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Single domain antibody-based and linker-free bispecific antibodies targeting FcγRIII induce potent anti-tumor activity without recruiting regulatory T cells

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Running title: anti-FcγRIII x CEA bispecific Fab-like antibodies

Keywords: bispecific antibody, CD16, CEA, Fc receptors, nanobody, single domain antibodies, VHH

Abbreviation list: ADCC, antibody-dependent cell-mediated cytotoxicity; bsAbs, bispecific antibodies; bsFabs, Fab-like bispecific antibodies; CEA, carcinoembryonic antigen; FcγRs, Fcγ receptors; IgG, Immunoglobulins G; mAbs, monoclonal antibodies; NK, natural killer cells; NSG, NOD SCID gamma; PBMC, peripheral blood mononuclear cell; sdAb, single domain antibody.

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ABSTRACT

Antibody-dependent cell-mediated cytotoxicity, one of the most prominent modes of action of anti-tumor antibodies, suffers from important limitations due to the need for optimal interactions with Fcγ receptors. In this work, we report the design of a new bispecific antibody format, compact and linker-free, based on the use of llama single domain antibodies that are capable of circumventing most of these limitations. This bispecific antibody format was created by fusing single domain antibodies directed against the carcinoembryonic antigen and the activating FcγRIIIa receptor to human Cκ and CH1 Immunoglobulin G1 domains, acting as a natural dimerization motif. *In vitro* and *in vivo* characterization of these Fab-like bispecific molecules revealed favorable features for further development as a therapeutic molecule. They are easy to produce in *E. coli*, very stable and elicit potent lysis of tumor cells by human natural killer cells at picomolar concentrations. Unlike conventional antibodies, they do not engage inhibitory FcγRIIb receptor, do not compete with serum Immunoglobulins G for receptor binding, and their cytotoxic activity is independent of Fc glycosylation and FcγRIIIa polymorphism. As opposed to anti-CD3 bispecific anti-tumor antibodies, they do not engage regulatory T cells since these latter cells do not express FcγRIII. Studies in NOD SCID gamma mice xenografted with carcinoembryonic antigen positive tumor cells showed that Fab-like bispecific molecules in the presence of human peripheral blood mononuclear cells significantly slow-down tumor growth. This new compact, linker-free bispecific antibody format offers a promising approach for optimizing antibody-based therapies.

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INTRODUCTION

Almost 30 monoclonal antibodies (mAbs) are now on the market, including fifteen mAbs for cancer therapy in Europe and the US. Most of these mAbs (9/15) are human Immunoglobulin G1 (IgG1) that trigger various mechanisms, such as target signaling inhibition, apoptosis, activation of the classical complement pathway and/or of immune effector cells expressing Fcγ receptors (FcγRs) (1, 2). While it is difficult to assess the contribution of each of these mechanisms in their *in vivo* efficacy, clinical trial results support an important role of antibody-dependent cell-mediated cytotoxicity (ADCC) for both lymphomas and solid tumors (3). The affinity between the Fc portion of human IgG1 and FcγRIIIa (CD16a), an activating receptor mostly expressed by natural killer (NK) cells, monocytes and macrophages, has a profound impact on ADCC exerted by antibodies (4, 5). Correlation of clinical responses to mAb therapy with FcγRIIIa polymorphism has been observed with more favorable response in patients homozygous for the high affinity FcγRIIIa (V158) (6, 7). The fact that about 80% of the Caucasian population is homozygous (F/F) or heterozygous (F/V) for low affinity FcγRIIIa (F158) is likely an important issue for antibody-based immunotherapy. Moreover, the *in vivo* efficacy of therapeutic antibodies is hindered by the presence of fucose residues in the N-glycosylation motif (N297 residue) of the Fc region that markedly decreases their affinity for FcγRIIIa (8-10). Therapeutic antibodies also compete with serum IgG for binding to the high-affinity FcγRI and to the intermediate affinity FcγRIIIa. Strikingly, most antibodies have to be injected at high doses to reach a serum concentration between 10 to 100 µg/ml, while they usually elicit a maximal cytotoxic activity at 10 ng/ml in *in vitro* ADCC experiments. Competition with patient IgG has been proposed to account for this large difference of concentration (11). Finally, the use of therapeutic antibodies may be hampered by their ability to engage the inhibitory FcγRIIb receptor. FcγRIIb possesses an inhibitory ITIM motif in its cytoplasmic domain, and has been shown to impact the anti-tumor efficacy of therapeutic antibodies in mouse models (12). Several approaches, such as site-directed mutagenesis, computational structure-based design,

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glycosylation engineering, and selection-based methods, have been used to increase Fc binding to activating receptors (FcγRI, FcγRIIa and FcγRIIIa), and to decrease their interaction with inhibitory FcγRIIb (3, 13, 14). Variants possessing up to 100-fold increased affinity for FcγRIIIa, resulting in 100-fold enhanced *in vitro* ADCC (3, 15) have been selected. Attractive alternative to recruit and activate effector cells is to use bispecific antibodies (bsAbs) capable of simultaneously binding to a target antigen and to an activating receptor such as FcγRI or FcγRIIIa (16, 17). Although the *in vitro* and *in vivo* anti-tumor efficiencies of bsAbs has been largely demonstrated over the last two decades, their development has been severely hindered by different factors, including immunogenicity and the difficulty to efficiently produce large amounts of active molecules (18). With the development of antibody engineering, several innovative recombinant formats have been proposed, including diabodies, tandem scFvs and minibodies (17, 19, 20), and some of these molecules are being tested in the clinics (21). However, most described recombinant bsAbs heavily rely on the use of peptide linkers. Although these linkers have obvious advantages in terms of antibody engineering, their hydrophilic nature makes them prone to proteolytic cleavage, potentially leading to production issues, poor antibody stability, aggregation and increased immunogenicity (22). Llama single domain antibodies (sdAbs), derived from heavy-chain antibodies naturally devoid of light chains (23) are small (13 kDa), well-expressed and extremely stable fragments. They are highly homologous to the VHIII subset family of human VH (24, 25), which should result in a low antigenicity in human, if any, and which makes the humanization process easier if needed (26). Due to their small size and single domain nature, sdAbs can recognize epitope usually not accessible to conventional antibodies. These fragments represent ideal molecular building units for bsAb construction (27). In a previous work, we have isolated two sdAbs capable of binding and activating FcγRIIIa, while showing no binding to FcγRI, FcγRIIa or inhibitory receptor FcγRIIb (28) as well as one sdAb able to bind carcinoembryonic antigen (CEA) with high specificity (29), a well-characterized tumor marker of interest for mAb-based cancer therapy.

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In the present work, we have exploited the natural affinity of human CH1 and Cκ IgG domains as an heterodimerization motif (30) to produce Fab-like bispecific antibodies (bsFabs), devoid of linker and capable of inducing a strong cytotoxicity against CEA positive tumor cells by human NK cells *in vitro,* and of inhibiting tumor growth in an *in vivo* mouse model.

MATERIALS AND METHODS

Expression vector design and generation of recombinant antibodies

Bicistronic expression vectors were designed to express bsFab molecules in periplasm. (for details see Supplementary Methods).

Production and purification of antibodies

BsFabs were purified from periplasm of *E. coli* DH5α and sdAb C17-Fc from culture supernatant of HEK293T cells (for details see Supplementary Methods).

