

Mitochondrial DNA segregation in hematopoietic lineages does not depend on MHC presentation of mitochondrially encoded peptides

Brendan J. Battersby, Margaret E. Redpath and Eric A. Shoubridge*

Department of Human Genetics, Montreal Neurological Institute, McGill University, Montreal, Quebec, Canada

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Mutations in mitochondrial DNA (mtDNA) are associated with a broad spectrum of clinical disorders. The segregation pattern of pathogenic mtDNAs is an important determinant of both the onset and the severity of the disease phenotype, but the mechanisms controlling mtDNA segregation remain poorly understood. To investigate this, we previously generated heteroplasmic mice containing two different mtDNA haplotypes and showed that BALB/c mtDNA was invariably selected over NZB mtDNA in blood and spleen. Here, we have characterized this process in hematopoietic tissues and tested whether it involves the presentation of mtDNA-encoded peptides by MHC class Ib molecules. Selection against NZB mtDNA was widespread across different hematopoietic cell lineages and proportional to heteroplasmy levels. Backcrossing heteroplasmic mice with CAST/Ei, a strain in which the MHC class Ib molecule H2-M3 is silent, completely abolished selection against NZB mtDNA in the spleen. To test whether this effect depended on an intact immune system, we generated heteroplasmic mice missing functional copies of *Tap1*, β_2m or *Rag1* to impair presentation or recognition of mtDNA-encoded peptides. The kinetics of selection against NZB mtDNA were unaltered in these mice compared with their wild-type littermates. We conclude that mtDNA selection in hematopoietic tissues is not based on an immune mechanism, but likely involves metabolic signaling.

INTRODUCTION

Mammalian mitochondrial DNA (mtDNA) is a small, maternally inherited genome that codes for 13 polypeptides in the mitochondrial respiratory chain (1). The copy number of mtDNA is high, ranging from hundreds to thousands of copies in most somatic cells. Typically, individuals harbor a single sequence variant of mtDNA (homoplasmy). Germline or somatic mutations in mtDNA lead to the co-occurrence of two or more sequence variants in a cell (heteroplasmy). Replication of mtDNA is not coupled with the cell cycle, therefore the segregation of two or more sequence variants during mitosis is a stochastic process. In the absence of selection, the process of mtDNA segregation is a function of mtDNA copy number and turnover (2). Selective pressures, such as mtDNA replication efficiency or oxidative phosphorylation function, can alter the segregation of mtDNA sequence variants.

Pathogenic mutations in mtDNA cause a heterogeneous group of multisystem disorders, often associated with neurological or neuromuscular disease (1). Most mtDNA mutations are heteroplasmic and the segregation pattern of mutants is an important factor in the onset and severity of the disease phenotype (1,3). In the female germline, the segregation of pathogenic mtDNA mutations appears to be a largely stochastic process (4). The factors that govern tissue-specific segregation of mtDNA sequence variants are not well-understood. In patient tissues, the segregation of mtDNA mutants varies with the specific mutation, cell type and nuclear background, and generally does not correlate well with the degree of mitochondrial dysfunction (1,3).

To investigate the mechanisms of mtDNA segregation, we previously constructed heteroplasmic mice segregating two polymorphic mtDNA sequence variants derived from two old, inbred strains of mice (BALB/c and NZB/BinJ, hereafter referred to as BALB/c and NZB) (5). We observed

*To whom correspondence should be addressed at: Department of Human Genetics, Montreal Neurological Institute, McGill University, 3801 University Street, Montreal, Quebec H3A 2B4, Canada. Tel: +1 5143981997; Fax: +1 5143981509; Email: eric@ericpc.mni.mcgill.ca

tissue-specific and age-dependent directional selection for different mtDNA haplotypes in the same animal: selection for the NZB haplotype in the liver and kidney, against the NZB haplotype in the spleen and blood, while all other tissues were neutral (6). In the liver, we demonstrated that selection for the NZB genotype occurs at the level of the mitochondrial genome and is independent of DNA replication efficiency or oxidative phosphorylation function (7). Moreover, we mapped three strong quantitative trait loci (QTLs) involved in tissue-specific mtDNA segregation, showing that nuclear-encoded genes can modulate the segregation of mtDNA sequence variants (8). These results suggest that the mechanisms regulating mtDNA segregation involve a complex nuclear-mitochondrial genetic interaction that is modulated in a tissue-specific manner.

Mitochondrially encoded peptides can be exported from mitochondria and presented on the cell surface as minor histocompatibility (H) antigens by MHC class Ib molecules. Originally described as the maternally transmitted antigen in mice, it was found that an mtDNA-encoded ND1 peptide could act as a target to cytotoxic T lymphocytes (CTLs) triggering an immune response (9,10). In total, four allelic variants of the ND1 peptide have been described among *Mus musculus domesticus* and wild mice, all of which differ at the sixth amino acid residue. The most common variant is the α form, which is present in most inbred *domesticus* strains, such as BALB/c, whereas the NZB mtDNA genotype encodes the β variant (10). Thus, an allograft of isogenic cells, carrying the β variant, into a BALB/c host should trigger a CD8+ T cell response. These observations suggested that directional selection against NZB mtDNA in our heteroplasmic mice could be an immune response mounted against the presentation of NZB mtDNA-encoded peptides in hematopoietic tissues. Such a mechanism would likely have to involve a differential, time-dependent expression of peptides, resulting in a T cell repertoire selected on and hence tolerant to one set of peptides (BALB/c) and subsequently confronted with and reacting to the other (NZB). An analogous mechanism has been evoked for autoimmune responses against the SV40 large T antigen driven off the insulin promoter in transgenic mice (11).

