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Redirecting NK cells mediated tumor cell lysis by a new recombinant bifunctional protein

Claire Germain[1,3], Emmanuelle Campigna[1], Imed Salhi[1], Sébastien Morisseau[1], Isabelle Navarro-Teulon[1], Jean-Pierre Mach[2], André Pèlegrin[1] and Bruno Robert[1,4]

[1]IRCM, Institut de Recherche en Cancérologie de Montpellier; INSERM U896; Université Montpellier1; CRLC Val d'Aurelle Paul Lamarque, Montpellier F-34298, France and [2]Department of Biochemistry, University of Lausanne, CH-1066 Epalinges, Switzerland

[3]Present address: IPMC, Institut de Pharmacologie Moléculaire et Cellulaire; CNRS/UNSA UMR 6097, Valbonne, 06560, France

[4]To whom correspondence should be addressed.
E-mail: brobert@valdorel.fnclcc.fr

Natural killer (NK) cells are at the crossroad between innate and adaptive immunity and play a major role in cancer immunosurveillance. NK cell stimulation depends on a balance between inhibitory and activating receptors, such as the stimulatory lectin-like receptor NKG2D. To redirect NK cells against tumor cells, we designed bifunctional proteins able to specifically bind tumor cells and to induce their lysis by NK cells, after NKG2D engagement. To this aim, we used the 'knob into hole' heterodimerization strategy, in which 'knob' and 'hole' variants were generated by directed mutagenesis within the CH3 domain of human IgG1 Fc fragments fused to an anti-CEA or anti-HER2 scFv or to the H60 murine ligand of NKG2D, respectively. We demonstrated the capacity of the bifunctional proteins produced to specifically coat tumor cells surface with H60 ligand. Most importantly, we demonstrated that these bifunctional proteins were able to induce an NKG2D-dependent and antibody-specific tumor cell lysis by murine NK cells. Overall, the results show the possibility to redirect NK cytotoxicity to tumor cells by a new format of recombinant bispecific antibody, opening the way of potential NK cell-based cancer immunotherapies by specific activation of the NKG2D receptor at the tumor site.

Keywords: bifunctional proteins/'knob into hole' strategy/natural killer cells/NKG2D/tumor targeting

Introduction

Natural killer (NK) cells are cytotoxic cells, belonging to innate immunity, which play a major role in host response against different pathogens, such as viruses (Bukowski *et al*., 1985), bacteria or parasites (Tay *et al*., 1998). It is also now well established that NK cells are involved in cancer immunosurveillance (Kim *et al*., 2000; Smyth *et al*., 2002). Despite no expression of antigen-specific receptors, such as T- or B-cell receptors (TcR and BcR), NK cells are able to recognize infected or malignant cells by several inhibitory or activating receptors, which act in balance either to induce NK cell tolerance towards normal cells or to promote NK cell activation and lysis of infected or tumor cells (Raulet and Vance, 2006). While inhibitory receptors, such as the killer immunoglobulin-like inhibitory receptors (KIR), are able to recognize the classical major histocompatibility class I (MHC I) proteins, whose expression is often lost or down-regulated on infected (Lodoen and Lanier, 2005) or malignant cells (Garrido *et al*., 1997), activating receptors, such as the natural cytotoxicity receptors (NCR) NKp30, NKp44 and NKp46 or the natural killer group 2 D (NKG2D) receptor, are able to recognize different ligands over-expressed on transformed cells (Moretta *et al*., 2001).

The activating NKG2D receptor has been particularly studied, both in humans and in mice. It is homogeneously expressed on all human and mouse NK cells (Bauer *et al*., 1999; Jamieson *et al*., 2002), but also on all human and mouse-activated CD8 + T cells, where it acts as a TcR co-activator (Groh *et al*., 2001; Jamieson *et al*., 2002). Several NKG2D ligands are now well described. The human NKG2D ligands comprise the MHC I chain-related proteins A and B (MICA and MICB) (Bahram *et al*., 1994) and the retinoic acid early transcripts (RAET1E, G, H, I, L, N) [IMGT Repertoire (RPI); http://imgt.cines.fr] (Cosman *et al*., 2001; Jan Chalupny *et al*., 2003). In mice, NKG2D ligands include the minor histocompatibility antigen H60 (Malarkannan *et al*., 1998), the retinoic acid early transcript 1 molecules α, β, γ, δ and ε (Rae1α–ε) (Cerwenka *et al*., 2000) and the murine UL16-binding protein-like transcript 1 molecule (Mult1) (Carayannopoulos *et al*., 2002). All these ligands are stress-induced proteins and have been identified on various tumor cell types, such as human epithelial carcinomas, melanomas, gliomas and leukemias (Pende *et al*., 2002; Vetter *et al*., 2002; Friese *et al*., 2003; Salih *et al*., 2003) or mouse epithelial carcinomas and lymphomas (Cerwenka *et al*., 2000; Diefenbach *et al*., 2000; Carayannopoulos *et al*., 2002). However, during tumor progression, malignant cells lose frequently NKG2D ligand expression, as a consequence of tumor variant selection (Vetter *et al*., 2004; Carbone *et al*., 2005), contact inhibition (Zou *et al*., 2006) or proteolytic shedding (Salih *et al*., 2002; Salih *et al*., 2003), and thus escape to NK cell recognition.

Several studies have established that transfection of human tumor cells with genes encoding NKG2D ligands sensitizes these cells to NK cell-mediated lysis *in vitro*, in an NKG2D-dependent pathway (Friese *et al*., 2003; Busche *et al*., 2006). It has also been demonstrated *in vivo* that transfected murine tumor cells expressing Rae1 or H60 are rejected by NK cells, while parental tumor cells critically grow, and that rejection is associated with T cell response priming (Diefenbach *et al*., 2001).

