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Chromosome choice for initiation of V-(D)-J recombination is not governed by genomic imprinting

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Abstract

V-(D)-J recombination generates the antigen receptor diversity necessary for immune cell function while allelic exclusion ensures that each cell expresses a single antigen-receptor. V-(D)-J recombination of the *Ig, Tcrb, Tcrg,* and *Tcrd* antigen receptor genes is ordered and sequential so that only one allele generates a productive rearrangement. The mechanism controlling sequential rearrangement of antigen receptor genes, in particular, how only one allele is selected to initiate recombination while at least temporarily leaving the other intact remains unresolved. Genomic imprinting, a widespread phenomenon wherein maternal or paternal allele inheritance determines allele activity, could represent a regulatory mechanism for controlling sequential V-(D)-J rearrangement. We used strain-specific single nucleotide polymorphisms (SNPs) within antigen receptor genes to determine if maternal vs. paternal inheritance could underlie chromosomal choice for the initiation of recombination. We found no parental chromosomal bias in the initiation of V-(D)-J recombination in T or B cells, eliminating genomic imprinting as a potential regulator for this tightly regulated process.

Introduction

Lymphocytes create diverse antigen-receptor repertoires by recombining germ line non-rearranged variable (V), diversity (D), and joining (J) gene segments that encode the ligand binding subunits of the T cell antigen receptor (TCR) or B cell antigen receptor (BCR) complexes. One important aspect of V-(D)-J recombination is the phenomenon of allelic exclusion, which limits productive antigen-receptor rearrangement to a single chromosome at most loci[1, 2]. Allelic exclusion ensures that each lymphoid cell expresses a single antigen receptor with a defined ligand binding specificity. Mechanistically, allelic exclusion requires

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Conflict of Interest

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that two distinct developmental events be tightly regulated. The first event is the decision to initiate rearrangement of only one of the two antigen receptor loci in the diploid genome. The second event is restriction of V-(D)-J recombination to a single allele if the product of the initial event is productive (i.e., leads to the expression of a functional TCR or BCR chain). Feedback mechanisms explain how successful recombination on one chromosome will prevent the initiation of recombination at the second allele[3–5]. However, it is not yet fully clear how the initial choice of one chromosome, and not the other or both, is initiated[3, 4, 6, 7]. "Stochastic" models emphasize mechanisms that decrease the efficiency of recombination on any allele, making it unlikely that recombination will ever occur simultaneously on both. In contrast, "deterministic" models suggest that the two chromosomes are somehow marked during early development so that they are not functionally equivalent substrates for rearrangement.

Several experimental models have been developed to study allelic choice. For example, Farago et al. provided strong evidence that asynchronous replication of receptor alleles is a marker for chromosome choice and demonstrated that the asynchrony is established stochastically in the early embryo, even before hematopoiesis[8]. On the other hand, results from Khor and Sleckman suggest that chromosome choice occurs late in lymphocyte development[9]. Thus, important issues remain unsettled.

Genomic imprints are epigenetic marks, established in germ cells that result in parent-of-origin differences in key aspects of chromosomal biology including transcription, recombination, DNA replication, establishment of long-range chromosomal interactions, and nuclear localization[10–12]. These are all chromosomal properties that are either known to or seem likely to play important roles in the initiation of V-(D)-J recombination. Therefore it was plausible that genomic imprinting might regulate the initiation of recombination at a single allele by biasing initiation of recombination toward the maternal or the paternal chromosomes.

Some previous studies of allelic exclusion have noted the parental origin of the expressed receptors as part of their analyses and the authors have not needed to invoke genomic imprinting to explain their results. (For examples, see [13–16].) However, because the experimental approaches employed in prior work were not designed to yield unequivocal results, we believe that this issue remains unresolved. First, previous studies analyzed the RNAs and/or proteins generated by the *recombined* alleles and found that both maternal and paternal chromosomes contribute to receptor repertoires. However, the high rate of failure for the first recombination event ensures the majority of cells will have rearranged the second allele. Thus, even an absolute parent-of-origin bias in the choice of chromosome substrates for initial rearrangement will still result in relatively modest deviations from a 1:1 ratio of maternal: paternal allele use in mature lymphocytes. To resolve this issue definitively, we developed a screening protocol for non-rearranged chromosomes that will detect even partial biases toward maternal or paternal chromosomes. Second, we analyzed all lymphocyte lineages that undergo V-(D)-J recombination and exhibit allelic exclusion for parent of origin bias including B cells, αβ T cells, and γδ T cells. Third, we analyzed cells directly after isolation from mice since recent data highlight the susceptibility of genomic imprinting marks to in vitro culture[17–19]. Finally, we analyzed both adult and fetal derived

