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The endogenous *Mtv8* locus and the immunoglobulin repertoire

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The vast diversity of mammalian adaptive antigen receptors allows for robust and efficient immune responses against a wide number of pathogens. The antigen receptor repertoire is built during the recombination of B and T cell receptor (BCR, TCR) loci and hypermutation of BCR loci. V(D)J recombination rearranges these antigen receptor loci, which are organized as an array of separate V, (D), and J gene segments. Transcription activation at the recombining locus leads to changes in the local three-dimensional architecture, which subsequently contributes to which gene segments are utilized for recombination. The endogenous retrovirus (ERV) mouse mammary tumor provirus 8 (*Mtv8*) resides on mouse chromosome 6 interposed within the large array of light chain kappa V gene segments. As ERVs contribute to changes in genomic architecture by driving high levels of transcription of neighboring genes, it was suggested that *Mtv8* could influence the BCR repertoire. We generated *Mtv8*-deficient mice to determine if the ERV influences V(D)J recombination to test this possibility. We find that *Mtv8* does not influence the BCR repertoire.

KEYWORDS

endogenous retrovirus, immunoglobulin repertoire, V(D)J recombination, MTV, B cell receptor (BCR) repertoire

Introduction

The extensive diversity of the jawed vertebrate adaptive immune response depends on the programmed assembly and hypermutation of antigen receptor (AgR) genes (1). The first stage of AgR assembly is V(D)J recombination, initiated by lymphocyte-specific recombination activating genes 1 and 2 (RAG1 and RAG2), during which immunoglobulin (Ig) and T cell receptor (TCR) genes are recombined from discrete variable (V), diversity (D), and joining (J) gene segments (2).

AgR assembly is a sequential process during lymphocyte development. In B cells, the Ig heavy-chain (*Igh*) locus recombines in early and pro B cells prior to the kappa and lambda light chain (*Igk, Igl*) loci in pre B cells (2, 3). *Igh* recombines in two phases: first, D_H-to-J_H

rearrangements occur in lymphoid progenitors; second, V_H-to-DJ_H rearrangements occur in pro-B cells (2, 4). After successful (i.e. in-frame without premature stop codons) *Igh* recombination, *Igk* undergoes V_K-to-J_K recombination (3). If neither *Igk* allele successfully rearranges, the *Igl* locus recombines in a V_L-to-J_L fashion. During V(D)J recombination, RAG (a heterotetramer composed of RAG1 and RAG2 molecules) accumulates at recombination centers (RCs) that encompass J or DJ gene segments (for D-to-J/V-to-J and V-to-DJ recombination, respectively) (4). In RCs, RAG binds to a recombination signal sequences (RSS) that flanks the rearranging gene segment (4). Then, a partner RSS of the second gene segment is brought into the RC for synapsis and RAG-mediated cleavage.

V(D)J recombination determines the V, (D), and J gene segments used in a particular AgR gene and is dependent on three-dimensional chromosomal architecture. Specifically, V(D)J recombination is constrained to AgR loci by chromatin loops, the bases of which are defined by CCCTC-binding factor (CTCF)-bound CTCF-Binding Elements (CBEs) (5–9). The number of loops formed is dependent on the recombining locus (10). In addition to chromatin loops defining the AgR, chromosomal architecture also defines the mechanism by which recombining gene segments are brought into the RC (11).

The *Igk* locus is comprised of 92 functional (163 total) V_K and four functional J_K spread across 3.2 Mb (Figure 1A) and contracts into a recombination-competent chromosomal structure in developing pro- and pre-B cells (12, 13). Once contracted, the *Igk* locus forms a rosette-like structure with five V_K-containing loops and one loop with J_K and C_K gene segments (10). *Igk* rearrangement occurs predominantly through the collision of, and subsequent recombination between, the J_K-containing and a V_K-containing loop (10). While the *Igk* loops are thought to be predominantly shaped by the CBEs throughout the locus, it is unknown whether other factors are involved.

