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Technical note

A method for rapid, ligation-independent reformatting of recombinant monoclonal antibodies

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ABSTRACT

Recombinant monoclonal antibodies currently dominate the protein biologics marketplace. The path from target antigen discovery and screening, to a recombinant therapeutic antibody can be time-consuming and laborious. We describe a set of expression vectors, termed mAbXpress, that enable rapid and sequence-independent insertion of antibody variable regions into human constant region backbones. This method takes advantage of the In Fusion™ cloning system from Clontech, which allows ligation-free, high-efficiency insertion of the variable region cassette without the addition of extraneous amino acids. These modular vectors simplify the antibody reformatting process during the preliminary evaluation of therapeutic or diagnostic candidates. The resulting constructs can be used directly for transient or amplifiable, stable expression in mammalian cells. The effectiveness of this method was demonstrated by the creation of a functional, fully human anti-human CD83 monoclonal antibody.

1. Introduction

Over the past 10–15 years there has been a surge of interest in the use of recombinant monoclonal antibodies (mAbs) as therapeutic agents. In 2007, mAb sales in the USA alone exceeded $14 billion, with a year on year growth rate of 22% (Aggarwal, 2008). With the number of approved mAbs approaching 30 and hundreds of new candidates in the pipeline, this trend shows no signs of slowing. Most therapeutic recombinant mAbs are members of the IgG family and owing to their large size and complex glycosylation patterns, these molecules are currently produced in mammalian cells, with the vast majority utilizing Chinese Hamster Ovary (CHO) cells as the production host (Wurm, 2004).

The path from discovery to the clinic for a therapeutic, recombinant mAb can be a long and tedious process, often taking several years. The first step of this process involves identification of a high-affinity binder to a target molecule, such as a surface antigen over-expressed during tumourigenesis. Considerable effort has been dedicated to elucidating methods that facilitate isolation of binding moieties to an antigen of interest. The first mAbs were produced utilizing hybridoma technology, however the resultant murine antibodies are not

Abbreviations: mAb, monoclonal antibody;CHO, Chinese hamster ovary; IgG, immunoglobulin G;V, variable region;scFv, single chain variable fragment;Fab, fragment antigen binding;ADCC, antibody dependent cellular cytotoxicity;SEC, size exclusion chromatography; LAK, lymphokine-activated killer.

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suitable for therapeutic applications (Berger et al., 2002). Subsequently, methods such as CDR grafting, phage, yeast and ribosome display were developed (for review see: Hoogenboom (2005)). Phage display is the most commonly used method. This technique identifies single chain variable fragment (scFv) or fragment antigen binding (Fab) elements, that bind to the target molecule isolated from libraries of high-complexity, emulating the naïve immune repertoire. This library may contain murine or human sequences and more recently, completely synthetic libraries have been created. Crucially, since these fragments contain antibody variable regions, they require "reformatting" into an expression vector containing both the requisite constant region sequences and the elements for high-level expression in mammalian cells. This reformatting step can be a protracted and complicated process since the sequences of the isolated fragments are by nature variable. This makes traditional PCR and/or restriction endonuclease cloning problematic. For example an anti-TNF antibody isolated from a naïve Fab immunoglobulin gene library was rebuilt as a complete antibody by a tripartite ligation; a fragment containing the leader sequence and the amino terminus of the V (variable) domain, a second fragment containing the remainder of the V domain and Cλ constant region, and the expression vector. The reformatting required PCR using fragment specific primers and appendage of compatible restriction sites (Mahler et al., 1997). Existing antibody reformatting vectors exhibit limited flexibility and the codons formed by restriction endonuclease recognition sequences result in the addition of several "foreign" amino acids into the primary sequence (Coloma et al., 1992; Persic et al., 1997; Jostock et al., 2004). These legacy vectors are also no longer commercially available and are difficult to source. In this paper we describe a set of novel vectors that will facilitate a high-throughput-compatible and sequence-independent method for rapid antibody reformatting. We demonstrate the effectiveness of this system by creating a functional, fully human anti-human CD83 mAb.

2. Methods and materials

2.1. Expression vector design

mAbXpress vectors were assembled using publically available human constant region heavy (IgG1 and IgG4 subtypes) and light chain (κ) sequences. Required DNA was synthesized and codon-optimized for mammalian expression by Geneart AG (Germany). These cassettes were then placed into mammalian expression vectors containing sequences for expression, selection and amplification in mammalian cells (Acyte Biotech, Australia) (Fig. 1). A single SacI site was included in the expression vector to facilitate linearization and In Fusion™ cloning of the variable region (see Section 3 for details). We plan to make these vectors available to academic research groups under a standard MTA and are available under license to commercial organizations.

