Trifunctional antibodies induce efficient antitumour activity with immune cells from head and neck squamous cell carcinoma patients after radio-chemotherapy treatment

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Abstract

Background Trifunctional antibodies, such as catumaxomab (anti-EpCAM×anti-CD3) and ertumaxomab (anti-HER-2/neu×anti-CD3), transiently link immune effector cells to tumour cells, which results in cellular cytotoxicity towards the tumour cells. A functional immune system is therefore essential for effective anti-tumour activity. However, the commonly observed haematotoxicity of chemotherapeutics and radiation therapy may be associated with some degree of immunosuppression. Combining chemotherapy and trifunctional antibodies in cancer treatment requires understanding of the impact of chemotherapeutics on immune cell function and, thus, on the activity of trifunctional antibodies.

Methods The effect of chemotherapeutic treatment on trifunctional antibody-mediated anti-tumour activity was

assessed *in vitro*. Blood samples were collected from 12 head and neck squamous cell carcinoma patients after chemotherapy (5-fluorouracil, cisplatin) and radiotherapy, and from one healthy control donor. The immune cell status was analysed and mononuclear cells (MNC) were isolated. The potency of catumaxomab and ertumaxomab was assessed in a cytotoxicity assay using MNC isolated from each patient sample in co-culture with a tumour target cell line. The release of inflammatory cytokines was also monitored in the cell culture supernatant.

Results Most patients included in this study had decreased immune cell counts during the course of chemotherapy. Nonetheless, an effective and concentration-dependent anti-tumour activity mediated by trifunctional antibodies was demonstrated using these patient immune effector cells. The immune response activity of the patient immune cells was not impaired one week after cisplatin administration or even three days after the last 5-fluorouracil treatment.

Conclusion This study shows for the first time that immune effector cells from cancer patients undergoing standard chemotherapy and radiotherapy can be activated by trifunctional antibodies for efficient killing of tumour cells.

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Introduction

Primary tumours are currently treated with a combination of different therapeutic strategies including surgery, local radiotherapy and chemotherapy. After the tumour appears to have been eradicated, micrometastases of dormant tumour cells may frequently lead to tumour relapse and therapeutic

failure. To gain a lasting tumour remission, it is necessary to develop strategies to kill all cancer cells efficiently, by using an appropriate combination and schedule of chemotherapeutic agents, surgery and irradiation, and also to stimulate an immune response against residual tumour cells.

The idea of recruiting host immune effector cells to the tumour site by using therapeutic antibodies to provoke an anti-tumour effect was first proposed in the late 1980s. Initial studies of antibody-based cancer therapy used monospecific monoclonal antibodies. Monospecific antibodies bind specifically to one particular antigen expressed on tumour cells and evoke an anti-tumour response via several mechanisms including complement-dependent cytotoxicity, induction of apoptosis, inhibition of receptor dimerisation and downstream signalling, and many others [1, 2]. Bispecific antibodies, which are able to bind two different antigens, were developed to further enhance efficacy. Suitable target antigens for bispecific antibodies include tumour-associated antigens and an antigen expressed on immune effector cells such as T cells or natural killer (NK) cells [3–7], to enable a transient linkage between immune effector cells and tumour cells leading to immune cell activation and lysis of tumour cells. Trifunctional antibodies combine the characteristics of classical monospecific antibodies and bispecific molecules: In addition to the two specific antigen binding sites, trifunctional antibodies retain their intact Fc region, which mediates recruitment and activation of accessory cells (macrophages, dendritic cells, NK cells) [11]. Thus, the mode of action of these trifunctional antibodies is based on the simultaneous recruitment of T cells and accessory cells to the tumour site leading to improved tumour cell elimination by various immunologic killing mechanisms [8–13]. The trifunctional antibody catumaxomab (anti-EpCAM×anti-CD3, Removab®) has been approved recently in the European Union for the intraperitoneal treatment of malignant ascites due to EpCAM-positive carcinoma where standard therapy is not available or no longer feasible [8, 9]. The related trifunctional antibody ertumaxomab (anti-HER-2/neu×anti-CD3) is in clinical development.

Due to their unique mode of action, a functional immune system is essential for trifunctional antibodies to exert their full activity. Haematologic toxicity is a known side effect of chemotherapy and radiation therapy, and results in transient immunodeficiency. The resulting impairment of cellular and humoral immunity can last up to several months after the end of treatment [14–19]. Combining chemotherapy and trifunctional antibody-based immunotherapy in cancer treatment therefore requires evaluation of the immune response activity that remains after chemotherapy treatment.

