Heterologous immunization strategy developed broadly reactive human monoclonal antibodies against the BK virus

J. Andrew Duty[1,2,+,*], Thomas Kraus[2,+], Madhu Kumar[1], Nicolo A. Tortorella[1], Tajudeen O. Jimoh[2], Diana V. Pastrana, Christopher B. Buck[3], Thomas Moran[1,2], and Domenico Tortorella[2,*]

[1]Center for Therapeutic Antibody Development, Drug Discovery Institute, Icahn School of Medicine at Mount Sinai, New York, NY

[2]Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, NY

[3]Lab of Cellular Oncology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA

+authors contributed equally to the study
*co-corresponding

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ABSTRACT

BK polyomavirus (BKV) causes polyomavirus-associated nephropathy (PyVAN) and polyomavirus-associated hemorrhagic cystitis (PyVHC) following kidney transplantation and allogeneic hematopoietic stem cell transplantation (HST). BKV strains fall into four distinct genotypes (BKV-I, -II, -III, and -IV) with more than 80% of individuals are seropositive against BKV-I genotype, while the seroprevalence of the other four genotypes is lower. PyVAN and PyVHC occurs in immunosuppressed (e.g. transplant recipients) or immunomodulated (e.g. pregnant women) individuals. In the case of transplant patients, the sole treatment is to reduce immunosuppression, which increases the chance of graft failure. Multiple investigations have shown that polyclonal antibodies have a role in preventing or treating BKV-mediated sickness, implying that a broadly reactive monoclonal antibody (mAb) regimen targeting BKV could be used to limit virus propagation in varied patient populations. Thus, we utilized a heterologous immunization strategy using BKV genotype I-IV major capsid protein VP1 DNA in transgenic VelocImmune@ mice to generate broadly reactive anti-BKV antibodies. Hybridoma clones from the immunized mice were screened using high-throughput binding assays against VP1 of the various BK genotypes. The binding clones were then assessed for neutralization of BKV pseudoviruses consisting of the VP1 protein of the BKV-I, -II, -III, or -IV genotypes. Overall, the screening identified more than 170 genotype-specific mAbs, as well as 15 broadly cross-neutralizing mAbs against BKV-I, -II, -III, and -IV PsVs. The unique panel of broadly neutralizing mAbs could be utilized prophylactically or therapeutically to prevent or treat BKV-induced illnesses.

INTRODUCTION

BK polyomavirus (BKV) is a member of the non-enveloped DNA virus that causes disease in immunosuppressed individuals. The BKV genome has three functional regions: early viral gene region (EVGR) that encodes the large and small T antigens, the late viral gene region (LVGR) encoding the capsid proteins and the non-structural agnoprotein; and the non-coding control region (NCCR). The structure of the BKV capsid is comprised of VP1, VP2, and VP3. The major capsid protein VP1 is responsible for attachment to target cells and organizes into 72 protruding pentamers (360 VP1 molecules) arranged in an icosahedral structure (1). VP2 and VP3 form part of the inner surface of the BKV capsid, located at the center of the VP1 pentamer (1, 2), 2). Cellular attachment and infectious entry are mostly mediated by interactions between VP1 and cell-surface glycosaminoglycans and sialylated glycans, such as the headgroups of the ganglioside GT1b (3, 4). Yet, BK genotype IV does not utilize gangliosides for entry and the JCV polyomavirus can use non-sialylated glycosaminoglycans as alternative attachment receptors (5, 6). BKV is generally categorized into genotypes I, II, III, and IV based on the sequence of VP1, with BKV-I being the most prevalent (60-80%) and BKV-IV (10-20%) next most common genotype (7).

BKV infection occurs through oral or respiratory routes and is nearly ubiquitous, with 75-90% seropositivity by the age of five. The virus is thought to establish a persistent latent infection of the epithelial cells of the kidney and bladder (8-10). As such, there are approximately 25,000 and 30,000 kidney transplants annually in the United States (11) and Europe (12), respectively. Uncontrolled BKV replication is observed in 30-50% of kidney transplant patients, with up to 8% of cases leading to graft dysfunction or loss (12). Extrapolations from these data suggest that over 2,000 allograft failures per year in the US and Europe can be linked to BKV replication. In HST patients, 10-25% develop PyVHC leading to pain, bleeding, obstruction and, in some cases, renal failure (13, 14). Detection of BKV DNA in the blood represents the primary method for diagnosis and levels of BKV DNAemia greater than 10^4/ml is an accepted proxy for polyomavirus nephropathy (PyVAN) (8, 15-17). BKV DNAemia also correlates with an increased risk of bladder cancer (18). Moreover, the estimated direct medical cost of renal allograft loss in the United States is $1.4 billion for all transplants or $78,000 per kidney transplant (19). The total economic burden of graft failures, including quality adjusted life years (QALY), ranges from $2.8-5.8 billion or $161,000-327,000 per kidney transplant recipient in 2017 (19). Thus, there is an urgent medical need for effective therapies to limit BKV replication in the immunosuppressed population.

Following primary infection, individuals with intact immunity are largely asymptomatic, and the virus persists or latently infects the renal urinary tract. BKV is frequently excreted in moderate amounts in the urine by the elderly. Several factors have been identified that predispose a recipient to get PyVAN. High virus levels in the urine or serum anti-BKV antibody titers in the donor are high risk factors for PyVAN development in the transplant recipient (20-23). The early appearance of BKV replication/viremia increases the risk of acute graft rejection (24). Importantly, if the graft recipient lacks antibodies against the viral genotype found in the donor organ (21-23) or there is a mismatch in viral genotype between donor and recipient, the risk of developing viremia and PyVAN is increased (8, 20). The combination of high antibody titers in the donor (indicating high viral load) and low or mismatched antibody titers in the recipient is a significant risk factor (20, 23). In fact, polyclonal human immunoglobulin treatment in clinical trials has been found to decrease the viral load in the transplant population, suggesting biologics as a therapeutic strategy to limit BKV-associate diseases (5, 25-28).

To develop broadly neutralizing antibodies to all BKV genotypes, we immunized *VelocImmune*® transgenic mice (29, 30) that encode for human antibodies using a heterologous immunization strategy using the VP1 cDNA of the diverse BKV genotypes. High-throughput binding and neutralization assays were able to identified monoclonal antibodies that broadly react to the diverse BKV-I, -II, -III, and -IV genotypes. Theses broadly reactive mAbs offer therapeutic potential to limit BKV DNAemia by preventing virus spread in the host regardless of the replicating BKV genotype.

METHODS AND MATERIALS

Cells and plasmids

HEK-293TT lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Corning, 10-013-CV) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1 mM HEPES (Corning, 25-060-CI), 100 U/mL penicillin, and 100 g/mL streptomycin (100X Pen/Strep, Corning, 30-002-CI). NIH EXPI293F and transfection systems were purchased from Invitrogen. The BKV genotypes of VP1: BKV-Ib2 (PittVR2; DQ989796), BKV-II (GBR-12; AB263920), BKV-III (KOM-3; AB211386), and BKV-IV (Novara) genotypes; VP2: BKV-IV (ph2b); and VP3: BKV-IV (ph3b) were cloned into mammalian expression plasmids (31, 32).

