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# Lysis of cancer cells by autologous T cells in breast cancer pleural effusates treated with anti-EpCAM BiTE antibody MT110

Juliane Witthauer · Bernd Schlereth · Klaus Brischwein · Hauke Winter · Ilona Funke · Karl-Walter Jauch · Patrick Baeuerle · Barbara Mayer

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**Abstract** In the present study, the efficacy of a new drug, i.e. the bispecific single-chain antibody MT110 targeting the epithelial antigen EpCAM and the T-cell antigen CD3 was tested ex vivo in malignant pleural effusions (MPEs). EpCAM<sup>+</sup> epithelial cells were found in 78% of the MPEs ( $n = 18$ ). Ex vivo treatment of seven MPEs resulted in a dose-dependent specific lysis of  $37 \pm 27\%$  ( $\pm$ SD) EpCAM<sup>+</sup> cells with 10 ng/ml ( $P = 0.03$ ) and  $57 \pm 29.5\%$  EpCAM<sup>+</sup> cells with 1,000 ng/ml MT110 ( $P = 0.016$ ) after 72 h. As a prerequisite for redirected lysis, stimulation of the autologous CD4<sup>+</sup> and CD8<sup>+</sup> cells in MPE by 1,000 ng/ml MT110 resulted in  $21 \pm 17\%$  CD4<sup>+</sup>/CD25<sup>+</sup> and  $29.4 \pm 22\%$  CD8<sup>+</sup>/CD25<sup>+</sup> cells ( $P = 0.016$ , respectively) after 72 h. This was confirmed by a 22-fold release of TNF- $\alpha$  and 230-fold release of IFN- $\gamma$  (1,000 ng/ml, 48 h,  $P = 0.03$ , respectively). Thus, relapsed breast cancer patients resistant to standard treatment might benefit from targeted therapy using MT110.

**Keywords** Malignant pleural effusion · Breast cancer · Bispecific antibody · EpCAM · CD3 · MT110 · Ex vivo therapy

## Introduction

Malignant pleural effusion (MPE) is frequent in advanced cancer, especially in lung, breast, ovarian and gastrointestinal carcinoma [1, 2]. One of the standard therapeutic strategies to prevent recurrent MPE is chemical pleurodesis using talcum. However, despite therapeutic intervention, recurrence rate is high and prognosis remains poor [3–5].

Although, MPEs are well characterized [6–14], the knowledge gathered by in vitro and in vivo studies, translated only into a few novel therapeutic strategies for clinical testing [15–17]. Thus, there is still a significant need for effective treatment strategies for the management of MPEs.

Targeted therapies based on monoclonal antibody technology may provide for such new therapeutic options. A widely accepted targeted therapy in metastatic breast cancer is the monoclonal antibody trastuzumab (Herceptin<sup>®</sup>) for patients that overexpress the target antigen HER-2/neu [18]. The important mode of action is antibody-dependent cellular cytotoxicity (ADCC), a reaction by which the cell-bound antibody recruits immune effector cells bearing Fc $\gamma$  receptors. Natural killer cells expressing Fc $\gamma$  receptor III (CD16) play a critical role in ADCC [19].

A different approach of redirecting cytotoxic immune effector cells are bispecific antibodies. One example is the class of single-chain bispecific antibodies, which is designed to recruit and simultaneously activate T cells against target cells expressing a particular surface antigen [20–22]. Hallmarks of single-chain bispecific antibodies

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are a high potency of redirected lysis in the femtomolar range [23, 24], a strictly target cell-dependent activation of T cells [25], serial lysis by activated T cells [26] and involvement of proper cytolytic synapses [27]. An EpCAM/CD3-bispecific antibody with specificity for the murine orthologs of EpCAM and CD3 has shown a significant therapeutic window, indicating that T cell-recruiting antibodies of this class can well distinguish between EpCAM antigen expressed on normal tissue and tumor tissue [28].

Several EpCAM (CD326)/CD3-bispecific antibodies have been shown in various mouse models to eradicate established tumors, to redirect tumor-resident T cells for lysis of metastatic human ovarian cancer tissue [24, 29] and to effectively treat lung metastasis [30–33]. In a phase I/II study, advanced ovarian cancer patients were treated with the trifunctional bispecific antibody catumaxomab (anti-EpCAM  $\times$  anti-CD3) resulting in a significant reduction of ascites flow rate [34].

In the present study we have investigated the EpCAM/CD3-bispecific antibody MT110 that is now in a clinical phase I study [35] for its activity against tumor cells in pleural effusates of breast cancer patients. Solely autologous immune cells present in these effusates were used as cytotoxic effector cells. Extent of target specific lysis and activation of different T cell subpopulations mediated by MT110 treatment were studied. Our data support the use of MT110 for the treatment of metastatic therapy resistant breast cancer patients.

## Patients and methods

### Patients and sample processing

MPE samples were collected from 18 patients with advanced breast cancer by chest drainage (Clinic of Surgery, Großhadern, Munich, Germany, ethics proposal number 278/04). Malignancy was confirmed by cytological analysis at the Institute for Pathology, University of Munich, Germany. Cellular fraction was isolated by two step centrifugation. Cell number and vitality were determined with the trypan blue exclusion test. Cytospins were prepared from the freshly isolated pleural effusion cells [36]. Briefly,  $5 \times 10^4$  isolated pleural effusion cells were spun on glass slides with an adhesive surface (Super-Frost plus, Menzel, Germany) using a cytospin centrifuge (Cytospin 2, Shandon, Frankfurt, Germany).

### Immunoperoxidase staining and antibodies

For target identification cytospins were stained using Avidin-Biotin Complex (ABC) immunoperoxidase staining

[37, 38]. Epithelial cells in the pleural effusion cells were identified with a mouse monoclonal antibody (mab) against human pan cytokeratin (clone KL-1, Immunotech, Marseille, France). The target antigen EpCAM was demonstrated using mouse mab against human EpCAM (clone BerEp4, Dako, Hamburg, Germany). Leukocytes and effector T cells were detected with a monoclonal mouse antibody against CD45 (clone 2B11 + PD7/26) and a polyclonal rabbit antibody anti CD3 (A0452), both purchased from Dako, Hamburg, Germany. Isotype controls were used in the corresponding concentrations. The percentage of positive cells was evaluated semi-quantitatively.