To bypass the need of a gene therapy, we designed here bifunctional proteins able to bind tumor cells through specific antibody structures and to coat tumor cell surface with NKG2D ligands. These new bifunctional proteins were produced according to the 'knob into hole' strategy, which allows the preferential heterodimerization of two heavy chains of distinct origin by changing two amino acids in their CH3 domains, and thus the production of recombinant bispecific antibodies (Ridgway *et al*., 1996; Merchant *et al*.,

1998). In the present study, we assembled through this strategy two different anti-tumor-associated antigen (TAA) scFv with the mouse NKG2D ligand H60. After showing the specificity of these new bifunctional proteins for two targeted TAAs, we demonstrated their capacity to stimulate NKG2D-dependent tumor cell lysis by murine NK cells.

Materials and methods

Tumor cell lines

Human acute monocytic leukemia THP-1 [expressing Fc receptors (FcR) FcγRI and FcγRII] and human breast carcinoma SK-BR-3 (expressing HER2) were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained as described by the manufacturer. The murine colon carcinoma MC38 expressing CEA (C15.4.3.AP) cell line [kindly provided by Dr J. Primus, Department of Pathology, Vanderbilt University Medical Center, Nashville, TN, USA (Clarke *et al*., 1998)], the murine melanoma B16 expressing HER2 cell line [kindly provided by Dr A. Donda, Department of Biochemistry, Faculty of Biology and Medicine, University of Lausanne, Switzerland (Stirnemann *et al*., 2008)] and the murine T lymphoma RMA expressing CEA cell line, obtained by electrotransfection (250 V, 960 μF, 5 μg of DNA) of RMA cells with plasmid-containing human CEA cDNA (Pelegrin *et al*., 1992), were maintained in DMEM/F12 (1:1) supplemented with 2 mmol/l L-glutamine, 10% fetal calf serum (FCS), penicillin (100 units/ml)/streptomycin (100 μg/ml) and geneticin (0.5 mg/ml; Life Technologies, Grand Island, NY, USA).

Vectors construction

The sequences of the different primers used for the construction of the bifunctional proteins are given in Table I. First, the genes encoding anti-human CEA scFv 511 (in pFLAG-1, kindly provided by Dr A.V. Terskikh, The Burnham Institute of Medical Research, La Jolla, CA, USA, derived from the mouse anti-human CEA mAb 511 hybridoma) and anti-human HER2 scFv 4D5 [in Bluescript® II KS +/−, kindly provided by Dr Alena Donda, Department of Biochemistry, Faculty of Biology and Medicine, University of Lausanne, Switzerland, derived from the humanized anti-human HER2 mAb 4D5 (Deyev *et al*., 2003)] were amplified by PCR using primers P1/P2 and P3/P4. The amplified fragments were next digested and inserted in frame in plasmid ps878 (kindly provided by Dr P. Schneider, Department of Biochemistry, Faculty of Biology and Medicine, University of Lausanne, Switzerland (Schneider, 2000)] encoding human IGHG1 Fc fragment (hinge, CH2 and CH3; GenBank/EMBL/IMGT/LIGM-DB accession No. X70421), with the signal sequence of the influenza virus hemagglutinin (HA) to allow protein secretion by mammalian cells. Knob variants of each construction were obtained by site-directed mutagenesis (PROMEGA Kit) in the IGHG1 CH3 sequence using primers P5/P6 (Xie *et al*., 2005). Plasmid ps521-rH60-Fc [kindly provided by Dr W. Held, Ludwig Institute for Cancer Research, Lausanne branch, Switzerland (Coudert *et al*., 2005)] contains the gene encoding the extracellular portion including the endogenous leader peptide of H60 (amino acids 1–213), amplified by polymerase chain reaction (PCR) from A20 B-cell cDNA, using the following primers (restriction sites are underlined): H60 5′ sense, actg AAGCTT tgagggaagacc ATG GCAAAG G; H60 3′ antisense, actg GTCGAC CTG GTT GTC AGA ATT ATG TCG GAA G. The PCR product was directionally cloned into modified PCR-3 vectors (Invitrogen, San Diego, CA, USA) to add a COOH-terminal FLAG epitope or to generate an Fc fusion protein (kindly provided by P. Schneider, Department of Biochemistry, University of Lausanne, Switzerland). A hole variant of this construction was obtained by site-directed mutagenesis using primers P7/P8 (Xie *et al*., 2005). Site-directed mutagenesis efficiency was verified by sequencing.

Table I. Sequence of the different primers used for vector constructions

Primers	Sequence (5′→3′)	Note
P1	atactcgagctggaggagtctggggag	anti-CEA scFv (scFv 511) 5′
P2	acagtcgacgatctccagtttggtccca	anti-CEA scFv (scFv 511) 3′
P3	gagctcgaggactacaaagatatc	anti-HER2 scFv (scFv 4D5) 5′
P4	tccgtcgacagaagaaacggtaac	anti-HER2 scFv (scFv 4D5) 3′
P5	ggtcagcctg**ta**ctgcctggtcaa	Fc knob variant T366Y 5′
P6	cag**ta**caggctgacctggttc	Fc knob variant T366Y 3′
P7	c**ac**cagcaagctcaccgtgga	Fc hole variant Y407T 5′
P8	tccacggtgagcttgctg**gt**gaggaagaa	Fc hole variant Y407T 3′

Underlined sequences represent XhoI and SalI restriction sites. Bold sequences represent nucleotides modified in sequence encoding CH_3 domain of human IGHG1 Fc.