Cell lines

Jurkat (ATCC TIB-152), LS174T (ATCC CL-188), BXPC3 (ATCC CRL-1687), LAN-1 (16), HT29 (ATCC HTB-38) and HEK293T (ATCC CRL-11268) cells purchased from ATCC were cultured in RPMI 1640+ Glutamax-I medium (Invitrogen) supplemented with 10 % FBS (PAA). SKOV3 (ATCC HTB-77) and CO115 (an established CEA-negative subclone from a human colon carcinoma cell line) (31, 32) were cultured in DMEM + Glutamax-I medium (Invitrogen) supplemented with 10 % FBS (PAA). MC38 and C15.4.3.AP (MC38-CEA) cells are murine colorectal cancer cells (33). MC38-CEA cells are MC38 cells transfected with a cDNA encoding CEA. MC38 were cultured in DMEM + Glutamax-I medium supplemented with 10 % FBS and MC38-CEA in DMEM + Glutamax-I medium supplemented with 10 % FBS and 0.5 mg/ml of geneticin. Jurkat-huFcγRIIIa/γ cells are Jurkat lymphoma T cells transfected with a cDNA encoding the extracellular domain of FcγRIIIa fused to the

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transmembrane and intracellular domains of the γ chain (generous gift of Prof. E. Vivier, Marseille, France) (34). These cells were cultured in RPMI 1640 + Glutamax-I medium supplemented with 10 % FBS and 0.5 mg/ml of geneticin. SKOV3-CEA-Luc are SKOV3 human ovarian carcinoma transfected with a cDNA encoding the extracellular domain of CEA and with a cDNA encoding luciferase; cells were cultured in DMEM + Glutamax-I medium supplemented with 10 % FBS, 0.5 mg/ml of geneticin and 0.4 mg/ml hygromycin B. All cell lines were grown in a humidified 37°C incubator containing 5% CO_2.

All cell lines purchased from ATCC were not cultured for more than 2 months. Other cell lines used in this study have not been authenticated.

Human NK cell purification

Human peripheral blood mononuclear cells (PBMCs) were isolated from fresh peripheral blood of healthy donors (Etablissement Français du Sang, Marseille and Hôtel-Dieu hospital, Paris, France) by Ficoll LSM 1077 (PAA) gradient centrifugation. Cells were counted and tested for viability by trypan blue exclusion assay. NK cells were isolated by depleting non-NK cells using the NK cell isolation kit (Miltenyi Biotec) as described by the manufacturer.

Flow cytometry assays

Increasing concentrations of bsFabs (1.2 to 100 nM) were used for staining $2x10^5$ Jurkat-huFcγRIIIa/γ or $2x10^5$ MC38-CEA cells in 50 µl PBS supplemented with 2% bovine serum albumin at 4°C for 1 h. Bound bsFabs were detected by incubating cells with either an anti-flag mAb M2 (4 µg/ml) (Sigma Aldrich) or anti-c-myc mAb 9E10 (10 µg/ml) (Santa Cruz Biotechnology) for 30 min at 4°C followed by a FITC-conjugated goat anti-mouse IgG $F(ab')_2$ (10 µg/ml) (Jackson ImmunoResearch) incubated 30 min at 4°C. After washing, labeled cells were analyzed by flow cytometry using a FACS Calibur (BD biosciences) or MACSQuant (Miltenyi Biotec) flow cytometers.

For serum stability assays, bsFabs were incubated at 1 µM in 90% human serum (not heat-inactivated) (Sigma Aldrich) at 37°C for various periods of time (7 to 168 h). Samples

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were collected at different time points, frozen and kept at -20°C. The remaining binding activity of the samples after storage was determined by flow cytometry using non-saturating bsFab concentrations (10 nM for bsFab C21 and 100 nM for bsFab C28 against Jurkat-huFcγRIIIa/γ, and 100 nM for both against MC38-CEA).

For binding competition assays with serum IgG, Jurkat-huFcγRIIIa/γ cells were pre-incubated with increasing concentrations of human serum (12.5 to 100 %) at 4°C for 1 h then incubated with a constant amount of bsFab C21 (4 nM) or bsFab C28 (36 nM) or biotinylated sdAb C17-Fc (200 nM). Bound antibodies were detected using either mouse anti-flag mAb M2 (4 µg/ml) (Sigma Aldrich) followed by an incubation with FITC-conjugated goat anti-mouse IgG $F(ab')_2$ (10 µg/ml) (Jackson ImmunoResearch Laboratories) for bsFab labeling or streptavidin-PE diluted 1:10 (Beckman Coulter) for biotinylated sdAb C17-Fc labeling. In another setting, Jurkat-huFcγRIIIa/γ cells were pre-incubated with constant concentrations of human serum (90 %) or PBS 1X at 4°C for 1 h and then incubated with various amounts of bsFab C21 or bsFab C28. Bound antibodies were detected as described previously.

For CEA detection, cells were pre-incubated with anti-CEA sdAb C17 (10 µg/ml) for 1 h at 4°C. Bound antibody was detected using mouse anti-6his mAb (1 µg/ml) (Novagen) followed by incubation with FITC-conjugated goat anti-mouse IgG $F(ab')_2$ (10 µg/ml) (Jackson ImmunoResearch).

Rosette formation assay

5×10^4 MC38-CEA and 25×10^4 Jurkat-huFcγRIIIa/γ were co-cultured in RPMI 1640+ Glutamax-I medium supplemented with 10 % FBS and 0.5 mg/ml of geneticin with or without bsFabs (2 µg/ml) at 37°C for 4h. Rosette formation was then observed by optical microscopy.

IL-2 secretion assay

Jurkat-huFcγRIIIa/γ cells (10^6) (or non-transfected Jurkat cells as negative control) were incubated for 18 h in EMS medium, containing 5% FBS, 10 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich) and bsFabs (50 nM) or anti-FcγRIII mAb 3G8 (35) used at

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50 nM as a positive control. In some experiments, anti-flag M2 mAb was added (4 µg/ml) to promote bsFab dimerization. In some experiments, 10^5 LS174T cells (or LAN-1 cells used as a negative control) were used as target cells. After incubation, cells were centrifuged for 5 min at 1200 x *g* and human IL-2 present in the culture medium was measured by ELISA using READY-SET-GO human IL-2 kit (eBioscience).

ADCC assay

Target cells (BxPC3, HT29, LS174T, SKOV3-CEA-Luc or SKOV3; $5x10^3$ cells/well) were mixed with $5x10^4$ freshly isolated human NK cells (Effector/Target ratio: 10:1). Variable concentrations of bsFabs or sdAb C17-Fc were added to the cells in a final volume of 200 µl. All procedures were done in triplicate with different donors. Following overnight incubation at 37°C, target cell viability was quantified with Cell titer Glo viability assay (Promega) according to manufacturer's protocol. The formula used for % lysis calculation was: % lysis= [T-(TEAb-E)]/[T- Tdead]×100 (T=Target, E= effector, Tdead= Target lysed with 1% triton solution, TEAb=Target + Effector + Antibody luminescent signal).

To investigate the effect of soluble CEA, target and effector cells were incubated as previously described with variable concentrations of bsFab and constant concentration (0.1 µg/ml or 1 µg/ml) of soluble CEA (Chemicon). FcγRIIIa genotyping of donors was performed as described (36). Dose response curves were treated by non-linear regression analysis using Prism software (GraphPad Software). Data were expressed as mean ± SEM.

Biodistribution assay.

Swiss female nude mice (Charles River Laboratories, France) bearing human colon carcinoma LS174T (CEA positive) or CO115 (for CEA negative control) tumors on the right and left flanks respectively were injected i.v. with ^{125}I labeled bsFab C21 (2.8 10^5 Bq, 3 µg) 14 days after s.c. tumor cell injection. Four groups of three mice were sacrificed at 3, 6, 15 or 24 h post-bsFab injection. Blood was collected and tissues and organs were weighted and dissected at each time point. Radioactivity was quantified using a γ- counter COBRAII

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(Packard) and results were expressed as % Injected Dose/g tissue (% ID/g). Mice housed in individual cages with laboratory chow and water. All experiments were performed according to the French Animal Protection Law with the permission from the local authorities.