### Ex vivo therapy of pleural effusion samples

Because of sample limitation MPEs obtained from seven patients were analyzable for ex vivo treatment using MT110. The MPEs were plated in duplicate in 96-well flat bottomed cell culture plates (BD Falcon, Heidelberg, Germany) with a density of  $1 \times 10^5/100 \mu\text{l/well}$ . After recovery overnight (37°C, 5% CO<sub>2</sub>) the pleural effusion cells were treated with the bispecific antibody MT110 (Micromet AG, Munich, Germany) at the concentrations 0.1, 1, 10, 1,000 ng/ml for 48 and 72 h, respectively. As controls, the growth medium (RPMI 1640 medium containing L-glutamine, 7.5% FCS and 1% NEAA and 1% sodium pyruvate), the non-specific antibody muS110 (anti-mouse EpCAM/anti-mouse CD3), the semi-specific antibodies Mec (anti-mouse EpCAM/anti-human CD3) and  $\alpha$ EpCAM (anti-human EpCAM/anti-mouse CD3) were used at the highest antibody concentration (1,000 ng/ml), respectively. The effect of MT110 on the malignant pleural effusion cells was measured using four different read out systems, namely: induction of morphologic changes, extent of specific cytotoxicity, T cell stimulation and induction of cytokine release.

### FACS analysis and cytotoxicity assay

Characterization of the MPE cells before treatment was done by FACS analysis [39]. For the detection of EpCAM<sup>+</sup> pleural cells a mouse anti-human EpCAM-APC antibody was used (clone EBA-1). The detection of the immune cell fractions was done by using an anti-CD4-FITC (clone RPA-T4) and anti-CD8-PERCP (clone SK1) antibody. Activation of immune cells was detected by anti-CD25-APC (clone M-A251) and anti-granzyme B-PE antibody (clone GB11). The E:T cell ratio (E: effector CD3<sup>+</sup> cells; T: target EpCAM<sup>+</sup> cells) in each MPE sample was determined as the ratio of CD3<sup>+</sup> to EpCAM<sup>+</sup> cells. Therefore, the anti-EpCAM-APC antibody was used in combination with an anti-CD3-PE antibody (clone UCHT-1). All antibodies were obtained by BD Pharmingen, Heidelberg,

Germany, except anti-granzyme-B (Invitrogen, Karlsruhe, Germany). As IgG1 isotype control for every antibody the clone MOPC-21 was used with the corresponding working concentration and labeling (BD Pharmingen, Heidelberg, Germany).

After *ex vivo* treatment, antibody induced redirected lysis was measured by FACS analysis. Double staining of lysed EpCAM<sup>+</sup> pleural effusion cells was performed using the anti-EpCAM-APC antibody and 7-amino actinomycin (7-AAD; BD Pharmingen, Heidelberg, Germany). Unlabeled standard calibration beads (BD Pharmingen, Heidelberg, Germany) in a volume of 50  $\mu$ l were added to every patient sample. In this way an equal number of cells was acquired in every sample. The MT110 mediated cell lysis was calculated according to the following equation:

$$\begin{aligned} & \text{Percent of specific lysis} \\ & = 100 - [(\% \text{ of EpCAM}^+ \text{ live cells in MT110} \\ & \quad \text{treated sample} / \% \text{ of EpCAM}^+ \text{ live cells in control}^*) \\ & \quad \times 100] \end{aligned}$$

\*control = mean of the growth medium and muS110 control value

#### T cell stimulation assay

MT110 induced T cell activation was measured detecting CD25 protein surface expression and intracellular granzyme B expression of CD4<sup>+</sup> and CD8<sup>+</sup> T cells by FACS [24]. Granzyme B levels were determined using the Cytofix/Cytoperm Fixation and Permeabilization Kit according to the manufacturer's protocol (BD Biosciences Pharmingen, Heidelberg). Calibration beads were added as described above. MT110 mediated stimulation of T cells was calculated according to the following exemplary equation:

$$\begin{aligned} & \text{Percentage of CD4}^+/\text{CD25}^+ \text{ expression} \\ & = [\text{number of CD4}^+/\text{CD25}^+ \text{ cells} / \\ & \quad \text{total number of CD4}^+ \text{ cells}] \times 100 \end{aligned}$$

Similar, using the same equation the number of CD4<sup>+</sup>/granzyme B<sup>+</sup>, CD8<sup>+</sup>/CD25<sup>+</sup> and CD8<sup>+</sup>/granzyme B<sup>+</sup> cells was calculated.

#### Cytokine analysis

The concentrations of the cytokines Interleukin-2 (IL-2), IL-4, IL-6, IL-10, Tumor necrosis factor alpha (TNF- $\alpha$ ) and Interferon- $\gamma$  (IFN- $\gamma$ ) were analyzed in duplets using the medium supernatant after MT110 treatment for 48 h. Cytokine analysis of the supernatants was performed by FACS analysis using the Human Th1/Th2 Cytokine

Cytometric Bead Array (CBA, BD Biosciences Heidelberg, Germany) kit following the manufacturer's instructions [24]. Quality control samples containing the relevant cytokines with a concentration of 200 and 500 pg/ml were measured in triplets as positive control for each cytokine. A cytokine concentration lower than 20 pg/ml was below the sensitivity of the CBA kit. If this was the case, 20 pg/ml was assessed for these values. The level of the MT110 dependent cytokine induction was calculated in relation to the mean value of the bispecific antibody controls Mec and  $\alpha$ EpCAM.

#### Statistics

Dose and time dependency of the MT110 mediated specific lysis, T cell stimulation and the MT110 induced increase of cytokine release was analyzed with the Wilcoxon matched-pairs signed-ranks test. The Spearman Rank Correlation Coefficient (*R*) was used to correlate the E:T ratio and the proportion of CD25 and granzyme B expressing CD4<sup>+</sup> and CD8<sup>+</sup> cells with the amount of specific tumor cell lysis and MT110 dependent cytokine detection.

A value of *P* < 0.05 was indicated as statistically significant.

