Gut Online First, published on September 27, 2005 as 10.1136/gut.2005.076208

A recombinant anti-CEA immunoreceptor with combined CD3ζ - CD28 signalling targets T cells from colorectal cancer patients against their tumor cells.

Andreas Hombach[1*], Claudia Schlimper[2*], Elisabeth Sievers[3], Susanne Frank[3], Hans H Schild[4], Tilman Sauerbruch[3], Ingo G H Schmidt-Wolf[3,5], Hinrich Abken[1]

[1] Tumorgenetik, Klinik I für Innere Medizin, and Zentrum für Molekulare Medizin Köln (ZMMK), Klinikum der Universität zu Köln, Köln, Germany
[2] Neurochirurgische Klinik, Rheinische Friedrich-Wilhelms-Wilhelms-Universität, Bonn, Germany
[3] Medizinische Universitätsklinik und Poliklinik I, Rheinische Friedrich-Wilhelms-Universität, Bonn, Germany
[4] Radiologische Klinik und Poliklinik, Rheinische Friedrich-Wilhelms-Universität, Bonn, Germany
[5] present address: Rebecca and John Moores Comprehensive Cancer Center, UCSD, La Jolla, U.S.A.

[*]Both authors contibuted equally

Address for correspondence:
Prof Hinrich Abken, Tumorgenetik, Klinik I für Innere Medizin, Klinikum der Universität zu Köln, D-50931 Köln, Germany, email: hinrich.abken@uk-koeln.de

key words:
immunotherapy, recombinant T cell receptor, CD28 costimulation, CEA

abbreviations:
CEA, carcinoembryonic antigen; scFv, single-chain antibody fragment; mAb, monoclonal antibody; TCR, T cell receptor; XTT, 2,3-bis(2-methoxy-4-nitro-5sulphonyl)-5[(phenyl-amino)carbonyl]-2H-tetrazolium hydroxide.

Abstract

Background and aims:
The prognosis of metastatic colorectal cancer is still poor rising the need for alternative therapeutic approaches, particularily by manipulating the anti-tumor immune response. Advanced tumor stages, however, are frequently accompanied by functional T cell defects which may be critical for a T cell based anti-cancer immunotherapy. The aim of this study was to address whether T cells from colorectal cancer patients with advanced tumor stages can be antigen-specifically activated against their autologous tumor cells.

Methods:
T cells were isolated from colorectal cancer patients and retrovirally transduced to express a recombinant immunoreceptor that has an extracellular binding domain for carcinoembryonic antigen (CEA) and an intracellular CD3ζ signalling domain with and without CD28 costimulation for T cell activation.

Results:
Peripheral blood T cells from colorectal cancer patients were successfully engineered to express the anti-CEA immunoreceptor on the cell surface. Upon coincubation with autologous CEA^+ tumor cells, T cells with anti-CEA immunoreceptor are specifically activated to secrete IFN-γ and to lyse the autologous tumor cells whereas T cells without immunoreceptor are not. T cells equipped with combined CD3ζ - CD28 signalling receptor are more efficiently activated to secrete IFN-γ compared to T cells with CD3ζ signalling receptor. Induction of IL2 secretion upon targeting towards autologous tumor cells requires triggering of T cells by the CD3ζ-CD28 costimulatory receptor.

Conclusions:
T cells from advanced colorectal cancer patients can be tumor-specifically activated with high efficiency by engraftment with a combined CD3ζ-CD28 immunoreceptor to break tolerance against autologous tumor cells.

Introduction

Despite continued advances in systemic and local treatment, colorectal cancer is still one of the common causes of cancer death in the western world without a significant improvement of the overall 5-year survival[1]. Many therapeutic options for advanced and metastatic colorectal cancer have evolved, most of them rely on adjuvant systemic chemotherapeutic regimens and surgery of hepatic metastases. Whereas only 5-10% of patients have operable disease[2], alternative therapeutic approaches are currently investigated including gene therapy[3] and immune therapeutic strategies[4]. The latter include the genetic manipulation of cells of the immune system thereby aiming to use the power and the specificity of the cellular immune response for the targeted elimination of cancer cells. In order to target T cells antigen-specifically towards tumor cells, strategies were developed to equip T cells with a recombinant T cell receptor of defined specificity. In particular, the immunoreceptor strategy is based on grafting immunological effector cells with a recombinant receptor molecule, i.e., immunoreceptor, that has a single-chain antibody fragment (scFv) derived domain for antigen binding in its extracellular moiety and the CD3 ζ signalling chain in its intracellular moiety for cellular activation[5]. Binding of the immunoreceptor to antigen causes crosslinking of the receptor molecules and CD3ζ mediated downstream signaling resulting in T cell activation with proliferation, induction of cytokine secretion and cytolysis of the target cell. Work from our laboratories[6, 8, 9], along that from others[10-13], have demonstrated that recombinant immunoreceptors can be engineered to activate cytotoxic T cells antigen-specifically towards colorectal cancer cells by targeting carcinoembryonic antigen (CEA), CA72-4 (TAG72) and CA19-9, respectively, resulting in tumor cell lysis.

CEA (CD66e) is a 180 kD glycoprotein and the name-giving member of the CEA family which belongs to the large immunoglobulin superfamily. CEA is physiologically expressed on the luminal surface of epithelial cells of the gastrointestinal tract and of respiratory organs and, moreover, on a variety of adenocarcinomas such as of colon, rectum, pancreas, gastric, breast and other organs. CEA is therefore well-established as a tumor and serum marker for adenocarcinomas[14]. In immunotherapy, different strategies have been developed to use CEA as vaccine, e.g., as recombinant CEA protein, CEA anti-idiotypic antibodies, and dendritic cells pulsed with agonist CEA epitopes, to induce a CEA-specific immune response[15]. The presence of CEA on the tumor cell surface is utilized to target cytotoxic drugs or radioisotopes coupled to CEA-specific antibodies to the tumor[16]. Indirect technologies aim to use CEA as a target antigen for cytotoxic T cells, e.g., by presenting CEA peptides bound to MHC class I alleles[17] or by CEAxCD3 bispecific antibodies[18].

