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► **To cite this version:**

Ghislaine Béhar, Sophie Sibénil, Agnès Groulet, Patrick Chames, Martine Pugnère, et al.. Isolation and characterization of anti-FcγRIII (CD16) llama single-domain antibodies that activate natural killer cells. *Protein Engineering, Design and Selection*, 2007, 21 (1), pp.1-10. <10.1093/protein/gzm064>. <hal-02454965>

HAL Id: hal-02454965

<https://hal.science/hal-02454965v1>

Submitted on 25 Jan 2020

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Isolation and characterization of anti-Fc γ RIII (CD16) llama single-domain antibodies that activate natural killer cells

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Fc γ RIII (CD16) plays an important role in the anti-tumor effects of therapeutic antibodies. Bi-specific antibodies (bsAbs) targeting Fc γ RIII represent a powerful alternative to the recruitment of the receptor via the Fc fragment, but are not efficiently produced. Single-domain antibodies (sdAbs) endowed with many valuable structural features might help to bypass this problem. In the present work, we have isolated anti-Fc γ RIII sdAbs (C21 and C28) from a phage library generated from a llama immunized with Fc γ RIIIB extra-cellular domains. These sdAbs bind Fc γ RIIIA⁺ NK cells and Fc γ RIIIB⁺ polymorphonuclear cells, but not Fc γ RI⁺ or Fc γ RII⁺ cells, as detected by indirect immunofluorescence. Competition experiments showed that C21 and C28 sdAbs bind different Fc γ RIII epitopes, with C21 recognizing a linear and C28 a conformational epitope of the receptor. Surface plasmon resonance experiments showed that C21 and C28 sdAbs bind Fc γ RIII with a K_D in the 10 and 80 nM range, respectively. Importantly, the engagement by both molecules of Fc γ RIIIA expressed by transfected Jurkat T cells or by NK cells derived from peripheral blood induced a strong IL-2 and IFN- γ production, respectively. These anti-Fc γ RIII sdAbs represent versatile tools for generating bsAbs under various formats, able to recruit Fc γ RIII killer cells to target and destroy tumor cells.

Keywords: CD16/Fc γ RIII/llama/phage display/single-domain antibodies

Introduction

Human receptors for IgG (Fc γ R) are divided into three classes designated Fc γ RI (CD64), Fc γ RII (CD32) and Fc γ RIII (CD16) (Sibériel *et al.*, 2006a). Two different Fc γ RIII isoforms have been identified. Fc γ RIIIA is a transmembrane isoform with an intermediate affinity for IgG, expressed on NK cells, a small subpopulation of T

lymphocytes, as well as on monocytes and macrophages. It is an activating receptor involved in antibody-dependent cell-mediated cytotoxicity (ADCC), phagocytosis, endocytosis and cytokine release. Fc γ RIIIB is attached to the outer leaflet of the plasma membrane of neutrophils by a glycosylphosphatidylinositol anchor and is involved in degranulation and generation of reactive oxygen intermediates by these cells (Gessner *et al.*, 1998).

Many anti-tumor therapeutic monoclonal antibodies (mAbs) have been shown to mediate ADCC *in vitro* and *in vivo* through Fc γ RIIIA recruitment. Mice invalidated for the γ chain (Fc γ R $\gamma^{-/-}$), which is associated with a binding unit of Fc γ RI and Fc γ RIIIA and is responsible for intracellular signaling, exhibit impaired ADCC and phagocytosis of IgG1-opsonized particles (Takai *et al.*, 1994). Furthermore, these mice fail to demonstrate protective tumor immunity in a model of passive immunization with an mAb directed against a tumor differentiation antigen expressed on melanoma (Clynes *et al.*, 1998). Other studies have shown that two widely used therapeutic anti-tumor mAbs, trastuzumab (a humanized IgG1, κ anti-HER2/Neu mAb) and rituximab (a chimeric IgG1, κ anti-CD20 mAb), engage in activating Fc γ R receptors. These therapeutic mAbs were unable to stop tumor growth in Fc γ RIII-deficient mice (Clynes *et al.*, 2000). Some other studies using Fc γ R $\gamma^{-/-}$ mice have also revealed that the anti-tumor effects of anti-CD25 or anti-CD52 (Campath-1H) mAbs are dependent on the expression of activating Fc γ R on polymorphonuclear (PMN) cells, monocytes and macrophages (Zhang *et al.*, 2003, 2004).

The crucial role of Fc γ RIIIA in the anti-tumor effect of IgG therapeutic mAbs has also been highlighted by the observation that Fc γ RIIIA polymorphism is associated with the therapeutic efficacy of rituximab in patients with non-Hodgkin lymphoma. Thus, patients homozygous for the *FCGR3A*-Val158 allele (IgG1 high binder) exhibit a better clinical response than those homozygous for the *FCGR3A*-Phe158 allele (IgG1 low binder) (Cartron *et al.*, 2002). A better response rate has also been shown in *FCGR3A*-Val158 and *FCGR2A*-His131 homozygous patients with follicular lymphoma treated with rituximab (Weng and Levy, 2003).

However, the use of therapeutic mAbs has been complicated by the fact that IgG, once complexed to target cells, engages in activating not only Fc γ R but also inhibitory Fc receptors (Fc γ RIIB1 and Fc γ RIIB2) expressed on B cells and on myeloid cells that represent an important anti-tumor cell compartment. The simultaneous engagement of both activating and inhibitory Fc γ R results in a decrease of the anti-tumor efficacy of antibodies, as exemplified by a study where a more pronounced tumor regression induced by trastuzumab or rituximab was observed in Fc γ RIIB-deficient mice compared with wild-type mice (Clynes *et al.*, 2000). It has led to the idea that the selective engagement of activating Fc γ R, in particular Fc γ RIII, might be beneficial to

antibody-based tumor treatments. Different approaches to selectively engage Fc γ RIII have been explored. First, point mutations in the human IgG1 Fc region that increase Fc γ RIII binding while decreasing Fc γ RII engagement have been defined (Shields *et al.*, 2001; Lazar *et al.*, 2006). Secondly, it has been reported that peculiar glycosylation profiles of human IgG1 (presence of a bisecting *N*-acetyl-glucosamine, lack of or decrease in fucose content) (Umana *et al.*, 1999; Shields *et al.*, 2002; Shinkawa *et al.*, 2003) provoke an increase in the ability of human IgG1 to trigger ADCC through Fc γ RIII engagement. However, a recent report has suggested that a decrease in the fucose content also affects the IgG1 binding to the inhibitory Fc γ RIIb1, although to a lesser extent (Siberil *et al.*, 2006b). Thirdly, bi-specific antibodies (bsAbs) that target Fc γ R on effector cells have been generated by using anti-human Fc γ R mouse mAbs as starting material (Perez *et al.*, 1985; Weiner and Adams, 2000). These bi-specific mAbs have been generated using either by biochemical approaches based mostly on the generation of Fab' fragments subsequently linked by hetero-bifunctional reagents used as spacers (Weiner *et al.*, 1993; Michon *et al.*, 1995) or by genetic engineering (Holliger *et al.*, 1993). However, these latter strategies have been made difficult to develop at an industrial scale due to the difficulty of producing bi-specific molecules with a high yield and at a low cost through complex biochemical and purification processes. In particular, bsAbs generated through genetic engineering have led to limited results due to both the difficulty of producing stable native molecules with a high yield and of defining a format that allows an adequate bridging between effector and target cells leading to an efficient tumor cell cytotoxicity. Thus, finding new easy-to-produce stable single domains that make it possible to build bi-specific molecules remains an important challenge.