## Results

### Characterization of pleural effusion samples

Malignant pleural effusion samples were obtained from 18 patients with advanced breast cancer. The pleural effusion volume ranged from 40 to 800 ml (mean: 350 ml). The number of effusion cells after cell isolation varied from  $3 \times 10^4$  to  $1.5 \times 10^6$  cells/ml (mean:  $4 \times 10^5$  cell/ml).

Immunohistochemical biomarker analysis revealed EpCAM<sup>+</sup> epithelial cells in 78% (14 out of 18) of pleural effusion samples. Most of the samples contained a high fraction of EpCAM<sup>+</sup> epithelial cells (mean 75%, range from 20% to 100%). A similar heterogeneity was observed for the CD3<sup>+</sup> cells, although most samples contained a high fraction of CD3<sup>+</sup> leukocytes (mean 75%, range 45–93%). Subgroup analysis of the T cells revealed a mean population of 53% CD4<sup>+</sup> and 21% CD8<sup>+</sup> cells (CD4:CD8 ratio: mean 2.5:1, range 10.6:1–1.4:1).

### Redirected lysis of EpCAM<sup>+</sup> pleural cells by MT110

Seven out of 14 pleural effusions containing EpCAM<sup>+</sup> epithelial cells (mean 78%, range from 30% to 100%) were amenable for *ex vivo* treatment with MT110. In this subset of samples, the fraction of CD3<sup>+</sup> leukocytes ranged from

**Table 1** The composition of MPE samples in % of CD45<sup>+</sup> cells before ex vivo therapy concerning the T-lymphocytes (CD3<sup>+</sup>), T-helper cells (CD4<sup>+</sup>), cytotoxic T-cells (CD8<sup>+</sup>), activated T-cells (CD3<sup>+</sup>/CD25<sup>+</sup>) and natural killer cells (CD3<sup>-</sup>/CD56/CD16<sup>+</sup>)

Patient no.	Immune cell composition of MPE in %				
	CD3 <sup>+</sup>	CD4 <sup>+</sup>	CD8 <sup>+</sup>	CD3 <sup>+</sup> /CD25 <sup>+</sup>	CD3 <sup>-</sup> /CD56/CD16 <sup>+</sup>
3745	60	32	23	4.8	26
3892	76	50	27	1.6	8.4
3823	78	59	15	3.7	13
3874	91	65	26	7.2	3.7
3888	93	85	8.3	1.4	2
3917	85	69	17	n.d.	n.d.
3924	78	50	27	2.7	0.4
Mean ± SD	80 ± 10	58.5 ± 16	20.5 ± 7	3.6 ± 2	8.9 ± 8.7

**Table 2** Evaluation of the E:T ratio of the malignant pleural effusions

Absolute cell numbers in %	The E:T ratio of the malignant pleural effusion samples						
	Patient samples						
	3745	3892	3823	3874	3888	3917	3924
E: CD3 <sup>+</sup>	4	18	28	24	70	69	62
T: EpCAM <sup>+</sup>	17.5	40	20	2.2	1.3	0.3	0.1
E:T ratio (CD3 <sup>+</sup> /EpCAM <sup>+</sup> )	1:4	1:2	1:1	11:1	54:1	230:1	620:1

The effector (E: CD3<sup>+</sup> cells) to target (T: EpCAM<sup>+</sup> cells) cell ratio of the malignant pleural effusion samples was calculated with the absolute numbers of both cell populations present in the MPE

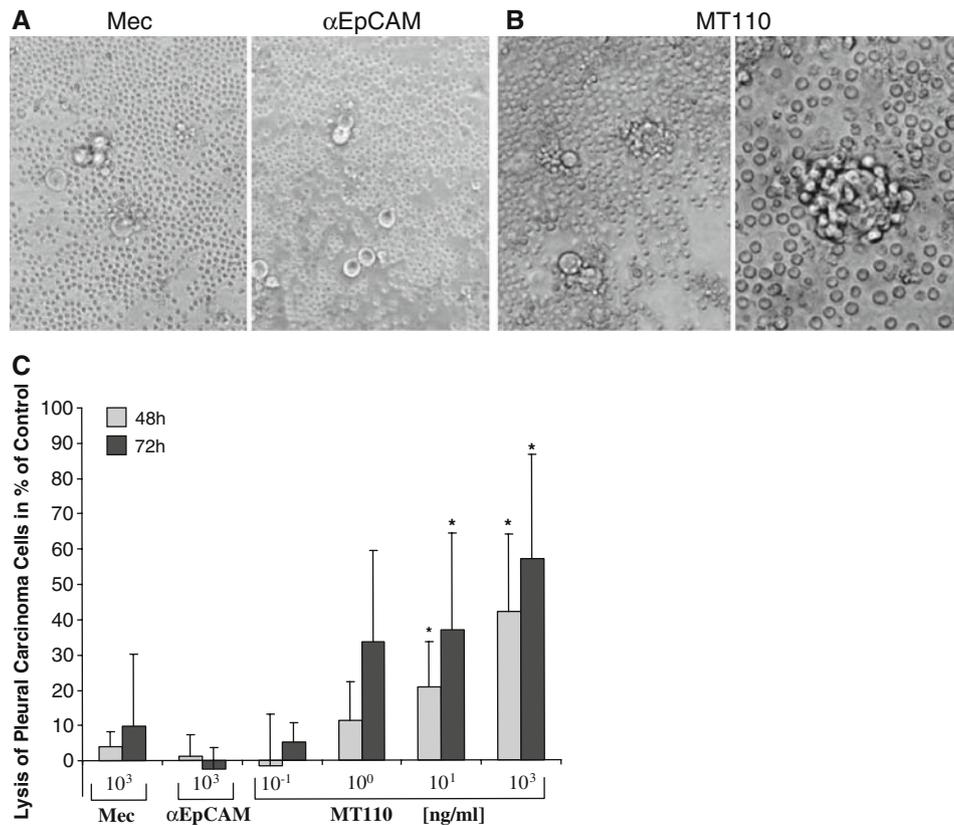
60% to 93% (mean 80%). The immune cell composition of this subset of MPEs was analyzed before MT110 treatment and is described in detail in Table 1. The E:T ratio (E: effector CD3<sup>+</sup> cells; T: target EpCAM<sup>+</sup> cells) varied between the patient samples from 1:4 to 620:1 (Table 2).

