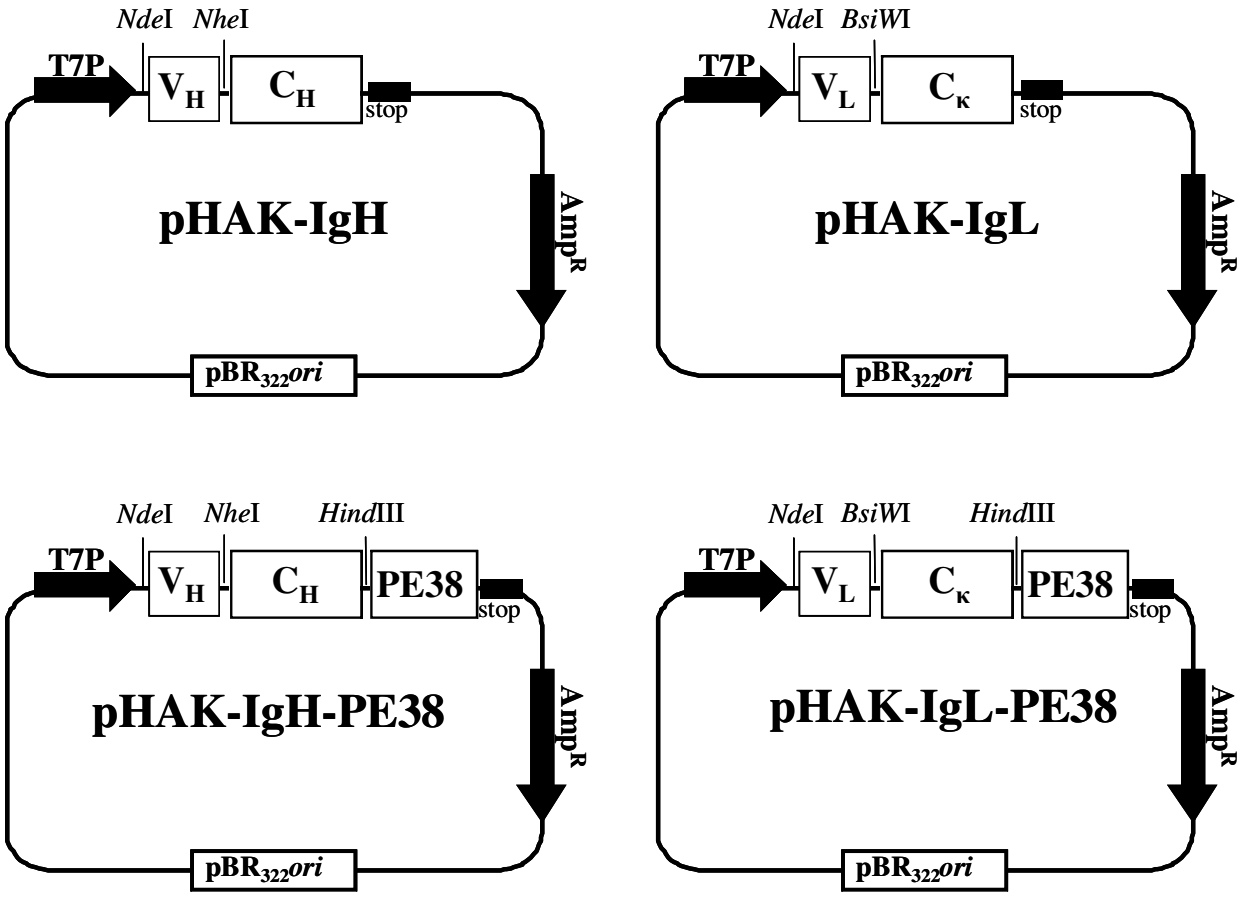
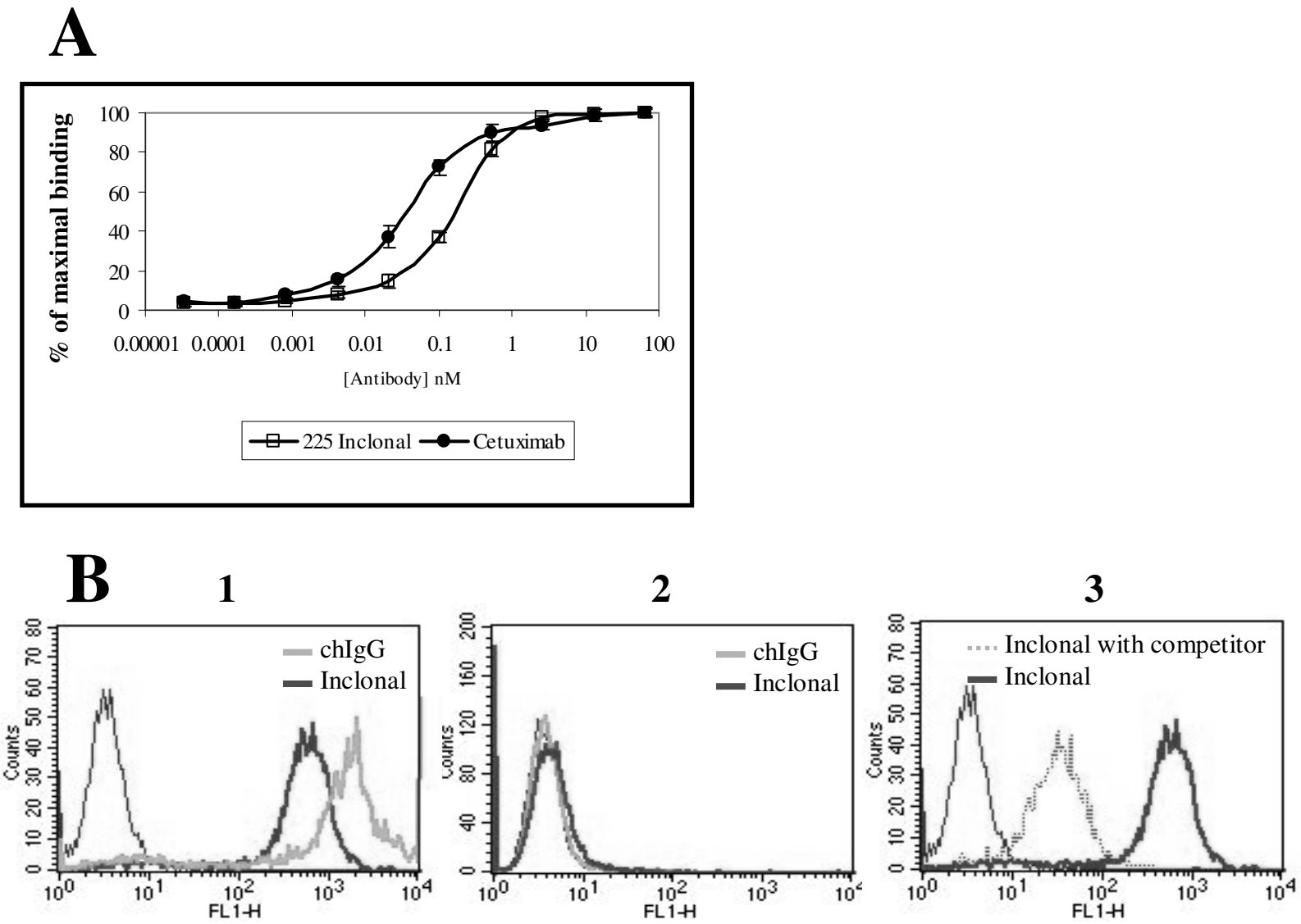