2.2. Phage display panning against CD83 and Ligation-Independent, In Fusion™ cloning of scFvs

The extracellular domain of human CD83 was expressed in CHO cells and purified by immobilized metal affinity chromatography. This preparation was used to isolate binders from the human scFv phage display library of Sheets et al. (1998), kindly provided by Dr James D. Marks (University of California, San Francisco). Several unique binders to recombinant CD83 were isolated and clone 3C12 was selected for cloning and expression.

Variable regions for both the heavy and kappa light chains were PCR amplified from the phagemid vectors using primers against the 5′ and 3′ conserved regions of each chain. An additional 15 bp was included on each primer corresponding to upstream and downstream bases of the destination vector to enable ligation-independent In Fusion™ cloning (Clontech). Example primers for the heavy chain were: 3C12_VhFor 5′-CAGGTGTCCACTCCGAGGTGCAGCTGCAGGAG-3′ and 3C12_VhRev 5′-GCGGAGGACACGGTGAGCGTGGTCCCTTGGCCC-3′, and for the kappa chain the primers were: 3C12_VkFor 5′-CCGGCGTGCACTCCGAGATCGTGATGACCCAG-3′ and 3C12_VkRev 5′-GCCACGGTCCGCTTGAGTTCCAGCTTGGTCCC-3′. Underlined regions represent the scFv-specific sequence, which varies from clone to clone. The unpurified PCR products were inserted into the mAbXpress IgG1 heavy and κ light chain vectors using the In Fusion™ system (Clontech), as per the manufacturer's instructions.

2.3. Mammalian cell expression and purification

For antibody expression we used suspension-adapted Chinese Hamster Ovary (CHO) cells maintained in CD-CHO (Invitrogen). Heavy and light chain plasmids were co-transfected using PEI-Max (prepared in water) (Polysciences Inc). The transfection complex is prepared at a ratio of PEI:DNA of 3.5:1. For transient transfection a 0.75:0.25 v/v ratio of cells:transfection complex is used, therefore each 750 μL of cells (at 1.5×10^6 cells/mL) in CD-CHO is transfected with 1.6 μg DNA and 5.6 μg PEI in 250 μL of OptiPro SFM medium (Invitrogen). The complex was incubated for 15 min at room temperature without disruption before addition to the cell suspension. At 4 h post transfection the cells were diluted by doubling the total volume with CD-CHO and IGF-1 was added at 0.1 mg/L before transferring the cultures to humidified incubators at 32 °C and 7.5% CO_2 for 7–14 days with shaking (160–250 rpm, depending on the vessel and the shaker throw ratio). Expression studies were typically performed at small (2 mL), medium (30 mL) or large (400 mL) scale.

Cellular debris was removed by centrifugation and secreted antibody was purified from the culture media using Protein-A chromatography. A 1 mL Protein-A HiTrap™ column from GE Healthcare was used. After loading, the column was washed with 20 mL of Phosphate buffered saline (PBS) and protein was eluted using 0.1 M glycine pH 2.7 and neutralized with 1 M Tris pH 9. Purified antibody (3C12) was then analyzed by SDS-PAGE (NuPAGE system, Invitrogen) and analytical size exclusion chromatography (SEC). For SEC we used a TSK-GEL G3000SWxl , 30 cm×7.8 mm column (Tosoh Bioscience) on an Agilent 1200 series LC. The mobile phase was 100 mM Phosphate pH 6.7, 200 mM Sodium Chloride, filtered through a 0.22 μm filter. Flow rate was 0.8 mL/min. Calibration was done using gel filtration standards (Bio-Rad). Typical yields from transient transfection experiments using this system ranged from 20 to 60 mg/L.