A number of studies indicate, however, that T cells from tumor-bearing mice[19] and from tumor patients[20] are not functional with respect to T cell receptor (TCR) signalling due to altered expression of proteins of the CD3 complex. T cells infiltrating hepatic metastases of colorectal cancer have reduced CD3ζ chain expression and lack tumor-specific activity in vitro[21]. In view of the immunoreceptor concept, the question arises whether T cells from colorectal cancer patients can be specifically activated by receptor signalling upon binding to tumor cells. Whereas previous studies demonstrated targeting of T cells from healthy donors towards tumor cells of established cell lines, we here asked whether primary T cells from the peripheral blood of colorectal cancer patients in advanced stages can be antigen-specifically activated by signalling through the recombinant immunoreceptor and whether these cells are activated upon binding to autologous tumor cells derived from surgery specimens from the same patient. The study thereby addresses the question whether engraftment with an immunoreceptor is capable to break antigen-specifically T cell tolerance against autologous colorectal cancer cells.

Materials and Methods

Cell lines and reagents.
293T cells are human embryonal kidney cells that express the SV40 large T antigen (Weijtens et al. 1998). LS174T is a CEA+ colorectal carcinoma line (ATCC, CL-188), Colo201 cells (ATCC CCL 224) were derived from an adenocarcinoma. OKT3 (ATCC CRL 8001) is a hybridoma cell line that produces the anti-CD3 monoclonal antibody (mAb) OKT3. 293T cells were cultured in DMEM medium supplemented with 10% (v/v) FCS, all other cell lines were cultured in RPMI 1640 medium, 10% (v/v) FCS (all Invitrogen Life Technologies, Paisly, U.K.). OKT3 mAb was affinity purified from hybridoma supernatants utilizing goat anti-mouse IgG2a antibodies (Southern Biotechnology, Birmingham, AL, USA) that were immobilized on N-hydroxy-succinimid-ester-activated sepharose as recommended by the manufacturer (Amersham Biosciences, Freiburg, Germany). Human IgG1 antibodies and the phycoerythrin-(PE)-conjugated anti-CD3 mAb UCHT1 was purchased from Dako, Hamburg, Germany. The goat anti-human IgG antibody and its FITC- and PE-conjugated $F(ab')_2$ derivatives were purchased from Southern Biotechnology. The anti-human IFN-γ mAb NIB42 and the biotinylated anti-human IFN-γ mAb 4S.B3 were purchased from BD Bioscience, San Diego, CA, USA.

Isolation of tumor cells.
Primary carcinoma cell cultures were established from colon carcinoma specimens by the single-cell isolation procedure as described[22]. In brief, biopsy fragments were incubated in HBSS buffer containing 100 U/ml DNase I (Roche, Mannheim, Germany), 50 U/ml collagenase III (Biochrome, Berlin, Germany), 150 U/ml hyaluronidase (Sigma, Deisenhofen, Germany) and 0,08 U/ml insulin (Hoechst, Bad Soden, Germany) at 37° C for 15 min while shaking. Cells in the supernatant were collected by centrifugation for 5 min at 400 x g, resuspended in 10 ml erythrocyte-lysation buffer (8,29 g/l NH_4Cl, 1 g/l $KHCO_3$, 0,0371 g/l EDTA in Aqua dest., all from Sigma) and incubated at room temperature for 15 min. The erythrocyte depletion step was performed three times. Cells were washed and cultured at a density of 10^6-10^7 cells per ml in Leibovitz medium containing 10% (v/v) FCS, 1 mM L-glutamine, 1x MEM vitamins, 2,5 mg/ml transferrin, 1 g/l sodium bicarbonate, 1 g/l glucose, 80 U/ml insulin, 10 mg/ml gentamycine (all from Invitrogen) giving rise to a homogenous and adherently growing cell culture. Cultures contaminated with fibroblasts were discarded. Tumor cell cultures were checked for mycoplasma contamination and found to be free.

Immunocytochemical analysis.
Cells grown in vitro were monitored for CEA expression by immunohistochemical analysis. Briefly, cells were spun onto microscope slides and fixed with icecold actone for 10 min. Non-specific binding was blocked by 10% (v/v) FCS in PBS for 30 min. The slides were incubated for 60 min. with either an anti-CEA mAb (1C3, Abcam, Cambridge, MA) or an isotype control mAb (BD Bioscienc, San Jose, CA) (10 µg/ml). Bound antibodies were detected by a peroxidase conjugated Fab anti-mouse Ab (1:50) (Roche Diagnostics) and visualized by 3-Amino-9-ethylcarbazole (AEC) (Sigma).

Recombinant immunoreceptors and FACS analysis.
The generation of the expression cassettes for the CEA-specific immunoreceptors BW431/26-scFv-Fc-ζ and BW431/26-scFv-Fc-CD28-ζ (cf. Figs. 1A, 6A) was previously described. Retroviral transduction of T cells with the pBullet vector was described in detail elsewhere[6, 7]. Recombinant receptor grafted T cells were identified by two colour immunofluorescence utilizing a PE- or FITC-conjugated F(ab')2 anti-human IgG1 antibody (1 µg/ml) and a FITC- or PE-conjugated anti-CD3 mAb (UCHT-1, 1:20). Immunofluorescence was analysed using a

FACScan™ cytofluorometer equipped with the CellQuest research software (Becton Dickinson, Mountain View, CA, USA). To identify T cells with recombinant receptor expression, we set markers with 99% of non-transduced T cells beyond.

Receptor mediated activation of grafted T cells.
T cells were grafted with recombinant anti-CEA immunoreceptors and cocultivated in round bottom 96-wellmicrotiter plates (1.25-10 x 10^4 grafted T cells/well) with CEA+ and CEA- tumor cells (5 x 10^4 cells/well), respectively. After 48 hrs, culture supernatants were analysed by ELISA for IFN-γ and IL-2. Briefly, IFN-γ was bound to the solid phase anti-human IFN-γ mAb NIB42 (1 µg/ml) and detected by the biotinylated anti-human IFN-γ mAb 4S.B3 (0.5 µg/ml). IL-2 was bound by a solid phase anti-IL-2 antibody (1:250) and detected by a biotinylated anti-human IL-2 antibody (1:250) (both BD Bioscience). The reaction product was visualized by a peroxidase-streptavidin-conjugate (1:10,000) and ABTS® (both Roche Diagnostics).
Specific cytotoxicity of receptor grafted T cells against target cells was monitored by a XTT based colorimetric assay[23]. Briefly, XTT (2,3-bis(2-methoxy-4-nitro-5sulphonyl)-5[(phenyl-amino)carbonyl]-2H-tetrazolium hydroxide) reagent (1 mg/ml) (Cell Proliferation Kit II, Roche Diagnostics) was added to the cells and incubated for 30-90 min at 37°C. Reduction of XTT to formazan by viable tumor cells was monitored colorimetrically at an adsorbance wavelength of 450 nm and a reference wavelength of 650 nm. Maximal reduction of XTT was determined as the mean of wells containing tumor cells only, and the background as the mean of wells containing RPMI 1640 medium, 10% (v/v) FCS. The non-specific formation of formazan due to the presence of effector cells was determined from triplicate wells containing effector cells in the same number as in the corresponding experimental wells. The number of viable tumor cells was calculated as follows:

$$\text{viability [\%]} = \frac{OD_{\text{(exp. wells – corresponding number of effector cells)}}}{OD_{\text{(tumor cells without effectors - medium)}}} \times 100$$

Results

We addressed the question whether T cells from colorectal cancer patients can be antigen-specifically activated towards their autologous tumor cells in vitro. In this study, we included three patients with advanced CEA^+ colorectal cancer and surgical tumor resection. Patient´s details are listed in Table 1.