The present study has therefore been conducted to provide first *in vitro* data in support of the treatment of cancer patients with trifunctional antibodies in combination with chemotherapy. Immune cells from head-and-neck squamous cell carcinoma (HNSCC) patients after cisplatin and 5-fluorouracil (5-FU) treatment were assessed for their remaining immune response activity. The functionality of these immune cells was evaluated by analysing the capac-

ity of trifunctional antibodies (catumaxomab and ertumaxomab) to mediate cellular cytotoxicity towards tumour cells *in vitro*. Patient immune cells were taken at different time points before, during and after the chemotherapy cycles, and after radiation therapy in order to assess a potential impact on the immune response activity in relation to the time interval to the previous chemotherapy application or in relation to the number of previous treatment cycles (potential cumulative effects of several treatment cycles). 5-FU, a pyrimidine antagonist that inhibits DNA synthesis, is commonly used in the treatment of patients with neoplastic disorders, e.g., of the colon, head and neck, and breast [20–22]. Cisplatin, an alkylating agent that binds to DNA and interferes with its repair mechanisms [23], is used in the treatment of patients with, e.g., ovarian cancer, colon cancer and head and neck cancer. Promising results have been reported from combining monoclonal antibody treatment and chemotherapy treatment in cancer patients. For example, the combination of anti-EGFR monoclonal antibodies with chemotherapy increased the median survival in colorectal cancer patients [24] and the anti-HER-2/neu antibody trastuzumab, combined with paclitaxel, was found to be an effective regimen as first-line chemotherapy in metastatic breast cancer [25].

This study included the analysis of each patient sample taken at different time points (before, during and after chemotherapy and after completion of radiation therapy, see Fig. 1 for details) for (i) characterisation of the status of immune cells, (ii) analysis of trifunctional antibody (catumaxomab and ertumaxomab)-mediated cellular cytotoxicity towards the EpCAM and HER-2/neu expressing SK-OV-3 human tumour cell line, and (iii) antibody-mediated release of cytokines (tumour necrosis factor alpha (TNF- α), interferon gamma (IFN- γ), interleukin 2 (IL-2), IL-4, IL-5, IL-10).

Patients and methods

Study approval

The study was approved by the Ethics Committee of the Johannes Gutenberg University, Mainz, Germany (institutional review board no. 837.266.06, 5364). Informed consent was obtained from all patients prior to blood withdrawal.

Patient population and blood sampling

Whole blood samples from a total of 12 HNSCC patients aged 51–78 were provided by the Laboratory of Tumor Biology, Department of Otolaryngology, Johannes Gutenberg-University, Mainz, Germany. Patients received cisplatin and 5-fluorouracil (5-FU) following surgical intervention (Fig. 1). Cisplatin was administered intravenously

Fig. 1 (A) Scheme of monthly blood sampling of studies I (catumaxomab) and II (ertumaxomab) (9 patients included) (B) Scheme of weekly blood sampling of study III (ertumaxomab) (3 patients included)

at 100 mg/m² body surface area on day 1 of the start of each 28-day treatment cycle. 5-FU was given intravenously at 1000 mg/m² surface area on days 1–5 of each cycle. Radiation therapy with 2 gray (Gy) daily was initiated three to four weeks after the third cycle of chemotherapy up to a cumulative dose of 60 Gy.

The *in vitro* study consisted of three parts: Studies I (catumaxomab; monthly sampling) and II (ertumaxomab; monthly sampling) comprised the same 9 patients, and study III (ertumaxomab; weekly sampling) comprised 3 additional patients. A total of four blood samples were taken from each patient and analysed within 24 h after blood collection.

In all three study parts, the first blood sample was taken following surgery and before the first application of chemotherapy. In studies I and II, the subsequent two blood samples were taken at monthly intervals immediately before application of the next chemotherapy cycle, about three weeks after the previous 5-FU application (Fig. 1A). The fourth and last blood sample was taken four weeks after completion of the radiation therapy (approximately 6 weeks duration) that was performed after the third cycle of chemotherapy.

