The mtDNA nt7778 G/T polymorphism affects autoimmune diseases and reproductive performance in the mouse

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Mitochondria are organelles of all nucleated cells, and variations in mtDNA sequence affect a wide spectrum of human diseases. However, animal models for mtDNA-associated diseases are rare, making it challenging to explore mechanisms underlying the contribution of mitochondria. Here, we identify a polymorphism in the mitochondrial genome, G-to-T at position 7778, which results in an aspartic acid-to-tyrosine (D-Y) substitution in the fifth amino acid of the highly conserved N-terminus of ATP synthase 8 (ATP8). Using a series of conplastic strains we show that this polymorphism increases susceptibility to multiple autoimmune diseases, including collagen-induced arthritis, autoimmune diabetes, nephritis and autoimmune pancreatitis. In addition, it impairs reproductive performance in females, but only in the MRL/MpJ strain. We also demonstrate that the mtAtp8 polymorphism alters mitochondrial performance, increasing H2O2 production and affecting mitochondrial structure. Functional analysis reveals that the polymorphism increase the CD4 T cell adaptive potential to an oxidative phosphorylation impaired condition. Our findings provide direct experimental evidence for the role of mitochondria in autoimmunity and reproduction.

INTRODUCTION

The mammalian mitochondrial genome (mtDNA) is a closed circular double-stranded DNA with genes encoding mitochondrial oxidative phosphorylation (OXPHOS) components (1). Compared with the nuclear genome, mammalian mtDNA is characterized by several unique features, including lack of recombination, exclusively maternal inheritance, high number of mtDNA copies per cell, high mutation rate and different codon usage (2). Human mtDNA variations are associated with a wide spectrum of diseases, either as specific mutations or deletions that cause particular disorders, or as predisposing factors in polygenic diseases (2,3). However, confirming the role of mtDNA variations in human complex diseases and exploring the underlying molecular mechanisms are challenging due to multiple factors, such as unpredictable consequence of heteroplasmic mtDNA variations, heterogeneity of the nuclear genome and interaction between mtDNA and nuclear DNA (nDNA) (4). Animal models represent a solution to the complexity of mtDNA variations in humans. However, due to unique genetic characters, e.g. lack of recombination, multiple copies and compartmentalization, mtDNA is much more difficult to be manipulated genetically than nuclear genes. Therefore, the identification of novel disease-related mtDNA variations in animals offers a valuable opportunity to investigate the biochemical and physiological roles of mitochondria in complex diseases. A good example is the role of mtDNA in type II diabetes. Many reports
suggested that mtDNA variations are associated with type II diabetes (5). Recently, Pravenec and his colleagues experimentally linked mitochondrial genome variations to type 2 diabetes using conplastic strains carrying spontaneous mtDNA mutations, thus providing an animal model to study the underlying molecular mechanism (4).

Here previous phylogeny studies demonstrated that mouse classic inbred strains were descended from a single female Mus. mus. domesticus mouse and that some mtDNA mutations occurred during their establishment (6,7). This provides a unique opportunity to investigate the impact of individual mammal mtDNA variations on complex diseases in vivo. Like nDNA-encoded quantitative trait genes, complex disease associated mtDNA polymorphism is a predisposition for common disease (2). Therefore, when in a heterogeneous nuclear genome background, detection of complex disease associated mtDNA variation demands large size samples. Previously, we performed two genome-wide linkage analyses in (DBA/1J × FVB/NJ)F2 and (B10.S/J × SJL/J)F2 mice to search for quantitative trait loci controlling collagen induced arthritis (CIA) and experimental autoimmune encephalomyelitis, respectively (8,9). The F2 progeny were generated by two reciprocal crosses with either parental strains as parental grandmothers. Thus, these large numbers of F2 progeny provide an opportunity to evaluate the effect of mtDNA variation on complex diseases. In this study, we investigated the effect of mtDNA variation in F2 progeny and in conplastic strains. We identified a novel mtDNA polymorphism affecting autoimmune diseases and female reproductive performance.