Table 1
Patient characteristics

patient #	initials	sex	age	tumor	tumor stage
1	HW	f	73	carcinoma of the sigmoid colon	Dukes C
2	US	f	66	carcinoma of the sigmoid & rectum	Dukes A
3	BS	m	50	rectum carcinoma	Dukes D

T cells were isolated from the peripheral blood of colorectal cancer patients and of a healthy donor and retrovirally transduced in vitro to express the recombinant, CEA-specific immunoreceptor BW431/26-scFv-Fc-ζ as described in Materials and Methods. Receptor

expression on the surface of $CD3^+$ T cells was monitored by FACS analysis utilizing an anti-IgG antibody directed against the extracellular IgG1 CH2CH3 domain of the receptor. As shown in Fig. 1B, about 30-40 % of $CD3^+$ T cells from the colorectal cancer patients express the recombinant immunoreceptor on the cell surface. For comparison, about 35 % of T cells from a healthy donor express the immunoreceptor on the cell surface upon retroviral transduction. At least in these examples, T cells from colorectal cancer patients are nearly equally effiently transduced to express the immunoreceptor as are T cells from a healthy individual.

We asked whether immunoreceptor grafted T cells from colorectal cancer patients are antigen-specifically activated upon binding to CEA^+ colorectal carcinoma cells. Thus, lymphocytes from colorectal cancer patient 1 and 2, respectively, were grafted with the CEA-specific immunoreceptor BW431/26-scFv-Fc-ζ and cocultured with cells of the CEA^+ colorectal carcinoma line LS174T and, as control, with cells of the CEA^- line Colo201. With an increasing ratio of engineered T cells to tumor cells, patient lymphocytes grafted with the BW431/26-scFv-Fc-ζ receptor are induced to secrete increasing amounts of IFN-γ whereas T cells without immunoreceptor are not activated (Fig. 2C, G). Induction of IFN-γ secretion is due to interaction of CEA^+ tumor cells with anti-CEA receptor grafted T cells because neither coincubation of receptor grafted T cells with CEA-negative Colo201 cells (Fig. 2D, H) nor of lymphocytes without the BW431/26-scFv-Fc-ζ receptor (Fig. 2C, D, G, H) did induce IFN-γ secretion.

To monitor the capacity of engineered T cells from colorectal cancer patients to lyse antigen-specifically established tumor cells, we recorded the viability of tumor cells in presence of increasing numbers of engineered T cells using a XTT based colorimetric assay as described in Materials and Methods. As summarized in Fig. 2, T cells grafted with the anti-CEA immunoreceptor BW431/26-scFv-Fc-ζ decreased the viability of CEA^+ LS174T tumor cells whereas coincubation with T cells without immunoreceptor did not (Fig. 2A, E). CEA-negative Colo201 tumor cells were not lysed by T cells neither with nor without anti-CEA immunoreceptor (Fig. 2B, F). Taken together, this indicates that the cytolytic activity of receptor grafted T cells towards LS174T cells is mediated by the CEA-specific immunoreceptor.

We addressed the question whether autologous, CEA^+ tumor cells can be specifically targeted by T cells from colorectal cancer patients. We therefore isolated tumor cells from colorectal carcinoma specimens obtained by surgery from the same patients 1 and 2. During short-term cultivation in vitro, the majority of colorectal carcinoma cells from both patients continue to express CEA in high densities on the cell surface (Fig. 3). For comparison, established SW948 colorectal carcinoma cells express CEA in lower levels and Colo201 cells do not express detectable amounts of CEA.

To test whether immunoreceptor grafted T cells from colorectal cancer patients are CEA-specifically activated by their autologous tumor cells, we coincubated increasing numbers of BW431/26-scFv-Fc-ζ receptor grafted lymphocytes from patient 1 and patient 2, respectively, with the autologous, CEA^+ tumor cells. As shown in Fig. 4, T cells with BW431/26-scFv-Fc-ζ immunoreceptor are dose-dependently induced to secrete increasing amounts of IFN-γ upon coincubation with the autologous tumor cells whereas T cells from the same patients without receptor did not. Obviously, T cells from colorectal cancer patients and grafted with anti-CEA immunoreceptor are CEA-specifically activated upon binding to their autologous tumor cells. To test whether T cell activation includes induction of cytolysis, we recorded the viability of patient tumor cells upon incubation with autologous T cells equipped with or without

BW431/26-scFv-Fc-ζ receptor. As exemplarily illustrated in Fig. 5, increasing numbers of BW431/26-scFv-Fc-ζ receptor grafted T cells lyse the autologous, CEA^+ tumor cells in a dose dependent fashion whereas T cells without immunoreceptor do not. Taken together, the data demonstrate that patient T cells with the BW431/26-scFv-Fc-ζ immunoreceptor are activated to IFN-γ secretion and cytolysis upon binding to autologous, CEA^+ tumor cells. This moreover indicates that engraftment with the CEA-specific immunoreceptor breaks T cell tolerance of colorectal cancer patients towards their autologous, CEA^+ tumor cells.