In study III, blood sampling was done weekly for 3 weeks (until one week before application of the second cisplatin and 5-FU cycle). The shortest time interval in

this schedule was assessed with sample #2 taken one week after the cisplatin administration on day 1 and three days after the last 5-FU dose on day 5 (Fig. 1B). As controls, blood samples from one healthy donor were drawn every two weeks, up to a total of three samples, and analysed in parallel to and in the same way as the patient samples.

All experimental analyses were performed at EUFETS GmbH, Idar-Oberstein, Germany, on behalf of Fresenius Biotech GmbH, Munich, Germany.

Staining of immune cells

Each blood sample was analysed for total cell counts and cellular composition. Leukocyte subsets of interest were analysed by flow cytometry. White blood cells were stained with specific fluorochrome-conjugated antibodies specific for CD3 (FITC, clone UCHT1, Beckman Coulter, Krefeld, Germany; Tritest T-cells, Becton Dickinson, Heidelberg, Germany) expressed on T cells, CD4 (PE, clone 13B8.2, Beckman Coulter; Tritest T-cells) on T-helper cells, CD8 (PE, clone B9.11, Beckman Coulter; Tritest T-cells) on cytotoxic T-cells, CD19 (PE, clone J4.119, Beckman Coulter, Tritest B-cells, Becton Dickinson) on B cells, CD16/CD56 (PE, clone 3G8+N901, Beckman Coulter; Tritest NK cells, Becton Dickinson) on NK cells, and CD14 (PE, clone RMO52, Beckman Coulter; Simultest LeucoGATE, Becton Dickinson) on monocytes. Cells were gated by CD45-staining (FITC, clone J.33, Beckman Coulter; Simultest LeucoGATE) to exclude dead cells and debris. T cells were defined as CD3+, cytotoxic T-cells as CD8+/CD3+, T-helper cells as CD4+/CD3+, B cells as CD19+/CD3–, NK cells as CD16+CD56+/CD3– and monocytes as CD14+. Expo 32 software (Beckmann Coulter) or CellQuest Pro software (Becton Dickinson) were used for data acquisition and analysis of immune status. The leukocyte subsets (positive cells for each antigen) were expressed as a percentage of the total nucleated cell count.

Cytotoxicity assay

Mononuclear cells (MNC) from patients or from a healthy donor (control blood samples) were isolated by density gradient centrifugation following a 1:2 dilution with PBS (Invitrogen) using lymphocyte separation medium (PAA).

The SK-OV-3 tumour target cell line (human ovarian tumour; EpCAM and HER-2/neu-positive) was purchased from ATCC (LGC Promochem, Wesel, Germany) and a primary and secondary cell bank was established. Cells were used within 15 passages resulting in a maximum culture period of 12 weeks. The cells were cultured in McCoy's 5A medium with 2 mM Glutamax (Invitrogen, Karlsruhe, Germany) and 10% foetal bovine serum (FBS; PAA, Pasching, Austria). SK-OV-3 cells were seeded (1×10^5 cells/ml) into 96-well microtitre plates and allowed to adhere overnight at 37°C, 5% CO₂ in a humidified atmosphere.

MNC were added to the tumour cell cultures at an effector to target ratio of 10:1, in RPMI-1640 culture medium (Invitrogen) supplemented with 10% FBS (PAA) and 2% penicillin and streptomycin (Invitrogen), and co-cultured in the presence of various concentrations of trifunctional antibodies (catumaxomab or ertumaxomab: 17.6 to 0.069 ng/ml in serial 2-fold dilutions) or without antibodies as control. Catumaxomab and ertumaxomab were provided by Fresenius Biotech (Munich, Germany). Each antibody concentration was assayed in replicates of 4–8, depending on the number of patient MNC available. Co-cultures of MNC and SK-OV-3 tumour cells were incubated for 3–4 days at 37°C, 5% CO₂ in a humidified atmosphere. After collection of cell-free supernatants for cytokine quantification, the immune effector cells (MNC) were removed by extensive washing with PBS. The residual tumour cells were quantified with the XTT cell proliferation kit II (Roche, Penzberg, Germany), according to the manufacturer's instructions. Absorbance was measured at a wavelength of 450 nm using a microplate reader (Spectrafluor Plus; Tecan, Crailsheim, Germany) and the software Magellan (Tecan) was used for data acquisition. Cytotoxicity *in vitro* was determined by calculating the percentage of residual tumour cells as follows:

$$\text{residual tumour cells (\%)} = \frac{\text{mean absorbance sample} - \text{mean absorbance medium control}}{\text{mean absorbance control without antibody} - \text{mean absorbance medium control}} \times 100$$

Tumour cell killing of >80% (residual tumour cells <20%) was defined as efficient killing, indicating functional activity of the patient MNC.

