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Partial dysfunction of STAT1 profoundly reduces host resistance to flaviviral infection

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ABSTRACT

The genetic basis for a dramatically increased virus susceptibility phenotype of MHC-II knockout mice acquired during routine maintenance of the mouse strain was determined. Segregation of the susceptibility allele from the defective MHC-II locus combined with sequence capture and sequencing showed that a Y37L substitution in STAT1 accounted for high flavivirus susceptibility of a newly derived mouse strain, designated *Tuara*. Interestingly, the mutation in STAT1 gene gave only partial inactivation of the type I interferon antiviral pathway. Accordingly, merely a relatively small impairment of interferon α/β signalling is sufficient to overcome the ability of the host to control the infection.

1. Text

In an investigation that addressed the contribution of antibody and T cells to the recovery from infection with the flavivirus, *Japanese encephalitis virus* (JEV), we reported that a strain of MHC-II k/o mice lacking the 4 classical MHC-II genes (*MHCII-Aα/β*$^{-/-}$ mice) (Madsen et al., 1999) were significantly more susceptible than congenic wt C57Bl/6 (B/6) mice to low-dose JEV infection; *MHCII-Aα/β*$^{-/-}$ mice uniformly succumbed to the infection with a mean survival time (MST) of 12 days (Larena et al., 2011). Surprisingly, a second strain of MHC-II k/o mice, in which only the Aα locus of the MHC-II complex was inactivated (*MHCII-Aα*$^{-/-}$ mice) (Kontgen et al., 1993), presented with a more severe phenotype than *MHCII-Aα/β*$^{-/-}$ mice in response to JEV infection (MST=8.7 ± 3 days; Fig. 1A). The difference in flavivirus susceptibility between *MHCII-Aα*$^{-/-}$ and *MHCII-Aα/β*$^{-/-}$ mice was even more pronounced when a low-virulence strain of *West Nile virus* (WNV_{KUN}) was used (Fig. 1B): while all B/6 wt and *MHCII-Aα/β*$^{-/-}$ mice survived the challenge, *MHCII-Aα*$^{-/-}$ mice showed 100% mortality (MST =9 ± 2.0 days). *MHCII-Aα*$^{-/-}$ mice infected with JEV or WNV_{KUN} presented with severe signs of encephalitis and high viral load in brain and spinal cord (data not shown). The increased susceptibility phenotype was consistently observed when *MHCII-Aα*$^{-/-}$ mice were challenged with a live-attenuated flaviviral vaccine, ChimeriVax-JE (Fig. 1 C,D).

To investigate whether the high sensitivity of *MHCII-Aα*$^{-/-}$ mice to the flaviviral infections was at least in part the result of an unknown defect other than the deficiency in the adaptive immune response mediated by the mutation in the MHC-II *Aα* gene, bone marrow-derived macrophages from *MHCII-Aα*$^{-/-}$ and B/6 wt mice were infected with JEV, WNV_{KUN} or the prototype flavivirus, *yellow fever virus* (YFV) and virus yield in culture supernatants measured by plaque assay (Fig. 1E). The three flaviviruses produced 50- to 100-fold higher titers in cultured cells from *MHCII-Aα*$^{-/-}$ than B/6 wt mice, suggesting the possibility of a spontaneous mutation that is independent of the MHC-II defect.

Collectively, these data show that *MHCII-Aα*$^{-/-}$ mice display an extraordinary sensitivity to flaviviral challenge, and that this susceptibility phenotype cannot be explained solely by an MHC-II defect. Moreover, the findings support the results from a second study published during the course of this investigation showing that the strain of *MHCII-Aα*$^{-/-}$ mice used is also more susceptible to alphavirus (Semliki Forest virus) and poxvirus (ectromelia virus) infections than *MHCII-Aα/β*$^{-/-}$ mice (Alsharifi et al., 2013). Accordingly, it was likely that the *MHCII-Aα*$^{-/-}$ mice had acquired an unidentified virus susceptibility allele as the result of a spontaneous mutation.