We now asked whether activation of T cells from colorectal cancer patients can be increased by CD28 costimulation simultaneously to CD3ζ signalling by the immunoreceptor. We therefore grafted by retroviral gene transfer T cells from patient 3 with the BW431/26-scFv-Fc-ζ receptor and the BW431/26-scFv-Fc-CD28-ζ receptor, respectively, that harbors the intracellular CD28 signalling domain in addition to the CD3ζ domain (Fig. 6A, B). Coincubation of receptor receptor grafted T cells with CEA^+ LS174T tumor cells specifically induced IFN-γ and IL-2 secretion (Fig. 7). Cyokine secretion, however, was significantly increased upon T cell stimulation via the BW431/26-scFv-Fc-CD28-ζ receptor with combined CD28 and CD3ζ signalling domains compared to stimulation via the CD3ζ signalling receptor without CD28 costimulatory domain (cf. Fig. 7 C, D). T cell activation is CEA-specific because coincubation with CEA-negative Colo201 tumor cells did not substantially induce IFN-γ and IL-2 secretion (Fig. 7 E, F). To address whether receptor-mediated CD28 costimulation increases T cell activation towards autologous tumor cells as well, we coincubated primary autologous, CEA^+ tumor cells together with receptor grafted T cells from the same patient. As shown in Fig. 7A, B, T cells grafted with the BW431/26-scFv-Fc-CD28-ζ receptor secrete substantially more IFN-γ and IL-2 than T cells grafted with the BW431/26-scFv-Fc-ζ receptor with CD3ζ signalling domain only. Moreover, receptor triggered CD28 costimulation simultaneously CD3ζ signalling is required to induce high IL-2 secretion of grafted T cells (Fig. 7B). This set of analyses demonstrates that receptor-mediated activation of T cells from colorectal cancer patients towards autologous tumor cells can be significantly increased by signalling through the combined C3ζ-CD28 costimulatory receptor.

Discussion

Previous studies from our laboratory and others demonstrated that T cells can be grafted with specificity for CEA by expression of a recombinant immunoreceptor on the cell surface[6, 10-12, 24]. The recombinant BW431/26-scFv-Fc-ζ receptor used in this study harbors the BW431/26 scFv domain in the extracellular moiety for CEA binding and the intracellular CD3ζ signalling domain for T cell activation. The vast majority of studies so far have used T cells from healthy donors to demonstrate the feasibility of receptor-mediated redirecting of T cells. In contrast to T cells from healthy donors, T lymphocytes from cancer patients were reported to be defective in the T cell activation machinery[20, 21, 25]. For clinical application of the immunoreceptor concept, however, it is crucial whether patient T cells can be effectively activated resulting in efficient redirecting towards autologous tumor cells. Data presented here, however, demonstrate that the conditions used to transduce by retroviral gene transfer T cells from patients with advanced colorectal cancer, at least in these examples, are nearly equally effective as for T cells from healthy donors (cf. Fig. 1). Upon binding to antigen, the BW431/26-scFv-Fc-ζ receptor drives activation of patient T cells similarly effective as of healthy donor T cells with respect to IFN-γ secretion and lysis of CEA^+ LS174T tumor cells (cf. Fig. 2). Receptor driven T cell activation is antigen-specific because CEA-negative Colo201 cells are not lysed in presence of receptor-grafted T cells and IFN-γ secretion by grafted T cells is not induced. This exemplarily demonstrates that T cells from colorectal

cancer patients can be activated against CEA^+ tumor cells by grafting with the appropriate immunoreceptor.

Our study moreover aims to elucidate whether patient T cells can be redirected towards autologous colon carcinoma cells. The tumor cells we isolated from surgical specimens and cultured in vitro for short time periods continued to express CEA on their cell surface as shown by immunohistochemical techniques. T cells isolated from the peripheral blood of these patients and grafted with the BW431/26-scFv-Fc-ζ receptor were efficently activated against the autologous tumor cells in vitro indicated by increase in IFN-γ secretion and cytolysis of tumor cells (cf. Fig. 4). Tumor cell lysis by receptor grafted T cells, in contrast, is only slightly greater than mediated by non-modified lymphocytes (cf. Fig. 5) which may be due to the heterogenous expression of CEA on the tumor cell surface and to tumor cells that lost CEA expression. Natural heterogeneity and particularly loss of CEA expression, however, is a significant limitation of the immunoreceptor strategy that is based on antigen directed target recognition. This limitation may be overcome by targeting more than one antigen on the tumor cell surface or by recruiting bystander T or NK cells via secreted cytokines.

T cell activation is more efficient when T cells were grafted with the BW431/26-scFv-Fc-CD28-ζ receptor with the costimulatory CD28 domain in addition to the CD3ζ domain compared to the BW431/26-scFv-Fc-ζ receptor without CD28 domain (cf. Fig. 7). The transduction efficiencies and the densities of the receptors on the T cell surface, however, were nearly similar. IL-2 secretion by patient T cells, moreover, is significantly induced by the the BW431/26-scFv-Fc-CD28-ζ costimulatory receptor in contrast to the BW431/26-scFv-Fc-ζ receptor as previously shown in allogeneic systems[26]. Enhanced IL-2 secretion triggered by the costimulatory receptor will furthermore result in improved recruiting of cytolytic bystander cells to the tumor site. Taken together, the study here exemplarily illustrates that tolerance of peripheral blood T cells from colorectal cancer patients towards autologous tumor cells can be antigen-specifically overcome by grafting with a CEA-specific immunoreceptor that drives T cell activation upon binding to CEA.

Soluble antigen in the serum of cancer patients, particularly in advanced stages, may prevent receptor-mediated activation of grafted T cells by blocking the recombinant immunoreceptor. In the case of the the BW431/26-scFv-Fc-ζ receptor we previously demonstrated[27] that presence of soluble CEA in concentrations up to 20 μg/ml does not block receptor-mediated T cell activation and does not inhibit induction of cytolytic activities. This suggests that high-serum CEA levels will not interfere with the activity of the anti-CEA immunoreceptor in colorectal cancer patients. Our data on BW431/26-scFv-Fc-ζ receptor driven T cell activation are in line with the recombinant anti-CEA receptor MFE23.CD3ζTDGA reported by Gilham and colleagues[25].

In vitro studies performed here, although including primary tumor cell isolates and autologous T cells, do certainly not reflect in toto the natural tumor micro-environment during a T cell attack. For example, tumor cells frequently upregulate the expression of death-receptor ligands and produce a panel of cytokines, such as TGF-β or IL-10, which inhibit T cell activation. Whereas these mechanisms may be partially active in the in vitro system, tumor secreted cytokines will be significantly diluted during culture of disaggregated tumor cells and will not accumulate to those concentrations present locally in the near vicinity of the tumor cells. Most importantly, tumor cells in these assays are not embedded into their network of stromal cells protecting the tumor cells from an immunological attack and providing the arichtectural superstructure that facilitates tumor growth. For an effective anti-

tumor response, it is anticipated that the T cells need to infiltrate the stromal network and to get in close contact to the tumor cells to allow tumor cell lysis, to proliferate and to secrete cytokines that in turn may recruit and/or activate additional effector cells.