lymphocyte populations since analyses of transcriptional regulation by genomic imprinting have shown that fetal parent-of-origin effects can be reduced or eliminated in post-natal animals[20–22].

Our results conclusively demonstrate that there is no influence of chromosome parental origin on the allelic choice for initiation of recombination at either the fetal or adult stages of lymphopoiesis in B cells, αβ T cells, or γδ T cells.

Results and Discussion

An inherent tenet of allelic exclusion is that V-(D)-J rearrangement must initiate on a single chromosome. Rearrangement of the second allele will only occur if the first recombination event is not productive. Thus, mature T and B cells should have either one or zero non-rearranged chromosomes depending upon whether the initial recombination event produces an mRNA that encodes a functional peptide.

We reasoned that we could test for the influence of genomic imprinting on this process by purifying cells where recombination had successfully occurred (i.e., mature, peripheral antigen receptor positive lymphocytes) and then analyzing the parental origin of the V-DJ interval on the *non-recombined* chromosome. If the same parental allele were always rearranged first, only the other parental allele, or no allele, would remain un-rearranged in each lymphocyte. Thus, measuring parent-specific single nucleotide polymorphisms (SNPs) from deleted regions of antigen receptor genes will reveal whether the maternal or paternal allele is recombined first (Figure 1).

Rearrangement of the *Tcrb* locus is tightly regulated by allelic exclusion such that the vast majority of mature T cells express only a single TCRβ chain, but is less strictly enforced for rearrangement of *Tcra*[4, 7]. In B cells, rearrangement of both the heavy and light chain *Ig* genes is subject to allelic exclusion[23] and in fact, rearrangement of the *kappa* light chain is the primary model system for studying allelic exclusion. In γδ T cells, allelic exclusion has been confirmed at the *Tcrg* locus, but it remains controversial whether it applies to *Tcrd*[24–26]. In this study, we assessed the parent-of-origin rearrangement of *Tcrb, Tcrg,* and *Igh* loci to determine if genomic imprinting underlies the initial selection of which chromosome is recombined.

To test for parental bias in the allelic choice for initial V-(D)-J rearrangement, we first identified SNPs that distinguish C57BL/6J (B6) from FVB/NJ (FVB) and/or DBA/2J (DBA) strains within DNA that is deleted upon V-DJ rearrangement (Supplemental Table 1). Mature, αβ T cells, γδ T cells, and B cells were isolated from spleen and lymph nodes from FVB × B6 and B6 × FVB adult heterozygote mice (for evaluation of *Tcrb and Igh* rearrangement) or from B6 × DBA and DBA × B6 F1 adult heterozygotes (for *Tcrg and Igh* rearrangement) by FACS cell sorting. The use of reciprocal crosses was deemed essential because it eliminates the possibility that parent-of-origin bias could be confused with allelic bias. After isolating cells, we prepared genomic DNA (gDNA), PCR amplified across the SNP, and then analyzed parental origin by two independent methods.

The first method was an assay that takes advantage of restriction length fragment polymorphisms (RFLPs) associated with the SNP. (See Methods and Supplemental Figures 1 and 2). Representative results are shown in Figure 2 and demonstrate absence of parent-of-origin bias for each of the genes examined; that is, neither the paternal nor the maternal *Tcrb*, *Tcrg* or *Igh* alleles were differentially depleted by the initial recombination event. For example, in the case of *Tcrb* rearrangement, in both $CD4^+$ T cells and $CD8^+$ T cells, the relative amounts of amplified DNA from each allele was not dependent on its parental origin and was not different from patterns we observed in other cell populations (tail epithelium, γδ T cells and B cells) that had not undergone *Tcrb* gene rearrangement (Figure 2A). Similarly, analysis of non-rearranged *Tcrg* or *Igh* genes from purified mature γδ T cells and B cells, respectively, also revealed absence of parent-of-origin bias for V-DJ recombination (Figure 2B, C).

