Supporting Information

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SI Materials and Methods

Construction, Expression, and Purification of C- and P-BiTE Antibodies. The sequence of the variable heavy- and light-chain domains of cetuximab and panitumumab were obtained from publicly available sources. After synthesis of VL and VH genes (Geneart), cDNA was genetically fused by a sequence encoding a serine/glycine linker and cloned into a vector encoding a scFv specific for human CD3 that is human in sequence and cross-reactive with nonhuman primates, and a sequence coding for a eukaryotic leader peptide (1). The verified nucleotide sequences encoding the C- and P-BiTE antibodies were cloned via two restriction sites into a plasmid designated pEF dihydrofolate reductase (DHFR), respectively. The resulting plasmids were transfected into DHFR-deficient CHO cells for eukaryotic expression of the constructs. The transfection was implemented with FuGENE 6 (Roche Applied Science) according to the instructions of the manufacturer. Eukaryotic protein expression in DHFR-deficient CHO cells is performed as described previously (1). The EGFR-specific BiTE antibodies were produced by stable expression in CHO cells and affinity-purified via a C-terminal hexa-histidine tag, and monomeric BiTE antibodies isolated by gel filtration (2). Specific EGFR binding of constructs was confirmed by flow cytometry using CHO cells transfected with human and macaque EGFR versus untransfected CHO cells. Binding to human and macaque CD3 was tested by flow cytometry using the human T cell line HPB-ALL and 4119 LnPx (3) (provided by Helmut Fickenscher, Hygiene Institute, Virology, Erlangen-Nuernberg, Germany).

Dose–Response Analyses of C- and P-BiTE for T Cell Activation and Redirected Target Cell Lysis. Human PBMCs with or without prior stimulation by an immobilized CD3 antibody (OKT-3; Janssen-Cilag), or CD3-selected human T cells (isolated by Dynabead untouched human T cell kit) were used as effector cells. For the assays with macaque T cells either stimulated or unstimulated cynomolgus PBMCs were deployed. Where indicated, the rhesus T cell line 4119 LnPx was also used as effector cell line (3). PBMCs were isolated from blood of healthy donors or cynomolgus monkeys by Ficoll (Biochrom) density gradient centrifugation using standard procedures. After centrifugation, cells were washed with PBS solution and resuspended in RPMI 1640 complete medium, and cell numbers adjusted to 5×10^5 cells/mL (chromium release) or 3×10^6 cells/mL (FACS assay). For chromium release assays, target cells were labeled by incubation with approximately 7 MBq ^{51}Cr for 1 h, followed by removal of free chromium with two washing steps. For FACS-based cytotoxicity assays, the fluorescent membrane dye PKH-26 (Sigma) was used to label target cells and to distinguish target from effector cells upon FACS analysis. PKH-26 labeling was performed using the PKH-26 Kit (Sigma) as described by the manufacturer.

CHO cells transfected with human or cynomolgus full-length EGFR cDNA, or SW480, HCT116, HT29, or KATO III cells were used as target cells. Labeled target cells were adjusted to 5×10^4 cells/mL (for chromium release) or 3×10^5 cells/mL (for FACS-based assay) in RPMI-1640 complete medium (Gibco). Equal volumes of target and effector cell suspension were then mixed, resulting in an effector-to-target (E:T) ratio of 10:1. Two hundred microliters (for chromium release) or 100 μL (for FACS-based assay) of this suspension were transferred to each well of a 96-well plate. Fifty microliters of serial BiTE antibody dilutions or complete RPMI medium as a background control were then added. For chromium release assays, the BiTE antibody-mediated cytotoxic reaction proceeded 18 h at 37 °C in a 5% CO_2 humidified incubator. After incubation, 100 μL of each well were removed and cytotoxicity was measured as relative values of released chromium in the supernatant related to the difference of maximum lysis (determined by addition of Triton X-100) and spontaneous lysis (in the absence of effector cells). All measurements were done in quadruplicate. Measurement of ^{51}Cr activity in the supernatants was performed with a Wizard 3" γ-counter (Perkin-Elmer).