Mouse vaccination and hybridoma fusion

VelocImmune® female mice were provided by Regeneron Pharmaceuticals and express human variable light and heavy chains in their antibodies as described (25). The VelocImmune® mice were immunized with expression plasmids for VP1, VP2 and VP3 by electroporation. Briefly, mice received a prime immunization with cDNAs with various protocols (**Figure 1**), followed by a boosting immunization at least 14 days post-priming. Mice received a total of three boosts, and blood was collected from the submandibular vein on different days post-priming for sera binding and neutralization analysis. Groups of mice were immunized and the mice displaying the highest sera titer of neutralizing antibodies against BKV-I, -II, -III, and -IV PsVs were selected for fusion. Boosts with BK PsV preparations were administered IP without adjuvant at 3-4 days prior to fusion. The mouse's spleen was processed to a single-cell suspension before hybridoma generation. Splenocytes from each mouse were fused using polyethylene glycol (PEG) with hypoxanthine-aminopterin-thymidine-sensitive F23.1 cells using the ClonalCell-HY system and protocol, as described previously (StemCell Technologies) (33). Briefly, individual B-cell clones were grown on soft agar and selected for screening using a robotic ClonaCell Easy Pick instrument (Hamilton/StemCell Technologies). Individual clones were expanded, and the supernatant was used to screen for virus neutralization. All animal studies were approved by the Icahn School of Medicine at Mount Sinai's Institutional Animal Care and Use Committee (IACUC); animal studies adhere to ARRIVE guidelines.

Flow cytometry analysis

Expi293F cells were transfected with expression plasmids encoding the VP1 proteins of each BK genotype along with VP2 and VP3 in the ratio of 3:1:1. Forty-eight hours post-transfection, cells were permeabilized [specify kit], washed, and stained with immunized mouse sera (1:100, 1:1000, 1:10,000) or hybridoma clone supernatant (1:2) in PBS with 0.5% BSA for 1 hr. After 2 washes in PBS/BSA, cells were incubated with allophycocyanin-conjugated goat anti-mouse IgG secondary (Jackson Immunoresearch) for 30 mins. Cells were washed once in PBS and resuspended in 50 µl PBS to be analyzed on a high throughput flow cytometry (HTFC) device (Intellicyt).

Pseudovirus generation

As previously described (5, 34), BK pseudoviruses (PsVs) were produced from HEK-293TT cells transfected with plasmids that express VP1, VP2, VP3, and the GFP reporter vector at ratios of 2:1:1:1 using Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, the cells were harvested at ~100 million cells/ml in PBS, washed 1X with PBS and lysed by addition of 0.5% Triton X-100, and RNase A/T1 cocktail (Ambion). The lysate was incubated at 37°C overnight and cell lysates were centrifuged at 5,000×g. The PsVs were isolated by ultracentrifugation through a 27–33–39% iodixanol (Optiprep, Sigma) step gradient at 300,000xg for 3.5 hrs at 16°C. The fractions from the Optiprep gradient were analyzed for detection of VP1/VP2/VP3 using SDS-PAGE (18, 30). PsV-transduced cells were visualized by fluorescent microscopy and, after harvesting with trypsin treatment, analyzed by flow cytometry using GFP as the readout for infection using an HTFC. Virus titer was determined based on the number of GFP-positive cells/ml of virus stock with a typical yield of ~2.5X10^{8} transducing units per ml.

PsV infectivity assay

Neutralization of BKV-I, -II, -III, and -IV PsVs was performed as follows: 15,000 HEK-293TT target cells were plated in 96 well plates. The next day, hybridoma supernatant or purified antibodies were serially diluted in complete DMEM and mixed with enough BKV-I, -II, -III, and -IV PsVs to yield ~30% of target cells transduced with GFP. The antibody/PsVs mixtures were added to the plated target cells and cultured for 72 hrs. Transduction was evaluated by flow cytometry using GFP as the readout for using the HTFC (Intellicyt). The overall mean fluorescent intensity (MFI) of the infected cells indicates the level of infection with non-treated PsV. The % GFP (+) cells with a 10-fold increase of MFI was determined and untreated or control-treated cells were determined as 100% infection.

Antibody sequence analysis of heavy chain variable region and junctional diversity

Sequencing of variable heavy and kappa chains was obtained using SMARTer 5' RACE technology (Takara Bio USA) adapted for immunoglobulins to amplify the variable genes from the heavy and kappa chains. Briefly, RNA was extracted from each hybridoma using a RNeasy Mini Kit (Qiagen, 74004), followed by first-strand cDNA synthesis using constant gene-specific 3' primers (GSP1) based on the specific mouse isotype of the hybridoma and incubation with the SMARTer II A Oligonucleotide and SMARTscribe reverse transcriptase (Takara, 634858). [GSP1 Primers (5'-3'): mG1-AGAGGTCAGACTGCAGGACA, mG2a- CTTGTCCACTTTGGTGCTGC, mG2b-GACAGTCACTGAGCTGCTCA, mG2b-GACAGTCACTGAGCTGCTCA, mcK-CCAACTGTTCAGGACGCCAT]. PCR amplification of the first-strand cDNA product was then performed using SeqAmp DNA Polymerase (Takara, 638504) with a nested 3' primer (GSP2 Primer) to the constant genes and a 5' universal primer (kit provided) based on universal primer sites added to the 5' end during cDNA generation. [GSP2 Primers (5'-3'): mG1- CCCAGGGTCACCATGGAGTT, mG2a-GGTCACTGGCTCAGGGAAAT, mG2b- CTTGACCAGGCATCCCAGAG, mG3-GACAGGGCTCCATAGTTCCATT, mCk- CTGAGGCACCTCCAGATGTTAAC] Purified PCR products were submitted for Sanger sequencing using 3' constant gene primers (GeneWiz, South Plainfield, NJ). Sequence results were BLASTed against the IMGT human databank of germline genes using V-Quest (http://imgt.org/).

Isotyping and monoclonal antibody purification

Isotyping for the constant gene of the antibodies was performed with the Mouse Immunoglobulin Isotyping Kit (BD, 550026) as per the manufacturer's protocol. Monoclonal antibodies were purified by FPLC on an ÄKTA pure FPLC systems on protein G affinity columns (HiTrap-1mL, GE/Cytiva, #17-0404-01). They were dialyzed against PBS and quantitated by both BCA and OD at 280 nm.

Quantitation of monoclonal antibodies

MAbs were quantitated using label-free, biolayer interferometry (BLI) on an Octet Red96 (ForteBio, Sartorius). Briefly, optically capable biosensors conjugated with either protein A or G were dipped into supernatants containing mAbs. Supernatants were measured undiluted and diluted 1:10 in conditioned media and compared to an isotype standard, diluted in conditioned media in a 1:2 dilution series ranging from 100ug/ml to 1.56ug/ml. Standard curves were analyzed in ForteBio Data Analysis Software v.10 (ForteBio) using a 5-parameter logistics (5PL) dose response curve fitting model on the initial binding slopes. MAb concentrations were then calculated from the standard curve. Diluted samples were compared to undiluted samples after the application of the respective dilution factors. Total concentrations were averaged together from the diluted and undiluted samples.

Statistics

All statistical tests were performed using GraphPad Prism 9 software (La Jolla, CA). The half (50%)-maximal inhibitory concentration (IC50) and IC80 values were calculated using three-parameter non-linear regression analysis, after antibody concentrations (x-axis) were transformed to log scale. Error bars represent standard deviation from the mean for all figures.