1.1. The unknown susceptibility allele is a recessive trait with full penetrance and is localized on chromosome 1

Genetic mapping enables localization of a spontaneous mutation to a particular chromosomal region by co-segregation of the mutation with genetic markers interspersed throughout the genome (reviewed in

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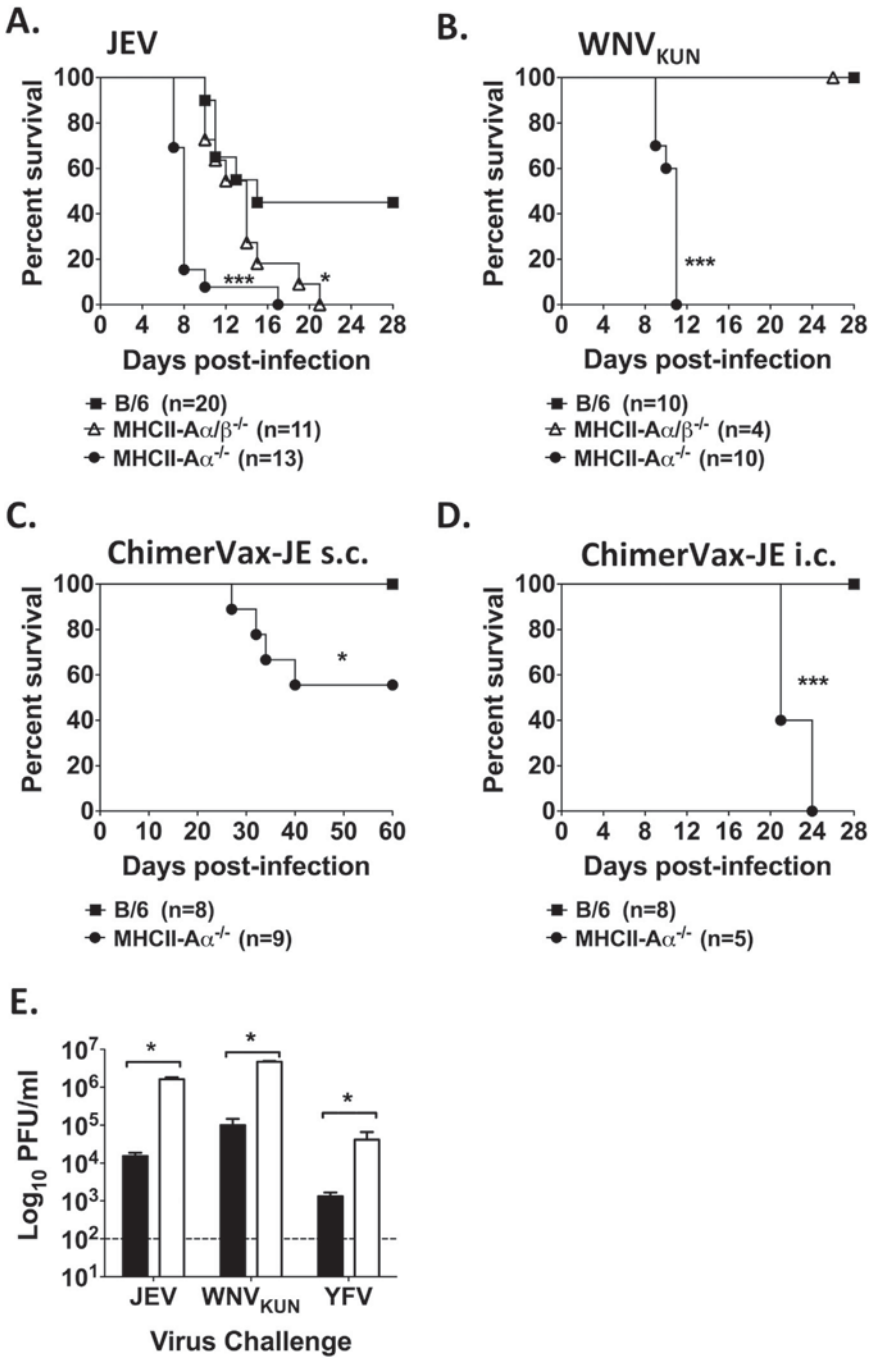