Since the discovery that functional heavy-chain γ -immunoglobulins lacking light chains occur naturally in *Camelidae* (Hamers-Casterman *et al.*, 1993), several groups have reported the isolation of single-domain antibodies (sdAbs) consisting of the variable domain of these heavy-chain antibodies, also named VHH (Muyldermans, 2001). These minimal antibody domains are endowed with a large number of properties making them very attractive for antibody engineering. Despite the reduced size of their antigen binding surface, VHH domains exhibit affinities in the range of those of conventional mAbs (Spinelli *et al.*, 2000; Alvarez-Rueda *et al.*, 2007). Strikingly, they often use CDR3 longer than regular mAbs, which allow them to bind otherwise difficult-to-reach epitopes within cavities present at the surface of antigens. Consequently, these fragments are a good source of enzyme inhibitors (Lauwereys *et al.*, 1998). Most importantly, the single-domain nature of VHH permits the amplification and subsequent straightforward cloning of the corresponding genes, without requiring the use of artificial linker peptide (as for single chain Fv fragments) or of bi-cistronic constructs (as for Fab fragments). This feature allows a direct cloning of large VHH repertoires from immunized animals, without being concerned by the usual disruption of VH/VL pairing faced when generating scFv and Fab fragments libraries. The VHH format is also likely responsible for the high production yield obtained when these domains or VHH-based fusion molecules are expressed.

A number of VHH and VHH-derived molecules have been produced in large amounts in prokaryotic (Pant *et al.*, 2006) and eukaryotic cell lines (Frenken *et al.*, 2000; Bazl *et al.*, 2007), and plants (Ismaili *et al.*, 2006).

Moreover, VHH fragments show exquisite refolding capabilities and an amazing physical stability (Dumoulin *et al.*, 2002). Finally, the genes encoding VHH show a large degree of homology with the VH3 subset family of human VH genes (Su *et al.*, 2002), which might confer a low antigenicity in humans, a very attractive feature for immunotherapeutic approaches. Altogether, these data make VHH excellent candidates to engineer multi-specific or multi-functional proteins for immunotherapy (Els Conrath *et al.*, 2001). Notably, sdAbs directed against activating Fc γ RIII and tumor-associated antigens could be used to generate bi-specific molecules suitable for bridging effector killer cells with tumor target cells.

Thus, as a first step toward the generation of anti-Fc γ RIII sdAb-based bi-specific molecules, we have isolated, purified and characterized llama sdAbs that specifically bind and engage Fc γ RIII. Four sdAbs, C13, C21, C28 and C72, have been selected from a phage-display llama VHH library generated from a llama (*Lama glama*) immunized against the purified soluble extra-cellular domains of human Fc γ RIIb (sFc γ RIIb). After sequencing and K_D determination by surface plasmon resonance (SPR), the most promising candidates C21 and C28 sdAbs have been further assessed *in vitro* for their capacity to bind both Fc γ RIIIa, present on genetically engineered Jurkat and NK cells, and Fc γ RIIb expressed by freshly isolated PMN cells. These sdAbs recognize close but different epitopes on Fc γ RIII and bind Fc γ RIII with a high affinity. They represent potent agonists, as they are able to induce a strong Fc γ RIIIa-dependent production of IL-2 by Fc γ RIIIa-transfected Jurkat cells and IFN- γ by NK cells. These results make C21 and C28 sdAbs excellent candidates for the building of bi-specific antibodies directed against activating Fc γ RIII and tumor antigens.

Methods

Recombinant sFc γ RIIb preparation and llama immunization

Recombinant human sFc γ RIIb was purified from culture medium of BHK cells transfected with a cDNA encoding the NA2 form of human Fc γ RIIb (JBIXA2) by S-Sepharose chromatography (Fast flow; GE Healthcare, Munich, Germany), affinity chromatography on rabbit IgG-Sepharose and gel exclusion chromatography on Superdex 200 column (GE Healthcare). These purifications steps gave a pure highly fucosylated sFc γ RIIb with an apparent molecular weight of 47 000–56 000 Da, as indicated by silver staining following SDS-PAGE (Teillaud *et al.*, 1994).

A young adult female llama (*Lama glama*) was then immunized subcutaneously at days 1, 30, 60, 90, 120 with 250 μ g of recombinant purified human sFc γ RIIb. Sera were collected 15 days prior to each injection to follow the immune response against the immunogen.

VHH library construction

Blood samples (100 ml) were taken 15 days after each of the three latest immunizations and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Histopaque-1077

(Sigma-Aldrich, Saint Louis, MO, USA) discontinuous gradient centrifugation. Total RNA was isolated by acid guanidinium thiocyanate–phenol–chloroform extraction and the synthesis of the cDNA was performed with Superscript II reverse transcriptase (GibcoBRL, Gaithersburg, MD, USA) using primer CH2FORTA4 (Arbabi Ghahroudi *et al.*, 1997). The VHH and part of the CH2 domain of immunoglobulin heavy chains were amplified by PCR with an equimolar mixture of four backward primers (5' VH1-Sfi: 5'-CATG CCATGACTCGCGGCCAGCCGGCCATGGCCAGGTG CAGCTGGTGCAGTCTGG-3'; 5' VH2-Sfi: 5'-CATGCCAT GACTCGCGGCCAGCCGGCCATGGCCAGGTACCTT GAAGGAGTCTGG-3'; 5' VH3-Sfi: 5'-CATGCCATGACTC GCGGCCAGCCGGCCATGGCCAGGTGCAGCTGGTG GAGTCTGG-3'; 5' VH4-Sfi: 5'-CATGCCATGACTCGCG GCCAGCCGGCCATGGCCAGGTGCAGCTGCAGGAG TCGGG-3') and one forward primer (CH2FORTA4). From these PCR fragments, VHH were re-amplified with the equimolar mixture of the four backward primers (5' VH1 to 4 -Sfi) and one 3' VHH-Not primer (5'-CCACGATTCTG CGGCCGCTGAGGAGACRGTGACCTGGGTCC-3') containing *Sfi*I and *Not*I restriction enzyme sites. The resulting VHH fragments were purified from 1% agarose gel by Qiaquick gel extraction kit (Quiagen, Hilden, Germany), digested with *Sfi*I and *Not*I and ligated into the pHEN1 phagemid (Hoogenboom *et al.*, 1991) digested with *Sfi*I and *Not*I. The ligated material was transformed into TG1 *Escherichia coli* electroporation-competent cells (Stratagen, Miami, FL, USA). Cells were plated on 2YT/ampicillin (100 μ g/ml)/glucose (2%) agar plates. Colonies (10^6) were scraped from the plates with 2YT/ampicillin (100 μ g/ml)/glucose (2%), and stored at -80°C in the presence of 20% glycerol.

Selection of binders

Selection was performed as described previously (Chames *et al.*, 2002). Briefly, $10\ \mu\text{l}$ of the phage library were grown in 50 ml of 2YT/ampicillin (100 μ g/ml)/glucose (2%) at 37°C to an $\text{OD}_{600\text{nm}}$ of 0.5. Five milliliters of the culture were then infected with 2×10^{10} M13KO7 helper phages for 30 min at 37°C without shaking. After centrifugation for 10 min at 3000g, the bacterial pellet was resuspended in 25 ml of 2YT/ampicillin (100 μ g/ml)/kanamycin (25 μ g/ml) and bacteria were grown for 16 h at 30°C with vigorous shaking. After centrifugation of the culture for 20 min at 3000g, 20% PEG 6000, 2.5 M NaCl (one-fifth of the volume) was added to the supernatant and the mixture was incubated for 1 h on ice to precipitate phage particles. The phage-containing pellet was then resuspended in 1 ml of PBS after centrifugation for 15 min at 3000g at 4°C and used for the selection of binders as described below.