Tumor growth inhibition.

Seven-week-old female NOD SCID gamma (NSG) mice (Charles River Laboratories, France) (n=8/group) were xenografted subcutaneously with human pancreatic carcinoma CEA positive BxPC3 ($2x10^6$ cells/mice) on the right flank and were injected with freshly purified human PBMCs ($30x10^6$/mice) (i.p.) and bsFab C21 (100 µg/mice) or PBS (i.p.) at Day 0. At Days 1 and 2, mice were injected i.p. with bsFab C21 (50 µg/mouse) or PBS. Tumor growth was measured with a Vernier caliper during 28 days to permit calculation of tumor volumes ($V=(L\times W^2)/2$, where L and W were length and width, respectively). All animals were sacrificed at the end of experiment in accordance with institutional guidelines. Mice housed in individual cages with laboratory chow and water. All experiments were performed according to the French Animal Protection Law with the permission from the local authorities.

Statistical analysis.

Data are presented as mean ± SEM. Cytotoxicity assays were statistically analyzed using a one-way ANOVA test. In vivo studies were analyzed using Student's t test. For all tests, $P< 0.05$ was considered statistically significant.

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RESULTS

BsFab design and production.

For construction of bsFabs, we designed a bicistronic expression vector allowing the co-expression of one sdAb directly fused to the human Cκ chain and another sdAb directly fused to the human CH1 chain in the periplasm of *E. coli* (Fig. 1A). We used the anti-CEA sdAb C17 previously characterized (Kd: 8 nM) (29) for targeting CEA positive cells and two different anti-FcγRIII sdAbs for targeting FcγRIIIa positive effector cells. The anti-FcγRIII sdAb C28 was previously shown (28) to display an apparent affinity for FcγRIII (Kd: 82 nM) in the range of the Fc portion of human IgG1 (>100 nM) whereas the anti-FcγRIII sdAb C21 displays a higher affinity of Kd: 10 nM for FcγRIII (28). The resulting bispecific Fab-like fragments were named bsFab C21 and bsFab C28, respectively (Fig. 1B). BsFabs were produced in *E. coli* periplasm and affinity-purified by anti-CH1 followed by anti-Cκ columns. As shown in Figure 1C, SDS-PAGE analyses of the second step of bsFab C21 purification reveal, under non reducing conditions, one band in the expected range of size (50-55 kDa) due to heterodimerization.

In parallel, we developed a human Fc fusion protein, sdAb C17-Fc, by fusing anti-CEA sdAb C17 to the hinge, CH2 and CH3 domains of IgG1 (Fig. 1B). Anti-CEA sdAb C17–Fc was produced by transient transfection in HEK293T cells and purified from culture supernatant by affinity chromatography on a G-protein column. This construction was designed to provide a molecule with the same epitope specificity of bsFabs, but triggering ADCC via a conventional Fc/FcγR interaction.

Binding analysis of bsFabs.

Binding specificities of bsFabs to FcγRIIIA and CEA were analyzed by flow cytometry using Jurkat-huFcγRIIIA/γ and MC38-CEA cells, respectively. As shown in Figure 2A, both bsFabs efficiently bound to these cells in a dose-dependent manner. To ensure that signals were generated by heterodimers, bsFab binding to MC38-CEA cells was assessed using the c-

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myc tag fused to the chain bearing the anti-FcγRIIIA sdAb and vice-versa. A marked shift in mean fluorescence intensity was observed when comparing bsFab C21 and bsFab C28 binding to Jurkat-huFcγRIIIA/γ, likely related to the 8-fold difference of affinity between these anti-FcγRIIIa sdAbs (28). As expected, bsFab C21 and C28, both bearing the same anti-CEA sdAb C17 exhibited a similar binding profile to MC38-CEA cells. No binding to MC38 and Jurkat cells was observed with any of these bsFabs (data not shown).

The ability of bsFabs to simultaneously bind to MC38-CEA and Jurkat-huFcγRIIIa/γ cells was demonstrated by rosette formation between the two types of cells. The addition of bsFabs in MC38-CEA and Jurket-huFcγRIIIa/γ co-culture led to rosette formation between Jurkat-huFcγRIIIa/γ and MC38-CEA cells (up to nine Jurkat-huFcγRIIIa/γ cells bound to one MC38-CEA cell have been observed) (Fig. 2B). In the absence of bsFab, no rosette formation was observed.

In vitro stability of bsFabs in human serum.

In vitro stability was analyzed by incubation of each bsFab in human serum at 37°C for up to 168 h and subsequent examination of CEA and FcγRIIIa binding by flow cytometry. Figure 2C shows that bsFab C21 and bsFab C28 retain their full binding on both target and effector cells, even after 168 h of incubation, demonstrating a remarkable stability. SDS-PAGE and Western-blot analyses showed the absence of breakdown products of both bsFabs (data not shown), further confirming that both bsFabs are stable up to 168 h in physiological conditions.

Competition with human IgG for FcγRIIIa binding.

The potential impact of serum, containing high amount of endogenous IgG, on FcγRIIIa binding by bsFabs was explored. Jurkat-huFcγRIIIa/γ cells were pre-incubated in the presence of various volumes of human serum and then stained with bsFabs. SdAb C17-Fc was used as control. As shown in Supplementary Fig. 1A, both bsFabs retain binding in the presence of human serum while sdAb C17-Fc binding is, as expected, strongly inhibited.

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Competition assays were also performed on Jurkat-huFcγRIIIa/γ cells in the presence of 90% human serum and various concentrations of bsFabs (Supplemental Fig. 1B). The interaction of bsFabs with FcγRIIIa was not hindered by the presence of human IgG, even at low bsFab concentrations. The same results were observed with purified human polyclonal IgG in a competition assay (data not shown). These results are in agreement with our previous data (28) showing that sdAbs C21 and C28 recognize FcγRIIIa epitopes different from those bound by human IgG Fc portion.

IL-2 release assays.

One possible side effect of antibodies targeting activating receptors is the activation of effector cells in the absence of target cells, potentially leading to a systemic inflammatory response (cytokine storm). To evaluate this possibility, Jurkat and Jurkat-huFcγRIIIa/γ cells were cultured in the presence of monovalent bsFab. Bivalent anti-FcγRIIIa mAb 3G8, able to crosslink the receptor and trigger activation was used as positive control. Monovalent bsFabs only led to a marginal IL-2 secretion, indicating that FcγRIIIa positive cells cannot be activated in the absence of target cells (Fig. 3), unlike bivalent anti-FcγRIIIa mAb 3G8. The low difference in IL-2 secretion observed between the two bsFabs could be explained by their different affinity for FcγRIIIa and/or different epitopes. The addition of a monoclonal antibody targeting the Flag tag fused to the C-terminus of Ck domain and thus leading to bsFab dimerization led to IL-2 secretion as expected (Fig. 3). These results demonstrate that monovalent bsFab do not induce effector cell activation in the absence of target cells.

However, the co-culture of Jurkat-huFcγRIIIa/γ with CEA positive LS174T tumor cells in the presence of bsFab C21 or C28 could lead to IL-2 secretion. This activation was not seen in the absence of bsFab, or when using non-transfected Jurkat cells or CEA negative LAN-1 human tumor cells in the presence of bsFab, as negative controls (Fig. 3). These results demonstrate in vitro that the efficient activation of effector cells by monovalent bsFabs is dependent on the presence of cells expressing the tumor antigen, and does not lead to unwanted cytokine release by FcγRIII positive effector cells in the absence of tumor cells.

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BsFab-dependent NK cell-mediated cytotoxicity.

