Supplementary Information for

Kappa-on-Heavy (KoH) bodies: A potential new class of fully-human antibody-like therapeutic agents with novel antigen-binding properties

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SI Materials and Methods

Mouse Construction & B-cell Analysis

Construction of BACvecs by bacterial homologous recombination and ligation.
Mouse and human BACs that span the 5′ and 3′ ends of the IgH and IgK locus were identified as previously described [1]. Using the Velocigene® technology [2], bacterial homologous recombination was performed as described previously to insert the human IgH and IgK Bacs into the mouse IgH and IgK locus, respectively, to make VI mice. To make the KoH mice, those same human IgK BACs were used to insert the human IgK locus into the mouse heavy chain locus. A detailed description of the steps for constructing all BAC vectors is provided in the SI Appendix (SI Appendix, Fig. S1, Tables S1-S3).

Modification of ES cells and generation of mice. Targeting of ES cells (F1H4) was performed using the VelociGene® method as previously described [2]. Derivation of mice from modified ES cells by eight-cell morula injection was as previously described [3]. Mice with humanized IgK variable loci [1] were bred together with mice that have humanized IgK variable on mouse IgH constant loci to make the KoH mice. The sequences of qPCR probes and primers used to screen for targeted ES cells and mice are included in the SI Appendix (Table S4). All mice were housed and bred in specific pathogen-free conditions at Regeneron Pharmaceuticals. All mouse studies were overseen and approved by Regeneron's Institutional Animal Care and Use Committee (IACUC).

***Analysis of bone marrow and spleen*.** Eight VelocImmune® (VI) control mice (23-26 week old males and females) and ten KoH mice (25 week old males and females) were sacrificed. Spleens and bone marrow were harvested. Bone marrow was collected from femurs by flushing with complete RPMI medium (RPMI medium supplemented with fetal calf serum, sodium pyruvate, HEPES, 2-mercaptoethanol, non-essential amino acids, and gentamycin). Red blood cells from spleen and bone marrow preparations were lysed with ACK lysis buffer, followed by washing with complete RPMI medium.

Isolated cells ($1x10^6$) were incubated with anti-mouse CD16/CD32 (2.4G2, BD) on ice for 10 minutes, followed by labeling with the following antibody cocktail for 30 min on ice. Bone marrow panel: anti-mouse FITC-CD43 (1B11, BioLegend), PeCy7-IgM (II/41, eBioscience), PerCP-Cy5.5-IgD (11-26c.2a, BioLegend), APC- B220 (RA3-6B2, eBioscience), APC-H7-CD19 (ID3, BD) and Pacific Blue-CD3 (17A2, BioLegend). Bone marrow and spleen panel: anti-mouse FITC-IgK (187.1, BD), PE-IgL (RML-42, BioLegend), PeCy7-IgM (II/41, eBioscience), PerCP-Cy5.5-IgD (11-26c.2a, BioLegend), Pacific Blue-CD3 (17A2, BioLegend), APC- B220 (RA3-6B2, eBioscience), APC-H7-CD19 (ID3, BD). Following staining, cells were washed and fixed in 2% formaldehyde. Data acquisition was performed on a BD LSRFortessa flow cytometer and analyzed with FlowJo. Bone marrow: immature B cells ($B220^{int}IgM^{+}$), mature B cells ($B220^{high}$ IgM^{+}), Pro-B cells (IgM^{-} $B220^{int}$ $CD43^{high}$), Pre-B cells (IgM^{-} $B220^{int}$ $CD43^{int}$). Spleen and bone marrow: B cells ($CD19^{+}$ $CD3^{-}$), T cells ($CD3^{+}$ $CD19^{-}$), IgK^{+} B cells ($CD19^{+}$ IgK^{+} IgL^{-}), IgL^{+} B cells ($CD19^{+}$ IgK^{-} IgL^{+}), mature B cells ($CD19^{+}$ IgD^{high} IgM^{int}), transitional/immature B cells ($CD19^{+}$ IgD^{int} IgM^{high}).

Next Generation Sequencing of antibody repertoires. Splenic B cells were positively enriched from total splenocytes by magnetic cell sorting using anti-CD19 (mouse) magnetic beads and MACS® columns (Miltenyi Biotech). Total RNA was isolated from the purified splenic B cells using an RNeasy Plus RNA isolation kit (Qiagen) according to manufacturer's instructions. Reverse transcription was performed to generate cDNA containing IgM or IgK constant region sequence, using a SMARTer™ RACE cDNA Amplification Kit (Clontech) and a IgM or IgK specific primer (SI Appendix, Table S5, RT primers). During this process, a DNA sequence corresponding to the reverse complement of primer PE2-PIIA (SI Appendix, Table S5), was attached to the 3′ end of the newly synthesized cDNAs. Purified IgM or IgK specific cDNAs were then amplified by the 1st round PCR using the PE2-PIIA primer and a corresponding constant primer listed in the SI Appendix (Table S5). PCR products between 450-700bp were isolated using Pippin Prep (SAGE Science) and these fragments were further amplified by a 2nd round PCR using the primers listed in the SI Appendix (Table S5). PCR products between 400bp-700bp were isolated, purified, and quantified by qPCR using a KAPA Library

Quantification Kit (KAPA Biosystems) before loading onto a Miseq sequencer (Illumina) for sequencing using Miseq Reagent Kits v3 (2x300 cycles).

For bioinformatic analysis, raw Illumina sequences were de-multiplexed and filtered based on quality, length, and perfect match to the corresponding constant region primer. Overlapping paired-end reads were merged and analyzed using IgBLAST (NCBI, v2.2.25+) to align rearranged light chain sequences to the human germline V and J gene database. Productive and non-productive joining events were noted along with the presence of stop codons. CDR3 sequences and likely non-template nucleotides were extracted using International Immunogenetics Information System (IMGT) boundaries.

Immunization and Antibody Isolation

Immunization. VI and KoH mice were immunized using a BSA conjugate of marinobufagenin (MBG) generated as previously described [4] or a KLH conjugate of nicotine. Pre-immune serum was collected from the mice prior to the initiation of immunization. The mice were boosted via footpad route twice a week for a total of 7-10 boosts using standard adjuvants. The mice were bled and anti-serum titers assayed. For KoH mice, after completion of 6 boosts, mice were allowed a resting phase of 4 to 5 weeks, and then 4 additional boosts with the immunogens were administered. Mice were bled and anti-serum titers assayed on biotin-MBG.

Anti-serum Titer Determination. Antibody titers in serum against the immunogen were determined using ELISA. 96-well microtiter plates (Thermo Scientific) were coated with streptavidin in phosphate-buffered saline (PBS, Irvine Scientific) overnight at 5 µg/ml. Plates were washed with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T, Sigma-Aldrich) and blocked with 250 µl of 0.5% bovine serum albumin (BSA, Sigma-Aldrich) in PBS for 1 h at room temperature. Biotin-MBG was then coated at 1 µg/ml in PBS, for 1 h at room temperature. The plates were washed with PBS-T. Pre-immune and immune anti-sera were serially diluted three-fold in 0.5% BSA-PBS and added to the plates for 1 h at room temperature. The plates were washed and goat anti-mouse IgG-Fc Horseradish Peroxidase- (HRP) conjugated secondary antibody (Jackson Immunoresearch) was added to the plates and incubated for 1 h at

room temperature. Plates were washed and developed using TMB/H_2O_2 as substrate by incubating for 20 min. The reaction was stopped with acid and plates read on a spectrophotometer (Victor, Perkin Elmer) at 450 nm. Antibody titers were computed using Graphpad PRISM software.

Quantitation of total serum IgM and IgG. Total serum IgM and IgG were determined by ELISA. Plates were coated with 1 μg/ml of either anti-mouse IgM or anti-mouse IgG to quantitate total IgM and IgG, respectively. Standard plots were generated using mouse IgG (Sigma) and mouse IgM (Biolegend) for quantitating IgM and IgG levels.

Antibody and KoH-body isolation. Splenocytes were stained with FITC-conjugated anti-mouse Fc, incubated with biotinylated MBG and then stained with PE-conjugated streptavidin. The stained cells were analyzed by flow cytometry. Single B-cells that were positive for both anti-mFc and MBG were sorted and plated. RT-PCR of antibody genes from these individual B cells was performed as described [5]. Briefly, cDNAs for each single B cell were synthesized via reverse transcriptase (RT) reaction (Superscript III, Invitrogen). Each resulting RT product was then split and transferred into two corresponding wells on two 384-well plates. One set of the resulting RT products was first amplified by PCR using a 5' degenerate primer specific for human IgG heavy chain variable region leader sequence (for antibodies) or a 5' degenerate primer specific for human kappa light chain variable region leader sequence (for KoH-bodies) and a 3' primer specific for mouse heavy chain constant region, to form an amplicon. The amplicons were then amplified again by PCR using a 5' degenerate primer set specific for framework 1 of human IgG heavy chain variable region sequence and a 3' degenerate primer set specific for framework 4 of human IgG heavy chain variable region sequence (for antibodies) or a 5' degenerate primer set specific for framework 1 of human kappa light chain variable region sequence and a 3' degenerate primer set specific for framework 4 of human kappa light chain variable region sequence (for KoH-bodies). The other set of the resulting RT products was first amplified by PCR using a 5' degenerate primer specific for human kappa light chain variable region leader sequence and a 3' primer specific for mouse kappa light chain constant region to form an amplicon. The amplicon was then amplified again by PCR using a 5' degenerate primer set specific for framework 1 of human kappa light chain variable region sequence and a 3'

degenerate primer set specific for framework 4 of human kappa light chain variable region sequence. The heavy chain and light chain derived PCR products were cloned into expression vectors containing human IgG4 heavy chain constant region and kappa light chain constant region, respectively. Recombinant anti-MBG antibodies and KoH-bodies were produced in CHO cells after stable transfection with paired expression plasmids containing heavy chain and light chain derived from the same B cell. The Fab fragments of KoH-body H4H14410P and the antibody 3E9 were cloned and expressed in the same manner. Antibodies, KoH-bodies, and Fabs secreted into conditioned culture media by the transfected cells were purified by Protein A or KappaSelect (GE Life Sciences) affinity chromatography.

Biophysical Characterization of KoH-bodies

SPR measurements of anti-marinobufagenin (MBG) mAbs and mKoH-bodies. Surface plasmon resonance (SPR) experiments were performed on a Biacore 2000 instrument using a dextran-coated (CM5) chip at 25°C. The running buffer was filtered PBS (8.1mM Na_2HPO_4, 1.9mM NaH_2PO_4, 2.7mM KCl, 137mM NaCl, 0.1% v/v DMSO, adjusted to pH7.4). A capture sensor surface was prepared by covalently immobilizing recombinant Protein A (Pierce, Rockford, IL) to the chip surface using (1Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride)/N-hydroxysuccinimide (EDC/NHS) coupling chemistry. Following surface activation, Protein A in coupling buffer (0.1 M acetate buffer, pH 4.5) was injected over the activated chip surface until a resonance unit (RU) signal of about 2000 RU was reached. The activated coupled chip surfaces were then washed and treated with 10 mM glycine-HCl, pH 1.5, to remove uncoupled residual Protein A. Anti-MBG mAbs and KoH-bodies were diluted into the running buffer and captured ~8,500 – 12,000 RU on the coupled Protein A chip surface. Following the capture step, a range of MBG concentrations (270.0 μM to 13.7 nM) were injected over the different anti-MBG mAb/mKoH-body captured surfaces. Association rate constant (k_a) was determined from data obtained at multiple ligand concentrations, while the dissociation rate constant (k_d), which is independent of test ligand concentration, was determined from the change in anti-MBG bound test ligand RU over time (~5 minutes) for MBG ligands. Specific Biacore kinetic sensorgrams were obtained by a double referencing procedure as described [6]. The data were then processed and kinetic analyses performed using Scrubber software (version 2.0,

BioLogic Software). The equilibrium dissociation constant (K_D) was calculated from the ratio of the dissociation rate constant divided by the association rate constant ($K_D = k_d/k_a$).

Isothermal titration calorimetry (ITC) measurements of anti-MBG mAbs and mKoH-bodies. Binding affinities (K_D's) in ITC are derived from sequential measurements of the heat production involved in titration of antigen (MBG) to antibody/KoH-body (anti-MBG). K_D is calculated from the slope of the resulting binding isotherm since heat production is directly proportional to amount of antigen bound. Enthalpy (ΔH) associated with binding interaction is measured as the accumulated heat over the course of titration and the binding stoichiometry (N) is determined from the inflection point in the binding isotherms. Free energy (ΔG) and entropy (ΔS) of binding are calculated from the K_D using the equations, $\Delta G = -RT/\ln K_D$, where R is the gas constant and T is the absolute temperature and $\Delta S = (\Delta H - \Delta G)/T$. Anti-MBG antibodies/KoH-bodies were dialyzed in degassed PBS (0.01M Na_2HPO_4/NaH_2PO_4, 0.15M NaCl, pH7.4) and diluted to a final concentration of 10 μM in titration buffer (PBS with 1.6% DMSO). Antibody/KoH-body stock concentrations were determined at 280 nm and concentrations determined using extinction coefficients calculated from anti-MBG antibody/KoH-body sequences. For MBG preparation, a 25 mM stock solution of DMSO was diluted to 150 μM in PBS. Final concentration of DMSO was adjusted to 1.6% for all MBG solutions to match the titration buffer (PBS with 1.6% DMSO).