As a second method of evaluating the parental origin of non-rearranged antigen receptor genes we performed pyrosquencing of the PCR amplified products. This method does not rely on restriction enzyme digestion, is highly quantitative, and is able to detect even very small changes in allelic usage (Supplemental Figure 3). The results in Figure 3 demonstrate two key points. First, the maternal: paternal ratio of alleles in control cells that have not undergone rearrangement is 1:1. This means that there is no PCR bias in allele amplification. More importantly, in cells that have undergone rearrangement, the maternal: paternal ratio remains 1:1 for the *Tcrb, Tcrg,* and *Igh* loci. Altogether these results indicated that parental origin has no detectable influence on the chromosome choice for initial V-DJ recombination at the *Tcrb, Tcrg or Igh* loci in adult derived immune cells.

We also tested DNA isolated from purified peritoneal B-1a cells that arise only during fetal development[27]. As shown in Figure 3C, similar to adult derived (B-1b and B-2) B cells, we observed no evidence of parent-of-origin bias for V-DJ recombination in the fetally derived B-1a cells.

Our study tested whether genomic imprinting marks receptor genes or their chromosomes in germ cells based on parent-of-origin and thereby determines which chromosome will initiate recombination first. The results conclusively demonstrate that imprinting does not contribute to the regulation of V-DJ recombination at the *Tcrb, Tcrg,* and *Igh* loci. Since we did not identify useful SNPs at *Igk*, we were unable to analyze parental origin effects at this locus. However, *Igk* has been the preeminent model for studying mechanisms for allelic exclusion and previous results, especially from the Cedar and Bergman groups already indicate that parent-of-origin bias was unlikely to play a role in monoallelic rearrangement at that locus[8].

It is important to note that our results do not help to distinguish between stochastic and deterministic models of initiation of V-(D)-J recombination in general, but instead definitively test one specific deterministic mechanism (genomic imprinting) that represents a known method for developmentally related allelic discrimination. Our results are not entirely a surprise, since previous studies already have evidence supporting stochastic models for initiating allelic exclusion at the *Igh* locus[14, 28]. Notwithstanding, our results are significant for several reasons. First, in addition to *Igh* we evaluated rearrangement of the *Tcr* genes. It was especially important to evaluate V-(D)-J rearrangement in γδ T cells since their biology

and ontology are distinct from αβ T cells and the rules governing allelic exclusion of *Tcrg* and *TCRb* could have differed. Second, we evaluated *Igh* rearrangements in B cells that occurred in the fetus and the adult. It was conceivable that different mechanisms might regulate allelic exclusion in immune cells at different stages of development particularly as it is now well established that imprinting is most important during embryonic development [29] and that imprinted regulation of transcription is sometimes reduced or even lost in postnatal animals[20–22]. Finally, most previous studies examined rearrangements in B cells after *in vitro* culture. Since recent reports show that imprinting can be rapidly lost during *in vitro* manipulations and culture[17–19], our results obtained with freshly harvested *ex vivo* cells definitively rule out a role for parental origin in both T and B cells.

In conclusion, the current study provides conclusive evidence that initiation of V-(D)-J recombination at a single chromosome is not controlled by genomic imprinting indicating that another method of regulation must regulate this developmental process.

Methods

Animals

C57BL/6J (B6), FVB/NJ (FVB), and DBA/2J (DBA) female and male mice were purchased from the Jackson Laboratory and interbred to generate F1 hybrids. All mice were bred and housed in accordance with National Institutes of Health and United States Public Health Service policy. Animal research was approved through the Eunice Kennedy Shriver National Institute of Child Health and Human Development Animal Care and Use Committee.

