SHORT COMMUNICATION

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

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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 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.

Immunology and Cell Biology (2017) 95, 473–477; doi:10.1038/icb.2017.1; published online 28 February 2017

INTRODUCTION

Lymphocytes create diverse antigen-receptor repertoires by recombining germline 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 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.8 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. On the other hand, results from Khor and Sleckman9 suggest that chromosome choice occurs late in lymphocyte development. 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 have 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 example, see refs 13–16). However, because the experimental approaches used 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

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Received 1 September 2016; revised 16 December 2016; accepted 18 December 2016; published online 28 February 2017
paternal chromosomes contribute to receptor repertoires. However, the high rate of failure for the first recombination event ensures that 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. 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 postnatal animals.

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 was always rearranged first, only the other parental allele, or no allele, would remain unrearranged 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 Terb 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 Tera. In B cells, rearrangement of both the heavy- and light-chain Ig genes is subject to allelic exclusion, and in fact, rearrangement of the κ-light chain is the primary model system for studying allelic exclusion. In γδ T cells, allelic exclusion has been confirmed at the Terg locus, but it remains controversial whether it applies to Tcrd. In this study, we assessed the parent-of-origin rearrangement of Terb, Terg and Igh loci to determine if genomic imprinting underlies the initial selection of which chromosome is rearranged.

To test for parental bias in the allelic choice for initial V–(D)–J rearrangement, we first identified SNPs that distinguish C57BL/6 (B6) from FVB/NJ (FVB) and/or DBA/2J (DBA) strains within DNA that is deleted upon V–DJ rearrangement (Supplementary 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 Terb and Igh rearrangement) or from B6×DBA and DBA×B6 F1 adult heterozygotes (for Terg rearrangement) by fluorescent-activated 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, 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 associated with the SNP (see Methods and Supplementary Figures 1 and 2). Representative results are shown in Figure 2 and demonstrate the absence of parent-of-origin bias for each of the genes examined; that is, neither the parental nor the maternal Terb, Terg or Igh alleles were differentially depleted by the initial recombination event. For example, in the case of Terb 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
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 TCRγ cells (γδ TCR⁺), purified TCRδ cells (TCRδ⁺), γδ TCR⁺, CD4⁺, 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, genomic DNAs were amplified across known SNPs, digested with informative restriction enzymes and electrophoresed. Four total analyses were carried out 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 gray boxes) relative to cells that did not undergo recombination. Recombination of the Tcrβ locus will have occurred in CD4⁺ and CD8⁺ cells, but not in tail or in TCRδ 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 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 heterozygotes as 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 ± s.e.m. (a) For Tcrg, n = 4 (2 B6 × FVB and 2 FVB × B6). (b) For Tcrg, n = 4 (2 B6 × DBA and 2 DBA × B6). (c) For IgH, n = 8 (2 B6 × FVB, 2 FVB × B6, 2 B6 × DBA and 2 DBA × B6). For each locus, the dark gray bars depict the cell population that will have undergone V–DJ recombination. Non-recombined cells are shown in light gray. 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.

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(D)J recombination at the Tcrb, Tcrd 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 have 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, as 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 as their biology and ontogeny are distinct from αβ T cells and the rules governing allelic exclusion of Terg 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 development29 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 (Bar Harbor, ME, USA) 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 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, San Jose, CA, USA) based on the following staining profiles: CD4 T cells (CD4*Tcrb+), CD8 T cells (CD8*Tcrb+), gdT cells (CD4*CD8*gTcr*), B cells (CD19* IgM*). Peritoneal lymphocytes were harvested as described and the following cell populations were purified by cell sorting: B1a (CD19*B20*CD5*), Bibb (CD19*B20*CD5*) and B2 (CD19*B20*CD5*).

SNPs and quantitation of alleles

Candidate SNPs at the Tcrb, Tcrd, Terg 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 (Supplementary 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 (Supplementary Figure 1) and to identify changes in allelic usage (Supplementary Figure 2). For Figure 2, PCR amplification was by OneTag Quick-Load 2× MM with Standard Buffer (NEB MO486S) (35 cycles with an annealing temperature of 58°C). PCR products were purified using the QiaQuick Purification Kit (Qiagen, Boston, MA, USA) and ~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 Supplementary Table 1. Pyrosequencing assays were performed by EpigenDx (Hopkinton, MA, USA). Sequencing was carried out using Pyrosequencing PSQ96HS System (Qiagen) and results were analyzed with the PSQ software (Qiagen). See Supplementary Table 1 for gene-specific details.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

Research was supported by the intramural program of the Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health. Project numbers: ZIA HD001804 (to KP) and ZIA HD001803 (to PEL).

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