Binding affinities (K_D's) of MBG and related glycosides to anti-MBG monoclonal antibodies/KoH-bodies were determined using a MicroCal 200 Auto Isothermal Titration Calorimeter (GE Healthcare) at 25°C. Titrations were performed with 15-fold excess of titrant for MBG or 20-fold excess of titrant for other glycosides via syringe, by injections (2 uL in 4 seconds) into the anti-MBG antibody/KoH-body cell with a stirring speed of 750 rpm. Reference power and intervals between injections were set at 6-10 μcal/second and 180 seconds. ITC-customized Origins 7.0 software was used to analyze data and resulting binding isotherms were fitted using a one-site model.

Isoelectric point determination. The isolectric point (pI) determination and charge variant profiling of anti-MBG antibodies/KoH-bodies was performed by imaged capillary isoelectric

focusing using a iCE3 analyzer equipped with Alcott 720 NV autosampler (ProteinSimple, San Jose, CA). The catholyte tank was filled with 0.1 M sodium hydroxide in 0.1% methyl cellulose and the anolyte tank was filled with 0.08 M phosphoric acid in 0.1% methyl cellulose. Antibodies/KoH-bodies were focused at 1500 V for 1 min and then at 3000 V for 7 min. Focused protein bands were detected by UV absorbance at 280 nm. The electropherograms were exported and processed in Empower 3 software (Waters Corp., Milford, MA, USA). Samples applied to the iCE3 analyzer contained a basic pI marker, an acidic pI marker, pharmalyte 3-10, 2M urea and 0.5 mg/mL of protein.

Thermal analysis by differential scanning calorimetry. The unfolding or denaturation (T_m) temperatures for VI antibodies and KoH-bodies were measured by differential scanning calorimetry (DSC). Antibodies/KoH-bodies were diluted to 1 mg/mL in reference buffer (10 mM histidine, pH 5.5). Diluted samples and buffer reference were degassed and equilibrated for 5 min at 10°C. Following degassing, samples were subjected to a temperature ramp at a scan rate of 90°C/hour to a maximum of 105°C on a MicroCal VP-DSC Capillary Cell MicroCalorimeter (Malvern Instruments, Westborough, MA, USA). Baseline correction and concentration normalization was applied to all data. Origin 7 (OriginLab, Northampton, MA) software was used to fit data to a No 2-State model (two transitions) to determine T_m values.

Solubility and viscosity studies of antibodies and KoH-bodies. KoH-bodies or mAb (50-52 mg/mL) in sample buffer (10mM histidine, pH 5.5) were added to Amicon ultra centrifugal filter tubes (Ultracel-30K) at room temperature. The filter tubes were centrifuged in a tabletop centrifuge at 7000 rcf for 30 min. Samples were then centrifuged for an additional 30 min at 7000 rcf or until no further reduction in volume was observed. Concentration of KoH-body or mAb in the supernatant was determined by measuring absorbance of the undiluted sample at 280 nm using a SoloVPE Slope Spectrophotometer (C Technologies, Bridgewater, NJ, USA). KoH-bodies or mAb at high concentration (>175 mg/ml) due to ultracentrifugation were diluted to the appropriate concentrations using sample buffer (10 mM histidine, pH 5.5). Viscosities were measured at 20°C using a m-VROC viscometer (Rheosense Inc, San Ramon, CA, USA). Reduction in viscosity by the addition of viscosity reducing agent was also evaluated at high antibody concentration (175 mg/mL).

Agitation and Stability studies. KoH-bodies or mAb at 10 mg/mL in Type I glass vials were agitated on an orbital shaker (250 rpm, Chemglass Life Sciences, IS-500 Incubator Shaker) at room temperature for 24 to 48 hr with or without the presence of a non-ionic surfactant. Soluble aggregates were analyzed by size-exclusion ultra-high performance chromatography (SE-UPLC). Chromatograms were integrated and processed in Empower 3 software (Water Corp, Milford, MA, USA).

Methods to make Marinobufagenin (SI Appendix, Fig. S7):

General Methods:

^{1}H NMR spectra were recorded on a Varian Inova 300 or 500 MHz NMR instrument. Chromatographic purities were determined on an Agilent 1200 Series or 1100 Series LC/MS system using a Merck Chromolith RP-18e analytical HPLC column (monolithic, 50 × 2 mm) and the following analytical HPLC method: injection volume 5 μL; flow rate 1 mL/min; 5→95% acetonitrile in water with 0.05% AcOH over 5 mins; Agilent diode array detector at λ= 254, 220 or 195 nm; room temperature.

14-Olefin (3)

A solution of Telocinobufagin (200 mg, 0.49 mmol) in MeOH (35 mL) and 35% hydrochloric acid (0.04 mL) was heated at reflux for 17 hrs. The reaction mixture was poured into ice-water (30 mL) and extracted with $CHCl_3$. The organic layer was then washed with brine, dried over Na_2SO_4, filtered, and concentrated on a rotary evaporator and evaporated to dryness via the use of a rotary evaporator. The crude residue was purified via column chromatography (40g, SiO_2, RediSep column) using a gradient of 0 → 50% EtOAc in hexanes, over 40 min. The desired product was obtained in 57% yield (110 mg, 0.29 mmol). LC/MS: retention time 2.93 min. (ESI) $C_{24}H_{32}O_4$ calculated for $[M+H]^+$ 385; found 385.

3β-Acetate (2)

To a round bottomed flask containing 14-Olefin (**3**) (80.0 mg, 0.20 mmol and a stir bar, was added acetic anhydride (1.35 mL) and pyridine (1.8 mL). The reaction mixture was allowed to stir overnight at room temperature. Upon completion of the reaction (as determined by LCMS),

the reaction mixture was directly loaded onto a pre-equilibrated silica column and eluted with a gradient of 0 → 100% EtOAc in hexanes. The desired 3β-Acetate (**2**) was isolated in 95% yield (86 mg, 0.20 mmol). LC/MS: retention time 3.20 min. (ESI) $C_{26}H_{34}O_5$ calculated for $[M+H]^+$ 427; found 427.

Marinobufagenin (1)

To a solution of 3β-Acetate (**2**) (60.0 mg, 0.14 mmol), in acetone (3.2 mL), was added a solution of NIS (1.9 equiv, 60.0 mg, 0.267 mmol) in acetone/water (1:1, 4.0 mL). The resultant solution was stirred at room temperature overnight. Upon complete formation of the desired iodohydrin (as determined by LCMS), K_2CO_3 (20 equiv) was added and the mixture was stirred at room temperature for an additional 17 hrs. Despite the reaction not achieving full conversion to the desired product, the reaction mixture was diluted with water and extracted with $CHCl_3$ (3x30 mL), dried over Na_2SO_4, filtered, and concentrated on a rotary evaporator. The crude residue was purified via column chromatography (40g, SiO_2, RediSep column) using a gradient of 0 → 100% EtOAc in hexanes, over 40 min. The desired product was obtained in 40% yield (23 mg, 0.0575 mmol). LC/MS: retention time 2.63 min. (ESI) $C_{24}H_{32}O_5$ calculated for $[M+H]^+$ 401; found 401.

***In vivo* characterization of KoH-bodies**

Mouse PK studies of KoH-bodies. The pharmacokinetic clearance rates of anti-MBG KoH-bodies and VI mAbs were determined in C57BL/6 mice (Taconic Biosciences). Cohorts contained five mice per test article and all mice received a single sub-cutaneous (1 mg/kg) dose. Blood samples were collected at 6 hours, 1, 2, 3, 4, 8, 11, 15, 21, 30, and 49 days post-dosing. Circulating drug levels were determined by total human antibody analysis using an ELISA immunoassay. Briefly, a goat anti-human IgG polyclonal antibody (Jackson ImmunoResearch Laboratory) was coated onto a 96-well Maxisorb plate (VWR) in order to capture the human IgG present in the sera. Plates were coated at 4°C overnight, followed by non-specific blocking by BSA (Sigma). The serum samples containing test articles were plated using a six-dose serial dilution and the reference standards of the dosed test articles were plated in 12-dose serial dilution and incubated for one hour at room temperature. Following a washing step, the plate bound antibodies were detected using a goat anti-human IgG polyclonal antibody conjugated with horseradish peroxidase (Jackson ImmunoResearch Laboratory) and incubated for one hour

at room temperature followed by development with a colorimetric substrate such as BD OptEIA (BD Biosciences). After the reaction was stopped with 1M phosphoric acid, optical absorptions at 450 nm were recorded. Drug antibody/KoH-body concentrations in the sera were calculated based on the reference standard curves generated using GraphPad Prism software. Pharmacokinetic parameters (elimination half-life, time of maximum concentration, maximum concentration and bioavailability) were calculated from the serum concentration-time data using non-compartmental analysis by Phoenix WinNonLin software (Pharsight).

Immunogenicity. VI mice were immunized with a prime at day 0 and a boost one week later without adjuvant with KoH-bodies against Targets C and D, a VI antibody with a human variable domain and a mouse Fc to protein E, and a mouse antibody with a mouse variable domain and a mouse Fc to protein F as immunogens via foot pad route. Pre-immune and immune sera were collected at days 7, 14, 21 following immunization. Immune responses were determined by ELISA using the respective immunogens as coating antigens. Anti-sera were diluted 1:300 with further 3-fold serial dilutions. IgM and IgG specific antibody responses were detected as indicated with HRP conjugated anti-mouse IgM or a cocktail of HRP conjugated anti-mouse IgG isotypes, excluding the isotype of the coating antigen. Anti-IgG isotype-HRP corresponding to each immunogen was used as positive control.

Crystallization and structure determination

Crystallization and structure determination of KoH Fab:MBG complex. The Fab fragment of a KoH-body (H4H14401P) was mixed with synthetic MBG in a 3:1 molar excess of MBG over Fab. Initial crystallization trials with the H4H14401P Fab:MBG complex were not successful, so an additional Fab (known to bind hCK, which is present on the light chain of the KoH Fab) was added to the complex. A 1:1 complex of the two Fabs was purified by size-exclusion chromatography, and MBG was added to this complex in a 3:1 molar excess of MBG over H4H14401P Fab. Diffraction-quality crystals of the 2 Fab + MBG complex grew in conditions containing 0.8 M ammonium sulfate and 0.1 M sodium citrate pH 5. These crystals were cryoprotected using 20% (v/v) ethylene glycol and cryo-cooled in liquid nitrogen. Data to 3.6 Å were collected at beamline 5.0.2 of the Advanced Light Source (Berkeley, CA). The structure was determined by molecular replacement [7] using Fab subdomains with high sequence identity

from PDB codes 4YHY, 5DQD, 4LRN, 1EEQ, and 4WCY. The C-terminal regions of the anti-kappa antibody (CK and CH1) are not particularly well ordered in this structure, since there are no stabilizing crystal contacts for this domain. This is reflected in the higher temperature factors for this portion of this Fab. The N-terminal domains of the anti-kappa antibody (VK and VH) are well ordered, and the CDR interaction surface between this Fab and the KoH Fab is clearly defined in the electron density. Once the two-Fab structure had been well refined, the difference electron density for MBG was clear, allowing placement and refinement of MBG molecules (SI Appendix, Fig. S10A,B). In addition to the primary bound MBG molecule depicted in Fig. 7A, a second MBG molecule sits nearby, making a crystal contact between the CDR surface of one KoH Fab and the C_K domain of a second KoH Fab. This second MBG molecule is most likely a crystal packing artifact, since it buries almost as much surface with the symmetry-related molecule as with the primary molecule (185 $Å^2$ and 193 $Å^2$ respectively) and our ITC experiments showed no more than one MBG bound per Fab arm of H4H14401P (SI Appendix, Table S6). We have chosen not to depict the second MBG in Figs. 7 and S7, but show it in Fig S9 for completeness (SI Appendix). All refinement was carried out using Refmac5 [8].

Crystallization and structure determination of comparator Fab:MBG complex. The Fab fragment of the 3E9 comparator antibody [9], expressed and purified as described above, was mixed with synthetic MBG in a 3:1 molar excess of MBG over Fab. In contrast to the KoH Fab, this conventional Fab crystallized readily in 0.2 M ammonium sulfate, 20% polyethylene glycol 3350. These crystals were cryoprotected using 20% (v/v) PEG 400 and cryo-cooled in liquid nitrogen. Data to 1.9 Å were collected at beamline 5.0.2 of the Advanced Light Source (Berkeley, CA). The structure was determined by molecular replacement using Fab subdomains with high sequence identity from PDB code 4PUB. As with the KoH Fab structure, the electron density for the bound MBG in this structure was clear (SI Appendix, Fig. S10C,D), permitting the molecule to be placed and refined with high confidence. Crystal structure data has been deposited in the PDB, with accession codes 6PYC (KoH antibody Fab + MBG) and 6PYD (3E9 antibody Fab + MBG).