Phages were selected using biotinylated sFc γ RIIIB and streptavidin-coated paramagnetic beads, Dynabeads M-280 (DynaL Biotech, Oslo, Norway). Briefly, 200 μl beads were mixed with 1 ml of PBS containing 2% skimmed milk powder (PBS/2% milk) for 45 min at room temperature in a siliconized Eppendorf tube. The beads were then washed with PBS/2% milk using magnetic particle concentrator and resuspended in 250 μl of PBS/2% milk. Two hundred microliters of sFc γ RIIIB, biotinylated using a biotin protein labeling kit (Roche, Basel, Switzerland), were then added and the mixture was gently rotated for 30 min at room temperature.

A volume of 150, 75 and 25 nM of biotinylated sFc γ RIIIB was used for the first, second and third rounds of selection, respectively. Four hundred fifty microliters of the phage preparation (10^{12} pfu), pre-incubated for 1 h in 500 μl of PBS/2% milk, were then added. The mixture was rotated for 3 h at room temperature and then washed five times first with 800 μl of PBS/4% milk, secondly with 800 μl of PBS–0.1% Tween and thirdly with 800 μl of PBS. sFc γ RIIIB-coated beads were then resuspended in 200 μl of PBS and incubated without shaking with 1 ml of log phase TG1 cells which were then plated on 2YT/ampicillin (100 μ g/ml)/glucose (2%) in 243×243 mm dishes (Nalgene Nunc, Roskilde, Denmark). Thirty colonies were picked for phage characterization (ELISA screening). The remaining colonies were harvested from the plates, suspended in 2 ml of 2YT/ampicillin (100 μ g/ml)/glucose (2%) and used for phage production for the next round of selection.

ELISA screening of binders

Single colonies resulting from infection of TG1 cells with phage were inoculated into 150 μl 2YT/ampicillin (100 μ g/ml)/glucose (2%) broth in 96-well plates and grown under shaking (400 rpm) for 16 h at 37°C . From each well, 5 μl of colonies were then mixed with 120 μl of fresh broth in new 96-well plates. Colonies were grown for 2 h at 37°C under shaking (400 rpm) and 35 μl 2YT/ampicillin (100 μ g/ml)/glucose (2%) containing 2×10^9 M13KO7 helper phage were added to each well and incubated for 30 min at 37°C without shaking. The plates were then centrifuged for 10 min at 1200g and the bacterial pellet was resuspended in 150 μl 2YT/ampicillin (100 μ g/ml)/kanamycin (25 μ g/ml) and grown for 16 h at 30°C under vigorous shaking.

Phage-containing supernatants were then tested for binding to sFc γ RIIIB by ELISA. Briefly, biotinylated sFc γ RIIIB (5 μ g/ml) was captured on streptavidin 96-well BioBind Assembly Streptavidin Coated microplates (Thermo Fischer Scientific, Waltham, MA, USA) saturated with PBS/2% milk. Fifty microliters of phage supernatant were then mixed with 50 μl PBS/4% milk/well and ELISA plates were incubated for 2 h at room temperature. Binding of phages to sFc γ RIIIB was detected with a peroxidase-conjugated anti-M13 mouse mAb (GE Healthcare, Munich, Germany). Measurement was done at $\text{OD}_{405\text{nm}}$.

SdAb sequencing, production and purification

DNA of positive phages ($\text{OD}_{405\text{nm}}$ three times above the blank) was sequenced using ABI PRISM® BigDye™ Terminators (Applied Biosystems, Foster City, CA, USA). The VHH encoding DNA of the binders selected by phage display were then amplified by PCR using primers 5' pJF-VH3-Sfi (CTTTACTATTCTCACGGCCATGGCGGCC GAGGTGCAGCT GGTGG) and 3' *c-myc*-6xhis/*Hind*III (CCGCGCGCGCCAAGACCCAAGCTTGGGCTARTGRTG RTGRTGRTGRTGTGC GGCCCCATTCAGATC) to add the *Hind*III site for further cloning and the 6xHistidine tag for purification and detection. PCR fragments were cloned into the pPelB55PhoA' (Le Calvez *et al.*, 1996) vector between the *Sfi*I and *Hind*III sites. The *E. coli* K12 strain TG1 was used for the production of the tagged sdAbs. An inoculum was grown overnight at 30°C in 2YT medium supplemented with 100 μ g/ml ampicillin and 2% glucose. Four hundred milliliters of fresh medium was inoculated to obtain an

OD_{600nm} of 0.1, and bacteria were grown at 30°C to an OD_{600nm} of 0.5–0.7 and induced with 100 µM IPTG for 16 h. The cells were harvested by centrifugation at 4200g for 10 min at 4°C. The cell pellet was suspended in 4 ml of cold TES buffer (0.2 M Tris–HCl, pH 8.0; 0.5 mM EDTA; 0.5 M sucrose), and 160 µl of lysozyme (10 mg/ml in TES buffer) were added. The cells were then subjected to an osmotic shock by the addition of 16 ml of cold TES diluted 1:2 with cold H₂O. After incubation for 30 min on ice, the suspension was centrifuged at 4200g for 40 min at 4°C. The supernatant was incubated with 150 µl DNase I (10 mg/ml) and MgCl₂ (5 mM final) for 30 min at room temperature. The solution was dialyzed against 50 mM sodium acetate, pH 7.0, 0.1 M NaCl, for 16 h at 4°C. sdAbs were purified by TALON metal-affinity chromatography (Clontech, Mountain View, CA, USA) and concentrated by ultra-filtration with Amicon Ultra 5000 MWCO (Millipore, Billerica, MA, USA). The protein concentration was determined spectrophotometrically using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

Sandwich ELISA

A sandwich ELISA was performed by incubating C21 or C28 biotinylated sdAbs (10 µg/ml) or 7.5.4 mAb (5 µg/ml) (Vely *et al.*, 1997) in the presence of recombinant purified sFcγ (1 µg/ml) on a plate previously coated with unlabelled C21 or C28 sdAb (10 µg/ml). sdAbs were biotinylated according to the manufacturer's instructions (Biotin Protein Labeling Kit, Roche Diagnostics, Mannheim, Germany). The binding of biotinylated sdAbs was detected using a streptavidin–Horse radish peroxidase (HRP) conjugate (Sigma-Aldrich) and that of 7.5.4 mAb using HRP–F(ab')₂ goat anti-mouse IgG antibodies (Southern Biotech, Birmingham, AL, USA).

Affinity measurements

Kinetic parameters were determined by real-time SPR using a BIACORE 2000 apparatus. The anti-*c-myc* 9E10 mAb (Evan *et al.*, 1985) was covalently immobilized (11 000 RU) on a flowcell of CM5 sensor chip (Biacore AB, Uppsala, Sweden) with EDC/NHS activation according to the manufacturer's instructions. A control flowcell surface was prepared with the same treatment, but without the antibody. All analyses were performed at 25°C, at a flow rate of 20 µl/min and using HBS-EP (Biacore AB; 10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA and 0.005% Biacore™ surfactant) as running buffer. Each anti-FcγRIII sdAb was injected during 180 s at the concentration of 50 µg/ml in HBS-EP and followed by an injection of sFcγRIIIB at seven different concentrations (0.4–25 µg/ml). A 400 s dissociation step was applied before a pulse of 5 mM HCl to regenerate the flowcell surfaces between each run. A cycle with a buffer injection instead of sFcγRIII was performed before each run to correct the decaying surface effect. No sFcγRIIIB binding to 9E10 was observed. All sensorgrams were corrected by subtracting the signal from the reference flowcell and were globally fitted to a Langmuir 1:1 binding isotherm using BIAevaluation 3.2 software.