RESULTS

Identification of the mtAtp8 polymorphism

We generated a (DBA/1J × FVB/NJ)F2 progeny to identify genetic loci regulating murine CIA. The incidence of the disease in the two parental strains were 100% (DBA/1J) and 0% (FVB/NJ), respectively (8). After backcross 33% of F2 mice developed arthritis (8). To evaluate the effect of mitochondrial genotype on CIA, we divided F2 mice into two groups according to their mtDNA. No significant difference in the disease incidence was observed between the two groups (data not shown). However, we observed that diseased mice carrying FVB/NJ mtDNA showed more severe arthritis than those with the DBA/1J mtDNA, especially in the chronic phase of the disease (Fig. 1A). This indicates that FVB/NJ mtDNA increases susceptibility to CIA. To confirm its arthritis-enhancing effect, we produced conplastic strains in a B10.Q/J genetic background which shows mild susceptibility to CIA (10). We then generated B10.Q/J-mtFVB/N conplastic strain by introducing FVB/NJ mtDNA into B10.Q/J background. We did not generated the reciprocal conplastic strains, FVB/NJ-mtB10.Q/J, because FVB/NJ background is completely resistant to CIA (8). The B10.Q/J-mtFVB/N conplastic strain developed more severe CIA than a B10.Q/J control strain (Fig. 1B), confirming that FVB/NJ mtDNA enhances susceptibility to CIA.

To investigate the molecular basis for this effect we sequenced the complete mitochondrial genomes of these strains. FVB/NJ mtDNA (GQ871746) differs from DBA/1J mtDNA (GQ871745) in two sequence variations, nt7778 G/T and nt9821 A-repeat. There is only a single mtDNA sequence variation, nt7778 G/T, between the FVB/NJ mtDNA and B10.Q/J mtDNA (GQ871744) (Table 1). Thus, we can conclude that the nt7778 G/T variation that results in the substitution of Asp-Tyr (D-Y) in the ATP8 protein may be causative for the arthritis-enhancing effect. We then screened 113 mouse inbred strains for this polymorphism and found that only the FVB/N strain carry the nt7778T allele, suggesting that from an evolutionary point of view it is of recent origin (Supplementary Material, Table S1).

The mtAtp8 polymorphism affects spontaneous autoimmune diseases

To investigate whether the mtAtp8 polymorphism affects spontaneous autoimmune diseases, we generated conplastic strains carrying the polymorphism on the NOD/LtJ (autoimmune diabetes) and MRL/MpJ (lupus nephritis, pancreatitis) genetic backgrounds. In the NOD/LtJ genetic background, a higher incidence of autoimmune diabetes was observed in female NOD/LtJ-mtFVB/N mice when compared with NOD/LtJ mice (Fig. 2A). We then studied the effect of the polymorphism on lupus nephritis in the MRL/MpJ genetic background. Mice were examined at the ages of 12, 18 and 24 weeks, respectively, and kidney specimens were evaluated. At 24 weeks, MRL/MpJ-mtFVB/N mice developed much more severe interstitial nephritis compared with MRL/MpJ controls (Fig. 2B). A previous study showed that MRL/MpJ mice develop autoimmune pancreatitis (AIP) characterized by destruction of pancreatic acinar cells with monocellular cell infiltrations. Moreover, the inflammatory lesions were transferable with spleen cells, but not with sera, to young healthy mice, suggesting that pancreatic inflammatory lesions in these mice are mediated by cellular autoimmune mechanisms (11). Therefore, we investigated the effect of the polymorphism on AIP by evaluating the pancreas in the same MRL/MpJ and MRL/MpJ-mtFVB/N mice. MRL/MpJ-mtFVB/N mice developed slightly more severe AIP than MRL/MpJ mice (Fig. 2C). We also evaluated the anti-nuclear antibody (ANA) levels in MRL/MpJ strains. In agreement with the difference in the clinical traits, MRL/MpJ-mtFVB/N mice showed higher ANA levels than the MRL/MpJ controls (Fig. 2D).