Bifunctional proteins expression and purification

HEK 293 cells (ATCC CRL-1573) were grown in DMEM/F12 (1:1) supplemented with 2 mmol/l L-glutamine, 10% FCS and penicillin (100 units/ml)/streptomycin (100 μg/ml) and transfected with each mutant vector using FuGene 6 reagent (Roche Applied Science, Indianapolis, IN, USA), as recommended by the manufacturer. Clones were selected by limit dilution with medium containing geneticin (0.8 mg/ml). Culture supernatant of each clone was assayed for bifunctional proteins production by western blotting, as below. As control, HEK 293 cells were also transfected with the original ps521-rH60-Fc vector encoding rH60-Fc, or with the original ps878 vector, encoding (scFv 511)-Fc or (scFv 4D5)-Fc, or untransfected. For the production of homodimeric and bifunctional proteins, selected clones were cultured for 12 days in medium without geneticin and FCS. Supernatant of these production batches were purified by affinity chromatography using a protein G column (Amerscham Pharmacia Biotech AB, Uppsala, Sweden) according to the manufacturer's instructions, and by gel filtration on a 16/60 Superdex 200 L column (Amerscham Pharmacia Biotech AB). Average yield of each of the recombinant proteins produced were: 3, 5.5 and 1.5 μg/ml for rH60-Fc, (scFv 4D5)-Fc and (scFv 511)-Fc, respectively, and 2 and 4 μg/ml for (scFv 511/rH60)-Fc and (scFv 4D5/rH60)-Fc, respectively. A schematic representation of the different homodimeric and bifunctional proteins produced is proposed in Fig. 1.

SDS–PAGE and western blot analyses of the produced bifunctional proteins

Purified bifunctional proteins, namely (scFv 511/rH60)-Fc and (scFc 4D5/rH60)-Fc, were analysed on a 6% SDS–PAGE gel, in comparison with homodimeric control proteins,

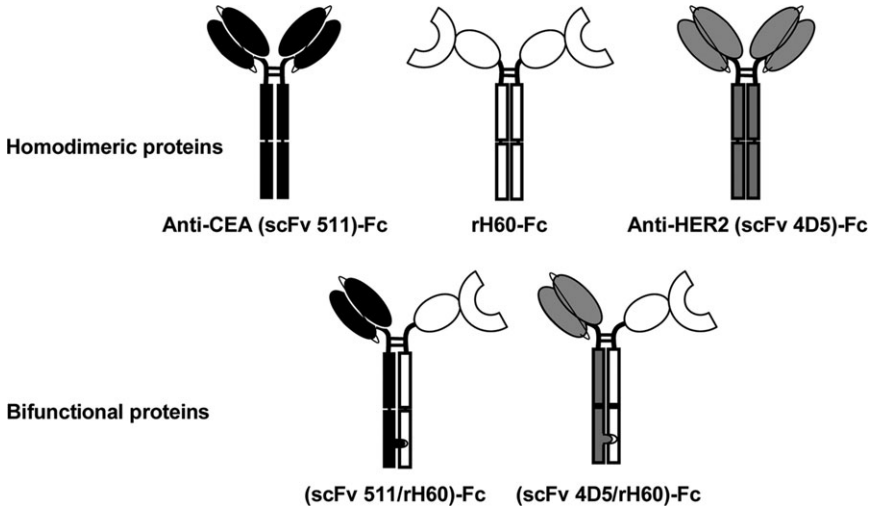

Fig. 1. Schematic representation of the different homodimeric and bifunctional proteins produced. Upper illustrations represent the parental homodimeric proteins (scFv 511)-Fc, rH60-Fc and (scFv 4D5)-Fc. Lower illustrations represent the two bifunctional proteins obtained after knob and hole variants generation, namely (scFv 511/rH60)-Fc and (scFv 4D5/rH60)-Fc.

rH60-Fc and (scFv 511)-Fc or (scFv 4D5)-Fc, under non-reducing and reducing conditions. For western blot analysis, reduced proteins were transferred to a nitrocellulose membrane. The membrane was saturated for 2 h in PBS-0.1% Tween 20 containing 5% non-fat dried milk at room temperature under agitation, incubated after washing with a rat anti-H60 mAb (R&D Systems Inc., Minneapolis, MN, USA) and then with an HRP-conjugated goat anti-rat IgG whole molecule mAb (Sigma Aldrich, St Louis, MO, USA), or directly incubated with an HRP-conjugated goat anti-human IgG Fc specific mAb (Sigma-Aldrich). Finally, membrane was revealed after washing using the ECL western blotting system (Amersham Pharmacia Biotech AB).

Flow cytometry analyses

TAAs and H60 expression on tumor cell lines was assessed by flow cytometry with mouse anti-CEA 511 mAb, humanized anti-human HER2 trastuzumab (Herceptin®, Genentech, Inc., San Francisco, CA, USA) and a rat anti-H60 mAb, revealed by a FITC-conjugated goat anti-mouse IgG Fc specific mAb (Sigma-Aldrich), a FITC-conjugated goat anti-human IgG Fc specific mAb (Sigma-Aldrich) or a FITC-conjugated mouse anti-rat IgG2a Fc specific mAb (BD Pharmingen, San Diego, CA, USA), respectively. NKG2D expression on murine NK cells was assessed with a rat anti-mouse NKG2D mAb (R&D Systems), revealed by a FITC-conjugated mouse anti-rat IgG whole molecule mAb (Sigma-Aldrich). Specificity of the homodimeric proteins (scFv 511)-Fc and (scFv 4D5)-Fc for CEA and HER2, respectively, was assessed as follows: tumor cells (5×10^5) were incubated with each of the two proteins for 1 h at 4°C, washed and then incubated with a FITC-conjugated goat anti-human IgG Fc specific mAb for 45 min at 4°C. Washed cells were finally analyzed on a FACScan (Becton Dickinson, San Jose, CA, USA). Capacity of the bifunctional proteins (scFv 511/rH60)-Fc and (scFv 4D5/rH60)-Fc to coat tumor cells with H60 was assessed as follows: tumor cells were first incubated with each of the two proteins for 1 h at 4°C, washed and then incubated with a rat anti-H60 mAb for 1 h at 4°C, washed and finally detected with a FITC-conjugated mouse anti-rat IgG2a Fc specific mAb. As controls, cells were first incubated with the parental homodimeric proteins, i.e. rH60-Fc and (scFv 511)-Fc or (scFv 4D5)-Fc, washed, then incubated with a rat anti-H60 mAb for 1 h at 4°C, washed and finally incubated with a FITC-conjugated mouse anti-rat IgG2a Fc specific mAb for 45 min at 4°C, or the cells were incubated with the different secondary antibodies. Washed cells were similarly analyzed on a FACScan.