For the correct processing of mitochondrial peptides as self-antigens, peptides need to be transported into the ER and then bound by the MHC class Ib molecule H2-M3, in order to migrate to the cell surface. This mechanism for mitochondrial peptide processing is similar to the pathway used by MHC class Ia molecules, except that H2-M3 remains trapped in the ER in the absence of peptide, even at low temperatures (12). To elicit the surface expression, H2-M3 requires peptide binding to stabilize the interaction with β_2 -microglobulin (β_2m) (13,14). Thus, the rate-limiting step for H2-M3 surface expression is peptide delivery (14). H2-M3 is also important in host defense against intracellular bacterial pathogens, such as *Listeria monocytogenes* (15,16). Most mouse strains have the wild-type allele of H2-M3, a notable exception being *Mus musculus castaneus*, in which a single missense mutation prevents peptide binding, rendering the allele functionally null (17).

In this study, we have investigated the basis for mtDNA segregation in hematopoietic tissues. We tested whether selection against NZB mtDNA is widespread across different cell

Table 1. Representative profiles of mtDNA segregation from heteroplasmic mice with age in different leukocyte cell populations

Tissue	Age (months)						
	4	4	4	9.5	9.5	13	21
	% NZB mtDNA						
Heart	62	68	46	29	34	36	25
Liver	86	89	83	88	89	92	92
Spleen	44	47	29	12	11	9	4
Leukocytes	37	42	24	7	5	4	2
CD45R+	37	40	22	6	5	6	2
CD3+	40	45	25	8	7	11	4
GR-1+	37	41	25	5	5	3	3
MAC-1+	38	43	23	8	5	3	1

Heteroplasmy levels for the heart (neutral for mtDNA segregation) and the liver (selects NZB genotype) are also listed.

lineages and tissues of the hematopoietic system. In addition, we tested the hypothesis that selection against the NZB mtDNA haplotype is dependent on a CTL-mediated response against hematopoietic cells presenting peptides encoded by NZB mtDNA. We demonstrate that selection against NZB mtDNA is proportional to the level of heteroplasmy, widespread in hematopoietic tissues, but independent of the MHC class Ib presentation of mitochondrial peptides.

RESULTS

Selection against the NZB mtDNA haplotype is widespread in hematopoietic cell lineages

In our heteroplasmic mouse model, tissue-specific mtDNA segregation does not begin until post-natal life. At birth, there are no significant differences in the level of mtDNA heteroplasmy in any tissues. In the spleen and peripheral blood, there is invariable selection against the NZB mtDNA haplotype with age. To test whether this selection was widespread or restricted to particular cell lineages of the hematopoietic system, we sorted leukocytes from the spleen into different cell populations and determined the mtDNA heteroplasmy level. Cells were sorted using surface markers into populations of T cells (CD3+), B cells (CD45R+), granulocytes (GR1+) and macrophages (CD11b/Mac1+). There was little variation in the heteroplasmy levels among any of these cell lineages (Table 1). As the mice aged, selection against the NZB mtDNA genotype was similar across all cell lineages even though the life cycle of these different cell lineages varies enormously, in both population size and rate of turnover. These data suggest a common mechanism for selection against NZB mtDNA in hematopoietic cells.

mtDNA selection in the peripheral blood is proportional to the level of heteroplasmy

To investigate the kinetics of mtDNA selection in the hematopoietic system, we serially sampled the peripheral blood from mice with different levels of mtDNA heteroplasmy every 2 weeks beginning at 30 days of age and ending at 128 days.

