Title: Optimized immunoglobulin knock-ins using Cas9 reveal peritoneal B cell lineage relationships *in vivo*

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ABSTRACT

Immunoglobulin (Ig) knock-in mice are valuable tools in basic and translational immunological research. Here we present “Speed-Ig,” a rapid Cas9-based method for generating Ig knock-in mouse lines with high on-target integration rates at both heavy and light chain alleles. With standardized target sites and promoter regions, Speed-Ig mice can be used for comparative studies of B cell biology and vaccine optimization *in vivo*. We used Speed-Ig to create panels of mice with Ig pairs derived from B-1a, B-1b, and B-2 cells. Surprisingly, B-1b and B-2 Ig pairs drove both B-1b and B-2 phenotypes, suggesting a previously unknown lineage relationship between these subsets. We then confirmed the B-1:B-2 relationship with transcription factor reporter lines and through adoptive cell transfer experiments. In summary, our Ig knock-in approach facilitated the discovery of previously unappreciated aspect of innate-like B cell biology.

INTRODUCTION

Mammalian humoral immune responses are critical in host protection against pathogen challenge.

B lymphocytes, the chief effector cells of humoral immunity, produce antibodies which facilitate recognition and clearance of pathogens in an antigen-specific manner. Eliciting protective humoral responses toward currently intractable infections, such as human immunodeficiency virus and influenza virus, is a major goal of current vaccine research.^{1–4} In addition to combating infections, antibodies are being used therapeutically to treat a variety of human diseases.⁵ Moreover, B cells and their antibody

exports play prominent roles in 1

autoimmune pathologies, allergic responses, and organismal homeostasis.^{6,7} Understanding the development and differentiation of B cells bearing defined immunoglobulin (Ig) pairs therefore holds tremendous value in basic and translational biological research. However, underlying population diversity in wild-type animals confounds analysis of clonal B cell behavior *in vivo*. The pioneering work of Klaus Rajewsky and colleagues developed targeted Ig integrations in mice such that virtually all B cells solely express the inserted Ig chains.⁸ Monoclonal Ig knock-ins preserve Ig locus-dependent features including affinity maturation and class switch recombination and thus closely approximate natural B cell biology, but are costly and time-consuming to create.⁹ These constraints may limit the scope of knock-in studies, and potentially lead to underrepresentation of heterogeneity in the B cell subsets being analyzed. Modern gene editing techniques incorporating programmable nucleases can greatly accelerate the generation of fixed-Ig mice while reducing costs, expediting the *in vivo* analysis of large Ig knock-in line panels.^{10,11} This is of direct relevance to understudied B cell populations such as those found in the peritoneal and pleural cavities of mice.

Innate-like B cells, in particular the B-1 lineages in mice, are thought to provide rapid, non-specific defense against infection and constitutive protection from autoimmunity^{12–14}. Primarily located in the cavities surrounding the lungs and intestine, B-1 cells phenotypically differ from conventional B cells in several ways. Both CD5⁺ B-1a and CD5⁻ B-1b cells are larger and more granular than B-2 cells and have a distinct set of surface markers¹⁵. Several transcription factors, including *Basic helix loop helix family, member e41* (*Bhlhe41*) and *Zinc finger and BTB domain-containing protein 32* (*Zbtb32*), have been associated with the B-1 phenotype^{16–18}. The majority of B-1 studies have focused on B-1a cells, which are at least in part seeded by precursors in early life and show a strong repertoire bias^{19–22}. The temporal dynamics of B-1a ontogeny evoked two competing but not necessarily exclusive models of B-1 cell differentiation²³. Succinctly, the “lineage” hypothesis predicts independent precursors for B-1 and B-2 cells, while the “induced differentiation” hypothesis predicts Ig-dependent cell fating from otherwise dually potent precursors. Transgenic and knock-in mice bearing “canonical” V_H11/V_K9 or V_H12/V_K4 Ig pairs drive strong B-1a lineage biases^{24,25}. Though clearly an Ig-dependent process, these Ig pairs typically recombine in fetal/neonatal progenitors. Modulation of BCR signaling strength alone can alter subset segregation^{26–28}. Additionally, analysis of cells from a Nur77 reporter line, serving as a proxy for BCR signaling, indicate that B-1a (but

not B-1b) have higher steady state BCR stimulation than B-2 cells²⁹. Recently, a mouse model in which mature B-2 cells are induced to switch expression from a B-2 Ig pair to one used by canonical B-1a cells, differentiation to the B-1a lineage was observed¹⁸. However, studies on canonical B-1a Ig pairs only account for a fraction of the B-1a repertoire and none of the B-1b subset, of which very little is understood besides a lack of surface CD5 expression. Whether non-canonical B-1a or B-1b cell biology support the lineage or induced differentiation hypotheses is an open question.

Here, we present “Speed-Ig,” a rapid and efficient method for generating Ig knock-in mice. Through microinjection of single-cell murine embryos with donor DNA, gRNA, and Cas9 protein followed by a single breeding, paired-chain monoclonal Ig mouse lines were developed in less than three months. Ig knock-in mice displayed robust Ig expression, B cell development, and subset differentiation. Our method serves as a platform for testing the development and differentiation of B cells directed by Ig pairs of interest. Using Speed-Ig, we observed that non-canonical B-1a Ig pairs, much like canonical B-1a receptors, imprint a B-1a phenotype in expressing cells. However, we observed unexpected phenotypic flexibility in B-1b and B-2 Ig knock-in mice. Both B-1b and B-2 cells were observed in either panel of knock-in lines, suggesting that factors outside of BCR signaling may be involved in fate decisions. Strikingly, a large fraction of peritoneal B cells in B-1b and B-2 knock-in lines took on a phenotype intermediate to either subset, potentially representing a transitional stage. Using a *Zbtb32*^{EGFP} reporter line, we observed that B-1/B-2 polarity correlates with *Zbtb32* expression. Furthermore, in a fate-mapping approach using *Bhlhe41*^{Cre}, a third of peritoneal B-2 cells and nearly all B-1/B-2 intermediate cells were labeled. Adoptive transfers revealed that both B-2 and intermediate cells can become bona fide B-1 cells, with little plasticity from B-1a/B-1b to B-2. Thus, we report additional complexity to B-1 cell differentiation not fully explained by existing models and propose that additional layers of B-1 subset seeding exist.

MATERIALS AND METHODS

Mice

C57BL/6J, B6.SJL-*Ptprca*^a*Pepcb*^b/BoyJ, *CD23*^{Cre} (B6.Cg-Tg(Fcer2a-cre)5Mbu/J)³⁰, *hCk* (B6;129-Igkctm1(IGKC)Mnz/J)³¹, *IgHa* (B6.Cg-Gpi1a Thy1a Igha/J), *R26R-EYFP* (B6.129X1-Gt(ROSA)26Sortm1(EYFP)Cos/J)³² and *Rag1*^{-/-} (B6.129S7-Rag1tm1Mom/J) mice were purchased from The Jackson Laboratory. *Rag2-EGFP* (FVB-Tg(Rag2-EGFP)1Mnz/J)³³ were purchased from the Jackson Laboratory and backcrossed to C57BL/6J. Ig knock-ins, the *Zbtb32*^{EGFP} reporter, and the *Bhlhe41*^{Cre} lines were generated for this study. Mice deficient for the IgH J segment loci were generated for this study using Cas9 and the following guide RNA sites: GCTACTGGTACTTCGATGTC and GCCATTCTTACCTGAGGAGA. For experiments, mice were analyzed at 3-8 weeks and compared to littermate controls. Mice were maintained in a specific pathogen-free environment at the University of Chicago. All experimental guidelines were approved by the Institutional Animal Care and Use Committee (IACUC).

