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RESEARCH ARTICLES

Transient lymphocyte decrease due to adhesion and migration following catumaxomab (anti-EpCAM x anti-CD3) treatment *in vivo*

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Abstract
Introduction In patients, a transient decrease in peripheral blood lymphocyte counts was observed following intraperitoneal administration of the trifunctional monoclonal antibody catumaxomab (anti-human EpCAM x anti-human CD3). The aim of this study was to clarify the observed effect in a preclinical mouse model and to analyse the related mechanism of action *in vitro*.
Materials and methods A related antibody, BiLu (anti-human EpCAM x anti-mouse CD3), was administered to mice and blood leukocytes were analysed. *In vitro* studies measured activation and cytokine secretion from human peripheral blood mononuclear cells (PBMC). For the analysis of T cell adhesion, PBMC were preincubated with catumaxomab and then co-cultured with human endothelial cells (HUVEC); T cell adhesion was assessed in the presence or absence of endothelial cell preactivation by TNFα. Adherent T cells were determined by flow cytometry.
Results Treatment of mice with BiLu resulted in a dose-dependent transient decrease in CD3+ T cells (both CD4+ and CD8+) that returned to the normal range within 48 h. Catumaxomab physiologically activated T cells *in vitro* (increased CD69 expression) and induced cytokine release (TNFα, IFNγ). TNFα increased expression of adhesion molecules CD54 and CD62E on endothelial cells. Furthermore, catumaxomab dose-dependently enhanced adhesion of T cells to endothelial cells. Adhesion was further increased when endothelial cells were preactivated with TNFα.
Conclusions Catumaxomab increases adhesion of T cells to endothelial cells due to antibody-mediated activation of T cells and production of T cell cytokines that up-regulate endothelial cell adhesion molecules. These results provide a mechanistic rationale for the transient, reversible decrease in lymphocyte counts observed following catumaxomab administration in patients, which is likely to be due to redistribution of lymphocytes.

Keywords Catumaxomab · Trifunctional antibody · T lymphocyte · Endothelium · Adhesion · Redistribution

Introduction

Catumaxomab is a trifunctional monoclonal antibody of mouse IgG2a and rat IgG2b origin. It is indicated for the intraperitoneal (i.p.) treatment of malignant ascites in patients with epithelial cancer [1, 2]. The antibody targets human CD3 (expressed on T lymphocytes) and EpCAM (human epithelial cell adhesion molecule), a tumour-associated antigen, and possesses a third functional binding site in its Fc-region, which preferentially binds and activates activating Fcγ-receptor-positive accessory cells. Thus, binding of catumaxomab to EpCAM-positive tumour cells results in simultaneous recruitment and activation of both T lymphocytes and Fcγ-receptor-positive accessory cells at the tumour site. The tumour cells and immune effector cells are brought into close proximity, and a complex "crosstalk" between T cell and accessory cell can occur resulting in effective killing of tumour cells [3–5]. Thus, catumaxomab leads to physiological cellular activation, cytokine release and proliferation of T cells.

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In patients a transient decrease in peripheral blood lymphocyte counts was observed following catumaxomab infusion [1, 2, 6]. Normalisation mostly occurred within 2–3 days after treatment. Due to the transient nature of the lymphocyte reduction and due to the observed short recovery period this reversible decrease is attributed to a redistribution of lymphocytes.

T lymphocytes are able to extravasate, migrate into organ parenchyma and re-appear in the circulation via the lymphatic system [7]. They can redistribute from the intravascular pool to the extravascular pool (e.g., bone marrow, spleen, lymph nodes, etc.). Activation of T lymphocytes results in up-regulation of adhesion molecules and increased cytokine production. The increased levels of T lymphocyte cytokines, such as tumour necrosis factor alpha (TNFα) and interferon gamma (IFNγ), up-regulate expression of endothelial cell adhesion molecules. This leads to increased adhesion of lymphocytes to endothelium and migration into the extravascular space [8, 9].