The endogenous retrovirus (ERV) mouse mammary tumor virus (MMTV), *Mtv8* was mapped to the *Igk* locus on chromosome 6 (Figure 1A) (14–16). *Mtv8* is a provirus with all open-reading frames (ORFs) intact, namely, *gag*, *pro*, *pol*, *env*, and superantigen (SAg) (Figure 1B). The provirus does not produce infectious virions and is silent in the mammary glands, the targeted tissue of all MMTVs, potentially due to hypermethylation of its promoter region (17–19). The *Mtv8* SAg has a specificity for Vβ11 and Vβ12 (20). Mice strains harboring *Mtv8* lack $V\beta11^+$ and $V\beta12^+$ T cells (21), indicating that *Mtv8* is expressed in antigen presenting cells, such as dendritic cells and B cells which present SAg to cognate T cells (22). As *Mtv8* is mapped within the *Igk* locus, it was hypothesized that it may contribute to *Igk* recombination by driving high levels of transcription in its vicinity (23). It was suggested that recombination to the first V_K gene segment downstream of *Mtv8* (V_K14-111, formerly V_K9M) could allow for enhancer activation of the *Mtv8* promoter (23). To identify whether *Mtv8* affects the *Igk* repertoire, the frequency of recombination of the J_K gene segments to V_K14-111 was analyzed in inbred mouse strains with [BALB/c, C58.C, A/J, and C57BL/6J (B6J)] and without (C58, C.C58, NZB, and PERA/Ei) *Mtv8* (23). These experiments demonstrated that mice inheriting *Mtv8* have higher recombination between V_K14-111 and all J_K gene segments compared to strains without *Mtv8*. While these analyses suggested differences in the usage of V_K14-111, *Ig* repertoire comparisons between inbred mouse lines is not an ideal approach, as polymorphisms within the loci other than *Mtv8* could also influence the repertoires.

To address whether *Mtv8* shapes the repertoire of mice with this ERV, we used CRISPR/Cas9 technology to generate B6J mice lacking *Mtv8* and compared their *Ig* repertoire to that of wild-type (WT) B6J mice. We found that the absence of *Mtv8* had no significant effects on the *Igh*, *Igk*, and *Igl* gene segment usage. Thus, contribution of *Mtv8* to the mouse *Ig* repertoires can be definitively ruled out.

Methods

Mice

Mice utilized in this study were bred and maintained at the animal facility of The University of Chicago. The studies described

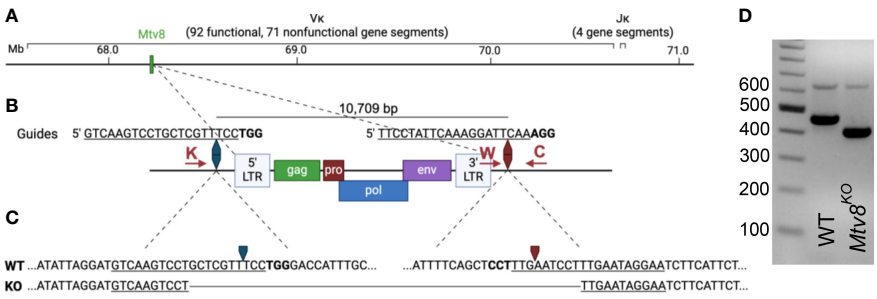

FIGURE 1
Generation of *Mtv8*^{KO} mice. **(A)** Schematic of B6J murine *Igk* locus on chromosome 6. Location of *Mtv8* is highlighted in green. **(B)** Schematic of *Mtv8* and guides used for CRISPR/Cas9 targeting of *Mtv8* to generate *Mtv8*^{KO} mice. LTR, long-terminal repeat; *gag*, group-specific antigen; *pro*, protease; *pol*, polymerase; *env*, envelope. Red arrows: genotyping primers (K: KOF, W: WTF, C: CommonR). **(C)** Genomic sequence of WT and KO *Mtv8* alleles. Only the flanking sequnces are shown for the WT allele. For **(B, C)**, underlined text indicates guide sequence, bold text indicates PAM sequence, arrows indicate Cas9-cut site. **(D)** Gel image of genotyping representative WT and homozygous *Mtv8*^{KO} mice. Mice were genotyped using a three-primer PCR with the KOF, WTF, and CommonR primers. WT product: 479 bp, KO product: 419 bp.

herein have been reviewed and approved by the Animal Care and Use Committee at the University of Chicago, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International).