Single-cell cloning of rearranged Ig genes

Single B cells were sorted as previously described³⁴⁻³⁶ from the following populations using the indicated markers: Splenic B-2 (B220⁺ CD19⁺ CD21⁺ CD23⁺), peritoneal B-1a (CD19⁺ CD23⁻ CD5⁺), and peritoneal B-1b (CD19⁺ CD23⁻ CD5⁻). Briefly, single cells were sorted into 96-well plates (Biorad # HSP9601) containing 10µl of 1% v/v β-mercaptoethanol TCL buffer (Qiagen # 1031576) using a BD AriaII cell sorter. RNA was purified using RNase-free SPRI beads^{37,38} (GE Healthcare #65152105050250) and followed by cDNA synthesis using Superscript IV cDNA synthesis kit (Invitrogen #18091050) as described³⁶. Rearranged Ig chains were amplified and sequenced using degenerate Ig primers³⁵. In-frame variable regions were screened in IMGT³⁹ then amplified with gene-specific overlap primers for subsequent Gibson assemblies⁴⁰ (Supplementary Table 1).

Guide site selection

Guide sites were identified using Integrated DNA Technologies' (IDT) selection tool (https://www.idtdna.com/site/order/designtool/index/CRISPR_CUSTOM) by inputting ~500bp surrounding

the region of interest. Individual guide sites were selected by a number of criteria, including on-target scores, off-target scores and proximity to the terminal stop codon (for non-Ig knock-ins). In general, on-target scores held primacy in selection, as off-target scores are skewed by non-PAM-led genomic hits and off-target integrations can be easily bred out unless falling near the intended target. Similar criteria were used when evaluating guide site options from CHOPCHOP (<https://chopchop.cbu.uib.no/>) and the Broad sgRNA designer (<https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design>).

Donor DNA construct generation

For Speed-Ig Donor DNA constructs, the pUC19 plasmid was digested with SacI and SbfI. Linearized plasmids were then assembled with overlapping homology arms encompassing both 5' and 3' arms to generate 'intermediate' targeting constructs. Promoter regions were amplified from genomic DNA (**Supplementary Table 1**), and full targeting constructs were obtained by assembling Sall-linearized intermediate constructs, promoter regions, and Ig variable regions. All assemblies were performed with NEB HiFi 2X Master Mix (NEB #E2621L). Assembled constructs were transformed into XL-10 Gold Ultracompetent *E. coli* (Agilent #200314). Colonies were miniprepmed and Sanger sequenced using a set of primers to ensure proper assembly and in-frame V regions (**Supplementary Table 1**). Glycerol stocks of desired clones were midiprepmed using anion-exchange columns (Invitrogen #K210015) following growth in 50ml LB + carbenicillin medium for 10-12 hours. Midiprepmed DNA was then ethanol precipitated, resuspended in nuclease-free water, and passed through 0.1um filters (EMD Millipore #UFC30VV25). DNA concentration was measured by spectrophotometry on a NanoDrop™; preparations falling below 1.8 A₂₆₀/A₂₈₀ were discarded.

Injection mix preparation

Three hours prior to microinjection, tracrRNA (IDT #1072532) and crRNA (IDT) were annealed and complexed with Cas9 protein (IDT #1081058) as described⁴¹. Briefly, 5µl of 1µg/µl crRNA was incubated with 10µl of 1µg/µl tracrRNA at 95°C for five minutes followed by cooling to 25°C with ramp speed of 5°C min⁻¹. In a sterile nuclease-free tube (Eppendorf #022600001), 5µl of annealed crRNA:tracrRNA was then complexed with 5µl of 1µg/µl Cas9 protein at 25°C for 15 minutes in 50µl nuclease-free ultrapure water (Thermo #J71786XCR). Following gRNA:Cas9 complexing, donor DNA was added along with ultrapure

water to give a final volume of 100µl and concentrations of 50ng/µl crRNA:tracrRNA (or sgRNA), 50ng/µl Cas9 protein (or 125ng/µl mRNA), and 15ng/µl targeting construct DNA. The complete injection mix was spun at 20,000 x g for ten minutes, and the top 65µl was transferred to a new sterile tube for transport to Transgenics core facilities. For injections with sgRNA and Cas9 mRNA, respective plasmids were linearized and transcribed in vitro prior to injection as described⁴².

Embryo microinjection and transgenesis

C57BL/6J female mice in diestrus between the age of 9 and 16 weeks of age were selected for superovulation. Depending on age and size of the mice, 5-7 IU of PMSG (Millipore #367222/BioVendor #RP1782721000) was injected (IP) at 2:50-3:05PM, followed by HCG 5-7 IU (Sigma CG5) 47 hrs. later. Mice were paired with C57BL/6J males one hour later. Females were checked the next morning for evidence of a mating plug; plugged mice were harvested for fertilized eggs. Eggs were cultured for 1-3 hrs. in KSOMaa Evolve Bicarb +BSA (Zenith Biotech #ZEKS-050). Glass needles were loaded with the described injection mix and placed on ice until used for injection. Eggs were placed on the injection slide in KSOMaa Evolve HEPES +BSA (Zenith Biotech #ZEHP-050). Fertilized eggs were injected using an Eppendorf FemtoJet apparatus. Injected eggs were returned to the incubator and cultured until embryo transfer into 0.5d.e. pseudopregnant CD-1 females. Implanted females were observed daily and separated 2 days before the pups were due to deliver. Tail samples were taken between 10 and 15 days for genotyping.

Ig knock-in genotyping

Tail clippings from potential founder animals were taken for genotyping. DNA was extracted in 300µl 50mM NaOH for 30' at 95°C. Samples were neutralized with 25µl 1M Tris-HCl pH7.5 and used as template DNA for PCR with targeting-specific primers (**Supplementary Table 1**). Reactions were carried out with OneTaq 2X master mix (NEB #M0488S) according to manufacturer instructions.

Southern blotting

DNA was extracted from spleen segments using DNeasy Blood & Tissue Kit (Qiagen #69504). 15ug of DNA were digested overnight with the indicated restriction enzymes and run on 0.8% agarose gel. Gels

were denatured in 0.5M NaOH 1.5M NaCl with gentle agitation for 15 minutes twice before neutralization in 0.5M Tris-HCl pH 7.5 1.5M NaCl for 15 minutes twice. DNA was transferred from gels to positively charged nylon membranes (Invitrogen #AM10102) overnight via capillary transfer in 20X SSC (Thermo # 15557036). Prior to transfer, nylon membranes were soaked in nanopure water for five minutes followed by 20X SSC for five minutes. Following transfer, nylon membranes were soaked in 2X SSC for five minutes followed by UV cross-linking. Cross-linked membranes were blocked in pre-warmed DIG EasyHyb (Roche # 11603558001) at 43°C for four hours rolling in hybridization tubes. Custom DIG-labeled probes were generated using PCR DIG Probe Synthesis Kit (Roche #11636090910) and blots were hybridized for 16 hours at 48°C with a final concentration of 25ng/ml probe in 10ml DIG EasyHyb buffer rolling in hybridization tubes. Following hybridization, membranes were washed twice with low stringency buffer (0.5X SSC 0.1% SDS) at 25°C for five minutes, then twice with high stringency buffer (2X SSC 0.1% SDS) at 68°C for fifteen minutes. Membranes were equilibrated in wash buffer (Roche #11585762001) at 25°C for five minutes with gentle shaking, then blocked in 100ml block buffer (Roche #11585762001) at 25°C for two hours with gentle shaking. Hybridized probe was detected using AP-conjugated anti-DIG Fab fragments (Roche #11093274910) via incubation at 1:10,000 in 50ml at 25°C for thirty minutes with gentle shaking. Membranes were washed three times in wash buffer at 25°C for fifteen minutes. Membranes were equilibrated (Roche #11093274910) and CDP-Star Substrate (Invitrogen #T2145) was used to detect AP.