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Fig S1

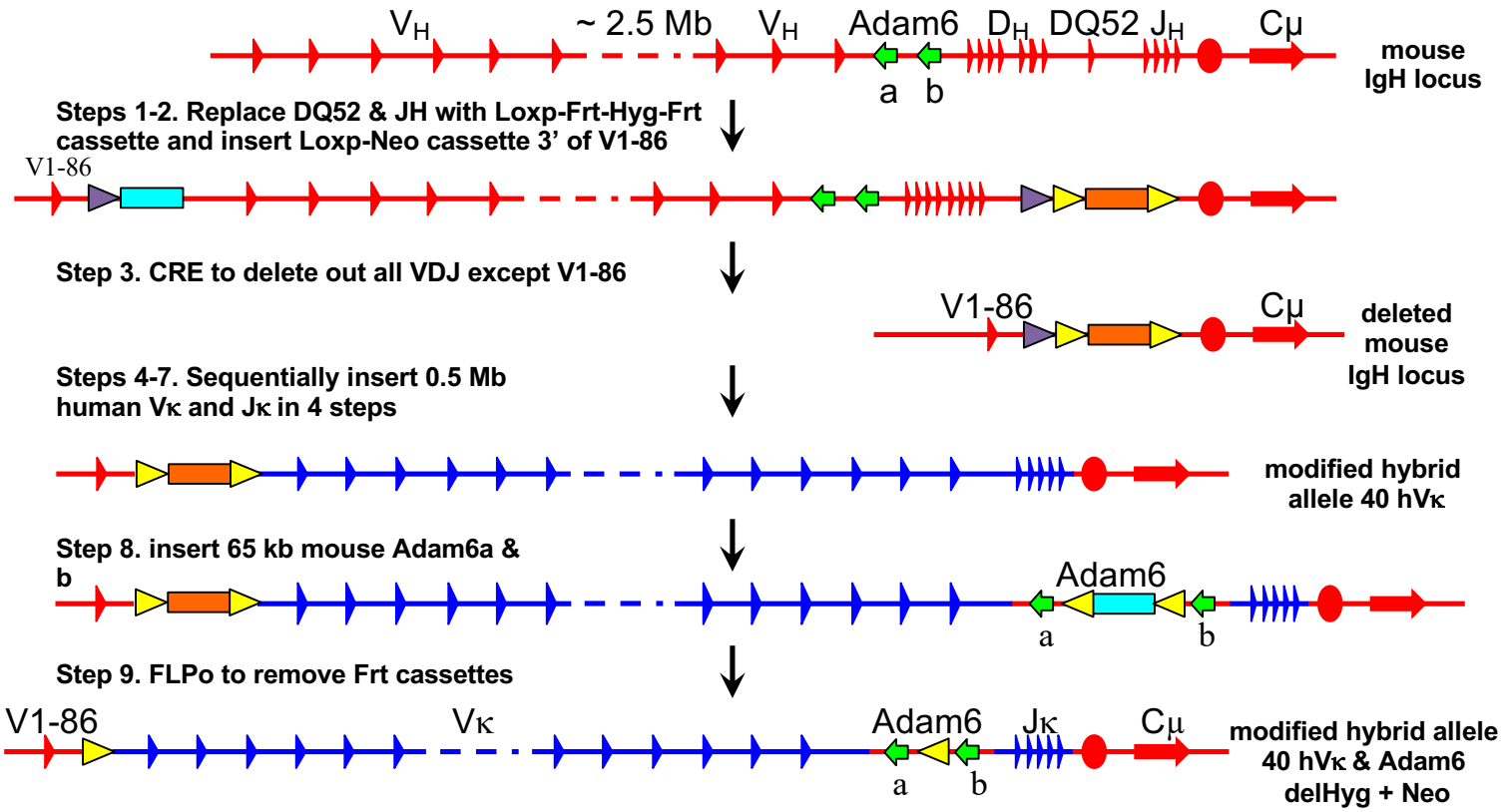


Figure S1. Detailed scheme for genomic replacement of mouse IgH V, D, and J segments with human IgK V and J segments. Steps 1-3: CRE-mediated deletion of 1.5 Mb containing all of the V, D, and J segments of the mouse Ig heavy chain locus. Steps 4-7: Sequential insertion of 0.5 Mb of human Ig Kappa sequence containing 40 V and 5 J segments. Step 8: insertion of 65 kb of mouse sequence containing the Adam6a and 6b genes required for male fertility. Step 9: FLPo-mediated deletion of the cassettes. Mouse IgH sequences are in red, human IgK sequences are in blue, red ovals indicate the mouse IgH intronic enhancer, green arrows indicate the mouse Adam6a and 6b genes, orange rectangles indicate Hygromycin selection cassettes, cyan rectangles indicate Neomycin selection cassettes, yellow triangles indicate Frt sites, and purple triangles indicate Loxp sites.

Table S1

Table S1. Replacement of mouse IgH V, D, and J genes with human IgK V and J genes

Modified allele name	Amount human sequence (kb)	Human genome coordinates(GRCh37)	Size of targeting construct (kb)	Percentage of total hVK use	Total V segments	No. of ES cell steps
IgH-PC	0		99			1
IgH-DC	0		74			2
IgH-cre	0					3
6hVKoH	110	2:89,159,668-88,270,090	148	14	6	4
16hVKoH	250	2:89,238,925-89,410,078	201	47	16	5
30hVKoH	390	2:89,388,839-89,549,402	190	70	30	6
40hVKoH	480	2:89,881,248-89,890,737; 2:89,487,788-89,630,436	182	100	40	7
40hVKoH+Adam6	480	2:89,881,248-89,890,737; 2:89,487,788-89,630,436 (Δ2:89,167,390-89,168,129)	158	100	40	8
40hVKoH+Adam6 dHyg+Neo	480	2:89,881,248-89,890,737; 2:89,487,788-89,630,436 (Δ2:89,167,390-89,168,129)		100	40	9

The KoH locus was made in nine sequential steps in F1H4 ES cells. The mouse IgH variable region locus was deleted in three sequential steps. The human IgK variable region locus was inserted in four sequential steps. The mouse Adam6a and 6b genes were inserted into the human Vk-Jk intergenic region, replacing human 2:89,167,390-89,168,129 (740 bp) with mouse 12:114,720,456-114,785,235 (64,779 bp, NCBI37/mm9 genome coordinates). A frt'd neo cassette was inserted between the two Adam6 genes at mouse genome coordinate 12:114,777,439. In the final step, transient Flp recombinase expression was used to delete the upstream Frt-Hyg-Frt cassette and the Frt-Neo-Frt cassette in Adam6. Amount of human sequence is the cumulative amount added for each allele. Note that the first 9490bp of the 40hVKoH allele map to 2:89,881,248-89,890,737 on the + strand just 5' of IGKV2D-40 (100% match). After the first 9490bp, beginning at IGKV2-40, the sequence matches 2:89,487,788-89,630,436 on the - strand as expected.

Table S2. BACvec construction

Table S2. BACvec construction

BACvec	Step	Description	Cassette	Recipient BAC	Process	Product name	Drug selection
PC	1	cassette at proximal end of mIgH locus	[5'mIgHp up homology/3'mIgHp up homology XmaI]-loxp-frt-pgk-em7-hyg-frt-[5'down mIgHp AvrII/3'down homology mIgHp]	CT7-302a07	BHR	VI1/1045	hyg cm
DC	1	cassette at distal end of mIgH locus	[mIgH dist 5' up-2/3' up mIgHd NotI]-loxp-pgk-em7-neo-loxp-[5' mighd 80kb down HindIII/3' mIgHd 80kb down]	CT7-253i20	BHR	VI243/1593	kan cm
6hVK on IgH	1	Replace CM of pBelo with PGK-Neo-PI-SceI	[3' pbelo del cm/5' pbelo del cm NsiI]-loxp-pgk-em7-neo-PI-SceI-[5' 302a07 ApaI (m56)/3' 302a07 (m57b)]	CT7-302a07	BHR	VI109	kan
	2	Replace loxp of pBelo with CM-I-CeuI	[5' 302 a07 (m53)/3' 302a07 NsiI (m54)]-Em7-CM-I-CeuI-[3' pbelo del loxp AvrII/5' pbelo del loxp]	VI109	BHR	VI111	kan cm
	3	Delete pgk-neo-PI-SceI plus 69kb of mIgH from 5' end, replace with Spec-PI-SceI. Leaves ~8kb proximal mIgH arm.	[3' pbelo del cm/5' pbelo del cm ApaI]-Spec-PI-SceI-[5' mIghp down HindIII (3d)/3' down homology mIgHp]	VI111	BHR	VI158	spec cm
	4	Ligate distal mIgH homology arm onto proximal hIgK 1167	VI76: NotI-20kb of mouse sequence upstream of mIgH locus-frt-UbCp-em7-hyg-frt-AscI	1167	Ligation	VI157	hyg spec
	5	Ligate proximal mIgH homology arm onto proximal hIgK	VI158: PI-SceI-8kb of mouse sequence downstream of IgHJ4-em7-cm-I-CeuI	VI157	Ligation	VI159/1430	hyg cm
	6	Replace frt-UbCp-em7-hyg-frt cassette with equivalent neo version	VI63: NotI-20kb of mouse sequence upstream of mIgH locus-frt-UbCp-em7-neo-frt-AscI	VI159	Ligation	VI295/6hVKoH	kan cm
16hVK on IgH	1	Ligate distal mIgH homology arm onto 16hVK (VI75/1182)	VI63: NotI-20kb of mouse sequence upstream of mIgH locus-frt-UbCp-em7-neo-frt-AscI	VI75/16hVK	Ligation	VI197	kan spec
	2	Replace frt-UbCp-em7-neo-frt cassette with equivalent hyg version	frt-UbCp-em7-hyg-frt	VI197	BHR	VI327/16hVKoH	hyg spec
30hVK on IgH	1	Ligate distal mIgH homology arm onto 30hVK (VI82/1205)	VI76: NotI-20kb of mouse sequence upstream of mIgH locus-frt-UbCp-em7-hyg-frt-AscI	VI82/30hVK	Ligation	VI198	hyg spec
	2	Replace frt-UbCp-em7-hyg-frt cassette with equivalent neo version	frt-UbCp-em7-neo frt	VI198	BHR	VI328/30hVKoH	kan spec
40hVK on IgH	1	Ligate distal mIgH homology arm onto 40hVK (1242)	VI63: NotI-20kb of mouse sequence upstream of mIgH locus-frt-UbCp-em7-neo-frt-AscI	1242/40hVK	Ligation	VI199	kan spec
	2	Replace frt-UbCp-em7-neo-frt cassette with equivalent hyg version	frt-UbCp-em7-hyg-frt	VI199	BHR	VI329/40hVKoH	hyg spec
40hVK on IgH + mouse Adam6	1	Replace 3' CM of CT7-929d24 with Spec cassette flanked by 5' PI-SceI and 3' I-CeuI sites	[5' 929d24 (m60)/3' 929d24 XbaI (m59)]-PI-SceI-Spec-I-CeuI-[5' pbelo del cm AscI/3' pbelo del cm]	CT7-929d24	BHR	VI108	spec
	2	Replace ~14.5 kb of 5' end of VI108 with NotI-Pgkp-em7-hyg-polyA-Hsvp-tk-AscI cassette	[5' pbelo del loxp /3' pbelo del loxp NotI]-pgkp-em7-hyg-polya-hsvp-tk-[5' 929d24 AscI (m78b)/3' 929d24 (m79)]	VI108	BHR	VI120	hyg spec
	3	NotI + AscI ligation to replace NotI-I-CeuI-Pgkp-em7-hyg-polyA-Hsvp-tk-AscI cassette with 20kb mouse IgH distal homology arm	VI63: NotI-20kb of mouse sequence upstream of mIgH locus-frt-UbCp-em7-neo-frt-AscI	VI120	Ligation	VI121	kan spec
	4	Replace neo in 5' pgkp-em7-neo-polya-PI-SceI cassette with hyg	[pgkp-em7]-hyg-[polya]	VI111	BHR	VI124	hyg cm
	5	PI-SceI + I-CeuI ligation to replace the 3' Spec cassette of VI121 with a 77 kb fragment of CT7-302a07 containing the mouse Adam6b, DH, JH, and IgM genes. PI-SceI site is 5.5 kb from end of Adam6b.	VI124: PI-SceI-77 kb from CT7-302a07-cm-I-CeuI	VI121	Ligation	VI122	kan cm
	6	Replace 5' distal mIgH arm, frt-UbCp-em7-neo-frt cassette, and ~44kb of mIgH sequence with loxp-spec-loxp cassette and I-CeuI site. I-CeuI is 751bp downstream of Adam6a gene.	[5' pbelo del loxp/3' pbelo del loxp SacI]-loxp-spec-loxp-[5' 929d24 Xba-ICeuI (md90c)/3' 929d24 mIgH (md90)]	VI122	BHR	VI148	spec cm
	7	CRE to delete loxp-spec-loxp cassette	none	VI148	CRE	VI149	cm
	8	Replace CM plus ~62kb from 3' end of VI149 (mouse DH, JH, and IgM) with AscI site and Spec cassette	[5' up VI149 (m56)/3' up VI149 AscI-EcoRI (m57b)]-spec-[5' pbelo del cm AvrII/3' pbelo del cm]	VI149	BHR	VI413	spec
	9	Insert frt-UbCp-em7-neo-frt cassette ~47kb downstream of Adam6b and ~4.8kb upstream of Adam6a	[5' up mAdam6/3' up mAdam6 AsiSI-AgeI]-frt-neo-UbCp-em7-frt-[5' down2 mAdam6 AsiSI-KpnI/3' down2 mAdam6]	VI413	BHR	VI422	kan spec
	10	Replace 5' distal mIgH homology arm, frt-UbCp-em7-neo-frt cassette, and ~35kb of human IgK of VI295 with em7-hyg cassette	[3' pbelo del cm/5' pbelo del cm MfeI]-em7-hyg-[5' down 1708 FseI-BamHI (h34b)/3' down 1708 (h35)]	VI295/6hVKoH	BHR	VI414	hyg cm
	11	Replace 3' CM with Spec cassette	[5' 302 a07 (m53)/3' 302a07 NsiI (m54)]-spec-[3' pbelo del loxp AvrII/5' pbelo del loxp]	VI414	BHR	VI425	hyg spec
	12	Replace 740bp of hIgK V-J intergenic region (~17kb downstream of IgKV4-1 and ~7kb upstream of IgKJ1) with CM cassette flanked by I-CeuI and AscI sites	[5' up hIgK V-J short del (h43)/3' up hIgK V-J short del AvrII (h44)]-I-CeuI-CM-[5' down hIgK short del AscI-BamHI (h46b)/3' down hIgK V-J short del (h47)]	VI425	BHR	VI426	hyg cm spec
	13	Replace CM cassette in hIgK V-J intergenic region with ~68kb Adam6a-frt-UbCp-em7-neo-frt-Adam6b cassette (GRCm37 coordinates 12:114,720,456-114,785,235)	VI422: AscI-9.6kb Adam6b-47.5kb intergenic region-frt-UbCp-em7-neo-frt-7.8kb Adam6a-I-CeuI	VI426	Ligation	VI428/40hVKoH + mouse Adam6	hyg kan spec