Cell lines and isolation of PMN and NK cells

Jurkat human lymphoma T cells (ATCC TIB 152) were cultured in RPMI 1640 (Sigma-Aldrich), supplemented with

10% heat-inactivated fetal calf serum (FCS; Hyclone, Logan, UT, USA), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (culture medium). Geneticin G418 (0.5 mg/ml) (GibcoBRL) was added to the culture medium of transfected Jurkat–huFcγRIIIA/γ cells (kindly provided by Prof. E. Vivier, CIML, Marseille, France) (Vivier *et al.*, 1992). The human FcγRIIA⁺ erythroleukemia K562 cells (ATCC no CCL-243) were cultured in culture medium. Human FcγRIIB1 transfected mouse IIA.1.6 lymphoma B cells (IIA1.6-huFcγRIIB1) (Siberil *et al.*, 2006b) were cultured in Click culture medium supplemented with 5 mM sodium pyruvate (GibcoBRL), 0.5 µM β-mercaptoethanol and G418 (0.5 mg/ml). Chinese Hamster ovary cells transfected with human FcγRI DNA (Tf2-13) were cultured as described previously (Tan *et al.*, 2003).

Polymorphonuclear cells were isolated by centrifugation from peripheral blood from healthy donors (Etablissement Français du Sang, EFS, Ile-de-France, France), using a discontinuous Percoll (Sigma-Aldrich) gradient of 55–70%. These cells were used in immunofluorescence binding experiments immediately after isolation.

The purification of NK cells was performed in two steps. PBMCs were first isolated from peripheral blood from healthy donors (EFS) by centrifugation on a Ficoll/Hypaque gradient (PAA Laboratories GmbH, Pasching, Austria). NK cells were then purified from the PBMC using the negative selection NK cell isolation kit II (Miltenyi Biotec, Bergishglaubach, Germany). Purified cells were at least 90% CD56⁺. Purified NK cells were then cultured for 15 days in the presence of IL-15 (20 ng/ml) (R&D Systems, Minneapolis, MN, USA) before being tested in immunofluorescence assays and for IFN-γ production.

Immunofluorescence assays

Immunofluorescence assays were performed by incubating 5×10^5 indicator cells with various concentrations of sdAbs (0.01, 1, 10, 20 and 50 µg/ml) for 30 min on ice. sdAbs binding to Jurkat–huFcγRIIIA/γ, PMN, FcγRIIA⁺ K562 and FcγR[−] Jurkat cells were then revealed by incubation with the anti-*c-myc* 9E10 mAb (10 µg/ml) followed by incubation with FITC-labeled mouse F(ab')₂ anti-mouse IgG (H+L) (FITC-GAM) antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

Biotinylated sdAbs were used with mouse IIA.1.6-huFcγRIIB1 lymphoma B cells since these cells express IgG2a that could be bound by FITC-GAM secondary antibodies. In these experiments, the binding was detected by incubation with FITC-conjugated streptavidin (BD Pharmingen, Le Pont-De-Claix, France). The binding of biotinylated sdAbs to FcγRIIIA⁺ NK cells was analyzed using the same method except that phycoerythrin (PE)-Cyan5 streptavidin (SAV-PE-Cy5) (BD Pharmingen) was used as revealing reagent. Immunofluorescence analyses were carried out by flow cytometry using a FACSCalibur (Becton Dickinson, San Jose, CA, USA) with CELLQuest Pro software (Becton Dickinson).

Competition immunofluorescence assays

Immunofluorescence competition assays were then performed by incubating 5×10^5 Jurkat–huFcRIIIA/γ cells with various concentrations of sdAbs (from 0.01 up to 50 µg/ml) and then with 0.5 µg/ml anti-human FcγRIII 3G8 (Fleit *et al.*, 1982) or

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
	10	30	40	50	60	70	80
	1	20	30	40	50	60	70
	100	110	120	130	140	150	160
	12345678901234567890123456	789012345678	90123456789012345	6789012345	678901234567890123456789012345	67890123456789012345678901234	5678901234567890123456789012345678
C28	EVQLVESGGGLVQPGESLTLSCVVA	GSIFSPA.....	MSWYRQAPGKERELVAR	IGSDDRV...	TYADSVK.GRFTISRDNIKRTAGLQMNLSLKPEDTAVYVC	NAQTDLR.DWTVREY	WGQGTQVTVSS
C72	-----G--A-----T-----R-----T--ET...	-----R-----N-----	M--K-----V-----	-----Y-----	-----R--Y-----	-----	-----
C13	-----G--R--SFP-----LT-----	-----TS	ATPGGDT...	N--F-----ARSLIY-----	Y--R--R-----	NWGTV	-----
C21	-----E--R--AAS--LT--SYN....	-----F--S	-----TWGG--DT..	F-----A--N--VY--S-----	A--NPWPVAAPRSGT-	-----	-----

Fig. 1. Amino-acid sequences of anti-Fc γ RIII sdAbs. The IMGT numbering is shown (Lefranc, 2003). The localization of frameworks (FR1 to FR4) and CDRs are indicated. Dashes indicate sequence identity. GenBank accession numbers: EF561290, C13 sdAb; EF561291, C21 sdAb; EF561292, C28 sdAb; EF561293 C72 sdAb.

7.5.4 mAbs. The binding of 3G8 or 7.5.4 mAbs was revealed by FITC-GAM. Inhibition of anti-Fc γ RIII mAb binding was calculated by considering as 100% binding value the percentage of labeled cells obtained in the absence of sdAb.

Western blot analysis of sdAb binding to sFc γ RIIIB

Soluble Fc γ RIIIB (1 μ g) was separated by 10% SDS-PAGE under reducing conditions and transferred onto nitrocellulose membrane. After saturation with PBS/5% milk, the membrane was cut and each strip was incubated with 5 μ g/ml of 7.5.4 mAb or sdAb as indicated. The binding of 7.5.4 mAb and sdAbs was then detected using F(ab')₂ rabbit anti-mouse IgG (RAM-PA) antibodies or the anti-*c-myc* 9E10 mAb+RAM-PA, respectively.

IL-2 and IFN- γ production assays

Jurkat-huFc γ RIIIA/ γ cells (5×10^5) were incubated for 18 h with 10 ng/ml phorbol myristate acetate (PMA), various doses of C21 or C28 biotinylated-sdAbs (diluted from 1 to 0.01 μ g/ml) and streptavidin (starting concentration: 10 μ g/ml), or not. After incubation, cells were centrifuged for 3 min at 1200g and the presence of human IL-2 in the culture medium was measured by ELISA with the DuoSet human IL-2 kit (DuoSet Human IL-2, R&D Systems).

In other experiments, freshly isolated NK cells from healthy donors were tested for the production of IFN- γ following cross-linking of Fc γ RIIIA by C21 and C28 biotinylated-sdAbs. Briefly, 10^6 NK cells/ml were cultured at 37°C in a 5% CO₂ humid atmosphere for 48 h in RPMI 1640 medium (Sigma-Aldrich) supplemented with 5% heat-inactivated FCS (Hyclone), 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine in the presence of various doses of sdAb (20, 10 or 1 μ g/ml) and of streptavidin (starting concentration: 10 μ g/ml), or not. Higher doses of sdAb were used in these experiments compared with the IL-2 production experiments, as the induction of IFN- γ production by freshly isolated NK cells through Fc γ RIII cross-linking has been proved difficult (Anegon *et al.*, 1988). Cells were then centrifuged and IFN- γ was detected in culture supernatants with a sandwich ELISA using the mouse 29.51A10 mAb as capture antibody (Stefanos *et al.*, 1985) and the biotinylated mouse 45-B3 mAb (BD Pharmingen) as revealing reagent. Purified recombinant human IFN- γ (R&D Systems) was used as standard for quantification. In these experiments, biotinylated anti-Fc γ RIIIA 3G8 antibody (10 μ g/ml) was included as positive control.