Lymphocyte isolation

Spleen and bone marrow were collected and passed through 70 µm cell strainers, centrifuged, and resuspended in flow cytometry buffer. For peritoneal lavage, 5ml PBS 3% FBS was injected with a 30g needle into the peritoneal cavity followed by gentle shaking. Fluid was then collected with a 25g needle, centrifuged, and resuspended in flow cytometry buffer. Small intestinal lamina propria isolation was performed as follows: tissues were excised and fat, Peyer's patches, and intestinal contents removed. Tissues were opened longitudinally, cut into ~1 cm pieces, and placed into a 50 mL tube with 10 mL RPMI 1% FCS 1 mM EDTA. Small intestines were processed as two halves in separate tubes and combined after lamina propria lymphocyte isolation as noted below. Samples were incubated at 37°C with shaking for 15

min. Pieces were collected on a 100 μ m cell strainer (Fisher) and placed in fresh RPMI 1% FCS 1mM EDTA for another 15 min with shaking at 37°C. Pieces were again collected on a 100 μ m cell strainer and flow-through was discarded. Intestinal pieces were then placed into 10 mL RPMI 20% FCS with 0.5 mg/mL Collagenase Type I (Gibco #17100017) and 0.1 mg/mL DNase I (Sigma #DN25) at 37°C with shaking for 30 min. Supernatant was collected by filtering through a fresh 100 μ m cell strainer and intestinal pieces were placed in fresh RPMI 20% FCS with collagenase and DNase for another 30 min with shaking at 37°C. Supernatant was again collected, remaining tissues mashed, and the cell strainer was washed with 30 mL ice-cold RPMI. The two lamina propria lymphocyte fractions were combined after centrifugation and further enriched by centrifugation in 40% Percoll® (Sigma #P1644) to remove epithelial cells and debris, washed in flow cytometry buffer, and resuspended for cell staining.

Flow cytometry

Cells were incubated with anti-CD16/32 (Fc block) prior to staining with the following fluorophore or biotin conjugated monoclonal antibodies purchased from Biolegend, eBioscience, or BD unless otherwise indicated (clone in parentheses): B220 (RA3-6B2), CD4 (GK1.5), CD8 α (53-6.7), CD11b, CD11b, CD19 (6D5), CD21 (7G6), CD23 (B3B4), CD43 (S7), IgD (11-26c.2a), IgM (R6-60.2), IgMa (DS-1), IgMb (AF6-78) Igk (187.1), F4/80 (BM8), NK1.1 (PK136), Ter119 (TER-119), goat anti-human Igk (Southern Biotech), goat anti-mouse IgA (Southern Biotech), goat anti-mouse Ig λ (Southern Biotech). Samples were run on a BD LSRII Cytometer.

Cell Sorting and Adoptive Transfer

Indicated populations of peritoneal B cells from multiple CD45.1-congenic wild type mice were sorted on a FACS Aria II cell sorter (Becton Dickinson). After post-sort purity analysis, 1e5 cells were transferred IP into adult (8-12 week) C57BL/6J mice. Recipient mice were analyzed one week following transfer.

Non-Ig construct cloning and genotyping

Targeting constructs for knock-ins other than Speed-Ig were prepared in largely the same manner. Homology arms exactly flanking the guide site were amplified from genomic DNA of the mouse strain used as embryo donors for microinjection (typically C57BL/6J). A second cloning PCR was performed to provide

Gibson overlaps to homology arms and cargo if necessary. Homology arms and cargo DNA were assembled into generic plasmids (pUC18 or pUC19) via Gibson assembly. Microinjections were carried out identically to Speed-Ig, but with crRNAs specific for the relevant guide site. Genotyping knock-in mice was performed by three separate PCRs on DNA samples from potential founder animals. First, PCRs to detect the cargo were performed to identify all mice which carried non-native DNA (e.g. EGFP). Then, cargo⁺ animals were genotyped for targeting PCR across the 5' and 3' homology arms to detect properly targeted insertions/replacements. Amplicons arising from targeting reactions were Sanger sequenced to confirm correctly targeted alleles.

RESULTS

Ig gene targeting utilizing a Cas9-based approach

To maximize the biological relevance of our Ig knock-in approach, we had the following objectives: (1) to remove the endogenous J segment loci, thereby preventing secondary Ig rearrangements after successful integrations; (2) to use fixed promoter regions which would allow for phenotypic comparisons across mouse lines in a truly Ig-dependent manner; and (3) to minimize targeting construct size and synthesis time such that panels of Ig knock-in mice could be generated quickly and efficiently.

To insert rearranged Ig genes into their corresponding loci, we first selected one guide RNA site each for J_H1 and J_κ1, directly upstream of the gene segment coding regions. We then generated targeting constructs with short (~800bp) “proximal” and long (~2.2kb) “distal” homology arms bookending all endogenous J segments (**Fig.1A**). Using a single Cas9 guide site, only the proximal homology arm directly abutted the double-strand break and served as an anchor for the intended synthesis-dependent strand annealing. The intervening genomic DNA between the guide RNA site and the beginning of the distal homology arm had no complementarity present in the donor DNA and was excised upon construct integration. Therefore, upon successful integration of the targeting construct, the entire J segment clusters were deleted, preventing secondary Ig rearrangements on the targeted allele. The distal homology arms were selectively increased in length as longer distal, but not proximal, homology arms were demonstrated to increase efficiency of

large gene replacements *in vitro*.⁴³ Promoter regions 800-1200 base pairs in size including split leader peptide sequences were cloned from the V_H1 family for heavy chain constructs and the V_κ10 family for kappa constructs based on usage patterns in wild type B cell repertoires.³⁴ Rearranged Ig chains amplified from cDNA of single sorted B cells were assembled seamlessly with promoters into respective targeting constructs using Gibson assembly⁴⁰ (**Supplementary Fig.1**). This modular construct assembly allowed for rapid and scalable Ig cloning combined with flexibility in promoter choice. Fertilized murine oocytes were microinjected at the one-cell stage with targeting constructs and either single-guide RNA (sgRNA) with Cas9 mRNA or pre-complexed gRNA:Cas9 ribonucleoproteins (RNPs) (**Fig.1B**). Potential Ig knock-in founders were screened for intact Ig insertions via ‘targeting’ polymerase chain reaction (PCR) which was accommodated by homology arms much shorter than those used in traditional knock-in transgenesis (**Fig.1C**). Ig inserts were further verified by southern blotting (**Fig.1D**). Rarely, some founder animals possessed off-target integrations (**Supplementary Fig.2**). After several rounds of optimization, successful Ig integrations/J cluster deletions ultimately reached 20.7% of live-born mice for heavy chains and 23.3% for kappa chains (**Table 1**). Successful knock-ins were only recovered from the injection of targeting constructs with longer 3’ homology arms (**Table 1**). Notably, the use of gRNA:Cas9 RNPs was superior to sgRNA/Cas9 mRNA, similar to recent reports.^{41,44}