Fig. 1. Susceptibility of MHC-II k/o and B/6 wt mice and ex vivo macrophage cultures to flavivirus infections. Groups of 8-week-old mice were infected into the footpad with 10^3 PFU of JEV (A) or WNV_{KUN} (B) as described (Purtha et al., 2012). Morbidity and mortality were recorded daily, and surviving mice were monitored for 28 days. Groups of 6-week-old mice were inoculated subcutaneously (s.c.) with 10^5 PFU of ChimeriVax-JE (C) as described (Purtha et al., 2012), and morbidity and mortality recorded daily for a 60-day-observation period. Groups of 4-week-old mice were inoculated intracranially (i.c.) with 10^3 PFU of ChimeriVax-JE (D), and morbidity and mortality recorded daily for a 28-day-observation period. The data shown were constructed from two independent experiments. Asterisks denote statistical significance in mortality between B/6 and various knockout mice (*, $P < 0.05$; ***, $P < 0.001$). (E) Bone marrow cells were isolated from lower extremity bones of B/6 wt and *MHCII-Aα*$^{-/-}$ mice, and cultured for 7 days towards macrophage differentiation. Mature macrophages were then infected with JEV, WNV_{KUN}, or YFV at MOI of 1. Bars represent mean viral load with SEM (n =3) in culture supernatants at 24 pi, determined by plaque titration in Vero cells as described (Lobigs et al., 2009). Asterisks denote statistical significance (*, $P < 0.05$), ***, $P < 0.001$).

(Nelms and Goodnow, 2001)). To identify the unknown virus susceptibility allele, *MHCII-Aα*$^{-/-}$ mice were first crossed with a mapping strain (CBA mice) to produce F1 (B6xCBA) progeny. CBA mice were known to be resistant to WNV_{KUN} infection. The virus susceptibility of a group of F1 (n=6) in comparison to CBA control mice (n =5) was tested by WNV_{KUN} challenge: all F1 and CBA mice survived the challenge, while 100% of a group of control *MHCII-Aα*$^{-/-}$ mice (n=5) succumbed to the infection (data not shown). This suggests that the unknown susceptibility allele is a fully penetrating recessive trait. Next, F1 mice were backcrossed to *MHCII-Aα*$^{-/-}$ mice to generate N2 progeny mice (*MHCII-Aα*$^{-/-}$xF1). Challenge of N2 (n=24) mice with WNV_{KUN} gave the expected outcome of resistant (46%) and susceptible (54%) mice, and showed that the virus susceptibility allele segregated independently from the MHC-II locus in a simple Mendelian pattern of inheritance (Table 1).

Meiotic recombination in the F1 and subsequent N2 generations acts as a genetic shuffle that can be used to distinguish regions of the genome that are closely linked to the susceptibility allele from regions

Table 1
Susceptibility of N2 (*MHCII-Aα*$^{-/-}$ x F1) to WNV_{KUN} infection.

MHCII genotype	Number (percentage) of affected	Number (percentage)of unaffected mice	Total
MHCII-Aα$^{-/-}$	7 (29%)	5 (25%)	12 (50%)
MHCII-Aα$^{+/-}$	6 (21%)	6 (25%)	12 (50%)
Total	13 (54%)	11 (46%)	24 (100%)

Twenty-four 8-week-old N2 mice were challenged with WNV_{KUN} (10^3 PFU injected into the footpad), and observed for 28 days to record mortality. All mice were genotyped by PCR for wt and k/o MHC-II-Aα as described in Materials and Methods.

that are unlinked. These regions were determined by linkage analysis in a last step of the mapping process by analyzing the recombinant chromosomes inherited by N2 mice with a panel of single-nucleotide polymorphisms (SNPs) that span the genome (Myakishev et al., 2001). N2 mice were grouped as affected or unaffected for the virus susceptibility phenotype based on the outcome of infection with WNV_{KUN} (death or survival after 28-day observation). DNA from tail samples of infected N2 mouse was pooled into affected or unaffected groups, and was analyzed with the use of ~110 SNPs genetic markers that can distinguish between B/6 and CBA strains. Thus, SNPs closely linked to the unknown susceptibility allele should only yield the homozygous B/6 allelic fragment in N2 mutant mice, whereas analyses with unlinked markers will yield heterozygous B6 and CBA strain alleles. The genome-wide screen of pooled DNA from affected and unaffected mice localized the unknown virus susceptibility allele to a ~50 MB region on chromosome 1 (Supplementary Table 1).