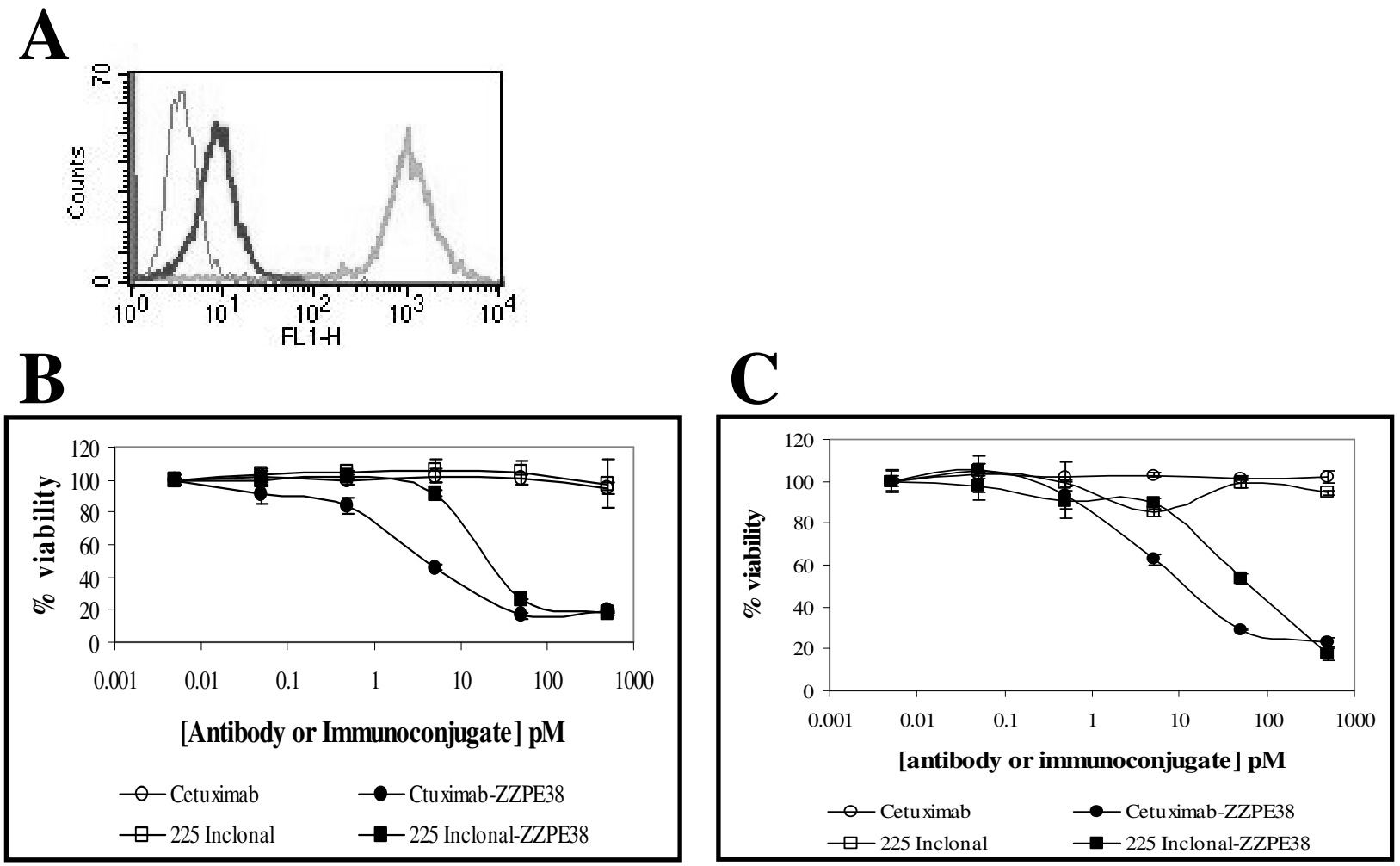
Hakim 2009 Supplementary Fig. 1



