Title

Tetramer Immunization and Selection Followed by CELLISA Screening to Generate Monoclonal Antibodies against the Mouse Cytomegalovirus m12 Immunoevasin.

Journal

The Journal of Immunology, 205(6)

ISSN

0022-1767

Authors

Aguilar, Oscar A
Tanaka, Miho
Balaji, Gautham R
et al.

Publication Date

2020-09-15

DOI

10.4049/jimmunol.2000687

Peer reviewed

The Journal of Immunology

Tetramer Immunization and Selection Followed by CELLISA Screening to Generate Monoclonal Antibodies against the Mouse Cytomegalovirus m12 Immunoevasin

Oscar A. Aguilar, Miho Tanaka, Gautham R. Balaji, Richard Berry, Jamie Rossjohn, Lewis L. Lanier and James R. Carlyle

J Immunol published online 19 August 2020
http://www.jimmunol.org/content/early/2020/08/13/jimmunol.2000687

The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Published August 19, 2020, doi:10.4049/jimmunol.2000687

Tetramer Immunization and Selection Followed by CELLISA Screening to Generate Monoclonal Antibodies against the Mouse Cytomegalovirus m12 Immunoevasin

Oscar A. Aguilar,[*,†,‡] Miho Tanaka,[*,†] Gautham R. Balaji,[§,¶] Richard Berry,[§,¶] Jamie Rossjohn,[§,¶,‖] Lewis L. Lanier,[†,‡] and James R. Carlyle[*]

The generation of reliable mAb of unique and desired specificities serves as a valuable technology to study protein expression and function. However, standard approaches to mAb generation usually involve large-scale protein purification and intensive screening. In this study, we describe an optimized high-throughput proof-of-principle method for the expanded generation, enrichment, and screening of mouse hybridomas secreting mAb specific for a protein of interest. Briefly, we demonstrate that small amounts of a biotinylated protein of interest can be used to generate tetramers for use as prime-boost immunogens, followed by selective enrichment of Ag-specific B cells by magnetic sorting using the same tetramers prior to hybridoma generation. This serves two purposes: 1) to effectively expand both low- and high-affinity B cells specific for the antigenic bait during immunization and 2) to minimize subsequent laborious hybridoma efforts by positive selection of Ag-specific, Ab-secreting cells prior to hybridoma fusion and validation screening. Finally, we employ a rapid and inexpensive screening technology, CELLISA, a high-throughput validation method that uses a chimeric Ag fused to the CD3ζ signaling domain expressed on enzyme-generating reporter cells; these reporters can detect specific mAb in hybridoma supernatants via plate-bound Ab-capture arrays, thereby easing screening. Using this strategy, we generated and characterized novel mouse mAb specific for a viral immunoevasin, the mouse CMV m12 protein, and suggest that these mAb may protect mice from CMV infection via passive immunity. ***The Journal of Immunology*****, 2020, 205: 000–000.**

The use of mAb has been fundamental in advancing our understanding of basic immunology and has revolutionized the fields of applied biotechnology, diagnostic medicine, and clinical therapeutics. mAb have proven to be effective agents to treat cancer, in which they elicit direct cytotoxic effects by guiding immune cells to more effectively recognize cells expressing tumor Ags or block inhibitory signals on immune cells, rendering them more active at eliciting effector functions via checkpoint inhibition (1–3). Broadly neutralizing Ab can also protect against pathogens and have been described for HIV, Ebola, dengue, and Zika virus (4).

The generation of mAb can often be a challenge attributable not only to immunization protocols that require large amounts of Ag but also due to labor-intensive procedures often involving screening of thousands of culture wells to identify suitable hybridomas. These approaches are often not only laborious but are also associated with high screening costs. We have previously described an efficient method to generate mAb using a reporter cell–based system (5, 6). Briefly, by constructing a chimeric Ag fused to the intracellular portion of the CD3ζ signaling domain, reporter cells can be generated that are capable of detecting and reporting Ab-mediated cross-linking; this eases the screening procedure to a low-cost, high-throughput assay whereby the reporter cells generate their own enzyme (5, 6). Initially, immunization protocols used the reporter cells as prime-boost immunogens themselves; however, in this study, we demonstrate in a proof-of-principle approach that it is also possible to effectively 1) immunize host animals and 2) selectively enrich Ag-specific B cells prior to hybridoma fusion using tetrameric forms of the Ag as both the immunogen and an antigenic bait for enrichment, followed by subsequent validation screening.

We recently reported that mouse CMV (MCMV) encodes a viral immunoevasin that targets and antagonizes the NKR-P1 family of

[*]Department of Immunology, University of Toronto, Toronto, Ontario M5S 1A8, Canada; [†]Department of Microbiology and Immunology, University of California, San Francisco, San Francisco, CA 94143; [‡]Parker Institute for Cancer Immunotherapy, University of California, San Francisco, San Francisco, CA 94143; [§]Infection and Immunity Program, Department of Biochemistry and Molecular Biology, Biomedicine Discovery Institute, Monash University, Clayton, Victoria 3800, Australia; [¶]Australian Research Council Centre of Excellence in Advanced Molecular Imaging, Monash University, Clayton, Victoria 3800, Australia; and [‖]Institute of Infection and Immunity, Cardiff University School of Medicine, Cardiff CF14 4XN, United Kingdom

ORCIDs: 0000-0002-7990-8745 (O.A.A.); 0000-0002-7436-4689 (G.R.B.); 0000-0002-2020-7522 (J.R.); 0000-0003-1308-3952 (L.L.L.).

Received for publication June 8, 2020. Accepted for publication July 19, 2020.

This work was supported by Canadian Institutes of Health Research Operating Grants MOP 106491 and PJT 159450 (to J.R.C.) and by Burroughs Wellcome Fund (BWF) Investigator in the Pathogenesis of Infectious Disease Award 1007761 (to J.R.C.) and a BWF Postdoctoral Enrichment Program award (to O.A.A). O.A.A. is a Cancer Research Institute Irvington Fellow supported by the Cancer Research Institute. O.A.A. and L.L.L. are supported by the Parker Institute for Cancer Immunotherapy. J.R. is a recipient of Australian Research Council Laureate Fellowship FL160100049. R.B. is a recipient of National Health and Medical Research Council of Australia Career Development Award APP1109901.

Address correspondence and reprint requests to Dr. Oscar A. Aguilar or Prof. James R. Carlyle, Department of Microbiology and Immunology, University of California, San Francisco, 513 Parnassus Avenue, Room HSE-1001B, San Francisco, CA 94143 (O.A.A.) or Department of Immunology, University of Toronto, 1 King's College Circle, Medical Sciences Building, Room 7307, Toronto, ON M5S 1A8, Canada (J.R.C.). E-mail addresses: oscar.aguilar@ucsf.edu (O.A.A.) or james.carlyle@utoronto.ca (J.R.C.)

The online version of this article contains supplemental material.

Abbreviations used in this article: ADCC, Ab-dependent cellular cytotoxicity; CPRG, chlorophenol-red-β-D-galactopyranoside; CTV, CellTrace Violet; DMEM-HG, high-glucose DMEM; HAT, hypoxanthine–aminopterin–thymidine; HT, hypoxanthine–thymidine; LAK, lymphokine-activated killer; MCMV, mouse CMV; P3, P3XAg8.653.1; rhIL-2, recombinant human IL-2; SA, streptavidin.

NK cell receptors (7, 8). Considering this, we sought to generate mAb specific for this viral decoy ligand, an NKR-P1B immunoevasin, to further characterize functional interactions. In this report, we describe an efficient approach for the immunization, isolation, and screening of hybridomas producing mAb of interest using tetramers of the viral m12 protein. Specifically, we used the MCMV-encoded m12 protein in tetrameric form as the prime-boost immunogen, leveraged the ability of m12 tetramers to enrich for Ag-specific B cells prior to hybridoma fusion, and subsequently validated and characterized several novel mAb specific for m12. Importantly, we last show that these mAb may have passive immunotherapeutic value against CMV infection.

Materials and Methods

Animals and cells

C57BL/6 (B6) mice were purchased from The Jackson Laboratory. Animals were housed and maintained according to approved animal protocols at the University of Toronto and Sunnybrook Research Institute.