*1.2. Production of a mouse strain (*Tuara*) with the virus susceptibility allele on wt MHC-II background*

Interpretation of the contribution of the spontaneous mutation identified in *MHCII-Aα*$^{-/-}$ mice to the phenotype of increased susceptibility to viral infection is complicated by the presence of the MHC-II defect. Therefore, a new mouse strain, designated *Tuara* mice, that is homozygous for the virus susceptibility trait and wt at the MHC-II locus on chromosome 17 was generated in a two-step breeding protocol: first, heterozygous *MHCII-Aα*$^{+/-}$ N2 mice carrying the unknown susceptibility allele based on SNP marker detection were intercrossed to produce an N3 progeny; next, *MHCII-Aα*$^{+/+}$ N3 mice were intercrossed to produce N4 mice, all of which carry the susceptibility allele and are wt for MHC-II. The exquisite susceptibility of *Tuara* mice to flavivirus infections was confirmed in challenge experiments with JEV or WNV_{KUN} (Fig. 2). All *Tuara* mice succumbed to the challenges, and the MST of the groups of JEV and WNV_{KUN} infected mice were very similar to those shown in Fig. 1 for infected *MHCII-Aα*$^{-/-}$ mice. The 100% mortality rate of JEV infected *Tuara* mice was accompanied by a high viremia on day 2 postinfection (pi) with a mean titer of 3.5×10^5 PFU/ml), and rapid and uncontrolled virus dissemination into the CNS, where the virus was clearly detectable on day 4 pi, and reached titers exceeding 10^9 PFU/g tissue in brain and spinal cord on day 7 pi (data not shown). Taken together, these data show that flavivirus challenge of *Tuara* mice produced a fulminating, lethal infection, and that despite the presence of functional MHC-II the severity of the disease phenotype was indistinguishable from that in *MHCII-Aα*$^{-/-}$ mice.

1.3. Sequencing analysis identifies a point-mutation in the STAT1 gene

To identify the virus susceptibility allele present in *Tuara* mice, we employed customized sequence capture (Nimblegen hard-array) and sequencing using the Illumina HiSeq system. A customized sequence capture array was designed to include a wide genomic area from 29 Mb to 79 Mb of chromosome 1. DNA samples from tails of three *Tuara* mice were used. Bioinformatics analysis revealed in each of the three independent samples a G→T transversion at reference position 52,179,547 bp in the STAT1 gene, which results in a Y37L substitution in the STAT1 protein (Fig. 3A). No other consistent sequence differences were found. Given that STAT1 is a key transcription factor in interferon (IFN)-α/β signalling (reviewed in (Sadler and Williams, 2008)), the result is consistent with the interpretation that the spontaneous mutation in the STAT1 gene is responsible for the high virus susceptibility phenotype of *MHCII-Aα*$^{-/-}$ and *Tuara* mice.

To further confirm the Y37L mutation, a specific SNP marker was designed to distinguish the G > T nucleotide change at reference position 52,179,547 in the STAT1 gene using amplifluour technology (Myakishev et al., 2001). We tested two batches of N2 mice, n=5 and n=4 for both affected and unaffected. The mutation was consistently present in all 9 mice that were identified as affected, while all the other 9 mice resistant to the WNV_{KUN} challenge did not display the G > T transversion (data not shown). Moreover, the presence of Y37L mutation in STAT1 was also confirmed in all the newly generated *Tuara* mice tested (n=17).