Quantification of cytokines in co-culture supernatants

Release of the cytokines TNF- α , IFN- γ , IL-2, IL-4, IL-5 and IL-10 was quantified in cell-free supernatants from co-cultures after the end of the incubation period (ertumaxomab studies II and III, only). Samples were stored at –80°C until analysis. Cytokines were quantified by cytometric bead array (CBA; Becton Dickinson), according to the manufacturer's instructions. Briefly, six bead populations with distinct fluorescence intensities were coated with cytokine-specific murine capture antibodies. The bead populations were mixed and incubated with recombinant cytokine standards or test samples and PE-conjugated anti-mouse antibodies. Acquisition and analysis of sample data was performed by flow cytometry and the manufacturer's CBA analysis software (Becton Dickinson).

Deviations to blood sampling schedule

In deviation to the proposed sampling scheme shown in Fig. 1A (monthly sampling), the second blood sample of two (out of nine) patients was taken immediately after the application of cisplatin. Therefore, results obtained from these two samples were not used in any calculation.

Fig. 2 Counts of immune cells from HNSCC patients (**A**) T cells and T-cell subtypes of studies I (catumaxomab, monthly interval) and II (ertumaxomab, monthly interval) (**B**) B cells, NK cells and monocytes of studies I and II (**C**) T cells and T-cell subtypes of study III (ertumaxomab, weekly interval) (**D**) B cells, NK cells and monocytes of study III. *** $p=0.0004$ (extremely significant), ** $p<0.0085$ (very significant), * $p=0.01$ (significant)

Statistics

GraphPad Prism software (version 5.0.2; GraphPad Software, Inc., La Jolla, CA, USA) was used for all statistical analyses. The cytotoxicity results were analysed using a non-linear fit of normalised data (as indicated in the GraphPad Prism software) and the logEC₅₀ (logarithmic effective concentration 50; the concentration that provokes a response halfway between the baseline and maximum response) was calculated. Any significant differences in logEC₅₀ values were determined by one-way analysis of variance (ANOVA) and any significant differences in immune cell counts were calculated using the unpaired Student's *t*-test ($p=0.01$ – 0.05 significant *; $p=0.001$ – 0.01 very significant **; $p<0.001$ extremely significant ***).

Results

Characterisation of immune cells from HNSCC patient samples

All patient samples were analysed for lymphocyte subsets at different time points before, during and after the chemotherapeutic treatment course in order to relate immune cell composition to the results obtained in the functional assay (cytotoxicity assay). The total numbers of T cells, T-helper

cells and cytotoxic T-cells of patient samples ($n=9$) from the monthly sampling schedule are presented in Fig. 2A. Total counts of B cells as well as NK cells and monocytes from these samples are shown in Fig. 2B. Compared to the pre-treatment samples, a slight increase in T cells was observed three weeks after the first and second courses of chemotherapy (CTX; before the second and third CTX cycles, respectively). At the end of the treatment period (after three CTX cycles and six weeks of radiotherapy) there was a significant decrease in T-helper cell counts compared to pre-treatment samples ($p=0.008$). At the end of radiochemotherapy, an inverse ratio of T-helper and cytotoxic T-cells was observed in two patient samples ($<1:1$; lower counts of T-helper than cytotoxic T-cells). In all other samples, the ratio of T-helper and cytotoxic T-cells was physiological; i.e., the T-helper cell count was higher than that of cytotoxic T-cells ($>1:1$). However, the abnormal ratio of T-helper and cytotoxic T-cells in the two samples did not affect antibody-mediated cytotoxicity of the trifunctional antibodies.

In study III blood samples from three HNSCC cancer patients were collected in weekly intervals up to three weeks following the first cycle of cisplatin and 5-FU. As depicted in Fig. 2C and D, the first application of 5-FU and cisplatin did not affect T-cell counts, T-cell subsets, NK cell counts or monocyte counts. However, B-cell counts were slightly increased one week after the first cycle of CTX. There were no changes in immune cell subsets in three

Fig. 3 Catumaxomab- (A) and ertumaxomab- (B) mediated cytotoxicity of MNC from HNSCC patients before, at several monthly time-points during, and at the end of radio-chemotherapy (studies I and II). (●: samples obtained before start of CTX, □: samples from 3 weeks after the first and immediately before the second cycle of CTX; △: samples obtained 3 weeks after the second and immediately before the third cycle of CTX; ⊗: samples obtained after the fourth cycle of CTX and after a cumulative radiation dose of 60 Gy) (C) Concentration of catumaxomab (●, study I) and ertumaxomab (□, study II) for killing 50% of the tumour target cells (EC_{50}) before, during and after the end of radio-chemotherapy. *** $p < 0.0001$ (extremely significant)

blood samples taken every two weeks from one healthy donor (data not shown).