For FACS-based cytotoxicity assays after 24 h of incubation, propidium iodide was added to a final concentration of 1 μg/mL and samples were analyzed in a FACSCanto II flow cytometer (Becton Dickinson). FACSDiva software was used to collect and analyze the data. Nonviable cells were excluded using forward and side scatter electronic gating. Quantification of cytotoxicity was based on the number of live target cells in the control samples (without antibody) compared with the number of live target cells in the test samples (with serial antibody dilutions). The specific cytotoxicity was calculated by the following formula:

$$[1 - \text{live target cells (sample)}/\text{live target cells (control)}] \times 100 \quad \textbf{[1]}$$

By determining the ratio of live target cells in the test sample to live target cells in the control sample, target cells undergoing non–BiTE-mediated death were excluded from the determination of BiTE-specific cytotoxicity. T cell activation was assessed by quantification of CD69-positive T cells. The control BiTE antibody bound to an irrelevant hapten antigen, the herbicide mecoprop (4), but otherwise shared its CD3-binding arm with C- and P-BiTE.

Analysis of the experimental data were performed with Prism 5 for Windows (GraphPad Software). Sigmoidal dose–response curves typically had R^2 values greater than 0.90 as determined by the software. EC_{50} values as calculated by the analysis program were used for comparison of bioactivity.

In Vivo Efficacy Analysis. HT-29 (2×10^6) and HCT116 (5×10^6) human colon carcinoma cell lines were injected s.c. with or without PBMCs from healthy human donors at an E:T cell ratio of 1:2 in the right dorsal flank of female NOD/SCID mice ($n = 8$ per group). Mice were treated from the day of tumor plus PBMC inoculation (d 1) with 5, 0.5, 0.05, and 0.005 mg/kg cetuximab BiTE for 8 (HT-29) or 10 (HCT116) d, respectively, by i.v. bolus injection in the lateral tail vein. Controls included two vehicle-treated groups (i.e., tumor cells injected with or without human PBMCs) and one group of animals treated with an irrelevant control BiTE antibody (at 5 mg/kg). Different doses and schedules for C-BiTE and cetuximab reflect are owed to the different pharmacokinetic properties of proteins. Because C-BiTE has a serum half-life of only a few hours, it was injected daily i.v. starting on day 1, a schedule that has been previously established for several other BiTE antibodies in SCID mouse models.

Cetuximab was administered by i.p. injection starting on d 1 at 50 mg/kg per dose twice weekly for a total of eight (HT-29) or 10 (HCT116) injections, respectively, as this dose and schedule was reported to be efficacious in nonmutated CRC cell lines (5). Animals in the corresponding control group were treated i.p. with human IgG (50 mg/kg). Progress of tumors was determined by external caliper measurements, and tumor volumes were calculated using a standard hemiellipsoid formula:

$$[\text{length(in mm)} \times \text{width (in mm)}^2]/2 \quad \textbf{[2]}$$

Pharmacokinetic Analysis of C-BiTE Serum Levels in Cynomolgus Monkeys. The assay for determining C-BiTE serum concentrations was based on electrochemiluminescence detection technology. C-BiTE contained in cynomolgus serum is immobilized by soluble EGFR protein coated to a high-bind microtiter plate. To this end, 5 μL soluble EGFR was placed between the electrodes of the carbon surface of the plate. After complete drying of the plate, 150 μL blocking solution (5% BSA) was added to the wells and incubated for 1 h at room temperature. After three washing steps with PBS solution plus 0.05% Tween-20, 10 μL cynomolgus serum sample was transferred to the wells. All serum samples were determined in duplicate. Calibration standard curve and three quality control (QC) samples containing high, medium, and low concentrations of the respective C-BiTE were prepared additionally. The plate was incubated for 1 h at room temperature on a rotation shaker. The detection of the respective immobilized C-BiTE occurred by addition of 25 μL penta-His-biotin (1 μg/mL) antibody and then 25 μL streptavidin SULFO-TAG (2 μg/mL; incubation 1 h at room temperature). After addition of reading buffer to the wells, the plate was measured by an SI2400 analyzer (Meso Scale Discovery). The values were transferred in the GraphPad Prism program wherein the QC samples as well as unknown C-BiTE concentrations were calculated with the help of the "point to point" function. The lower limit of quantification of the assay was 0.5 ng/mL, lower limit of detection was 0.01 ng/mL, and upper limit of quantification was 100 ng/mL (Fig. S4).