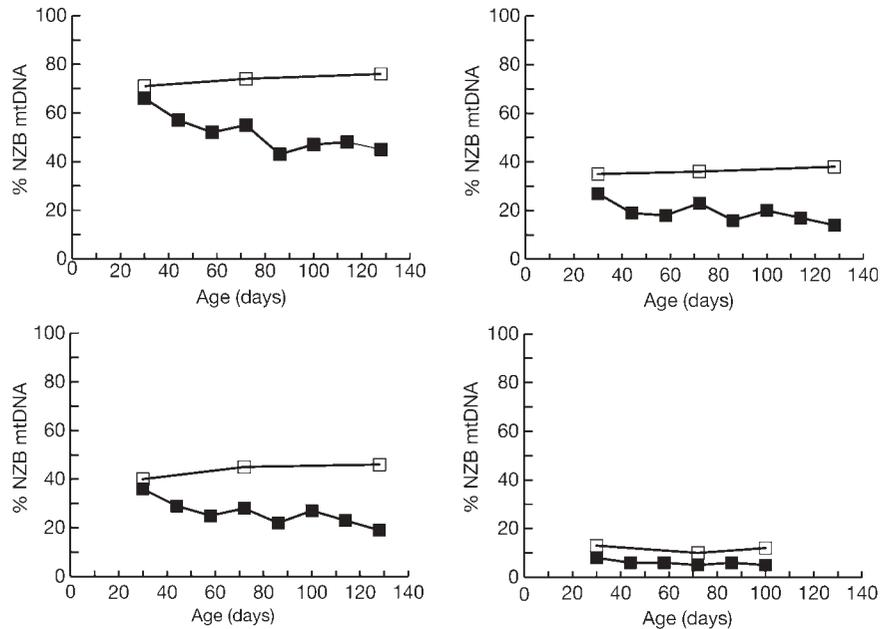


Figure 1. Representative profiles of mtDNA segregation in the peripheral blood of serially sampled heteroplasmic mice within the first 130 days of post-natal life. Filled boxes represent the NZB heteroplasmy level in the blood and open boxes represent the NZB heteroplasmy level in the tail.

We also collected tail biopsies at 30, 72 and 128 days as a measure of neutral mtDNA segregation. Selection against the BALB/c mtDNA haplotype was not detectable until after weaning (day 30). At post-natal day 86, the proportion of NZB mtDNA in peripheral leukocytes had decreased by half when compared with the initial starting frequency (Figs 1 and 2). Between day 86 and 128, there was little change in the heteroplasmy level in the blood, suggesting that selection against NZB mtDNA had reached an asymptote (Fig. 1). At day 128, we collected samples from the peripheral blood, spleen, thymus and bone marrow to determine the mtDNA heteroplasmy level. The decrease in NZB mtDNA across these hematopoietic tissues was similar (data not shown), suggesting that the mechanism for selecting against NZB mtDNA occurs with the same kinetics across all tissues.

To assess how the rate of selection against NZB mtDNA continued beyond 128 days, we sampled the spleen and heart from mice between 91 and 270 days to compare the proportion of NZB mtDNA in these tissues (Fig. 2). Plotting the proportion of NZB in the spleen to the heart (a neutral tissue) showed an $\sim 50\%$ decrease within a 100 days, similar to that observed in the peripheral blood, but over the next 200 days, the decrease in the proportion of NZB mtDNA was more gradual than that observed in the first 100 days (Fig. 2). One of the peculiar aspects of mtDNA segregation in the hematopoietic tissues of these mice is that the cells or tissues of the hematopoietic system never fix for the BALB/c mtDNA haplotype, even in mice born with a high percentage of the BALB/c genotype (Fig. 1), or in very old mice (data not shown). Using both data sets, we can model the selection against the NZB mtDNA haplotype as a decaying exponential function ($y = 0.8136e^{-0.0038x}$, $r^2 = 0.833$; where y is the proportion of NZB and x is the age in days).

Selection against NZB mtDNA in the spleen is lost on a *M. musculus castaneus* nuclear background

We previously demonstrated that there is nuclear-genetic control of tissue-specific mtDNA segregation. Using an inter-cross mating strategy with *M. musculus castaneus* (CAST/Ei), we were able to map a QTL (*Smdq3*) on chromosome 6 that controlled the rate of mtDNA segregation in the spleen (8). The CAST/Ei *Smdq3* allele was found to reduce the rate of selection against NZB mtDNA in the spleen. To further investigate the behavior of the CAST/Ei allele, we outcrossed our BALB/c heteroplasmic females with CAST/Ei males and then backcrossed with CAST/Ei males. After N8 backcross generations, we analyzed the tissues of heteroplasmic mice. Surprisingly, we found that in the spleen, selection against NZB mtDNA was completely lost (Fig. 3) demonstrating that in the spleen, mtDNA segregation can be regulated in a binary as well as a quantitative manner. In the liver (Fig. 3) and kidney (data not shown), selection for the NZB mtDNA haplotype occurred as in the *domesticus* background, although at a slower rate. This result suggests that the genetic regulation of mtDNA segregation in hematopoietic tissues is a more complex process when compared with that of the liver or kidney.

Presentation of mitochondrial peptides has no role in mtDNA segregation

As mitochondrially encoded peptides can be presented as self-antigens by the MHC class Ib molecule H2-M3 and as mtDNA selection was lost in the spleen on the CAST/Ei background, we hypothesized that the silent CAST/Ei H2-M3 allele (17) might underlie the phenotype in the CAST/Ei nuclear background. The CAST/Ei H2-M3 allele (L95Q