The mtAtp8 polymorphism affects female reproductive performance

An unexpected finding was observed when we monitored reproductive performance of the conplastic strains and...
controls in four genetic backgrounds. The ATP8 polymorphism reduced the litter size in the MRL/MpJ genetic background by 40% (Fig. 3A). This effect was not observed in any of the other genetic backgrounds, indicating a specific interaction between mtDNA and the nuclear genome (nDNA) of MRL/MpJ. Further analysis of the control MRL/MpJ-mtB10.Q/J conplastic strain suggested that the mtAtp8 polymorphism is the causal variant in the impaired reproductive performance. Besides reducing the litter size, the polymorphism also lengthened the time period from mating to birth (Fig. 3B). We conclude that this is a maternal effect because male MRL/MpJ mice were used in matings with MRL/MpJ, MRL/MpJ-mtB10.Q/J and MRL/MpJ-mtFVB/N females.

The mtAtp8 polymorphism affects mitochondrial performance

Previous studies in yeast demonstrated that the N-terminal domain of ATP8 is located in the inter-membrane space of mitochondria and is part of the Fo subunit (12,13). Thus, we hypothesized that the polymorphism might affect the phenotype and the function of mitochondria. We firstly investigated the morphology of mitochondria in kidney cells using transmission electron microscopy. Compared with B10.Q/J control, kidney cells from B10.Q/J-mtFVB/N mice showed an altered mitochondrial morphology with a slightly swollen shape (Fig. 4A). Morphometric analysis revealed that the size of mtAtp8 mitochondria was 10% larger than that of control mitochondria (Fig. 4B). However, there was no difference in the numbers of mitochondria per cell between the two genotypes. Also, the protein expression level of ATP synthase and copies of mtDNA were not altered by the polymorphism (Supplementary Material, Fig. S1).

We then investigated whether the polymorphism affected mitochondrial functions. We first assessed the activity of mitochondrial complex I–III and complex V (ATP synthase) in isolated spleen mitochondria. No difference of these parameters was observed between mutant and wild-type mitochondria (Supplementary Material, Fig. S2a and b). In the next step we measured mitochondrial respiration, in terms of state 3:state 4 ratio. The mtAtp8 polymorphism had also no effect on mitochondrial respiration (Supplementary Material, Table 1.

**Table 1. mtDNA variations in common inbred strains**

<table>
<thead>
<tr>
<th>Variation</th>
<th>Gene and amino acid change</th>
<th>C57BL/6J</th>
<th>AKR/J</th>
<th>DBA/1J</th>
<th>B10.Q/J</th>
<th>FVB/NJ</th>
<th>MRL/MpJ</th>
<th>NOD/LtJ</th>
</tr>
</thead>
<tbody>
<tr>
<td>nt7778 G/T</td>
<td>ATP8 Asp-Tyr</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>T</td>
<td>G</td>
<td>G</td>
<td></td>
</tr>
<tr>
<td>nt9348 G/A</td>
<td>COX3 Val-Ile</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>nt9461 T/C</td>
<td>ND3 Met-Met</td>
<td>C</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>nt9821 A repeat</td>
<td>tRNA-Arg</td>
<td>8A</td>
<td>9A</td>
<td>10A</td>
<td>9A</td>
<td>9A</td>
<td>11A</td>
<td>10A</td>
</tr>
</tbody>
</table>

The mtDNA sequence of AKR/J strain was taken as reference sequence. mtDNA sequences of C57BL/6J (NC005089), AKR/J (AB042432), NOD/LtJ (EF108340) and MRL/MpJ (FJ374651) are retrieved from Genebank.
Although there was no difference in the cellular ATP level between the two genotypes, the ATP/ADP ratio was slightly higher in the cells with mutant mitochondria than in control cells (Supplementary Material, Fig. S2d). Finally, we determined the H$_2$O$_2$ production, a by-product of the electron transfer system of OXPHOS. Interestingly, in ATP8 mutant mitochondria H$_2$O$_2$ production increased by $\sim$60% compared with control mitochondria (Fig. 4C). Therefore, we conclude that the ATP8 D-Y polymorphism alters mitochondrial physiology primarily on the level of ROS production with concomitant effects on structure and function.