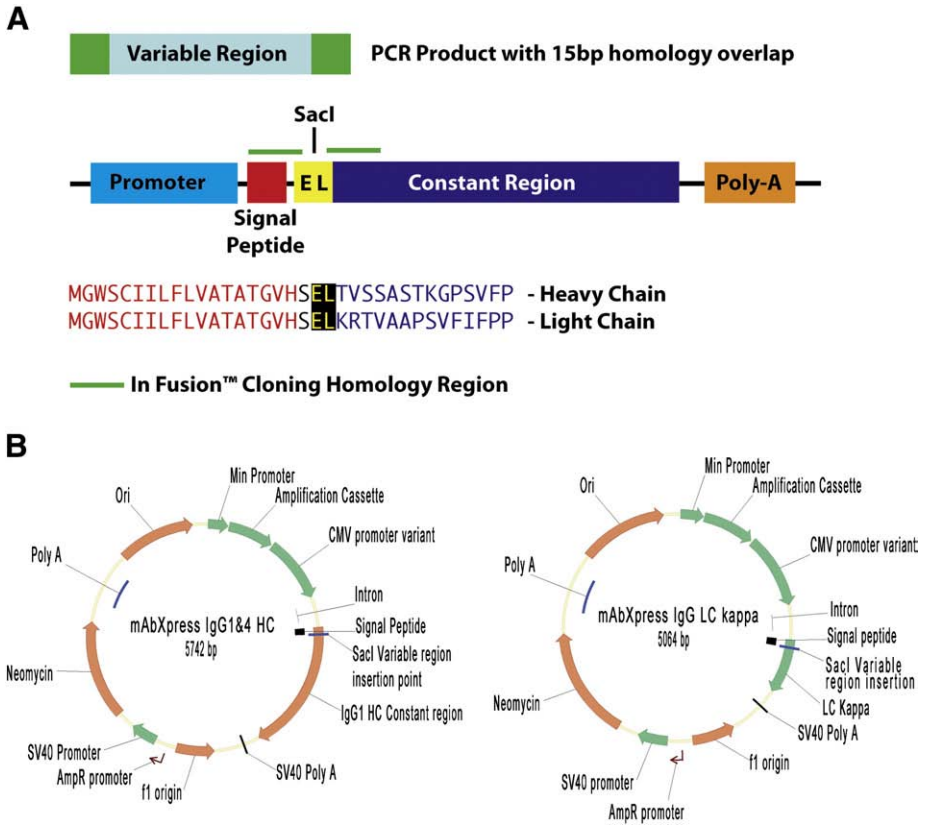

Fig. 1. mAbXpress vector system. Vectors contain all required elements for high-level expression in mammalian cells as well as the backbone sequence of the IgG including a secretory signal peptide. (A) The E–L codons form a SacI site for vector linearization prior to In Fusion™ mediated cloning of the variable region. The variable region PCR product contains 15 bp at the 3′ and 5′ ends with exact homology to the destination vector insertion site flanking the SacI site. (B) Vector maps of the complete heavy chain and light chain constructs.

2.4. Analysis of antibody binding by flow cytometry

One million live cells (KM-H2, L428 and FDCP1) were stained with 2.5 μg/mL purified 3C12 mAb or isotype control (human IgG1; Sigma) for 1 h at 4 °C. Bound antibody was detected with a FITC-conjugated anti-human Fc antibody (Cappel, ICN Pharmaceuticals Inc) diluted 1:50 with PBS. Flow cytometric analysis was performed on a FACS Calibur (Becton Dickinson), and analyzed in FCS Express Version 3 (De Novo Software).

2.5. Generation of lymphokine-activated killer (LAK) cells

Ficoll–Paque density gradient separation was used to isolate peripheral blood mononuclear cells (PBMCs). NK cells were purified using CD56 Microbeads (Miltenyi) on a VarioMACS separator as per manufacturer's specifications. Cells were cultured in RPMI-10 (100 U/mL penicillin, 100 μg/mL streptomycin, 1 x GlutaMAX and 10% fetal calf serum (all from Invitrogen)) with 6000 IU/mL human IL-2 (Boehringer Mannheim) at 37 °C, 5% CO_2 for 48 h. Cells were harvested by incubation for 30 min on ice before supernatant removal, followed by 30 min incubation in ice cold PBS containing 2% EDTA; all harvested cells were washed twice before re-suspension in RPMI-10.

2.6. ^{51}−Chromium release assay

Functional assays were performed with a $CD83^+$ human cell line to determine whether LAK cells could induce antibody dependent cellular cytotoxic (ADCC) lysis in the presence of human anti-hCD83 IgG1. KM-H2 cells (1×10^6 cells/mL) were labeled for 45 min at 37 °C with 100 μCi ^{51}Cr in TD buffer (140 mM NaCl, 5 μM KCl, 25 μM Tris–HCl [pH 7.4], 0.6 μM Na_2HPO_4, 1% human serum albumin). Cells were washed twice with complete RPMI-10.