C57BL/6J (B6J) mice were purchased from The Jackson Laboratory. *Mtv8*^{KO} B6J mice were generated using CRISPR/Cas9 technology. Two guide RNAs targeting the flanking up- and downstream sequences of *Mtv8* (5' guide: 5'-GTCAAGTCC TGCTCGTTTCC; 3' guide: 5'- TTCCTATTCAAAGGATTCAA) were co-injected into a single cell B6J embryos along with Cas9 (Figure 1B). Founder mice and subsequent offspring in which *Mtv8* was eliminated were identified using PCR with primers flanking the guide cut sites (KOF: 5'-GAATTTGGGTGCTCTTGCAT; CommonR: 5'-AACACAAATGGAGGCAAAGC; KO product size: 419 bp). To identify mice with the WT allele, a separate PCR was used using the CommonR primer and a WT-specific reverse primer that lies in the excited region (WTF: 5'- CAGTC CTAACATTCACCTCT; WT product size: 479 bp). A founder line was established in which *Mtv8* was eliminated with a 10,688 bp deletion (Figure 1C). The founder mouse was bred to a WT B6J mouse and the resulting F1 offspring were intercrossed to generate a homozygous KO line (Figure 1D). The deletion was confirmed at the DNA level by sequencing the KO allele using the KO-specific PCR primers.

RNA isolation from splenic B cells

CD19^{+} splenocytes were isolated from three WT and three *Mtv8*^{KO} 10.5-week-old B6J female mice. Red blood cell lysed splenocytes were labeled with microbeads conjugated to monoclonal anti-mouse CD19 antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany) and positively sorted as detailed by the manufacturer. RNA was isolated from sorted cells using guanidine thiocyanate extraction and CsCl gradient centrifugation (24).

Library preparation

Immunoglobulin libraries were generated from 1 µg of RNA using the NEBNext Immune Sequencing Kit (New England Biolabs, Ipswich, Massachusetts, USA) according to manufacturer's instructions, specifically enriching for B cell receptor (BCR) chains during the first PCR step using the mouse NEBNext IS BCR primers included in the kit (Mus-IgGb: 5'-GATGGG GCTGTTGTTKTRGC; Mus-IgGa: 5'-GTGTCGTTTTGGCCTG; Mus-IgE: 5'-GGTTCCTGATAGAGGC; Mus-IgD: 5'-GTTCCTTTTTATCACC; Mus-IgM: 5'-TGACTCTC CTGMRGARAC; Mus-IgA: 5'-GTGGGTAGATGGTGGG; Mus-IgK: 5'-RCATCAGCCMGGTWT; Mus-IgL: 5'-ATGG HGWRGMCTTGGG). The libraries generated from the six individual mice were pooled in equimolar amounts and sequenced by paired-end 300 bp sequencing on an Illumina MiSeq by The University of Chicago Genomics Facility.

B cell receptor sequence processing and analysis

Preprocessing of BCR sequences was performed using the open-source workflow pRESTO NEBNext Immune Sequencing Kit Workflow (v3.2.0) on Galaxy (25). Reads with a Phred quality score <20 were removed for quality control. Reads that did not match to the constant region primer (maximum error rate 0.2) were removed. Reads that did not match to the template switch sequence (maximum error rate 0.5) were removed. The first 17 bp following the template switch site were a unique molecular identifier (UMI) on each read. Sequences with identical UMIs were collapsed into consensus sequences with sequences found in less than 60% of reads removed. Positions with more than 50% gap sequences were removed. Mate-pairs were assembled with a minimum of 8 bp overlap (maximum error rate of 0.3). Assembled reads were assigned isotype-constant region identities based on local alignment of the 3' ends of the reads (maximum error rate of 0.3). Using the *Mus musculus* reference C57BL/6J genome (GRCm38/mm10), V, (D), and J gene segments were assigned using MiGMAP mapper (Galaxy Version 1.0.3+galaxy2). Subsequent analyses were done using R Studio. Statistically significance was calculated using unpaired Welch's *t* test with Bonferroni correction. Graphing was done using GraphPad Prism version 10.0.1 for Mac (GraphPad Software, Boston, Massachusetts, USA).