By contrast, lymphocytopenia due to decreased production or increased destruction of lymphocytes can persist for months to years after therapy [7, 10, 11]. Lymphocytopenia is defined as a lymphocyte count of $\leq 1.0 \times 10^9$/l. In peripheral blood, approximately 65–80% of lymphocytes are T cells and 8–15% are B cells, so lymphocytopenia is more likely to be associated with changes in the T cell count. Lymphocytopenia may be a result of decreased production of lymphocytes (e.g., due to inherited immunodeficiency or zinc deficiency), increased destruction of lymphocytes (e.g., due to radiotherapy, chemotherapy, HIV), redistribution of lymphocytes (e.g., due to steroids, anaesthesia, surgery, infections), or various unknown or multifactorial pathogeneses [7].

The aim of this study was to determine whether the transient lymphocyte decrease following catumaxomab administration might be due to antibody-induced adhesion of lymphocytes. We conducted an *in vivo* study in mice to evaluate the effect of CD3 binding by the closely related trifunctional antibody BiLu (anti-human EpCAM x anti-mouse CD3) on lymphocytes. In addition, *in vitro* studies investigated the effect of catumaxomab on human T cell activation, cytokine release and adhesion to endothelial cells.

Materials and methods

Effect on lymphocytes *in vivo*

The trifunctional antibody BiLu (anti-human EpCAM x anti-mouse CD3; TRION Research GmbH) binds to mouse CD3. Female BALB/c mice (Elevage Janvier, France) received intravenous (i.v.) doses of BiLu (diluted in PBS) as bolus application of 10 ml/kg. Mice (48 per dose group with 6 per time point) received single doses of 0 (vehicle control), 30, 100 or 300 μg/kg. Blood samples were taken from all mice before BiLu administration and a second sample was taken either at 4, 8, 16, 24, 48, 72, 96 or 120 h after administration. CD3, CD4, CD8 and CD45+ leukocytes were measured by flow cytometry (Coulter EPICS XL/MCL, Beckman Coulter, Germany) using monoclonal rat-anti-mouse antibodies (BD Pharmingen, Germany): CD45-PECy5/CD3-FITC/CD4-PE, CD45-PE-Cy5/CD3-FITC/CD8-PE and CD3-FITC/CD4-PE/CD8-PE-Cy5. The mean fluorescence of the CD3 staining was comparable in samples after vehicle or BiLu administration, which confirms that binding of BiLu did not affect the detection of T cells by the anti-CD3 antibodies used in this study.

T cell preparation and activation *in vitro*

Peripheral blood mononuclear cells (PBMC) from healthy donors were isolated from EDTA-blood by density gradient centrifugation. PBMC (10^6 cells/ml) were incubated in RPMI-1640 (Gibco/Invitrogen, Germany) plus 10% foetal bovine serum (Sigma-Aldrich, Germany) in the presence or absence of catumaxomab (2–200 ng/ml; Fresenius Biotech GmbH, Germany) for 24 h at 37°C. The cells were harvested, washed with phosphate-buffered saline (PBS), stained with anti-CD69-PE (Becton Dickinson, Germany) together with the T cell marker anti-CD3-FITC (Becton Dickinson), and analysed by flow cytometry (FACSCalibur, BD Bioscience).

Cytokine secretion from T cells *in vitro*

PBMC (10^5 cells) were co-cultured with EpCAM-positive HCT-8 tumour cells (10^4 cells) in the presence or absence of catumaxomab (0.1–20 ng/ml; Fresenius Biotech GmbH, Germany) for 20 h at 37°C. Cell culture supernatants were collected and analysed for catumaxomab-induced TNFα and IFNγ release using a cytometric bead array (CBA Cytokine Kit II; Becton Dickinson) according to the manufacturer's instructions.

Endothelial cell preparation and activation *in vitro*

Human vascular endothelial cells (HUVEC) were isolated from umbilical vein following informed consent. The umbilical vein (within 24 h after birth) was flushed with PBS, filled with 0.02% collagenase (Sigma-Aldrich) buffer, incubated for 12 min at 37°C, and cells were collected and cultured in ECGM-Medium (Promocell, Germany) at 37°C. The cells were sub-cultured to a maximal passage number of 5 before use. HUVEC were incubated in the presence or absence of 25 ng/ml TNFα (Sigma-Aldrich) for 24 h before analysis.