Fluorescent-Activated Cell Sorting (FACS) and DNA extraction

Cells from spleen and lymph nodes were combined, enumerated and surface stained as described[30]. Fluorochrome-conjugated antibodies against CD4(RM4.5), CD8 (53-6.7), TCRb (H57), TCRd (GL3), CD19 (1D3), B220 (RA3-6B2) IgM (DS-1) CD5 (53-7.3) were obtained from BD Biosciences. Cells were sorted on a FACSAria cytometer (BD Biosciences) based on the following staining profiles: CD4 T cells ($CD4^+TCRb^+$), CD8 T cells ($CD8^+TCRb^+$), gdT cells ($CD4^-CD8^-gdTCR^+$), B cells ($CD19^+IgM^+$). Peritoneal lymphocytes were harvested as described and the following cell populations were purified by cell sorting: B1a ($CD19^+B220^-CD5^+$), B1b ($CD19^+B220^-CD5^-$), B2 ($CD19^+B220^+CD5^-$).

Single nucleotide polymorphisms (SNPs) and quantitation of alleles

Candidate SNPs at the *Tcrb*, *Tcrd*, *Tcrg*, and *IgH* loci were identified using the Mouse Genome Informatics database (http://www.informatics.jax.org/strains_SNPS.shtml) and primers were designed (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) to span the SNP and yield a single PCR amplicon when analyzed on a 2% agarose gel (Supplemental Table 1). Specificity of the primers and presence of the SNPs were confirmed by DNA sequencing. In preliminary studies, we confirmed the ability of restriction enzyme digestion to distinguish B6, FVB, and DBA amplicons (Supplemental Figure 1) and to identify changes in allelic usage (Supplemental Figure 2). For Figure 2, PCR amplification was by OneTaq Quick-Load 2× MM with Standard Buffer (NEB MO486S) (35 cycles with an annealing

temperature of 58C). PCR products were purified using the QiaQuick Purification Kit (Qiagen) and approximately 100 ng amplicon were digested with restriction enzymes before analysis by electrophoresis on a 2% agarose gel (1X TBE buffer). Additional details are included in Supplemental Table 1. Pyrosequencing assays were performed by EpigenEx. Sequencing was done using Pyrosequencing PSQ96HS System (Qiagen) and results were analyzed with PSQ software (Qiagen). See Supplemental Table 1 for gene specific details.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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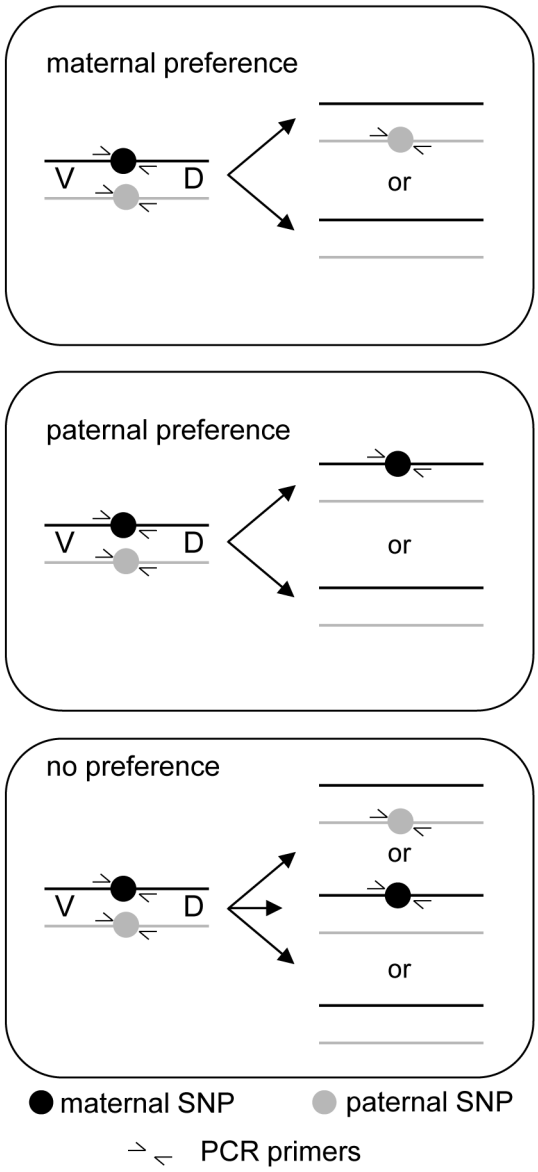