Results

Isolation of anti-sFc γ RIIIB sdAbs

A female llama was immunized subcutaneously five times with 250 μ g of recombinant, purified sFc γ RIIIB per

injection. Anti-sFc γ RIIIB serum titer reached 1:10 000 after 3 months following the first immunization. A library of 10^6 clones was then obtained by RT-PCR amplification and cloning of VHH genes from RNA purified from the llama peripheral blood cells. Binders were selected by panning the phage library onto streptavidin beads coated with decreasing amounts of biotinylated sFc γ RIIIB from one cycle to another one (see the 'Methods' section). After only three rounds of affinity selection, 27 out of 30 clones were found positive by phage-ELISA using biotinylated-sFc γ RIIIB. Sequence analyses of the 27 clones revealed that 10 different sdAbs had been selected. From these 10 clones, 4 clones leading to the highest ELISA signals, termed C13, C21, C28 and C72, were chosen for further characterization. The amino-acid sequences of their CDRs were different, except clones C28 and C72 that exhibited a 90% amino-acid homology (Fig. 1). The presence of an Arg at position 50 confirmed the *Camelidæ* nature of these sdAbs (Harmsen *et al.*, 2000).

SdAb affinity determination

To characterize these sdAbs further, the cDNA-encoding sequences were cloned into the expression vector pPelB55PhoA' (Le Calvez *et al.*, 1996), allowing an efficient production and purification of the molecules. sdAbs harboring a 6xHistidine tag at the C-terminus part were produced in the periplasm of *E. coli* and purified by immobilized ion metal affinity chromatography. The affinity of the purified sdAb was then analyzed by SPR using a BIACORE by capturing the sdAb on the chip via the anti-*c-myc* 9E10 mAb, and by injecting sFc γ RIIIB into the soluble phase. Analyses revealed that three out of the four sdAbs have a K_D of ~ 100 nM (C13, C28 and C72), whereas the latter sdAb (C21) has a K_D of 10 nM, reflecting a higher association rate ($2.86 \pm 0.02 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$) and a lower dissociation rate ($2.79 \pm 0.006 \times 10^{-3} \text{ s}^{-1}$) (Fig. 2). Since C13 sdAb aggregated during the purification process and C28 and C72 sdAb exhibited strongly homologous CDR sequences and similar affinities, further experiments were performed with C21 and C28 sdAbs only.

Binding of sdAbs to human Fc γ R

The specificity of the binding of purified C21 and C28 sdAbs to human Fc γ RIII was assessed by immunofluorescence assays using NK cells, PMN cells, and cells from cell lines expressing different human Fc γ R or not (Fig. 3). As expected, since sFc γ RIIIB had been used for llama immunization, C21 and C28 sdAbs bound human Fc γ RIIIB expressed on freshly purified human PMN cells (Fig. 3A, lower panel). They also strongly bound Fc γ RIIIA⁺ NK cells and Jurkat-huFc γ RIIIA/ γ cells that express huFc γ RIIIA (Fig. 3A, middle and upper panels). The binding of C21 and C28 sdAbs to Fc γ RIIIA could be observed in these experiments even at a dose as low as 0.1 μ g/ml (data not shown). In

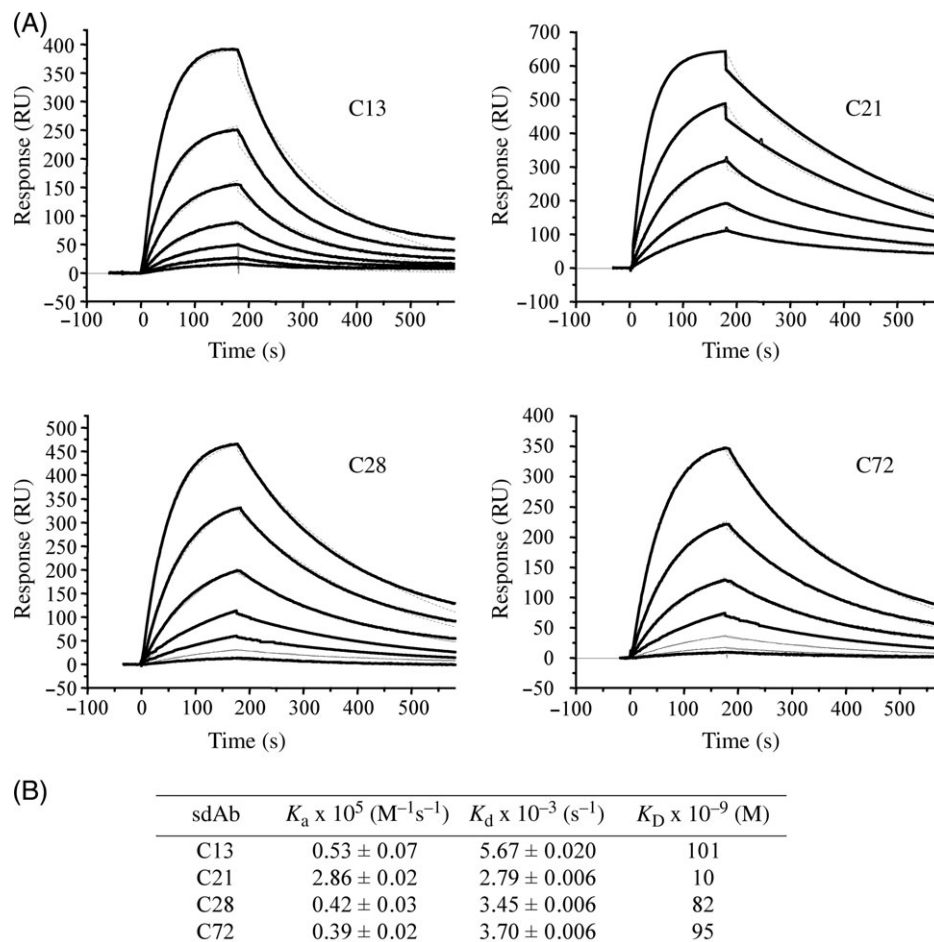


Fig. 2. Surface plasmon resonance analysis of sdAb binding to sFc γ RIIIB. SdAbs were captured on a CM5 sensor chip using the anti-c-myc 9E10 mAb and sFc γ RIIIB was then injected in solution (see ‘Methods’ section). (A) Kinetic analysis of sdAb. Dashed line represent a global fit of the response data to a 1:1 interaction model. (B) All constants were calculated from seven sensorgrams obtained with different concentrations of sFc γ RIIIB (from 0.4 to 25 μ g/ml) by a global Langmuir 1.1 method (BIAevaluation 3.2 software). No binding of sFc γ RIIIB was observed on the immobilized anti-c-myc mAb in absence of sdAb.

contrast, no binding of C21 and C28 sdAbs (used at 20 μ g/ml) to Jurkat, Tf2-13-huFc γ RI, huFc γ RIIA⁺ K562 and IIA.1.6-huFc γ RIIB1 cells was detected (Fig. 3B). These results show that C21 and C28 sdAbs bind human Fc γ RIIIA and Fc γ RIIIB, but not Fc γ RI and Fc γ RII.