Phase contrast light microscopy demonstrated, that tumor cells formed rosettes with leukocytes in the presence of MT110 (Fig. 1b). This opsonization was seen in five out of seven treated samples (71%). The cytotoxic effect of MT110 was measured by FACS analysis, detecting non-viable EpCAM<sup>+</sup> cells. The mean specific lysis is shown in Fig. 1c after 48 and 72 h treatment with the concentrations of MT110 0.1, 1, 10, 1,000 ng/ml. One patient (3917) had pleural effusion cells sufficient for testing the 72 h time point and three doses of MT110 (0.1, 1, 1,000 ng/ml), only. Compared to the control antibodies Mec and  $\alpha$ EpCAM, MT110 induced a significant specific lysis of target cells depending on the doses of MT110. Ex vivo treatment with 10 ng/ml MT110 for 48 h resulted in 21% cell lysis (\**P* = 0.03) and 1,000 ng/ml in 42% cell lysis (\**P* = 0.03). Similar, treatment with MT110 for 72 h resulted in a specific lysis of 37% with 10 ng/ml MT110 (*P* = 0.03), and of 57% with 1,000 ng/ml MT110 (*P* = 0.016). Contrary, the length of treatment (48 h vs. 72 h) had no significant impact (Fig. 1c).

The high standard deviations seen in Fig. 1c suggested strong differences between individual pleural effusion samples (Fig. 2). Specific lysis differed among the patients from 13.5% to 79% after 48 h for the highest concentration of MT110 (1,000 ng/ml). After 72 h with 1,000 ng/ml MT110, the extent of the specific lysis varied from 24% to 100%. High responders such as samples 3823 (Fig. 2a and b), 3917 (Fig. 2b) and 3924 (Fig. 2a and b) responded to 1 ng/ml MT110 and revealed a high fraction of dead cells after 72 h MT110 treatment (1,000 ng/ml). Other patient samples, such as 3888, 3874 and 3892 were less susceptible and required higher concentrations of MT110.

#### Activation of T cells by MT110

A significant increase of the CD25<sup>+</sup> fraction of CD4<sup>+</sup> and CD8<sup>+</sup> cells for the seven MPE samples was seen after treatment with 1,000 ng/ml MT110 for 48 h (\**P* = 0.03) and 72 h (\**P* = 0.016), respectively (Fig. 3a and b, Table 3). At this MT110 concentration, the percentage of CD4<sup>+</sup>/CD25<sup>+</sup> T cells reached 18% and 21% and that of CD8<sup>+</sup>/CD25<sup>+</sup> T cells 33% and 29% after both 48 h and 72 h. Contrary, there was no significant increase detected for granzyme B expression in CD4<sup>+</sup> and CD8<sup>+</sup> cells after MT110 treatment (Fig. 3c and d, Table 3). The proportion



**Fig. 1** Treatment of malignant pleural effusion cells with the bispecific antibody MT110. (a, b) Ex vivo antibody treatment of malignant pleural effusion cells obtained from patient 3888 for 72 h in 96-well cell culture plate. (a) The bispecific control antibodies Mecoprop Mec (left panel) and  $\alpha$ EpCAM (right panel) in a concentration of 1,000 ng/ml respectively had no impact on the pleural effusion cells (magnification 200 $\times$ , Olympus IX 50; Olympus Camera SC 35). (b) The bispecific antibody MT110 (1,000 ng/ml)

induced the opsonization of the tumor cells by the immune cells (left panel, magnification 200 $\times$ ; right panel, magnification 400 $\times$ ). (c) Mean specific lysis of malignant pleural effusions treated ex vivo with MT110 at different doses (0.1, 1, 10 and 1,000 ng/ml) and time points (bright columns, 48 h,  $n = 6$  patients; dark columns, 72 h,  $n = 7$  patients). Significant specific lysis of EpCAM<sup>+</sup> pleural effusion cells ex vivo treated with MT110 are pointed out (\*  $P < 0.05$ )

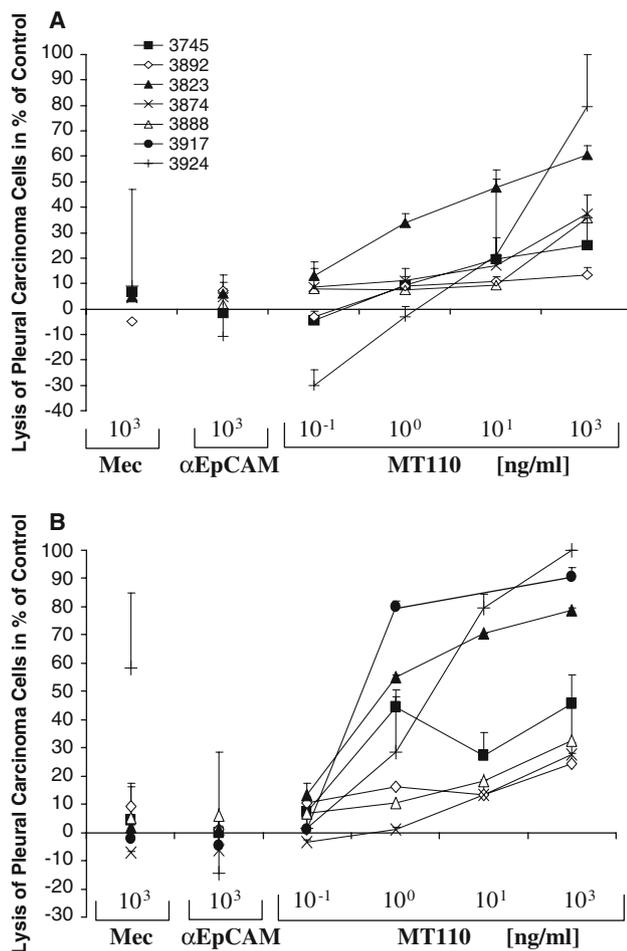
of CD4<sup>+</sup>/granzyme B<sup>+</sup> T cells maximally increased 5-fold (23%) after 72 h in the presence of 1,000 ng/ml MT110 (Fig. 3c). Under the same condition, the maximum increase of CD8<sup>+</sup>/granzyme B<sup>+</sup> T cells was 1.7 (44%, Fig. 3d). However, the initial percentage of granzyme B<sup>+</sup>/CD8<sup>+</sup> cells was significantly higher compared to the CD4<sup>+</sup> cells after 48 h ( $P = 0.03$ ) and 72 h ( $P = 0.016$ ) (Fig. 3d). There was no significant difference between 48 and 72 h, regarding the MT110 depending activation of both CD4<sup>+</sup> and CD8<sup>+</sup> cells.