Figure 1. Rationale for experimental approach

Models of V-(D)-J recombination where the choice of initial substrate is biased toward the maternal (dark gray) chromosome, biased toward the paternal (light gray) chromosome, or not influenced by chromosome parental origin. Maternal and paternal alleles are distinguished by SNPs within the region that is always deleted by V-DJ (or V-J) recombination. By assaying the relative proportions of maternal and paternal alleles in mature antigen-receptor cell populations we can distinguish these three mutually exclusive models. Alleles are distinguished and then quantitated by restriction enzyme digestion and also by pyrosequencing. Analyzing mice generated by reciprocal crosses eliminates the possibility that allelic bias will mask parent-of-origin bias in the selection of the initial chromosome that will undergo recombination.

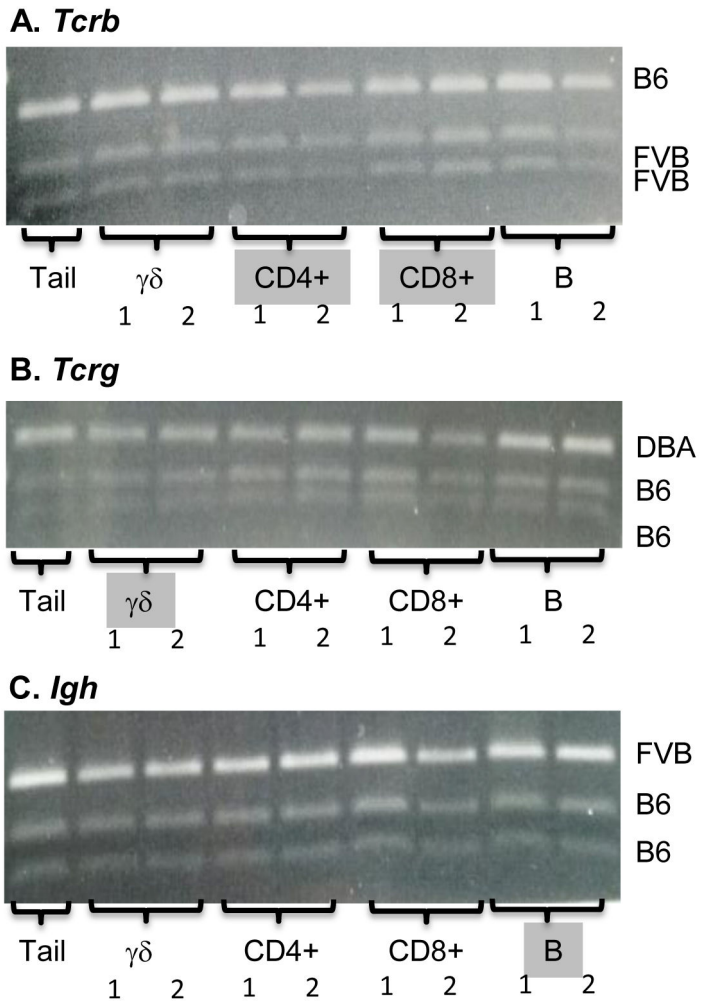

Figure 2. Analysis of parent-of-origin bias by restriction enzyme digestion

(A) *Tcrb* locus: genomic DNA was prepared from tail biopsy and from purified TCRγδ cells (γδ TCR+), purified TCRαβ CD4+ cells (TCRβ+, γδ TCR−, CD4+, CD8−), purified TCRαβ CD8+ cells (TCRβ+, γδ TCR−, CD8+, CD4−), and purified B cells (TCRβ−, γδ TCR−, CD19+, B220+) from adult mice generated by intercrosses of B6 × FVB (lane 1) or FVB × B6 (lane 2). To determine allelic frequency, gDNAs were amplified across known SNPs, digested with informative restriction enzymes, and electrophoresed. Four total analyses were done and representative data are shown. To identify a parent-of-origin bias in the choice of chromosome to initiate V-DJ recombination, we looked for distinct patterns in cells