Results

Generation of Mtv8-deficient B6J mice

Mtv8 knockout (KO) B6J mice were generated using a CRISPR/Cas9 approach. To target *Mtv8* without disturbing the V_K gene segments in its vicinity, we designed two guides to precisely delete the ERV: one 645 bp upstream of the 5' LTR and one 79 bp downstream of the 3' LTR (Figure 1B). A founder was identified using PCR with primers flanking the predicted deleted region and it was determined that a 10,688 bp deletion occurred, resulting in the elimination of the entire *Mtv8* locus (Figures 1C, D). The founder was crossed to a wildtype (WT) B6J mouse and heterozygous, mutant allele-carrying progeny were interbred to generate a homozygous *Mtv8*-deficient line (Figure 1D).

Mtv8 does not contribute to light and heavy chain recombination

To investigate whether *Mtv8* alters *Ig* gene segment usage, we analyzed the Ig repertoires of WT and *Mtv8*^{KO} B6J mice (Figures 2-4, Supplementary Table 1). Accordingly, RNA isolated from CD19^{+} splenocytes was used to prepare Ig heavy and light chain-specific 5'-RACE libraries, which were analyzed to determine the BCR repertoire using the pRESTO toolkit using the *Mus musculus* reference C57BL/6J genome (GRCm38/mm10) (25). The repertoire of productive transcripts was analyzed. Productive transcripts were defined as in-