Trifunctional antibodies mediate efficient tumour cell killing using immune cells from cancer patients after cisplatin and 5-FU treatment

Patient immune cells obtained at monthly intervals in the context of chemotherapy treatment induced efficient anti-tumour activity by the trifunctional antibodies catumaxomab and ertumaxomab; activity was comparable to the pre-treatment samples (immune cells before first cycle). Catumaxomab-mediated tumour cell killing by patient immune effector cells is shown in Fig. 3A (study I, catumaxomab) and results obtained with ertumaxomab are presented in Fig. 3B (study II, ertumaxomab). Both trifunctional antibodies mediated dose-dependent cytotoxicity towards human SK-OV-3 tumour cells in the presence of patient immune cells taken at monthly intervals. Immune cells obtained three weeks after the first and second cycles of cisplatin and 5-FU resulted in efficient, concentration-dependent tumour cell killing in both studies (>80%). The dose-dependent killing was maintained even after a third cycle of chemotherapy and a cumulative radiation dose of 60 Gy. Although the number of T cells was strongly reduced in all patient samples obtained at the end of radio-chemotherapy, the remaining immune effector cell function was still potent enough to result in efficient cytotoxicity mediated by trifunctional antibodies.

As controls, three blood samples from one healthy donor taken at 2-weekly intervals were analysed in cytotoxic-

ity assays using ertumaxomab. All three control samples showed comparable antibody concentration-dependent cytotoxicity ($\log EC_{50}$: -0.24 ± 0.24 ng/ml; $n=3$).

The results further indicate that the patient immune cells may become more susceptible to activation by trifunctional antibodies for efficient tumour cell killing during treatment with cisplatin and 5-FU. In Fig. 3C, the $\log EC_{50}$ values of studies I (catumaxomab; monthly sampling) and II (ertumaxomab; monthly sampling) are shown. The $\log EC_{50}$ values for ertumaxomab were significantly reduced when comparing patient immune cells taken three weeks after the first cycle (study II) vs. $\log EC_{50}$ values obtained with immune cells from patients before chemotherapy (* $p < 0.0001$). Following the second cycle of chemotherapy the $\log EC_{50}$ of ertumaxomab was even further reduced to a significant level (* $p < 0.0001$) compared to pre-treatment. Experiments with catumaxomab show that the antibody concentration required for the killing of 50% of SK-OV-3 tumour cells was similar in samples obtained before the treatment and three weeks after the first and second cycle of cisplatin and 5-FU, respectively. However, after the third chemotherapy cycle and radiation therapy the $\log EC_{50}$ was significantly increased in both studies when compared to the pre-treatment $\log EC_{50}$ values (I, catumaxomab and II, ertumaxomab; $p < 0.0001$), indicating that radiotherapy mediates a stronger immunosuppressive effect than chemotherapy under these conditions.

Impaired immune cell function due to chemotherapy treatment was observed for the patient immune cells taken immediately after the intravenous infusion of cisplatin (two samples). There was no antibody-dependent specific anti-tumour activity with catumaxomab or ertumaxomab

Fig. 4 (A) Ertumaxomab-mediated cytotoxicity of MNC from HN-SCC patients obtained before, and at weekly intervals between the first and second cycles of chemotherapy (study III). (●: samples obtained before start of CTX; □: samples from 1 week after the first cycle of CTX; △: samples obtained 2 weeks after the first cycle of CTX; ×: samples obtained 3 weeks after the first cycle of CTX) (B) Concentration of ertumaxomab (study III) for killing 50% of the tumour target cells (EC₅₀) before and at weekly intervals between the first and second cycles of chemotherapy

against the SK-OV-3 tumour target cells in the presence of MNC isolated from these samples (data not shown).