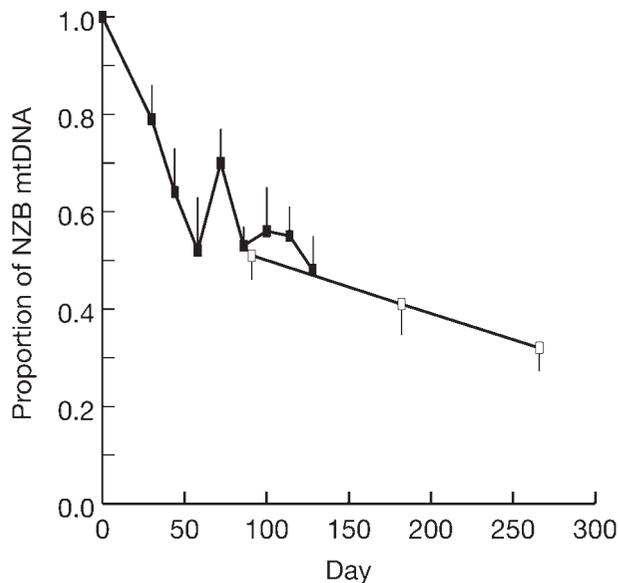


Figure 2. Selection against NZB mtDNA in hematopoietic tissues of heteroplasmic mice. Proportion of NZB heteroplasmy in hematopoietic tissues when compared with neutral tissues with age. Filled boxes represent the mean ($n = 9$; SD) proportion of NZB in the blood compared with the tail of serially sampled mice. Open boxes represent the mean ($n = 7$; SD) proportion of NZB mtDNA in the spleen compared with the heart in independent sets of heteroplasmic mice.

polymorphism) can be transcribed and translated efficiently; however, it is unable to bind peptides and remains in its docked position in the ER (12,13,17). The expression of the *domesticus* H2-M3 variant on the cell surface is undetectable in most cell types, because peptide delivery to this MHC class Ib molecule is either rate limiting or non-existent (12). However, H2-M3 can be found on the cell surface of a variety of hematopoietic cell types (12), suggesting that the presentation of NZB mtDNA-encoded ND1 in these cell lineages could trigger a CD8⁺ T cell response that might drive mtDNA segregation.

It is currently unknown how the kinetics of mitochondrial peptide processing to the ER is regulated when there are two mtDNA haplotypes in a cell. Whether peptides from both mtDNA haplotypes (BALB/c and NZB) are used during positive and negative selection of T cells is unclear, although cultured heteroplasmic cybrid (cytoplasmic hybrid) cells can present N-formylated peptides from both mtDNA haplotypes (18).

To test the mtDNA peptide presentation hypothesis, we generated heteroplasmic mice missing genes essential for presentation and recognition of mitochondrially encoded peptides. The presentation of mitochondrial peptides is similar to that of classical MHC class Ia molecules (12). Mitochondrial peptide transfer into the ER is dependent on the heterodimer ATP binding cassette transporter, TAP (12,14,19). The binding of N-formylated peptides by H2-M3 stabilizes the interaction with β_2m , allowing this oligomeric complex to be released from its docked position in the ER and migrate to the cell surface (12,14). To generate mice, incapable of peptide transport or MHC trafficking to the cell surface, we outcrossed heteroplasmic mice on a BALB/c background to

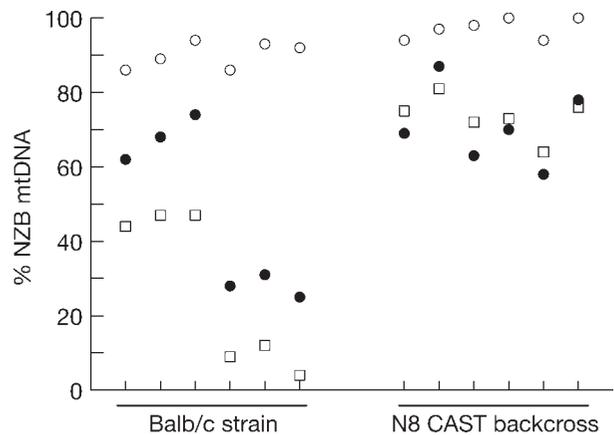


Figure 3. Selection against NZB mtDNA in the spleen is lost on a CAST/Ei nuclear background. Representative profiles of mtDNA heteroplasmy levels in tissues from mice on a BALB/c or a CAST/Ei (after N8 of a backcross) nuclear background. All mice on the BALB/c nuclear background demonstrate a lower level of NZB mtDNA in the spleen when compared with the initial heteroplasmy level (heart). This decrease in NZB mtDNA in the spleen is shown for representative mice on a BALB/c background with either a high initial starting frequency of NZB (60–75%, heart) or a low initial level (25–30%, heart). In contrast, mice on the CAST/Ei background have no decrease in the NZB mtDNA heteroplasmy level of the spleen when compared with the heart. Filled circles, heart; open circles, liver and open squares, spleen.

mice homozygous null for *Tap1* (20) or β_2m (21). The resulting F1 progenies were intercrossed, and F2 littermates were compared to reduce any potential nuclear background effects (although we have previously demonstrated that different *domesticus* backgrounds have no effect on mtDNA segregation).