YAC-1 cells were obtained from the American Type and Culture Collection. BWZ.36 cells were provided by Dr. N. Shastri (University of California, Berkeley). HEK293T and P3XAg8.653.1 (P3) cells were obtained from Dr. D. H. Raulet (University of California, Berkeley). Cells were cultured in complete high-glucose DMEM (DMEM-HG) or RPMI 1640 supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin, 110 μg/ml sodium pyruvate, 50 μM 2-ME, 10 mM HEPES, and 10–20% FCS. Splenic lymphokine-activated killer (LAK) cells were cultured in complete RPMI 1640 supplemented with 1000 U/ml recombinant human IL-2 (rhIL-2; Teceleukin).

Transfections

For transfections, HEK293T cells were plated 1 d prior to transfection in six-well plates (4×10^5 per well). Transfections were performed using Lipofectamine 2000 according to the manufacturer's protocol (Thermo Fisher Scientific). Cells were analyzed 48 h posttransfection using flow cytometry. All mammalian expression vectors used in this study have been previously described (7, 9).

Protein expression and generation of tetramers

Tetrameric forms of $m12^{Smith}$ were generated as previously described (7, 10, 11). Briefly, DNA encoding m12 from the $MCMV^{Smith}$ strain (residues 29–208) was ligated into the pFastbac vector (Thermo Fisher Scientific) upstream of 6× histidine and BirA tags and used to generate recombinant baculovirus according to the manufacturer's instructions. Recombinant baculovirus was produced by transfection into Sf9 cells, and the supernatant was used to infect Hi5 insect cells. Protein was harvested 48–72 h later from cell media and was then purified using nickel affinity and size-exclusion chromatography using HisTrap and Superdex 200 columns 16/60 (GE Healthcare), respectively. For biotinylation, purified m12 was buffer exchanged into 10 mM Tris and enzymatically biotinylated overnight at 20°C prior to removal of free biotin via size-exclusion chromatography. Tetramers were generated by incubating biotinylated $m12^{Smith}$ monomeric protein with streptavidin (SA)–PE or SA–allophycocyanin in a 4:1 M ratio, aliquoted in 1/10 volumes over a period of 3 h at 4°C.

Immunizations, hybridoma selection, and CELLISA screening

On day 0, 6–8-wk-old B6 mice were immunized with $m12^{Smith}$–allophycocyanin tetramer (25 μg) emulsified in CFA by i.p. injection. MCMV $m12^{Smith}$ tetramers have previously been described (7). Twenty-eight days later, the mice were boosted using $m12^{Smith}$–allophycocyanin tetramer (50 μg) emulsified in IFA. On day 42, the mice received one final boost of purified $m12^{Smith}$–biotin protein (25 μg, no SA–allophycocyanin) in IFA, then sacrificed 3 d later. On the day of sacrifice, cardiac puncture blood was collected for purification of polyclonal antisera. Splenocytes were harvested, exposed to 1× ammonium–chloride–potassium lysis buffer for 5 min on ice, thoroughly washed with ice-cold PBS, then stained with $m12^{Smith}$–PE tetramers (no SA–allophycocyanin) before enrichment using anti-PE magnetic selection (STEMCELL Technologies), as previously described (12). These enriched $m12^{Smith}$–PE^+ splenocytes were fused with P3X63.Ag8.653.1 (P3) hybridoma fusion partners at a 2:1 ratio using polyethylene glycol 1500 (MilliporeSigma) according to an established protocol (13). Hybridomas were resuspended in 20% DMEM-HG and aliquoted into a 96-well plate (one plate per fusion). The following day, selection using 20% DMEM-HG supplemented with 1× hypoxanthine–aminopterin–thymidine (HAT) was initiated. After 7 d, the medium was switched to 20% DMEM-HG with 1× hypoxanthine–thymidine (HT), and 3–5 d later, the cells were screened for mAb of interest.

Screening of hybridomas was initially accomplished by using Ig-capture plate-bound stimulations of CELLISA arrays using BWZ.CD3ζ–$m12^{Smith}$ reporter cells, as described below. Once candidate hybridomas were identified and subcloned, mAb were further validated and characterized using several approaches described below.

Flow cytometry

Cells were stained with primary mAb supernatants (100 μl of hybridoma supernatant) in FACS buffer (HBSS, 0.5% BSA, and 0.03% NaN_3) on ice for 30 min, washed, incubated with secondary Ab for another 30 min, then analyzed using an FACSCanto II or LSR II (BD Biosciences). Cells were gated by forward and side light scatter properties and propidium iodide or DAPI exclusion for viability. Data were analyzed using FlowJo software (FlowJo). Alexa Fluor 647–conjugated goat anti-mouse IgG, IgG_1, IgG_{2a}, IgG_{2b}, IgG_{2c}, IgG_3, IgM, and IgG + IgM secondary reagents were purchased from Jackson ImmunoResearch Laboratories. The anti-mouse $NKR\text{-}P1B^{B6}$ mAb (2D12) was a kind gift from Drs. K. Iizuka and W. M. Yokoyama (14). Anti-mouse NK1.1/$NKR\text{-}P1C^{B6}$ (PK136), NKp46 (29A1.4), and CD3ε (145-2C11) mAb were purchased from BioLegend.

BWZ reporter cell assays

BWZ.CD3ζ–$NKR\text{-}P1B^{B6}$, BWZ.CD3ζ–$NKR\text{-}P1B^{129}$, BWZ.CD3ζ–$NKR\text{-}P1B^{FVB}$, BWZ.CD3ζ–$NKR\text{-}P1C^{B6}$, and BWZ.CD3ζ–$m12^{Smith}$ reporter cells have previously been described (7, 15). Briefly, the ectodomains of the NKR-P1B receptor or $m12^{Smith}$ were fused with intracellular mouse CD3ζ signaling domains in the murine stem cell virus vector expressing type II and type I fusion cassettes, respectively, and used to transduce BWZ.36 cells, as previously described (6). Transduced cells were then sorted for GFP^+ expression using FACS.

For plate-bound stimulations (CELLISA assays), anti-mouse IgG + IgM capture plates were prepared by incubating high-binding chemistry, flat-bottom, 96-well plates (Corning) with polyclonal goat anti-mouse IgG + IgM Ab (Jackson ImmunoResearch Laboratories) at 10 μg/ml in 75 μl PBS per well, then incubated overnight at 37°C in a tissue culture incubator. Plates were washed three times with 200 μl of PBS, followed by Ab capture via addition of 100 μl of hybridoma supernatant directly to the wells in 96-well arrays and incubated overnight. Plates were triple washed with 200 μl of PBS, then reporter cells (BWZ.CD3ζ–$m12^{Smith}$, 5×10^4 in 200 μl medium/well) were added and incubated overnight. The following morning, cells were pelleted, washed with PBS, and then resuspended in 150 μl of 1× chlorophenol-red-β-D-galactopyranoside buffer (90 mg/l CPRG [MilliporeSigma], 9 mM $MgCl_2$, and 0.1% NP-40 in PBS). Plates were incubated at room temperature, then analyzed using a Varioskan microplate reader (Thermo Fisher Scientific) with a signal–background subtraction set at OD signal 595–655-nm background ($\Delta OD_{595-655}$).

For cell-based stimulation assays, 5×10^4 BWZ reporter cells were cocultured with 5×10^4 stimulator cells overnight in a tissue culture incubator. Hybridoma supernatants (100 μl) were preincubated with stimulator cells for 1 h at 37°C to test blocking potential. Plates were developed as described above. As positive controls, reporter cells were stimulated with 10 ng/ml PMA and 0.5 μM ionomycin in complete DMEM-HG.

Hybridoma isotyping

mAb were isotyped using a mouse Ig isotyping ELISA kit following the manufacturer's protocol (BD Biosciences). Because the ELISA kit detects BALB/c strain Ab isotypes, we confirmed Ab cross-reactivity using secondary anti-mouse IgM and IgG reagents by flow cytometry.

Ab purification

Monoclonal IgG Ab were purified from hybridoma supernatants using Capturem Protein G Maxiprep Columns according to manufacturer's protocols (Takara Bio). mAb were concentrated using Amicon Ultra columns (MilliporeSigma).