Murine natural killer cells preparation

Murine NK cells used as effectors in chromium release assays were prepared from a C57BL/6 spleen. Briefly, spleen was dilacerated, aggregates were eliminated on a 30 μm filter (Miltenyi Biotec, Auburn, CA, USA) and splenic cells were cultured for 5 days in DMEM/F12 (1/1) supplemented with 2 mmol/l L-glutamine, 10% FCS, penicillin (100 units/ml)/streptomycin (100 μg/ml), non-essential amino acids, 1 μmol/L sodium pyruvate (Life Technologies), 50 μmol/L β-mercaptoethanol and recombinant human interleukin-2 (5000 units/ml) (Proleukin, Chiron, Emeryville, CA, USA). After 5 days, adherent cells, corresponding to lymphokine activated killer (LAK) cells were recovered and used as effectors in chromium release assays.

Chromium-release assays

NK cell-mediated cytotoxicity was measured by the chromium release assay. Tumor target cells (2×10^5 in 100 μl) were co-incubated for 1 h at 37°C with, at the same time, 100 μCi of ^{51}Cr and 5 μg, or less if specified, of the studied bifunctional protein or antibody. After washing, the labeled tumor target cells were plated in a 96-well plate (2000 cells per well) and incubated with LAK cells at different effector-to-target cell ratios (E:T ratios). For the comparison of trastuzumab antibody with our anti-HER2 bifunctional protein, an E:T ratio of 30:1 was used. After incubation for 4 h at 37°C, 100 μl of supernatant was taken to measure the amount of ^{51}Cr released, reflecting the number of target cells

killed by LAK cells. Spontaneous release of ^{51}Cr was determined by incubating the target cells with medium alone. Maximum release was determined by adding 0.1 M HCl. The percentage of specific lysis was calculated as: $100\times$ [(experimental − spontaneous release)/(total − spontaneous release)] (Ogg *et al*., 2000). In control experiments, ^{51}Cr-labeled tumor target cells were pre-incubated without any bifunctional protein, with the irrelevant bifunctional protein, or with the parental homodimeric protein scFv-Fc, and tested with LAK cells pre-incubated or not for 30 min at room temperature with a rat anti-mouse NKG2D mAb used at 20 μg/ml. In the experiment comparing trastuzumab antibody and anti-HER2 bifunctional protein, the spontaneous lysis of SK-BR-3 cells alone was subtracted to each value. In a reverse antibody-dependent cell-mediated cytotoxicity lysis assay, THP-1 cells were incubated with 100 μCi of ^{51}Cr and rH60-Fc, after or without pre-incubation with an FcR blocking reagent which is composed of intact rat IgG2 antibodies (Miltenyi Biotec), to prevent rH60-Fc binding to the Fc receptors expressed on THP-1 cells. They were then incubated with LAK cells.

Results

Production of bifunctional proteins

Supernatants of several G418-resistant HEK 293 clones, expressing the soluble homodimeric and bifunctional proteins designed (Fig. 1), were analysed by western blot to identify the ones producing the attempted proteins in terms of molecular weight (MW). Production supernatant batches for five different selected clones were purified on protein G columns, followed by a gel filtration chromatography.

When loaded on a SDS–PAGE gel under non-reducing conditions, purified (scFv 4D5/rH60)-Fc bifunctional protein appeared as a single band of ∼160 kDa, i.e. at an intermediate MW between the homodimeric rH60-Fc and (scFv 4D5)-Fc proteins, with MW of ∼180 and 120 kDa, respectively (Fig. 2A, left part of the gel). Under reducing conditions, bifunctional protein appeared as two bands of 90 and 60 kDa, corresponding to the respective MW of the reduced parental homodimeric proteins, rH60-Fc and (scFv 4D5)-Fc (Fig. 2A, right part of the gel). Western blot analysis (Fig. 2B), using an HRP-conjugated goat anti-human IgG Fc specific mAb (left membrane) and a rat anti-H60 mAb (right membrane), confirmed that purified bifunctional (scFv 4D5/rH60)-Fc protein comprises the mouse NKG2D ligand H60 associated by an Fc part to the considered scFv. The same results were obtained for the purified bifunctional (scFv 511/rH60)-Fc protein (data not shown).

Functional evaluation of the recombinant homodimeric proteins

Homodimeric anti-CEA or anti-HER2 scFv-Fc proteins were tested for their capacity to bind specifically CEA or HER2 on tumor cells by flow cytometry. Using a FITC-conjugated anti-human IgG Fc specific mAb, an efficient and specific binding of the two homodimeric proteins (scFv 511)-Fc and (scFv 4D5)-Fc to MC38 expressing CEA and B16 expressing HER2 cells, respectively, was obtained (Fig. 3A, right panels). Flow cytometry signals of similar magnitudes to those obtained with specific monoclonal antibodies, 511 mAb and trastuzumab, were observed (Fig. 3A, left panels).