5×10^4 LAK cells were plated per well in a V-bottom 96-well plate (Nunc) with 1×10^3 ^{51}Cr labeled KM-H2 cells. Cells were treated with 5 μg/mL 3C12 or Herceptin (Roche) as a human IgG1 isotype control. Each well contained either 15 μg/mL anti-human CD16 clone 3G8 or mouse IgG1 isotype control (both from BD Biosciences) to a final volume of 150 μL. Additional wells containing 1×10^3 KM-H2 cells in 150 μL of either RPMI-10 (*spontaneous release*) or 1.67% Triton-X-100 (*total release*) were prepared. Each condition was run with five replicates. Each plate was incubated for 4 h at 37 °C in 5% CO_2 before centrifugation at $300\times g$ for 5 min at 24 °C. 50 μL supernatant was mixed with 150 μL OptiPhase "SuperMix" and assayed for ^{51}Cr counts per minute (cpm) with a 1450-MicroBeta scintillation counter (both from Wallac). Specific cell lysis was calculated using the standard formula: % lysis = [(test sample cpm − spontaneous cpm)/

(total cpm − spontaneous cpm)*100]. GraphPad Prism Version 5.01 software was used to perform a two way ANOVA.

3. Results and discussion

The vectors described here (Fig. 1) overcome several major challenges confronting the reformatting of antibody fragments with regards to the insertion of variable sequences into a constant region backbone. Firstly, this method is sequence-independent. Since scFv constructs contain semi-conserved framework adjacent to hypervariable regions, the semi-conserved framework sequence can be used as template for PCR. This potentially allows the use of a single primer set to construct the complete, fully assembled antibody. Crucially, this feature means the system is directly applicable to high-throughput applications and automation. Secondly, unlike many reformatting vectors, the site of insertion does not require any extraneous bases. Introduction of such additional amino acids to the primary sequence has the potential to interfere with antibody folding, and/or molecule function, immunogenicity and stability. We have identified semi-conserved glutamate (E) and leucine (L) residues,