Our study here is line with previous studies from our group and others using T cells from cancer patients and autologous tumor cells[17, 28, 29]. We reported[29] that T cells from a patient with $CD30^+$ cutaneous lymphoma can be effectively redirected in vitro towards the autologous tumor cells freshly isolated from a tumor biopsy resulting in highly efficient tumor cell lysis. Sheen and colleagues[28] demonstrated the efficient transduction of T cells from colorectal cancer patients to express an anti-CEA immunoreceptor and the successfull activation of T cells against autologous tumor cells. These reports together with the data presented here clearly demonstrate that T cells from tumor patients can be genetically manipulated by grafting with a recombinant immunoreceptor to break tolerance against autologous tumor cells. The recombinant BW431/26-scFv-Fc-CD28-ζ receptor with combined CD28 and CD3ζ signalling domains used in this study substantially enhances the cellular immune response against autologous tumor cells thereby counteracting the induction of tolerance against autologous tumor cells. This represents an important step towards the development of the immunoreceptor strategy for use in the immunotherapy of malignant diseases by specific manipulation of effector cells.

Acknowledgements

Work in the Cologne laboratory is kindly supported by grants from the Deutsche Forschungsgemeinschaft, the Deutsche Krebshilfe - Mildred Scheel-Stiftung, Bonn, and the Wilhelm Sander-Stiftung, Munich.

Ethics approval and patient consent.
This study was approved by the Ethics Committee of the University of Bonn, Bonn, and written consent from the patients were obtained.

Competing interest statement.
All authors declare that they do not have any competing interests.

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Legends to Figures

Fig. 1
Expression of the recombinant, CEA specific immunoreceptor BW431/26-scFv-Fc-ζ on the surface of T cells from colorectal cancer patients and a healthy donor.
(A) Schematic diagram of the expression cassette coding for the BW431/26-scFv-Fc-ζ receptor (#439) used in this study. scFv: BW431/26 scFv; IgG1-Fc: IgG1 CH2CH3; TM: transmembrane region; CD3ζ: CD3ζ intracellular chain with the signalling domains.
(B) Lymphocytes were isolated from the peripheral blood of colorectal patient 1 and 2 and from a healthy donor (A, B, C) and transduced by retroviral gene transfer to express the BW431/26-scFv-Fc-ζ receptor (D, E, F) as described in Materials and Methods. The receptor expressed on $CD3^+$ T cells was recorded by two-color FACS analysis using the anti-CD3 antibody OKT3 and the anti-human IgG-Fc antibody that detects the extracellular IgG1 CH2CH3 spacer domain of the receptor.

Fig. 2
Antigen specific activation of patient T cells grafted with the BW431/26-scFv-Fc-ζ immunoreceptor.
Peripheral blood lymphocytes (PBL) from patient 1 and 2 were grafted with the anti-CEA immunoreceptor BW431/26-scFv-Fc-ζ (PBL-439) as shown in Fig. 1B and cocultivated for 48 hrs (1.25-10 x 10^4 receptor grafted T cells/well) with CEA^+ LS174T colorectal carcinoma and CEA^- Colo201 tumor cells (each 2.5 x 10^4 cells/well). Patient`s lymphocytes without BW431/26-scFv-Fc-ζ receptor served as control. Viability of target cells was determined colorimetrically by a tetrazolium salt based XTT assay (A, B, E, F) and the concentration of IFN-γ in the culture supernatants was recorded by ELISA (C, D, G, H) as described in Materials and Methods.

Fig. 3
Primary tumor cells isolated from colorectal cancer biopsies express CEA in vitro.
Tumor cells were isolated from tumor specimens obtained during surgery and cultured for short time periods in vitro. Expression of CEA was monitored by means of immunohistochemical procedures using the anti-CEA antibody 1C3 as described in Material and Methods. Note the natural heterogeneity of CEA expression within the tumor cell population. Monitoring of CEA on established CEA^+ LS174T colorectal cancer cells and CEA^- Colo201 cells is shown for comparison.

Fig. 4
Engineered patient´s T cells with anti-CEA receptor are specifically activated by autologous tumor cells.
Lymphocytes from the peripheral blood of colorectal cancer patients 1 and 2 were grafted with the anti-CEA immunoreceptor BW431/26-scFv-Fc-ζ (PBL-439) (cf. Fig. 1B) and 1.25-10 x 10^4 grafted cells per well were coincubated with autologous, CEA^+ primary tumor cells (5 x 10^4 cells/well) for 48 hrs. Patient´s lymphocytes (PBL) without BW431/26-scFv-Fc-ζ receptor served as controls. IFN-γ in the culture supernatant was determined by ELISA.

Fig. 5
Patient´s lymphocytes grafted with anti-CEA receptor lyse autologous CEA^+ tumor cells. Lymphocytes from the peripheral blood of patient 1 were grafted with the BW431/26-scFv-Fc-ζ receptor (PBL-439) and coincubated (1.25-10 x 10^4 cells/well) with autologous CEA^+ tumor cells (5 x 10^4 cells/well) for 48 hrs. Coincubation with patient´s lymphocytes (PBL) without BW431/26-scFv-Fc-ζ receptor served as control. Viability of tumor cells was determined colorimetrically by a tetrazolium salt based XTT assay as described in Materials and Methods.

Fig. 6
Lymphocytes from colorectal cancer patient 3 were grafted with an anti-CEA receptor with combined CD3ζ and CD28 signalling domains.
(A) Schematic diagram of the expression cassettes for the recombinant BW431/26-scFv-Fc-ζ receptor (#439) with CD3ζ signalling domain and the BW431/26-scFv-Fc-CD28-ζ.receptor (#607) with combined CD3ζ and CD28 signalling domains. scFv: BW431/26 scFv; IgG1-Fc: IgG1 CH2CH3; TM: transmembrane region; CD3ζ: CD3ζ intracellular chain; CD28: CD28 intracellular chain.
(B) Peripheral blood lymphocytes from colorectal patient 3 (a) were retrovirally transduced to express the BW431/26-scFv-Fc-ζ (b) and the BW431/26-scFv-Fc-CD28-ζ. receptor (c), respectively. FACS analysis using the anti-CD3 antibody OKT3 and the anti-human Ig-Fc antibody that detects the extracellular IgG1 CH2CH3 spacer domain of the receptors demonstrates the expression of the recombinant receptors on the surface of $CD3^+$ T cells.

Fig. 7
Enhanced activation of T cells from a colorectal cancer patient against autologous tumor cells by the combined CD3ζ-CD28 costimulatory receptor.
Lymphocytes from the peripheral blood of colorectal cancer patient 3 were grafted by retroviral gene transfer with the CEA-specific receptors BW431/26-scFv-Fc-ζ.(#439) and BW431/26-scFv-Fc-CD28-ζ (#607), respectively (cf. Fig. 6B). Receptor grafted lymphocytes (PBL-439; PBL-607) as well as non-transduced lymphocytes (PBL) from the same patient ($1x10^5$ cells/well) were coincubated for 48 hrs with autologous, primary CEA^+ colon cells (A, B), CEA^+ LS174T colorectal tumor cells (C,D), and CEA^- Colo201 (E, F) ($5x10^4$ cells/well each), respectively. IFN-γ (A, C, E) and IL-2 (B, D, F) were monitored in the culture supernatants by ELISA.