Although MT110 treatment resulted in a dose-dependent stimulation of the T cell response in all MPE samples, interindividual differences were observed (Fig. 4). With respect to the CD4<sup>+</sup>/CD25<sup>+</sup> subpopulation, the highest fraction of CD4<sup>+</sup>/CD25<sup>+</sup> cells was obtained in patient sample 3823, while no increase was observed in effusion sample 3745 (Fig. 4a). Concerning the proportion of CD8<sup>+</sup>/CD25<sup>+</sup> cells, all seven pleural samples showed a dose-dependent increase after MT110 treatment (Fig. 4b).

The malignant pleural effusions 3745, 3823 and 3892 reached much higher levels of CD8<sup>+</sup> T cells expressing CD25 in comparison to the other samples (Fig. 4b). The proportion of granzyme B-expressing CD4<sup>+</sup> T cells was highest in sample 3823 reaching almost 100% in a MT110 dose-dependent fashion (Fig. 4c), while it remained below 20% for five out of seven samples. The initial proportion of CD8<sup>+</sup>/granzyme B<sup>+</sup> cells was high (>20%) for the three pleural effusion samples 3745, 3823 and 3917 (Fig. 4d). The patient samples 3823 and 3745 showed a strong increase in the number of CD8<sup>+</sup>/granzyme B<sup>+</sup> cells stimulated by MT110 treatment (Fig. 4d).

#### Impact of the immune system on the specific lysis induced by MT110

The extent of redirected specific lysis of EpCAM<sup>+</sup> cells varied from 24% (3892) to 100% (3924) after MT110 therapy using 1,000 ng/ml for 72 h (Figs. 2b, 5). The E:T



**Fig. 2** Specific lysis of individual MPE patient samples treated ex vivo with the bispecific antibody MT110 at different doses for 48 h (a) and 72 h (b). (a) Redirected lysis of EpCAM<sup>+</sup> pleural effusion cells induced by MT110 after 48 h treatment ( $n = 6$ , because of sample limitation of patient 3917). (b) Redirected lysis of EpCAM<sup>+</sup> pleural effusion cells induced by MT110 after 72 h treatment ( $n = 7$ )

ratio (E: effector CD3<sup>+</sup> cells; T: target EpCAM<sup>+</sup> cells), which individually differed from 1:4 (3745) to 620:1 (3924) did not significantly correlate with the amount of specific cell lysis ( $P = 0.1$ ;  $R = 0.67$ ). Moreover, concerning the fraction of the CD3<sup>+</sup> effusion cells, ranging from 4% (3745) to 70% (3888) no correlation was observed with the extent of the MT110 induced specific lysis ( $P = 0.35$ ;  $R = 0.4$ ). Similar, the activation status of both CD4<sup>+</sup> and CD8<sup>+</sup> cells had no significant impact on the extent of the MT110 dependent specific lysis (CD4<sup>+</sup>/CD25<sup>+</sup>:  $P = 0.3$ ,  $R = -0.5$ ; CD8<sup>+</sup>/CD25<sup>+</sup>:  $P = 0.6$ ,  $R = -0.3$ ; CD4<sup>+</sup>/granzyme B<sup>+</sup>:  $P = 0.9$ ,  $R = -0.045$ ; CD8<sup>+</sup>/granzyme B<sup>+</sup>:  $P = 0.39$ ,  $R = 0.4$ ). Nevertheless, highest efficacy of MT110 was seen for the patient samples 3917 (lysis: 90%) and 3924 (lysis: 100%), which had a 230- and 620-fold excess of CD3<sup>+</sup> over EpCAM<sup>+</sup> cells, respectively. Samples with E:T ratios as low as 1:4 (3745)

and 1:1 (3823) also showed high efficacy of lysis (3745: 46%; 3823: 79%) and in addition revealed a high proportion of CD8<sup>+</sup>/CD25<sup>+</sup> (3745: 69%; 3823: 49%; Fig. 5a) and CD8<sup>+</sup>/granzyme B<sup>+</sup> (3745: 81%; 3823: 99%; Fig. 5b) cells. Moreover, the patient sample 3823 had a strong MT110 dependent activation of CD4<sup>+</sup> cells showing both a high fraction of CD4<sup>+</sup>/CD25<sup>+</sup> (60%; Figs. 4a, 5a) and CD4<sup>+</sup>/granzyme B<sup>+</sup> (96%; Figs. 4c, 5b) cells. Samples with much higher E:T ratios of 11:1 (3874) and 54:1 (3888) were not more responsive (3874: 28%; 3888: 33%) but additionally had a much lower fraction of granzyme B/CD8<sup>+</sup> cells (3874: 11%; 3888: 24%; Fig. 5b).