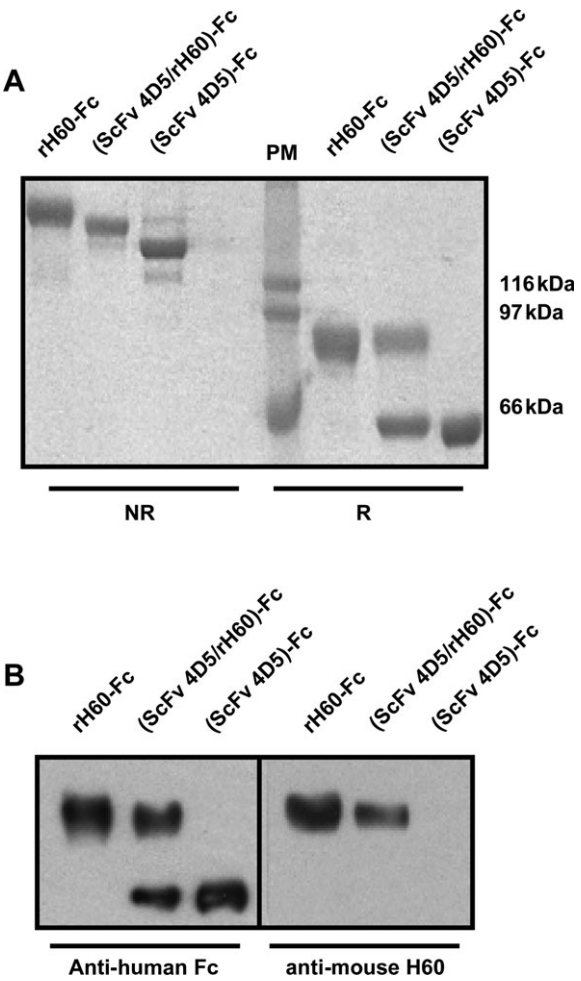

Fig. 2. SDS–PAGE and western blot analyses of the bifunctional protein (scFv 4D5/rH60)-Fc, in comparison with the two parental homodimeric proteins rH60-Fc and (scFv 4D5)-Fc. (**A**) SDS–PAGE analysis. Under non-reducing conditions (left part of the gel) (scFv 4D5/rH60)-Fc appeared as a single band of intermediate MW compared with the bands obtained for rH60-Fc and (scFv 4D5)-Fc. Under reducing conditions (right part of the gel) (scFv 4D5/rH60)-Fc appeared as two bands, each one of MW similar to the single bands obtained for the two related homodimeric parental proteins. (**B**) Western blot analysis. Left membrane, revealed by an HRP-conjugated goat anti-human IgG Fc specific mAb, confirmed that these two different chains contain a human Fc part, and that only one (the heavier) contains H60, as demonstrated in right membrane, first incubated with a rat anti-H60 mAb and then revealed by an HRP-conjugated goat anti-rat IgG whole molecule mAb. Similar profiles were obtained for (scFv 511/rH60)-Fc SDS–PAGE and western blot analyses.

Recombinant H60-Fc protein was tested in a reverse antibody-dependent cell-mediated cytotoxicity (ADCC) lysis assay for its capacity to induce NK-mediated tumor cell lysis. In a chromium release assay using NKG2D-expressing IL-2 activated murine NK cells (LAK cells) as effectors (Fig. 3B), pre-incubation of THP-1 cells, which are known to express FcRs, with rH60-Fc induced a specific increase of THP-1 cells lysis (Fig. 3C, black bars). This lysis was reduced to background level (Fig. 3C, white bars) when THP-1 cells were pre-incubated with an FcR blocking reagent (Fig. 3C, hatched bars), confirming that this lysis was exclusively triggered by the coating of these cells with H60.