Epitope analysis

To determine whether C21 and C28 sdAbs recognize different Fc γ RIII epitopes, their binding to sFc γ RIIIB was assayed by sandwich ELISA, where the binding of biotinylated-C21 or -C28 sdAbs or of a mouse anti-human Fc γ RIII mAb, 7.5.4 to sFc γ RIIIB (10 and 1 μ g/ml) was evaluated in plates coated with C21 or C28 sdAbs (Fig. 4A). 7.5.4 mAb inhibits the binding of IgG to Fc γ RIII only marginally and strongly binds sFc γ RIIIB immobilized on ELISA plate, as opposed to 3G8 mAb that was not used in these experiments (Vely *et al.*, 1997). Figure 4A shows that C21 and C28 sdAbs did not cross-compete at all for sFc γ RIIIB binding, indicating that their corresponding epitopes are different. However, 7.5.4 mAb was able to compete with both sdAbs, suggesting that these two sdAbs epitopes are spatially related. In these experiments, biotinylated sdAb molecules competed with the corresponding soluble unlabeled molecules, showing that biotinylation did not hamper the binding efficiency of C21 and C28 sdAbs.

The binding of C21 and C28 sdAbs to Fc γ RIII was further analyzed in immunofluorescence competition assays (Fig. 4B). In these experiments, the binding of the two anti-human Fc γ RIII mAbs, 3G8 or 7.5.4, to Jurkat–huFc γ RIIIA/ γ cells in the presence or absence of different amounts of C21 or C28 sdAb was evaluated. In contrast to 7.5.4 mAb, 3G8 mAb inhibits the binding of IgG to human Fc γ RIII (Fleit *et al.*, 1982). In agreement with the ELISA results, both C21 and C28 sdAbs interfered in a dose-dependent manner with the binding of 7.5.4 mAb. The binding of 7.5.4 mAb to Jurkat–huFc γ RIIIA/ γ cells was almost abrogated in the presence of 50 μ g/ml of C21 or C28 sdAbs (Fig. 4B). In contrast, the binding of 3G8 mAb to Jurkat–huFc γ RIIIA/ γ cells was not affected by the presence of C21 sdAb (Fig. 4B, left panel), whereas the binding of C28 sdAb inhibited the binding of 3G8 mAb to Fc γ RIIIA only slightly (Fig. 4B, right panel).

Altogether, ELISA and immunofluorescence assays demonstrate that sdAbs C21 and C28 recognize different but closely related epitopes on Fc γ RIII. Both epitopes are shared with 7.5.4 mAb, whereas only the C28 sdAb epitope on Fc γ RIII is shared in part with 3G8 mAb, as summarized in Fig. 5.

C21 and C28 sdAbs were also analyzed for their ability to bind denatured sFc γ RIIIB in western blot experiments. The binding of C21 and C28 sdAbs to sFc γ RIIIB was detected

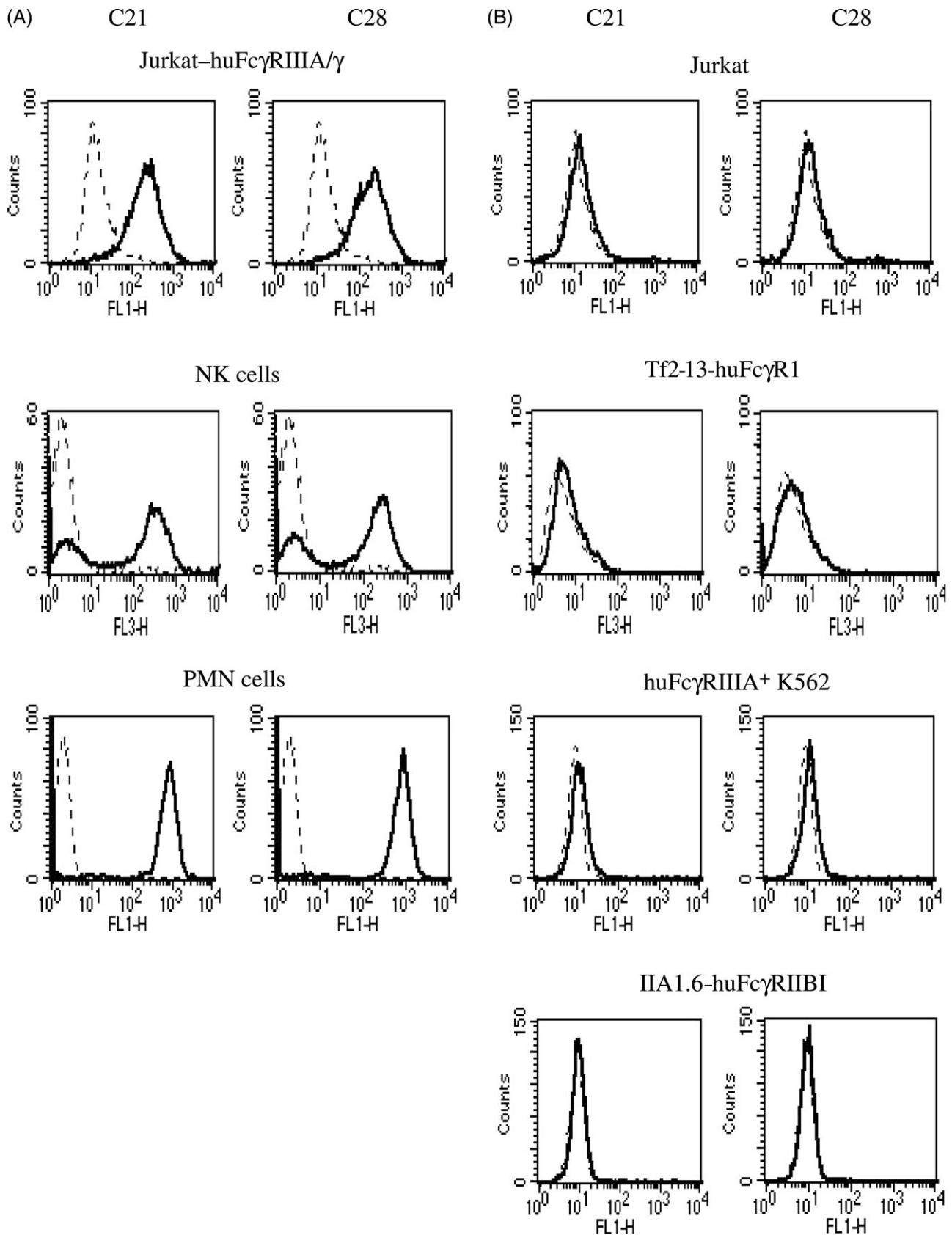


Fig. 3. Analysis of the binding of C21 and C28 sdAbs to human FcγRI, FcγRIIA, FcγRIIB1, FcγRIIIA and FcγRIIIB by indirect immunofluorescence. (A) Binding of C21 and C28 sdAbs to Jurkat-huFcγRIIIA/γ, NK and PMN cells. FcγRIIIA⁻ and FcγRIIIA⁺ NK cell subpopulations can be observed, as seen with mouse anti-FcγRIII mAbs. (B) No binding to FcγR⁻ Jurkat (upper panel), Tf2-13-huFcγRI (upper middle panel), FcγRIIA⁺ K562 (lower middle panel) and IIA1.6-huFcγRIIB1⁺ cells (lower panel) was observed. Binding of sdAbs (black curves) was revealed as described in the 'Methods' section. Shaded histograms show background fluorescence.