Toxicology Study. Animal studies were conducted in accordance with the guiding principles in the care and use of animals (http://www.the-aps.org/about/opguide/appendix.htm#care). Male cynomolgus monkeys (*M. fascicularis*) were maintained under housing conditions accredited by the American Association for Accreditation of Laboratory Animal Care, or the European equivalent. Approximately 2 wk before the start of treatment, for each animal on test, a catheter was implanted into the posterior vena cava via a femoral vein and tunneled s.c. to exit at the interscapular region. The catheter was attached to the delivery system via a tether system and a swivel joint.

Groups of two animals received C- or P-BiTE antibodies or the vehicle by continuous 24-h i.v. infusion. Animals were observed at least daily during the study for mortality or moribundity as well as clinical signs or reactions to treatment. Other evaluations conducted before the test and on one or more occasions during the study included body temperature, ophthalmology, body weight, food consumption, cardiovascular function, hematology, coagulation, serum chemistry, urine analysis, lymphocyte subtyping (FACS), cytokine release, and toxicokinetics. Serum was also prepared before the test and at necropsy for immunogenicity evaluation. Animals were also subjected, at this latter occasion, to a thorough macroscopic assessment of external and internal surfaces, orifices and cavities, and tissues and organs as well as the implantation site(s). Selected organs were weighed and an extensive list of tissues from each animal sampled for subsequent histopathological examination.

1. Kaufman RJ (1990) Selection and coamplification of heterologous genes in mammalian cells. *Methods Enzymol* 185:537–566.
2. Lutterbuese R, et al. (2009) Potent control of tumor growth by CEA/CD3-bispecific single-chain antibody constructs that are not competitively inhibited by soluble CEA. *J Immunother* 32:341–352.
3. Knappe A, et al. (2000) Herpesvirus saimiri-transformed macaque T cells are tolerated and do not cause lymphoma after autologous reinfusion. *Blood* 95:3256–3261.
4. Li Y, Cockburn W, Kilpatrick JB, Whitelam GC (2000) High affinity ScFvs from a single rabbit immunized with multiple haptens. *Biochem Biophys Res Commun* 268:398–404.
5. Wild R, et al. (2006) Cetuximab preclinical antitumor activity (monotherapy and combination based) is not predicted by relative total or activated epidermal growth factor receptor tumor expression levels. *Mol Cancer Ther* 5:104–113.

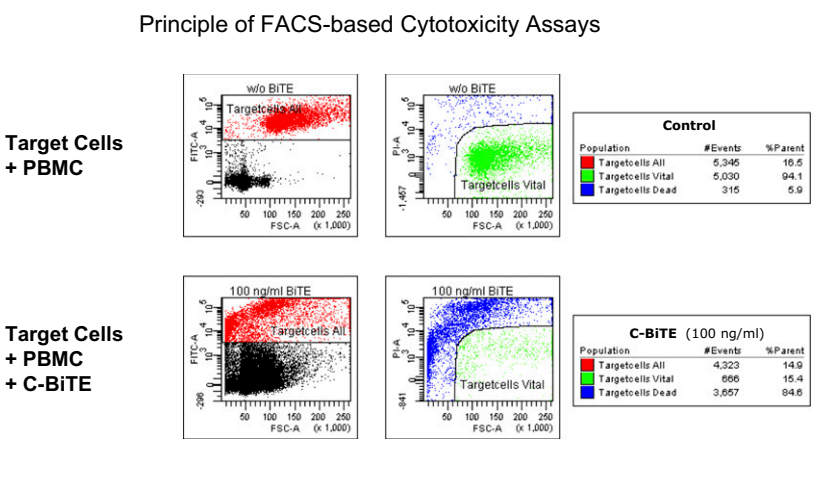

Fig. S1. Redirected lysis of human EGFR-expressing CHO cells by a BiTE antibody based on cetuximab. Dose-dependent redirected lysis of CHO cells transfected with human EGFR is shown for one example as raw data in the form of dot plots of one representative donor. The left column depicts a dot plot that discriminates between PKH26-positive tumor cells (FITC-positive cells) and unlabeled effector cells (FITC-negative cells). The gated tumor cells (red) are further analyzed in the right column where propidium iodide (PI)–positive dead cells are separated from the living PI-negative tumor cells (green). The event numbers of vital target cells are used for analysis of the BiTE-mediated cytotoxicity.