For analysis of adhesion molecule expression, HUVEC were detached with 1 mM EDTA, washed twice with PBS, stained with anti-CD62E-FITC (Becton Dickinson), anti-CD54-FITC (Becton Dickinson) and anti-CD31-FITC (Dianova, Germany), and analysed by flow cytometry

(FACSCalibur). A multiparametric analysis was carried out to detect expression of CD62E and CD54 by CD31+ endothelial cells.

T cell adhesion to endothelial cells *in vitro*

Analysis of adhesion of T cells to endothelial cells was performed using co-cultures of PBMC and adherent HUVEC. PBMC were preincubated with catumaxomab (2–200 ng/ml) for 24 h. Subconfluent monolayers of HUVEC in 6-well plates were preincubated in the absence/presence of TNFα for 24 h. Following preincubation, PBMC (2×10^6 cells/well) were transferred to the HUVEC cultures and incubated for 2 h at 37°C. Non-adherent cells were removed by extensive washing; and adherent PBMC and HUVEC were detached using trypsin/EDTA (Invitrogen). The relative amount of T cells (CD3+) attached to HUVEC (CD31+) was determined by flow cytometry (FACSCalibur, BD Bioscience).

Results

Effect on lymphocytes *in vivo*

Female BALB/c mice (48 per group, 6 per time point) received i.v. doses of BiLu, a trifunctional antibody (anti-human EpCAM x anti-mouse CD3) that binds to mouse CD3. The relative amount of total CD3+ cells was comparable in all groups of mice before application of BiLu (44–52% of total CD45+ leukocytes). Application of vehicle control (phosphate-buffered saline) resulted in no relevant change in the relative amount of CD3+ cells (mean 43%).

The investigation of the effect of CD3-binding on peripheral blood lymphocytes revealed that treatment of BALB/c mice with BiLu resulted in dose-dependent transient decreases in T cell counts (Fig. 1). A dose-dependent transient decrease in total CD3+ T cells (% of total CD45+ leukocytes) was apparent 4 h after application of BiLu and persisted up to 24 h, after which CD3+ cells returned to baseline values within 48 h (Fig. 1A). At 4–8 h after application of 30, 100 or 300 µg/kg BiLu, the CD3+ T cells declined to levels of 17–18%, 4–5% and 2–4%, respectively (control 39–46%).

Figures 1B and 1C show the effect of BiLu on CD4+ and CD8+ T cell subsets (% of total CD3+ T cells). There was no relevant effect of 30 µg/kg BiLu compared to vehicle control: throughout the experiment (up to 120 h), CD4+ levels were 70–75% vs. 72–79% in control and CD8+ levels were 20–28% vs. 22–26% in control. At higher doses of BiLu, 100 and 300 µg/kg, the relative amount of both CD4+/CD3+ and of CD8+/CD3+ cells decreased. As with CD3+ T cells, there was a dose-dependent decrease in the proportion of CD4+/CD3+ cells up to 8–24 h post-application of 100 or 300 µg/kg BiLu (to a minimum of 44% and 15%, respectively), after which the levels of these cells slowly increased back to normal levels after 48 h. There was a similar transient decrease in the relative amount of