The cell-mediated cytotoxicity of bsFabs was investigated using a panel of four different human tumor cells expressing various levels of CEA, as shown in Figure 4A. Freshly purified human NK cells were used as effector cells at an E:T ratio of 10:1. Dose-response curves allowed the determination of EC_{50} values (Fig.4B) and percentages of maximal lysis. Both bsFabs triggered a strong cytotoxicity against SKOV3-CEA cells in a dose-dependent manner but were ineffective when CEA-negative SKOV3 cells were tested (Fig. 4B). Cells from all CEA positive tumor cell lines were killed with a similar efficiency, leading to EC_{50} in the 10 pM range using three different donors. In all three cases, a slightly higher efficiency was observed with bsFab C21, potentially due to its higher affinity for FcγRIIIa (Table 1).

Sensitivity to FcγRIIIa polymorphism.

Since the efficiency of ADCC is affected by FcγRIIIa polymorphism at position 158, experiments were performed using SKOV3-CEA cells as target cells and purified NK cells from FcγRIIIa 158V/V, F/F or V/F donors and bsFab C21 or sdAb C17-Fc. As shown in Figure 5A, the cytotoxic activity triggered by sdAb C17-Fc was lower than that of bsFab C21, both in terms of EC_{50} (values in the nM range) and of maximal lysis (< 50%), despite its bivalent binding to CEA. This result is likely due to the weaker interaction of the Fc portion of sdAb C17-Fc with FcγRIIIa, as compared to bsFab C21. More importantly the lytic activity of sdAb C17-Fc varied according to FcγRIIIa polymorphism, NK cells from 158F/F donor being significantly less efficient than NK cells from 158V/V and 158V/F donors. In contrast, the cytotoxic activity of bsFab C21 was very similar whatever the *FCGR3* genotype of the donor. These results confirm that the epitope recognized by sdAb C21 is not affected by the nature of residue 158, and demonstrate that bsFab can trigger highly potent tumor killing at very low doses, independently of FcγRIIIa polymorphism.

Sensitivity to the presence of soluble CEA.

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It is well known that CEA can be released by phospholipases from the cell surface through cleavage of its glycosyl-phosphatidyl-inositol-linkage, leading to the appearance of a soluble form in the blood (37). It represents a valuable tumor marker because its serum level correlates disease progression. However, this soluble form of CEA could also interfere with antibody-based immunotherapy by competing with membrane-bound CEA for antibody binding. Thus, we performed ADCC assays using BxPC3 tumor cells in presence of intermediate (0.1 µg/ml) or high (1 µg/ml) concentrations of soluble CEA. As shown on Figure 5B, soluble CEA has a detectable but moderate effect on the anti-tumor efficacy of bsFab C21. EC_{50} values increased to around 100 pM at the highest CEA concentration, although it did not affect the maximal lysis value. These results suggest that circulating CEA should not critically impact the effect of bsFab therapy, in particular in clinical settings where levels of soluble CEA are low.

Biodistribution of bsFab in xenografted mice.

The biodistribution of bsFab C21, which exhibited the most favorable *in vitro* characteristics in terms of cell binding and cytotoxicity, was studied using xenografted nude mice. BsFab C21 was labeled with ^{125}I and was injected i.v. in mice bearing both LS174T cells (CEA positive colorectal tumor) (right flank) and CO115 cells (CEA negative colorectal tumor) (left flank, as negative control). Mice were sacrificed 3, 6, 15, 24 h post bsFab injection to quantify the presence of bsFab in blood, various organs and tumors. Best tumor-to-normal organ ratios were observed 15 h post-injection (Fig. 6A) with more than 4.5% ID/g of bsFab being localized in the CEA positive tumor, whereas less than 0.8% ID/g remained in the blood or other organs and CEA negative tumor, except for kidneys (around 1%). Non-tumor infiltrated organs with noticeable level of bsFab were kidneys, probably due to bsFab clearance.

BsFab *in vivo* efficacy in xenografted mice.

To determine if the potent *in vitro* activity of bsFab would translate into inhibition of tumor growth *in vivo*, an adoptive transfer model was used. At Day 0, NOD SCID gamma (NSG)

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mice were engrafted s.c. with CEA positive human pancreatic cancer BxPC3 cells and with human PBMCs from healthy donors and treated with 100 µg of bsFab C21 by i.p injection. Injections of bsFab C21 (50 µg) were performed the two following days (cumulative dose/mouse= 200 µg). Mice treated only with either PBS, bsFab C21 or PBMC + PBS were used as control groups (n=8). As shown in Figure 6B, a significant tumor growth inhibition was visible in mice treated with PBMCs and bsFab C21 as compared with PBS or bsFab treatment alone. A slight reduction of tumor growth was observed with human PBMCs treatment alone.

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DISCUSSION

Redirecting the cytotoxic potential of leukocytes to eliminate tumor cells has been a major impulse for the development of bsAbs for cancer immunotherapy. FcγRIIIa is an attractive candidate to recruit effector cells. It is strongly expressed by NK cells that play a critical role in the induction of ADCC, one of the major modes of action of anti-tumor antibodies. In addition, FcγRIIIa is also expressed on monocytes and macrophages that are important actors of anti-tumor immunity. Anti-FcγRIIIa bsAbs have the potential to bypass several important limitations faced by therapeutic antibodies. However, difficulties in producing sufficient amounts of functional and stable bsAbs at reasonable costs have strongly hampered their development for many years, although early clinical trials had been promising (38, 39). Molecular constructs, such as bispecific diabodies, single chain diabodies, tandem scFvs and F(ab') obtained by recombinant DNA technology, might circumvent the above limits. These formats can be expressed in bacteria or mammalian cells, and may show benefits due to their small size, although they can present manufacturing challenges related to their production and *in vitro* and *in vivo* stability. Notably, the variable and unpredictable expression yields observed for different scFvs hamper their widespread use. Moreover, the linker connecting the VH and VL in the scFv and that between different scFvs can provoke aggregation of the molecule. Shortening the linkers to produce diabodies does not always guarantee success, since the linkers can induce an erroneous angle between the VH-VL pair, thus forming poorly functional antibodies (40).

An important motivation of the work reported herein was therefore to design a new linker-free bispecific format to avoid aggregation and stability issues and easy to produce. Thus, we have studied a format relying on the use of llama sdAbs that possess valuable functional and structural properties and of human CH1/Cκ domains as heterodimerization motif, to produce Fab-like bispecific antibody fragments.

As a proof of concept, we constructed bsFabs targeting CEA positive tumor cells and human FcγRIIIa positive immune effector cells, using previously isolated anti-FcγRIIIa sdAbs

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binding epitopes located outside the Fc binding site, and an anti-CEA sdAb chosen for its affinity comparable to conventional anti-CEA mAbs, despite its lack of bivalency.

These molecules were easily produced in *E. coli* with routine yield ranging from 0.5 and 2 mg/l of culture in non-optimal shaker flask conditions in an academic laboratory, which compares favorably with other formats such as tandem scFv (the BiTE format) requiring eukaryotic cells for production (41). The high stability of these bsFabs conferred by the absence of linkers is illustrated by the fact that they retained their full binding activity at least for 7 days in human serum at 37°C, even at low concentration.