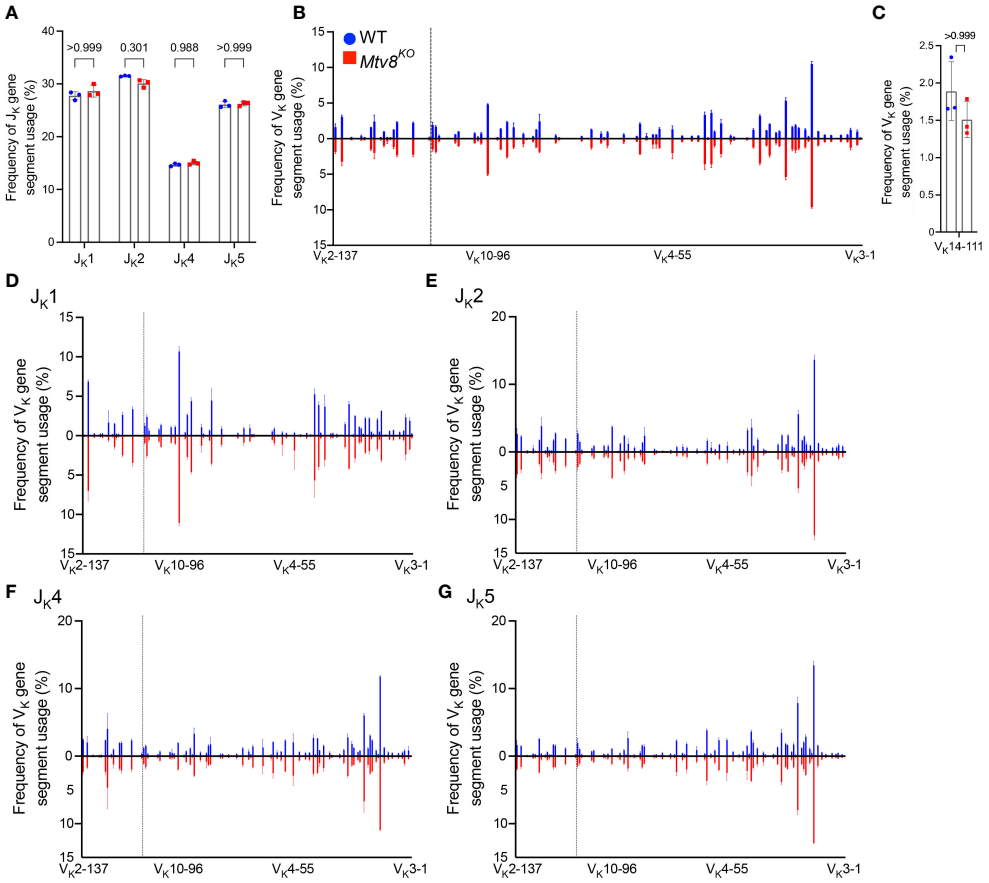

FIGURE 2
Igk repertoire of CD19$^+$ splenocytes of *Mtv8*^{KO} mice. Frequency of J_K **(A)** and V_K **(B)** gene segments in productive *Igk* transcripts from CD19$^+$ splenocytes. Productive recombination events are in-frame without premature stop codons. **(C)** Frequency of V_K14-111 (previously termed VK9M) in and productive *Igk* transcripts. **(D-G)** Frequency of V_K gene segments in total *Igk* transcripts recombined to J_K1 **(D)**, J_K2 **(E)**, J_K4 **(F)**, and J_K5 **(G)**. **(B, D-G)** V_K gene segments are arranged from 5′ distal to 3′ proximal to J_K gene segments and the vertical dotted line represents chromosomal location of *Mtv8*. n=3 mice per group. Data presented as mean with error bars indicated SD. Statistical significance was determined by unpaired Welch's *t* test with Bonferroni correction (numbers above bars indicate adjusted P value).

frame without premature stop codons and are likely those transcripts that are translated into expressed Ig proteins.

To determine whether *Mtv8* influences *Igk* recombination, we examined the J_K and V_K usage in productive *Igk* transcripts from CD19$^+$ splenocytes (Figure 2). There was no difference in J_K usage (Figure 2A). Furthermore, the loss of *Mtv8* led to no alterations in the total V_K repertoire (Figure 2B). Notably, there was no change in usage of V_K gene segments mapped in the close proximity of *Mtv8* (dotted line; Figure 2B). An increased usage of the V_K gene segment, V_K14-111 (formerly called V_K9M) directly downstream of *Mtv8* in *Mtv8*$^+$ mouse strains compared to *Mtv8*$^-$ strains was previously reported (23). We observed no difference in frequency of V_K14-111 usage in *Igk* transcripts between WT and *Mtv8*^{KO} B6J mice (Figure 2C). Thus, the previously observed disparities in V_K14-111 usage between *Mtv8*$^+$ and *Mtv8*$^-$ mice from distinct genetic backgrounds are independent of *Mtv8*.

The same *Igk* locus can undergo multiple rounds of recombination. While *Mtv8* does not affect the overall frequency of V_K usage, we wanted to test the possibility that it might affect either the first or subsequent recombination in distinct ways. Recombination of any V_K to J_K1 can only occur during a primary recombination event, as recombination to any other J_K gene segment would remove J_K1 from the *Igk* locus. Recombination to J_K2, J_K4, or J_K5 can either occur during a primary or secondary recombination event. As such, we calculated the frequency of V_K gene segment usage in total recombination events with each J_K gene segment. We found that, in line with globally unaffected J_K gene segment usage, frequency of any particular V_K gene segment recombining to J_K1, J_K2, J_K4, and J_K5 was unchanged with the loss of *Mtv8* (Figures 2D–G). Taken together, these data show that the *Igk* repertoire of *Mtv8*^{KO} B6J mice does not differ from WT B6J and that *Mtv8* does not influence the *Igk* recombination.

We also found no difference in the J_L and V_L usage among productive *Igl* transcripts between WT and *Mtv8*^{KO} B6J mice (Figure 3). Similarly, we found no alterations in J_H, D_H, and V_H gene segment usage between WT and *Mtv8*^{KO} mice (Figure 4).

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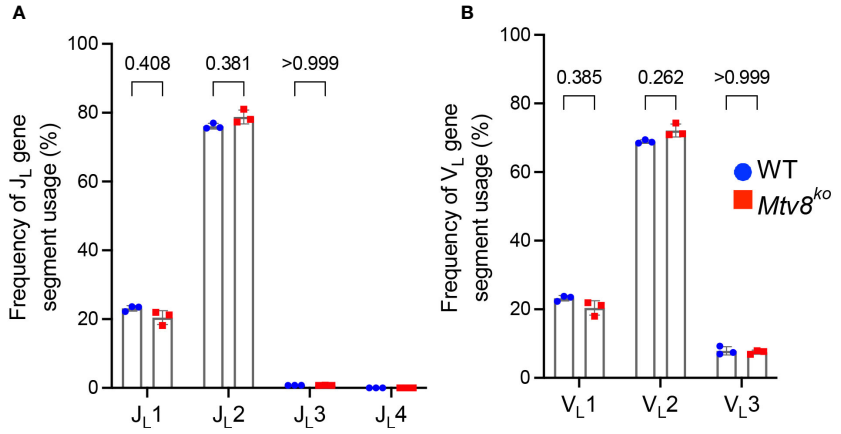

FIGURE 3
Igl repertoire of CD19$^+$ splenocytes of *Mtv8*^{KO} mice. Frequency of J_L **(A)** and V_L **(B)** gene segments in and productive *Igl* transcripts from CD19$^+$ splenocytes. Productive recombination events are in-frame without premature stop codons. n=3 mice per group. Data presented as mean with error bars indicated SD. Statistical significance was determined by unpaired Welch's *t* test with Bonferroni correction (numbers above bars indicate adjusted P value). No P value is indicated for JL4 as values for all samples are 0.