Cytokine levels in culture supernatants after ex vivo therapy with MT110

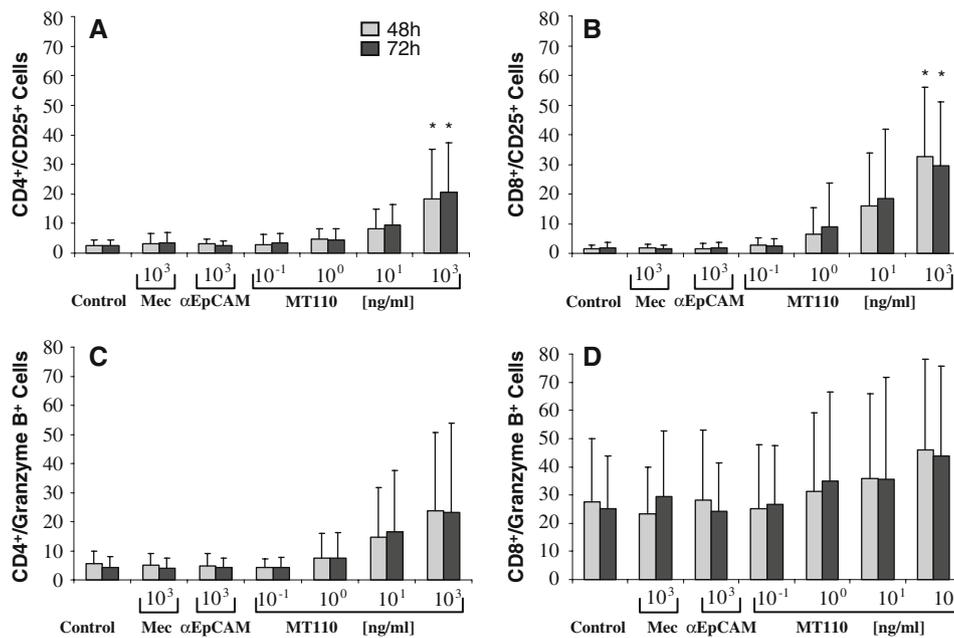
The cytokine release induced by MT110 treatment (1,000 ng/ml; 48 h) into the culture supernatant was analyzed by FACS analysis (Table 4). MT110 induced a significant release of TNF- $\alpha$  ( $P = 0.03$ ) and IFN- $\gamma$  ( $P = 0.03$ ) with a mean of a 22-fold (426 pg/ml) and 230-fold (4,634 pg/ml) increase in comparison to the control antibodies Mec and  $\alpha$ EpCAM. The induced level of IFN- $\gamma$  release correlated positively with the proportion of CD4<sup>+</sup>/granzyme B<sup>+</sup> cells after 48 h ( $P = 0.03$ ;  $R = 0.88$ ).

IL-10 showed a mean increase of 13-fold (485 pg/ml) and IL-2 a mean increase of 7-fold (141 pg/ml) after MT110 treatment (1,000 ng/ml; 48 h) which was however not significant. The concentration for IL-4 was below the sensitivity level of the kit, i.e., less than 20 pg/ml for all but one patient sample (3823). IL-6 showed a high initial concentration with a mean level of 5,738 pg/ml (range 2,400–10,757 pg/ml) in four out of seven patient samples (3924, 3823, 3892 and 3745) which did not increase during MT110 treatment.

## Discussion

Advanced breast cancer metastasized to the pleural cavity is associated with poor prognosis [2, 4, 40, 41]. In the present study, all but one patient developed recurrent malignant pleural effusions despite multimodal therapy and died within a mean time of 3 months (range, 3 weeks to 9 months). This grave situation indicates the urgent need of more efficient treatment strategies for MPE, such as targeted therapies.

The present study shows that breast cancer cells in pleural exudates frequently express the tumor-associated antigen EpCAM (CD326) providing a rationale for treatment of metastatic breast cancer with EpCAM-directed antibody-based therapeutic approaches [42, 43]. As one example, a bispecific single-chain antibody called MT110



**Fig. 3** Mean stimulation of the CD4<sup>+</sup> and CD8<sup>+</sup> T cells present in the MPE effusions after ex vivo treatment with MT110 after 48 h (bright columns,  $n = 6$  patients,) and 72 h (dark columns,  $n = 7$  patients). (a, b) Stimulation of the T cells was demonstrated by CD25 expression using FACS analysis. In both, CD4<sup>+</sup> (a) and CD8<sup>+</sup> (b) cell populations, the fraction of CD25<sup>+</sup> cells significantly correlated with

increased doses of MT110 ( $* P < 0.05$ ). (c, d) The cytoplasmic expression of granzyme B was analyzed in the CD4<sup>+</sup> and CD8<sup>+</sup> populations by FACS analysis. The initial expression of granzyme B in CD4<sup>+</sup> cells was significantly lower compared to CD8<sup>+</sup> cells after 48 h and 72 h ( $* P < 0.05$ ) and did not significantly change under treatment with MT110

**Table 3** Changes of the CD25 and granzyme B expression of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells during MT110 ex vivo therapy (48 h, 72 h) with 1,000 ng/ml MT110

Phenotype	Phenotypic changes of the effector cells induced by MT110 treatment in % ( $n = 7$ )					
	48 h			72 h		
	–MT110 <sup>a</sup>	+MT110 <sup>b</sup>	<i>P</i> -value	–MT110 <sup>a</sup>	+MT110 <sup>b</sup>	<i>P</i> -value
CD4 <sup>+</sup> /CD25 <sup>+</sup>	2.7 ± 1.6	18 ± 11	0.03	2.5 ± 1.9	21 ± 17	0.016
CD8 <sup>+</sup> /CD25 <sup>+</sup>	1.6 ± 1.3	33 ± 23	0.03	1.8 ± 1.9	29 ± 22	0.016
CD4 <sup>+</sup> /granzyme B <sup>+</sup>	6 ± 4.3	24 ± 27	n.s.	4 ± 3.6	23 ± 31	n.s.
CD8 <sup>+</sup> /granzyme B <sup>+</sup>	28 ± 22	46 ± 32	n.s.	25 ± 19	44 ± 32	n.s.