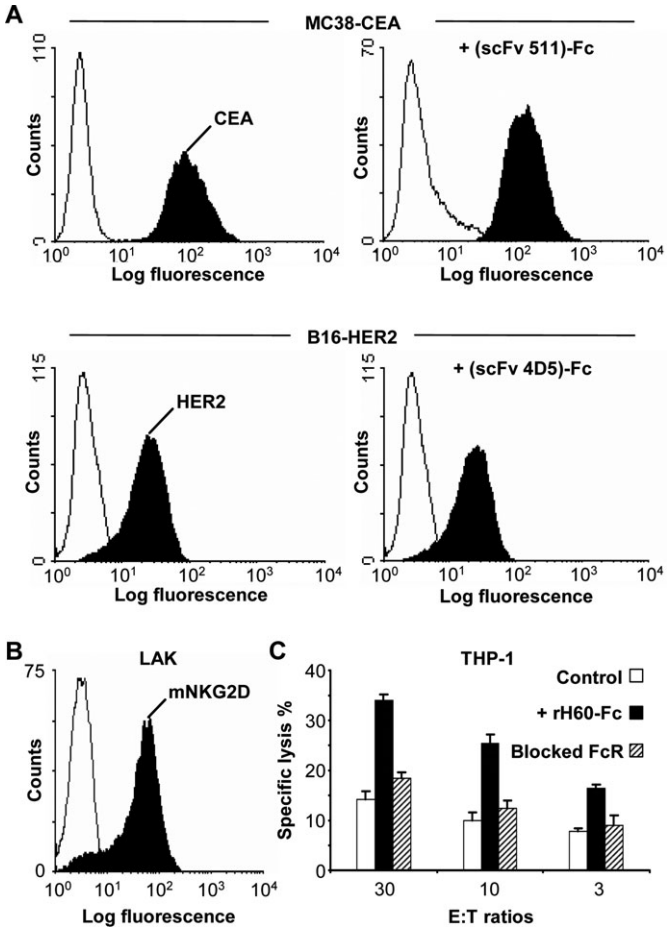

Fig. 3. Functional evaluation of the three homodimeric parental molecules. (**A**) Flow cytometry evaluation of the control molecules (scFv 511)-Fc and (scFv 4D5)-Fc on CEA expressing MC38 cells and HER2 expressing B16 cells. Cells were first incubated with scFv-Fc proteins, then with a FITC-conjugated goat anti-human IgG Fc specific mAb and finally analysed on a FACScan. Each scFv-Fc protein efficiently bound the TAA considered (black-filled peaks, right panels). As control, CEA expression on MC38-CEA cells and HER2 expression on B16-HER2 cells (black filled peaks, left panels) were evaluated using the specific monoclonal antibodies 511 mAb and trastuzumab, respectively. Cells were first incubated with monoclonal antibodies, then with a FITC-conjugated goat anti-mouse IgG Fc specific mAb or with a FITC-conjugated goat anti-human IgG Fc specific mAb, respectively and finally analysed on a FACScan. (**B**) NKG2D expression on murine LAK cells. Cells were first incubated with a rat anti-mouse NKG2D mAb, then with a FITC-conjugated mouse anti-rat IgG whole molecule mAb and finally analysed on a FACScan. LAK cells, obtained after incubation of murine splenocytes for 5 days in presence of rhIL-2 (5000 units/ml), significantly expressed NKG2D (black-filled peak). (**C**) Chromium release evaluation of the control homodimeric rH60-Fc. Pre-incubation of THP-1 cells with rH60-Fc (black bars) significantly increased targets specific lysis by murine LAK cells in comparison with control THP-1 cells (white bars). Addition of an FcR blocking reagent reduced targets specific lysis to control (hatched bars).

TAA binding capacity of the bifunctional proteins

Binding capacity of the two purified bifunctional proteins to tumor cells expressing relevant antigens was assessed by flow cytometry. MC38 cells expressing CEA and B16 cells expressing HER2 (Fig. 4, first column) did not express endogenous H60, as assayed with a rat anti-H60 mAb (Fig. 4, second column). These two cell lines clearly became positive for H60 detection when incubated with the relevant bifunctional protein, (scFv 511/rH60)-Fc for MC38-CEA$^+$ cells and (scFv 4D5/rH60)-Fc for B16-HER2$^+$ cells (Fig. 4, fifth column). In contrast, no detection of this ligand could be observed when tumor cells were first incubated with the different homodimeric proteins (Fig. 4, third and fourth column), neither when tumor cells were first incubated with the irrelevant bifunctional protein (data not shown). These results thus clearly demonstrate the capacity of these two bifunctional proteins to specifically bind and coat tumor cell lines expressing CEA or HER2 with H60.

Cytotoxicity assays

Among all the cell lines negative for endogenous H60 expression tested (the human, LS174T, LoVo and HT29 colon carcinomas; SK-BR-3 breast carcinoma; SK-OV-3 ovarian carcinoma, and the murine, MC38 colon carcinoma; RMA T lymphoma, both transfected with CEA), SK-BR-3 expressing HER2 and CEA-transfected RMA cells (Fig. 5A, black-filled peaks) were chosen for their high resistance to lysis mediated by NK cells. Lysis of these two tumor cell lines was evaluated in the presence of the different bifunctional and control proteins (Fig. 5B–D). In the presence of the (scFv 4D5/rH60)-Fc bifunctional protein, SK-BR-3 cells were much more lysed by murine-activated NK cells (Fig. 5B, black bars). In contrast, no increase of SK-BR-3 cells lysis was observed when these cells, which do not express CEA (Fig. 5A, left panel, black-dotted peak), were incubated with the irrelevant bifunctional protein (scFv 511/rH60)-Fc (Fig. 5B, dark grey bars), neither in presence of the homodimeric protein (scFv 4D5)-Fc (Fig. 5B, light grey bars), excluding any Fc receptor-engagement by the Fc part of the protein and so any antibody-dependent cell cytotoxicity (ADCC). Similarly, (scFv 511/rH60)-Fc bifunctional protein significantly sensitized RMA cells expressing CEA antigen to lysis by activated murine NK cells (Fig. 5D, black bars). Finally, specific lysis induced by the two bifunctional proteins studied was abrogated by pre-incubation of murine NK cells with an anti-mouse NKG2D antibody (Fig. 5B and D, hatched bars). This clearly demonstrated that the increase in NK cell-mediated lysis was only the result of H60 engagement, brought exogenously thanks to these two bifunctional proteins, by the activating receptor NKG2D present on NK cells. Moreover, stimulation of NKG2D by our anti-HER2 (scFv 4D5/rH60)-Fc bifunctional protein seems to be more efficient than activation of Fc receptors by anti-HER2 trastuzumab antibody to induce NK-mediated SK-BR-3 tumor cell lysis, whatever the dose tested (Fig. 5C).

Discussion

The *in vivo* rejection of several types of tumor cells has been previously described when these ones are genetically manipulated to express at the cell surface ligands of NKG2D, an activating receptor expressed on NK and T cells (Cerwenka *et al*., 2001; Diefenbach *et al*., 2001; Friese *et al*., 2003; Oppenheim *et al*., 2005; Busche *et al*., 2006).

In this study, we designed and purified new bifunctional proteins, associating a ligand of NKG2D to anti-tumor antibody fragments, and demonstrated their capacity to specifically coat this ligand on different tumor cells and, more importantly, to induce their lysis by NK cells. Here, actually, we demonstrate that the exogenous coating of tumor cells with NKG2D ligands by our bifunctional proteins can induce