The mtAtp8 polymorphism affects T cell activation in an OXPHOS impaired condition

Given that CD4 T cells play an essential role in all the four autoimmune diseases affected by the mtAtp8 polymorphism, we were interested whether the mtAtp8 polymorphism affects CD4 T cell activation. It is well-known that oxygen tension varies in different compartments of the body, i.e. $\sim$100 mm of Hg (14% O$_2$) in arterial blood, 20–40 mm of Hg (3–6% O$_2$) within tissues and even lower (<1% O$_2$) in tissues under hypoxia in chronic inflammatory conditions, such as rheumatoid arthritis (14,15). The low oxygen could impair the OXPHOS which is the major source providing ATP for then energy-consuming signal cascades of T cell activation. To investigate the role of the mtAtp8 polymorphism in T cell activation, we cultured cells in the presence of myxothiazol, a chemical inhibitor of complex III (16). We determined the expression of the T cell activation markers CD25, CD69 and CD71. Under T cell stimulation by phorbol myristate acetate (PMA), the only difference between the Atp8 and control genotype was expression of CD71, with a $\sim$10% higher expression level in controls. However, when the PMA stimulation was performed in the presence of myxothiazol, there was a tendency ($P < 0.11$) that CD4 T cells with the mtAtp8 polymorphism had $\sim$20% higher expression levels of CD25, CD69 and CD71 than controls (Fig. 5A). Then we calculated the adaptive potential to the myxothiazol inhibition for cells with both genotypes. T cells with the mtAtp8 polymorphism showed a higher adaptive potential than controls, in terms of expression of CD69 and CD71 (Fig. 5B). Taken together, these data suggested that the mtAtp8 polymorphism could increase the CD4 T cell adaptive potential to a conditions of impaired OXPHOS.

DISCUSSION

In this study, we report a novel mtDNA polymorphism, nt7778 G/T, resulting in a conservative amino acid substitution in the ATP8 protein. We demonstrated that this polymorphism increased the susceptibility to multiple autoimmune diseases. Our data are in line with previous reports showing that mutations affecting mtDNA- or nDNA-genes encoding mitochondrial proteins are associated with experimental autoimmune diseases (17–19). Also, it provides direct experimental support for the observation that variations in
mtDNA genes or nDNA-encoded mitochondrial genes are associated with autoimmune diseases (20–24).

Unexpectedly, the mtAtp8 polymorphism affected female fecundity in the MRL/MpJ strain by decreasing the litter size and increasing the time period from mating to delivery. To our knowledge this is the first report of an association of mtDNA with impaired female reproductive performance. We did not determine if the reduced litter size is due to decreased conception rate, loss of a portion of the embryos during development or post-natal death of mutant animals. However, we did not observe any loss of fetuses or post-natal death in MRL/MpJ-mtFVB/N mice. This suggests that the impairment in female fecundity may be likely explained by reduced implantation rates and/or very early fetal loss, pointing to immunological rejection as an underlying mechanism. The connection between autoimmunity and female fecundity is supported by additional evidence. Firstly, it is consistent with observations in humans, where autoantibodies are involved in individual cases of female reproductive failure, e.g. in systemic lupus erythematosus patients (25). Secondly, the effect could only demonstrated in the MRL/MpJ strain which is a lupus-prone strain that produces a wide spectrum of autoantibodies. The mtAtp8 polymorphism might have changed the autoantibody pattern or enhanced autoantibody production in those mice ultimately resulting in autoimmune reproductive failure. Finally, genetic loci regulating fetal reproduction and both arthritis and lupus often co-localize in mice (10,26).