1.4. The Y37L mutation in STAT1 results in partial inactivation of the IFN-α/β antiviral pathway

Type I IFNs are induced when pathogen-associated molecular patterns on viral proteins and nucleic acids activate host pattern recognition receptors, and the response is amplified via both autocrine and paracrine signalling through the IFN-α/β receptor and the JAK/STAT pathway to promote expression of IFN stimulated genes (Diamond and Gale, 2012). STAT1, as a homodimer or in complex with STAT2 and IRF9, can act as an ISG transcription factor. Thus, a

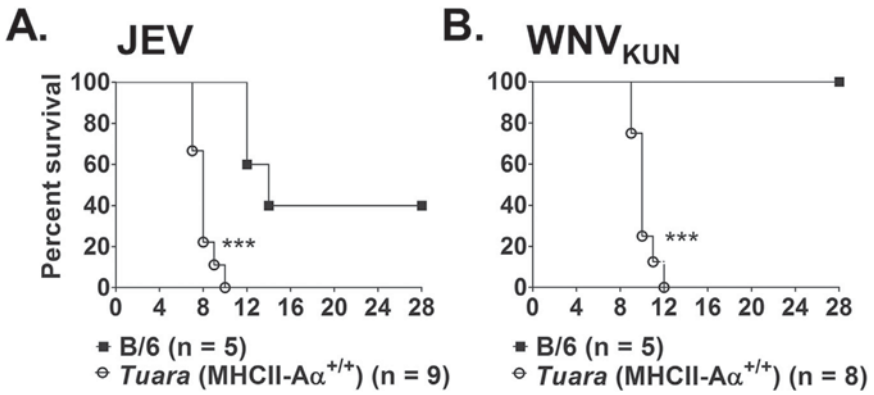

Fig. 2. Susceptibility of *Tuara* mice to challenge with JEV and WNV_{KUN}. Groups of 8-week-old mice were infected into the footpad with 10^3 PFU of JEV (A) or WNV_{KUN} (B). Morbidity and mortality were recorded daily, and surviving mice were monitored for 28 days. The data shown were constructed from two independent experiments. Asterisks denote statistical significance (***, $P < 0.001$).

A.

Reference amino acid sequence	Tyr	Leu	Ala	Gln	Trp	Leu	Glu	Lys	Gln
Reference genome	CTG	CTG	GCC	CAG	TGG	CTG	GAA	AAG	CAA
Sample 1	CTG	CTG	GCC	CAG	TTG	CTG	GAA	AAG	CAA
Sample 2	CTG	CTG	GCC	CAG	TTG	CTG	GAA	AAG	CAA
Sample 3	CTG	CTG	GCC	CAG	TTG	CTG	GAA	AAG	CAA
Predicted amino acid sequence	Tyr	Leu	Ala	Gln	Leu	Leu	Glu	Lys	Gln