Hakim 2009 Supplementary Fig. 2



Hakim 2009 Supplementary Fig. 3



Supplementary Methods

Preparation of MBP-CD30 as a soluble antigen. Recombinant CD30 was expressed as a maltose-binding protein fusion in *E. coli*. A DNA fragment corresponding to the extracellular domain of human CD30 was recovered by PCR using plasmid pHR30HNB [1] as template with primers CD30(N)-BspHI-FOR and CD30(N)-NotI-REV (All the PCR primers are described in upplementary Table 1). The PCR product was digested with *Bsp*HI and *Not*I and cloned into a pMALc-NHNN vector (pMALc-NHNN is a plasmid for the expression of MBP fusion proteins which was modified from pMALc-NN [2] by the addition of a HIS-tag on the 5' end of the MBP coding sequence). The protein was produced and purified essentially as described for other MBP fusion proteins. [2]

Preparation of the recombinant 225(scFv)-PE38 immunotoxin. The variable domains of anti EGFR mAb 225 were recovered by PCR using the plasmid pCMV/myc/ER-225(scFv) [3] as template with the primers 225-NdeI-FOR and 225-NotI-REV. The PCR product was digested with *Nde*I and *Not*I and cloned into a derivative of the pRB98-Amp expression vector [4] that was linearized using the same enzymes. (In this derivative, we replaced the *Hind*III site used as 3' end site for cloning scFvs with a *Not*I site to make it compatible for subcloning from common phage display vectors). The resulting plasmid, named pRB98-Amp-225(scFv)-PE38 was used for expression as a scFv-PE38 single-chain immunotoxin in BL21 (DE3) pUBS500 cells. The expression, refolding and purification of the recombinant single-chain immunotoxin were preformed as described. [5, 6]

