in the different groups. Data are given in mean and SD.

IFN- $\gamma$ expression levels		
Pleural tissue		
Groups	Site of injection	Far from site of injection
Control	99.45 $\pm$ 99.1	99.45 $\pm$ 99.2
CpG 100 $\mu$ g	244.12 $\pm$ 422.8	295.62 $\pm$ 59.1
CpG 500 $\mu$ g	397.74 $\pm$ 274.6	259.22 $\pm$ 82.3
CpG 1000 $\mu$ g	258.26 $\pm$ 172.7	199.98 $\pm$ 79.1
IL-6		
Control	997.23 $\pm$ 1068.1	997.23 $\pm$ 1068.1
CpG 100 $\mu$ g	14680.17 $\pm$ 22692.1	2930.88 $\pm$ 3363.2
CpG 500 $\mu$ g	9798.3 $\pm$ 10409.1	1861.93 $\pm$ 2056.4
CpG 1000 $\mu$ g	12433.41 $\pm$ 5744.6	1790.94 $\pm$ 2391.3

Table 2

Dose-escalating pilot study: TNF- $\alpha$  levels in the serum. Data are given in mean and SD.

TNF- $\alpha$				
Serum (pg/ml)				
Groups	Baseline	1 day	2 days	6 days (autopsy)
Control	3 $\pm$ 0.2	3 $\pm$ 1.1	3 $\pm$ 0.7	6.5 $\pm$ 1.9
CpG 100 $\mu$ g	4.9 $\pm$ 2.4	4.9 $\pm$ 1.9	3 $\pm$ 2.1	3 $\pm$ 1.3
CpG 500 $\mu$ g	4.8 $\pm$ 2.1	7.25 $\pm$ 1.9	3 $\pm$ 1.8	3 $\pm$ 3.1
CpG 1000 $\mu$ g	3 $\pm$ 2.8	14.4 $\pm$ 4.2	3 $\pm$ 0.9	3.35 $\pm$ 2.6

### 3.3. Treatment strategy

All animals survived until the end of the observation period of six days. Six days after inoculation, all animals had a tumour nodule of a mean diameter of  $5.5 \pm 0.7$  mm at the injection site. The rats were sacrificed and tumour recurrence was evaluated six days after the experimental treatment, which consisted of pneumonectomy, pleural abrasion and application of the therapy solution according to the experimental design. All rats presented recurrence at the resection site whereas they showed no signs of tumour recurrence or metastases in the contralateral chest cavity or abdomen. The volume of tumour recurrence was significantly reduced in the treatment groups (Kruskal–Wallis test  $p = 0.001$ ) (Fig. 1). Further analysis showed that cisplatin-fibrin and cisplatin-fibrin + CpG had significantly reduced tumour volume with  $12 \text{ mm}^3$  ( $\pm 15$ ) ( $p = 0.004$ ) and  $22 \text{ mm}^3$  ( $\pm 22$ ) ( $p = 0.004$ ), respectively, in comparison to the control group with  $610 \text{ mm}^3$  (Fig. 2) but also in comparison to immunotherapy alone with  $442 \text{ mm}^3$  ( $p = 0.004$  and  $p = 0.004$ , respectively).

The histopathological analysis revealed only small tumour islets remaining in the group of animals treated with chemotherapy and/or immunotherapy. The difference between cisplatin-fibrin and cisplatin-fibrin + CpG treated rats was statistically not significant.

SRY gene determination for differentiation between host and tumour cells showed higher percentage of host cells (45%) in the group of animals which received the combination treatment cisplatin-fibrin + CpG than in the group treated with cisplatin-fibrin alone (27% of host cells,  $p = 0.06$ ) (Fig. 3).