Table S3. BACvec primers

Table S3. BACvec primers

MAID	Step	Name	Sequence
mIgH-PC	1	5'mIgHp up homology	CCAGCAATCATGGTGGGTGTC
		3'mIgHp up homology xmai	CCTCCCCGGGGATATGTGTTTTTGAATGCTT
		5'down mIgHp avrII	CCTAGGCTGAGCATTGCAGACTAATC
		3'down homology mIgHp	CCCTATGCATCGGATACTGT
mIgH-DC	1	mIgH dist 5' up-2	CTATGGGATACAATTCCTCAG
		3' up mIgHd not	GTTCATGGCGGCCGCTAGAATCAGTGTCTTCAGGGA
		5' mighd 80kb down hindiii	CACCTGGAAGCTTATCACTGGATCCTGAGTGACCA
		3' mIgHd 80kb down	TACATGCTTGTGCATTCTGAT
6hVK	1	3' pbelo del cm	CAATCCAGGTCCTGACCGTTC
		5' pbelo del cm NsiI	CTGTCATGCATGCCCGGTAGTGATCTTATTTC
		5' 302a07 ApaI (m56)	CCTCGGGCCCTTGCTCTGTAGTAAAGCTTTA
		3' 302a07 (m57)	TAACTAAGGAAGTGAAAGATC
		3' 302a07 (m57b)	TGGCTGTCTTGCCAAAAGGAA
	2	5' 302a07 (m53)	AGGCCACGAGGCCCTGCCACA
		3' 302a07 NsiI (m54)	CCTCATGCATGAGTTCACACACAAGGAGGAA
		3' pbelo del loxp AvrII	CCTCCCTAGGCTCGCTTTCAGCACCTGTCGT
		5' pbelo del loxp	ATCCGATGCAAGTGTGTCGCT
	3	3' pbelo del cm	CAATCCAGGTCCTGACCGTTC
		5' pbelo del cm ApaI	GCTGCGGGCCCGCCCGGTAGTGATCTTATTTC
		5' mIghp down hind3 (3d)	CTGCTAAGCTTCTGAGCATTGCAGACTAATC
		3' down homology mIgHp	CCCTATGCATCGGATACTGT
40hVK-Adam6	1	5' 929d24 (m60)	CCACTTTCTCCTCTATAAGTT
		3' 929d24 XbaI (m59)	CCTCTCTAGATTGTGATAAAAACTGCATGGTA
		5' pbelo del cm AscI	CCTAGGCGCGCCGCCCGGTAGTGATCTTATTTC
		3' pbelo del cm	CAATCCAGGTCCTGACCGTTC
	2	5' pbelo del loxp	ATCCGATGCAAGTGTGTCGCT
		3' pbelo del loxp NotI	CCTCTGCGGCCGCCTCGCTTTCAGCACCTGTCGT
		5' 929d24 AscI (m78b)	CCTCGGCGCGCCCCCTGTACTTCTCTTCCCTGG
		3' 929d24 (m79)	GCTTATATCTCATAAAACCTC
	6	5' pbelo del loxp	ATCCGATGCAAGTGTGTCGCT
		3' pbelo del loxp SacI	GCTAGAGCTCTCGCTTTCAGCACCTGTCGT
		5' 929d24 Xba-ICeuI (md90c)	CCTCTCTAGACTTAACTATAACGGTCCTAAGGTAGCGAGGGATGACAGATTCTCTGTTC
		3' 929d24 mIgH (md90)	TTGATGGTTCCCATCTCCTAT
	8	5' up VI149 (m56)	ACCACACAGCCTTCCACAAGAG
		3' up VI149 AscI-EcoRI (m57b)	CTCCGAATTCGGCGCGCCTGCAGGTGTAGAGAGCATTGG
		5' pbelo del cm AvrII	CTCCCCTAGGCCGGTAGTGATCTTATTTC
		3' pbelo del cm	CAATCCAGGTCCTGACCGTTC
	9	5' up mAdam6	TGGTTTTCTGGAGTCTAACG
		3' up mAdam6 AsiSI-AgeI	CTCCACCGGTGCGATCGCGACCTCAGCACAATAATAGAGAC
		5' down2 mAdam6 AsiSI-KpnI	CTCCGGTACCGCGATCGCCCTTGATCCACTTGAACTTGAGCA
		3' down2 mAdam6	GGAATGACCATCCTTGACCTCA
	10	3' pbelo del cm	CAATCCAGGTCCTGACCGTTC
		5' pbelo del cm MfeI	CTCCCAATTGCCCGGTAGTGATCTTATTTC
		5' down 1708 FseI-BamHI (h34b)	CTCCGGATCCGGCCGGCCAGTGAAGCCAAGAACCTGCC
		3' down 1708 (h35)	CCTCATACCCAGAAGAATCAGC
	11	5' 302 a07 (m53)	AGGCCACGAGGCCCTGCCACA
		3' 302a07 NsiI (m54)	CCTCATGCATGAGTTCACACACAAGGAGGAA
		3' pbelo del loxp AvrII	CCTCCCTAGGCTCGCTTTCAGCACCTGTCGT
		5' pbelo del loxp	ATCCGATGCAAGTGTGTCGCT
	12	5' up hIgK V-J short del (h43)	AGACGCTGGACAAAGGGATG
		3' up hIgK V-J short del AvrII (h44)	CTCCGAGCTCAAAGTTGGTTCAAAGGACGATG
		5' down hIgK short del AscI-BamHI (h46b)	CTCCGGATCCGGCGCGCCCTCTGAAGTACGGCTTTTCTGG
		3' down hIgK V-J short del (h47)	TAAGAAGGAGGATAGGAGGCG

Table S4. qPCR probes

Table S4. qPCR probes

					Probe copy numbers									
Probe name	Description	Forward primer	Probe	Reverse primer	IgH PC het	IgH DC het	PC/DC cre het	6hVK het	16hVK het	30hVK het	40hVK het	40hVK ho	40hVK-Adam6 het	40hVK-Adam6 ho
mIgHp2	prox mIgH deletion	GCCATGCAAGGCCAAGC	CCAGGAAAATGCTGCCAGAGCCTG	AGTTCTTGAGCCTTAGGGTGCTAG	1	1	1	1	1	1	1	0	1	0
mIgHp1	prox mIgH deletion	ATGGGTCTGGGTCAGGGAC	TCTGCCTGCTGGTCTGTGGTGACATT	TGGCAGATCCTTCATCATACTTCAG	1	1	1	1	1	1	1	0	1	0
mIgHd10	distal mIgH deletion	GGTGTGCGATGTACCCTCTGAAC	CTAAAAATGCTACACCTGGGGCAAAACACCTG	TGTGGCAGTTTAATCCAGCTTTATC	2	1	1	1	1	1	1	0	1	0
mIgHd57-1	distal mIgH deletion	CACAGAATGCTTGCTTTTACCAGC	ATGGCATATGATAGATAGGTAGCGATTCACAACCC	AGGAGACATGCTCACTGAATAGAGAAG	2	1	1	1	1	1	1	0	1	0
mIgHA7	mIgH deletion	TGGTCACCTCCAGGAGCCTC	AGTCTCTGCTTCCCCCTTGTGGCTATGAGC	GCTGCAGGGTGTATCAGGTGC	2	2	1	1	1	1	1	0	1	0
887-10	mIgH deletion	GATGGGAAGAGACTGGTAACATTTGTAC	CCTCCACTGTGTTAATGGCTGCCACAA	TTCCTCTATTTCACTCTTTGAGGCTC	2	2	1	1	1	1	1	0	1	0
hIgK5	6 hVK insertion	CCCCGTCCTCCTCCTTTTTC	TCATGTCCATTAACCCATTTACCTTTTGCCCA	TGCAAGTGCTGCCAGCAAG	0	0	0	1	1	1	1	2	1	2
hIgK6	6 hVK insertion	GTCAAGCACTGCTGGCACAC	AACCCTTGTGCTATTGAATTGCTATGCTGTCAG	TGTTGTAGACCCTCCGCCAC	0	0	0	1	1	1	1	2	1	2
hIgK11	16 hVK insertion	GCAAAGAAGCTGTGTGAACGC	CACCCTGGGGCTGAAACCTGCA	CAGTCACAGGAAAGGCAGGG	0	0	0	0	1	1	1	2	1	2
hIgK12	16 hVK insertion	TTGCCTTTCTCACACCTGCAG	CAGCCCATCCTGTCACTTCGCTGGA	TGGCCCAACAGTACAGCTCAG	0	0	0	0	1	1	1	2	1	2
hIgK15	30 hVK insertion	CAGGTGCAAAGGTGACCACAG	TGGGTCCTGCCCATCCATGCA	GGCAGCCTGAGTGTCAGAGC	0	0	0	0	0	1	1	2	1	2
hIgK25	30 hVK insertion	GTTCAGGCCCCACAGACTCTC	TCCTCTCTGGAGCAACCATGAAGTTCCCT	CCTGAAGCCATGAGGGCAG	0	0	0	0	0	1	1	2	1	2
hIgK26	40 hVK insertion	TGGGCCGTTATGCTAGTACCA	TGGCTTTACCCCTTTTGAAGGGCCC	CACAGCTGAAGCAGGATGAGC	0	0	0	0	0	0	1	2	1	2
hIgK32	40 hVK insertion	AGCACTGCTGCATGGCTTC	TGGGTGGAGCCAAGTCTTCGCAC	TGAGTAATAAAGGAGAGAGAAAGTTTGGA	0	0	0	0	0	0	1	2	1	2

Fig S2A

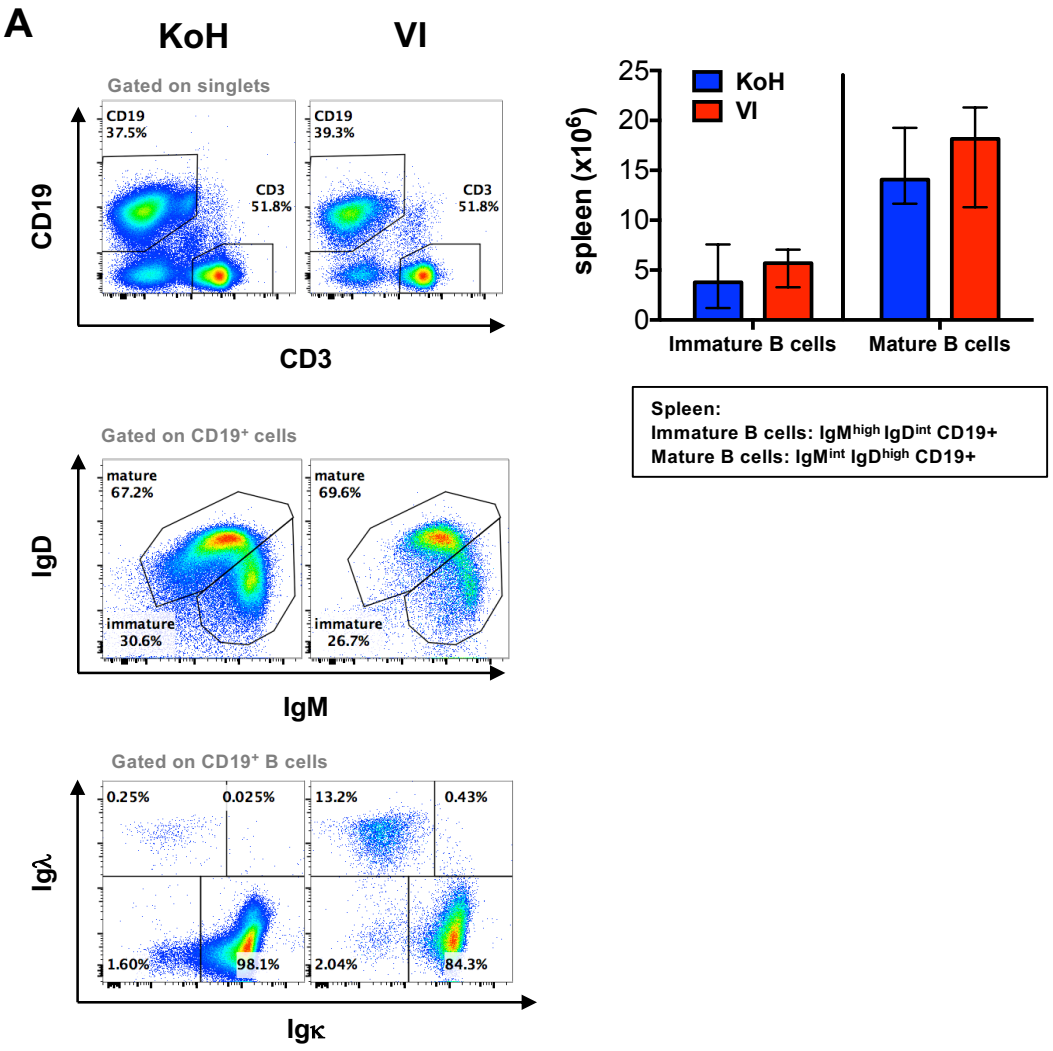

A
KoH
VI
Gated on singlets
CD19
37.5%
CD3
51.8%
CD19
39.3%
CD3
51.8%
CD19
CD3
Gated on CD19⁺ cells
mature
67.2%
immature
30.6%
mature
69.6%
immature
26.7%
IgD
IgM
Gated on CD19⁺ B cells
0.25%
0.025%
1.60%
98.1%
13.2%
0.43%
2.04%
84.3%
Igλ
Igκ
spleen (x10⁶)
25
20
15
10
5
0
KoH
VI
Immature B cells
Mature B cells
Spleen:
Immature B cells: IgM^high IgD^int CD19+
Mature B cells: IgM^int IgD^high CD19+