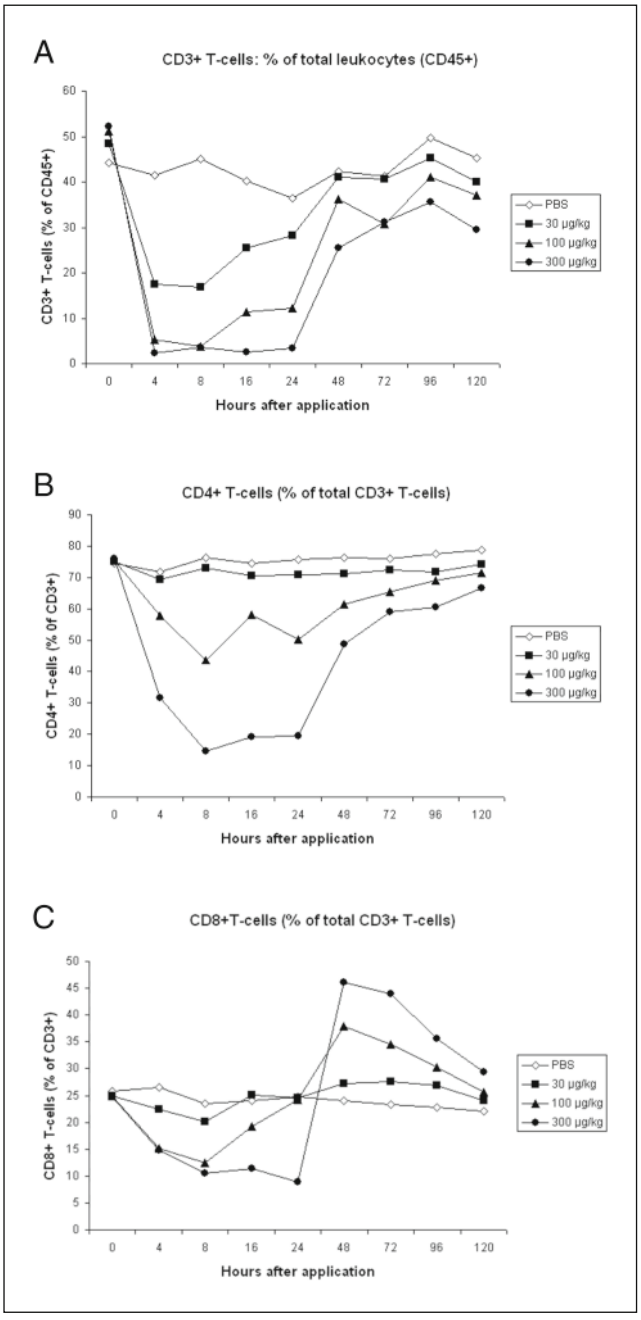

Fig. 1 Effect of BiLu (anti-human EpCAM x anti-mouse CD3) on T cells in mice. **A** CD3+ T cells as a % of total leukocytes (CD45+). **B** CD4+CD3+ T cells as a % of total T cells (CD3+). **C** CD8+CD3+ T cells as a % of total T cells (CD3+). Groups of female BALB/c mice received i.v. doses of BiLu in a bolus application of 10 ml/kg at doses of 0, 30, 100 or 300 µg/kg. Blood samples (0.2 ml) were taken from all mice before administration of BiLu and a single sample was taken from each mouse at 4, 8, 16, 24, 48, 72, 96 or 120 h after the last application. CD3+, CD4+, CD8+ and CD45+ leukocytes were measured by flow cytometry. Data are shown as mean values from 6 animals per time point (48 animals at time 0)

Table 1 Effect of catumaxomab on CD69 expression on human T cells

Catumaxomab (ng/ml)	CD69 expression (% positive T cells) Mean±SE (n)
0	7.1±2.9 (10)
2	23.4±9.0 (7)
20	34.3±7.5 (10)
200	34.4±14.5 (3)

Human PBMC were incubated with catumaxomab for 24 h and CD69 expression was determined by flow cytometry
n, number of independent donors; SE, standard error

CD8+/CD3+ cells in animals treated with 100 or 300 µg/kg BiLu (to a minimum of 13% and 9%, respectively) that persisted for 24 h, after which the levels rebounded to values greater than baseline (to 38% and 46%, respectively) before recovering to baseline values by 48 h.

Overall, treatment of mice with BiLu resulted in only a transient decrease in CD3+ T cells (both CD4+ and CD8+) that returned to the normal range, mostly within 48 h.