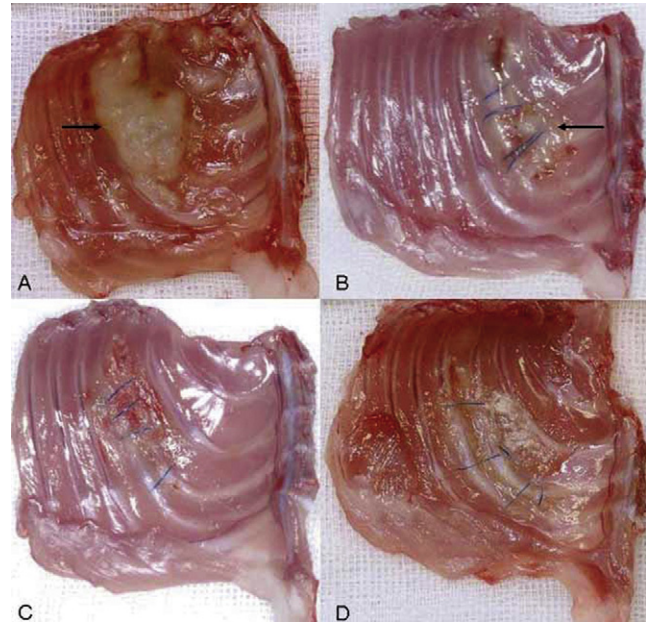


Fig. 1. Successful treatment of mesothelioma tumour with cisplatin-fibrin and cisplatin-fibrin + CpG. Photographs show ipsilateral chest wall at the time point of autopsy six days after intrapleural treatment; (A) control group: arrow indicating tumour recurrence, (B) CpG 500  $\mu$ g group: arrow indicating tumour recurrence, (C) cisplatin-fibrin group: tumour recurrence macroscopically not visible, and (D) cisplatin-fibrin + CpG 500  $\mu$ g group: tumour recurrence macroscopically not visible.

The animals treated with combination treatment cisplatin-fibrin + CpG showed a higher number of CD8+ T-cells than animals treated with cisplatin-fibrin alone ( $p = 0.07$ ). Two representative figures of the infiltration with CD8+ T-cells into the recurrent tumour are shown (Fig. 4). While the number of NK-cells ( $p = 0.08$ ) and macrophages ( $p = 0.07$ )

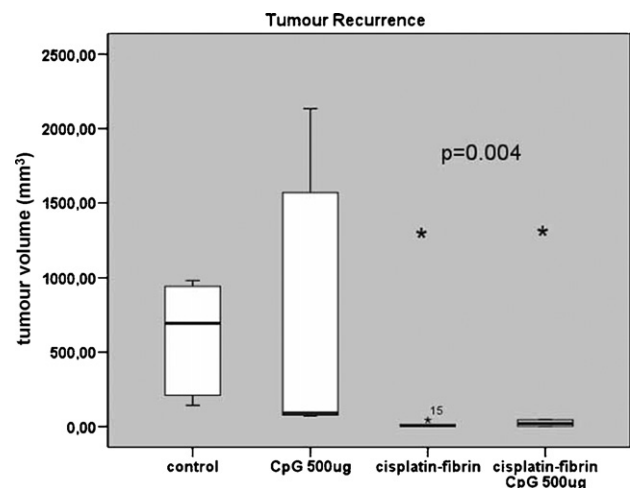


Fig. 2. Minimal tumour recurrence with cisplatin-fibrin or cisplatin-fibrin + CpG. In each group of rats  $n = 6$ . Results presented as box-plot of volume of tumour recurrence. Initially a Kruskal–Wallis test was performed ( $p = 0.001$ ) and subsequently a Mann–Whitney test was performed for multiple comparison between the different groups: comparison between cisplatin-fibrin to control ( $p = 0.004$ ) and cisplatin-fibrin + CpG to control ( $p = 0.004$ ). Control group: mean volume  $610 \text{ mm}^3$  ( $\pm 284$ ); CpG group: median volume  $442 \text{ mm}^3$  ( $\pm 1002$ ); Cisplatin-fibrin group: median volume  $12 \text{ mm}^3$  ( $\pm 15$ ); Cisplatin-fibrin + CpG group: median volume  $22 \text{ mm}^3$  ( $\pm 2$ ).