Cellular cytotoxicity assay

NK-mediated cytotoxicity was measured using a flow cytometry–based assay. Briefly, NK-LAK cells were generated by processing spleens into single-cell suspensions then culturing them in complete RPMI 1640 supplemented with 1000 U/ml rhIL-2 (Teceleukin; National Cancer Institute). On day 7, cells were harvested and used as effectors in cytotoxicity assays. YAC-1 targets expressing $m12^{Smith}$, $m12^{MW97}$, or

control vector were labeled with CellTrace Violet (CTV; Thermo Fisher Scientific), and then incubated with NK-LAK effectors at different E:T ratios in the presence or absence of purified Ab. Cells were cocultured for 4 h at 37°C and then resuspended in FACS buffer containing propidium iodide. For control wells, bleach or media were used to determine maximal and spontaneous cellular death in the assay, respectively. Percentage of specific lysis was calculated by determining the percentage of live CTV^+ targets in each well relative to control cells as follows:

$$\%\ \text{Specific lysis} = \%\ CTV^+PI^-\ \text{in sample} - \%\ CTV^+PI^-\ \text{in spontaneous control} / \%\ CTV^+PI^-\ \text{in maximal control} - \%CTV^+PI^-\ \text{in spontaneous control}$$

Statistical analysis

Data were analyzed using Prism 7 (GraphPad) employing one-way ANOVA analysis (see figure legends). All graphs show mean ± SEM (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). All data are representative of at least three independent experiments.

Results

Prime-boost immunization and Ag-specific B cell enrichment using m12^{Smith} tetramers

We have recently described how MCMV targets the NKR-P1B/Clr-b missing-self NK cell recognition axis, including the evolution of a virally encoded decoy immunoevasin, m12, which inhibits NK cell responses via NKR-P1B (7–9, 16, 17). Importantly, we also observed that the activating NKR-P1A and NKR-P1C paralogs could also bind this ligand (7). To further characterize m12 function, we set out to generate m12-specific mAb. Using our previously generated $m12^{Smith}$ tetramers (Fig. 1A) (7), we initiated an immunization protocol using these tetramers as the prime-boost immunogen emulsified in CFA adjuvant (Fig. 1B). Briefly, B6 mice were immunized with 25 μg of $m12^{Smith}$–allophycocyanin tetramer in CFA, boosted on day 28 using 50 μg of $m12^{Smith}$–allophycocyanin tetramer emulsified in IFA, then finally boosted on day 42 using 25 μg of purified $m12^{Smith}$–biotin monomers (to avoid stimulating

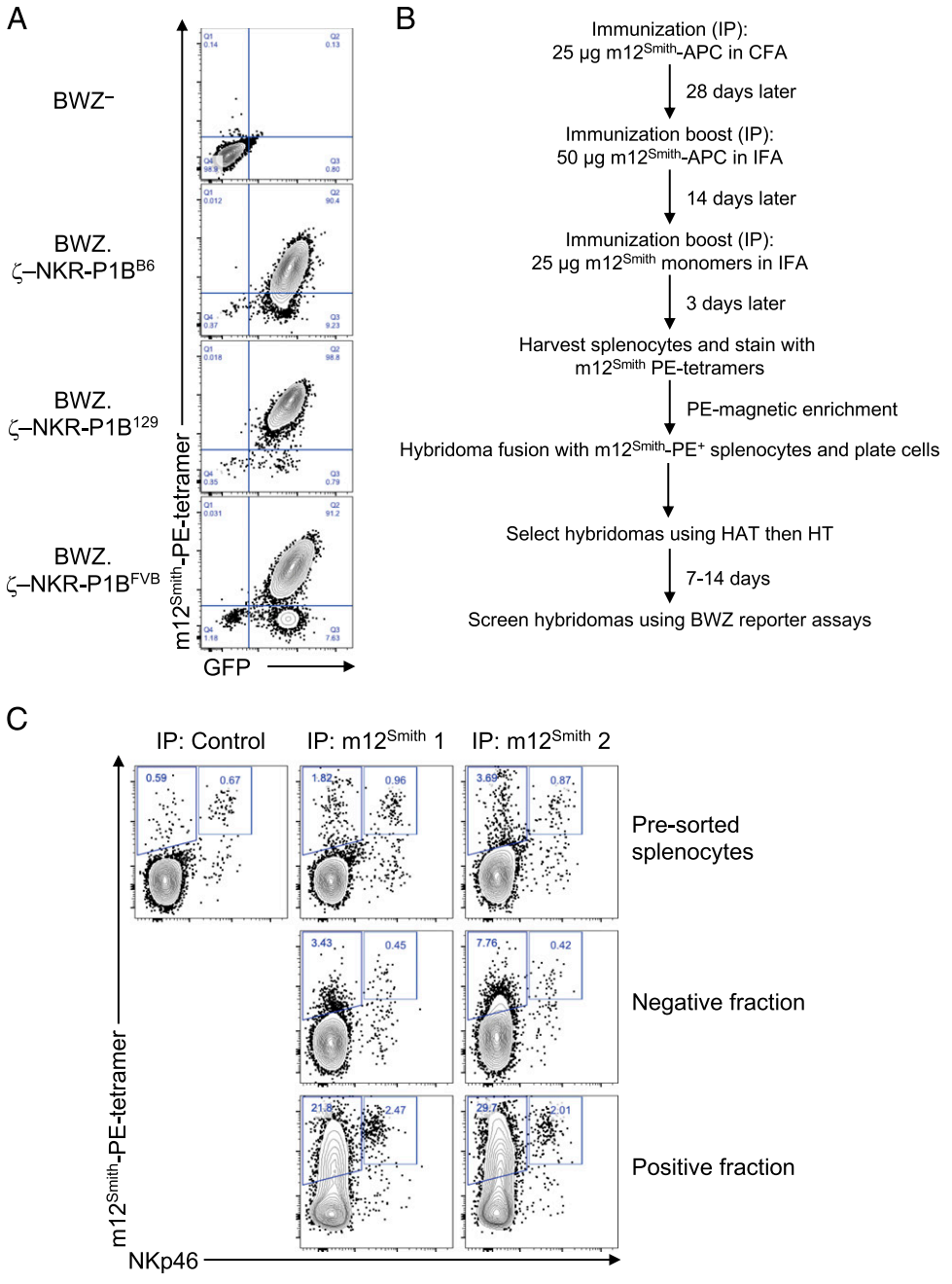

FIGURE 1. Prime-boost immunizations and B cell enrichment using $m12^{Smith}$ tetramers. (**A**) BWZ reporter cells bearing chimeric CD3ζ fusions of NKR-P1B alleles were stained with $m12^{Smith}$-PE tetramers. (**B**) Prime-boost immunization strategy. (**C**) Analysis of pre- and post-MACS–sorted splenocytes using $m12^{Smith}$-PE tetramer. Splenocytes from mice immunized with $m12^{Smith}$ Ag allophycocyanin tetramers or PBS control were stained with $m12^{Smith}$-PE tetramers then positively enriched for PE using an anti-PE MACS kit. These cells were then costained with anti-NKp46 Ab and analyzed by flow cytometry. Flow plots demonstrate pre- and postsorted splenocytes (negative and positive fractions, respectively).

SA–allophycocyanin carrier-specific B cells) in IFA. Mice were sacrificed 3 d later, spleens were harvested, and m12^{Smith}–PE–tetramer^{+} splenocytes (to avoid allophycocyanin-reactive B cells) were isolated using an anti-PE^{+} enrichment kit, akin to a previously described method (12). As seen in Fig. 1C, the anti-PE enrichment using m12^{Smith}-PE tetramers resulted in 20–30% of the splenocytes being NKp46^{-}m12^{Smith}–tetramer^{+}, corresponding to roughly a 10-fold enrichment of m12-reactive B cells. Interestingly, this approach also allowed coenrichment of m12^{Smith}-tetramer^{+} NK cells, which largely represents the NKR-P1B^{+} subset (NKp46^{+}m12–tetramer^{+}; Fig. 1C). These cells were then fused with P3 fusion partners, selected using HAT followed by HT media and then tested for production of mAb of interest.

Screening hybridomas for m12 reactivity

First, to confirm the successful generation of anti-m12 Ab, we tested the polyclonal antisera from immunized mice. Importantly, only the antisera from animals immunized with m12^{Smith} reagents were capable of staining BWZ.CD3ζ–m12^{Smith} reporter cells, but not parental BWZ^{-} cells, using flow cytometry (Fig. 2A), thus validating our immunization approach. We then proceeded to isolate hybridomas for m12-specific mAb.