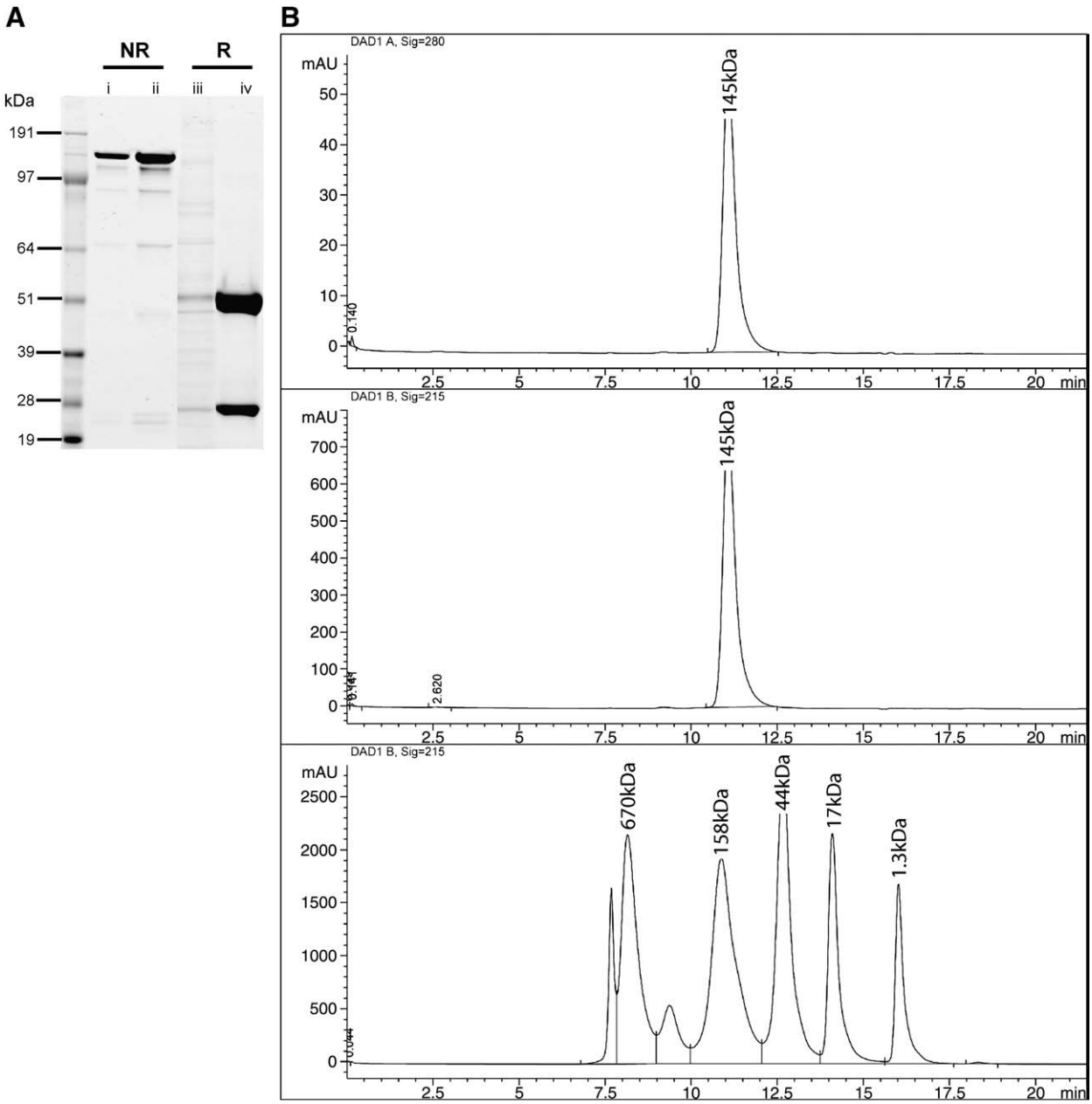

Fig. 2. Analysis of the expression and purification of 3C12 IgG1. (A) Non-reduced (NR) and 2-mercaptoethanol reduced (R) samples of culture supernatants (i, iii) and 5 μg affinity purified material (ii, iv) were separated on 4–12% SDS-PAGE and stained with Coomassie Blue R250. (B) Analytical size exclusion chromatography of protein-A purified recombinant 3C12 antibody at both 280 nm, top panel and 215 nm middle panel, and gel filtration standards at 215 nm bottom panel. The sample shows no detectable aggregation and an as predicted molecular weight of 145 kDa.