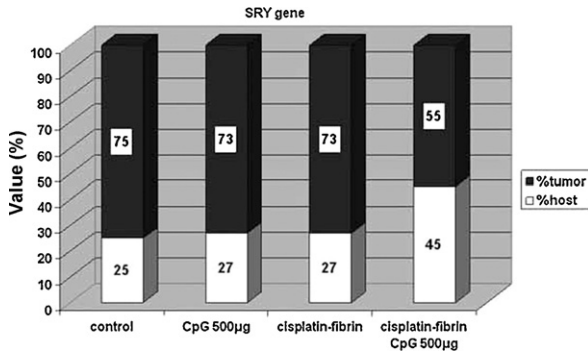


Fig. 3. Treatment with cisplatin-fibrin + CpG results in a higher content of host cells in the tumoural mass in comparison to the other groups. The content of host versus tumoural cells was determined by means of qPCR analysis of SRY gene.

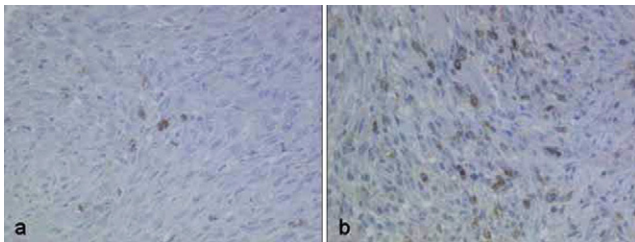


Fig. 4. Immuno-chemotherapy results in a prominent infiltration of the residual tumour mass with CD8+ T-cells. Immunohistochemistry of tumour tissue obtained from rats treated with cisplatin-fibrin (a) and cisplatin-fibrin + CpG (b). Representative photographs shown.

was not significantly higher in the treatment groups, there was a trend of increased number in the tumour tissue. NK-cells were in any case very rare, while macrophages were very much present independently on the treatment.

We found that rats treated with cisplatin-fibrin, had a lower level of IFN- $\gamma$ , IL-6 and IL-12 in the pleural space as compared to the control group. Notably, while statistically not significant, the rats treated with cisplatin-fibrin + CpG had a higher level of IFN- $\gamma$ , IL-6 and IL-12 in the pleural space as compared to rats treated with cisplatin-fibrin alone (Figs. 5 and 6). In the serum, we found high levels of IL-6, IFN- $\gamma$  and TNF- $\alpha$  in the cisplatin-fibrin + CpG group as compared to untreated animals. Blood count, renal and hepatic parameters

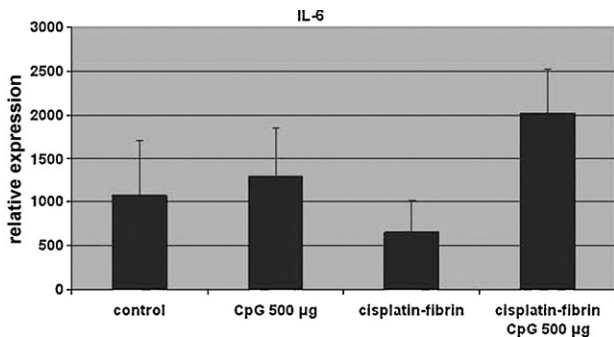


Fig. 5. The combination cisplatin-fibrin + CpG resulted in more important stimulation of IL-6 expression in the tumour tissue as quantified by qPCR compared to cisplatin-fibrin alone but differences were not statistically different (Kruskal–Wallis test:  $p = 0.07$ ).

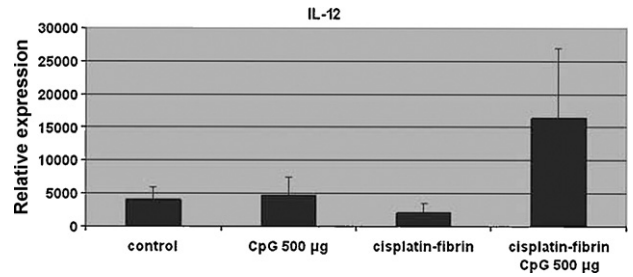


Fig. 6. The combination cisplatin-fibrin + CpG resulted in strong stimulation of IL-12 expression in the tumour tissue as quantified by qPCR which was not the case in the other treatment groups but differences were not statistically different (Kruskal–Wallis test  $p = 0.06$ ).

in the blood samples taken at different time points were similar between treatment and control group as related to renal and hepatic chemistry and complete blood count. Thrombocytes were significantly increased at day 3 in the group of rats treated with CpG 500  $\mu$ g alone (data not shown).