Speed Ig expression, allelic exclusion, and BCR-driven phenotypes

We performed a series of experiments to evaluate the utility of Speed-Ig mice in studying B cell biology, a hallmark of prior Ig knock-in approaches. Heavy chain and light chain expression were measured independently by flow cytometry. Progeny from H knock-in mice (on the *IgH^b* C57BL/6J background) bred to *IgH^a* allotype mice demonstrated knock-in chain expression at the protein level (**Fig.2A**, left panels). As expected, the knocked-in chain, due to its rearranged state and ensuing expression kinetics, prevented endogenous rearrangements and therefore excluded *IgH^a* allele expression⁴⁵. On an *IgH^A* background, knocked-in heavy chains were capable of driving normal B cell development (**Supplementary Fig.3A**). Furthermore, the surface expression of IgM and IgD were comparable between wild type B cells and B cells from *IgH^{KI/Δ}* mice (**Supplementary Fig.3A**). The *IgH^A* background also provided an opportunity to assess class-switch recombination in Speed Ig mice. As expected, B cells forced to use knocked-in H chains were

able to switch to the IgA isotype in mucosal tissue, a firm indication that our Speed-Ig lines retain this important capability (**Supplementary Fig.3B**). These data confirm the functionality of the targeting approach, promoter regions, and Ig integrations for H knock-ins. Similar experiments were performed using κ knock-in mice. κ knock-ins were bred to mice possessing the human kappa constant region to allow for allele-specific flow cytometry staining.³¹ κ knock-in mice with V κ 10-96 promoters had normal mIgk surface expression and outcompeted the human kappa allele (**Fig.2A**, right panels). This contrasted with κ knock-in mice generated with V κ 4-53 promoter regions, which had poor kappa expression (**Supplementary Fig.4**). Although the V κ 4-53 promoter region was selected based on published work⁴⁶, we did not observe adequate Igk expression in our lines, prompting our use of a larger promoter region from a different Igk family. We then examined full (H + κ) Ig knock-in mice (henceforth with V κ 10-96 promoters) for B cell developmental progression. It has been previously noted that, in Ig knock-in mice, developing B cells transit the pre-B stage faster than wild-type cells due to pre-rearranged Ig expression.^{9,46–48} Our knock-in lines demonstrated a reduction in pre-B frequency, consistent with prior knock-in approaches and further validation of knocked-in Ig expression (**Fig.2B**). Taken together, these results suggest Speed-Ig mice display physiologic Ig chain expression levels and that knocked-in Igs predominate over endogenous chains. In summary, our results demonstrated that the Speed-Ig system is a robust tool for *in vivo* studies of monoclonal B cell biology.

Peritoneal B cell relationships in Ig knock-in mice

Having validated our knock-in system, we sought to understand the ontogeny of the less well-studied innate-like B cell populations: non-canonical B-1a and B-1b cells. To do so, we created mouse lines with Ig pairs from randomly selected B-1b clones or non-expanded B-1a clones (**Table 2**)³⁴. Additionally, we created a canonical B-1a V_H11-V κ 9 Ig line “321C6” to serve as a control for BCR-driven cell fating^{25,49}. Both canonical and non-canonical B-1a Ig knock-in mice harbored populations of B cells strongly biased towards the B-1a fate (**Fig.3A-B**). B-1b Ig knock-in mice, on the other hand, gave rise to both B-1 and B-2 cell subsets in the peritoneal cavity, albeit with a reduction in B-1a (**Fig.3A-B**). The same result was observed in B-2 Ig knock-in lines, such that B-1b and B-2 Ig knock-in lines were phenotypically indistinguishable. Given the possibility that some endogenous early-life B-1a precursors escaped allelic exclusion, we bred one B-1a line, “416C1, and B-1b line, “301G2”, to *Rag1*-deficient backgrounds. In this setting, virtually all

peritoneal B cells in 416C1/*Rag1*^{-/-} mice were of the B-1a phenotype as expected (**Fig.3C**). Conversely, 301G2/*Rag1*^{-/-} mice had minimal B-1a cells, confirming that the knocked-in Ig pair was incapable of giving rise to the B-1a subset (**Fig.3C**). However, there was no loss of peritoneal cavity B-2 cells, suggesting that some Ig pairs can seed both B-1b and B-2 subsets. Interestingly, ~15% of the B cells present in B-1b and B-2 Ig knock-in lines did not fully conform to either the B-1 or B-2 lineages, appearing as an “intermediate” (Int) cells. As Int cells persisted in the *Rag1*-deficient 301G2 line, we hypothesized that shared origins between the B-1b and B-2 subsets might exist in wild type animals. However, due to the lack of markers used to identify B-1b cells, it was possible that immature or transitional B cells were present in our samples. To rule out the presence of young CD23^{variable} B cells which might appear in the Int gate, we utilized the *Rag2-EGFP* reporter line. In these animals, ~80% of splenic transitional stage 1 (T1) and stage 2 (T2) cells still had residual GFP signal, indicative of recent development (**Supplementary Fig.5A**). Splenic follicular B cells (FO B) and marginal zone B (MZ B) cells were largely GFP⁻, in concordance with their temporal separation from lymphopoiesis (**Supplementary Fig.5A**). Virtually all cells in the peritoneal cavity were GFP⁻, suggesting that the large fraction of poorly defined Int B cells present are not recent bone marrow emigrants (**Supplementary Fig.5A**). As another determination of lineage relationships, we pursued a fate-mapping experiment by crossing *CD23-Cre* with *R26R-EYFP* mice. CD23 expression initiates at the T2 stage of development and is ubiquitously expressed by B-2 cells. In the peritoneal cavity, approximately 98% of B-2 phenotype cells were fate-mapped, demonstrating efficient and robust Cre activity driven by the CD23 promoter (**Supplementary Fig. 5B**). Nearly 90% of Int cells were also YFP⁺, in line with the variable but apparent surface CD23 expression. Notably, ~70% of B-1b cells were also fate-mapped, suggesting prominent seeding of the B-1b compartment by cells that had expressed CD23 during a precursor stage (**Supplementary Fig.5B**). B-1a cells displayed the lowest fate-mapping by CD23, with approximately 30% YFP⁺ (**Supplementary Fig.5B**). Taken together, these data suggest that some fraction of each B-1 lineage has origins in mature, CD23⁺ precursors. Furthermore, the ability of a single Ig pair to drive B-1b, B-2, and intermediate cell phenotypes establishes the possibility of shared B cell lineages in the peritoneal cavity.