Effects of catumaxomab on T cell activation and cytokine secretion *in vitro*

Next, we addressed the effect of catumaxomab on human T cell activation and cytokine secretion. Table 1 shows that incubation of PBMC for 24 h with 2, 20 and 200 ng/ml catumaxomab increased expression of the activation marker CD69 on T cells. Figure 2 shows that incubation of PBMC with tumour target cells (EpCAM-positive HCT-8 cells) and catumaxomab (0.1 to 20 ng/ml) resulted in a concentration-dependent increase in secretion of both TNFα (Fig. 2A) and IFNγ (Fig. 2B).

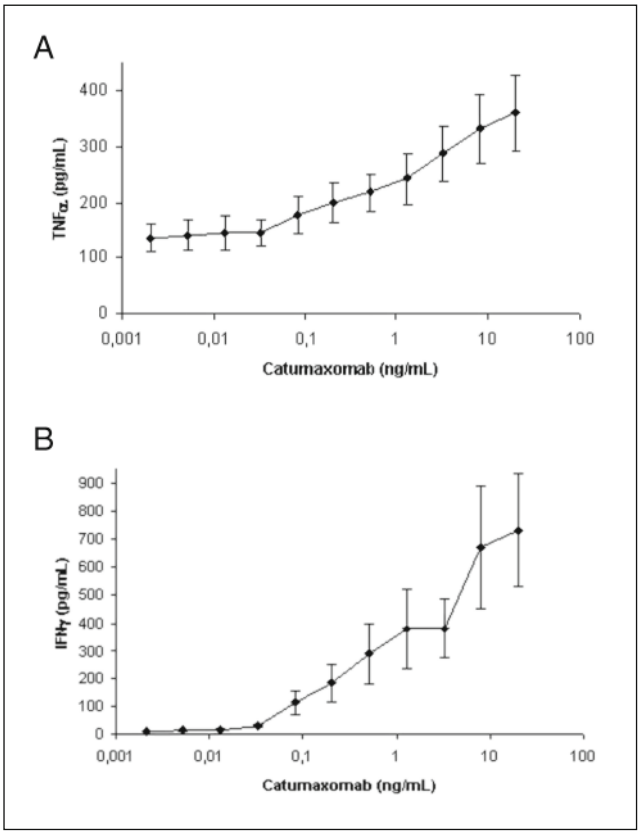

Fig. 2 Effect of catumaxomab on cytokine secretion from PBMC *in vitro*. **A** TNFα secretion; **B** IFNγ secretion. PBMC were co-cultured with EpCAM-positive HCT-8 tumour cells for 20 h at 37°C. Cell culture supernatants were collected and analysed for catumaxomab-induced TNFα and IFNγ release using a cytometric bead array. The limit of quantification was 20 pg/ml. Both cytokines were measured in the same samples. Data are shown as mean values±standard error from 12 replicates

Effect of TNFα on endothelial cell activation *in vitro*

Then, the effect of TNFα on HUVEC activation was investigated. Table 2 shows that incubation of HUVEC for 24 h with 25 ng/ml TNFα increased expression of the adhesion molecules CD54 and CD62E on HUVEC. Although there were variations between donors, the number of positive cells and the expression level (mean fluorescence intensity, MFI) of these adhesion markers markedly increased compared to control without TNFα.

Effect of catumaxomab on T cell adhesion to endothelial cells *in vitro*

Figure 3 shows that activation of T cells by catumaxomab (2–200 ng/ml) resulted in a concentration-depen-

Table 2 Effect of TNFα on expression of activation markers on human umbilical vein endothelial cells

TNFα (ng/ml)	CD62E		CD54	
	% positive cells	MFI	% positive cells	MFI
0	7.0±6.1	27±11	49.9±11.3	288±106
25	38.2±9.7	130±49	75.5±9.0	4138±1385

HUVEC were incubated with TNFα for 24 h and adhesion molecule expression was determined by flow cytometry. Data are shown as mean±standard error from 6 independent donors
MFI, mean fluorescence intensity