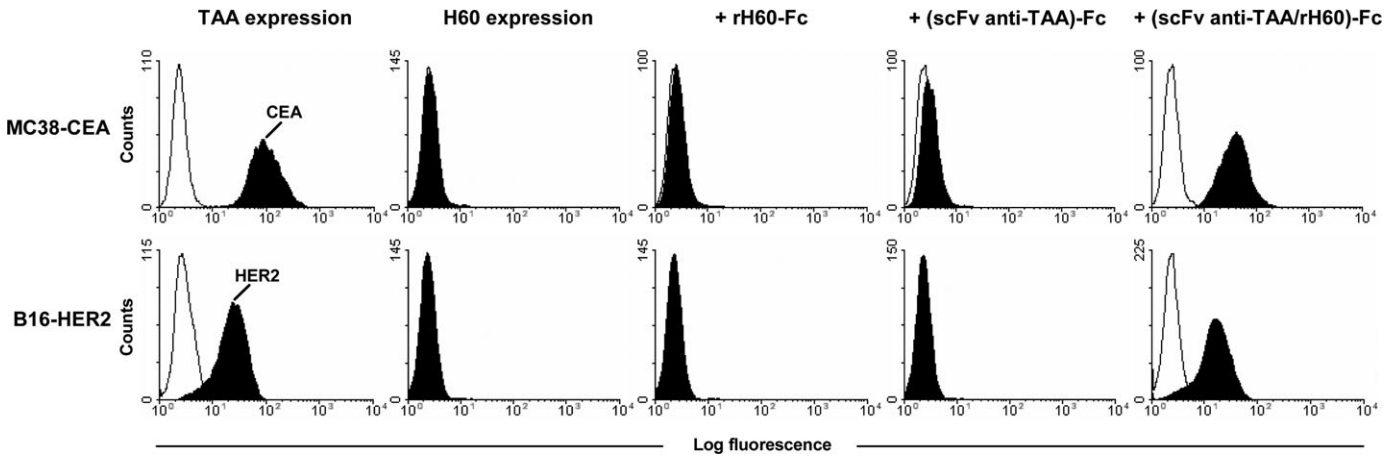

Fig. 4. Flow cytometry evaluation of the two bifunctional proteins produced. CEA, HER2 and H60 expressions on tumor cells were evaluated using anti-CEA 511 mAb, anti-HER2 trastuzumab or a rat anti-H60 mAb, revealed by a FITC-conjugated goat anti-mouse IgG Fc specific mAb, a FITC conjugated goat anti-human IgG Fc specific mAb or a FITC conjugated mouse anti-rat IgG2a Fc specific mAb, respectively. Neither CEA expressing MC38 cells nor HER2 expressing B16 cells (black-filled peaks, first column) did express H60 (black-filled peaks, second column). In a second time, tumor cells were first incubated with the homodimeric parental proteins, rH60-Fc, (scFv 511)-Fc or (scFv 4D5)-Fc, or with the relevant bifunctional molecule, (scFv 511/rH60)-Fc or (scFv 4D5/rH60)-Fc, then with a rat anti-H60 mAb and finally with a FITC-conjugated mouse anti-rat IgG2a Fc specific mAb. As opposed to control molecules (black-filled peaks, third and fourth columns), each bifunctional molecule specifically coated tumor cells with H60 (black-filled peaks, fifth column).