RESULTS

Analysis of humoral response from VelocImmune mice immunized with BKV VP1 genotypes.
To generate a panel of neutralizing human monoclonal antibodies (mAbs) against BK virus, VelocImmune® mice (25) were immunized with different combinations of cDNAs encoding the VP1 proteins of each of the four BKV genotypes with VP2 and VP3 (**Figure 1**). Three immunization regimens (Group 1, 2, and 3) were utilized to develop mAbs to diverse BKV serotypes. VelocImmune mice encode chimeric immunoglobulins consisting of human Vh and Vl chains and a mouse Fc domain. They can be made fully human by a simple cloning step (25). The immunization strategy utilized the VP1 proteins of different BKV genotypes in hopes of enriching for broadly neutralizing mAbs against conserved epitopes (**Figure 1**). Following immunization, the sera from the respective animals were collected and analyzed for VP1 reactivity by flow cytometry on Expi293F cells transfected with the VP1. The sera from immunized mice were also assessed for neutralizing activity using a high-throughput infectivity assay against BKV-I, -II, -III, and -IV PsVs (**Figure 2**). BKV PsVs were generated from HEK-293TT cells transfected with BKV VP1 cDNAs of the respective BKV genotypes and BKV-I VP2 and VP3 cDNA to mimic intact virus (27). In addition, a GFP expressing vector was included in the transfection to evaluate PsV infectivity in a high-throughput manner. The diluted sera (1:200, 1:1,000, and 1:5,000) from the immunized animals (tertiary immunization) were preincubated with the respective PsVs and incubated with HEK-293TT cells for up to 24hrs. Infectivity was normalized to the percentage of GFP+ cells observed with normal mouse serum. Interestingly, the neutralization profiles of animals immunized using different combinations of BKV VP1 genotypes were quite distinct. In all cases, the sera from the immunized mice demonstrated inhibition of at least two BKV serotypes in a dilution dependent manner. The sera from animals immunized with BKV-I VP1 (M-5, -6, -7, and -9) was effective at inhibiting BKV-I PsV infection with varied reduction of BKV-II, -III, or -IV infectivity in different individual animals (**Figure 2A**). Group 2 animals demonstrated varied inhibition among the BKV PsVs (**Figure 2B**). Most animals in Group 3 (immunization with BKV-I, -II, -III, and -IV VP1) showed a broad response against all four genotypes (**Figure 2C**). Collectively, these studies show the immunogenicity of BKV VP1 protein to inhibit infection of diverse BKV genotypes.

Generation and identification of anti-BKV monoclonal antibodies
The VelocImmune® immunized mice whose sera demonstrated a robust cross-neutralizing activity against diverse BKV genotypes were selected for the generation of hybridomas. Briefly, the spleens of mice 9, 12, 14, and 25 were used to generate hybridomas. The supernatants from the hybridoma clones were screened by high-throughput binding assay on HEK-293 cells transfected with BKV-I VP1 cDNA (**Figure 3A-D**). The hybridoma clones were incubated with VP1-transfected cells followed by anti-mouse IgG conjugated with FITC and then analyzed for mean fluorescence intensity (MFI) on a flow cytometer. Controls included normal mouse sera from non-immunized mice and polyclonal sera from the immunized animals. Collectively, Mouse (Ms)9 (693 clones), Ms12 (1628 clones), Ms14 (clones 1443), and Ms25 (clones 823) clones were evaluated for binding to VP1. Clones selected for further evaluation demonstrated at least a 2-fold MFI on VP1-transfected cells (Ms9 (169 clones), Ms12 (clones 823), Ms14 (clones 135), Ms25 (clones 144).

Analysis for broadly neutralizing anti-BK virus mAbs
The anti-BKV VP1 binding clones were subjected to isotype analysis to identify IgG1, IgG2a, and IgG2b while excluding IgM and IgG3 isotypes because they are more prone to proteolysis and aggregation in solution, respectively, and are generally considered less viable for development as therapeutics (26). Based on Ig isotype, 353 binding clones (Ms9 (75 clones), Ms12 (96 clones), Ms14 (123 clones), and Ms25 (59 clones) were examined in a BKV PsV infectivity assay (**Figure 4**). Neutralizing antibodies to BKV-I were highly representative in Group 1 and 2 immunization strategies with several clones that reduced BKV-I, -II, -III, and -IV PsV infectivity (**Figures 4 and 5A-C**). Most of the clones from Fusion 4 (mouse 25) reduced transduction of BKV-I, -II, -III, and -IV PsVs (**Figure 5D**). The overall analysis of specificities shows that most clones are selective for limiting transduction of BKV-I PsV, with 37 clones targeting BKV-I, -II, -III, and -IV PsVs (**Table 1**). These data support the heterologous immunization strategy to generate mAbs that broadly react to VP1 of BKV-I, -II, -III, and -IV and block infection of the

respective PsVs. The hybridoma clones demonstrating the most robust neutralization of at least two genotypes were subjected to nucleotide sequencing of the immunoglobulin variable segment. Sequence analysis was utilized to eliminate identical clones and categorize the remaining unique clones into clonotype families. Clonotype families were defined by heavy sequences that shared homologous CDR3 regions (exact length and >95% nucleotide identity) with shared V(D)J gene usage and homologous light chain pairs (light chain CDR3 homology and VJ gene usage). Clones also averaged 5-8% nucleotide mutations in their V-gene segments when compared to the closest germline gene by blasting the sequences against the IMGT human germline databank, indicative of antigen-matured, somatically hypermutated antibodies. Based on the sequences and mutation frequency, we propose that these antibodies have evolved through affinity maturation by our immunization strategy to improve their specificity and binding affinity (35, 36). Collectively, we found 12 diverse clonotypes.

Evaluation of the inhibitory capacity of anti-BKV mAbs

We next evaluated the neutralization capacity of the cross-reactive anti-BKV mAbs using a BKV PsV infectivity assay (**Table 2**). Clones demonstrating the highest inhibitory activity in the preliminary screening (**Figures 4 and 5**) were grown, and the approximate concentration of antibody was measured using biolayer label-free protein interferometry on an Octet Red 96 compared to isotype matched standard controls. Clones from Fusion 1, 3, and 4 and four different mAb genotype families (A-D) were selected for analysis based on their broad specificity and neutralization capacity.. The supernatants were examined in the BKV-I, -II, -III, and -IV PsV infectivity assay to determine the antibody concentration at 50% (IC50) and 80% (IC80) inhibition (**Table 2**). The mAb clones demonstrated broad inhibition across BKV-I, -II, -III, and -IV PsVs, ranging from IC50 values of 9-975 pM and IC80 values of 20-1307 pM. Several mAbs showed broad cross-neutralization at low pM concentrations, while others neutralized two or three of the genotypes. An IC50 above 1000 is considered a weak neutralizing mAb and such mAbs were excluded from further characterization. Several clones (4F7, 7A4, 15C10, and 14F5) effectively reduced PsV infection with IC50 <50 pM for all BKV genotypes. These results demonstrate the effectiveness of a panel of broadly effective monoclonal antibodies to prevent infection of BKV-I, -II, -III, and -IV genotypes.

To further evaluate the inhibitory properties of the anti-BKV mAbs, a representative clone of each mAb Family was recombined with a human IgG1 Fc domain and amplified to produce a purified antibody. The purified 2B9 (Family C), 7A4 (Family J), 7H8 (Family K), and 15C10 (Family H) mAbs were examined in the BKV-I, -II, -III, and -IV PsV infectivity assays (**Figure 6**). In addition, a previously identified anti-BKV mAb, MAU868 (32), was examined to compare the neutralization profiles of the newly characterized anti-BKV mAbs. All mAbs demonstrated robust neutralization of all BKV PsV with varying degrees of neutralization ranging with IC50 values of 7-30 pM for BKV-I, 12-985 pM for BKV-II, 45-187 pM for BKV-III, and 85-337pM for BKV-IV PsVs (**Figure 6A-D**). Monoclonal antibodies 7H8 and 15C10 were the most effective across all BKV genotypes and demonstrated similar or lower IC50 values compared to the MAU868 mAb (**Figure 6C-E**). Notably, the IC80 values of the newly characterized mAbs were dramatically lower than MAU868 mAb suggesting these mAbs may be more effective at completing blocking infection at low mAb concentrations. Collectively, these studies demonstrate the proof of concept that mAbs targeting VP1 of the BK virus can broadly limit infection of all genotypes.