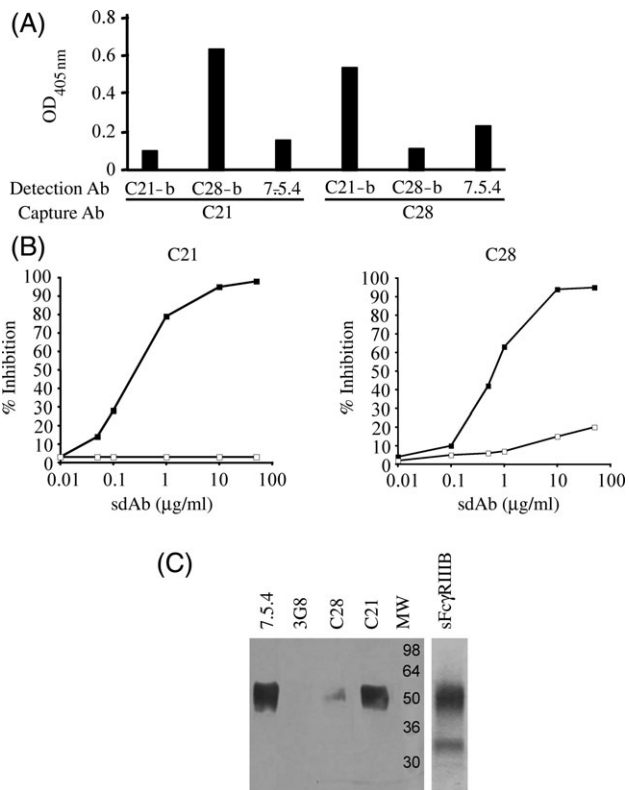


Fig. 4. sdAb epitope analysis. (A) Soluble Fc γ RIII sandwich ELISA using immobilized C21 and C28 sdAb and biotinylated sdAb or anti-Fc γ RIII 7.5.4 mAb. Recombinant sFc γ RIIIB was incubated in presence of biotinylated sdAbs (C21-b and C28-b) or 7.5.4 mAb on an ELISA plate previously coated with unlabelled C21 or C28 sdAb (see 'Methods' section). The binding of biotinylated sdAb was detected with a streptavidin–HRP conjugate and that of 7.5.4 mAb with HRP–F(ab')₂ goat anti-mouse IgG antibodies. (B) Immunofluorescence competition assays. Inhibition of 3G8 (open squares) or 7.5.4 (black squares) anti-Fc γ RIII mAbs binding to Jurkat–huFc γ RIIIA/ γ cells by various doses of the C21 (left panel) or C28 (right panel) sdAb. Results are expressed as described in the 'Methods' section. (C) Western blot analysis of sdAb binding to sFc γ RIIIB. After SDS–PAGE, sFc γ RIIIB was transferred onto nitrocellulose membrane. The membrane was cut and each strip was incubated with a particular mAb or sdAb as indicated. The binding of antibodies and sdAb was detected using F(ab')₂ rabbit anti-mouse IgG (RAM-PA) antibodies or the anti-*c-myc* 9E10 mAb and then RAM-PA, respectively. MW: molecular weight (kDa). The right lane corresponds to a SDS–PAGE gel showing the protein used for llama immunization and binder selection (sFc γ RIIIB).

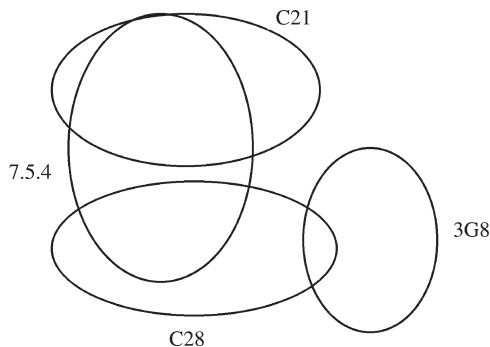


Fig. 5. Schematic representation of the Fc γ RIII binding relationship between 3G8 and 7.5.4 mAbs and C21 and C28 sdAb. Overlapping areas symbolize the observed competitions between antibodies for Fc γ RIII binding.

using the mouse anti-*c-myc* 9E10 mAb followed by alkaline phosphatase-F(ab')₂ rabbit anti-mouse IgG antibodies (RAM-AP). The binding of 7.5.4 and 3G8 mAbs was detected using RAM-AP. Figure 4C shows that, as previously published (Fleit *et al.*, 1982; Vely *et al.*, 1997), 7.5.4 mAb, but not 3G8 mAb, strongly binds the denatured form of sFc γ RIIIB. In this assay, C21 sdAb gave a strong signal, demonstrating the linear nature of the sFc γ RIIIB epitope recognized by this sdAb. In contrast, C28 sdAb yielded only a faint signal, suggesting that the epitope recognized by this sdAb is a conformational epitope, as for mAb 3G8.

Fc γ RIIIA-dependent induction of IL-2 and IFN- γ production by C21 and C28 sdAbs

Next, we analyzed whether the binding of C21 or C28 sdAbs to Fc γ RIIIA⁺ Jurkat–huFc γ RIIIA/ γ or by NK cells could induce the production of IL-2 or IFN- γ , respectively. Since the triggering of cytokine production by Fc γ RIII⁺ cells requires the aggregation of the receptor, biotinylated C21 or C28 sdAbs were first incubated with streptavidin prior to incubation with effector cells in order to generate multivalent (at the best tetraivalent) sdAb molecules. As shown in Fig. 6, both multivalent sdAbs could induce the secretion of IL-2 by Jurkat–huFc γ RIIIA/ γ cells (Fig. 6A) or of IFN- γ by NK cells (Fig. 6B) in a dose-dependent manner, indicating that the binding to the two different epitopes defined by

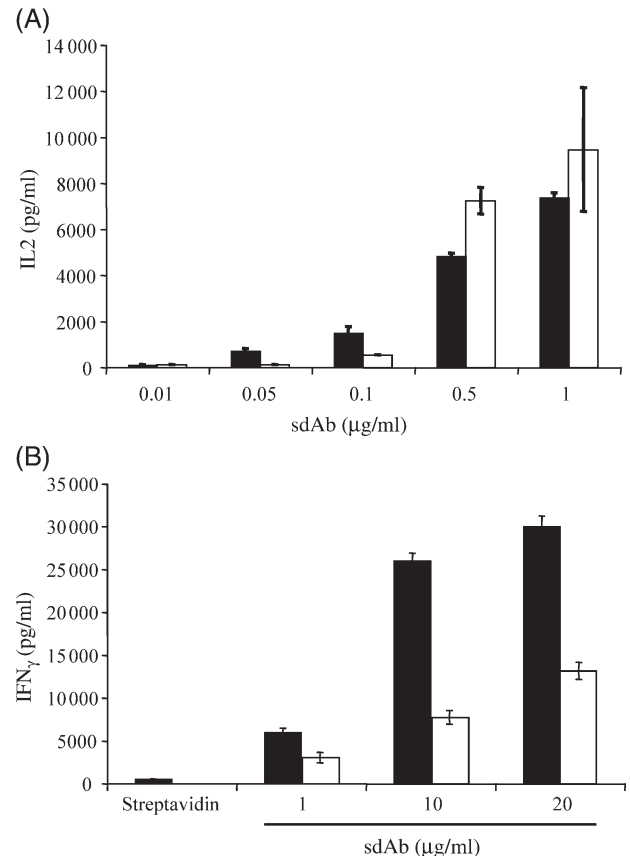


Fig. 6. Secretion of IL-2 by Jurkat–huFc γ RIIIA/ γ cells (A) or of IFN- γ by NK cells (B) after incubation with streptavidin/biotinylated sdAb complexes. The indicated concentrations on abscissa refer to the sdAb concentrations. The ratio streptavidin/biotinylated sdAb was maintained constant (see 'Methods' section). Jurkat–huFc γ RIIIA/ γ cells alone or in the presence of PMA alone did not produce any detectable amount of IL-2. Black bars represent C21 sdAb, whereas white bars represent C28 sdAb.

multivalent C21 and C28 sdAbs allows an efficient cross-linking of surface Fc γ RIIIA that triggers downstream intracellular events leading to cytokine production. In experiments where NK cells were tested for the induction of IFN- γ production by sdAbs, biotinylated anti-Fc γ RIIIA 3G8 antibody was used as positive control (data not shown). In both experiments, streptavidin, or sdAb alone, did not induce any cytokine production.