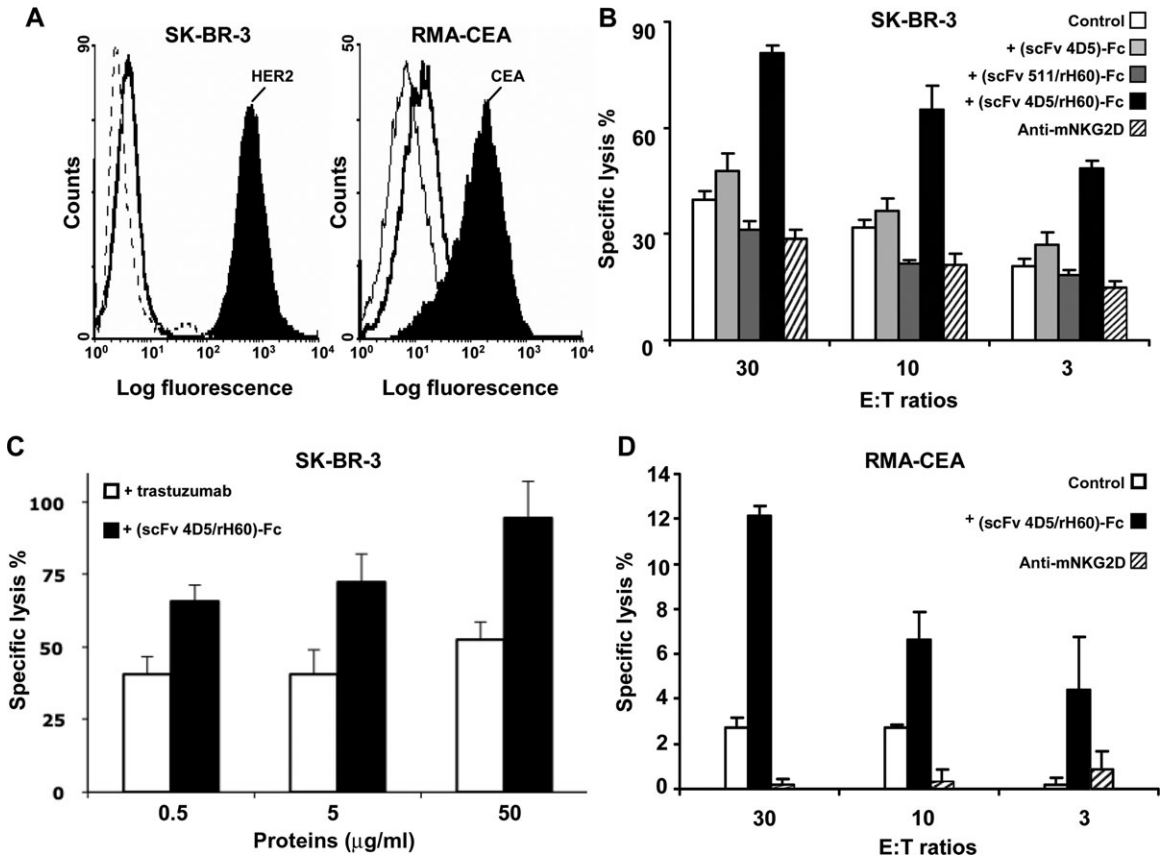

Fig. 5. Antigenic profile and susceptibility to LAK cells lysis, without and with recombinant bifunctional proteins (scFv anti-TAA/rH60)-Fc, of the different tumor cell lines tested. (**A**) CEA, HER2 and H60 expressions on SK-BR-3 and CEA transfected RMA cell lines. Neither HER2 expressing SK-BR-3 cells nor CEA expressing RMA cells (black-filled peaks) did express H60 (black-thick peaks). SK-BR-3 cells neither expressed CEA (black-dotted peak). (**B**–**D**) Functional evaluation in chromium release assay of the bifunctional proteins (scFv 4D5/rH60)-Fc and (scFv 511/rH60)-Fc. (B) Pre-incubation of SK-BR-3 cells with (scFv 4D5/rH60)-Fc (black bars) significantly increased targets lysis by murine LAK cells in comparison with SK-BR-3 alone (white bars), as opposed to SK-BR-3 cells pre-incubated with the homodimeric parental protein (scFv 4D5)-Fc (light grey bars) or with the irrelevant bifunctional protein (scFv 511/rH60)-Fc (dark grey bars). (**C**) Higher cytotoxicity is obtained when SK-BR-3 cells are pre-incubated with (scFv 4D5/rH60)-Fc (black bars) than with Herceptin (traztuzumab; white bars) at every doses tested in presence of murine LAK cells. (D) Pre-incubation of RMA-CEA cells with the bifunctional protein (scFv 4D5/rH60)-Fc (black bars) significantly sensitized targets to lysis by murine LAK cells in comparison with control (white bars). Pre-incubation of murine LAK cells with a rat anti-mouse NKG2D mAb completely prevented tumor cell lysis increase induced by the bifunctional protein considered (B, D hatched bars).

an efficient and specific NK-mediated lysis of these cells, in an NKG2D-dependent pathway.

Bispecific antibodies and bifunctional molecules have been largely exploited in cancer immunotherapy (Koelemij *et al*., 1999; Peipp and Valerius, 2002; Kontermann, 2005), notably in order to favor the recruitment and/or the activation of effector cells to the tumoral site, by immuno-targeted cytokines (Gillies *et al*., 1991; Hoogenboom *et al*., 1991) or agonist antibodies against activating receptors expressed on cytotoxic cells (Van Dijk *et al*., 1989), thanks to antibody structures specific for TAAs. Many strategies have been developed to this aim, such as hybrid hybridomas [quadromas (Milstein and Cuello, 1983)] or chemical conjugation (Karpovsky *et al*., 1984; Robert *et al*., 1996). Nevertheless, production of this type of molecules is frequently limited in terms of quality and/or quantity, two major criteria for their use in humans as therapeutic agents. While quadroma method leads to the formation of many different contaminant forms [up to nine (Carter, 2001)] via uncertain rearrangement of the two heavy chains and the two light chains in presence, chemical conjugation, using for example Fab' fragments of monoclonal antibodies (Robert *et al*., 1999), is often associated with poor production outputs. Genetic engineering allowed the development of other formats such as diabody (Arndt *et al*., 1999), single-chain bispecific antibody (Loffler *et al*., 2000) or single-chain diabody, but unfortunately frequently associated with short half-lives *in vivo* (Kontermann, 2005). To bypass this last point, however, an original study recently proposed the fusion of these small molecules with human serum albumin, strongly improving their pharmacokinetic properties (Muller *et al*., 2007).

In a previous study (Germain *et al*., 2005), we demonstrated the ability of chemical conjugates associating recombinant MICA molecules with Fab' fragments of different monoclonal anti-tumor antibodies to specifically bind TAAs and to stimulate NKG2D-dependent lysis of resistant tumor cells by human NK cells. In the same way, an antitumor activity against human myeloma has been described with a recombinant ULBP2-scFv anti CD138 molecule (von Strandmann *et al*., 2006). Here, in the perspective to produce higher amounts of *in vivo* usable bifunctional proteins, we chose to use the 'knob into hole' strategy, which was developed to produce bispecific antibodies easily purified from culture supernatant (Ridgway *et al*., 1996; Merchant *et al*., 1998), based on the preferential heterodimerization of two distinct heavy chains of human immunoglobulin. Recently, Xie *et al*. applied similarly this technology for the production of a bispecific antibody associating an anti-HER2 scFv to an anti-CD16 scFv to stimulate *in vitro* CD16-dependent tumor cell lysis by PBMC (Xie *et al*., 2005). In contrast to this report, the (scFv anti-TAA)/rH60-Fc bifunctional proteins produced here associate an anti-tumor scFv to a recombinant form of one of the natural ligands of the mouse NKG2D receptor, H60, able as shown for MICA in humans to induce mouse NK cells cytotoxicity when expressed on tumor cells (Cerwenka *et al*., 2000; Diefenbach *et al*., 2000). We postulated that the intermediate affinity of H60 for mouse NKG2D, compared with other mouse NKG2D ligands (O'Callaghan *et al*., 2001; Carayannopoulos *et al*., 2002), is high enough to bind and activate NK cells when coated on tumor cells, in correlation with TAA expression degree, and not sufficiently high to induce a dramatic, or ineffective, NK cell activation in the whole blood circulation. This hypothesis is supported by results previously obtained during the evaluation of the ability of Fab-HLA-A2/Flu conjugates to activate T-lymphocytes, in soluble form or coated on tumor cells (Robert *et al*., 2001). Using Ca_2^+ mobilization assays, we demonstrated that these conjugates, associating monomeric HLA-A2/Flu complexes to anti-TAA Fab' fragments, can efficiently activate CTL only when oligomerized on the surface of tumor cells.

In the present study, we demonstrated that the two produced (scFv anti-TAA)/rH60-Fc bifunctional proteins are able to specifically coat CEA- or HER2-expressing tumor cells with H60, and to induce their lysis by mouse-activated NK cells in an NKG2D-dependent manner.

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