DISCUSSION

Multiple adjunctive therapies have been explored for the treatment of BKVAN in small case series by the addition of intravenous immunoglobulin (IVIG), cidofovir, leflunomide, and fluoroquinolones to the standard of care (37). Several studies using IVIG treatment demonstrated a reduction of viremia in transplant patients (27, 29-32). Notably, of 30 patients with BKV DNAemia treated with IVIG, viral DNA was cleared from blood in 27 of the patients and only one of the grafts (from a patient that failed to respond to the IVIG) was lost (30). A retrospective cohort analysis of kidney transplant recipients with histologically established BKVAN shown that IVIG, in addition to rigorous BKVAN therapy, dramatically reduced BKV viremia but did not significantly improve allograft survival (27). Recently, a study by Benotmane *et al.* demonstrated that IVIG can prevent BKV reactivation and disease (32). A monoclonal antibody, MAU868 (Vera Therapeutics, licensed from Pfizer in 2021), has completed a Phase 1 clinical trial (38) and has been used to treat BKV DNAemia in one patient under expanded access (39). Currently, in a Phase 2 clinical trial for MAU868 treatment (Clinical trial#NCT04294472), subjects had a 1.14 $\log_{10}$ decrease ($p=0.051$) in BKV viral load but no change in the estimated glomerular filtration rate was observed (40). These studies provide proof-of-concept for interventions that increasing the neutralizing antibody titer in serum results in reduced BKV viremia, thereby potentially decreasing the incidence of BKVAN.

We have identified a panel of genetically distinct mAbs that broadly react to the VP1 of BKV-I, -II, -III, and -IV genotypes and effectively block the infectivity of cognate PsVs. Specifically, clones from four genetically diverse mAb genotype families effectively reduced infection with BKV-I, -II, -III, and -IV PsVs with low pM values (**Figure 6**). Further, these mAbs completely limited infection at low IC80 values of 13-231pM. Importantly, we compared the capacity of the newly identified cross-reactive mAbs and MAU868 mAb to limit infection of the BKV-I, -II, -III, and -IV PsVs (**Figure 6**). Clones 2B9, 7A4, 7H8, and 15C10 had comparable IC50 values to MAU868 in neutralizing BKV-I and BKV-III PsVs. Yet, clones 2H1, 7H8, and 15C10 had similar or lower IC50 against BKV-II and BKV-IV PsVs in relation to MAU868. Notably, the IC80 values of clones 2H1, 7H8, and 15C10 against BKV-I-IV PsVs were lower than the MAU868 mAb. This could be owing to antibodies' affinity for VP1 or binding to a specific epitope essential for virus infection. These data indicate that these new human mAbs have the potential therapeutics for preventing BKV infection and limiting viral load in at-risk patient populations.

These newly identified broadly neutralizing mAbs were generated from the heterologous immunization of VP1 cDNA from the different genotypes (**Figure 1**). The immunization strategy generated cross-reactive neutralizing mAbs against common epitopes within the four genotypes. Interestingly, mice immunized initially with BKV-II and -III VP1 followed by BKV-I and -IV VP1 generated the largest percentage of cross-reactive mAbs implying the order of immunization may dictate the broad specificity of anti-BKV neutralizing antibodies. Yet, initial immunizations with BKV-I and BKV-IV VP1 genotypes mostly generated mAbs to BKV-I and -IV with only a small number of cross-reactive mAbs to all four genotypes. The VP1 amino acid sequence identity among the genotypes is ~95% with the exception of residues 61-83, which have only ~65% (7). Thus, these newly identified broadly reactive mAbs target a conserved epitope within all genotypes that likely blocks virus binding to the cell.

The primary use for these mAbs would be as prophylactic treatment given to the recipient before to transplantation to decrease the viral load by preventing dissemination within the host. The mAbs could also be useful in a therapeutic capacity for patients in the early stages of BKVAN development (i.e., initial low-level DNAemia). The ideal antibody(s) will be capable of neutralizing all BKV genotypes. Such antibodies would avoid the need to identify genotypes in donors and recipients prior to transplant. While genotype I and IV account for most BKVAN cases in the US and Europe, worldwide the hierarchy of the genotypes may vary (8). In a prospective study of 168 transplant patients by Solis et al (20), the authors showed that high neutralizing antibody titer against the donor genotype led to a very significant reduction in risk of viral BK viremia and each $\log_{10}$ rise in antibody titer reduced the risk by 50%. The observations that genotype mismatch increased the risk significantly support a primary role for

antibodies in the prevention of disease and argue that broadly neutralizing monoclonal antibodies may be utilized prophylactically/therapeutically to lessen the risk of PyVAN (8, 20, 23).

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Competing Interests
A.D, D.T., T.K., T.M., and Mount Sinai Innovation Partners (MSIP) hold the patent for antibody sequences.

FIGURE LEGENDS

Figure 1. Immunization regimen for generating anti-BKV antibodies. (**A**) VelocImmune mice were immunized with expression plasmids encoding BKV VP1 from genotypes I, II, III, and IV together with BKV-IV VP2 and VP3 in diverse combinations with a final boost of the respective PsVs. (**B**) The components of immunization Groups 1, 2, and 3 are outlined.

Figure 2. Analysis of the neutralization activity of sera from VelocImmune mice immunized with BKV antigens. The sera from VelocImmune mice immunized with BKV antigens based on regimen of Group 1, 2, or 3 (Figure 1) were evaluated for neutralization of BKV-I, -II, -III, and -IV PsVs. The diluted sera (1:200, 1:1,000, and 1:5,000) was pre-incubated with PsVs and added to HEK-293TT cells. Virus infectivity was evaluated 24 hours post infection (hpi) using flow cytometry to determine the mean fluorescence intensity of the GFP positive cells. These values were normalized using normal mouse sera as 100%.

Figure 3. Binding profile of hybridoma clones from BKV-immunized VelocImmune mice. Hybridoma clones generated from BKV-immunized VelocImmune mice were analyzed for binding to the VP1 protein of BKV-I, -II, -III, and -IV. HEK-293 cells transfected with BKV-I, -II, -III, or -IV VP1 cDNA were incubated with the hybridoma supernatants (1:100) from Fusion 1 (**A**), Fusion 2 (**B**), Fusion 3 (**C**), and Fusion 4 (**D**) followed by analysis using a high-throughput flow cytometer. The mean fluorescence intensity (MFI) was measured from each sample and plotted as a heatmap of MFI for each Fusion. What are the rows and columns? I am assuming each individual line is a clone, but how do you know if that line is reacting to BKI, II, III or IV?

Figure 4. Neutralization capacity of VP1-binding hybridoma clones to limit infection of BKV genotypes. Hybridoma clones that bind to BKV-I in intracellular staining assays were evaluated in PsV-based infectivity assays. The hybridoma supernatant from the VP1-binding clones from Fusion 1 (**A**), Fusion 2 (**B**), Fusion 3 (**C**), and Fusion 4 (**D**) was pre-incubated (1:500) with the indicated PsV followed by incubation with HEK-293TT cells. Infectivity was evaluated 48hpi using flow cytometry to determine the as % GFP positive cells. These values were normalized using supernatant from a non-binding clone as 100%.

Figure 5. Summary of neutralization results of the VP1 binding hybridoma clones. Based on the neutralization data in Figure 4, the VP1-binding hybridoma clones with <75% infectivity from Fusion 1 (**A**), Fusion 2 (**B**), Fusion 3 (**C**), and Fusion 4 (**D**) were categorized in groups that selectively limit infection of one of the BKV genotype, several genotypes, and all four genotypes.