It is important to note that the effect of the mtDNA polymorphism on complex traits depends on the nuclear genome background, indicating an interaction between nDNA and mtDNA. The effect of the nDNA–mtDNA interaction could...
be explained in two ways: Firstly, a distinct nuclear genome background confers susceptibility to a disease and the mtDNA polymorphism synergistically aggravates this susceptibility. Secondly, adaptive coevolution of nDNA and mtDNA might be the reason for nDNA–mtDNA interaction-induced defects as verified for OXPHOS function in human/primate cybrids (27).

Our functional studies demonstrated that the mtAtp8 polymorphism increases the mitochondrial ROS production and alters the mitochondrial morphology, but neither affected the activity of complexes on the OXPHOS chain nor the cellular ATP level under normal metabolic conditions. This indicates that mitochondrial ROS might be the essential factor linking the ATP8 variation to enhanced autoimmunity. In that case, two aspects should be considered in more detail: Firstly, how does a polymorphism in the ATP synthase affect ROS production. The most plausible explanation is based on the fact that the coding protein of the Atp8 allele plays an
important role for the proper assembly of the ATP synthase Fo subunit. This may result in variations of the proton flux and concomitant variations of the electron flux in particular under state 4 conditions limited by ADP. These variations in the electron flux may result in disproportionate transfer of electrons on oxygen in complex IV as a prerequisite for generation of superoxide radicals. This proposed mechanism needs to be experimentally addressed by comprehensive functional characterization of the different complexes in direct comparison to ROS generation.

Secondly, what are the cellular and molecular pathways involved in mitochondrial ROS contribution to autoimmunity? Our data demonstrated that mitochondrial ROS production is associated with severity of autoimmune diseases. They are in agreement with previous reports indicating that mitochondrial ROS are associated higher immune response (28). Mitochondrial ROS are toxic by-products of OXPHOS chain when accumulating at very high concentrations. At moderate concentrations, however, ROS can act as signaling molecules and are essential for cell proliferation (5,29,30). In hypoxia, mitochondrial ROS activate cytoprotective gene transcription and thus support cell survival (31). In this report, we show that the mtAtp8 polymorphism increases CD4 T cell adaptive potential to an OXPHOS impaired environment. In inflamed tissues the cellular microenvironment is characterized by low availability of oxygen and consequently impaired OXPHOS (14,15). As T cells recruited to those sites are important for the subsequent inflammatory cascade, it appears plausible that the mtAtp8 polymorphism aggravates autoimmune diseases through CD4 T cell activation under hypoxic conditions in inflamed tissues.

Although the effect of the mtAtp8 polymorphism on CD4 T cells could be considered as one of several possible mechanisms, we could not exclude the possibility that the polymorphism also affects target tissues. For example in diabetes, a mtDNA mutation in mtAtp6 gene has been reported to be associated with type II diabetes, and further studies showed that the polymorphism enhanced ROS production and impaired insulin secretion in beta-cells (32,33). As beta cell crucially depends upon metabolic stimulus-secretion coupling functionally-relevant mtDNA polymorphisms significantly affect nutrient-regulated insulin release. In this study, we showed that the mtAtp8 polymorphism increases ROS production in mitochondria, which may subsequently favor beta cell death under conditions of metabolic stress. It is therefore possible that increased ROS production amplifies trigger pathways of autoimmunity through presentation of beta cell antigens to autoaggressive T cells and a proinflammatory cytokine/chemokine milieu from islet-invading macrophages. The effect of the mtAtp8 polymorphism on the effector arm of autoimmune diabetes as well as the other autoimmune diseases will be investigated in future studies.