Construction of vectors for expression of chimeric IgG1 in mammalian cells

Heavy chain vector. The VH variable domain of anti CD30 antibody T427 [7] was recovered by PCR using as template plasmid pRB98Amp-T427VH(C44)-PE38 (an expression vector for the VH-cys-PE38 component of the dsFv-immunotoxin [4]). The 5' end half of T427 VH was amplified using primers T427VH-BssHII-FOR and T427VH-C44G-REV that restore G44 (that was mutated to cys in the dsFv configuration). The 3' end half of T427 VH was amplified using primers T427VH-

C44G-FOR and T427VH-NheI-REV. The resulting PCR products were combined and assembled into an intact VH domain using primers T427VH-BssHII-FOR and T427VH-NheI-REV. The VH PCR product was digested with *Bss*HII and *Nhe*I and cloned into a pMAZ-IgH vector [8] that was linearized using the same enzymes. The resulting plasmid, pMAZ-IgH-T427 was used to express the heavy chain of T427 in a chimeric IgG1 format in mammalian cells.

Light chain vector. The V-Kappa variable domain of anti CD30 antibody T427 was recovered by PCR using as template plasmid pRB98Amp-T427VL(C105) (an expression vector for the VL-cys component of the dsFv-immunotoxin [4]). The T427 VL was amplified using primers T427VL-BssHII-FOR and T427VL-BsiWI-REV that restored G105 (that was mutated to cys in the dsFv configuration). The VL PCR product was digested with *Bss*HII and *Bsi*WI and cloned into a pMAZ-IgL vector[8] that was linearized using the same enzymes. The resulting plasmid, pMAZ-IgL-T427 was used to express the light chain of T427 in a chimeric IgG1 format in mammalian cells.

Expression of chimeric IgGs in mammalian cells. The chimeric T427 IgG1 was expressed in HEK293 cells that were co-transfected with plasmids pMAZ-IgH-T427 and pMAZ-IgL-T427 and selected with G418 and hygromycin essentially as described. [8] After a highly-expressing clone was selected, it was expanded in DMEM supplemented with 10% FBS and antibiotics. 72 hours before harvesting, the cells were transferred into DCCM1 (serum free) medium (Beit-Haemek, Israel). Medium was collected several times at 48-72 h intervals. The IgG was purified from the conditioned medium by protein-A chomatography as described[8]. Protein concentrations of the purified proteins were determined by a Bradford assay (Coomassie Plus; Pierce, Rockford, IL) with BSA as the standard. Purified IgG was stored at 4ºC. Cetuximab was purchased from Merck.

Supplementary figure legends

Supplementary Figure 1. Schematic representation of Inclonals expression vectors. Maps of plasmids pHAK-IgH for expression of antibodies with human γ1 heavy chain; pHAK-IgL for expression of antibodies with human κ light chain; pHAK-IgH-PE38 for expression of antibodies with human γ1 heavy chain fused to a truncated form of *Pseudomonas* exotoxin A (PE38); pHAK-IgL-PE38 for expression of antibodies with human κ light chain fused to PE38.

Supplementary Figure 2. Binding properties of the anti EGFR 225 Inclonal. (A) Binding to EGFR expressed on A431 cells tested by whole cell ELISA. Detection is with HRP-conjugated anti human IgG. (B) FACS analysis: (B1) A431 cells were incubated with 10 nM of 225 Inclonal or the commercial anti EGFR antibody cetuximab used as control. (B2) FACS analysis as in B1 on G43 melanoma cells that do not express EGFR. (B3) FACS analysis of 225 Inclonal binding in the presence of ×30 molar excess of 225(scFv)-PE38 immunotoxin as competitor. Binding was detected using FITC-conjugated anti human antibody.

Supplementary Figure 3. Analysis of 225 Inclonal-ZZ-PE38. (A) FACS analysis of EGFR expression levels of the cell lines: A431 (thick light grey), HEK293 (thick dark grey) and control G43 (thin light grey). EGFR levels were detected by staining with cetuximab mixed with FITC-conjugated anti human antibody. (B) Cell-killing assay of 225 Inclonal-ZZ-PE38 on A431 cells (expressing a high level of EGFR). (C) Cell-killing assay of 225 Inclonal-ZZ-PE38 on HEK293 cells (expressing a low level of EGFR). cells were incubated for 48 h with the indicated concentration of IgG-ZZ-PE38 immunoconjugates or the IgGs alone. The relative number of viable cells was determined using an enzymatic MTT assay. Each point represents the mean of a set of data determined in triplicate in three independent experiments. Error bars represent the standard deviation of the data.