#### 4. Discussion

In this work we studied the effects of triggering TLR9 on tumour recurrence in a mesothelioma rat model. We found no beneficial effect when TLR ligands were applied alone or no additional benefit when TLR ligands were added in concert with chemotherapy. In any case, we observed a significant reduction of tumour volume after resection and intrapleural treatment with chemotherapy or combined immuno-chemotherapy.

The lack of any additional benefit with immuno-chemotherapy may be explained by the very aggressive sarcomatoid tumour cells, which result in development of a huge tumour recurrence in only six days after surgery and the strong anti-tumour effect of cisplatin-fibrin alone. Indeed, cisplatin results in a near entire disappearance of the tumour. In addition, the short observation period of six days after treatment may not allow for the detection of immuno-mediated effects. These results were obtained after a single local injection of 500  $\mu$ g of CpG after resection and different results might be obtained if repeated or systemic application were used. Indeed, in several human and experimental studies, chemotherapy and immuno-chemotherapy with CpG have shown synergistic effects [10, 12–14]: van der Most et al. [10] reported an enhanced anti-tumour efficacy when combining intraperitoneal chemotherapy with coramsine together with CpG-containing oligodeoxynucleotides in a murine model of malignant peritoneal mesothelioma. Similarly, Carpentier and co-workers [12–14] reported very impressive tumour reduction and long-term survival without secondary side effects when repeated injections of CpG were given intra-tumourally and thereafter subcutaneously in murine glioma model. Thus, we speculate that repeated local or systemic injections, intraperitoneally or subcutaneously, of immunomodulatory CpG oligodeoxynucleotides may potentiate their anti-tumour response; a single dose of CpG may be insufficient.

Although the volume of tumour recurrence in rats treated with immuno-chemotherapy was similar to the rats treated with chemotherapy, the immuno-chemotherapy resulted in a

very pronounced recruitment of inflammatory host cells to the site of tumour. In fact, almost half of the cells in the tumour bed were found to be inflammatory cells and not neoplastic cells. The local increase of pro-inflammatory cytokines after immunotherapy observed, when given alone or in combination with chemotherapy reflects an activated immune system. The mesothelioma cell line was derived from female rats. Since we exclusively used male rats, we were able to distinguish within the tumour recurrence between tumour cells and chemotactically attracted host cells by using primers specific for the 'sex determining region Y gene' and a reference gene. We found that the percentage of host cells in animals treated with cisplatin-fibrin + CpG was 45% in comparison to the control rats and rats treated with cisplatin-fibrin alone with 27%. Immunohistochemical analysis revealed an increased influx of CD8+T-cells into the tumour recurrence of rats treated with immuno-chemotherapy compared to rats treated with chemotherapy alone. Similarly, the immuno-chemotherapy resulted in a pronounced increase of inflammatory cytokine such as IL-6, IL-12 and IFN- $\gamma$  in the vicinity of the tumour, which was not the case in the other therapy groups.

Notably, persistent TLR stimulation may break tolerance against tumour associated antigens; synthetic compounds may be an ideal adjuvant to the existing chemotherapeutic approaches. While we have not found an effect of CpG alone on tumour recurrence, the potent inflammatory reaction may be beneficial for anti-tumour killing and perhaps for generating an adaptive immune response against tumour antigens, which may limit or slow down tumour growth or metastasis over a longer time period.

In summary, TLR9 ligands are very promising as an adjunct to chemotherapy for treating neoplasia. However, we need a better and more detailed knowledge about the molecular mechanism(s) of these immunomodulatory compounds and how we have to apply them. In this study we contributed to this ever growing field by gathering information on the inflammatory profile present in chemotherapy versus immuno-chemotherapy: briefly, we found that local sub-pleural injection of CpG in combination with chemotherapy induces a local inflammatory reaction which is not present with chemotherapy alone.

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