Fig S2B

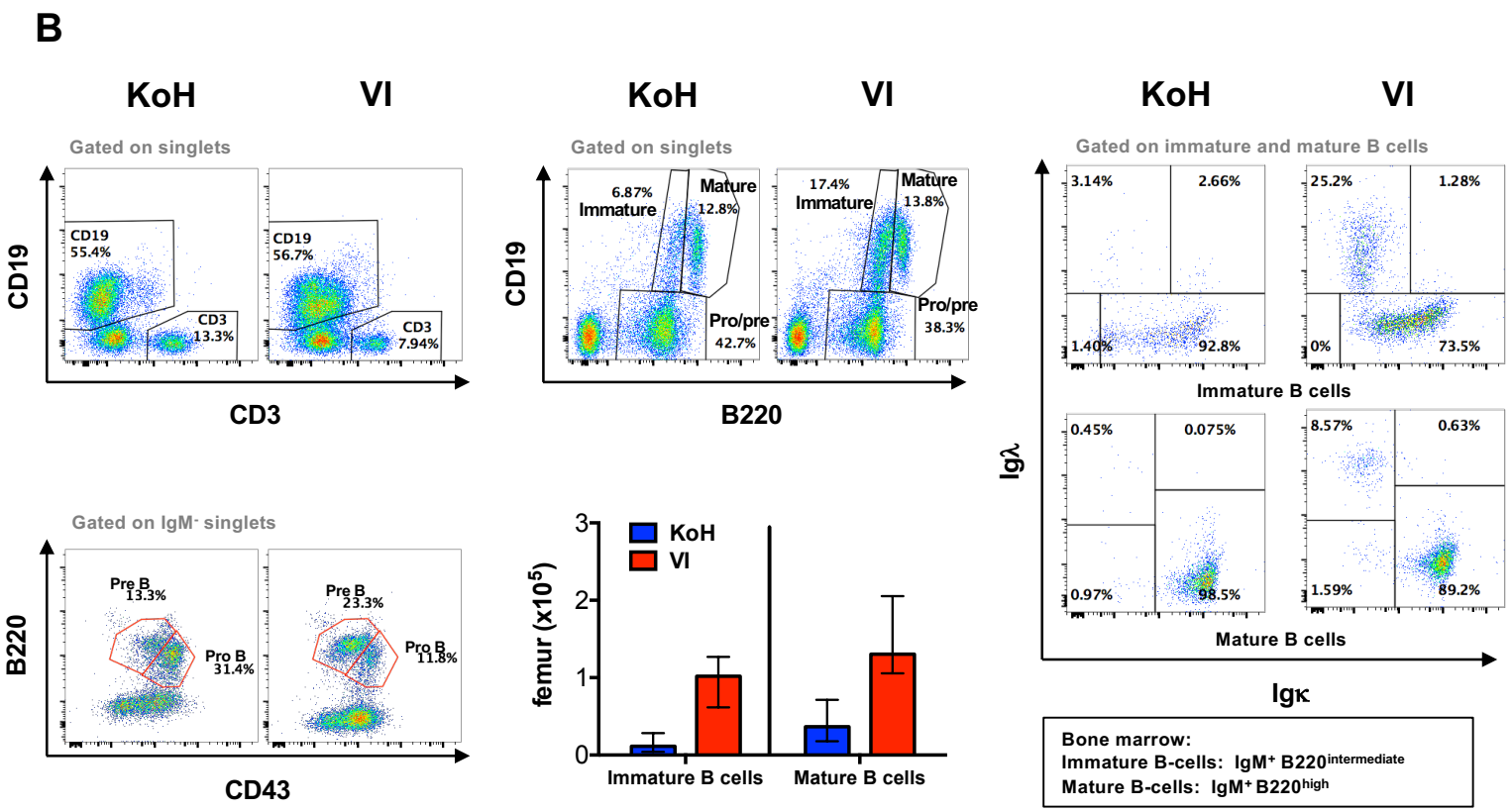


Figure S2. Normal B cell development in the spleen of KoH mice, compared to VI mice, despite altered B cell development in the bone marrow. (A) Flow cytometry analysis of the spleen and quantitation of CD19+ IgMhigh IgDint immature and CD19+ IgMint IgDhigh mature B cells per spleen of KoH vs. VI. (B) Flow cytometry analysis of bone marrow B cells, including IgM- CD43high B220int Pro B cells and IgM- CD43int B220int Pre-B cells, and quantitation of IgM+ B220int immature and IgM+ B220high mature B cells per femur of KoH vs. VI mice. Cross bar shows median with interquartile range. *n*=10 KoH and *n*=8 VI mice.

Fig S3

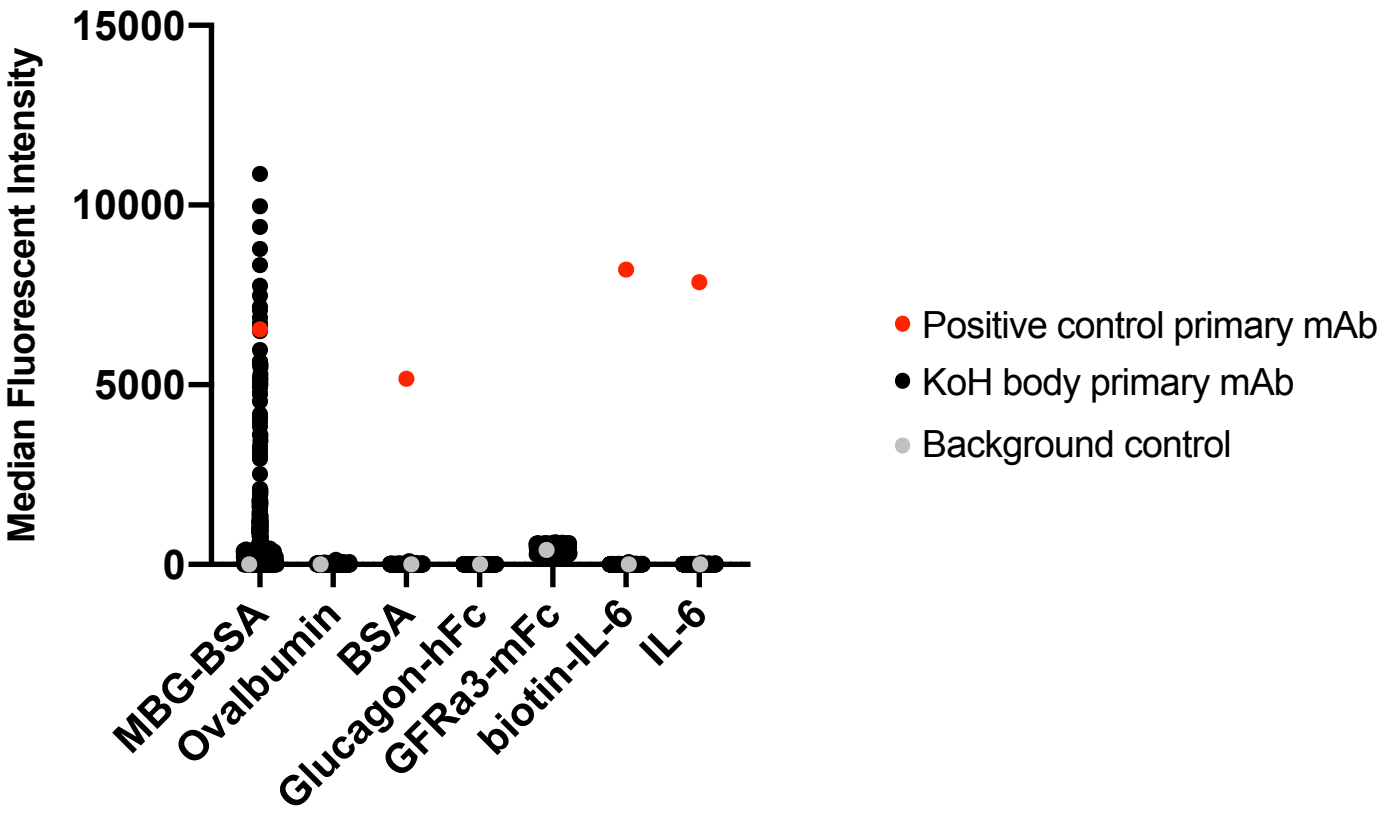


Figure S3: KoH-bodies do not bind irrelevant proteins. Luminex screening was performed by coupling the immunogen (MBG-BSA) or irrelevant proteins (ovalbumin, BSA, Glucagon-hFc, GFRa3-mFc, biotin-IL6 or IL-6) to Luminex beads, followed by incubation with KoH bodies (n=339), before staining with PE-conjugated anti-mouse IgG F(ab')2 specific secondary antibody (black circles). Background control values were determined following staining of beads coupled to above MBG-BSA or irrelevant proteins, then incubated with buffer control, before staining with above mentioned secondary antibody (grey circles). Positive control values were determined following staining of beads coupled to MBG-BSA, BSA or -biotin-IL-6 (or -IL-6 alone) with anti-MBG (mIgG1), anti-BSA (mIgG1) or anti-IL-6 (mIgG1) primary antibody, respectively, before staining with above mentioned secondary antibody (red circles).

Fig S4

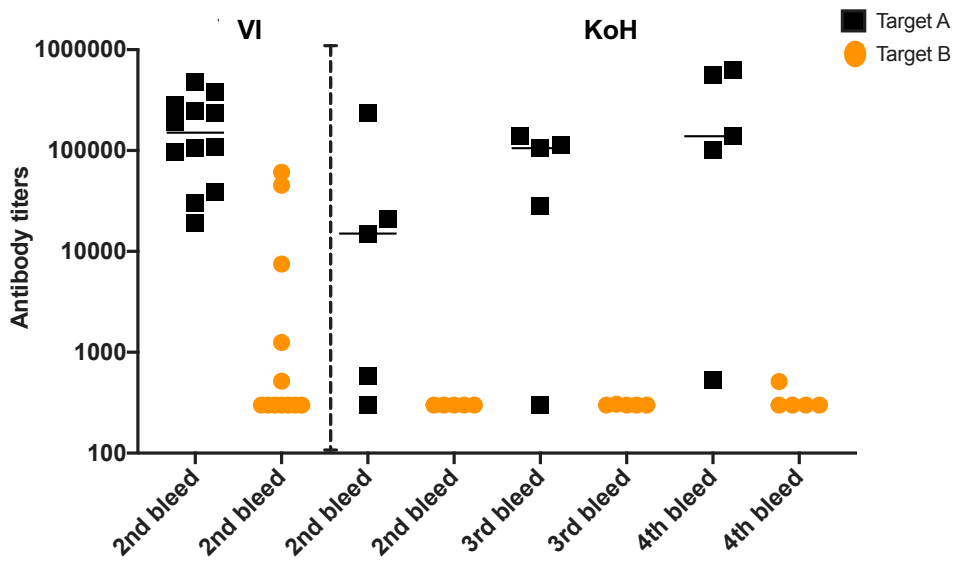


Figure S4. KoH mice elicit high anti-Target A antibody titers post-immunization. Mice were immunized with Target A protein (containing His tag) as immunogen via f.p. (VI) and i.p route followed by f.p. boosts (KoH) respectively. Antibody titers against Target A was assayed by a standard ELISA with Target A and irrelevant Target B protein (containing a His tag) coated plates

Table S5

Table S5. Oligonucleotide primers used for antibody repertoire sequencing

RT primers	**IgM**	5′ – TCTTATCAGACAGGGGGCTCTC – 3′
	IgK	5′ – AAGAAGCACACGACTGAGGCAC– 3′
1st round PCR primers	**IgM**	5′ – ACACTCTTTCCCTACACGACGCTCTTCCGATCT GGGAAGACATTTGGGAAGGAC – 3′
	IgK	5′ – ACACTCTTTCCCTACACGACGCTCTTCCGATCT GGAAGATGGATACAGTTGGTGC – 3′
	PE2-PIIA	5′ – GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT AAGCAGTGGTATCAACGCAGAGT – 3′
2nd round PCR Primers	**Forward**	5′ – AATGATACGGCGACCACCGAGATCTACACXXXXXX ACACTCTTTCCCTACACGACGCTCTTCCGATCT– 3′
	Reverse	5′ – CAAGCAGAAGACGGCATACGAGATXXXXXX GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT– 3′

Table S6

Table S6. Binding constants (25ºC) and thermodynamic parameters for anti- MBG monoclonal antibody interactions determined using ITC and Biacore