Figure 1

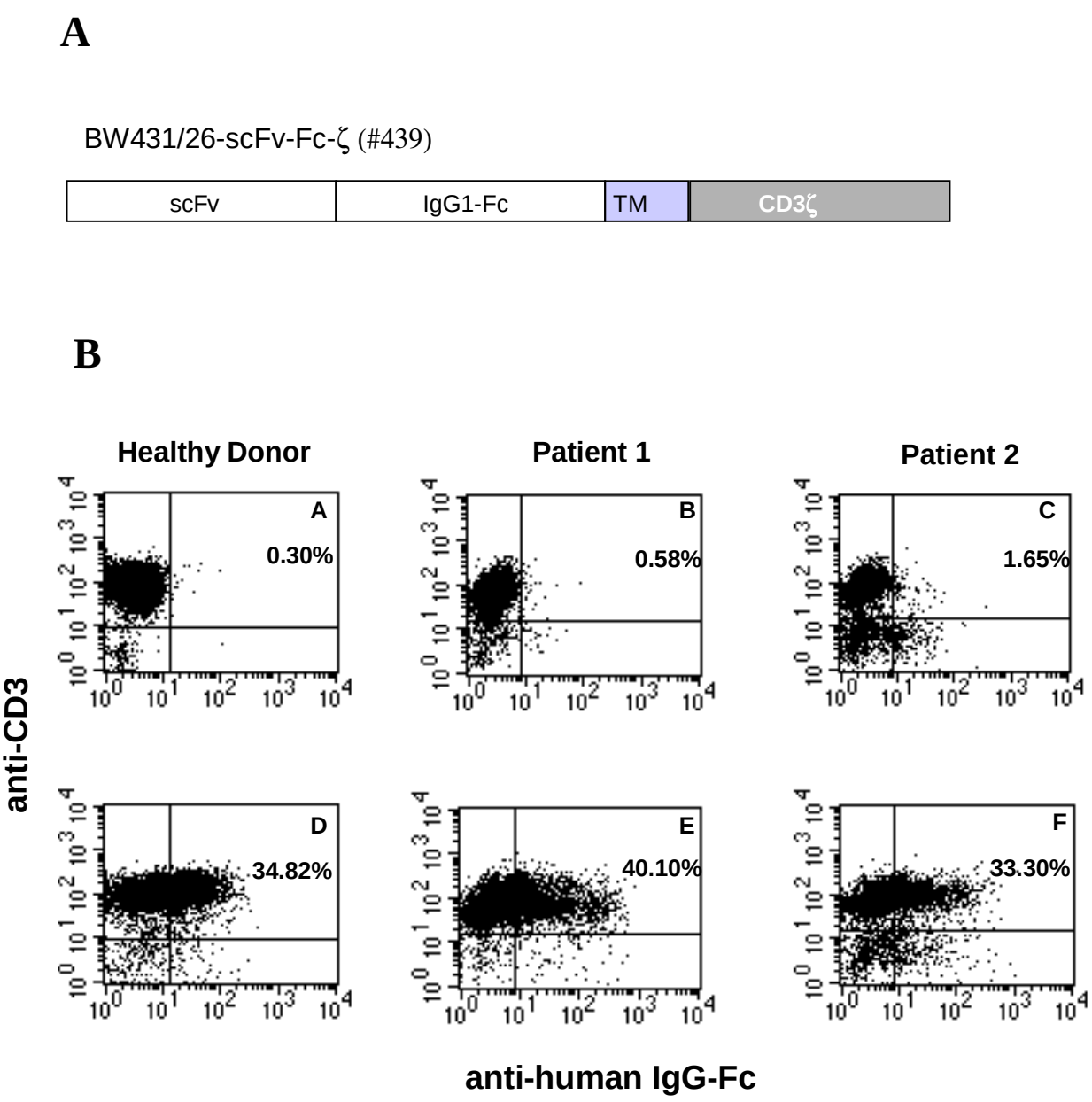

Figure 2

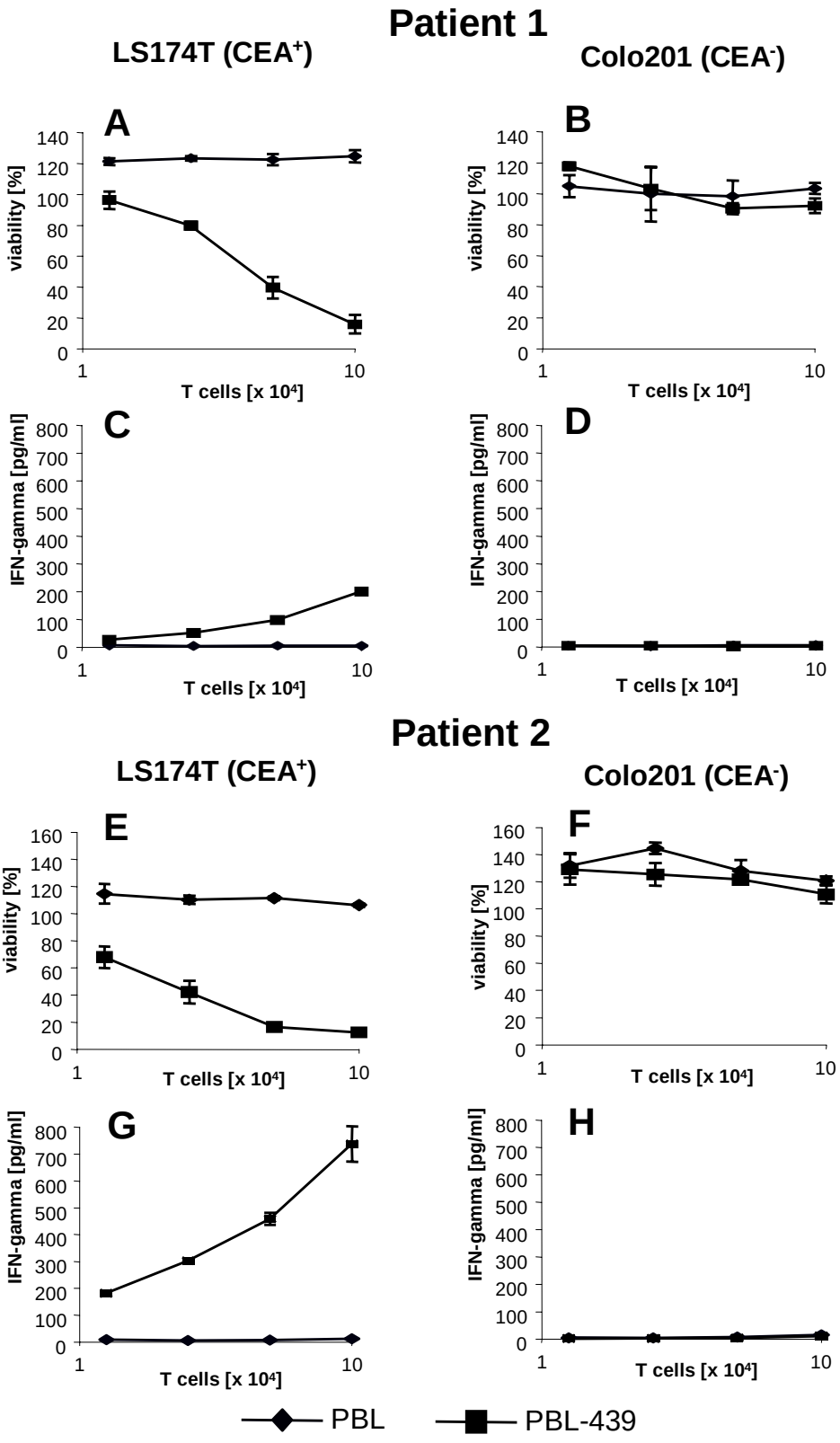