that have undergone recombination (marked by grey boxes) relative to cells that did not undergo recombination. Recombination of the β locus will have occurred in CD4+ and CD8+ cells but not in tail or in TCRγδ or B cells. However, the relative frequencies of B6 and FVB are not different in those cell types. The use of reciprocal crosses ensures that allelic biases (in either recombination or in detection) did not obscure parent-of-origin biases. N = 4. (B) *Tcrg* locus was analyzed as in (A) except for the heterozygotes being B6 × DBA (lane 1) and DBA × B6 (lane 2). (C) *Igh* locus was exactly analyzed as described in panel A.

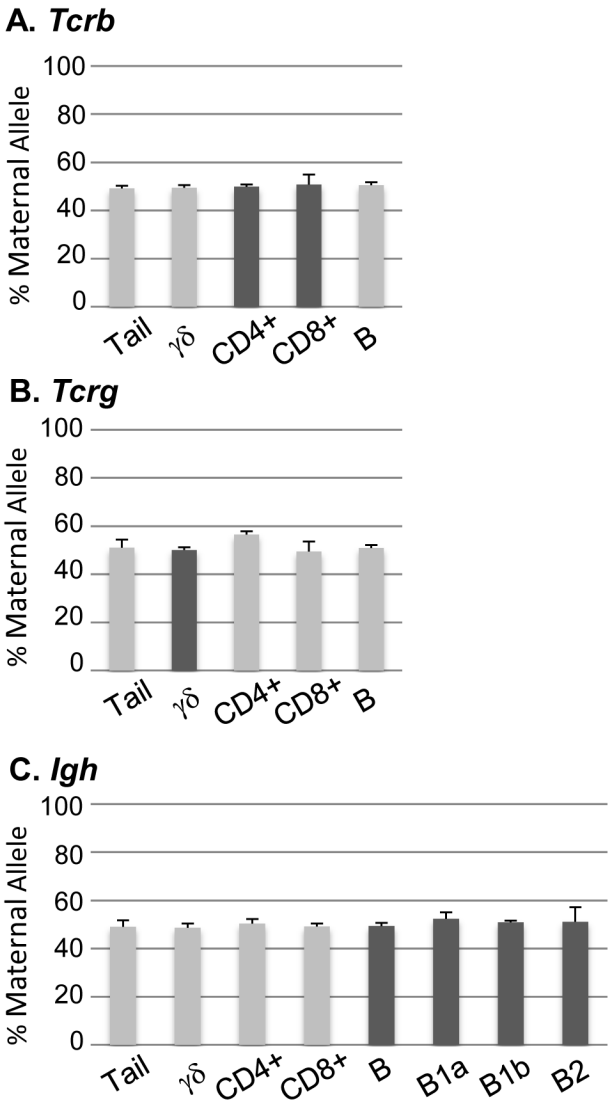

Figure 3. Analysis of parent-of-origin bias by pyrosequencing

Genomic DNAs were prepared essentially as described for Figure 2 except that for *Igh,* we included samples from B6 × FVB, FVB × B6, B6 × DBA, and DBA × B6 F1 heterozgyotes since both crosses included informative SNPs. After amplification across known SNPs, allelic frequency was analyzed by pyrosequencing. The relative frequency of the maternally inherited allele is depicted as mean ± standard error of the mean. For *Tcrb*, n=4 (2 B6 × FVB and 2 FVB × B6). For *Tcrg*, n=4 (2 B6 × DBA and 2 DBA × B6). For *Igh*, n = 8 (2 B6 × FVB, 2 FVB × B6, 2 B6 × DBA, and 2 DBA × B6). For each locus, the dark grey bars depict the cell population that will have undergone V-DJ recombination. Non-recombined cells are shown in light grey. The absence of detectable differences between these two types of cells and maternal allele frequencies of 50% together indicate that there was no parent-of-origin bias in the choice of chromosome to initiate recombination.