***Zbtb32* and *Bhlhe41* identify B-1-like precursors in the peritoneal cavity**

To further analyze the ontogeny of B-1 cells, we created a *Zbtb32* transcription factor reporter line (**Supplementary Fig.6A**). *Zbtb32* is highly upregulated in B-1 cells, but has minimal expression in follicular B cells^{17,18}. As expected, virtually all peritoneal B-1 cells were EGFP⁺ (**Fig.4A-B**). Interestingly, Int cells were largely positive for EGFP expression and a small fraction of B-2 also had expression (**Fig.4A-B**). The mean fluorescence intensity of GFP across the three populations suggests a gradual phenotypic transition from B-2 to B-1 (**Fig.4B**, right panel). We then compared B-2 cells grouped by the presence or absence of EGFP expression. Across a variety of markers and cell size and granularity, EGFP⁺ B-2 cells were skewed toward a B-1 phenotype compared to GFP⁻ B-2 cells (**Fig.4C**). *Zbtb32* expression therefore directly correlates with B-1 phenotype polarization, and putative B-1 precursors present within the B-2 subset can be identified by *Zbtb32* expression.

We further probed peritoneal B cell lineage relationships by generating a *Bhlhe41*^{Cre} line (**Supplementary Fig.6B**). *Bhlhe41* is a master factor in B-1 differentiation and like *Zbtb32* is highly expressed in B-1 cells with negligible expression in other B cell subsets^{16–18}. Like *CD23*^{Cre}, we crossed the *Bhlhe41*^{Cre} line to *R26R-EYFP* mice to delineate the ontogeny of B-1 and Int cells. We observed that all B-1 were fate-mapped by *Bhlhe41*^{Cre}, as were ~90% of Int cells (**Fig.4D-E**). Consistent with the *Zbtb32* reporter data, a fraction of peritoneal B-2 cells were fate-mapped by *Bhlhe41*Cre, providing a second line of evidence for transcriptional heterogeneity of the peritoneal B-2 compartment (**Fig.4D-E**). Gating fate-mapped peritoneal B-2 cells from EYFP⁻ B-2 cells confirmed that fate-mapped B-2 appear more B-1-like across a variety of markers in addition to cell size and granularity (**Fig.4F**). These data were consistent with observations in the *Zbtb32* reporter line. Taken together with results from Speed-Ig lines, we hypothesized some peritoneal B-2 cells were triggered to differentiate to a B-1 phenotype, and that Int cells represent ex-B-2 cells en route to the B-1 cell fate.

Peritoneal Cavity B-2 and Int cells seed the B-1 compartment

To test the ability of peritoneal B-2 and Int cells to seed the B-1 compartment, we performed an adoptive transfer experiment using congenically marked but otherwise wild type B cells (**Fig.5A**). Cells were FACS-purified and assessed for purity prior to intraperitoneal injection into C57BL/6J hosts (**Fig.5A-B**). After one week, cells were collected from recipient animals by peritoneal lavage and for stained for B cell makers.

Both B-1a and B-1b subsets largely retained their original phenotypes following transfer, consistent with a previous report⁵⁰ (**Fig.5B-C**). Transferred Int cells closely resembled the phenotype of transferred B-1 cells, with most cells now falling into a CD23^{lo/-} gate (**Fig.5B-C**). A large fraction of transferred B-2 cells also fell into the B-1 gate at a frequency in line with the *Zbtb32*^{EGFP} and *Bhlhe41*^{Cre} results (**Fig.5B-C**). The presence of host B cells allowed us to directly compare the marker profiles of donor cells. Not only had Int and B-2 cells now appearing in the B-1 gate lost surface expression of CD23, they had gained a marker profile consistent with the B-1 phenotype (**Fig.5D**). Regardless of original cell phenotype, the donor cells falling into a B-1 gate were indistinguishable from bona fide host B-1 cells, allowing us to conclude that some B-2 and most Int cells can differentiate to the B-1 fate. In summary, the Speed-Ig knock-in lines, transcription factor genetic approaches, and adoptive transfers all suggest an unappreciated shared origin of peritoneal B cell subsets, with previous instructive and induced-differentiation models only accounting for B-1a cells.

DISCUSSION

Here, we reported an efficient and robust Cas9-mediated method to generate Ig knock-in mice. Our method retains all the biologically useful features of targeted Ig mouse lines while increasing throughput and reducing generation time. Knocked-in Igs expressed at levels similar to wild-type mice, strongly excluded endogenous rearrangements, and recapitulated physiologically relevant BCR signaling. Using identical promoter regions across Speed-Ig lines provides several advantages over using V region-specific promoters. The promoters themselves are now well validated to provide robust expression. Phenotypic comparisons between Speed-Ig lines can be directly attributed to different V regions, as only these will differ among lines.

Our approach complements the elegant method recently reported by Victora and colleagues¹¹. Their strategy effectively halves the number of mouse lines required to study any given Ig pair by virtue of integrating both Ig chains into the heavy chain locus. Such mouse lines are a valuable tool to study mature B cell responses to cognate antigens. Our method, while still requiring separate integration events at the *IgH* and *Igk* loci, represents a closer approximation of endogenous Ig expression kinetics and amplitude. Depending on the biological question, each strategy has clear advantages and disadvantages. Our heavy chain knock-in approach is similar to that of Batista and colleagues; Lin et al. demonstrated impressive

integration efficiencies at the *IgH* locus¹⁰. The main difference between our approaches is the length of targeting construct homology arms. Increasing the homology arms to lengths commonly used for embryonic stem cell constructs might afford the greatest efficiency. Our chief comparative advantage is the ability to genotype via targeting PCR across both homology arms to ensure correct integrations, in addition to our description of *Igk* knock-ins. Nevertheless, there is clearly a relationship between cargo size and homology arm lengths that affects integration efficiency. The Speed-Ig strategy stands to be improved by further studies into optimal homology arm length, especially for the longer distal arm. Guide site selection was originally conducted by analyzing loci with CHOPCHOP and the Broad Institute's sgRNA Designer^{51–53}. These algorithms are based off screening results of sgRNA libraries in cell lines. Later guide site selection was through the IDT design tool for crRNA:tracrRNA bipartite gRNA which may behave differently from sgRNA *in vivo*⁴⁴. While all targeted integrations were achieved regardless of predicted score, our highest efficiencies came from guides with on-target scores above 60 per IDT. However, guide site quality is only one factor in determining knock-in rates, as the length of inserted cargo can also have an effect⁵⁴. Our results are informative on the insertion of cargo up to at least two kilobases. This range includes most if not all reporter genes and a variety of genetic tools, including Cre recombinase and luciferases. In conclusion, our method may serve as a platform for universally successful gene editing in the mouse.

Our novel insights into B-1 plasticity principally arose from observations in Speed-Ig mice. The ability of a single Ig pair to drive cells into the B-2 and B-1b subsets. Recent evidence for lineage plasticity *in vivo* using an inducible Ig expression system demonstrated the ability of B-2 cells to acquire a B-1 phenotype¹⁸. While elegant, this approach was still limited to the biology of canonical B-1a signaling and differentiation. In a related study, BCR signaling blockade prevented B-1a Ig transgenic cells from assuming a full B-1a phenotype²⁸. The apparent heterogeneity of peritoneal B cell subsets limits extrapolation of these findings. We expanded on existing data by showing that a monoclonal knocked-in Ig pair can give rise to diverse B cell subsets, and that a phenotypic spectrum is apparent in wild type mice. The polarity of cells toward the B-1 phenotype was directly correlated with the expression of hallmark B-1 transcription factors, regardless of CD23 surface expression. Furthermore, we observed rapid differentiation of peritoneal B-2 and Int cells into B-1 cells following adoptive transfer. Similar results were observed in an adoptive transfer experiment using peritoneal B-2 cells, but not splenic B-2 cells⁵⁵. These data suggest that BCR-independent signaling

events occurring in the peritoneum might drive acquisition of the B-1 phenotype in a broad range of B cells. This argument is supported by the fact that peritoneal B-2 cells have no repertoire bias²¹. The continual presence of Int cells in wild type mice opens the possibility that at steady-state, there is perpetual seeding of the B-1 compartment by non-B-1 precursors. Moreover, in combination with data from B-1b and B-2 Ig knock-in mice, there appears to be some factor(s) limiting the size of the B-1 niche. Both BCR-dependent and -independent mechanisms are possible, and further studies are needed to identify the rules governing innate-like B cell fating.