In conclusion, we have identified an mtDNA polymorphism in the murine mtAtp8 gene that increases susceptibility to multiple autoimmune diseases, and impairs female reproductive performance. These observations link mitochondrial dysfunction and ROS production to autoimmune disorders and compromised reproductive capacity.

MATERIALS AND METHODS

Mice

All mouse inbred strains used in this study, including FVB/NJ, C57BL/6J, AKR/J, B10.D1-H2q/SgJ (B10.Q/J), DBA/1J, NOD/LtJ and MRL/MpJ, were obtained from Jackson Laboratory (Bar Harbor, USA). To generate the conplastic strains, we crossed females of donor strains with males of recipient strains and then backcrossed the female offspring to males of recipient strains. Such backcrosses were performed for at least 10
H₂O₂-production was measured under respiratory state 4 con-
commitochondria were adjusted to the same total protein concen-
tration using an Amplex Red kit as described (37). H₂O₂ levels
were calculated from a H₂O₂ standard curve. ATP and ADP
levels were measured using an ADP/ATP ratio Kit (Biovision,
USA), in spleen cells from age-matched male conplastic mice
expressing MT-ΔROS. Mice were sacrificed at ages 12, 18 and 24 weeks,
and kidney and pancreas specimens were evaluated for nephritis
and AIP. Kidneys were scored by two experienced patholo-
gists for the intensity of the interstitial mononuclear cell infiltr-
ate. Severity of interstitial nephritis was graded blindly on a
0–4 scale as follows: 0, no mononuclear cell infiltration; 1, slight
lymphocyte infiltration (borderline changes); 2, mild
interstitial nephritis; 3, moderate interstitial nephritis
and 4, severe interstitial nephritis. The infiltrates were
graded according to the diameter. Severity of AIP was evalu-
ated as described previously (11). Briefly, severity of AIP was
scored on a 0–4 grade based on the histopathologic changes as
follows: 0, no mononuclear cell infiltration; 1, mononuclear
cell infiltration without parenchymal destruction; 2, mild
destruction with mononuclear cell infiltration; 3, moderate
parenchymal destruction but retained some intact parenchymal
residue; and 4, almost whole pancreatic tissue, except pancreati-
cells, destroyed. The anti-nuclear antibodies were
measured using quantitative Mouse ANA I’s ELISA Kit
(Alpha Diagnostic Intl. Inc., San Antonio, TX, USA).

Transmission electron microscopy
Tissue samples from age-matched conplastic strains and controls
were collected and fixed in 4% glutaraldehyde in 0.1 M
sodium phosphate buffer, pH 7.2 for 1 h. Tissue blocks were
prepared, washed in buffer, post-fixed in 1% OsO₄ for 1 h,
dehydrated in alcohol and embedded in epoxy resin Araldite.
Ultra-thin sections were cut with an ultramicrotome (Ultracut
SWS, Leica) using a diamond knife, contrasted with uranyl
acetate and lead citrate and studied with an electron micro-
scope EM 902 A (Zeiss, Germany). We analyzed mitochon-
drial area by the software Image J (http://rsb.info.nih.gov/ij/).
In total >800 individual mitochondria from kidney tissue
were analyzed per genotype.

Mitochondrial traits evaluation
Spleen mitochondria were isolated by homogenization in
10 mM Tris–HCl, pH 7.8, 0.25 M sucrose, 0.2 mM EDTA
with the addition of protease inhibitors, followed by low-speed
centrifugation (1000 g, 10 min, +4°C) and high-speed cen-
trifugation (12 000 g, 15 min, +4°C) of the supernatant.
The mitochondria were adjusted to the same total protein concentra-
tion determined by the BCA protein assay kit (Pierce). The
ATPase activity (complex V) was analyzed as described (36).
H₂O₂-production was measured under respiratory state 4
condition using an Amplex Red kit as described (37). H₂O₂ levels
were calculated from a H₂O₂ standard curve. ATP and ADP
levels were measured using an ADP/ATP ratio Kit (Biovision,
USA) in spleen cells from age-matched male conplastic mice
and controls. The concentrations of ATP and ADP were calcu-
lated by comparison to a standard curve of defined amounts of
ATP.