In summary, immune cells taken three weeks after the respective previous chemotherapy cycle (monthly sampling schedule) mediated effective anti-tumour activity *in vitro* induced by the trifunctional antibodies catumaxomab and ertumaxomab, indicating a potent immune response activity of the patient cells that remains even after several cycles of chemotherapeutic treatment.

We were then interested to further evaluate the effect of a single cycle of cisplatin and 5-FU, and to investigate a time interval closer to the previous chemotherapy (<three weeks). For this purpose, blood samples were taken weekly between the first and second cycles of chemotherapy (study III, ertumaxomab; Fig. 1B). With all samples from the weekly sampling schedule, concentration-dependent

antibody-mediated killing of tumour target cells was found (Fig. 4A). Furthermore, there were no differences in tumour cell killing activity (log EC₅₀) between the different sampling time points (Fig. 4B). This finding, observed also for sample 2 (1 week after first CTX cycle), indicates that even one week after the cisplatin administration (day 1) and as soon as three days after the last 5-FU dose (day 5), there was no functional impairment of the immune cells of these cancer patients due to the previous chemotherapeutic treatment.

Trifunctional antibodies induce the release of inflammatory cytokines *in vitro*

Cell-free supernatants were collected from the cytotoxicity assays from studies II (ertumaxomab; monthly sampling schedule) and III (ertumaxomab; weekly sampling schedule) and cytokines were quantified using a flow cytometric bead array. Ertumaxomab mediated concentration-dependent release of the inflammatory cytokines IFN-γ (Fig. 5A), TNF-α (Fig. 5B), IL-5 (Fig. 5C) and IL-10 (Fig. 5D) in all patient samples taken at monthly intervals (study II). However, antibody-induced release of IL-2 and IL-4 was minor in the majority of samples (data not shown). Higher IFN-γ levels were found in blood samples obtained during chemotherapy (three weeks after the first and second cycles of chemotherapy) compared to the levels obtained before any chemotherapeutic treatment. Additionally, high levels of TNF-α were observed and increased in samples obtained at the end of the radio-chemotherapy.

Cytokine levels in samples from study III (weekly sampling schedule) increased with increasing ertumaxomab concentrations in the *in vitro* assay. However, in this weekly sampling interval, no significant changes in cytokine levels were observed over time (data not shown). The control sample using MNC from a healthy donor also showed an increase in IFN-γ, TNF-α, IL-10 and IL-5 dependent on the antibody concentration used *in vitro*; lower levels of IL-4 and IL-2 were detected (data not shown).

Discussion

Standard chemotherapy and radiation therapy may have inhibitory effects on immune cells. For example, cisplatin and 5-FU are known to induce lymphocytopenia, resulting in transient immunosuppression in cancer patients. These drugs are also reported to enhance or permit efficient tumour cell killing *in vitro* at concentrations comparable with the maximally achieved therapeutic concentration *in vivo* in humans [26]. There is recent evidence indicating that some cytotoxic compounds and radiotherapy promote specific anticancer immune responses that contribute to the overall therapeutic effect [27, 28]. This study investigated whether trifunctional antibodies are able to induce efficient

Fig. 5 Release of IFN- γ , TNF- α , IL-5 and IL-10 in response to increasing ertumaxomab concentrations (study II; ertumaxomab, monthly interval, 8 patients included; ●, before CTX; □, 3 weeks after first CTX cycle; △, 3 weeks after second CTX cycle; ⊗, end of radio-CTX)

anti-tumour activity with immune effector cells obtained from patients with HNSCC before, during and after chemotherapy, and following radio-chemotherapy.

In our study, most patients showed a decrease in T cell, B cell, NK cell and monocyte counts during the course of chemotherapy treatment, suggesting a suppressive effect of chemotherapy on the immune system. However, regardless of the chemotherapeutic treatment cycle, both trifunctional antibodies mediated efficient concentration-dependent antitumour activity with immune cells from these patients. Even after three previous chemotherapeutic cycles of cisplatin (cumulative dose 300 mg/m²) and 5-FU (cumulative dose 15,000 mg/m²) and a cumulative radiation dose of 60 Gy, the patient immune effector cells retained their potential for efficient antibody-mediated tumour cell killing. Moreover, the killing efficacy of ertumaxomab was comparable using either effector cells from a healthy donor or from chemotherapy patients. Notably, using effector cells obtained three weeks after the first and second courses of cisplatin and 5-FU administration, antibody-mediated antitumour activity *in vitro* was enhanced (lower EC₅₀) compared to pre-treatment, indicating that the patient immune cells may become even more susceptible to the activation by trifunctional antibodies. Our results are supported by the *in vitro* data of Wu and colleagues [29], who showed that the percentage of T-regulatory cells and IFN- γ secreting CD8-positive T cells decreased remarkably during the course of chemotherapy when blood samples were taken from ovarian cancer patients at different time points during paclitaxel and carboplatin chemotherapy. Thus, chemother-