Mean values ± standard deviation (SD) and *P*-values are given (n.s. = not significant)

<sup>a</sup> –MT110 = control, as described in “Patients and methods”

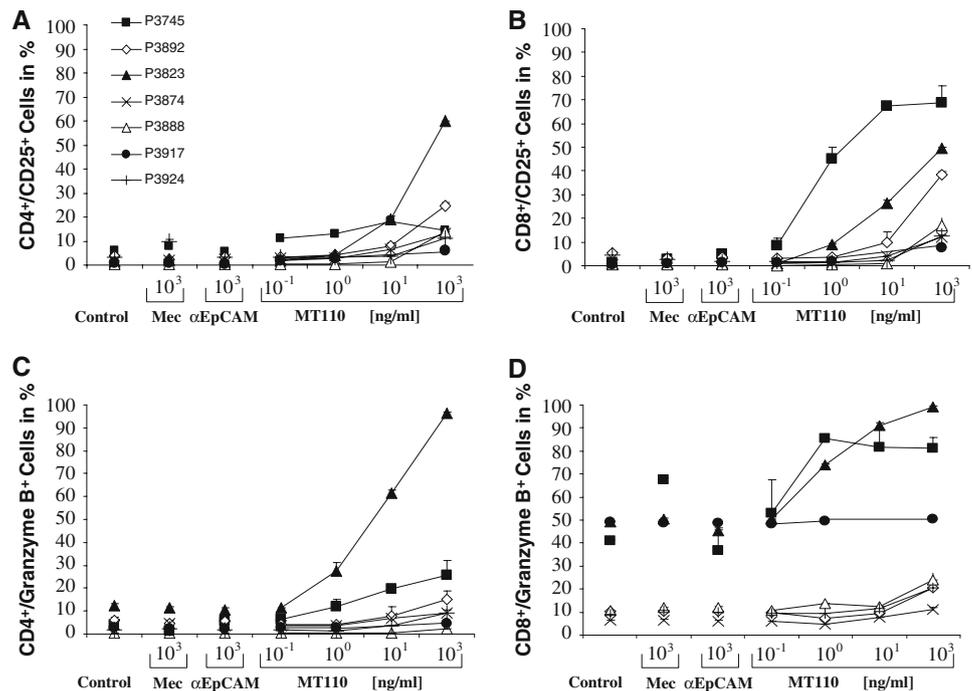
<sup>b</sup> +MT110 = 1,000 ng/ml

with the capacity of recruiting and activating T cells against EpCAM-expressing cancer cells [24] is here shown to redirect autologous T cells—as are found in the cellular milieu of malignant pleural effusions—for efficient lysis of carcinoma cells. This lysis was highly specific, dependent on the dose of MT110, similar as shown for the anti-EpCAM treatment of primary ovarian cells [44]. In addition, specific lysis appeared to be highest in the presence of either a favourable E:T ratio or granzyme B-expressing

CD8<sup>+</sup> cells but needs to be confirmed in a higher number of patient samples.

MT110 was found to potently activate previously unstimulated T cells in pleural effusates. However, unlike with T cells from the peripheral blood of healthy donors [24], not all samples reached a high proportion of CD25 expressing T cells. It is likely that not all T cells in exudates can be activated by MT110 because they are anergic due to escape mechanisms of the pleural tumor cells, due to

**Fig. 4** Stimulation of CD4<sup>+</sup> and CD8<sup>+</sup> T cell populations in the individual pleural effusion samples ( $n = 7$ ) ex vivo treated with the bispecific antibody MT110 after 72 h. (a, b) MT110 dependent T cell activation was reflected by the expression of the activation marker CD25. The low fraction of CD25 expressing T cells in the control samples indicates that the CD4<sup>+</sup> cells were unstimulated before MT110 treatment. (c, d) Cytotoxic potential of the T cells was reflected by the expression of the apoptosis inducing protease granzyme B in CD4<sup>+</sup> (c) and CD8<sup>+</sup> (d) cells. A high initial extent of granzyme B was found in CD8<sup>+</sup> cells of patient samples 3823, 3745 and 3917, but was completely missing in CD4<sup>+</sup> cells



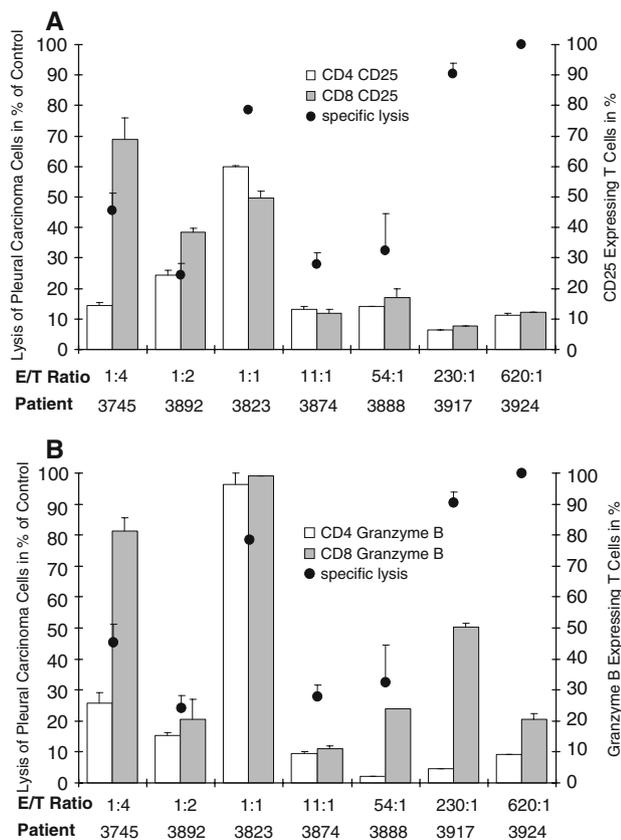
exposure to tumor cell-derived TGF- $\beta$ , or expression of PD receptor ligands B7-H1 or B7-H4, or the tryptophan degrading enzyme indole 2,3-dioxygenase (IDO) by cancer cells [45, 46]. Because T cell activation by MT110 is dependent on the presence of target cells [25], it is also conceivable that low numbers of EpCAM-expressing cells have limited the polyclonal T cell activation by MT110. Our finding that the extent of redirected target cell lysis in some patients was associated with a high percentage of granzyme B<sup>+</sup>/CD8<sup>+</sup> cells, as reached after a 72 h reaction with MT110, is well consistent with the recruitment of lysis-competent, cytotoxic CD8<sup>+</sup> T cells by MT110. In vitro experiments have shown that MT110-mediated lysis of tumor cell lines is most effective with CD8<sup>+</sup> T cells [24]. This predominant role of CD8<sup>+</sup> cells was also observed with the anti-EpCAM/CD3 trifunctional antibody catumaxomab [47]. However, isolated CD4<sup>+</sup> T cells may also significantly contribute to lysis as seen for patient sample 3823. CD4<sup>+</sup> cells were reported to require a lag phase during which granzyme B and perforin expression is induced before they can engage in MT110-mediated redirected target cell lysis [24].