Figure 3

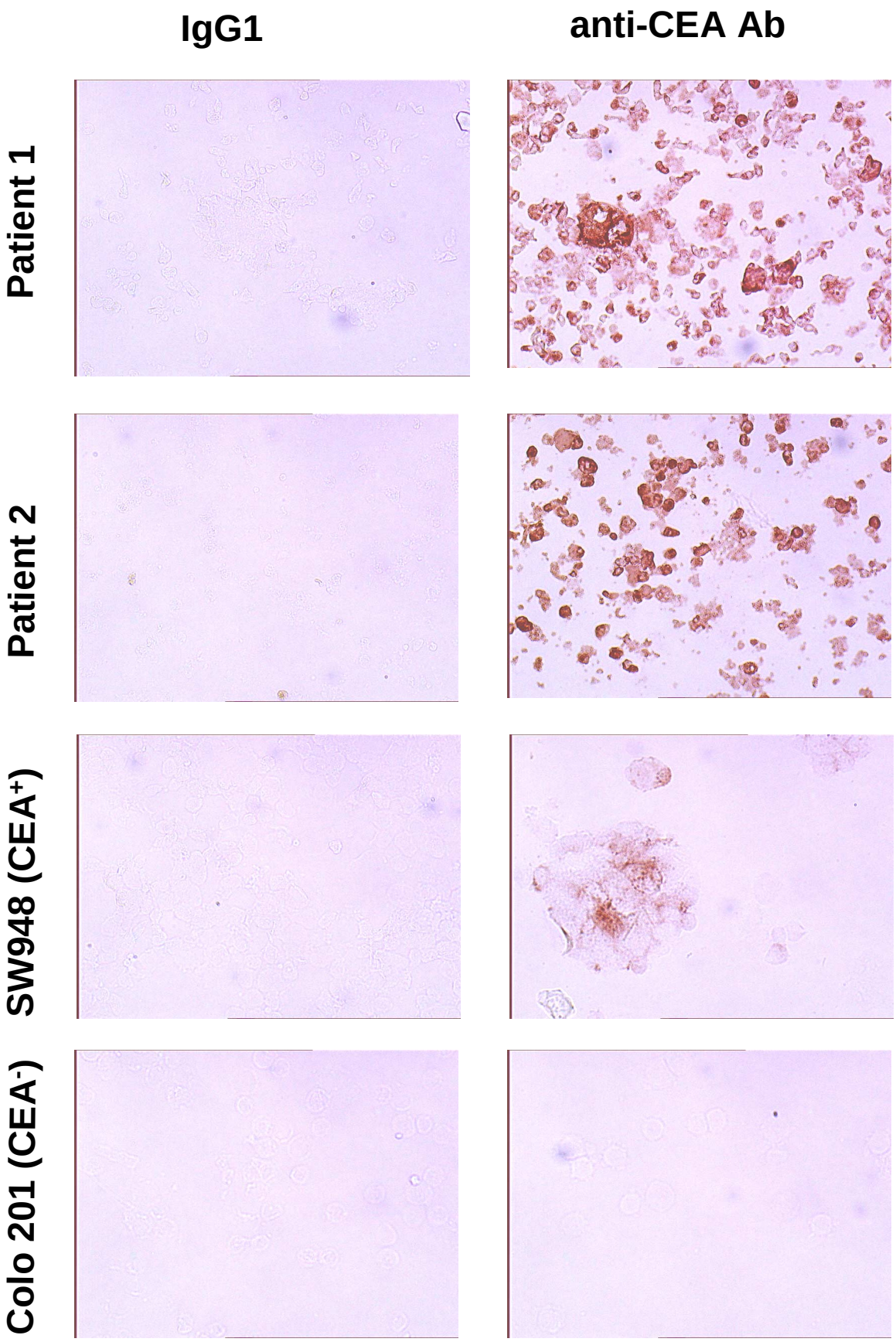

Figure 4

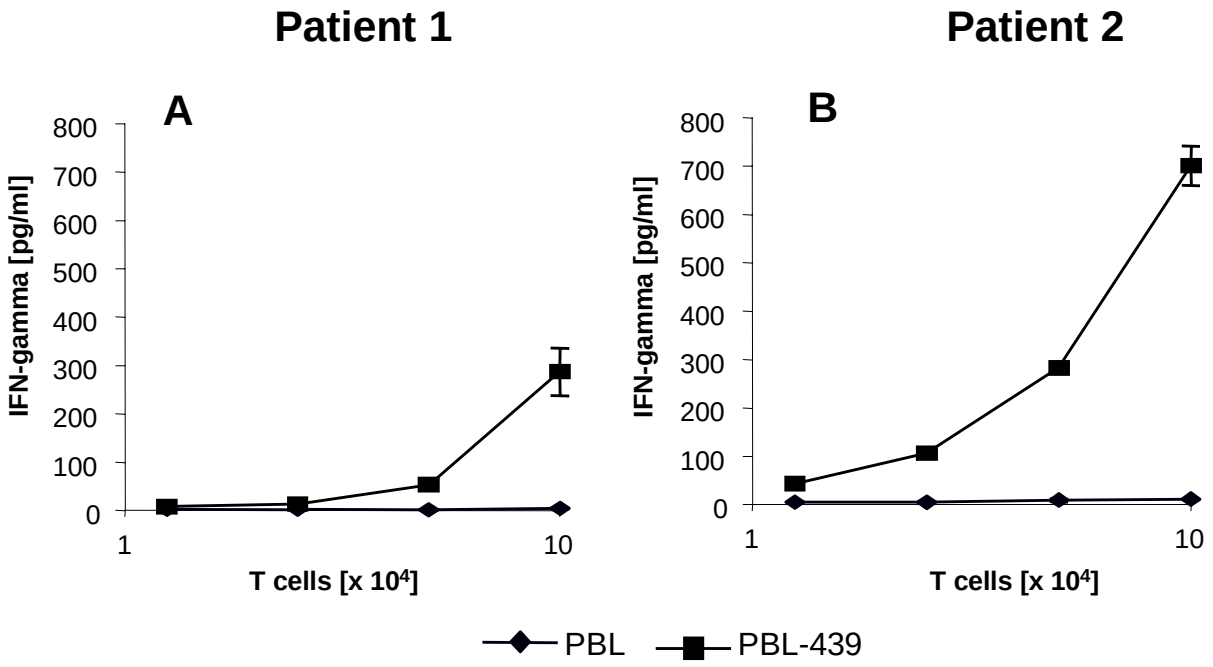

Figure 5