Supplementary Table 1: **PCR primers (restriction sites are underlined)**

Primer name	Sequences
CD30(N)-BspHI-FOR	5′ TTTAAATCATGACCTTCCCACAGGATCGACCC
CD30(N)-NotI-REV	5′ ATATATGCGGCCGCTTAATCCAGAACGGGCTTCCC
225-NdeI-FOR	5′ GATATACATATGGAGGTCCAACTGCAGCAG
225-NotI-REV	5′ CCGGATGCGGCCGCCCGTTTGATCTCCAGCTTGG
T427VH-BssHII-FOR	5′ CCACAGGCGCGCACTCCCAGGTCCAACTGCAGCAGCCG
T427VH-C44G-REV	5′ CCACTCAAGGCCTTGTCCAGGCC
T427VH-C44G-FOR	5′ GGACAAGGCCTTGAGTGGATTGG
T427VH-NheI-REV	5′ CTTGGTGCTAGCTGAGGAGACGGTGACTGAG
T427VL-BssHII-FOR	5′ CCACAGGCGCGCACTCCGACATTGTGCTGACCCAATCTC
T427VL-BsiWI-REV	5′ AGCCACCGTACGTTTGATTTCCAGTTTGGTGCCTCCACC GAACGTC
CMV-Seq	5′ TGGGCGGTAGGCGTGTACGG
CMV-antiseq-EcoRI-REV	5′ TTTAAAGAATTCCAACAGATGGCTGGCAACTAG
225VH-NheI-REV	5′ TTTAAAGCTAGCTGAGGAAACGGTGACCAGGGTCCCT TGGCCC
225VK-BsiWII-REV	5′ TTTAAACGTACGTTTGAGCTCCAGCTTGGTCCCAGCAC
RGD/TAT-BsrGI-FOR	5′ GACGTGAGCCACGAAGACCCTGAGGTC
CH_3-HindIII-EcoRI-REV	5′ AAATTTGAATTCACCTCCGGAAGCTTTACCCGGGGAC A GGGAG
BsiWI-Back-IgL	5′ AAACGGCGTACGGTGGCTGCACCATCTGTCTTC
C□-HindIII-EcoRI-REV	5′ AAATTTGAATTCACCTTCGGAAGCTTTTCCACCGCCA CACTCTCCCCTGTTGAAG

Supplementary references

1. Rozemuller H, Chowdhury PS, Pastan I, Kreitman RJ. Isolation of new anti-CD30 scFvs from DNA-immunized mice by phage display and biologic activity of recombinant immunotoxins produced by fusion with truncated pseudomonas exotoxin. Int J Cancer 2001; 92:861-70.
2. Bach H, Mazor Y, Shaky S, Shoham-Lev A, Berdichevsky Y, Gutnick DL, Benhar I. Escherichia coli maltose-binding protein as a molecular chaperone for recombinant intracellular cytoplasmic single-chain antibodies. J Mol Biol 2001; 312:79-93.
3. Shaki-Loewenstein S, Zfania R, Hyland S, Wels WS, Benhar I. A universal strategy for stable intracellular antibodies. J Immunol Methods 2005; 303:19-39.
4. Nagata S, Onda M, Numata Y, Santora K, Beers R, Kreitman RJ, Pastan I. Novel anti-CD30 recombinant immunotoxins containing disulfide-stabilized Fv fragments. Clin Cancer Res 2002; 8:2345-55.
5. Benhar I, Pastan I. Cloning, expression and characterization of the Fv fragments of the anti-carbohydrate mAbs B1 and B5 as single-chain immunotoxins. Protein Eng 1994; 7:1509-15.
6. Buchner J, Pastan I, Brinkmann U. A method for increasing the yield of properly folded recombinant fusion proteins: single-chain immunotoxins from renaturation of bacterial inclusion bodies. Anal Biochem 1992; 205:263-70.
7. Nagata S, Numata Y, Onda M, Ise T, Hahn Y, Lee B, Pastan I. Rapid grouping of monoclonal antibodies based on their topographical epitopes by a label-free competitive immunoassay. J Immunol Methods 2004; 292:141-55.
8. Mazor Y, Barnea I, Keydar I, Benhar I. Antibody internalization studied using a novel IgG binding toxin fusion. J Immunol Methods 2007; 321:41-59.