One of the key issues with bsAbs is their capacity to trigger an efficient cell-mediated cytotoxicity. Our *in vitro* data demonstrated a strong and specific NK cell-mediated lysis of CEA expressing tumor cells (from pancreatic and colorectal cancers) at picomolar concentrations, i.e., orders of magnitude lower than conventional mAbs (10, 42). These results suggest that a high affinity for FcγRIIIa translates into improved cytotoxic activity of effector cells *in vitro*, a finding also described with mutated and glyco-engineered antibodies (43, 44). As opposed to classical mAbs such as rituximab, target antigen density did not significantly impact bsFab-mediated ADCC as shown by the use of tumor cell lines expressing different amounts of CEA. The significant inhibition of tumor growth observed upon human PBMCs adoptive transfer to CEA positive tumor-bearing NSG mice supports the *in vivo* efficacy of the bsFab format. The low level of INF-γ secretion by NK cells induced by bsFab in the absence of target cells *in vitro* suggests in addition that bsFabs should not induce any systemic activation of NK cells *in vivo* and should not provoke cytokine storm.

Importantly, by carefully choosing the anti-FcγRIII sdAb, we have constructed bsFabs whose action is insensitive to FcγRIIIa polymorphism and that do not bind inhibitory FcγRIIb.

Because of their high efficiency and the absence of competition with serum IgG, these molecules should be clinically active at much lower concentrations than those used for conventional mAbs as already reported for the bispecific T-cell engager format (21). These properties, added to the possibility to produce easily these fragments in *E. coli* in large

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amounts, have the potential to substantially reduce the high manufacturing costs associated with mAb therapy.

The retargeting of FcγRIII expressing cells has another advantage over $CD3^+$ T cell retargeting by bsAb. Anti-CD3 bsAbs have been shown to lead to the recruitment and the activation of regulatory T cells (Treg) (45) possibly leading to a downregulation of the anti-tumor response. FcγRIII retargeting does not activate the Treg subset, and does not trigger immunosuppression.

A possible limitation of anti-CEA bsFabs might be the presence of shed extracellular domains of CEA in the serum of cancer patients, possibly blocking the anti-CEA binding site. Here, we demonstrate that bsFab-dependent cytotoxicity is only slightly impacted by concentration of soluble CEA exceeding concentrations found in the majority of cancer patients (46). Thus, as already suggested by some clinical studies using anti-CEA antibodies (47, 48), soluble CEA should not have a significant impact on the clinical efficacy of the bsAb.

Another important issue faced by therapeutic antibody fragments devoid of Fc portion is their pharmacokinetics property. As a likely consequence of their small size and of their lack of interaction with FcRn, Fab fragments have a mean retention time in the body 35-fold lower than full-length IgG (49). Despite this disadvantage, it has been shown that their smaller size as compared to whole IgG limits their retention in non-targeted organs and increases their tumor penetration. Consistent with those data, biodistribution experiments performed in nude mice bearing CEA positive tumor xenografts revealed a rapid increase of CEA positive to CEA negative tumor ratio up to almost 7-fold at 15 h, demonstrating a specific and efficient tumor targeting of bsFabs. Nevertheless, the bsFab format authorizes the linker-free addition of single domain antibodies at the C-terminus of CH1 or Cκ domains (data not shown). It can therefore be envisaged to construct tri-specific formats by adding an anti-human serum albumin sdAb, a method that has been shown to significantly increase tumor retention and half-life (50, 51).

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In conclusion, we have designed a new class of single domain-based bispecific antibodies that combines easy production, high stability and potent *in vitro* ADCC activity irrespective of FcγRIIIa polymorphism, glycosylation and competing endogenous IgG issues. Altogether, these properties associated to a significant cytotoxic activity in a preclinical *in vivo* model confer to this bispecific Fab-like antibody format the potential to lead to a new generation of highly active therapeutic antibodies for tumor therapy.

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Table 1. Half maximal effective concentration of bsFabs in ADCC assay

Cell lines	EC_{50} (pM)	
	bsFab C21	bsFab C28
SKOV3-CEA	4 ± 0.2	6 ± 0.5
LS174T	3 ± 1.5	4 ± 0.4
BxPC3	8 ± 3	17 ± 6
HT29	5 ± 1.5	27 ± 10

FIGURE LEGENDS

Figure 1. Antibody formats.

A) A biscistronic construct was designed to allow the production of bsFab in the periplasm of *E. coli*. Dark grey: portion of hinge (H) and Cκ and CH1-3 constant domains of human IgG1. Light grey: sdAbs. Ptac: tac promoter. RBS1 and RBS2: ribosome binding sites. 55: Optimized PelB signal sequence. WT: PelB wild-type signal sequence. F: flag tag. C: c-myc tag. 6H: hexahis tag. Pß-act: chicken beta-actin promoter. Kozac: ribosome binding site. µp: micro phosphatase signal sequence. B) Scheme of antibody formats used in this study. C) Analysis of purified bsFabs by Coomassie blue stained SDS-PAGE after affinity chromatography on IgG-CH1 matrix followed on LC-kappa matrix. MW: molecular weight ladder, FT: flow through.

Figure 2. BsFab binding analysis.

A) BsFabs were incubated with antigen positive cells at various concentrations. Binding on MC38-CEA and Jurkat-huFcγRIIIa/γ was detected using anti-c-myc or anti-flag antibodies, respectively, followed by goat anti-mouse labeled antibodies. Filled black: isotype control. Filled grey: bsFab C21. Open black: bsFab C28. B) MC38-CEA (black arrow) and Jurkat-huFcγRIIIa/γ (dotted arrow) were co-cultured with or without bsFabs C21 or bsFab C28.

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Rosette formation was observed by optical microscopy. C) BsFab stability in human serum. bsFabs were incubated at 1 μM in 90% serum for up to 168 h. Binding experiments were next performed by flow cytometry using non saturating concentrations (10 nM for bsFab C21 and 100 nM for bsFab C28 against Jurkat-huFcγRIIIa/γ, and 100 nM for both against MC38-CEA) . Filled black: isotype control. Open black: bsFab incubated for various times at 37°C in PBS. Filled grey: bsFab incubated for various times at 37°C in 90% human serum. CEA: MC38-CEA cell line. FcγRIIIa: Jurkat-huFcγRIIIa/γ cell line.

Figure 3. IL-2 release assays.

Jurkat or Jurkat-huFcγRIIIa/γ cells were pre-stimulated with PMA and incubated with 50 nM of bsFab or anti-FcγRIIIa bivalent mAb 3G8. In some case, anti-Flag tag mAb was added to induce bsFab dimerization. The addition of CEA positive LS174T tumor cells could induce IL-2 secretion while CEA negative LAN-1 human tumor cells did not. IL-2 secreted in the medium was quantified by ELISA. Error bars represent the standard deviation of experiments performed in triplicates.

Figure 4. BsFab-dependent NK cytotoxicity.

A) CEA expression of various tumor cell lines was assessed by flow cytometry (open black: isotype control, filled grey: anti-CEA sdAb C17) on SKOV3, SKOV3-CEA, BxPC3, LS174T or HT29 cells. B) Human NK cells were mixed at ratio 10:1 with CEA positive cells in the presence of indicated concentrations of bsFabs. Target cell viability was measured by Cell titer Glo viability assay after overnight incubation at 37°C in the presence of indicated concentrations of bsFab C21 (open circle), bsFab C28 (open square), except for SKOV3 cells for which bsFab C21 is represented by open triangle and bsFab C28 by open diamond. A table summarizes EC_{50} (pM) of each bsFab determined for every tumor cell line. Error bars represent the standard deviation of experiments performed in triplicates.

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Figure 5. Influence of FcγRIIIa polymorphism or soluble CEA on bsFab-dependent NK cell cytotoxicity.