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Contributions

S.E. conceived the study, designed and performed experiments, analyzed data, and wrote the paper. E.Z. performed experiments, L.D helped generate the knock-in models, A.B. conceived the study and wrote the paper.

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Figure 1. Targeted integration of rearranged V regions at the J_H/J_κ loci. **A)** Targeting approach to replace endogenous J segments with rearranged VDJ_H/VJ_κ. Dashed lines indicate areas of complementarity for homology arms. **B)** Schematic diagram of pronuclear injection **C)** Genotyping of H and κ knock-in founders using paired targeting PCRs. Corresponding primers are depicted in panel A. **D)** Southern blotting of H and κ knock-in mice. Genomic DNA was digested with Apol (for H) or Accl (for κ) and probed within the 5' homology arm. A: Apol/Accl sites.

Figure 2. Speed-Ig gene expression and B cell development. **A)** Knocked-in H and κ chains dominate over endogenous alleles. [Left panels] Flow cytometry of splenic follicular B cells (CD19⁺ CD21⁺ CD23⁺) from *IgH^{b/a}* and *IgH^{KI/a}* littermates stained with allotype-specific anti-IgM antibodies. *IgH* locus genotype indicated above plots. [Right panels] Flow cytometry of splenic follicular B cells from *Igk^{m/h}* and *Igk^{KI/h}* littermates stained with human and mouse-specific anti-IgK antibodies. *Igk* locus genotype indicated above plots. **B)** Bone marrow development of Speed-Ig B cells expressing H, k, or both Ig knock-in chains. All panels are representative of n ≥ 3 mice.

Figure 3. B-1b and B-2 Ig knock-ins display unpredicted phenotypic flexibility. **A)** [Left] Representative plots of peritoneal B cells in wild type, non-canonical B-1a, and B-1b Ig knock-in mice. [Right] Frequencies of B-1 cells of all peritoneal B cells in Ig knock-in lines. Significance determined by one-way ANOVA with post-hoc Tukey test. **B)** [Left] Breakdown of B-1a and B-1b cells present in the B-1 gate of representative Ig knock-in lines as in A. [Right] Frequencies of B-1a and B-1b cells in all Ig knock-in lines. Significance determined by one-way ANOVA with post-hoc Tukey test. **C)** Comparison of a non-canonical B-1a line and a B-1b line on Rag1-deficient backgrounds. **D)** frequency of B-1 among all B cells and B-1a among B-1 cells in Rag1-deficient Ig knock-in lines. Significance determined by Student's T-test. n ≥ 3 biological replicates for all panels.

Figure 4. A *Zbtb32* reporter and *Bhlhe41* Cre-driver reveal B-1-like phenotypic changes in peritoneal B cells. **A)** *Zbtb32* expression reported by EGFP in peritoneal B cells. **B)** EGFP⁺ frequency and mean fluorescence intensity in peritoneal B cell subsets. Significance determined by one-way ANOVA with post-hoc Tukey test. **C)** Histograms of scatter values and fluorescence intensities for indicated cell subsets. Mean fluorescence intensities given as 1x10³. **D)** Fate-mapping of peritoneal B cells in *Bhlhe41^{Cre} x R26R-EYFP* mice. **E)** EYFP⁺ frequency in peritoneal B cell subsets. Significance determined by one-way ANOVA with post-hoc Tukey test. **F)** Histograms of scatter values and fluorescence intensities for indicated cell subsets. Mean fluorescence intensities given as 1x10³. n ≥ 4 biological replicates for all panels.

Figure 5. Adoptively transferred peritoneal B-2 and Intermediate cells give rise to B-1 cells. **A)** adoptive transfer scheme. **B)** Post-sort and d7 analysis of donor cells. Significance determined by Student's T-test. **C)** Breakdown of B-1a and B-1b subsets within the B-1 gate of donor cells. Significance determined by

one-way ANOVA with post-hoc Tukey test. **D)** Histograms of scatter and mean fluorescence intensities for donor cells present in the B-1 gate compared to host B-1 and B-2 cells. $n \geq 3$ biological replicates for all panels.

Supplementary Figure 1. Rapid *IgH* targeting construct generation. A generic plasmid backbone was digested with *SacI* and *SbfI*, allowing assembly of homology arms into an intermediate targeting construct. Intermediate constructs were digested with *Sall*, followed by final assembly with an Ig-specific promoter region and Ig variable regions of interest. Regions of assembly overlaps at the ends of inserts are color-matched to construct sequence. A similar assembly pipeline was done for *Igk* targeting constructs. Not depicted to scale.

Supplementary Figure 2. Off-target analysis of H and κ knock-in founder animals. Genomic DNA was digested with *ApoI* (for H) or *AccI* (for κ) and probed within the 5' homology arm. Wild type, on-target, and off-target bands are indicated by arrows. Panels include representatives from four different knock-in lines.

Supplementary Figure 3. H knock-ins are sufficient in the absence of endogenous alleles and can class switch. **A)** [Left panels] Flow cytometry of developing B cells in the bone marrow and [right panels] mature B cells in the spleen of an H knock-in mouse on an IgH-deficient background. **B)** Class switching to IgA in the gut lamina propria of an *IgH^{KI/Δ}* mouse. All panels are representative of $n \geq 3$ mice.

Supplementary Figure 4. A $V_{\kappa}4$ -53 promoter fragment is insufficient to drive Igk knock-in expression. [Left panels] B cell development in the bone marrow gated on CD19⁺ cells past the Pro-B (CD43⁺) stage. [Right Panels] surface expression of CD21 and CD23 on splenic CD19⁺ B cells. All panels are representative of $n \geq 3$ mice from 5 separate lines using the $V_{\kappa}4$ -53 promoter

Supplementary Figure 5. CD23⁺ precursors contribute to B-1 subsets with minimal contribution from recent bone marrow emigrants. **A)** [Top panels] gating strategy for splenic T1, T2, FO B and MZ B subsets in *Rag2-EGFP* mice. [Bottom panels] frequency of GFP⁺ (recent bone marrow emigrants) in splenic and peritoneal B cell subsets. Significance determined by one-way ANOVA with post-hoc Tukey test. **B)** Peritoneal B cells from *CD23-Cre x R26R-EYFP* mice. Significance determined by one-way ANOVA with post-hoc Tukey test. $n \geq 3$ biological replicates for all panels.