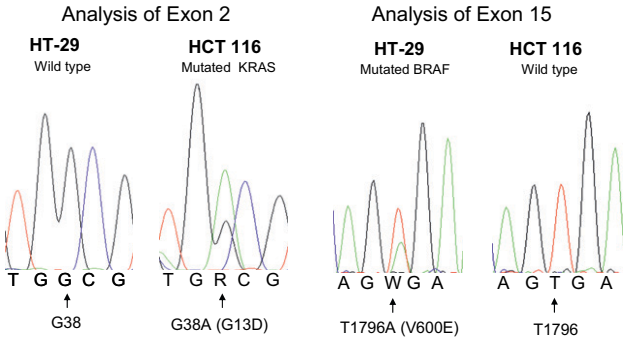

Fig. S2. Characterization of the mutational status of HT-29 and HCT 116 CRC lines Confirmation of mutation in KRAS and BRAF genes was obtained by genomic DNA sequencing. Genomic DNA was extracted from cells using the DNeasy Blood and Tissue kit (Qiagen) following the manufacturer's instructions. Exon 2 of KRAS and exon 15 of BRAF were amplified with primers complementary to surrounding sequences: K-RAS EXON 2 FW 5′-AGGCCTGCTGAAAATGACTGAATA-3′; K-RAS EXON 2 BW 5′-CTGTATCAAAGAATGGTCCTGCAC-3′; BRAF EXON 15 FW 5′-AACACATTTCAAGCCCCAAA-3′; and BRAF EXON 15 BW 5′-GAAACTGGTTTCAAAATATTCGTT-3′. PCR products were purified with the QIAquick Gel Extraction Kit (Qiagen) and sequenced.

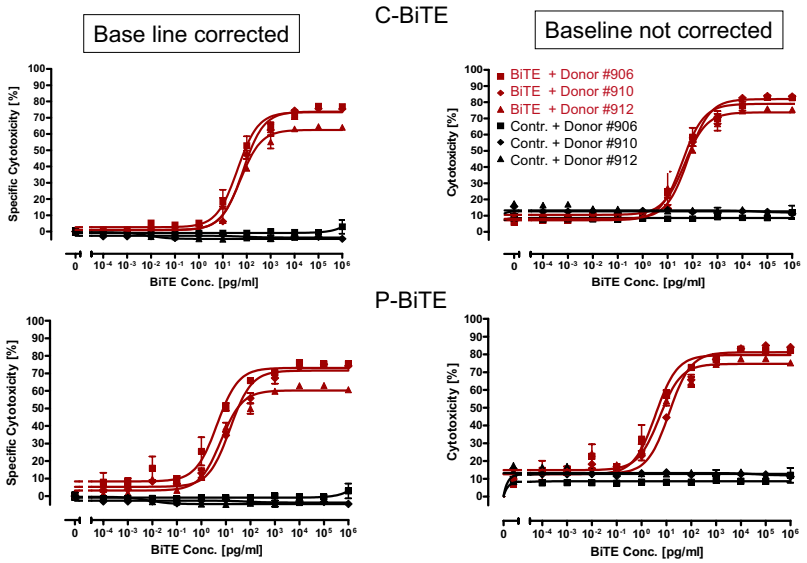

Fig. S3. Redirected lysis of human EGFR-expressing CHO cells by a BiTE antibody based on cetuximab. The influence of baseline correction for spontaneously lysed target cells is shown for two dose–response analyses.

Qualification of C-BiTE ECL Assay for Determination of Serum Levels in Cynomolgus Monkeys