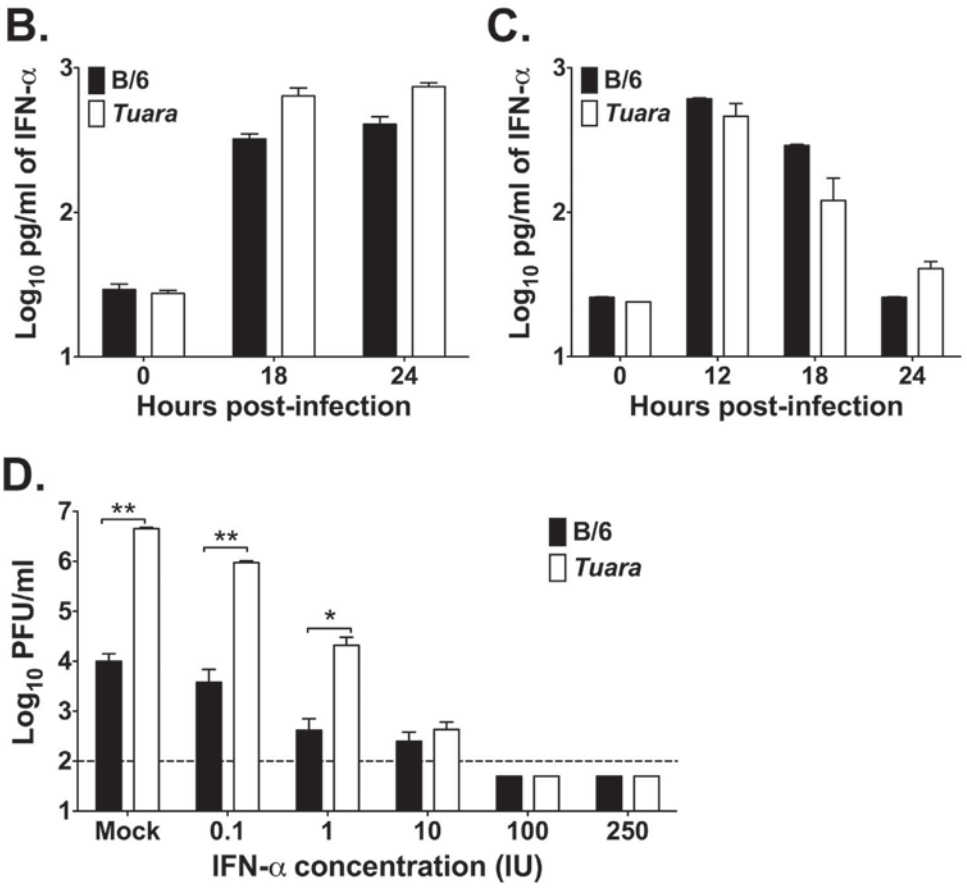

Fig. 3. Indentification of G > T transversion in STAT1 gene and the effect of resultant Y37L mutation on IFN-α secretion and responsiveness. Genomic DNA (≥500 μg/sample at ≥1 μg/μl) isolated from tails of three *Tuara* mice was used for customized sequence capture using the Nimblegen hard-array and sequencing using the Illumina HiSeq system. A customized sequence capture array was designed to include the region from 29 Mb to 79 Mb of chromosome 1. Bioinformatics teams from Australian Genomics Resource Facility and Australian Phenomics Facility independently provided analysis of the raw sequence data generated from Illumina HiSeq re-sequencing. The analyses consistently showed a G > T transversion at reference position 52,179,547 bp in the STAT1 gene, resulting in a tryptophan to leucine substitution at amino acid 37 (shaded gray) of the STAT1 protein. (B). Mature macrophages were infected with JEV at MOI of 1. IFN-α levels in culture supernatants at indicated time points were determined following the manufacturer's recommended protocol (PBL InterferonSource). Each bar represents means with SEM. (C) IFN-α production in serum in JEV-infected B/6 wt and tuara mice at indicated time points. Each bar represents means with SEM of 3 biological samples. (D) Mature macrophages were pre-treated for 16 h with 250, 100, 10, 1, 0.1 IU of IFN-α or mock-treated with tissue culture medium. Macrophages were then infected for 24 h with JEV at MOI of 1. Viral load in culture supernatants were measured by plaque titration in Vero cells. Each bar represents mean with SEM of 3–6 samples from two independent experiments. Asterisks denote statistical significance (*, $P < 0.05$; **, $P < 0.01$).

deficiency in STAT1 prevents amplification of the type I IFN response, and leads to significantly increased susceptibility to viral infection (reviewed in (Boisson-Dupuis et al., 2012)), including that with flaviviruses (Chen et al., 2012; Perwitasari et al., 2011). To investigate the effect of the Y37L mutation in STAT1 on the IFNα/β response, IFN-α secretion in JEV infected *Tuara* macrophage cultures and mice was compared to that in wt; surprisingly, the analysis did not show a significant difference (Fig. 3B,C). Next, integrity of downstream IFN signalling and antiviral activity were tested. Bone marrow-derived macrophages from *Tuara* and B/6 control mice were pre-treated with IFN-α prior to infection with JEV, and virus growth inhibition determined by plaque titration (Fig. 3D). Pre-treatment with a high dose of IFN-α completely abolished growth of JEV in cells from *Tuara* and wt mice, while sham-treated cells showed as expected a > 2 logs difference in virus yield. However, pre-treatment with lower doses of IFN-α revealed a significant difference in the antiviral response between *Tuara* and wt macrophages, which is consistent with a partial dysfunction of the mutant STAT1 protein. Consistent with this interpretation, others have shown that residue Y37 is located in a domain of STAT that has been implicated in protein-protein interactions affecting transcription, and that mutagenesis of the invariant tryptophan abrogates cooperative DNA binding resulting in the reduction of

transcriptional response following cytokine stimulation (Vinkemeier et al., 1998).