apy may induce temporary immune reconstitution and may augment an anti-tumour immune response. Radiotherapy may also contribute to enhance killing activity of immune cells since irradiation mediates many effects on cells and tissues that may stimulate an immune response [30]. For example, low doses of ionising irradiation upregulate expression of MHC class I molecules [31] and tumour-associated antigens [32], as well as adhesion molecules by endothelial cells [33], thereby boosting cytotoxic T-cell activity [34].

Data from our study showed that even one week after cisplatin administration (day 1) and as soon as three days after the last 5-FU dose (day 5), trifunctional antibodies are able to activate immune effector cells of cancer patients for efficient tumour cell killing, indicating that the chemotherapeutic substances did not cause any relevant functional loss of the immune effector cells. Effector function was only impaired in two samples taken immediately after the intravenous infusion of cisplatin. Nonetheless, the observed lack of antibody-dependent specific cytotoxicity of these two samples demonstrates the suitability of this *in vitro* assay for testing the functionality of patient immune effector cells.

Inflammatory cytokine release is part of the mode of action of the trifunctional antibodies catumaxomab and ertumaxomab [10]. In a phase I trial of ertumaxomab in metastatic breast cancer, measurements of cytokines (IL-6, IL-2, TNF- α and IFN- γ) suggest a strong T-helper cell type 1-associated immune response [35]. In addition, a randomised phase II/III trial of catumaxomab in malig-

nant ascites has demonstrated that cytokine-release-related symptoms may be positively correlated with efficacy and may serve as a predictive factor for the efficacy of catumaxomab [8, 36]. IFN- γ is an inflammatory cytokine released from activated T cells and NK cells, and is known to exert anti-proliferative effects on neoplastic cells [37]. In our *in vitro* assays IFN- γ levels were increased using patient blood samples taken three weeks after the first and second cycles of chemotherapy correlating to the increased cytotoxic activity mediated by these immune effector cells. Based on this observation, trifunctional antibody-induced release of IFN- γ may be further enhanced in combination with cisplatin therapy. The efficient anti-tumour activity in parallel with the enhanced release of anti-neoplastic, pro-inflammatory cytokines such as IFN- γ and TNF- α in our assays may suggest synergistic effects of the combination of trifunctional antibody and chemotherapy. Further supportive results for the combination of chemotherapeutics and trifunctional antibodies have been obtained in other preclinical models. In two *ex vivo* studies using the chicken embryo chorioallantoic membrane assay, the combination of catumaxomab and cisplatin enhanced the anti-tumour activity of immune effector cells from squamous cell carcinoma patients against autologous tumour cells [38, 39]. Notably, the effect of each individual drug alone was lower. Furthermore, in a multicellular tumour spheroid model, combined administration of catumaxomab and cisplatin resulted in a synergistic anti-tumour effect with a strong reduction of tumour spheroid volume [40]. These

reports indicate synergy for the combination of trifunctional antibodies with different chemotherapeutic drugs. In our study, no negative influence of the alkylating agent cisplatin and the pyrimidine antagonist 5-FU was observed on the trifunctional mode of action *in vitro* using immune cells from patients undergoing chemotherapy. Catumaxomab and ertumaxomab are able to mediate effective killing of tumour cells by immune cells from patients treated with chemotherapy. The patient immune cells can be activated by these bispecific antibodies already one week after chemotherapy with cisplatin and already three days after 5-FU treatment, resulting in significant tumour cell killing *in vitro*. Furthermore, our preliminary results indicate that the patient immune cells may become even more susceptible to activation by trifunctional antibodies for efficient tumour cell killing during the course of chemotherapy treatment.

These first results provide a preclinical starting point for combining chemotherapeutic drugs with trifunctional antibodies in the clinical setting. Thus, the appropriate selection of a chemotherapeutic drug or drug combination in an appropriate sequence could further enhance the anti-tumour activity of trifunctional antibodies in cancer patients.

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