To test the efficiency of generating Ab, we initially screened hybridomas using our CELLISA approach (5, 6). This high-throughput screen was accomplished using BWZ.CD3ζ–m12^{Smith} reporter cells (BWZ.ζ–m12^{Smith}) in Ab-capture, plate-bound stimulation arrays. These reporter cells display the m12^{Smith} extracellular domain fused with the intracellular signaling domain of CD3ζ and express the *LacZ* gene (β-galactosidase enzyme) upon downstream NFAT activation, thus facilitating measurements of receptor ligation using an inexpensive colorimetric assay whereby the responding cells make their own enzyme (6). Briefly, high-binding chemistry plates were initially coated with anti-mouse IgG + IgM capture Ab, thoroughly washed, then incubated with target hybridoma supernatants. Wells were then washed, BWZ.ζ–m12^{Smith} reporter cells were incubated on plates overnight, and assays were developed by adding CPRG solution containing substrate and lysis buffer following the removal of media and washing using PBS. As shown in Fig. 2B, this approach yielded a significant frequency and number of hybridomas capable of stimulating BWZ.ζ–m12^{Smith} reporter cells. In this initial screen, the first and second host mice gave rise to 5 and 46 anti-m12 mAb^{+} clones, respectively. We then froze down replicates of these initial plates while expanding 50 of the strongest responding wells for further validation and characterization.

Characterization of anti-m12 mAb

We next validated and characterized the expanded hybridoma clones. Of the 50 chosen candidates, only 40 grew as viable

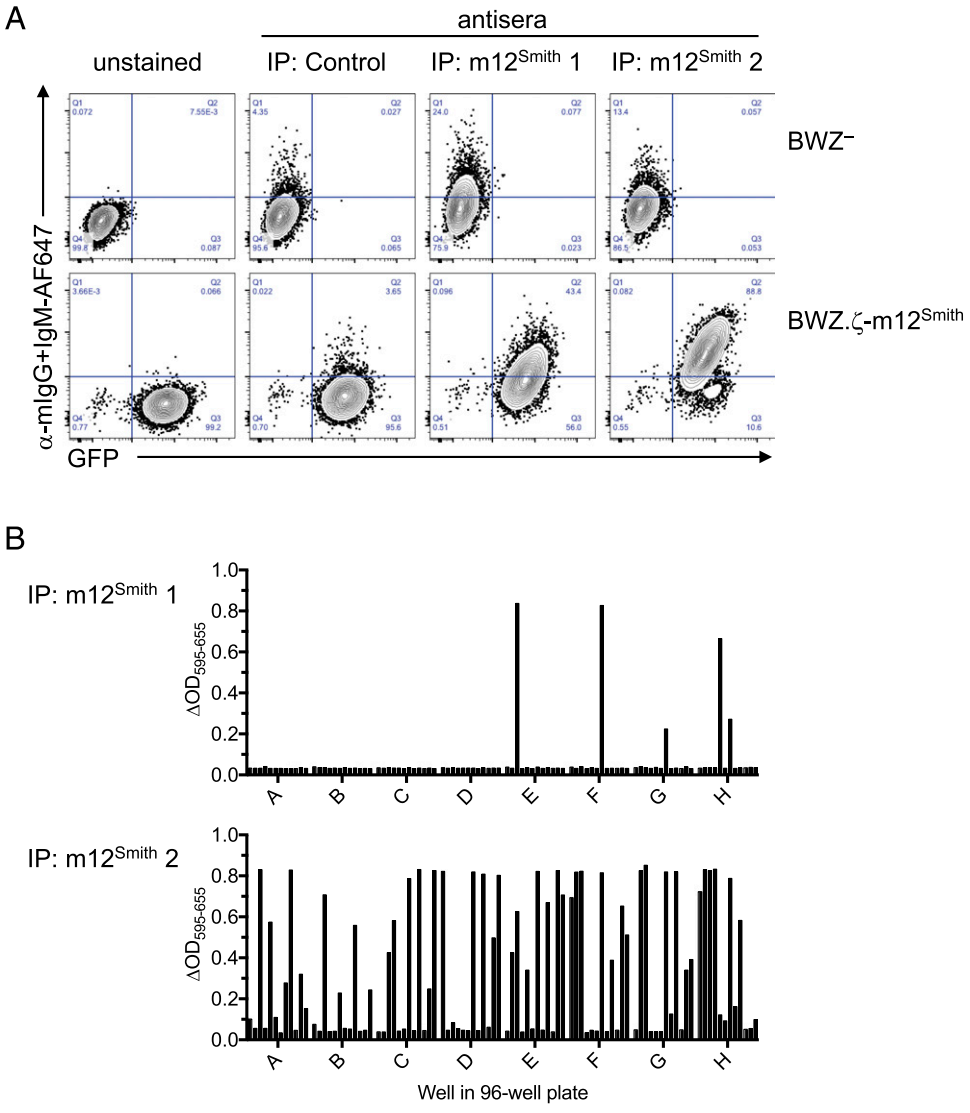

FIGURE 2. Screening antisera and hybridomas for m12 reactivity by flow cytometry and CELLISA. (**A**) BWZ^{-} and BWZ.CD3ζ–m12^{Smith} reporters were stained with polyclonal antisera from immunized mice using flow cytometry. (**B**) Hybridoma supernatants were screened using Ab-capture, plate-bound reporter cell stimulations. mAb isolated from supernatants of HAT/HT-selected hybridomas were captured with anti-mouse Ig–coated wells of 96-well plates and were then used to screen for m12-specific Ab by stimulating BWZ.CD3ζ–m12^{Smith} reporters. Screening results from two individual mice are shown.

long-term stable cultures, and of these, only 18 consistently produced m12-reactive mAb once stably established. These hybridoma clones were then characterized in several biological assays. First, we confirmed that their mAb were capable of stimulating BWZ.ζ–m12^{Smith} reporters in plate-bound assays (Fig. 3A). Second, we tested their ability to stain BWZ.ζ–m12^{Smith} cells. As shown in Fig. 3B, all candidate m12 mAb stained m12^{Smith}-bearing reporter cells, but not parental BWZ^{-} cells. Using anti-mouse IgG– and anti-mouse IgM–specific secondary reagents, we observed that most of the mAb were IgG isotype, whereas two clones produced IgM isotypes (data not shown). This was subsequently confirmed using a mouse mAb isotyping kit (Table I).

We investigated which mAb were capable of blocking the m12^{Smith}/NKR-P1B interaction in a cellular context. To this end, we used our previously generated BWZ target cells transduced with constructs expressing two different m12 alleles (BW.m12^{Smith} and BW.m12^{MW97}), which differ in a single amino acid (E91K). To confirm that these cells were surface m12^{+}, we used hybridoma supernatants and observed that ~5–10% of the BW.m12^{Smith} and ~0.5–1% of the BW.m12^{MW97} were m12^{+}, respectively. Therefore, we sorted these cells for m12 expression using a selected 2C12 hybridoma (Supplemental Fig. 1). Interestingly, the BW.m12^{Smith} allele consistently had higher 2C12 anti-m12 expression versus the BW.m12^{MW97} allele (observed with all hybridoma stains; data not shown). Next, we performed reporter cell assays using CD3ζ–NKR-P1B fusion receptor–bearing reporter cells (BWZ.ζ–NKR-P1B) with BW.m12 targets as stimulator cells. We preincubated hybridoma supernatants with these stimulator cells for 1 h prior to coculture with BWZ.ζ–NKR-P1B^{B6}, BWZ.ζ–NKR-P1B^{129}, and BWZ.ζ–NKR-P1B^{FVB} reporter cells. These alleles cover a range from low to medium to high affinities for m12^{Smith} (B6 < 129 < FVB) (7). In this study, we observed that 2C12 was capable of blocking interactions between m12^{Smith} and NKR-P1B^{B6}, NKR-P1B^{129}, and NKR-P1B^{FVB}; notably, 2D1 partially blocked B6 and