The comparison of heterozygous and homozygous, *Tap1* and β_2m , mutants with their wild-type littermates showed that neither mutation had an effect on mtDNA segregation in the spleen, thymus or bone marrow of heteroplasmic mice (Figs 4 and 5). The data were plotted as a scatter plot between the hematopoietic tissue and the heart, which is neutral with respect to mtDNA segregation. If selection against NZB mtDNA was lost, then we would predict that the slope of the data would be 1 (plotted as a straight line in Figs 4 and 5). A slope of less than 1 would be predicted for negative selection of NZB mtDNA. In all cases, the slope of the data from the knockout mice is less than 1 and indistinguishable from that of heterozygous or wild-type littermates (Figs 4 and 5). In the liver, there was no effect on the relative fitness of the NZB mtDNA haplotype (data not shown).

Mitochondrially encoded peptides are presented by a MHC class Ib molecule and hence could potentially trigger an immune response by CD8⁺ T cells. A cytotoxic response of CD8⁺ T cells targeting hematopoietic cells with a high content in NZB-derived mtDNA and, consequently, a high number of H2-M3/NZB-derived peptide complexes on their surface could result in the progressive disappearance of such hematopoietic cells. To rule out this possibility, we determined whether the elimination of mature T cells played a role in the segregation of mtDNA. To generate heteroplasmic mice missing a complete T cell repertoire, we outcrossed our

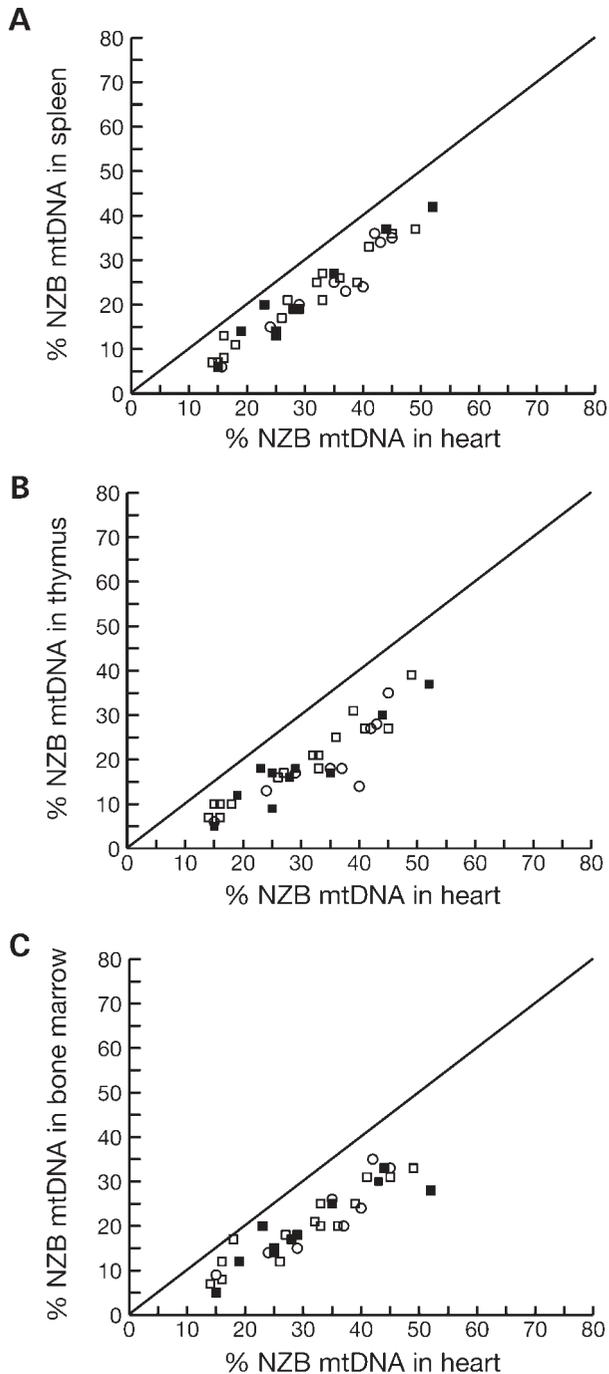


Figure 4. Loss of mitochondrial peptide transport into the ER of F2 *Tap1* mutant mice has no effect on mtDNA segregation. mtDNA heteroplasmy levels in the spleen (A), thymus (B) and bone marrow (C) compared with the heart in F2 littermates at 6 weeks of age. A slope of 1 (straight line) would indicate no segregation of mtDNA. Open circles, wild-type; open squares, heterozygous and filled squares, mutant.

heteroplasmic mice with *Rag1* knockout males (22) on a BALB/c nuclear background, then intercrossed F1 offspring and compared with F2 littermates. Even though the number of non-erythroid cells in the spleen of mutant *Rag1* mice is 5–9-fold lower when compared with wild-type littermates (22), there was still selection against the NZB haplotype