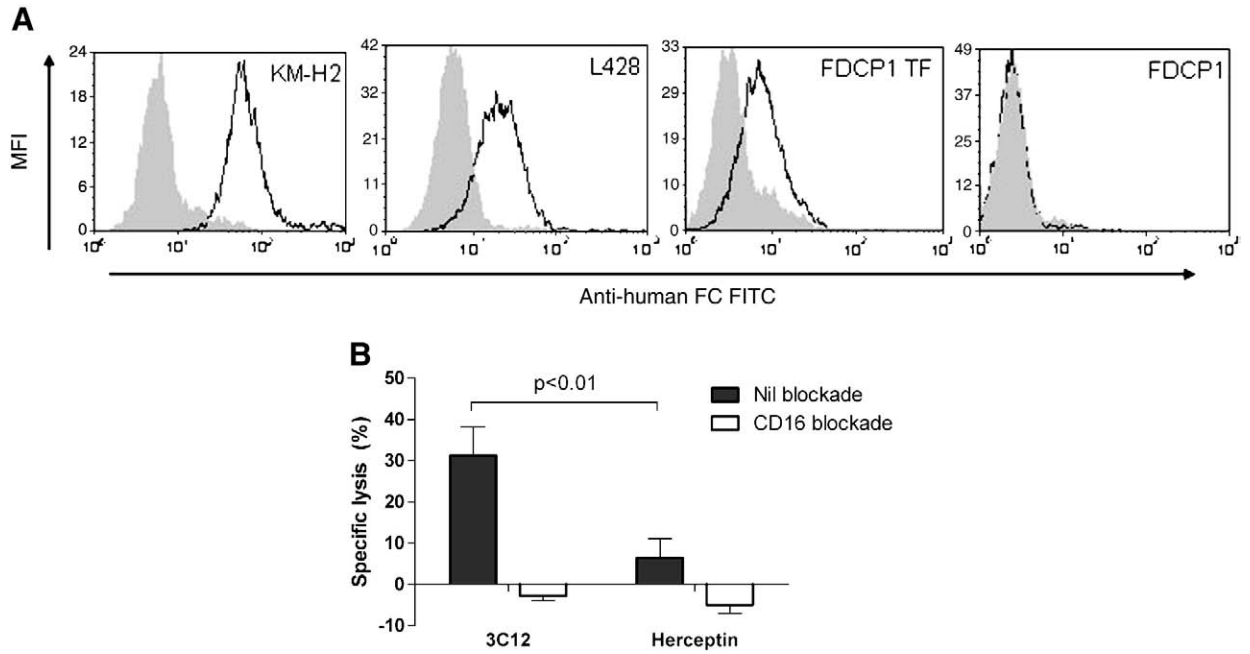

Fig. 3. Functional analysis of purified 3C12, an anti-human CD83 IgG. (A) 25 μg/mL 3C12 IgG1 binds CD83$^+$ cell lines KM-H2, L428 and FDCP1 cells transfected with human CD83. No difference between 3C12 and an isotype IgG1 control is seen on un-transfected FDCP1 cells (CD83$^-$). MFI = mean fluorescence intensity. (B) 3C12 IgG1 induced significant lysis of the KM-H2 cell line relative to Herceptin (negative control) via a CD16-dependent mechanism. Error bars represent standard error of the mean.

which are present in many IgGs at the N- and C-termini of the variable region, respectively (Fig. 1). The sequence encoding these two amino acids (GAG CTC) forms the recognition site for the enzyme SacI. This creates an ideal way to linearize the expression vector and facilitate insertion of the variable region PCR product using Ligation-Independent Cloning, via the In Fusion™ system (Clontech). The In Fusion™ system utilizes an enzyme that facilitates insertion of target DNA into a linearized vector by homologous recombination. Typically this is achieved by adding a 15 base pair sequence at the 3′ and 5′ ends of a PCR product with 100% homology to the site of insertion (Fig. 1). This highly efficient method allows for the rapid reformatting of antibodies into the final expression construct.

A scFv phage clone was obtained by biopanning a human scFv immunoglobulin gene library (Sheets et al., 1998) by three rounds of selection against recombinant hCD83 extracellular domain (AA1-144). This clone demonstrated specific binding to cell surface CD83 expressed by the human Hodgkin's Lymphoma derived cell line, KM-H2 (Fig. 3). Using primers that bind the semi-conserved flanking framework region for each variable region, and which also contain the required vector overlap, this clone was amplified by PCR and cloned into the mAbXpress vectors using the In Fusion™ system (Section 2.2). We expressed the reformatted IgG1 mAb in CHO cells, followed by protein-A based purification. Analysis by SDS-PAGE and SEC (Fig. 2) showed that the molecule was expressed well in our transient expression system, with no observable degradation or aggregation.

We have created IgG1 κ and IgG4 κ versions of these vectors as those variants are commonly used in therapeutic applications. In this initial study we chose to reformat this molecule as an IgG1 κ specifically to provide an ADCC response. In order to show the resulting antibody was functional, we used a purified sample of the recombinant anti-CD83 molecule (3C12 mAb) to demonstrate binding to CD83$^+$ human cell lines and hCD83-transfected cells (Fig. 3A). Additionally, in a chromium release functional assay, 3C12 mAb induced significant cytolysis of KM-H2 cells in the presence of activated natural killer (NK) effector cells (Fig. 3B). This antibody-induced lysis, however, was abrogated upon blockade of FcγIIIRa (CD16) with anti-CD16 mAb, 3G8. This indicates the purified 3C12 mAb is capable of mediating ADCC, as the role of FcγIIIRa expression in this mechanism is well characterized (Perussia and Trinchieri, 1984). Additionally, to test the robustness of this system, we also repeated the process using a scFv clone isolated from a murine display library, and created an intact chimeric CD83-specific binding mAb (data not shown).

At present there are no simple, generic methods for reformatting antibody fragments (Fabs, scFvs, dAbs) as complete, fully assembled antibodies. Traditional approaches of antibody reformatting are antibody/laboratory specific and rely on careful, time intensive, sequence analysis and restriction enzyme cutting and ligation-mediated cloning. These approaches can also lead to the introduction of extraneous amino acids, which may have profound effects on protein folding and/or bioactivity. Moreover there is limited public availability of the required vectors ((Persic et al., 1997), personal communication Mahler). Here we have described a vector system for the rapid reformatting and expression of functional recombinant monoclonal antibodies that operates essentially independently of the variable region sequence. This is particularly attractive for applications that require cloning

of a large number of variable regions during drug discovery and screening.

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