Figure 6. Anti-BKV mAbs are broadly neutralizing against BKV genotypes. Analysis of purified anti-BKV mAbs in the BKV PsV infectivity assay. Increasing concentrations (0-6670 pM) of the anti-BKV mAb clones 2B9 (**A**), 2H1 (**B**), 7H8 (**C**), 15C10 (**D**), and MAU868 (**E**) were incubated with BKV-I, -II, -III, and -IV PsVs followed by addition to HEK-293TT cells. Virus infectivity was evaluated 48hpi using flow cytometry to determine the mean fluorescence intensity of the GFP positive cells. The values were normalized using non-treated PsVs 100%.

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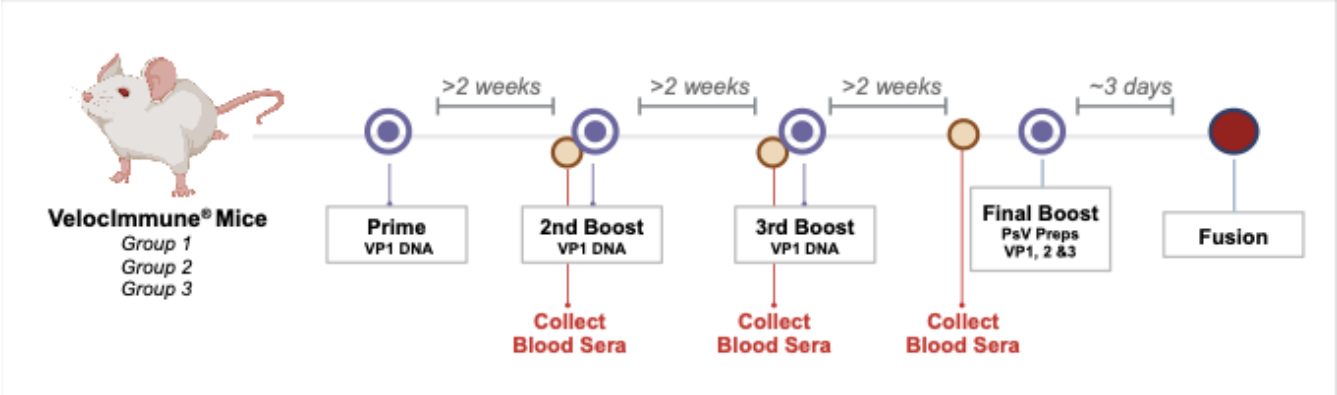
Figure 1

A



B

Immunization strategy to generate BK virus neutralizing monoclonal antibodies					
Group	**Primary (VP-1 cDNA)**	**Secondary (VP-1 cDNA)**	**Tertiary (VP-1 cDNA)**	**Final Boost (pseudovirus)**	**Fusion: Mouse**
1	BKV-I	BKV-IV	BKV-IV	BKV-I and -IV	Fusion 1: Mouse 9
2	BKV-I and -IV	BKV-I and -IV	BKV-I –II, and -III	BKV-I and -IV	Fusion 2: Mouse 9 Fusion 3: Mouse 12
3	BKV-II and -IIII	BKV-II and -IIII	BKV-I and -IV	BKV-I, -II, -III, and -IV	Fusion 4: Mouse 25

Figure 2

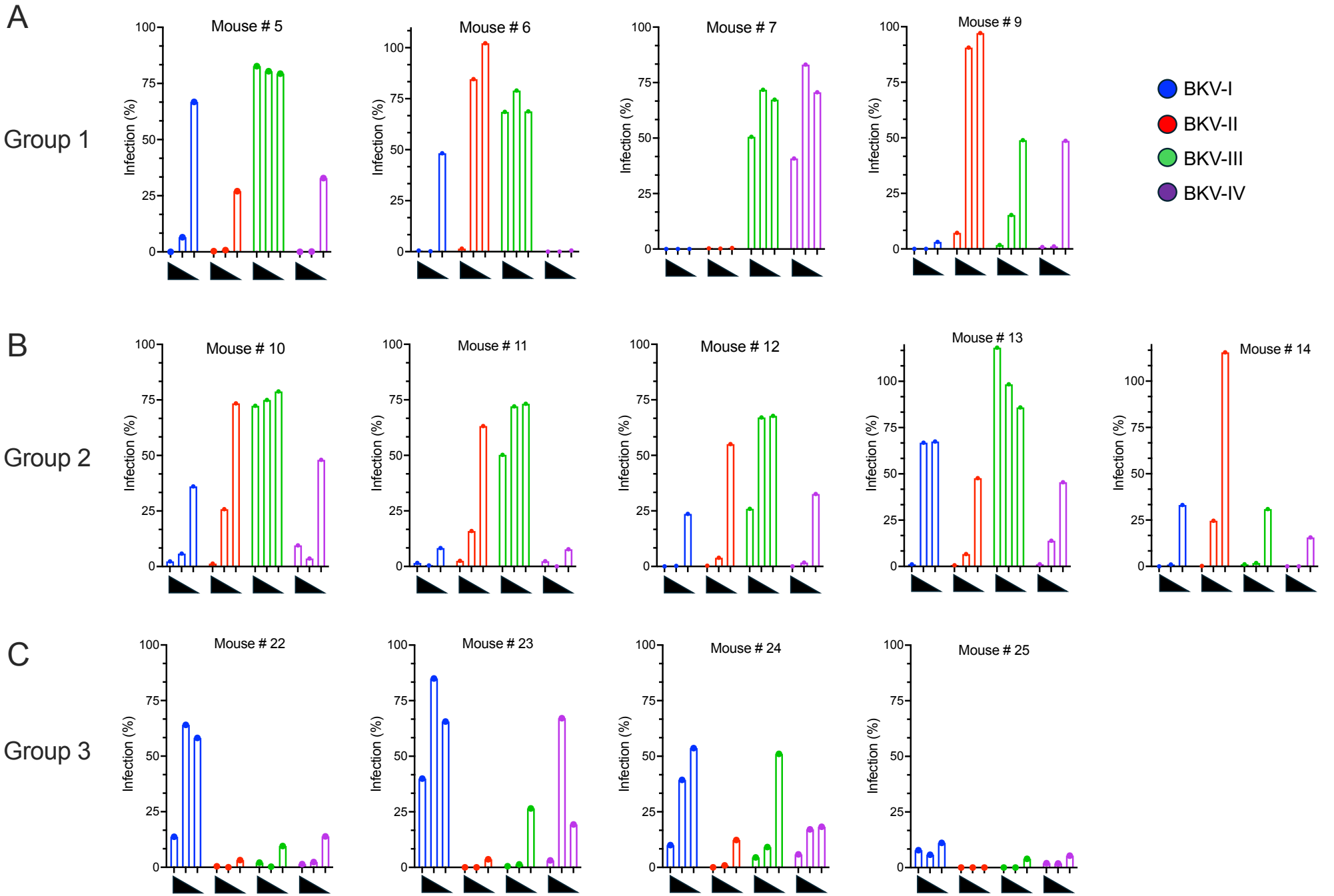

A
Group 1
Mouse # 5
Mouse # 6
Mouse # 7
Mouse # 9
Infection (%)
BKV-I
BKV-II
BKV-III
BKV-IV
B
Group 2
Mouse # 10
Mouse # 11
Mouse # 12
Mouse # 13
Mouse # 14
C
Group 3
Mouse # 22
Mouse # 23
Mouse # 24
Mouse # 25