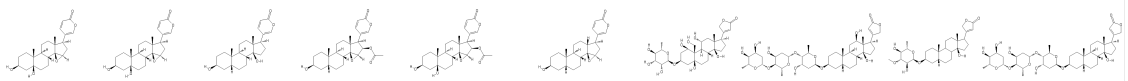
			ITC				Biacore			
Antibody	mouse strain	VL Protein /mAb	*N*	K_D (M) ^{ITC}	ΔH (cal/mol)	(-)TΔS (cal/mol)	ka (M-1s-1)	kd (s-1)	K_D (M) ^{SPR}	t1/2 (seconds)
mAb 3E9	BALB/C	Control mAb 1	2.1	2.49E-07	-7073	-1931	1.03E+06	5.85E-02	5.68E-08	12
H4H14357P	KoH	VL Protein 1	1.97	3.48E-08	-9135	-1037	4.82E+06	4.58E-02	9.50E-09	15
H4H14362P	KoH	VL Protein 2	1.59	1.79E-08	-14460	3904	6.40E+05	7.43E-03	1.16E-08	93
H4H14371P	KoH	VL Protein 3	1.72	4.81E-08	-11820	1842	1.35E+06	5.98E-03	4.43E-09	116
H4H14372P	KoH	VL Protein 4	1.63	6.62E-08	-11480	1690	1.19E+06	7.11E-03	5.97E-09	97
H4H14373P	KoH	VL Protein 5	2.03	4.59E-08	-12690	2682	8.50E+05	7.41E-03	8.72E-09	94
H4H14401P	KoH	VL Protein 6	1.81	6.29E-09	-18850	7659	1.01E+06	4.46E-03	4.42E-09	156
H4H14407P	KoH	VL Protein 7	1.85	7.87E-07	-9984	1660	1.04E+05	2.02E-01	1.94E-06	3
H4H14416P	KoH	VL Protein 8	1.9	4.74E-08	-12690	2697	2.42E+06	8.10E-02	3.35E-08	9
H4H14453P2	VI3	Control mAb 2	2.06	8.70E-07	-9778	1508	nd	nd	*6.3E-06	nd

nd: not determined

*: Steady state K_D analysis

Table S7

Table S7. Cross Reactivity of anti-MBG KoH Antibodies

	ITC K_D [M], 10uM									
Ab PID #	**Bufadienolides**						**Cardenolides**			
										
	Natural MBG	Synthetic MBG	Bufalin	Cinobufagin	Cinobufotalin	Resibufagenin	Ouabain	Digoxin	Nerifolin	Digitoxin
H4H14371P	5.88E-08	7.41E-08	WB	WB	7.63E-08	7.69E-08	NB	NB	NB	NB
H4H14401P	3.61E-08	8.55E-08	WB	5.03E-08	6.33E-08	7.69E-08	NB	NB	NB	NB
H4H14357P	3.58E-08	7.75E-08	WB	5.29E-06	9.01E-08	2.04E-06	NB	NB	NB	NB
3E9	1.56E-07	3.27E-07	NB	NB	1.69E-07	NB	NB	NB	NB	NB
Iso type control	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB

NB: No Binding

WB: Weak Binding observed, but K_D could not be determined under these experimental conditions

Fig. S5

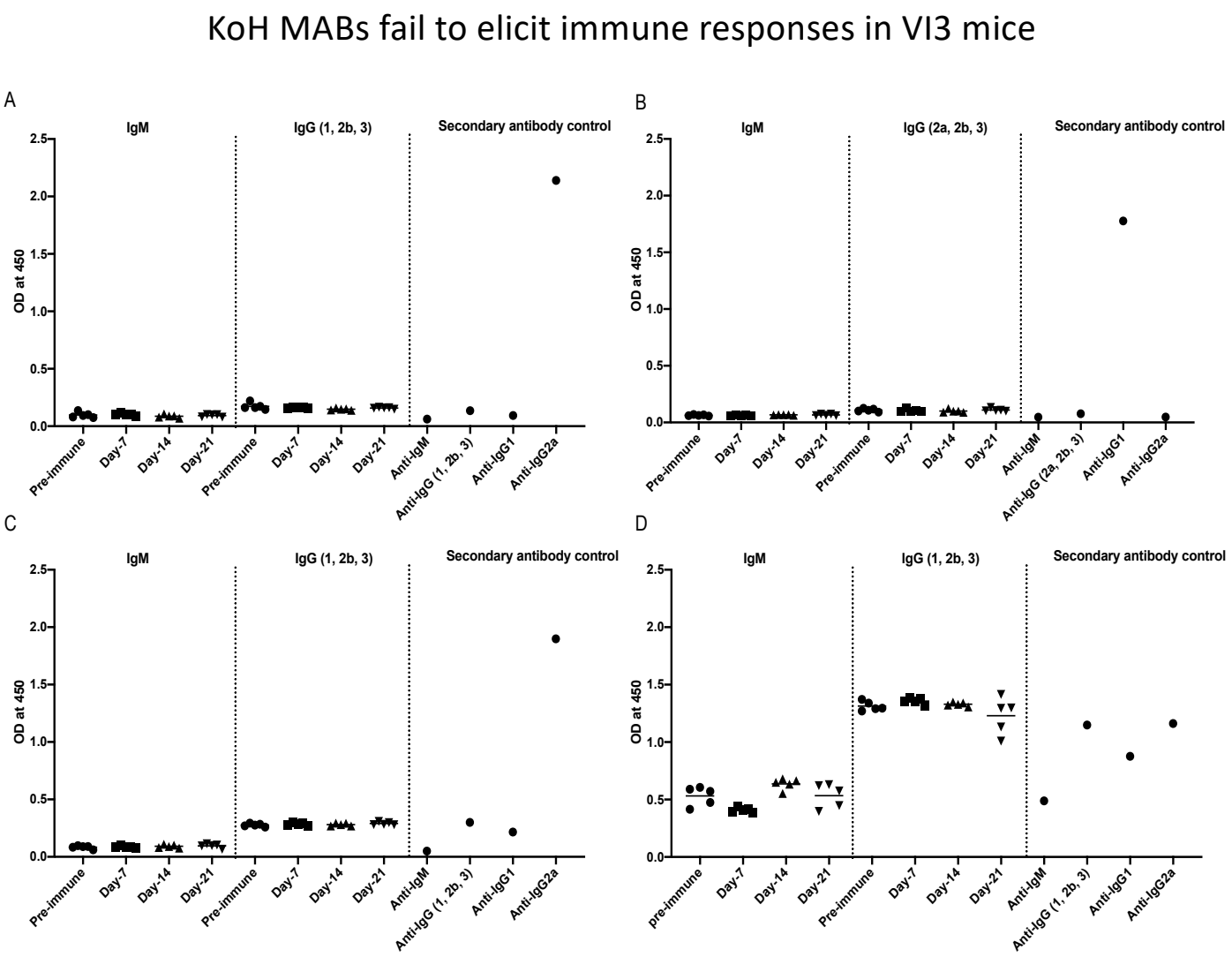


Figure S5. KoH-bodies do not elicit immune responses in VI mice. VI mice were immunized with KoH-bodies against Targets C and D (panels A, B, respectively), a VI antibody with a human variable domain and mFc to protein E (panel C), and a mouse antibody with mouse variable domain and mFc to protein F (panel D) as immunogens without adjuvant via f.p.route. Immune responses were determined by ELISA using the respective immunogens as coating antigen. IgM and IgG antibody responses were detected as indicated with HRP-conjugated anti-mouse IgM or a cocktail of HRP-conjugated anti-mouse IgG isotypes, excluding the isotype of the coating antigen.

Fig S6A

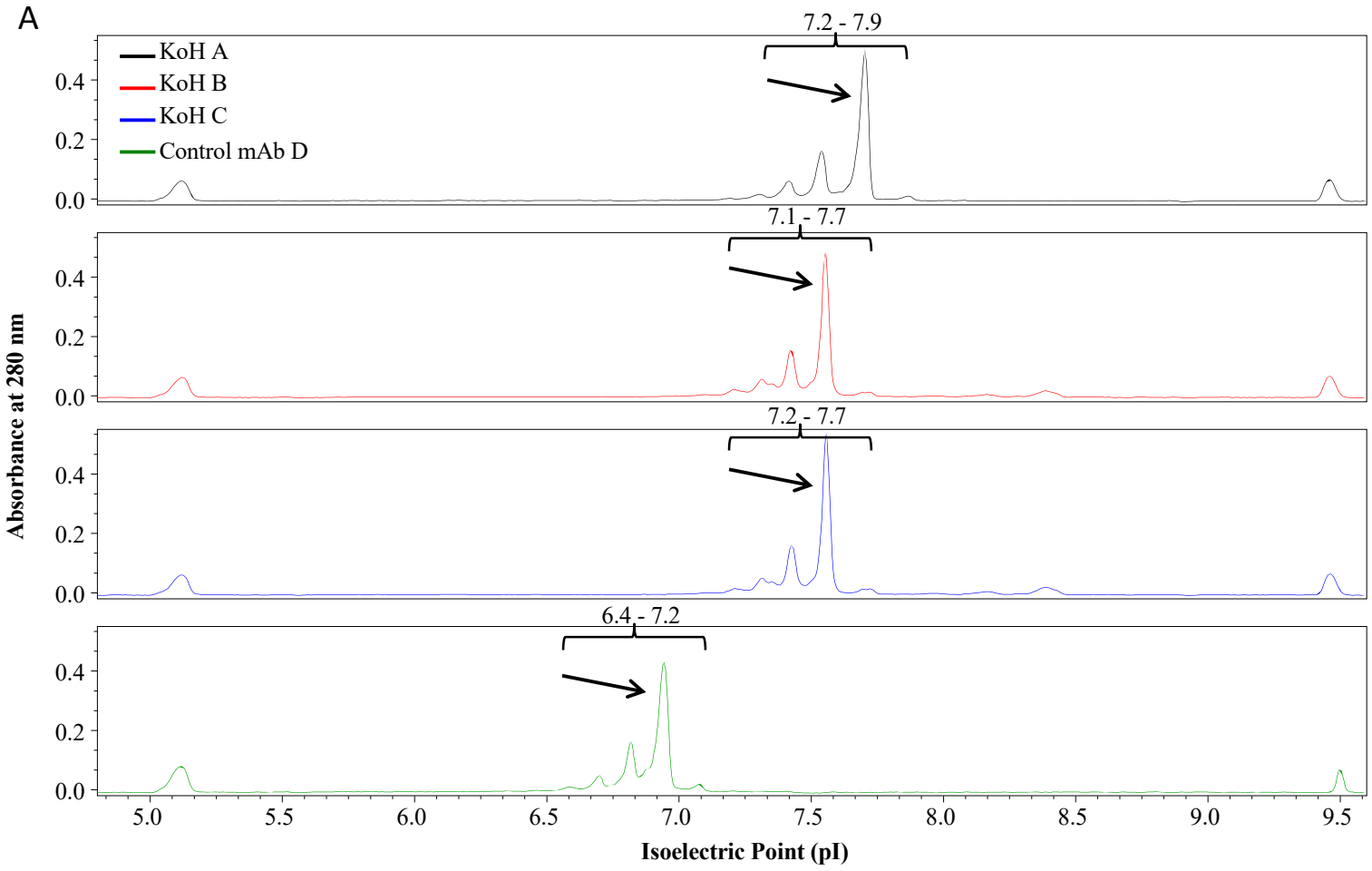
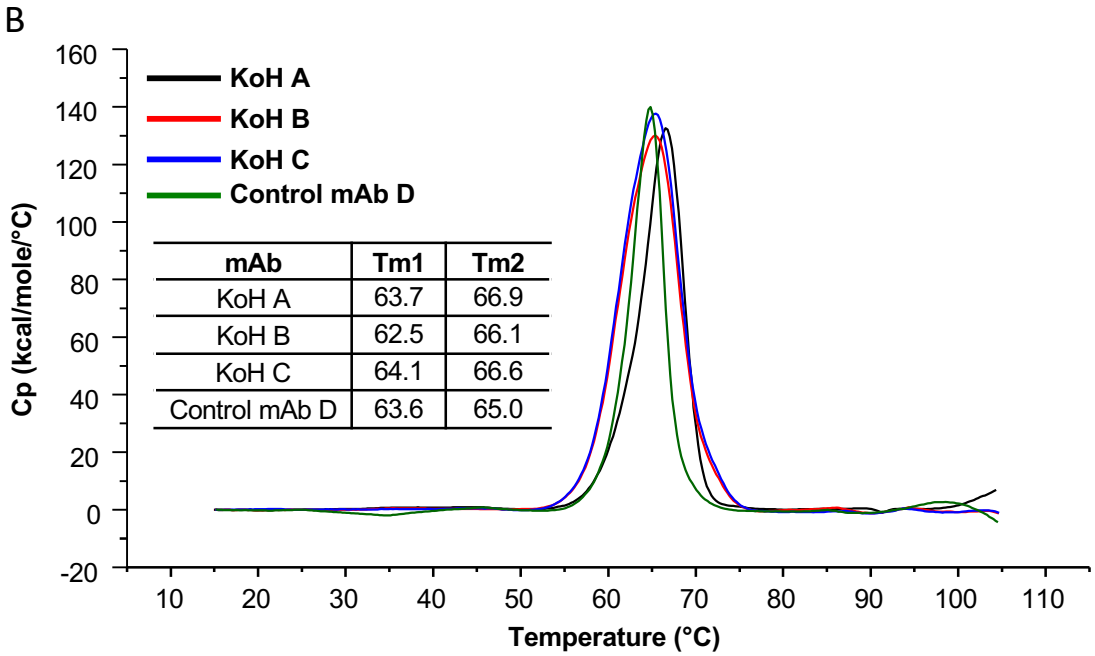

A
KoH A
KoH B
KoH C
Control mAb D
7.2 - 7.9
7.1 - 7.7
7.2 - 7.7
6.4 - 7.2
Absorbance at 280 nm
0.0
0.2
0.4
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
Isoelectric Point (pI)

Fig S6B



mAb	Tm1	Tm2
KoH A	63.7	66.9
KoH B	62.5	66.1
KoH C	64.1	66.6
Control mAb D	63.6	65.0