Various parameters were identified as candidates to predict efficacy of MT110, but need to be tested in an enlarged patient cohort. One is a high CD3<sup>+</sup> to EpCAM<sup>+</sup> cell (E:T) ratio in effusions, which was found associated with a high redirected specific tumor cell lysis. Another one is a high fraction of granzyme B expressing CD8<sup>+</sup> cells, which also correlated with a high degree of specific

tumor cell lysis. Lastly, the combination of both may be important to predict the therapeutic effect of MT110.

It is conceivable that samples with a high proportion of T cells also contain a higher number of regulatory T cells that can potentially impede the activity of BiTE-activated cytotoxic T cells. Future studies need to further analyze pleural effusion samples for factors potentially modulating the activity of MT110, including the percentage of regulatory FoxP3<sup>+</sup> T cells. We however assume that the cellular composition of pleural effusates in patients with respect to immune cells is a highly dynamic one. Indeed, in patient 3888 the extent of immune cells changed in recurrent effusions (data not shown). In patients, new T cells may be recruited after an initial lytic reaction. Moreover, differences among samples in the extent of redirected lysis seen here after 72 h of MT110 incubation may vanish in patients where the bispecific antibody is present for several weeks. This exposure is currently realized for another bispecific antibody, called MT103/MEDI-538, which is targeting the CD19 antigen on non-Hodgkin lymphoma cells [48]. MT103/MEDI-538 induced confirmed partial and complete responses in lymphoma patients, which required continuous infusion for 4–8 weeks using a portable minipump. A similar duration may be necessary for treatment of metastatic breast cancer with MT110 and may equalize differences seen in the present ex vivo experiment lasting up to 72 h.

EpCAM appears as an attractive target for treatment of breast cancer. Firstly, a negative prognostic impact has



**Fig. 5** Impact of the E:T ratio and the activation of the immune cell subpopulations CD4<sup>+</sup> and CD8<sup>+</sup> on the amount of specific lysis of EpCAM<sup>+</sup> effusion cells after 72 h MT110 treatment (1,000 ng/ml). (a) Expression of the activation marker CD25 is shown for CD4<sup>+</sup> (bright columns) and CD8<sup>+</sup> (dark columns) cells. In addition, the E:T ratio of every patient sample (*x*-axis) and the specific lysis of EpCAM<sup>+</sup> cells is pictured (dark dots). (b) Expression of the apoptotic protease granzyme B is shown for CD4<sup>+</sup> (bright columns) and CD8<sup>+</sup> (dark columns) cells. As in a, the E:T ratio of every patient sample (*x*-axis) and the specific lysis of the EpCAM<sup>+</sup> cells is pictured (dark dots)

been shown for the level EpCAM expression on node-positive primary breast cancer tissue [49]. Secondly, up to 42% of breast cancer patients express high levels of EpCAM and another 40% of patients express intermediate to low levels of the antigen [50]. An increase in high level EpCAM expression to 55% has been reported to occur in metastatic tissue [51]. Thirdly, EpCAM is expressed on so-called cancer stem cells in breast cancer and only those stem cells expressing EpCAM are highly tumorigenic in immunodeficient mice [52]. Fourthly, knockdown of EpCAM expression by a specific small inhibitory RNA in breast cancer cell lines inhibited proliferation, migration and invasiveness of cancer cells [53], and overexpression of EpCAM in quiescent cells increased these oncogenic parameters [54]. Based on the present data and the emerging biology of EpCAM in breast cancer, MT110 appears as an attractive therapeutic option for treatment of metastatic and therapeutic resistant stages of the disease.

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**Table 4** Cytokine measurement in the medium supernatant after treatment with MT110 (1,000 ng/ml) for 48 h (*n* = 6 patient samples)

Patient samples	Cytokine release in medium supernatant of MPE samples after MT110 ex vivo therapy											
	IL-2		IL-4		IL-6		IL-10		TNF $\alpha$		IFN $\gamma$	
	pg/ml	Fold	pg/ml	Fold	pg/ml	Fold	pg/ml	Fold	pg/ml	Fold	pg/ml	Fold
3745	565 $\pm$ 34	28	<20 <sup>a</sup>	1	14,043 $\pm$ 1,689	1.3	137 $\pm$ 7	6	344 $\pm$ 15	17	2,985 $\pm$ 115	149
3892	124 $\pm$ 4	6	<20	1	5,371 $\pm$ 267	1	95 $\pm$ 19	5	34 $\pm$ 3	2	359 $\pm$ 19	18
3823	23 $\pm$ 2	1	185 $\pm$ 6	9	3,345 $\pm$ 71	1.4	2,458 $\pm$ 76	55	1,170 $\pm$ 45	59	23,711 $\pm$ 912	1,186
3874	74 $\pm$ 7	4	<20	1	151 $\pm$ 5	1.4	<20	1	153 $\pm$ 3	8	228 $\pm$ 10	11
3888	<20	1	<20	1	642 $\pm$ 8	2	74 $\pm$ 12	4	510 $\pm$ 1	26	208 $\pm$ 42	10
3924	42 $\pm$ 4	2	<20	1	2,412 $\pm$ 90	1	127 $\pm$ 8	6	342 $\pm$ 2	17	311 $\pm$ 2	4
Median $\pm$ SD	141 $\pm$ 193		48 $\pm$ 61		4,327 $\pm$ 4,675		485 $\pm$ 883		426 $\pm$ 366		4,634 $\pm$ 8,589	
<i>P</i> -values	0.06		1		0.5		0.06		<b>0.03</b>		<b>0.03</b>	

Sample 3917 was excluded because only the 72 h time point was available. The data show the concentration  $\pm$  standard deviation (SD) of the cytokines Interleukin-2 (IL-2), IL-4, IL-6, IL-10, Tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) and Interferon  $\gamma$  (IFN  $\gamma$ ) in pg/ml after MT110 treatment.

<sup>a</sup> Cytokine concentration lower than 20 pg/ml was below the sensitivity of the Cytokine Bead Array (CBA) kit. In addition, the fold increase of every cytokine is displayed

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