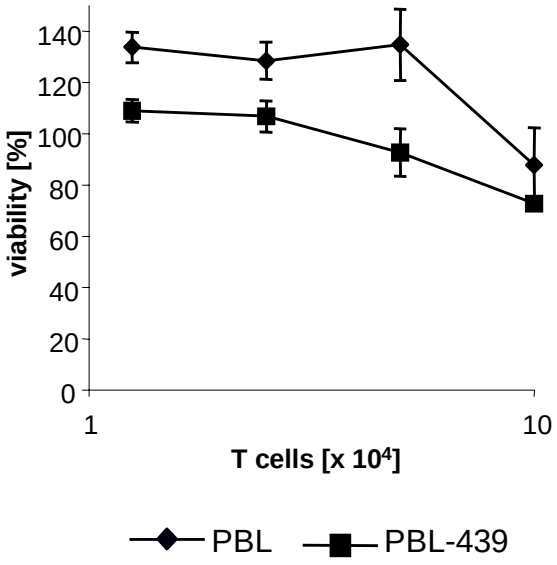

Figure 6

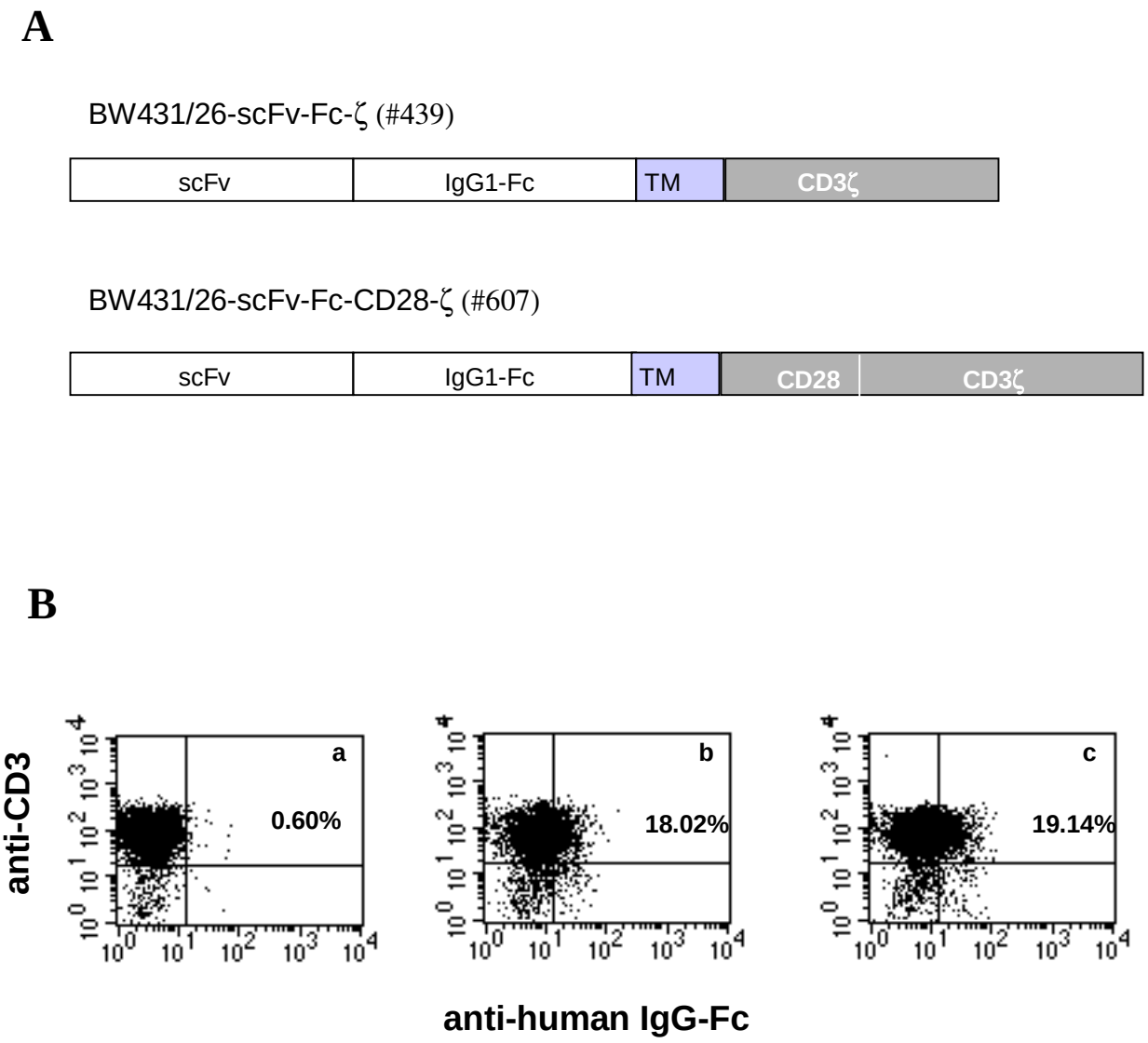

Figure 7