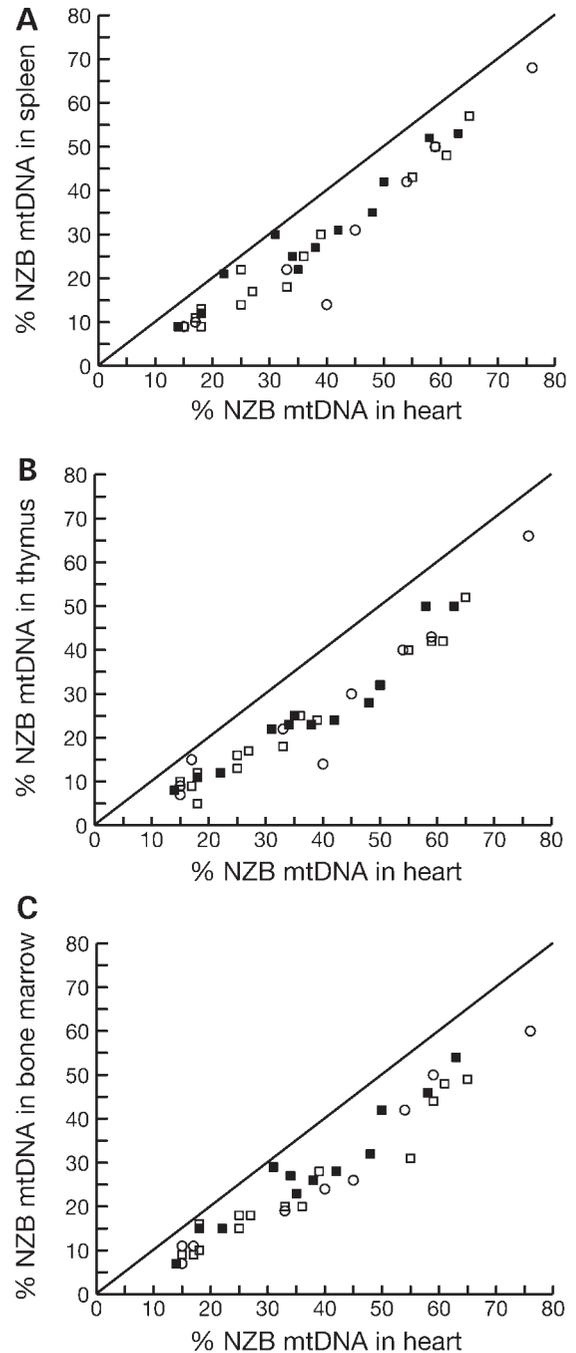


Figure 5. Loss of MHC class I presentation on the cell surface of F2 β_{2m} mutant mice has no effect on mtDNA segregation. mtDNA heteroplasmy levels in the spleen (A), thymus (B) and bone marrow (C) compared with the heart in F2 littermates at 6 weeks of age. A slope of 1 (straight line) would indicate no segregation of mtDNA. Open circles, wild-type; open squares, heterozygous and filled squares, mutant.

(Fig. 6). Enrichment of the remaining splenocytes from the spleens of mutant mice revealed that selection against NZB mtDNA was no different than that in wild-type and heterozygous littermates (Fig. 6). These data were also plotted as a scatter plot. The slope of the *Rag1* knockout mice was less than 1 and indistinguishable from wild-type and heterozygous

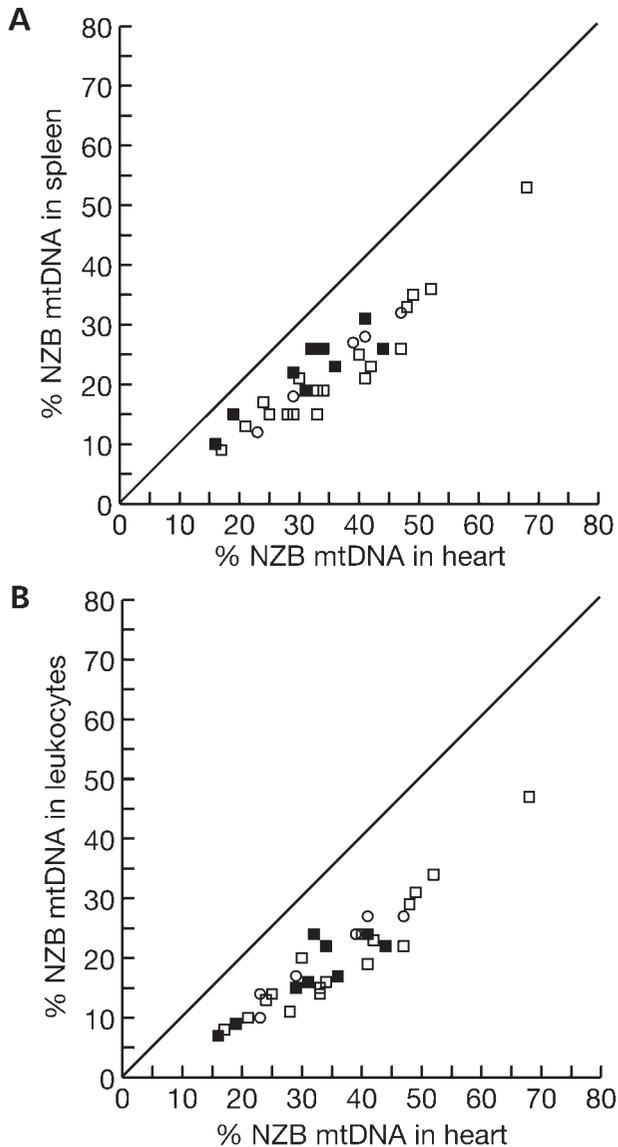


Figure 6. Loss of mature B cell and T cell repertoires in F2 *Rag1* mutant mice has no effect on mtDNA segregation. mtDNA heteroplasmy levels in the spleen (A) and leukocytes (B) compared with the heart in F2 littermates at 20 weeks of age. A slope of 1 (straight line) would indicate no segregation of mtDNA. Open circles, wild-type; open squares, heterozygous and filled squares, mutant.