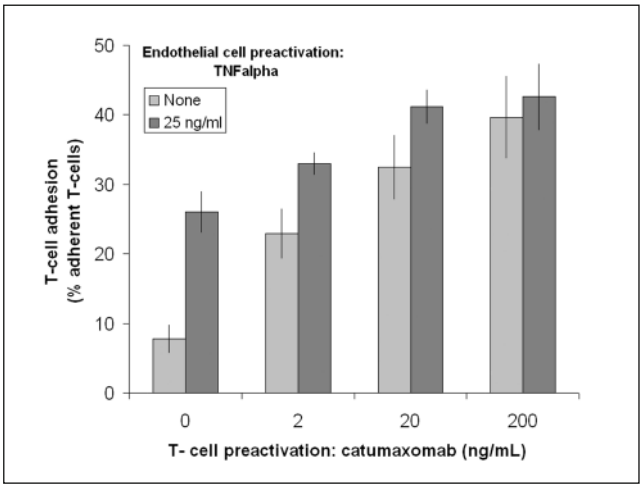

Fig. 3 Effect of catumaxomab and TNFα on adhesion of human T cells to human umbilical vein endothelial (HUVEC) cells. PBMC prestimulated with catumaxomab (0–200 ng/ml) were incubated with HUVEC prestimulated with TNFα for 2 h at 37°C, and adherent T cells were determined by flow cytometry. Data are shown as mean±standard error from 12 (0 and 20 ng/ml), 9 (2 ng/ml) or 3 (200 ng/ml) independent experiments with different donors

dent increase in T cell adhesion to endothelial cells. This catumaxomab-induced adhesion of T cells was seen in the presence or absence of endothelial cell preactivation by TNFα. Preactivation of endothelial cells with TNFα induced increased T cell adhesion in the absence of catumaxomab, and adhesion was further increased in a concentration-dependent manner when T cells were activated with catumaxomab (2 and 20 ng/ml). No clear differences in the catumaxomab-induced adhesion between samples with or without TNFα preactivation were detected at 200 ng/ml.

Discussion

We have shown that treatment of mice with the trifunctional antibody BiLu (anti-human EpCAM x anti-mouse CD3) resulted in a dose-dependent transient decrease in CD3+ T cells (both CD4+ and CD8+) that returned to the normal range, mostly within 48 h. These results are consistent with findings of a transient decrease in lymphocyte counts after infusion of catumaxomab (anti-human EpCAM x anti-human CD3) in patients [1, 2, 6]. The decrease observed in patients was transient, returning to normal within 2–3 days, mild to moderate in intensity and in general fully reversible. Furthermore, there was no increase in the number or nature of infections.

The short recovery interval argues against an antibody-mediated destruction of lymphocytes. We, therefore, conducted *in vitro* studies to investigate the potential mechanism behind this adhesion induced by catumaxomab.

We showed that catumaxomab physiologically activates T cells (increased expression of CD69) and stimulates the release of cytokines, including TNFα and IFNγ, from human PBMC *in vitro*. T cell activation was observed starting at the lowest concentration of catumaxomab studied (2 ng/ml) and an increase in cytokine release was observed at catumaxomab *in vitro* concentrations from 0.1 ng/ml. This is in accordance with previous *in vitro* studies which underline that catumaxomab activates PBMC and stimulates the release of cytokines, including TNFα and IFNγ, from human blood cells in the presence of target tumour cells [12–15] as part of its mode of action. Release of TNFα and IFNγ peaked at 24 h [14]. Furthermore, a clinical study has shown that catumaxomab increases serum TNFα in patients with malignant ascites [6].

Pharmacokinetic data demonstrate systemic availability of catumaxomab after intraperitoneal administration with a median C_{max} of approximately 0.4 ng/ml in plasma. Thus, even this low systemic exposure would be sufficient to activate PBMC and increase levels of TNFα, which provides an explanation for the increased levels of serum TNFα observed in catumaxomab-treated patients [6].

We showed that exogenous TNFα increases expression of adhesion molecules, ICAM-1 (CD54) and E-selectin (CD62E) on endothelial cells. Since catumaxomab-activated PBMC produce TNFα, this observation is consistent with the hypothesis that cytokines (e.g., TNFα) released by immune cells due to anti-CD3-antibody binding can activate endothelial cells, resulting in increased expression of adhesion molecules on the endothelial cell surface.