Supplementary Figure 6. Generation of *Zbtb32^{EGFP}* and *Bhlhe41^{Cre}* lines. **A)** [Left] Targeting strategy for Cas9-mediated insertion of IRES-led EGFP into the 3' UTR of the *Zbtb32* locus. [Right] Genotyping PCR bands of a founder animal. **B)** [Left] Targeting strategy for Cas9-mediated insertion of IRES-led Cre into the 3' UTR of the *Bhlhe41* locus. [Right] Genotyping PCR amplicons of a founder animal.

Table 1. Speed-Ig knock-in efficiencies.

Table 2. Ig pairs used for knock-in lines.

Supplementary Table 1. Sequences of primers and crRNAs.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Supplementary Figure 1

Supplementary Figure 2

Supplementary Figure 3

Supplementary Figure 4

Supplementary Figure 5

Supplementary Figure 6

Table 1

Heavy Chain							
Version	Protospacer	CRISPR/Cas9 Modality	Homology Arms (5':3')[bp]	Promoter family/size [bp]	Total Pups	Positive	%
1	GCTACTGGTACTTCGATGTC	sgRNA + Cas9 mRNA	800:800	IgHV1-18, 874	33	0	0
2	GCTACTGGTACTTCGATGTC	sgRNA + Cas9 mRNA	800:2200	IgHV1-18, 874	222	6	2.7
3	GCTACTGGTACTTCGATGTC	crRNA:tracrRNA + Cas9 Protein	800:2200	IgHV1-18, 874	161	10	6.2112
4	AGCCAGCTTACCTGAGGAGA	crRNA:tracrRNA + Cas9 Protein	800:2200	IgHV1-18, 874	163	9	5.5215
5	ACCACGGTCACCGTCTCCTC	crRNA:tracrRNA + Cas9 Protein	800:2200	IgHV1-18, 874	198	41	20.707
Kappa Chain							
Version	Protospacer	CRISPR/Cas9 Modality	Homology Arms (5':3')[bp]	Promoter family/size [bp]	Total Pups	Positive	%
1	GTGGAGTACTACCACTGTGG	sgRNA + Cas9 mRNA	800:800	IgKV4-53, 729	15	0	0
2	GTGGAGTACTACCACTGTGG	sgRNA + Cas9 mRNA	800:2200	IgKV4-53, 729	214	9	4.2056
3	GTGGAGTACTACCACTGTGG	crRNA:tracrRNA + Cas9 Protein	800:2200	IgKV4-53, 729	129	30	23.256
4	GTGGAGTACTACCACTGTGG	crRNA:tracrRNA + Cas9 Protein	800:2200	IgKV10-96, 1179	479	70	14.614

Table 2

Ig Knock-in Line Receptor Sources							
Ig Name	Source Cell Type	VH	DH	JH	CDR3H Junction AAs	VK	CDR3K Junction AAs
321C6	B-1a	11-2	2	1	CMRYSNYWYFDVW	14-126	CLQHGESPFTH
320F7	B-1a	5-15	4	2	CARHAPNWWYFDYW	17-127	CLQSDNMPLTF
416C1	B-1a	3-8	1	4	CARCPLYYYGSSPYAMDYW	17-127	CLQSDNMPYTF
301G2	B-1b	2-6	2	3	CASTNYDYDRFAYW	8-24	CQQHYSTPYTF
319F1	B-1b	5-17	2	4	CAYGNYYAMDYW	6-23	CQQYSSYPYTF
307C9	B-2	14-4	1	3	CTTDYGSRGFAYW	4-62	CQQYSGYPLTF
335A3	B-2	6-3	2	2	CTGYDYDFDYW	4-55	CQQWSSYPLTF

Supplementary Table 1

Name

IgH 5' homology arm-F
IgH 5' homology arm-R
IgH 3' homology arm-F
IgH 3' homology arm-R
IgH promoter-F
IgH promoter-R
IgK 5' homology arm-F
IgK 5' homology arm-R
IgK 3' homology arm-F
IgK 3' homology arm-R
IgK promoter-F
IgK promoter-R

Cloning Primers

Sequence

CCAGTGAATTCGAGCTGGTTTAAGCCTCAGAGTCCCTGT
GGAGACTTGTGCGACCGAGACGGTGACCGTGGT
CGGTGCGACAAGTCTCCTCAGAAGAATGGCC
GCTTGCATGCCTGCACCAGAATAGATTCAAAATTCAAACC
CGGTACCGTCTCGGCAACAATAAGCAAGGAT
AGAGAGGACACCTGTGAAGAGAAAGACAG
CCAGTGAATTCGAGCTGGTATCATGAATGTGGTTGATCTTGGTG
GTCAACTGGTCGACTGACCACAGTGGTAGT
CAGTCGACCAGTTGACGTGCGCATACAGTGT
GCTTGCATGCCTGCACTGAACACAAGCACATAAGGTAAG
TACTACCACTGTGGTCAGAGTGGGTAATAGTCTCTGGCAGG
ACATCTGGTACCTAGGAGTGGAGACATAATAAATACCCACA

IgH variable region primers:

Speed-Ig_mVH01
Speed-Ig_mVH02
Speed-Ig_mVH03
Speed-Ig_mVH04
Speed-Ig_mVH05
Speed-Ig_mVH06
Speed-Ig_mVH07
Speed-Ig_mVH08
Speed-Ig_mVH09
Speed-Ig_mVH10
Speed-Ig_mVH11
Speed-Ig_mVH12
Speed-Ig_mVH13
Speed-Ig_mVH14
Speed-Ig_mVH15
Speed-Ig_mVH16
Speed-Ig_mVH17
Speed-Ig_mVH18
Speed-Ig_mVH19
Speed-Ig_mVH20
Speed-Ig_mVH21
Speed-Ig_mVH22
Speed-Ig_mVH23
Speed-Ig_mJH01
Speed-Ig_mJH02
Speed-Ig_mJH03
Speed-Ig_mJH04

ACAGGTGTCCTCTCTCAGGTGCAGCTGCAGCAGCCTGG
ACAGGTGTCCTCTCTCAGGTGCAGCTGCAGCAGTCTGG
ACAGGTGTCCTCTCTCAGGTGCAGCTGAAGCAGTCTGG
ACAGGTGTCCTCTCTCAGGTGCAGCTGAAGGAGTCTGG
ACAGGTGTCCTCTCTGAGGTGAAGCTGGAGGAGTCTGG
ACAGGTGTCCTCTCTGAGGTGCAGCTGGTGGAGTCTGG
ACAGGTGTCCTCTCTGAAGTGCAGCTGTTGGAGACTGG
ACAGGTGTCCTCTCTGAGGTGCAGCTGCAGCAGTCTGG
ACAGGTGTCCTCTCTGAGGTGCAGCTGCAGGAGTCTGG
ACAGGTGTCCTCTCTAGGTGCAGCTGCAGCAGTCTGTG
ACAGGTGTCCTCTCTGAGGTGAAGCTGGTGGAGTCTGG
ACAGGTGTCCTCTCTCAGATCCAGCTGCAGCAGTCTGG
ACAGGTGTCCTCTCTCAGGTTCCAGCTGCAACAGTCTGA
ACAGGTGTCCTCTCTGAGTTCCAGCTGCAGCAGTCTGG
ACAGGTGTCCTCTCTGATGTACAGCTTCAGGAGTCAGG
ACAGGTGTCCTCTCTGAGGTGCAGCTTGTGAGTCTGGTGGAGG
ACAGGTGTCCTCTCTCAGCGTGAGCTGCAGCAGTCTGG
ACAGGTGTCCTCTCTGACGTGAAGCTGGTGGAGTCTGG
ACAGGTGTCCTCTCTGAAGTGATGCTGGTGGAGTCTGG
ACAGGTGTCCTCTCTCAGGTGCAGCTTGTAGAGACCGG
ACAGGTGTCCTCTCTCAGATGCAGCTTCAGGAGTCAGG
ACAGGTGTCCTCTCTCAGGCTTATCTACAGCAGTCTGG
ACAGGTGTCCTCTCTGAGTTCCAGCTGCAGCAGTCTGG
ttcTGAGGAGACTTGGacttacCTGAGGAGACGGTGACC
ttcTGAGGAGACTTGGacttacCTGAGGAGACTGTGAGA
ttcTGAGGAGACTTGGacttacCTGCAGAGACAGTGACC
ttcTGAGGAGACTTGGacttacCTGAGGAGACGGTGACT