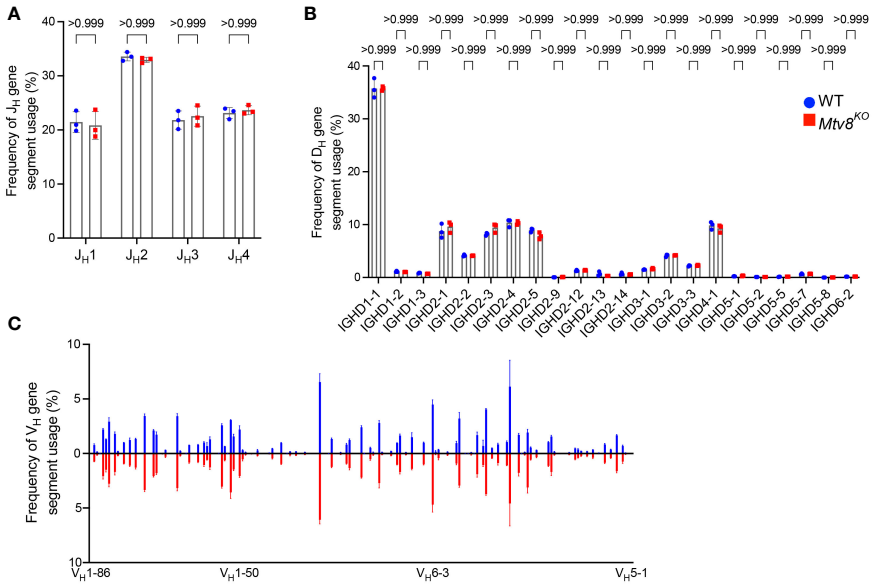

FIGURE 4
Igh repertoire of CD19$^+$ splenocytes of *Mtv8*^{KO} mice. Frequency of J_H **(A)**, D_H **(B)**, and V_H **(C)** gene segments in productive *Igh* transcripts from CD19$^+$ splenocytes. Productive recombination events are in-frame without premature stop codons. **(B)** D_H gene segments are arranged from 5′ distal to 3′ proximal to J_H gene segments. **(C)** Frequency of V_H gene segments in productive *Igh* transcripts that are statistically different between WT and *Mtv8*^{KO} mice. V_H gene segments are arranged from 5′ distal to 3′ proximal to D_H gene segments. n=3 mice per group. Data presented as mean with error bars indicated SD. Statistical significance was determined by unpaired Welch's *t* test with Bonferroni correction (numbers above bars indicate adjusted P value).

Discussion

It is now accepted that the collisions of the rosette-like loops of the *Igk* locus are the major mechanism of V_K-to-J_K recombination (10). However, the identification of an ERV, *Mtv8*, in the middle of the V_K array on the *Igk* locus led to the hypothesis that it may influence the Ig repertoire (14–16). Differences in V_K usage in mouse strains with and without *Mtv8* initially supported this hypothesis (23). Now, advancements in genome-editing technologies allow us to definitely address whether a genetic loss of *Mtv8* can change the *Ig* repertoire within a mouse line. We show that *Mtv8* has no effect on the *Ig* repertoire by analyzing the *Igh, Igk*, and *Igl* transcriptional repertoires of B cells isolated from WT and *Mtv8*^{KO} B6J mice. These data demonstrate that while *Mtv8* is an intact ERV at the *Igk* locus, it has no influence on *Igk* recombination.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE248410, GSE248410.

Ethics statement

The animal study was approved by University of Chicago Animal Resources Center. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

HB: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing. SE: Methodology, Writing – review & editing. TG: Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1345467/full#supplementary-material

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