A) Human FcγRIIIa-158V/F NK cells were mixed at ratio 10:1 with SKOV3-CEA positive Luc positive cells in the presence of various concentrations of bsFab C21 or sdAb C17-Fc. Target cell viability was measured by Cell titer Glo viability assay after overnight incubation at 37°C. BsFab C21 (black circle) or sdAb C17-Fc (open circle) with 158V/V NK cells, bsFab C21 (black square) or sdAb C17-Fc (open square) with 158F/V NK cells, bsFab C21 (black triangle) or sdAb C17-Fc (open triangle) with 158F/F NK cells. Asterisk indicates a significant difference between sdAb C17-Fc with 158V/V NK cells group and sdAb C17-Fc with 158F/F NK cells group (asterisks indicates significant difference between groups $p < 0.05$). Error bars represent the standard deviation of experiments performed in triplicates. B) Human NK cells were mixed at a ratio of 10:1 with BxPC3 cells in the presence of various concentrations of bsFab C21 and of soluble CEA. Target cell viability was measured by Cell titer Glo viability assay after overnight incubation at 37°C. BsFab C21 (open circle) or bsFab C21 with 1 µg/ml (open square) or 0.1 µg/ml (open triangle) of soluble CEA. No significant difference was found between bsFab C21 with 1 µg/ml of soluble CEA group or between bsFab C21 with 0.1 µg/ml of soluble CEA group versus control bsFab C21 group. Error bars represent the standard deviation of experiments performed in triplicates.

Figure 6. BsFab in xenografted mice.

A) *Biodistribution*: ^{125}I-labeled bsFab C21 was injected i.v. into LS174T (CEA positive) and Co115 (CEA negative) xenografted nude mice. Mice (n=3/group) were sacrificed at indicated times 3 h (black), 6 h (dark grey), 15 h (light grey), 24 h (white) and radioactivity of each organ was measured and plotted as % injected dose/g of tissue. The significance of bsFab retention in CEA positive tumor versus CEA negative tumor was compared at each time. Asterisks indicate significant difference between groups: two asterisks= $p< 0.005$, one asterisk= $p< 0.05$; and n.s= non significant. Error bars represent the standard deviation of experiments performed in triplicates. B) *Tumor growth inhibition assay*: At Day 0, NSG mice

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(n=8/group) were xenografted (s.c.) with BxPC3 cells and injected i.p. with human PBMCs and bsFab C21 (100 µg) or PBS, followed at Days 1 and 2 by 50 µg of bsFab C21 or PBS (i.p.). Tumor growth was followed using a Vernier caliper. PBS (open circle), bsFab C21 (open square), PBMCs and PBS (open triangle), PBMCs and bsFab C21 (open diamond). The asterisk indicates a significant difference between PBMCs and bsFab C21 group and PBMCs and PBS group ($p < 0.05$). Error bars represent the standard deviation of experiments performed with 8 mice per group.