Fig S6C

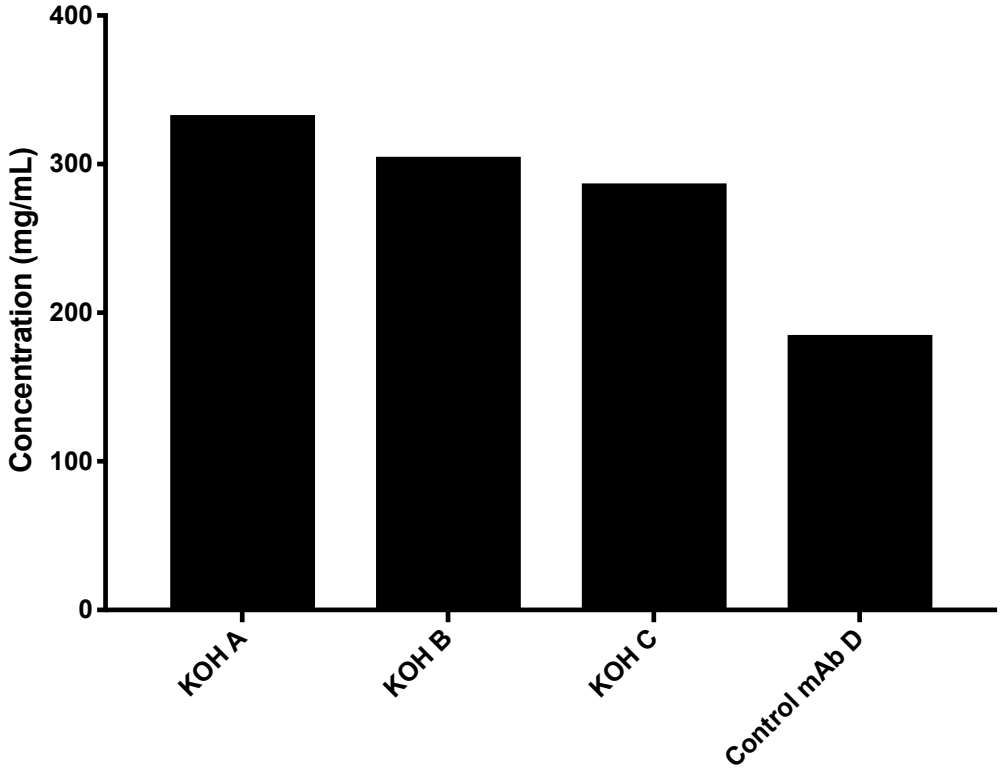

400
300
200
100
0
Concentration (mg/mL)
KOH A
KOH B
KOH C
Control mAb D

Fig S6D

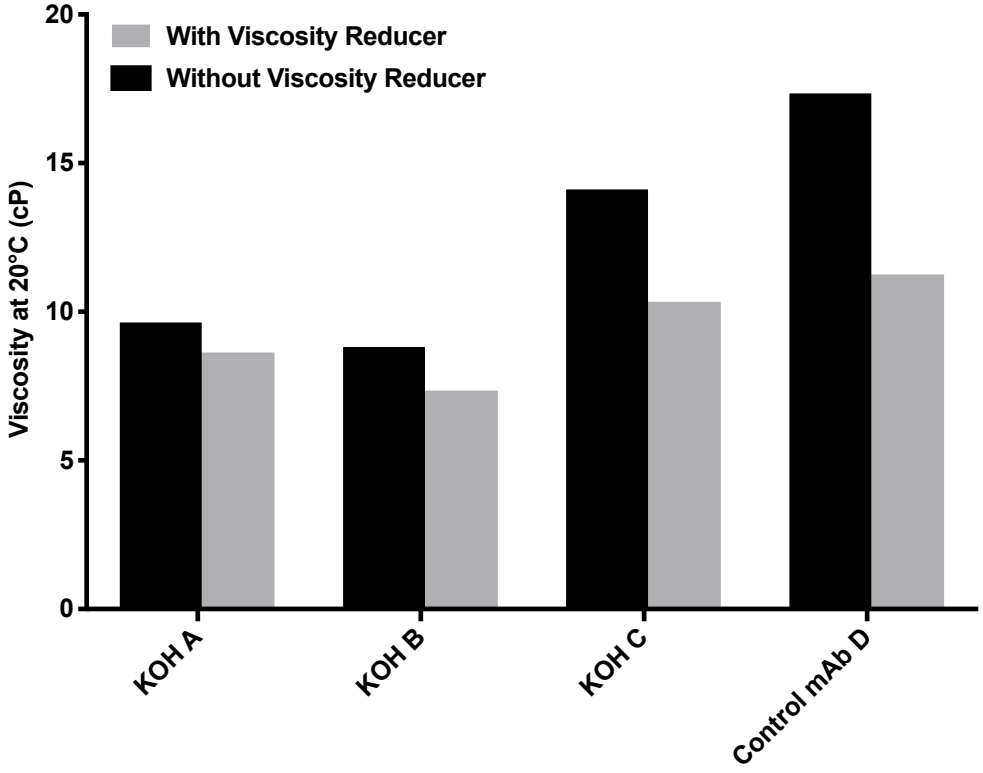

With Viscosity Reducer
Without Viscosity Reducer
Viscosity at 20°C (cP)
20
15
10
5
0
KOH A
KOH B
KOH C
Control mAb D

Fig S6E

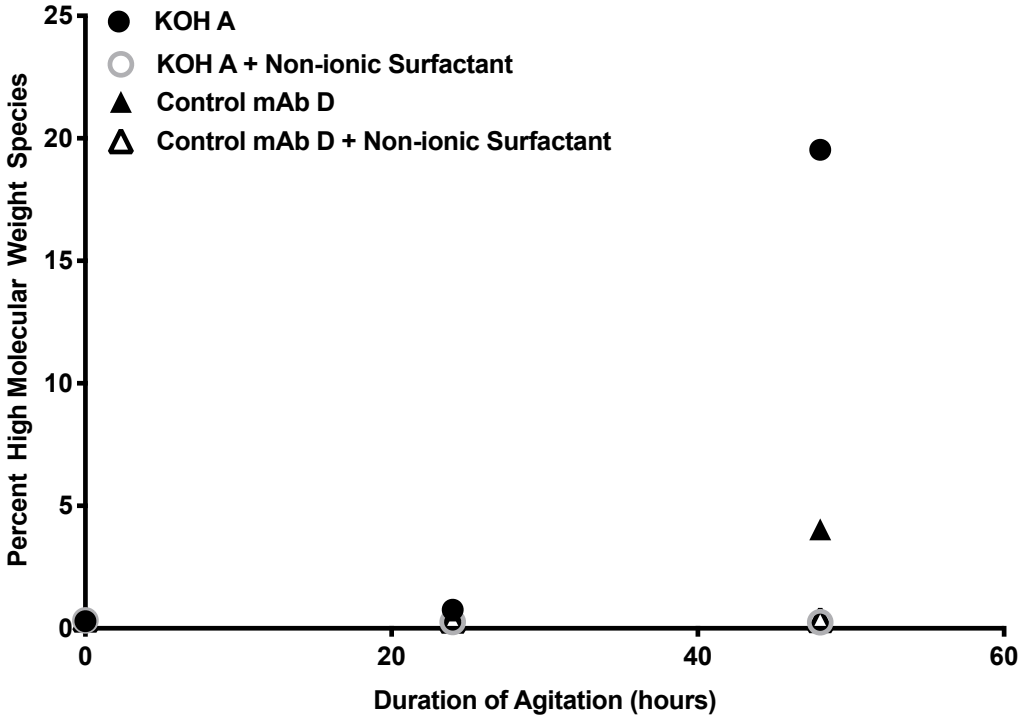


Figure S6. Biophysical properties of three KoH-bodies (KoH A: H4H14357P, KoH B: H4H14371P, KoH C: H4H14401P) compared to a normal VI antibody (mAb D: REGN1193) (A) Differential Scanning Calorimetry thermograms of monoclonal antibody molecules as a plot of heat capacity (Cp) and temperature (° C). A single transition was observed with a weak shoulder indicating a possible second transition. Data was fitted to MN2- State model with two transitions to calculate the midpoint of two melting transitions defined as Tm1 and Tm2. (B) Determination of isoelectric point (pI) and charge variant profile of mAb molecules by imaged capillary isoelectric focusing (iCIEF). The brackets define the observed charge variant species and the range of pI values. The arrow indicates the species (defined as the main peak) that was used to determine the pI of the mAb molecule. (C) Solubility of KoH-bodies. Centrifugal based concentration was performed to assess the highest concentration achievable without any visible appearance of precipitate. Protein concentration was determined using a UV spectrophotometer. (D) Viscosity of KoH-bodies and the effect of viscosity reducer. Viscosity was measured at protein concentration of 175 mg/mL with or without the viscosity reducing agent. Samples were measured using m-VROC viscometer maintaining the sample temperature at 20° C. (E) Agitation Studies of KoH-bodies. High molecular weight species were determined by SE-UPLC analysis of KoH bodies after agitation for upto 48 hours at room temperature.

Fig S7

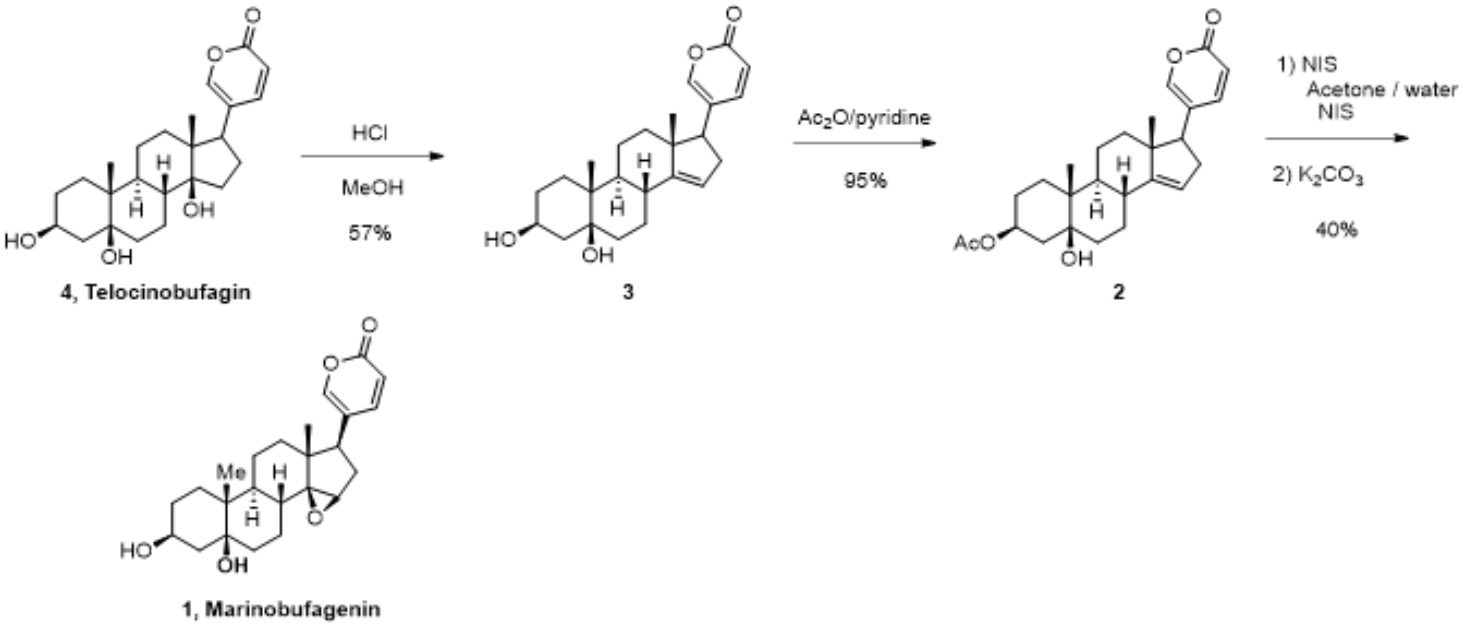


Fig S7 Synthesis of Marinobufagenin from Telocinobufagin

Marinobufagenin was synthesized by a modification of the method described by Pettit and Kamano [43].