Figure 3

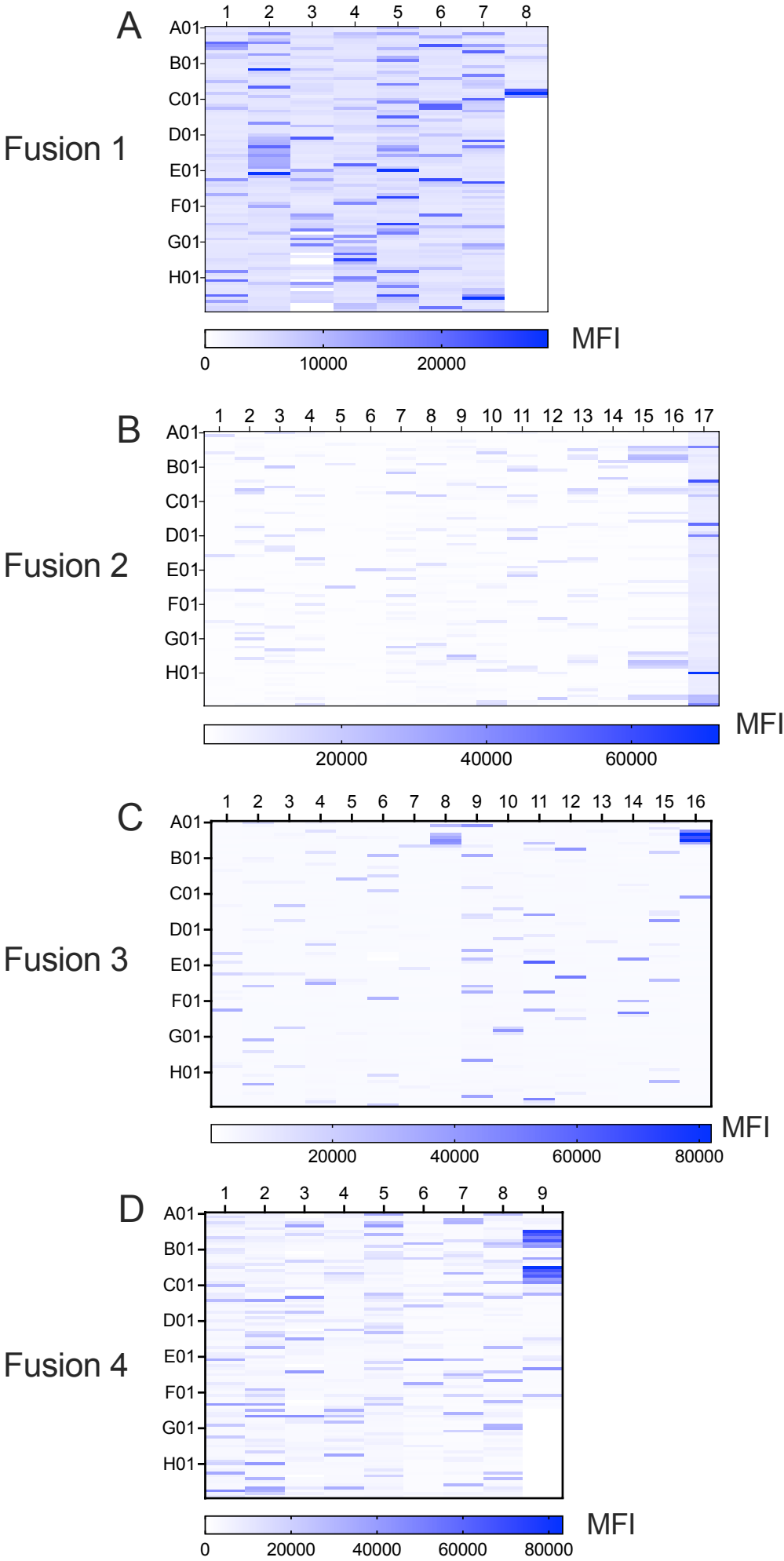

A
Fusion 1
1 2 3 4 5 6 7 8
A01 B01 C01 D01 E01 F01 G01 H01
MFI
0 10000 20000
B
Fusion 2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
A01 B01 C01 D01 E01 F01 G01 H01
MFI
20000 40000 60000
C
Fusion 3
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
A01 B01 C01 D01 E01 F01 G01 H01
MFI
20000 40000 60000 80000
D
Fusion 4
1 2 3 4 5 6 7 8 9
A01 B01 C01 D01 E01 F01 G01 H01
MFI
0 20000 40000 60000 80000