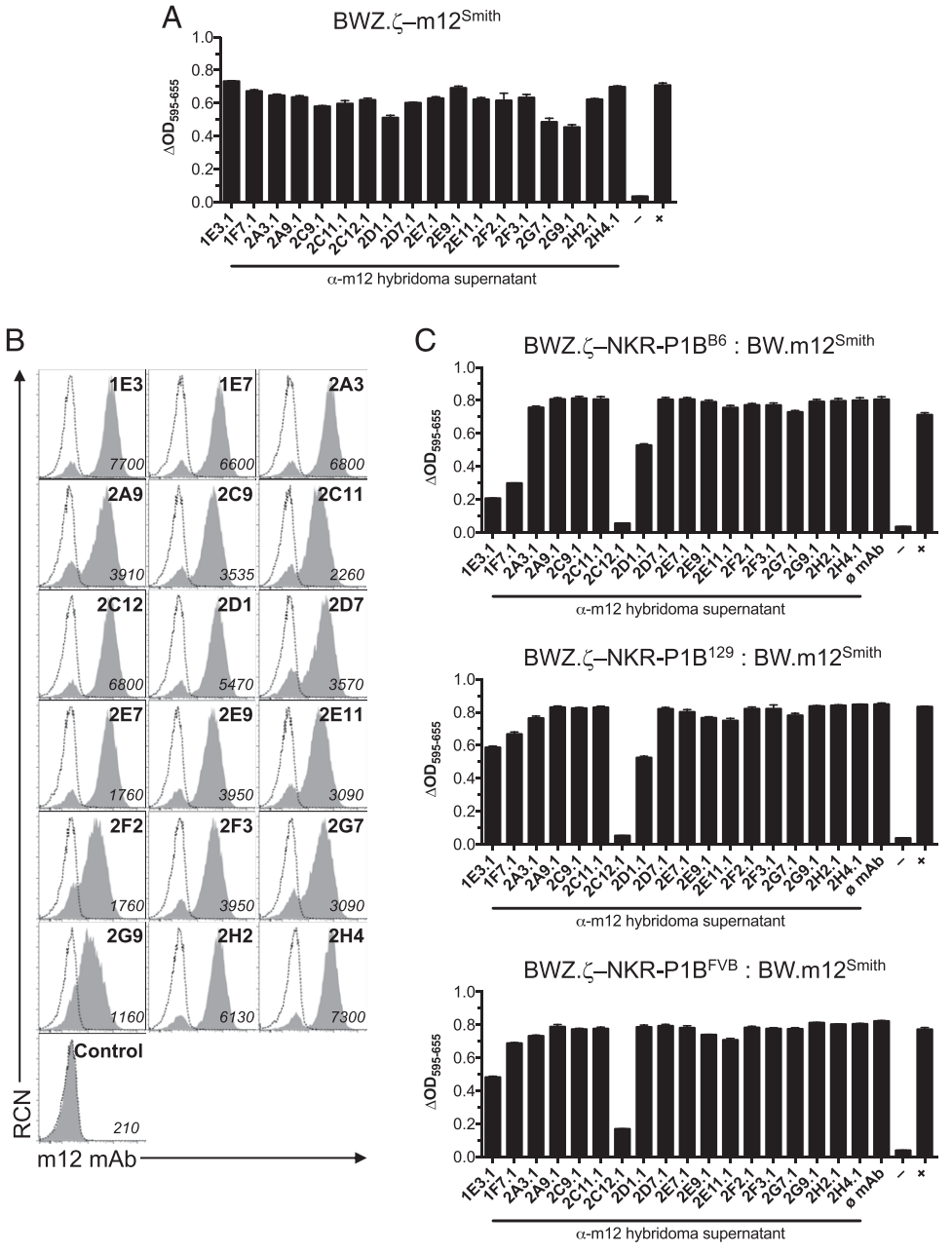

FIGURE 3. Characterization of m12 mAb. (**A**) Plate-bound stimulations. mAb from hybridoma supernatants of HAT/HT-selected hybridomas were captured with anti-mouse Ig–coated wells of 96-well plates then used to stimulate BWZ.CD3ζ–m12^{Smith} reporter cells to confirm anti-m12 specificity using a colorimetric assay. (**B**) Hybridoma supernatants were used to stain parental BWZ^{-} (dotted line) and BWZ.CD3ζ–m12^{Smith} (shaded histograms), which were then analyzed using flow cytometry. Labels on top right and bottom right correspond to hybridoma clone and mean fluorescence intensity (MFI), respectively. (**C**) Blocking assays. Hybridoma supernatants were tested to determine if they could block NKR-P1B:m12^{Smith} interactions in reporter/stimulator cell assays. BWZ.CD3ζ–NKR-P1B reporter cells were cocultured with BW.m12^{Smith} stimulator cells in the presence or absence (ø) of hybridoma supernatants. Media and PMA + ionomycin correspond to negative (–) and positive (+) controls, respectively. Graphs show mean ± SD. All data are representative of $n = 3$.

Table I. Ab isotypes of polyclonal hybridoma clones

Hybridoma Clone	Ab H Chain Isotype; L Chain
1E3.1	IgM; κ
1F7.1	IgM; κ
2A3.1	IgG_{2a}, IgG_{2b}; κ
2A9.1	IgG_1, IgG_{2a}; κ, λ
2C9.1	IgG_1; κ, λ
2C11.1	IgG_1, IgM; κ, λ
2C12.1	IgG_1; κ
2D1.1	IgG_1; κ, λ
2D7.1	IgG_{2a}; κ
2E7.1	IgG_1, IgG_{2a}; κ
2E9.1	IgG_1; κ
2E11.1	IgG_1; κ, λ
2G2.1	IgG_1; κ, λ
2F3.1	IgG_1; κ, λ
2G7.1	IgG_1; κ, λ
2G9.1	IgG_1; κ
2H2.1	IgG_1; κ, λ
2H4.1	IgG_{2a}; κ

Mice immunized were C57BL/6, and therefore do not encode IgG2a locus but instead have IgG_{2c}.

129 alleles, whereas 1E3 and 1F7 were only capable of partially blocking NKR-P1B (Fig. 3C).

We next determined if anti-m12 mAb could recognize other allelic variants of m12. In this study, we transfected HEK293T cells with constructs expressing $m12^{Smith}$, $m12^{MW97}$, $m12^{C4A}$, or $m12^{G4}$ and tested the ability of our mAb to detect these alleles at the cell surface. Interestingly, we observed that all mAb were capable of recognizing $m12^{Smith}$, $m12^{MW97}$, and $m12^{C4A}$, but not $m12^{G4}$. This was not surprising given that $m12^{G4}$ is highly polymorphic relative to $m12^{Smith}$ (26-aa differences) (7). Worth noting, $m12^{G4}$ has 3-aa differences in residues that contact NKR-$P1B^{B6}$, whereas $m12^{MW97}$ and $m12^{C4A}$ do not have any differences in contact residues (7). Also, consistent with our observations using BW.m12 targets, the $m12^{MW97}$ transfectants displayed lower staining levels compared with their $m12^{Smith}$ counterparts (Fig. 4A; Supplemental Fig. 1). We next determined if any mAb could cross-react with other m02 or m145 family MCMV immunoevasins by transfecting the various family members into HEK293T cells then staining them using m12 mAb supernatants. In this study, we confirmed that m12 mAb were uniquely specific for the $m12^{Smith}$ among all other m02 and m145 family members (Fig. 4B). Because some of these initial parental hybridomas were very likely to be oligoclonal, we further selected for monoclonal hybridoma subclones by limiting dilution. In this study, we obtained 19 mAb that retained their anti-m12 specificity using CELLISA assays (Table II; data not shown). We then reassessed these mAb in their ability to block NKR-P1B/m12 interactions in BWZ reporter cell assays. To further evaluate these mAb, we also performed assays with either $m12^{Smith}$- or $m12^{MW97}$-expressing stimulator cells. Importantly, $m12^{MW97}$ only interacts with the 129 and FVB, but not B6 NKR-P1B alleles, whereas $m12^{Smith}$ interacts with all with varying affinities. Consistent with previous findings (Fig. 3C), 1E3, 1F7, and 2D1 partially blocked NKR-$P1B^{B6}$ interactions, whereas these mAb were less effective at blocking binding to NKR-$P1B^{FVB}$ (Figs. 3C and 5A). All 2C12 subclones were very effective in blocking engagement of $m12^{Smith}$ to all NKR-P1B alleles, whereas 2D1 only completed blocked NKR-$P1B^{129}$ (Fig. 5A). Importantly, 2C12 also blocked BW.$m12^{MW97}$ interactions with