Discussion

In the present work, we have isolated anti-Fc γ RIII sdAbs from a phage library of VHH fragments derived from an immunized llama, as building units to construct BsAbs able to redirect NK cells toward tumor cells. Four candidates have been selected and characterized. The amino-acid sequences of their CDRs are different, except clones C28 and C72 that exhibit a 90% amino-acid homology. As expected for VHH domains, sequence analysis using IMGT/V-QUEST (Giudicelli *et al.*, 2004) showed that the four corresponding genes are homologous to human IGHV3 subgroup genes (C13, 70% homology with IGHV3-66; C21, 84% with IGHV3-23; C28 and C72, 69% with IGHV3-74). The principal sequence differences between the four llama VHH and human IGHV3 are localized in the CDR regions, and in the former VL and CH1 interfaces (residues 40, 42, 49, 50 and residues 15 and 96, respectively). Moreover, the llama VHH domains belong to subfamilies VHH1 (C21) and VHH2 (C13, C28 and C72) (Harmsen *et al.*, 2000). As described earlier for VHH belonging to these families (Muyldermans *et al.*, 1994), CDR3 from these four VHH do not exceed the mean CDR3 length of classical VH and do not contain an additional disulfide bond, in contrast to most camel VHH.

Here, we show that C21 and C28 sdAbs specifically bind human Fc γ RIII and induce the production of cytokines upon cross-linking of Fc γ RIIIA expressed by NK cells and Jurkat-huFc γ RIIIA/ γ cells. Although the llama was immunized with recombinant soluble Fc γ RIIIB, sdAbs that were isolated bind both Fc γ RIIIA and Fc γ RIIIB isoforms, as most of the anti-Fc γ RIII mAbs raised in mice. Also, similarly to mouse anti-human Fc γ RIII mAbs, these sdAbs do not react with other human Fc γ R (Fc γ RI and Fc γ RIIA/B). Extra-cellular domains of Fc γ RIIIA and Fc γ RIIIB differ in only six residues, and it has been proved difficult to generate mAbs specific for only one isoform. Thus, since a highly purified recombinant sFc γ RIIIB, previously characterized in details (Teillaud *et al.*, 1994), was available in large amounts in the laboratory, we used it as an immunogen. Whether these sdAbs show differential reactivity with the Fc γ RIIIB NA1/NA2 polymorphic forms as the murine GRM-1, CLB-GRAN11 and B73.1 mAbs (van der Herik-Oudjik, 1996) remains to be established.

The two selected sdAbs, C21 and C28, exhibit different characteristics. C21 sdAb has a good affinity with a K_D in the 10 nM range. It binds to a linear epitope also detected on the native protein expressed by NK and PMN cells, as for 7.5.4 mouse mAb (Vely *et al.*, 1997). In contrast, C28 sdAb binds a conformational epitope of Fc γ RIII. However, both sdAbs strongly inhibit the binding of 7.5.4 mAb to sFc γ RIIIB and the binding of this mouse mAb to Jurkat-huFc γ RIIIA/ γ cells. Only C28 sdAb could slightly compete with the binding of 3G8 mAb to these cells. Interestingly,

since 3G8 mAb binds the Fc binding site of Fc γ RIII (Fleit *et al.*, 1982), it suggests that C21 and C28 sdAbs will not compete *in vivo* with circulating Ig for binding to the receptor. Figure 5 summarizes the binding relationship between the two sdAbs and the two murine mAbs and suggests the presence of a least three different, although partly overlapping, epitopes.

C21 and C28 sdAbs, once multimerized via streptavidin/biotin interactions, are able to activate cells expressing Fc γ RIIIA to make them producing IL-2 and IFN- γ , which makes them good candidates to be used in bi-specific formats for therapeutic use. A rather similar efficacy of C28 and C21 sdAbs for inducing the production of IL-2 was observed (Fig. 6A), despite a near 10-fold difference of affinity between the two molecules as measured by SPR (Fig. 2B). In contrast, the induction of IFN- γ production by NK cells by C21 was higher than C28 (Fig. 6B). This could be related to the weaker expression of Fc γ RIII by NK cells than transfected Jurkat cells. The ability to trigger cell activation through Fc receptor clustering depends on more complex parameters than the sole mere affinity. In particular, the epitopes recognized by C21 and C28 sdAbs might lead to different efficacies. Ultimately, the conformation of the sdAb expressed in a bsAb format will be critical to determine which one of these two sdAbs exhibits the best efficacy to activate effector killer cells.

The use of a bsAb to recruit Fc γ RIII⁺ NK cells and monocytes/macrophages rather than a human IgG1 antibody has various advantages. Anti-Fc γ RIII bsAbs make it possible to avoid difficult issues faced by Fc containing therapeutic antibodies, such as (i) variation in glycosylation from batch to batch or from cell line to cell line used for the production, a critical parameter that can dramatically affect the binding to FcR (Umana *et al.*, 1999; Shields *et al.*, 2002; Shinkawa *et al.*, 2003; Siberil *et al.*, 2006b), (ii) competition with circulating IgG, (iii) Fc γ RIII polymorphism in position 158 (Cartron *et al.*, 2002), (iv) toxicity issues due to binding to other Fc-binding receptors and, not but not the least, (v) binding to Fc γ RIIB leading to inhibitory signals that repress the therapeutic effects of conventional antibodies (Clynes *et al.*, 2000).

The stability and ease of expression of sdAbs make them very good candidates as binding units for more elaborate constructs such as bsAbs or any multivalent or multifunctional constructs (Muyldermans, 2001; Omidfar *et al.*, 2004; Coppieters *et al.*, 2006). Notably, the availability of sdAbs directed against activating Fc γ RIII and tumour-associated antigens such as Her2/Neu or CEA opens the way to the generation of bsAbs that can be tested in preclinical models. Ultimately, the success of this new generation of antibody-based molecules will also strongly depend on multiple factors including general conformation, size, stability, immunogenicity and serum half-life, as well as the nature of the targeted molecule on cancer cells. The availability of ADCC-triggering sdAbs might offer innovative solutions leading to new antibody-based cancer therapeutics.

Acknowledgements

We thank Martine Chartier and Françoise Banères-Roquet for their excellent technical assistance, Christiane and Bernard Giudicelli for generously

providing llama for immunizations and Dr Serge Muyldermans for helpful discussion and scientific advice.

Funding

This work was supported by CNRS, INSERM and by the GDR No. 2352 CNRS ‘Tumor immuno-targeting’. Sophie Sib ril was supported by the Pierre and Marie Curie – Paris 6 University.

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Received June 26, 2007; revised September 4, 2007;
accepted October 8, 2007

Edited by Andrew Bradbury