Figure 4

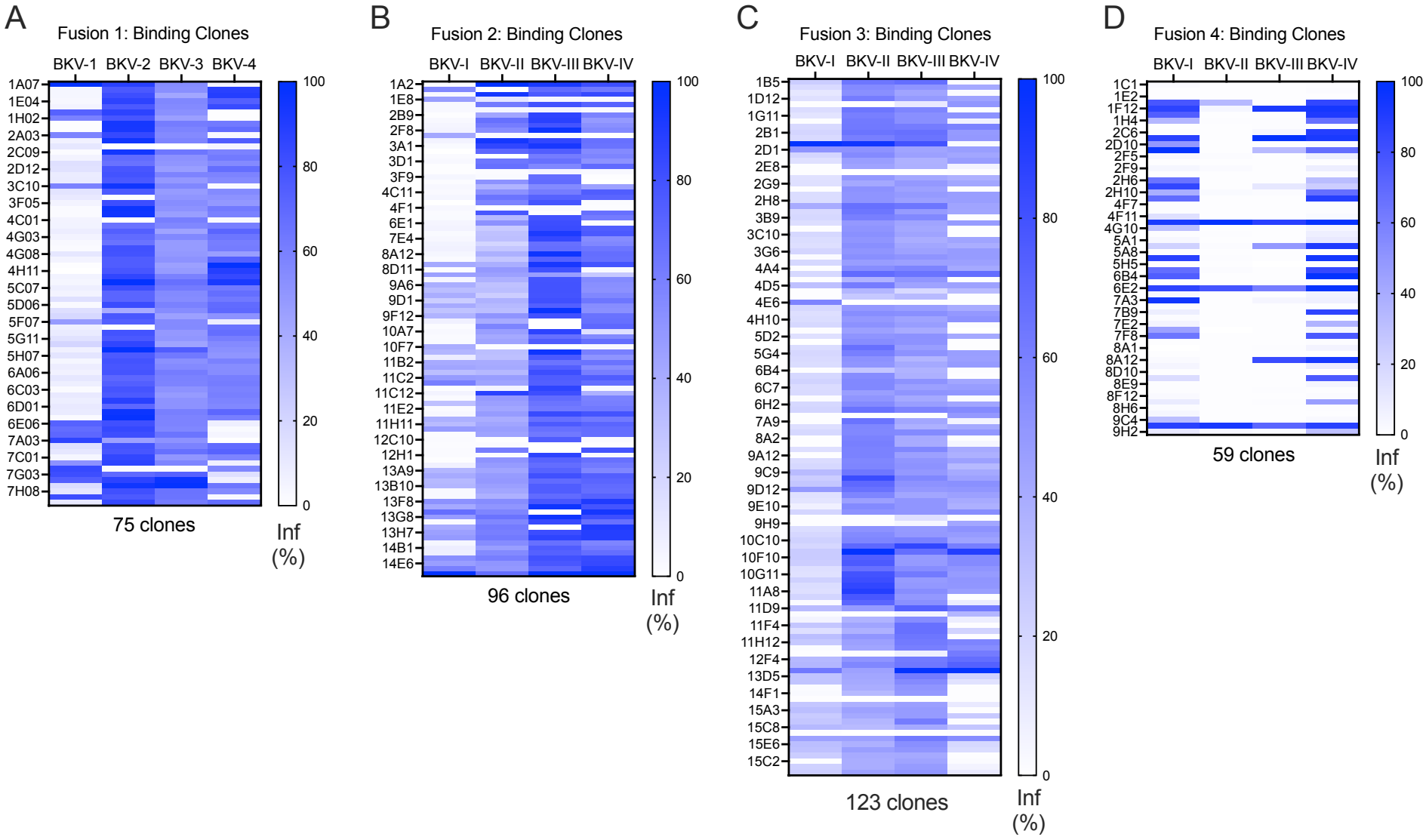

A
Fusion 1: Binding Clones
BKV-1 BKV-2 BKV-3 BKV-4
1A07 1E04 1H02 2A03 2C09 2D12 3C10 3F05 4C01 4G03 4G08 4H11 5C07 5D06 5F07 5G11 5H07 6A06 6C03 6D01 6E06 7A03 7C01 7G03 7H08
75 clones
100 80 60 40 20 0
Inf (%)
B
Fusion 2: Binding Clones
BKV-I BKV-II BKV-III BKV-IV
1A2 1E8 2B9 2F8 3A1 3D1 3F9 4C11 4F1 6E1 7E4 8A12 8D11 9A6 9D1 9F12 10A7 10F7 11B2 11C2 11C12 11E2 11H11 12C10 12H1 13A9 13B10 13F8 13G8 13H7 14B1 14E6
96 clones
100 80 60 40 20 0
Inf (%)
C
Fusion 3: Binding Clones
BKV-I BKV-II BKV-III BKV-IV
1B5 1D12 1G11 2B1 2D1 2E8 2G9 2H8 3B9 3C10 3G6 4A4 4D5 4E6 4H10 5D2 5G4 6B4 6C7 6H2 7A9 8A2 9A12 9C9 9D12 9E10 9H9 10C10 10F10 10G11 11A8 11D9 11F4 11H12 12F4 13D5 14F1 15A3 15C8 15E6 15C2
123 clones
100 80 60 40 20 0
Inf (%)
D
Fusion 4: Binding Clones
BKV-I BKV-II BKV-III BKV-IV
1C1 1E2 1F12 1H4 2C6 2D10 2F5 2F9 2H6 2H10 4F7 4F11 4G10 5A1 5A8 5H5 6B4 6E2 7A3 7B9 7E2 7F8 8A1 8A12 8D10 8E9 8F12 8H6 9C4 9H2
59 clones
100 80 60 40 20 0
Inf (%)

Figure 5

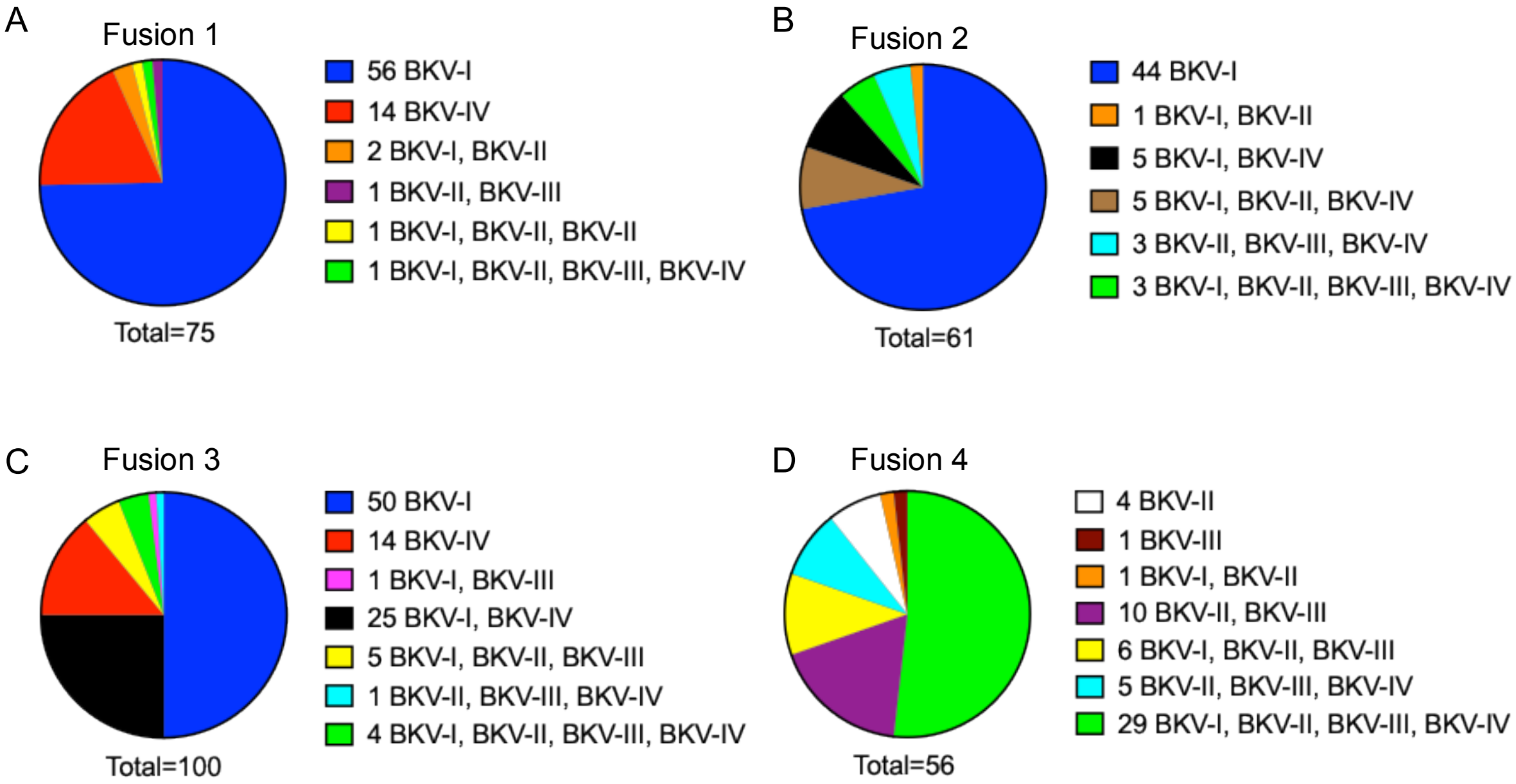
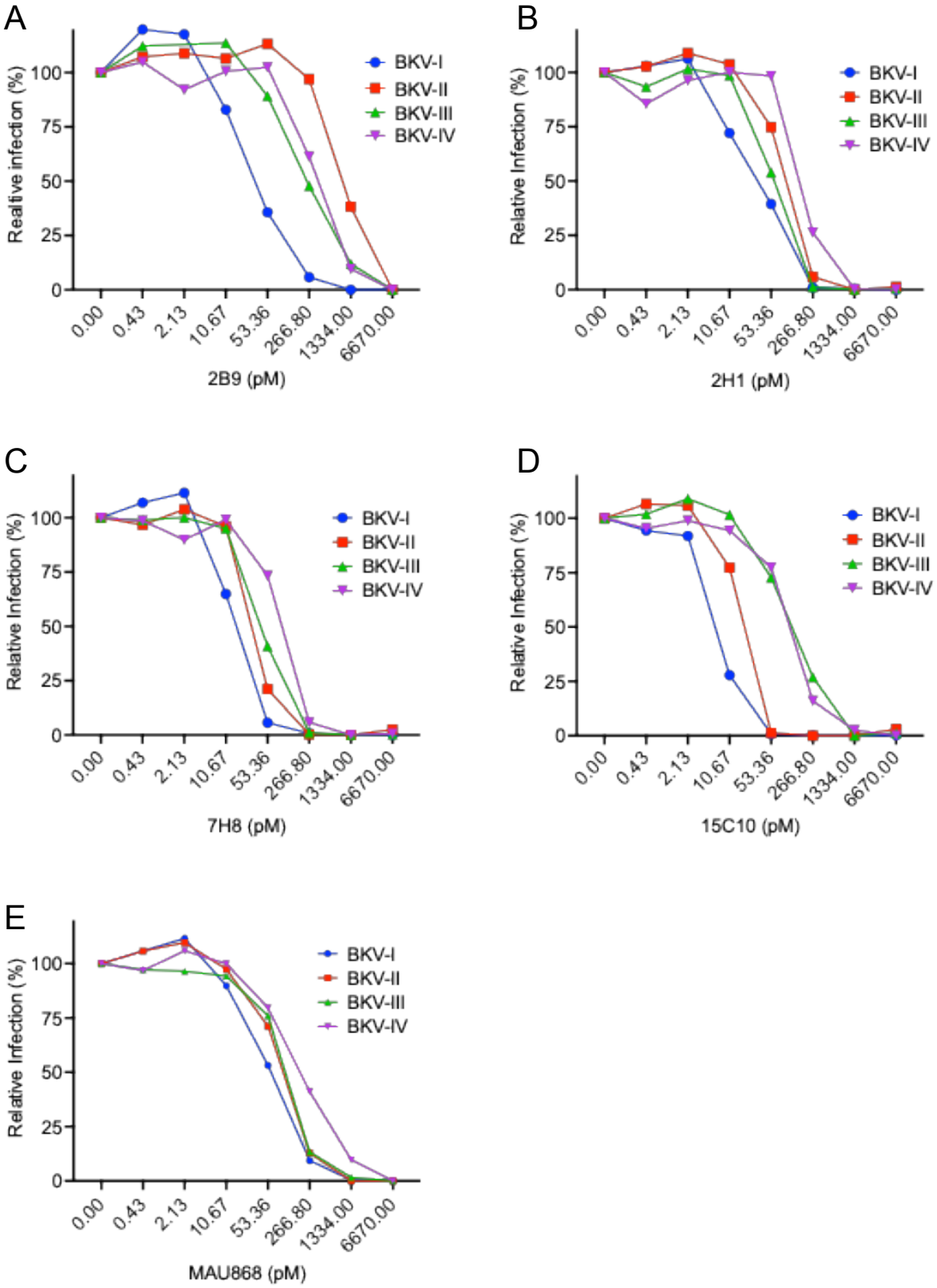