IgK variable region primers:

Speed-Ig_mVK01
Speed-Ig_mVK02
Speed-Ig_mVK03
Speed-Ig_mVK04
Speed-Ig_mVK05
Speed-Ig_mVK06

ctaggtaccagatgtAACATTATGATGACACAGTCGCCA
ctaggtaccagatgtAACATTGTGCTGACCCAATCTCCA
ctaggtaccagatgtCAAATTGTTCTCACCCAGTCTCCA
ctaggtaccagatgtCAAATTGTTCTCTCCAGTCTCCA
ctaggtaccagatgtGAAAATGTTCTCACCCAGTCTCCA
ctaggtaccagatgtGAAACAACCTGTGACCCAGTCTCCA

Speed-Ig__mVK07	ctaggtaccagatgtGAAATTGTGCTCACTCAGTCTCCA
Speed-Ig__mVK08	ctaggtaccagatgtGACATCAAGATGACCCAGTCTCCA
Speed-Ig__mVK09	ctaggtaccagatgtGACATCCAGATGAACCAGTCTCCA
Speed-Ig__mVK10	ctaggtaccagatgtGACATCCAGATGACTCAGTCTCCA
Speed-Ig__mVK11	ctaggtaccagatgtGACATTGTGATGACTCAGTCTC
Speed-Ig__mVK12	ctaggtaccagatgtGACATTGTGATGTCACAGTCTCCA
Speed-Ig__mVK13	ctaggtaccagatgtGACATTGTGCTGACCCAATCTCCA
Speed-Ig__mVK14	ctaggtaccagatgtGATATCCAGATGACACAGACTACA
Speed-Ig__mVK15	ctaggtaccagatgtGATGTTGTGATGACCCAACTCCA
Speed-Ig__mVK16	ctaggtaccagatgtGAAATCCAGATGACCCAGTCTCCA
Speed-Ig__mVK17	ctaggtaccagatgtGACATCCAGATGACACAATCTTCA
Speed-Ig__mVK18	ctaggtaccagatgtGACATCCAGATGACCCAGTCTCCA
Speed-Ig__mVK19	ctaggtaccagatgtGACATCCTGATGACCCAATCTCCA
Speed-Ig__mVK20	ctaggtaccagatgtGACATTGTGCTCACCCAATCTCC
Speed-Ig__mVK21	ctaggtaccagatgtGATGTTGTGGTGACTCAAACCTCCA
Speed-Ig__mVK22	ctaggtaccagatgtAACATTGTAATGACCCAATCTCCC
Speed-Ig__mVK23	ctaggtaccagatgtGATGTTTTGATGACCCAACTCCA
Speed-Ig__mVK24	ctaggtaccagatgtGATATTGTGATGACTCAGGCTGCA
Speed-Ig__mVK25	ctaggtaccagatgtGACATCCAGATGATTCAGTCTCCA
Speed-Ig__mVK26	ctaggtaccagatgtGACATCTTGCTGACTCAGTCTCCA
Speed-Ig__mVK27	ctaggtaccagatgtGATGTCCAGATGATTCAGTCTCCA
Speed-Ig__mVK28	ctaggtaccagatgtGATGTCCAGATAACCCAGTCTCCA
Speed-Ig__mJK01	TGCGACGTCAACTGGacttacGTTTGATTTCCAGCTT
Speed-Ig__mJK02	TGCGACGTCAACTGGacttacGTTTTATTTCCAGCTT
Speed-Ig__mJK03	TGCGACGTCAACTGGacttacGTTTTATTTCCAACCTT
Speed-Ig__mJK04	TGCGACGTCAACTGGacttacGTTTCAGCTCCAGCTT

Supplementary Table 1

Name

IgH-seq1
IgH-seq2
IgH-seq3
IgH-seq4

Construct Sequencing Primers

Sequence

GAGAGCCTCACTCCCATTCTC
CCTCAGGGACAAATATCCAAGATTAGTCTG
CTCAACCACCAACCAGCATG
CCAGAATAGATTCAAATTCAAACCCACATG

IgK-seq1

IgK-seq2

IgK-seq3

IgK-seq4

GGATGGTGACCATAGTATTAATTACTTTCTC

GCTCTGTTCTATCACTGTGC

GGTTTTAAATTACAGATGTCATTTTGAAGG

CAGAAAACACTGCTACTGGGTA

Supplementary Table 1

Name

Speed-IgH 5'-F
Speed-IgH 5'-R
Speed-IgH 3'-F
Speed-IgH 3'-R
Speed-IgK 5'-F
Speed-IgK 5'-R
Speed-IgK 3'-F
Speed-IgK 3'-R
IgHJ wt-F
IgHJ wt-R

Genotyping Primers

Sequence

CCCCACCATCACAGACCTTT
CTTTGTTGTGTCCCAGCTCC
CTCTCCACAGGCACTGAACA
ATTGCCTTCTTAGCCTGGGT
GCAGGATTGGAAGGACTTTG
GCTGAGAGGAAGCCCTCAT
AATGGGAATTTTGCTGTTGC
CCATGCTTCAGACAGAGAT
GTCCTTACAACCTCTCTCTTCTATTTCAGC
CCTGACAATAAATGATCCTTGGCTAGG

IgHJ del-F	CTCCACCAACACCATCACACAG
IgHJ del-R	CTGGGGACTGTGGGGTTCAG
Bhlhe41 5'-F	AAAGCCGAGCTCACTGTAGG
Bhlhe41 5'-R	TGCATGATCTCCGGTATTGA
Bhlhe41 3'-F	CGTACTGACGGTGGGAGAAT
Bhlhe41 3'-R	GCTGTGGTTTCCAACACCTT
Zbtb32 5'-F	CAGGAGTCCAGCCTTAGCAG
Zbtb32 5'-R	TCCTTGAAGAAGATGGTG CG
Zbtb32 3'-F	AAGTTCATCTGCACCACCG
Zbtb32 3'-R	GCCGGAGAAGTTCAGTCAGT

Supplementary Table 1	Protospacer Sequences
Target	Protospacer
IgH	ACCACGGTCACCGTCTCCTC
IgK	GTGGAGTACTACCACTGTGG
IgH J deletion	GCTTTAGAAGCATGTCACAT
	GCCATTCTTACCTGAGGAGA
Bhlhe41	CTTTAGAGGACGTTTGAAC
Zbtb32	ACAGGAGCTACGAACCCTTC