littermates (Fig. 6). Collectively, these data suggest that negative selection of the NZB mtDNA haplotype in the hematopoietic system does not involve an MHC class I-mediated immune response directed against mitochondrial peptides.

DISCUSSION

In this study, we characterized a type of tissue-specific mtDNA segregation in the hematopoietic system of heteroplasmic mice and tested the hypothesis that selection against one of the mtDNA haplotypes (NZB) is driven by an immune response directed against the presentation of mitochondrially-encoded

peptides from the NZB mtDNA haplotype, a known maternally inherited antigen.

In the spleen, we found that selection against the NZB mtDNA haplotype occurred in both myeloid and lymphoid cell populations. The level of NZB mtDNA heteroplasmy was similar in these cell lineages and in other hematopoietic tissues, suggesting that there exists a common mechanism for selection against the NZB mtDNA haplotype. It is unlikely that selection results from a genetic program activated during hematopoietic development, as mtDNA segregation is only observed post-natally in this model (6).

The kinetics of mtDNA segregation in the peripheral blood and spleen suggest that the rate of selection against NZB mtDNA is proportional to the level of heteroplasmy and does not require a particular threshold level of NZB mtDNA. Thus, mice with a high level of NZB mtDNA experience a greater decrease in the absolute level of NZB when compared with mice with a low level, but the proportional decrease is the same in both cases. Because selection is proportional to the level of heteroplasmy, hematopoietic cells never fix the BALB/c mtDNA haplotype. This pattern of mtDNA segregation contrasts sharply with that observed in the liver of the same heteroplasmic mice, where selection for the NZB mtDNA haplotype is independent of initial haplotype frequency and constant with time (7). Moreover, the hepatocytes of these mice eventually become homoplasmic for the NZB mtDNA haplotype. We have argued that a constant rate of selection for one or the other haplotype implies a mechanism that acts directly on the mtDNA genome itself. *In vivo* BrdU-labeling of mtDNA suggests that this does not result from differential rates of synthesis, but rather acts at the level of the turnover of mtDNA (7). The proportional decrease in NZB mtDNA that we observe in the hematopoietic system suggests that selection occurs at the level of the organelle or cell. We favor a mechanism that operates at the level of the organelle for two reasons. First, the same pattern is observed in cells with very different life cycles. Secondly, we have been unable to demonstrate a functional difference in oxidative phosphorylation efficiency, as assessed by coupled respiration rates with a variety of substrates, in cells essentially homoplasmic for either of the two mtDNA haplotypes (7).

There are few longitudinal studies on mtDNA segregation in patients harboring pathogenic mtDNA mutations. In one report of the A3243G mtDNA mutation usually associated with MELAS (mitochondrial encephalomyopathy, lactic acidosis, stroke-like episodes), there was a slow decrease of the mutant load in the blood of patients over many years, which was attributed to selection against cells with respiratory chain dysfunction (23). However, the same A3243G mutation has also been shown to increase with age in post-mitotic tissues and leads to clearly distinct clinical presentations based on the segregation pattern of mtDNA and the nuclear background of the patients (24–27). Further, there is one reported case in which a decrease in the proportion of the A3243G mutation in muscle with time was associated with marked clinical improvement (28). Thus, no clear segregation patterns can be discerned for pathogenic mtDNA sequence variants, as they can be variously influenced by mtDNA haplotype, tissue and nuclear background.

As BALB/c and NZB mtDNA encode two different ND1 allelic variants, we wanted to test whether the presentation of the NZB ND1 peptide in hematopoietic tissues was responsible for selection of the BALB/c mtDNA haplotype. Our results illustrate that if we impair the presentation or recognition of mitochondrial peptides on the cell surface, there is no effect on mtDNA selection in this model. These data also support the idea that peptides from both mtDNA genotypes are used during positive and negative selection of T cells and result in immune tolerance for both sets of peptides. Thus, the loss of mtDNA segregation in the spleen of CAST/Ei mice is not due to the silent allele of H2-M3, but must involve another genetic mechanism.

Recently, a number of human births have been reported that have resulted from assisted reproductive technologies involving transfer of oocyte cytoplasm prior to *in vitro* fertilization (29). These children are demonstrably heteroplasmic for two different, presumably neutral, sequence variants that are likely to differ by at least 50 bp, similar to the mice we constructed. As this situation is never observed in normal human biology, concern has been raised about the physiological consequences of this genetic manipulation. It is possible that tissue-specific segregation of different mtDNA haplotypes could occur in these individuals; however, the aforementioned results suggest that it is unlikely that they will develop immune intolerance to peptides, derived from one or the other mtDNA haplotypes.