We further showed that catumaxomab dose-dependently increases adhesion of T cells to endothelial cells and that adhesion is further increased when endothelial cells are preactivated with TNFα.

These results are supported by previous studies with other bispecific anti-CD3 antibodies: *In vitro* studies with a related trifunctional antibody (BiUII) showed that antibody-stimulated production of TNFα by PBMC depends on the presence of target tumour cells, with approximately four times more TNFα produced by PBMC in the presence of target cells, which contributes to the antitumour activity of the antibody [16]. Administration of an EpCAM/CD3-bispecific BITE antibody to mice resulted in a transient increase in serum levels of TNFα and IFNγ [17]. Another bispecific anti-CD3 antibody also caused a rapid increase in plasma levels of TNFα and IFNγ in carcinoma patients [18, 19].

Thus, the transient decrease in lymphocytes observed with CD3-binding antibodies is interpreted as an antibody-induced redistribution phenomenon as a result of adhesion to the endothelial cells and migration from the blood stream into the tissues, and possibly into the tumour.

Peripheral blood lymphocytes isolated from patients after administration of monospecific anti-CD3 antibodies had increased expression of T cell adhesion molecules (including LFA-1 [CD11a/CD18]) and increased adhesion to endothelial cells *in vitro* [20]. The *in vitro* adhesion of T cells to endothelial cells was mediated by T cell LFA-1 binding to endothelial cell ICAM-1, and was blocked by anti-LFA-1 antibodies.

An *in vitro* study showed that preincubation of PBMC with a bispecific antibody increased adhesion to endothelial cells followed by increased trans-endothelial migration [21]. The antibody increased expression of lymphocyte adhesion molecules, including VLA-4 and high affinity LFA-1. Furthermore, the antibody-activated PBMC induced expression of endothelial cell adhesion molecules (E-selectin, VCAM-1 and ICAM-1) during adhesion and trans-endothelial migration. The authors showed that these phenomena were related to the CD3-recognising antibody fragment of the antibody and were dependent on lymphocyte–endothelial cell contact.

Decreases in lymphocyte counts in patients were also reported following administration of other anti-CD3 antibodies. Administration of a monospecific anti-CD3 antibody or polyclonal anti-T cell antibody to patients after renal transplantation resulted in transient decreases in CD3+ lymphocyte counts [22]. A further study also showed that administration of a monospecific anti-CD3 antibody to patients after renal transplantation resulted in decreases in lymphocyte counts [20]. Administration of the bispecific antibody BIS-1 (anti-CD3 x anti-EGP-2) to renal cell carcinoma patients caused a rapid decrease in circulating mononuclear cells in the peripheral blood [18, 19]. The effect was dose-dependent and there was a preferential decrease in LFA-1+CD3+/CD8+ lymphocytes [18].

Overall, our results, supported by previous studies, demonstrate mechanisms that are involved in anti-CD3 antibody-induced migration of lymphocytes into the tissue. T cells that become activated by anti-CD3 antibody binding and cytokines (e.g., TNFα) released by immune cells due to anti-CD3 antibody binding activate endothelial cells resulting in increased expression of adhesion molecules on the endothelial cell surface. Both mechanisms are likely to contribute to the initiation of the adhesion cascade, involving tethering, rolling and firm adhesion of T cells to endothelial cells; a prerequisite for migration into tissues [7–9].

In conclusion, catumaxomab can increase adhesion of T cells to endothelial cells, which is due to antibody-mediated activation of T cells and antibody-mediated production of cytokines that up-regulate endothelial cell adhesion molecules. These results provide a rationale for the transient, reversible decrease in lymphocyte counts following catumaxomab administration in patients, which is likely due to redistribution of lymphocytes and not due to damage of circulating lymphocytes or stem cells.

Conflict of interest Kirsten Dettmar, Isabell Seitz-Merwald, Diane Seimetz and Judith Atz are full-time employees of Fresenius Biotech GmbH. Carsten Lindemann and Petra Schroeder are full-time employees of EUFETS GmbH.

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