A
Fusion 1
56 BKV-I
14 BKV-IV
2 BKV-I, BKV-II
1 BKV-II, BKV-III
1 BKV-I, BKV-II, BKV-II
1 BKV-I, BKV-II, BKV-III, BKV-IV
Total=75
B
Fusion 2
44 BKV-I
1 BKV-I, BKV-II
5 BKV-I, BKV-IV
5 BKV-I, BKV-II, BKV-IV
3 BKV-II, BKV-III, BKV-IV
3 BKV-I, BKV-II, BKV-III, BKV-IV
Total=61
C
Fusion 3
50 BKV-I
14 BKV-IV
1 BKV-I, BKV-III
25 BKV-I, BKV-IV
5 BKV-I, BKV-II, BKV-III
1 BKV-II, BKV-III, BKV-IV
4 BKV-I, BKV-II, BKV-III, BKV-IV
Total=100
D
Fusion 4
4 BKV-II
1 BKV-III
1 BKV-I, BKV-II
10 BKV-II, BKV-III
6 BKV-I, BKV-II, BKV-III
5 BKV-II, BKV-III, BKV-IV
29 BKV-I, BKV-II, BKV-III, BKV-IV
Total=56

Figure 6



Summary of IC_{50} and IC_{80} values (pM)								
Clone	**BKV-I**		**BKV-II**		**BKV-III**		**BKV-IV**	
	IC_{50}	IC_{80}	IC_{50}	IC_{80}	IC_{50}	IC_{80}	IC_{50}	IC_{80}
2B9	22.4	81	985	2237	187	759	337	679
2H1	30	104	78.5	139	56.3	84.2	243	280
7H8	12.5	22.5	32.8	53.8	45.4	85.1	85.2	148
15C10	7.2	13.1	12.5	15.2	112	331	109	231
MAU868	35	170	82.3	203	104	211	189	686

Table 1. Summary of the specificity of the neutralizing clones

	Number of neutralizing clones based on Fusion				
Genotype	**Fusion 1**	**Fusion 2**	**Fusion 3**	**Fusion 4**	**Total**
BKV-I	56	44	50	0	150
BKV-II	0	0	0	4	4
BKV-III	0	0	0	1	1
BKV-IV	14	0	14	0	28
BKV-I, -II	2	1	0	1	4
BKV-I, -III	0	0	1	0	1
BKV-I, -IV	0	5	25	0	30
BKV-II, -III	1	0	0	10	11
BKV-I, -II, -III	1	0	5	6	12
BKV-I, -II, -IV	0	5	0	0	5
BKV-II, -III, -IV	0	3	1	5	9
BKV-I, -II, -III, -IV	1	3	4	29	37

Table 2. Summary of IC_{50} and IC_{80} (pM) values of anti-BKV neutralizing monoclonal antibodies*

Fusion	Clone	BKV-I IC_{50} (IC_{80}) (pM)	BKV-II IC_{50} (IC_{80}) (pM)	BKV-III IC_{50} (IC_{80}) (pM)	BKV-IV IC_{50} (IC_{80}) (pM)	Isotype	Clonotype Family
1	2B9	32 (179.1)	154 (315.9)	80.8 (238)	148.2 (349.7)	IgG2b	A
1	7E5	24.9 (122.1)	975.5 (1307)	414.9 (1097)	133 (349.3)	IgG2a	A
3	14F5	10.7 (25.8)	14.6 (26.4)	20.0 (44.5)	43.6 (120)	IgG2b	B
3	15C10	9.4 (21.5)	13.1 (23)	21.1 (44.6)	42.3 (103.0)	IgG2b	B
4	1E2	62.4 (76.4)	12.7 (26.1)	14.8 (29.6)	99.9 (181.7)	IgG2a	C
4	1H10	69.1 (110.1)	12.3 (34)	57.1 (67.7)	108.2 (214.6)	IgG2a	C
4	2F7	58.4 (70.6)	8.4 (6.2)	12.1 (19.8)	69.3 (86.5)	IgG2a	C
4	2H1	20.2 (59.4)	26.1 (51.7)	21.9 (40.4)	72.8 (190.9)	IgG2a	C
4	4F7	32.4 (71.7)	31.9 (53.1)	14.3 (26.1)	41.6 (165.5)	IgG2a	C
4	5A8	33 (106.1)	7.7 (14.1)	16.1 (29.5)	85 (330.3)	IgG2a	C
4	7A4	9.1 (20.2)	13.2 (22.9)	8.1 (14.2)	25.1 (83.2)	IgG2a	C
4	7H8	61.5 (74.4)	50 (109.4)	33.4 (124.8)	94.3 (188.6)	IgG2a	D
4	8A11	93.8 (120.1)	9.4 (20.2)	47.9 (56.4)	105.9 (195.4)	IgG2a	C
4	8D10	64.2 (79.2)	49.2 (104.1)	29 (93.8)	89.4 (180.4)	IgG2a	D
4	9B4	70.4 (88.2)	15.2 (27.3)	11.8 (21.5)	46.9 (271.7)	IgG1	D

*monoclonal antibodies in hybridoma supernatant