Fig S8

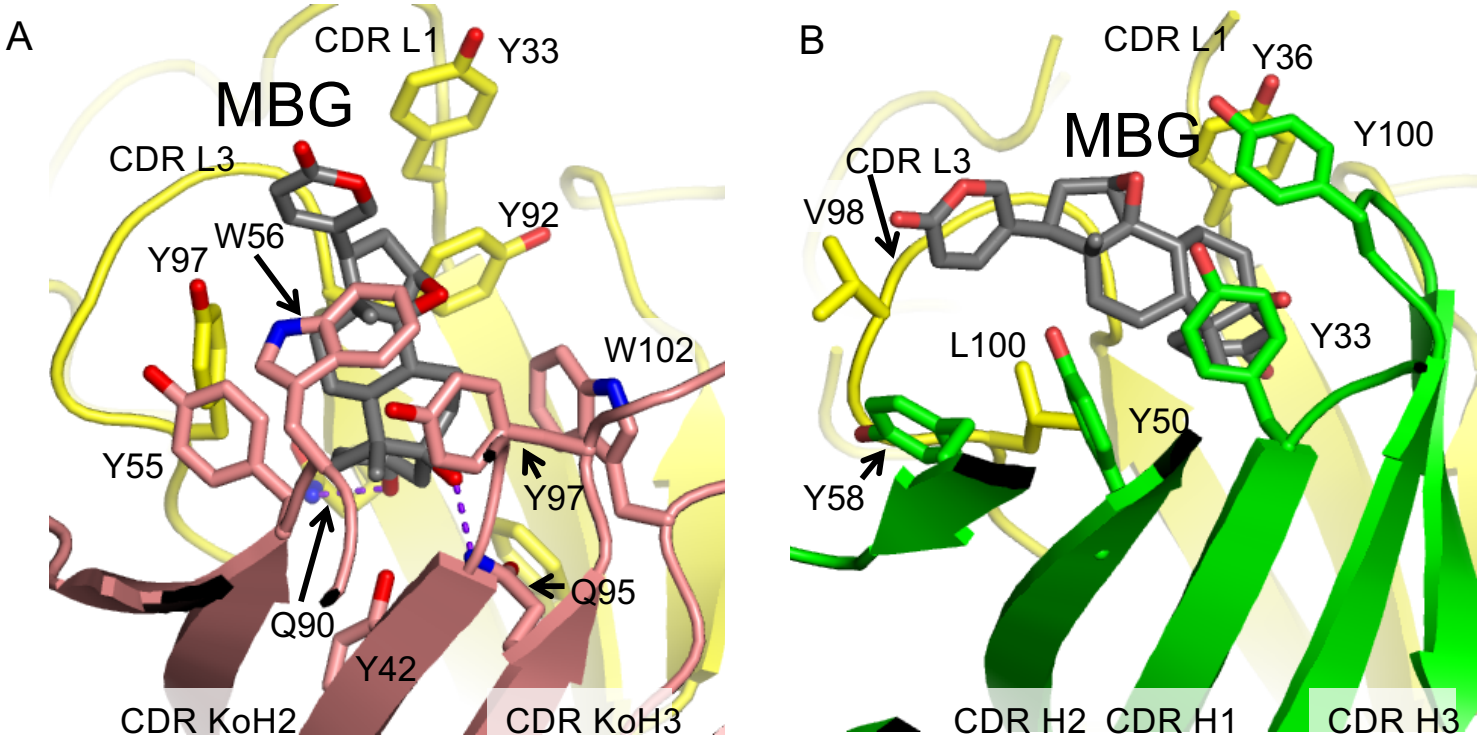


Figure S8. Comparison of crystal structures of MBG bound to the Fab fragment of KoH-body H4H14401P (A) or to the conventional Fab 3E9 (B). Bound MBG and selected amino acid side chains from the Fabs are shown in stick form, colored as in Fig. 7, along with a cartoon representation of the C backbone of the Fab. Two hydrogen bonds between MBG and KoH-body Fab are shown as purple dashed lines in panel A.

Fig S9

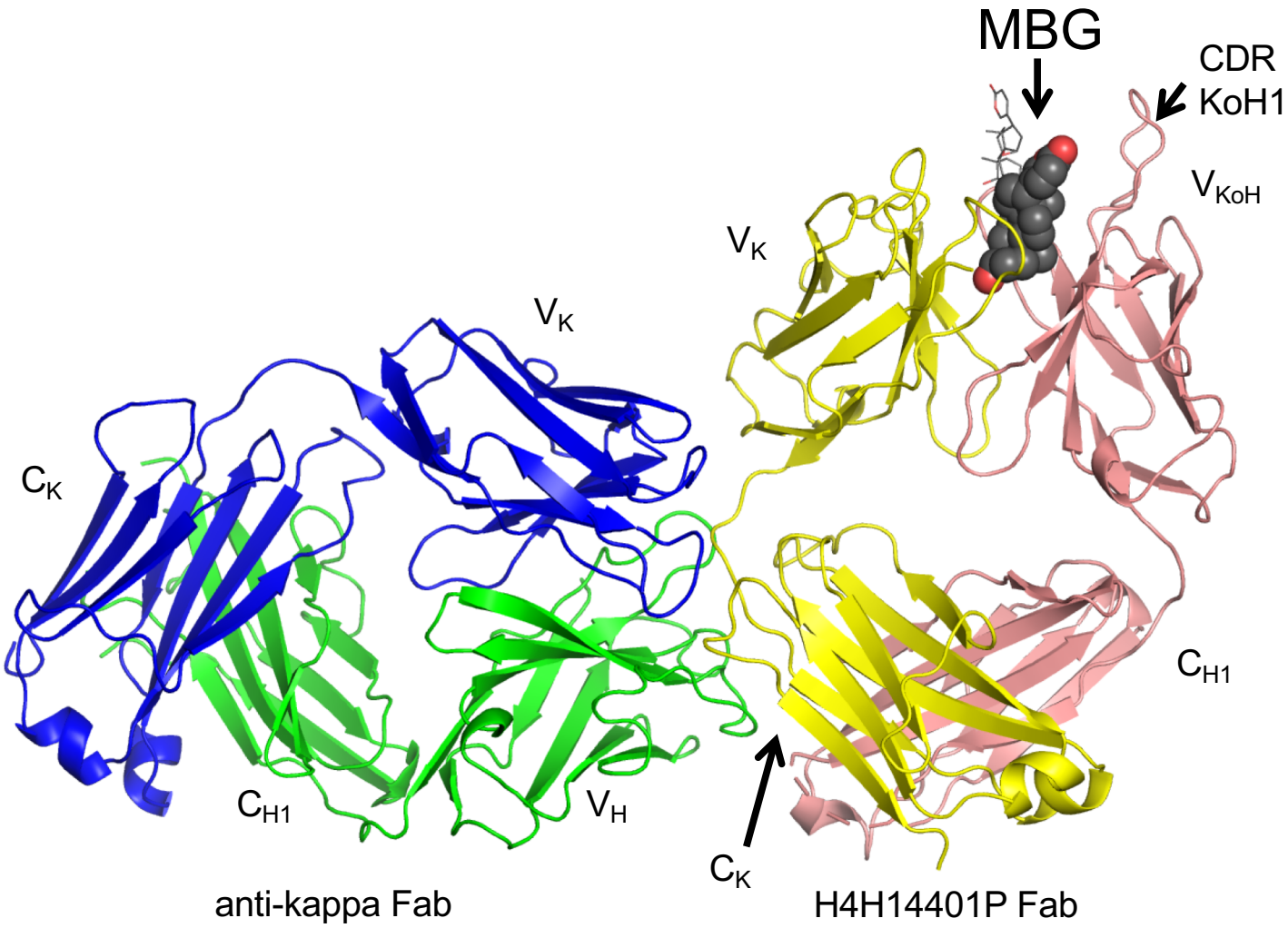


Figure S9. Complete depiction of the KoH Fab crystal structure. The complex of MBG and KoH-body Fab is shown exactly as in Fig. 7A, but the bound anti-kappa Fab is not omitted. The heavy chain of the anti-kappa Fab is colored green, and the light chain is colored blue. A second MBG molecule is shown in thin lines; this molecule bridges a crystal contact with a symmetry-related copy of the KoH Fab C_K domain.

Fig S10

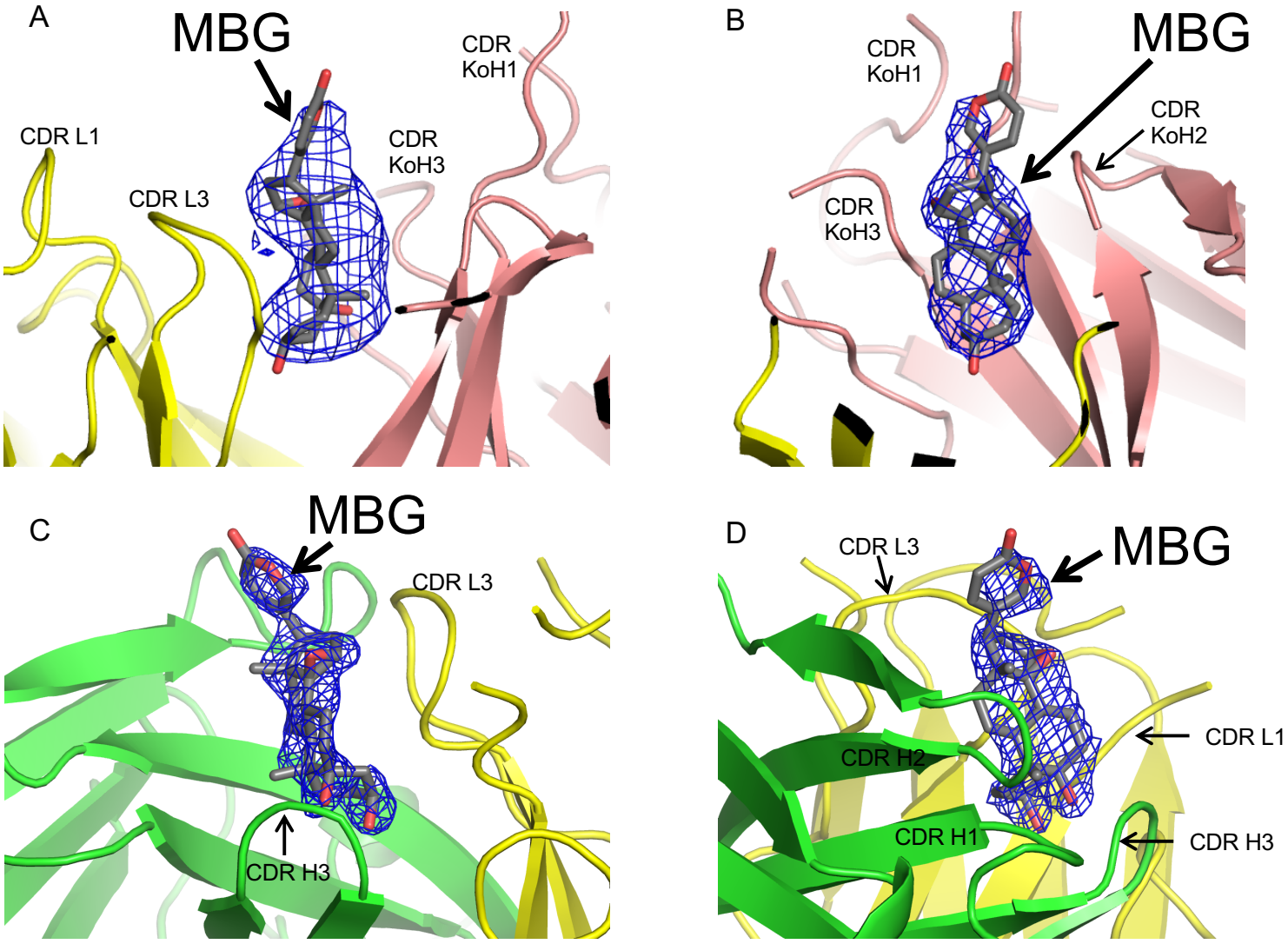


Figure S10. Electron density defining the position and orientation of the MBG molecule. (A) and (B): An m(Fo) - D(Fc) difference electron density map is depicted in blue mesh, superimposed on the final KoH Fab + MBG crystal structure, with the KoH Fab depicted as in Figs. 7B and S6. This map was calculated at the last round of refinement before MBG was added to the structure, and is contoured at +2.5 sigma, and drawn in the area around the final, refined MBG molecule, shown in stick form. Panels A and B show two different orientations of the same map and model. (C) and (D): The same depiction as in panels A and B, but for the 3E9 Fab + MBG structure. The electron density contour is +2.0 sigma.

Table S8

Supplementary Table S8. Data Collection and Refinement Statistics

Complex	**KoH Fab + MBG**		**3E9 Fab + MBG**	
Data Collection Statistics				
Space group	I422		P1	
Unit cell (Å / °)	128.48 128.48 333.33 / 90.0 90.0 90.0		49.92 67.69 71.99 / 97.37 106.35 107.00	
Data collection location	Beamline 5.0.2, Advanced Light Source, LBNL		Beamline 5.0.2, Advanced Light Source, LBNL	
Resolution (Å)	100 - 3.6	3.66 - 3.60	80 - 2.0	2.03 - 2.00
Unique reflections	15341	766	51985	1404
R_{merge} / R_{meas} / R_{pim}	0.298 / 0.320 / 0.111	1.118 / 1.215 / 0.452	0.031 / 0.043 /0.031	0.324 / 0.458 / 0.324
$CC_{1/2}$	---	0.54	---	0.827
Completeness (%)	91.9	93.4	89.7	47.5
Average redundancy	8.4	7.9	1.9	1.6
$I/\sigma(I)$	5.5	2.0	17.4	1.4
Refinement Statistics				
Resolution (Å)	47 - 3.6		67 - 2.0	
Reflections used (free)	14000 (711)		41801 (2269)	
R factor	0.234		0.229	
R_{free}	0.287		0.276	
RMSD				
bonds (Å)	0.004		0.008	
angles (°)	1.39		1.33	
Ramachandran statistics				
Most favored (%)	92.8		98.1	
Additional allowed (%)	5.3		1.3	
Outliers (%)	1.9		0.6	
Rotameric outliers (%)	2.0		1.6	
Asymmetric unit contents	KoH antibody Fab (chains H and L) bound to MBG, and an anti-kappa light chain antibody Fab (chains A and B) bound to the KoH Fab		Two copies of 3E9 antibody Fab bound to MBG	
Average B factors*	KoH Fab: 77; anti-kappa Fab: 124; MBG: 71		3E9 Fabs: 32 and 32; MBGs: 30 and 36	
PDB code	**6PYC**		**6PYD**	

*TLS parameters and residual isotropic B factors were combined to produce anisotropic displacement parameters for each atom; the ADPs were then converted to an equivalent isotropic B factor for each atom; the isotropic B factors for all atoms were then averaged to produce the numbers listed here.