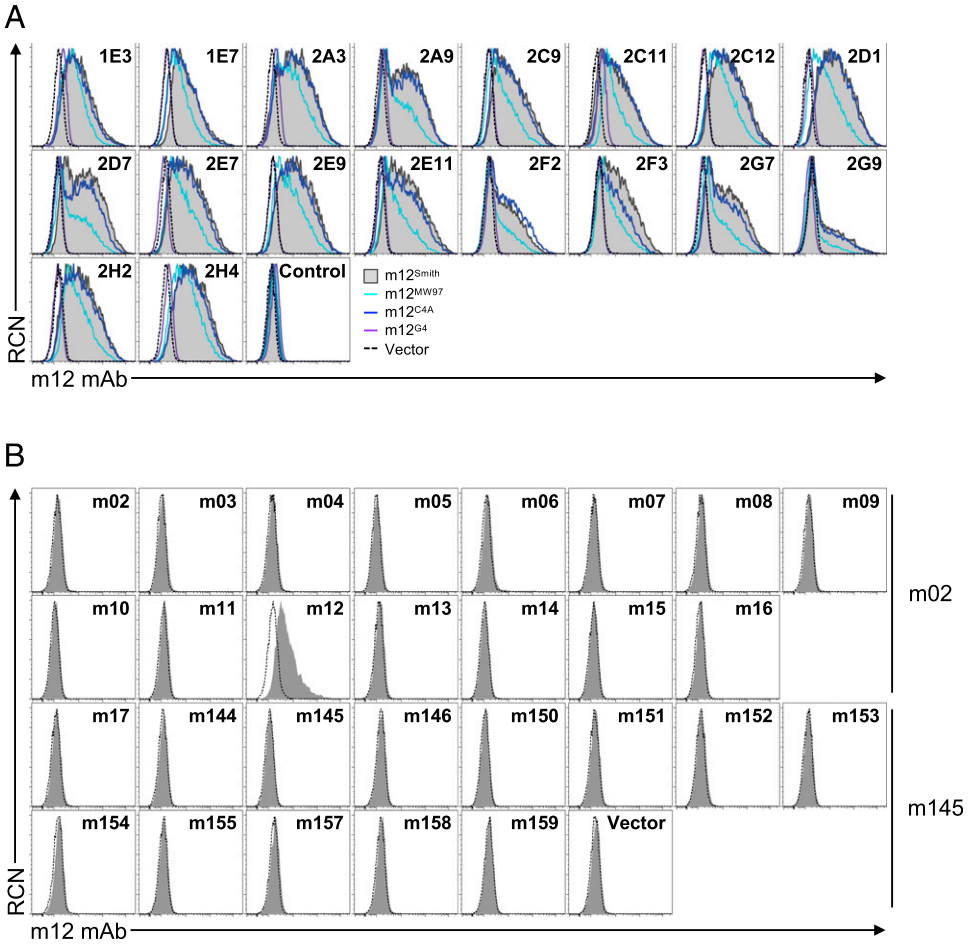

FIGURE 4. m12 mAb are uniquely m12 specific and do not cross-react with other MCMV m02 or m145 family members. (**A**) HEK293T cells were transfected with mammalian expression vector (pIRES2-EGFP) expressing m12 alleles ($m12^{Smith}$, $m12^{MW97}$, $m12^{C4A}$, or $m12^{G4}$). After 48 h, cells were stained with anti-m12 hybridoma supernatants and then analyzed by flow cytometry. (**B**) HEK293T cells were transfected with pIRES2-EGFP vectors expressing m02 or m145 family of immunoevasins, and 48 h later, cells were stained with a mixture of anti-m12 hybridoma supernatants and analyzed using flow cytometry. Shaded histogram, anti-m12 mAb mixture, followed by secondary reagent; dashed line, anti-mIgG + IgM secondary Ab control.

Table II. Ab isotypes of monoclonal hybridoma clones

Hybridoma Clone (mAb)	Ab Isotype
1E3.1.1	IgM
1E3.1.3	IgM
1E3.1.5	IgM
1F7.1.7	IgM
1F7.1.10	IgM
1F7.1.12	IgM
2A3.1.1	IgG_{2b}
2A3.1.4	IgG_{2b}
2C12.1.3	IgG_1
2C12.1.4	IgG_1
2D1.1.4	IgG_1
2D7.1.1	IgG_{2c}
2D7.1.5	IgG_{2c}
2D7.1.7	IgG_{2c}
2D7.1.10	IgG_{2c}
2E9.1.1	IgG_1
2H4.1.1	IgG_{2c}
2H4.1.2	IgG_{2c}
2H4.1.3	IgG_{2c}

Note: these are all subclones of the hybridomas in Table I. For example, 2C12.1.4 is a subclone of 2C12.1, which is a subclone of well C12 from plate 2.

BWZ.ζ–NKR-P1B^{129} and BWZ.ζ–NKR-P1B^{FVB} (Fig. 5B). Interestingly, in BW.m12^{MW97}:BWZ.ζ–NKR-P1B^{129} cocultures, we observed some mAb actually increased reporter cell stimulation, which may be the result of mAb stabilizing this m12 allele at the cell surface (Fig. 5B). Consistent with our previous findings, cocultures of the immunogen variant BW.m12^{Smith}:BWZ.ζ–NKR-P1C^{B6,129,FVB} alleles did not yield significant signals (7) (data not shown). Collectively, these data demonstrate that m12 reactive mAb hybridoma supernatants could recognize the original m12^{Smith} prime-boost immunogen, all subclones cross-reacted with the m12^{MW97}, and m12^{C4A}, but not m12^{G4} alleles, and some subclones were capable of blocking m12 interactions with multiple NKR-P1B receptor alleles.

Anti-m12 mAb clone 2C12 blocks m12-mediated inhibition of NK cell cytotoxicity

To determine if some of these Ab were capable of influencing target recognition by NK cells, cytotoxicity assays were performed. For these experiments, we selected one mAb for each IgG isotype (2A3 = IgG_{2b}, 2C12 = IgG_1, and 2H4 = IgG_{2c}) and purified the mAb from hybridoma supernatants. Splenic NK-LAK cells (Fig. 6A) from B6 mice were cultured and used as effectors against NK cell–sensitive YAC-1 targets expressing m12 alleles (Fig. 6B). As expected, there was no difference in killing of YAC-1 controls (YAC.Vector) when incubated with any of the mAb. Similar results were observed with m12^{MW97}-expressing targets because m12^{MW97} is poorly engaged by the NKR-P1B^{B6} allele (Fig. 6B) (7). In contrast, in cocultures with YAC-1–expressing