DNA and sequencing
The mtDNA sequences of the strains of NOD/LtJ, AKR/J, C57BL/6j and MRL/MpJ have been published previously,
with the accession number of EF108340, AB042432, NC005089 and FJ374651, respectively (7,19,34,35). We
sequenced the whole mtDNA of FVB/NJ, B10.Q/J and DBA/1J mice as previously described (19). DNA of all
mouse strains was obtained from Jackson laboratory (Bar
Harbor, USA) with except STR/orf (Taconic Farms, Inc.,
Denmark), FVB/NCl (Charles River Laboratories Inter-
national Inc., USA); FVB/NHanTMHsd (Harlan Laboratories Inc.) and FVB/N (Janvier, Le. Genest-St-Isle, France).

Disease models
CIA in F2 was induced as previously described (8). To induce
CIA in B10.Q/J mice, an additional boost with 100 μg col-
genon II in incomplete Freund’s adjuvant was performed. The arthritis score was determined blindly using a protocol
developed previously (38). Type I diabetes was investigated in
NOD/LtJ and NOD/LtJ-mtFVB/N mice simultaneously.
Mice were bred and fed conventional rodent chow and water ad
libitum. Blood glucose levels were then followed (bi-weekly during weeks 6–25 and every 4 weeks during
weeks 25–40). Blood glucose was measured by freestyle
mini equipment (Abbot), and the threshold of diabetes was
set as 9 mm at two subsequent measure time points. Both
nephritis and AIP were investigated in the MRL/MpJ mice.
Mice were sacrificed at ages 12, 18 and 24 weeks, and
kidney and pancreas specimens were evaluated for nephritis
and AIP. Kidneys were scored by two experienced patholo-
gists for the intensity of the interstitial mononuclear cell infiltr-
ate. Severity of interstitial nephritis was graded blindly on a
0–4 scale as follows: 0, kidney without lymphocyte infiltr-
ation; 1, slight lymphocyte infiltration (borderline changes);
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residue; and 4, almost whole pancreatic tissue, except pancreati-
cells, destroyed. The anti-nuclear antibodies were
measured using quantitative Mouse ANA I’s ELISA Kit
(Alpha Diagnostic Intl. Inc., San Antonio, TX, USA).

Evaluation of reproductive performance
Female mice, 6–12 weeks old, were mated with mature males.
We noticed that MRL/MpJ strains (both MRL/MpJ and MRL/
Mpl-mtFVB/N) had a significant reduction (~30%) in the litter
size of the second litter as compared with the first litter. There-
fore, the number of offspring of the first litter was taken as the
litter size. Time to delivery represents the time period from
mating to birth and the fertility rate represents a ratio
between number of female mice giving birth and those used
for mating.

Analysis of T cell activation
Spleen cell suspensions were freshly prepared from 12-week-old
C57BL/6j-mtFVB/N (n = 4) and C57BL/6j-mtAKR/J mice (n = 4).
Erythrocytes were lysed by brief incubation with distilled water.
Leukocytes were cultured at 10⁶ cells/ml (1 ml per well in a
48-well plate) in RPMI-1640 (Invitrogen, Karlsruhe, Germany)
supplemented with 10% fetal calf serum (Biochrom, Berlin,
Germany), 100 U/ml penicillin/100 μg/ml streptomycin (PAA,
Pasching, Austria), 2 mM l-glutamine (PAA) and 50 μM 2-
mercaptoethanol (Sigma-Aldrich, Munich, Germany). Cells
were stimulated by 10 ng/ml PMA and 1 μg/ml ionomycin
We used Student’s t-test for normally distributed quantitative traits and Mann–Whitney U test for non-normally distributed quantitative traits. The difference in the diabetes incidence was compared using Gehan–Breslow–Wilcoxon test.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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