QC-samples 100%Cynoserum	expected concentration [ng/ml]	calculated C-Bite conc. [ng/ml] exmeasured conc. 1	measured conc. 2	measured conc. 3	Arithm. Mean	STDEV	CV [%]	% recovery	
1	100,00	116,25	135,13	115,97	122,45	10,98	8,97	**122,45**	[100 ng/ml]
	Prism	2,07	2,13	2,06					
2	50,00	44,73	42,70	41,63	43,02	1,57	3,66	**86,04**	[50 ng/ml]
	Prism	1,65	1,63	1,62					
3	25,00	19,55	19,12	19,86	19,51	0,37	1,89	**78,04**	[25 ng/ml]
	Prism	1,29	1,28	1,30					
4	12,50	12,72	9,32	8,99	10,34	2,06	19,96	**82,75**	[12,5 ng/ml]
	Prism	1,10	0,97	0,95					
5	6,25	5,37	5,23	5,51	5,37	0,14	2,59	**85,93**	[6,25ng/ml]
	Prism	0,73	0,72	0,74					
6	3,13	2,51	3,50	3,80	3,65	0,21	5,78	**116,93**	[3,13ng/ml]
	Prism	0,40	0,54	0,58					
7	1,56	1,34	1,40	1,19	1,31	0,11	8,50	**83,99**	[1,56ng/ml]
	Prism	0,13	0,15	0,07					
8	0,78	0,73	0,62	0,90	0,75	0,14	19,24	**96,09**	[0,78ng/ml]
	Prism	-0,13	-0,21	-0,04					
9	0,39	0,40	0,49	0,49	0,46	0,05	11,48	**116,65**	[0,39ng/ml]
	Prism	-0,40	-0,31	-0,31					
10	0,20	0,46	0,42	0,32	0,40	0,07	17,61	**204,60**	[0,2ng/ml]
	Prism	-0,34	-0,38	-0,49					
11	0,10	0,31	0,00	0,31	0,21	0,18	85,92	**213,67**	[0,10ng/ml]
	Prism	-0,51	-2,78	-0,51					
12	0,05	0,50	0,00	0,00	0,17	0,29	171,22	**344,26**	[0,05ng/ml]
	Prism	-0,30	-2,78	-2,66					

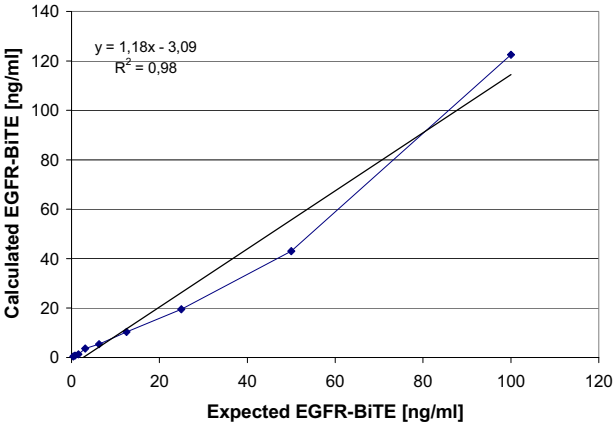

Fig. S4. Determination of serum concentrations of C-BiTE dose levels during continuous i.v. infusion up to the maximum infusion period of 3 wk. The lower limit of quantification of 0.5 ng/mL was determined during assay development with respect to accuracy and linearity of the ECL assay. Serum samples with drug concentrations higher than 100 ng/mL were diluted accordingly. The mean recovery values of the QC samples used with acceptable CV values (<20%) are shown and were determined in triplicate.