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Figure 1

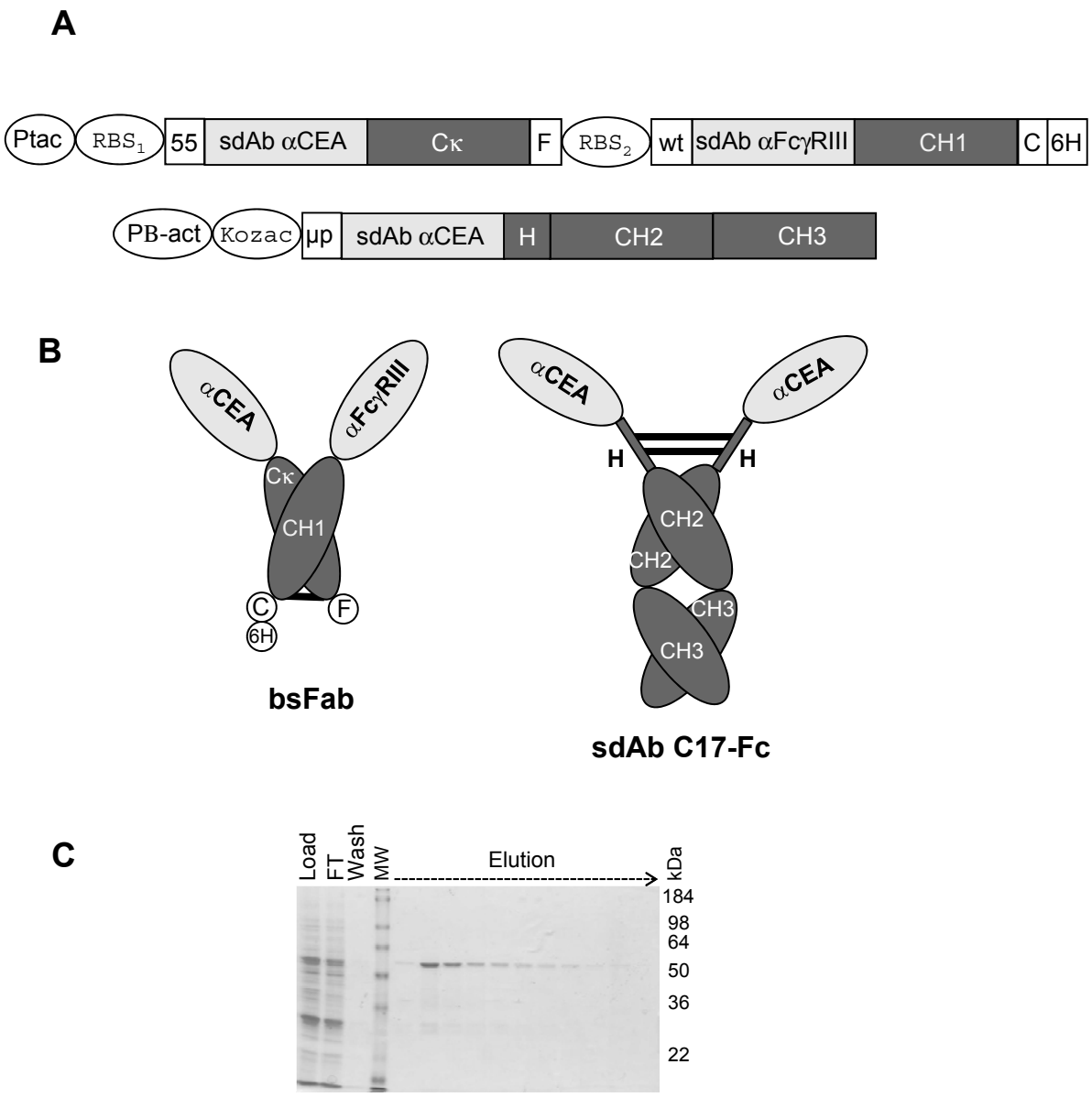

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Figure 2

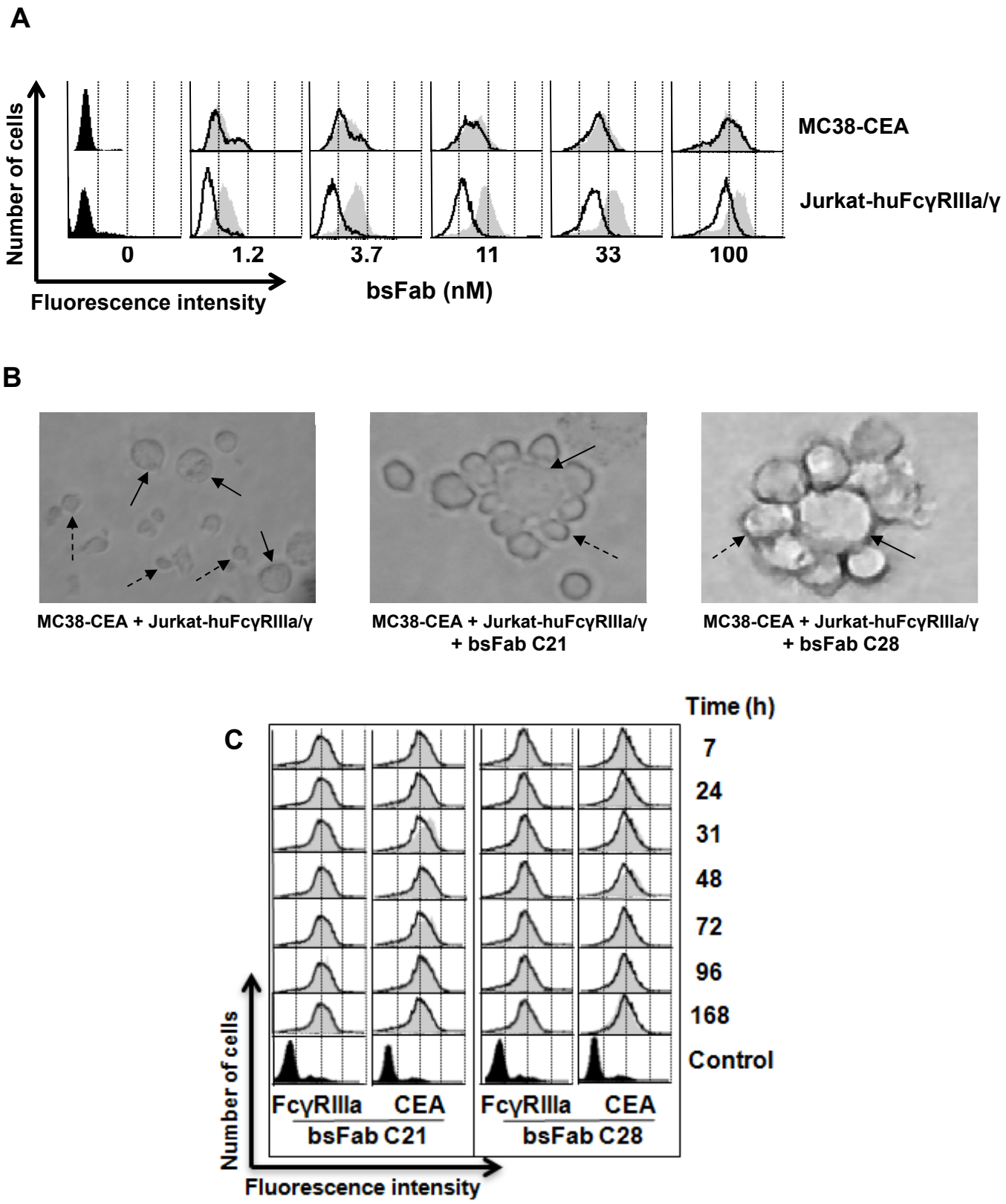

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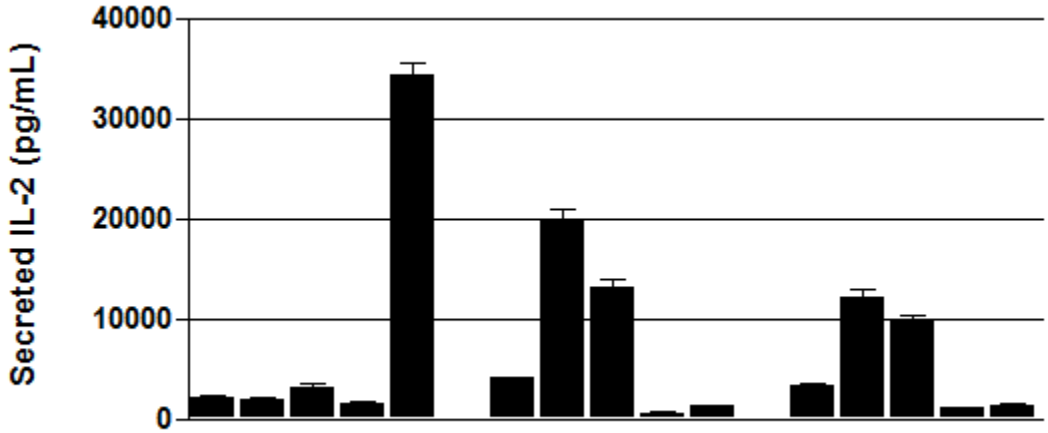
Figure 3



Jurkat						+										+							+
Jurkat-huFcγRIIIa/γ	+		+		+			+		+		+		+				+		+		+	
bsFab C21								+		+		+		+		+							
bsFab C28																		+		+		+	+
Anti Flag mAb										+										+			
3G8							+																
LS174T (CEA+)			+			+						+				+						+	+
LAN-1 (CEA-)					+										+						+		