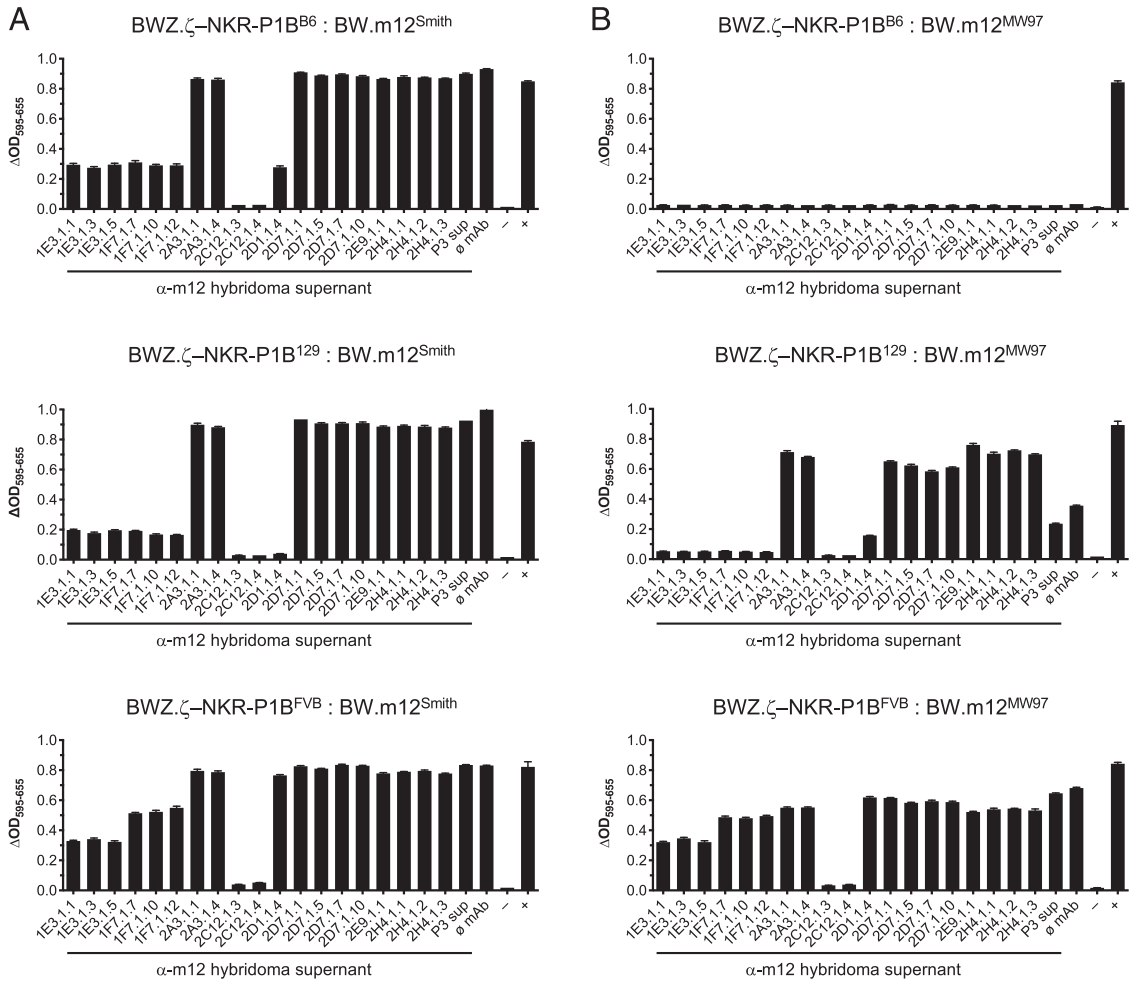

FIGURE 5. Assessment of the ability of m12 mAb subclones to block m12 and NKR-P1B allelic interactions. BWZ.CD3ζ–NKR-P1B^{B6}, BWZ.CD3ζ–NKR-P1B^{129}, and BWZ.CD3ζ–NKR-P1B^{FVB} reporters were cocultured with (**A**) BW.m12^{Smith} or (**B**) BW.m12^{MW97} stimulators in the presence of m12 mAb supernatants, supernatant from P3 fusion partner cells (P3 sup), or media (ø mAb). After overnight cocultures, reporter assay was developed using colorimetric BWZ assay. Media and PMA + ionomycin correspond to negative (–) and positive (+) controls, respectively. Graphs show mean ± SD. All data are representative of $n = 3$.

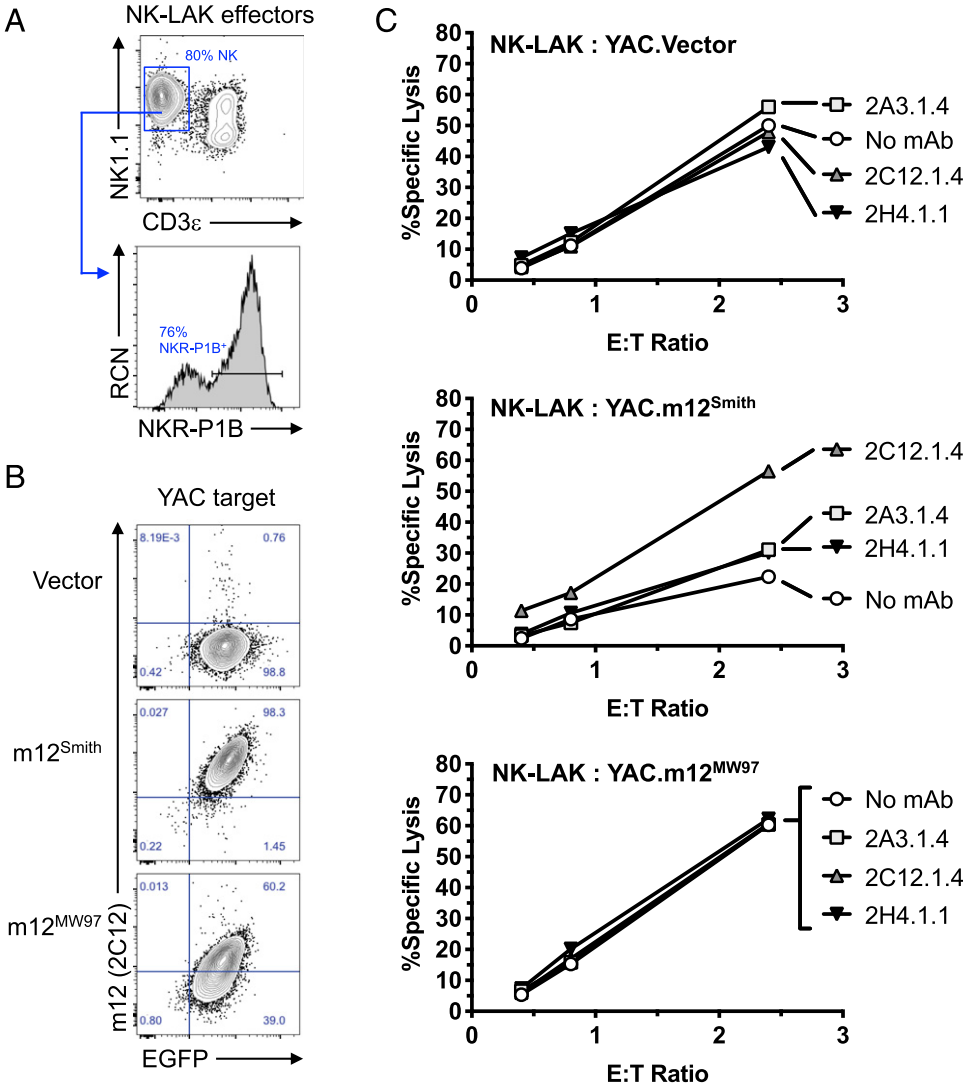

FIGURE 6. m12 mAb clone 2C12 blocks m12-specific NKR-P1B–mediated inhibition of NK cell cytotoxicity. Splenic NK-LAK cells were generated by culturing splenocytes from B6 mice in the presence of rhIL-2. After 7 d of culturing, cells were harvested, stained for cell surface expression of (**A**) NK1.1, CD3ε, and NKR-P1B and then used as effectors in cytotoxicity assays. (**B**) YAC-1 cells expressing $m12^{Smith}$ or $m12^{MW97}$ alleles were stained to confirm m12 expression then used as targets in cytotoxicity assays. (**C**) Cytotoxicity assays using NK-LAK as effectors and YAC.Vector, YAC.$m12^{Smith}$, and YAC.$m12^{MW97}$ as targets. After 4 h of coculture, cells were analyzed to measure percentage of live target cells.

$m12^{Smith}$, there was a slight increase in killing with 2A3 and 2H4 (possibly because of FcγRIII-mediated, Ab-dependent cellular cytotoxicity [ADCC]); however, 2C12 yielded efficient killing of these targets most likely because of its superior ability to block $m12^{Smith}$ engagement with NKR-P1B. Collectively, these results demonstrate that the 2C12 is strikingly effective at blocking m12-mediated NKR-P1B–dependent decoy immunoevasion (in multiple cellular contexts), thereby restoring NK cell function and perhaps additionally enhancing cytotoxicity via ADCC.

Discussion

In this study, we describe a novel sensitive high-throughput protocol for the efficient generation of Ag-specific hybridomas and mAb. We used tetrameric forms of the candidate protein of interest for both prime-boost immunization and selective enrichment of candidate-responding B cells to generate hybridomas and Ag-specific mAb along with a cost-effective, high-throughput assay for the screening and validation of mAb specificities (CELLISA). We demonstrate that this method enhances immunization and eases screening while greatly diminishing the effort required to fuse and screen hybridomas; CELLISA also facilitates a rapid and sensitive high-throughput assay for identification of positive mAb-secreting clones to cell surface Ags.