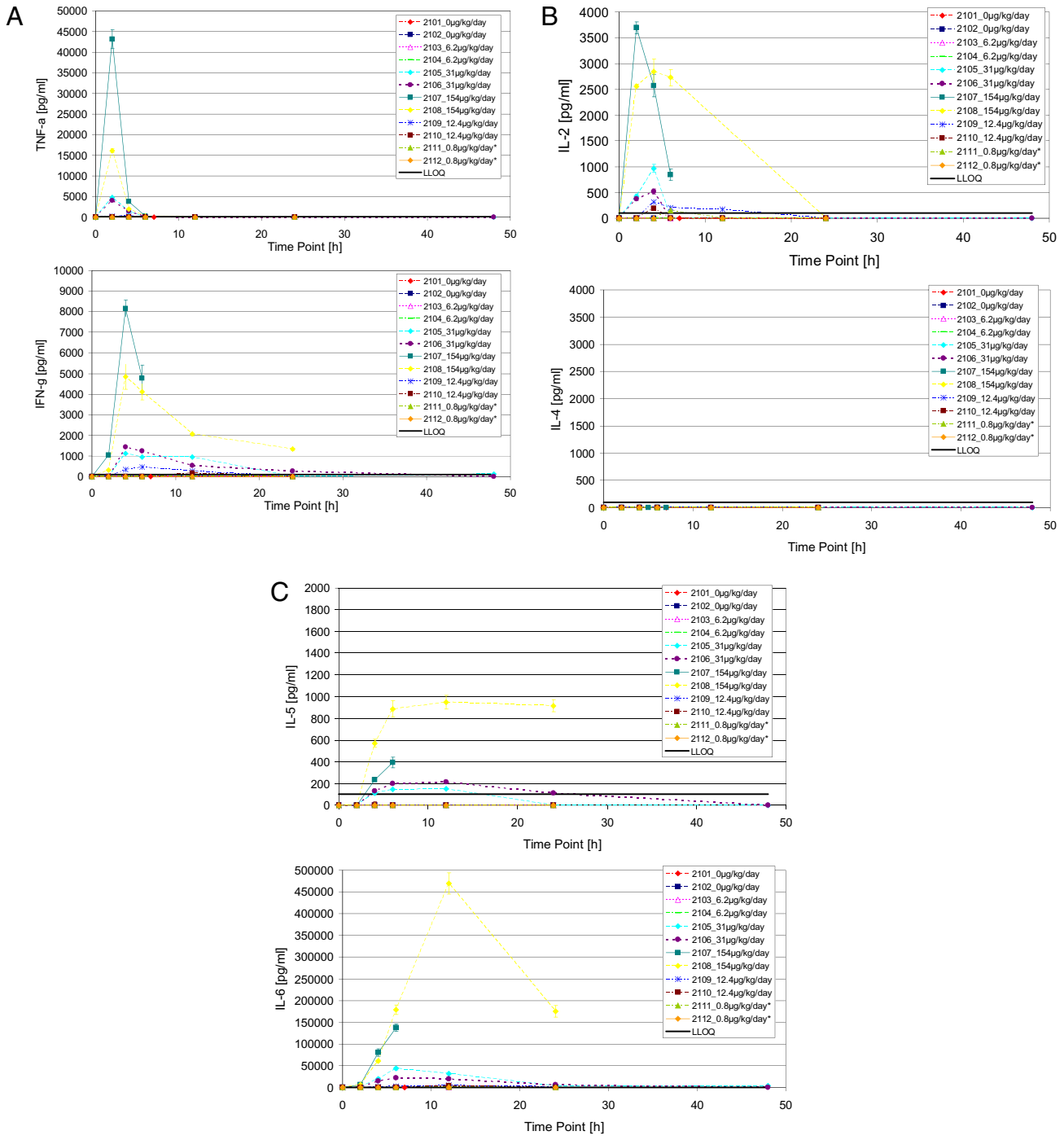

Fig. S5. Serum cytokine levels in cynomolgus monkeys treated with C-BiTE and P-BiTE. Cytokine levels in the serum samples were measured using the commercially available FACS-based Becton Dickinson Cytometric Bead Array system. The system consisted of six bead populations with discrete fluorescence intensities, each bead population precoated with cytokine specific capture antibodies. The beads, each specific for one cytokine (*A*) TNF-α and IFN-γ, (*B*) IL-2 and IL-4, and (*C*) IL-5 and IL-6, could be distinguished via fluorescence-based emission and flow cytometric analysis. Bound analytes were quantified using a secondary specific phycoerythrin (PE)–conjugated antibody. Samples and a set of cytokine standards were prepared and analyzed in accordance with the manufacturer's instructions. The assay was conducted in a 96-well V-bottom plate (Nunc). All washing steps were accomplished by centrifugation at 250 × *g* for 5 min at 25 °C. Briefly, the standards were reconstituted in provided assay diluent, allowed to dissolve completely and mixed together to form a bulk standard mixture. This was serially diluted to produce the calibration curve. The individual capture beads were mixed together to form a bulk stock, as was the specific PE-conjugated secondary detection antibody. Once all of the reagents have been prepared, the assay was conducted using 25 μL per well diluted test sera or cytokine standard dilution, 25 μL per well bead mixture, and 25 μL of detection antibody mix. The prepared plate was sealed with adhesive film and incubated for 3 h in the dark at room temperature. Finally, samples were washed and the bead pellet was resuspended in wash buffer and then measured using a FACSCalibur flow cytometer (Becton Dickinson), running Cell Quest Pro software or FACSCanto II instrument (Becton Dickinson), using FACSDiva software (version 5.0.2; Becton Dickinson).The electronic files generated by the analysis were exported in the manufacturer's FCAP Array software (version 1.0; Softflow) in Excel (Microsoft). The lower limit of detection of the assay was 20 pg/mL, the lower limit of quantification 100 pg/mL, and the upper limit of quantification 5,000 pg/mL.