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Figure 4

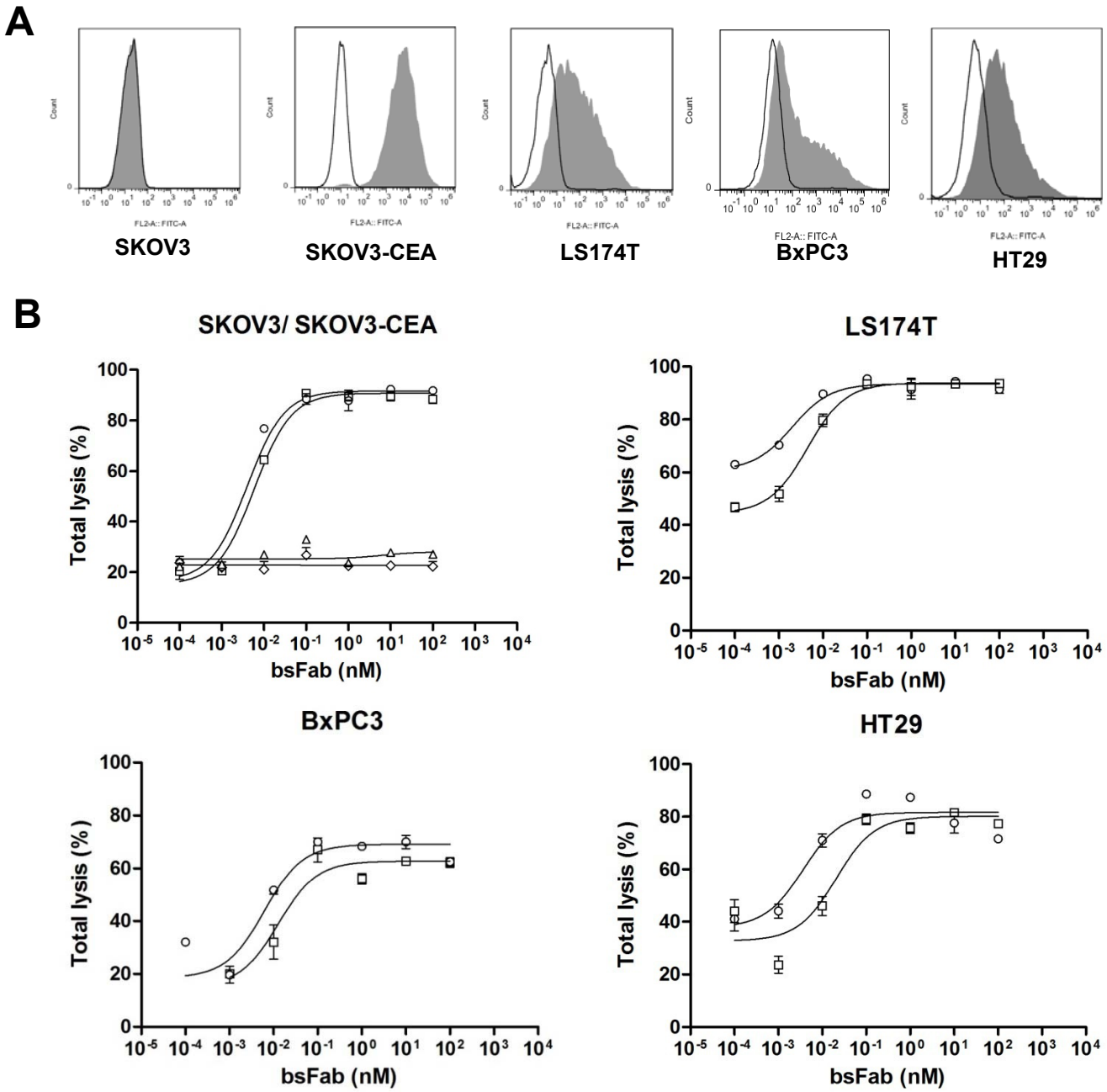

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Figure 5

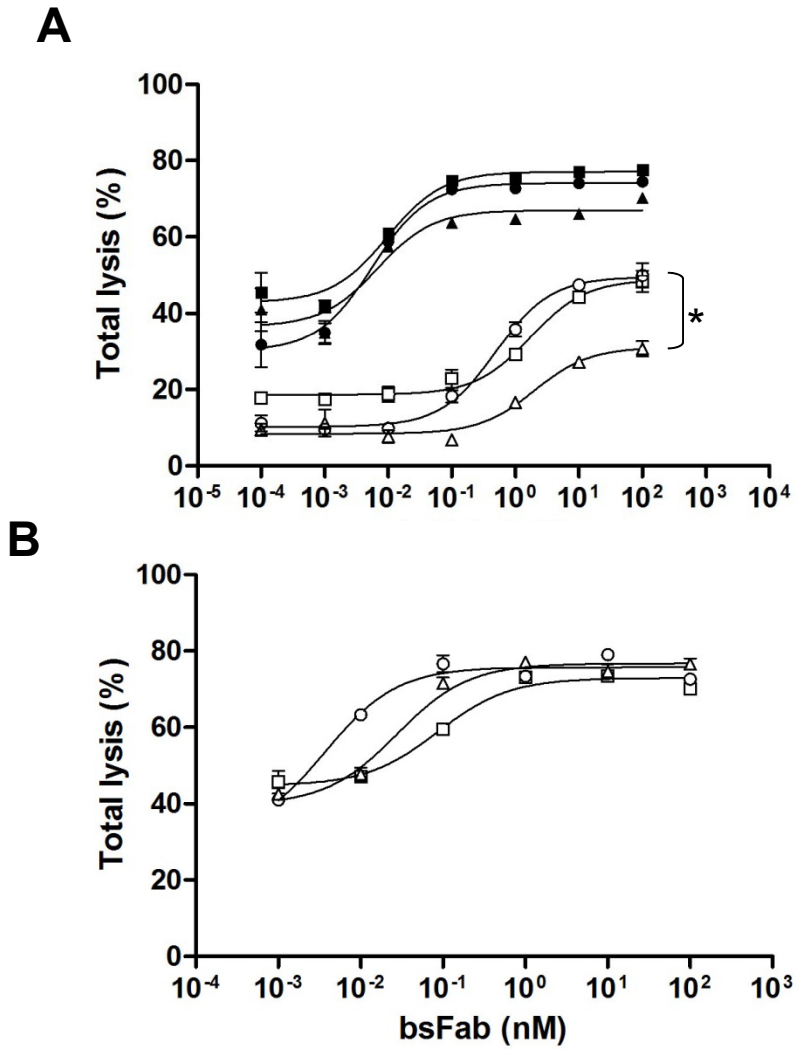

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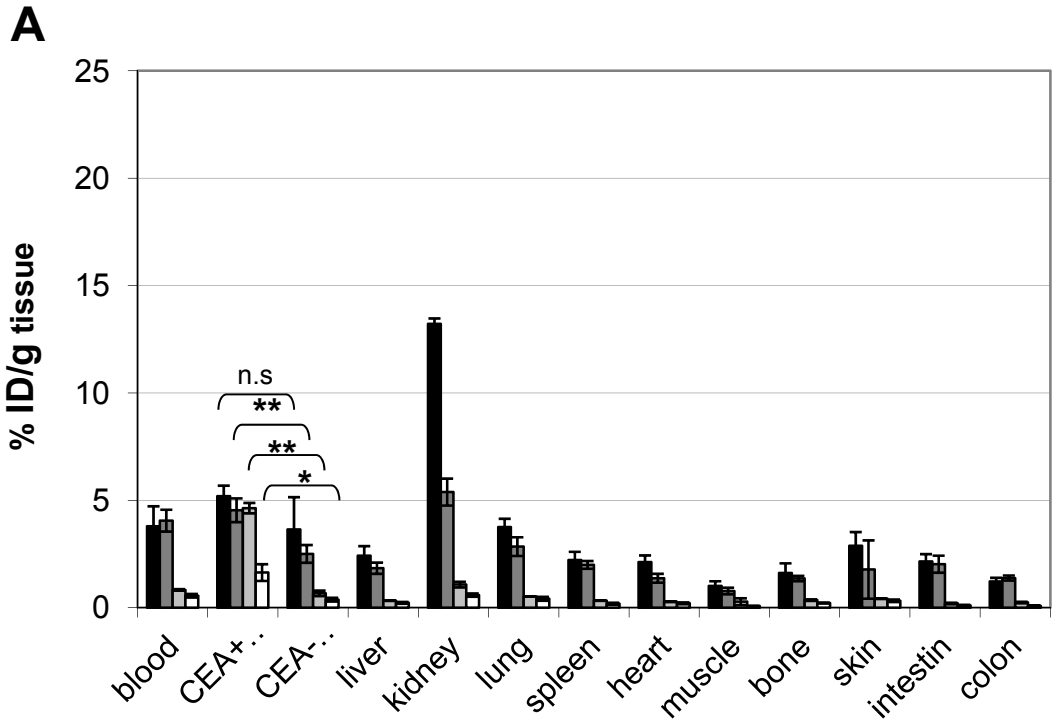
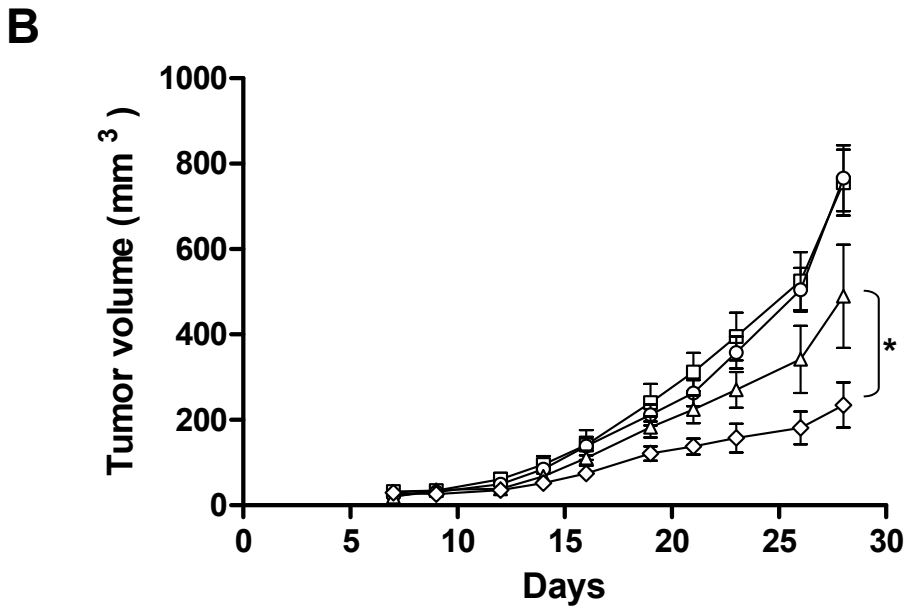
Figure 6





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