The utility of this assay is supported by the following theoretical and real advancements: 1) tetramers of the protein of interest facilitate the activation of both low-affinity and high-affinity B cells during prime-boost immunization and should also permit greatly reduced amounts of protein immunogen upon further experimentation; 2) the final boost using purified tetramers free of SA–allophycocyanin carrier facilitates the selective reactivation of Ag-specific B cells prior to fusion, minimizing cross-reactive, carrier-specific B cells; 3) magnetic (or perhaps enhanced FACS based) SA–PE^{+} tetramer enrichment of only Ag-reactive B cells prior to hybridoma fusion greatly minimizes the number of fusion partner cells required (logarithmically lower numbers), as well as the plating and screening efforts required to generate Ag-specific hybridomas while at the same time minimizing or eliminating undesired or allophycocyanin-reactive clones; 4) the BWZ.CD3ζ–fusion reporter cells bearing the Ag of interest facilitate rapid, efficient, and high-throughput screening of Ag-specific hybridomas in a single day using an inexpensive colorimetric assay because they produce their own enzyme. Using this approach, our fusions yielded one plate per spleen rather than the 30 plates per spleen, as is commonly used in the absence of enrichment, resulting in an economy of labor, materials, and time. The use of reporter cells, which drive potent β-galactosidase enzyme production in response to Ag–CD3ζ chimeric receptor stimulation, in plate-bound stimulation colorimetric assays greatly enhances the sensitivity and ease of screening (5, 6). In addition, our technique facilitates the detection of Ab that engage an Ag in its native state.

Importantly, although in this study we used anti-mouse IgG and anti-mouse IgM Ab for capturing hybridoma-produced mAb, this could easily be adapted for targeting specific Ab isotypes, such as IgA or IgE. Therefore, the combined use of reporter cells, tetrameric proteins for both immunization and enrichment and hybridoma supernatants allow for a rapid, efficient, and specific validation of several Ab parameters in a short time period, including Ag expression levels, affinity and avidity measurements (6), comparative allelic reactivities, functional blocking assays, cross-reactivities with antigenic variants, mAb isotype variants, and theoretically complement activation, Fc receptor binding, ADCC function, etc. Although prior studies have used FACS to enrich Ag-specific B cells for hybridoma production (18), our method combines this approach with tetrameric Ag immunization and a sensitive functional reporter screening technique. We are now testing whether this protocol will allow for similar generation and screening of mAb specific for intracellular protein targets in addition to cell surface proteins.

Importantly, in light of the global COVID-19 pandemic, this approach may be particularly useful as a basis for the rapid generation of novel neutralizing or diagnostic mAb to emerging pathogens, such as the SARS-CoV2 coronavirus, the causative agent behind COVID-19. We are currently pursuing this endeavor.

Disclosures

The authors have no financial conflicts of interest.

References

1. Weiner, L. M., R. Surana, and S. Wang. 2010. Monoclonal antibodies: versatile platforms for cancer immunotherapy. *Nat. Rev. Immunol.* 10: 317–327.
2. Khalil, D. N., E. L. Smith, R. J. Brentjens, and J. D. Wolchok. 2016. The future of cancer treatment: immunomodulation, CARs and combination immunotherapy. [Published erratum appears in 2016 *Nat. Rev. Clin. Oncol*. 13: 394.] *Nat. Rev. Clin. Oncol.* 13: 273–290.
3. Mahoney, K. M., P. D. Rennert, and G. J. Freeman. 2015. Combination cancer immunotherapy and new immunomodulatory targets. *Nat. Rev. Drug Discov.* 14: 561–584.
4. Walker, L. M., and D. R. Burton. 2018. Passive immunotherapy of viral infections: 'super-antibodies' enter the fray. *Nat. Rev. Immunol.* 18: 297–308.
5. Chen, P., A. Mesci, and J. R. Carlyle. 2011. CELLISA: reporter cell-based immunization and screening of hybridomas specific for cell surface antigens. *Methods Mol. Biol.* 748: 209–225.
6. Mesci, A., and J. R. Carlyle. 2007. A rapid and efficient method for the generation and screening of monoclonal antibodies specific for cell surface antigens. *J. Immunol. Methods* 323: 78–87.
7. Aguilar, O. A., R. Berry, M. M. Rahim, J. J. Reichel, B. Popovic, M. Tanaka, Z. Fu, G. R. Balaji, T. N. Lau, M. M. Tu, et al. 2017. A viral immunoevasin controls innate immunity by targeting the prototypical natural killer cell receptor family. *Cell* 169: 58–71.e14.
8. Balaji, G. R., O. A. Aguilar, M. Tanaka, M. A. Shingu-Vazquez, Z. Fu, B. S. Gully, L. L. Lanier, J. R. Carlyle, J. Rossjohn, and R. Berry. 2018. Recognition of host Clr-b by the inhibitory NKR-P1B receptor provides a basis for missing-self recognition. *Nat. Commun.* 9: 4623.
9. Aguilar, O. A., I. S. Sampaio, M. M. A. Rahim, J. D. Samaniego, M. E. Tilahun, M. Krishnamoorthy, B. Popović, M. Babić, A. Krmpotić, B. Treanor, et al. 2019. Mouse cytomegalovirus m153 protein stabilizes expression of the inhibitory NKR-P1B ligand Clr-b. *J. Virol.* 94: e01220-19.
10. Berry, R., N. Ng, P. M. Saunders, J. P. Vivian, J. Lin, F. A. Deuss, A. J. Corbett, C. A. Forbes, J. M. Widjaja, L. C. Sullivan, et al. 2013. Targeting of a natural killer cell receptor family by a viral immunoevasin. *Nat. Immunol.* 14: 699–705.
11. Deuss, F. A., G. M. Watson, K. J. Goodall, I. Leece, S. Chatterjee, Z. Fu, M. Thaysen-Andersen, D. M. Andrews, J. Rossjohn, and R. Berry. 2019. Structural basis for the recognition of nectin-like protein-5 by the human-activating immune receptor, DNAM-1. *J. Biol. Chem.* 294: 12534–12546.
12. Spanier, J. A., D. R. Frederick, J. J. Taylor, J. R. Heffernan, D. I. Kotov, T. Martinov, K. C. Osum, J. L. Ruggiero, B. J. Rust, S. J. Landry, et al. 2016. Efficient generation of monoclonal antibodies against peptide in the context of MHCII using magnetic enrichment. *Nat. Commun.* 7: 11804.
13. Yokoyama, W. M., M. Christensen, G. Dos Santos, D. Miller, J. Ho, T. Wu, M. Dziegelewski, and F. A. Neethling. 2013. Production of monoclonal antibodies. *Curr Protoc Immunol.* 102: 2.5.1–2.5.29.
14. Iizuka, K., O. V. Naidenko, B. F. Plougastel, D. H. Fremont, and W. M. Yokoyama. 2003. Genetically linked C-type lectin-related ligands for the NKRP1 family of natural killer cell receptors. *Nat. Immunol.* 4: 801–807.
15. Chen, P., S. Bélanger, O. A. Aguilar, Q. Zhang, A. St-Laurent, M. M. Rahim, A. P. Makrigiannis, and J. R. Carlyle. 2011. Analysis of the mouse 129-strain Nkrp1-Clr gene cluster reveals conservation of genomic organization and functional receptor-ligand interactions despite significant allelic polymorphism. *Immunogenetics* 63: 627–640.
16. Weizman, O. E., E. Song, N. M. Adams, A. D. Hildreth, L. Riggan, C. Krishna, O. A. Aguilar, C. S. Leslie, J. R. Carlyle, J. C. Sun, and T. E. O'Sullivan. 2019. Mouse cytomegalovirus-experienced ILC1s acquire a memory response dependent on the viral glycoprotein m12. *Nat Immunol.* 20: 1004–1011.
17. Aguilar, O. A., A. Mesci, J. Ma, P. Chen, C. L. Kirkham, J. Hundrieser, S. Voigt, D. S. Allan, and J. R. Carlyle. 2015. Modulation of Clr ligand expression and NKR-P1 receptor function during murine cytomegalovirus infection. *J. Innate Immun.* 7: 584–600.
18. Parks, D. R., V. M. Bryan, V. T. Oi, and L. A. Herzenberg. 1979. Antigen-specific identification and cloning of hybridomas with a fluorescence-activated cell sorter. *Proc. Natl. Acad. Sci. USA* 76: 1962–1966.