What type of mechanism could trigger-specific mtDNA segregation in hematopoietic tissues in which selection apparently acts on the cell or on the organelle? We have ruled out mechanisms that can act at the level of the cell, such as an immune response against NZB-encoded peptides. Another possible mechanism could still involve mitochondrial peptides, but as intracellular signaling molecules and not as self-antigens. In *Saccharomyces cerevisiae*, there is a continual efflux of mitochondrial peptides from mitochondria, even though mitochondrial proteases are capable of digesting mitochondrial peptides to completion (30). This is not a random process, as 40% of the total peptide efflux derive from two proteins, Nde1 which is encoded in the nucleus and Cox1 which is mtDNA-encoded (31). This mitochondrial peptide export in yeast suggests a mechanism for relaying information, perhaps in a regulatory role, as yeast cells do not need to present antigens on the cell surface. Bacteria are known to use peptide export–import as a control circuit to regulate processes such as nutrient uptake and sporulation (32). It is possible that mitochondria also use peptide export in some regulatory capacity, which has been conserved among eukaryotes.

In conclusion, we demonstrate that selection against NZB mtDNA in the hematopoietic system is widespread, proportional to the level of heteroplasmy and does not involve an MHC class I-mediated immune response against mtDNA-encoded peptides. Although the genetics of mtDNA segregation appear to be a complex nuclear–mitochondrial interaction that is regulated in a tissue-specific manner, the loss of mtDNA selection in the spleen on a CAST/Ei background should enable us to genetically dissect additional nuclear genes involved in the genetic regulation of mtDNA segregation.

MATERIALS AND METHODS

Mouse breeding and genotyping

Heteroplasmic mice were maintained on a BALB/c nuclear background by brother–sister matings. To generate heteroplasmic mice on a *castaneus* background, we outcrossed female BALB/c mice, heteroplasmic for NZB and BALB/c mtDNA, with male *M. musculus castaneus* (CAST/Ei, Jackson Labs) to produce F1 pups and then successively backcrossed female pups with CAST/Ei males until N8. Heteroplasmic mice defective in MHC class I presentation were generated by outcrossing female heteroplasmic mice with *Tap1* (B6.129S2-*Tap1*^{tm1Atp}) and β_2m mutants (B6.129P2-B2 *m*^{tm1Unc}) (Jackson Labs) to produce F1 pups. Heteroplasmic mice missing a full B cell and T cell repertoire were generated by mating female heteroplasmic mice with mutant *Rag1* males (C.129S7(B6)-*Rag1*^{tm1Mom}). Mutant mice were genotyped according to the procedures at Jackson Labs. To control the background effects, F1 pups were intercrossed and F2 littermates were analyzed. Tissues were collected from mice and DNA was extracted by conventional methods or with a Qiagen DNAeasy kit. Peripheral blood was sampled every 2 weeks from the tail vein of mice and DNA was extracted by conventional methods. These studies were approved by the McGill University Animal Care Committee.

To determine the mtDNA heteroplasmy level in tissues, we used polymerase chain reaction (PCR) to amplify a fragment that encompasses a polymorphic site at position 3691 in the ND1 gene present in BALB/c, but absent in NZB mtDNA. This polymorphism eliminates an *RsaI* site, which allows a restriction fragment length polymorphism analysis of the PCR product. Total genomic DNA was amplified using standard conditions and the following primers: forward, nt 3571–3591, 5'-GAGCATCTTATCCACGCTTCC-3'; reverse, nt 4079–4059, 5'-CTGCTTCAGTTGATCGTGGGT-3' as follows: 94°C, 30 s; 55°C, 30 s and 72°C, 30 s for 30 cycles. In the last cycle, 1.5 μ Ci of [α -³³P]dCTP was added to the reaction to radiolabel the PCR product. A 10 μ l aliquot of this reaction was cut with 10 U of *RsaI* overnight and run on a 10% non-denaturing polyacrylamide gel. Gels were analyzed on a Molecular Dynamics Storm Phosphorimager.

Cell sorting

Leukocyte cell suspensions were prepared from spleens from heteroplasmic mice at different ages as described (33). Cells (5×10^6) were incubated with one of the following primary antibodies (PharMingen) anti-CD3, anti-CD45R, anti-Gr1 and anti-CD11b/Mac1+ in phosphate-buffered saline (PBS) and 5% bovine serum albumin (BSA) for 10 min at room temperature. Cells were washed in PBS, 5% BSA and resuspended in PBS, 0.5% BSA, 2 mM EDTA, pH 7.2 and incubated with anti-rat IgG Microbeads (Miltenyi Biotec) for 10 min at 4°C. Cells were washed and separated on a MACS-MS column (Miltenyi Biotec) and then analyzed.

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Conflict of Interest statement. None declared.

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