1.5. Conclusion

Flaviviruses have evolved various strategies to suppress the IFN response that include blocking phosphorylation, enhancing degradation or down-regulating expression of major components of the JAK/STAT pathway (reviewed in (Munoz-Jordan and Fredericksen, 2010)). Nevertheless, type I IFN remains vital in the host defence against most flaviviruses (Samuel and Diamond, 2005; Lobigs et al., 2003; Chen et al., 2008). The results of this study, using *Tuara* mice, show that only a partial impairment of STAT1 and an associated moderate reduction in responsiveness to IFN-α/β is sufficient to overcome the ability of the host to control the infection. Further investigations are necessary to uncover the molecular mechanisms of partial STAT1 dysfunction in *Tuara* mice. It still remains to be known whether there is a difference in STAT1 phosphorylation or nuclear translocation and/or STAT1-mediated ISG transcription following treatment of WT vs *Tuara* macrophages with type I or type II interferons. In spite of this study's limited scope, we show that *Tuara* mice constitute the first suitable animal model that resembles the clinical picture of partial STAT1 deficiency recently described in humans (Chapgier et al., 2009; Kristensen et al., 2011; Kong et al., 2010). Similar to a previous finding (Purtha et al., 2012), this work highlights that spontaneous mutation in inbred knockout mouse strains can occur and can have profound effects on the immunological phenotype. Thus, routine screening and backcrossing of mouse strains used for research in viral immunology is necessary to guard against misleading experimental outcomes.

2. Materials and methods

2.1. Cells and viruses

Vero cells (African green monkey kidney) were grown in Eagle's minimal essential medium plus nonessential amino acids (MEM, Invitrogen) supplemented with 5% fetal bovine serum (FBS). A murine fibrosarcoma cell line, L-929, were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% FBS. All cell lines were obtained from the American Type Culture Collection, and were maintained at 37 °C with 5% CO_2 atmosphere. Single-use working stocks of viruses were stored at −70 °C, and were prepared from infected Vero cell culture supernatants (JEV Nakayama strain at 2×10^8 PFU/ml and ChimeriVax-JE at 1×10^7 PFU/ml), infected C6/36 cell culture supernatant (Yellow Fever Virus 17-D strain at 1×10^7 PFU/ml), and 10% suckling mouse brain homogenates (WNV strain Kunjin at 1×10^9 PFU/ml).

2.2. Mice

C57Bl/6 (B/6), CBA, *MHCII-Aα/β*$^{-/-}$, and *MHCII-Aα*$^{-/-}$ mice were bred under specific pathogen-free conditions, and supplied by the Animal Breeding Facility at the John Curtin School of Medical Research, The Australian National University (ANU), Canberra. All animal experiments were conducted with approval from ANU Animal Ethics Committee.

2.3. Mouse inoculation, tissue collection, and viral load determination

For subcutaneous (sc) and intracranial (ic) inoculation, mice were anesthetized with 50 μl of a 10% ketamine–xylazole solution in phosphate-buffered saline (PBS) by intramuscular injection. Mice were infected sc via the footpad or ic with a single injection of a defined dose of a virus in 20 μl Hanks' balanced salt solution (HBSS) containing 20 mM HEPES buffer (pH 8.0) and 0.2% bovine serum albumin. Mice were monitored twice daily. Daily changes in 5 parameters including hair coat, posture, breathing pattern, activity, and movement were recorded (scored) as normal (0), mild (1), moderate (2), or severe (3), and severely moribund mice and/or mice with hindlimb paralysis were euthanized by rapid cervical dislocation. For tissue processing, mice were euthanized at the time points indicated and a sterile midline vertical thoracoabdominal incision was made to expose the internal organs. After cardiac puncture for blood collection, animals were perfused with 10 ml sterile PBS. The brain and spinal cord were excised intact and were snap frozen on dry ice. Determination of viral load was by plaque assay on Vero cell monolayers as described previously (Licon Luna et al., 2002).

2.4. Preparation of bone marrow-derived macrophages

B/6 or knock-out mice were euthanized by cervical dislocation. Aseptically, skin in the lower extremities was removed, and muscles dissected to exposed femur, tibia, and fibula. Bones were excised through a cut in the proximal hip and distal ankle joints, and placed in dish containing complete Mixed Lymphocyte Culture media (MLC media supplemented with 1% L-glutamine, 1% NEAA, 1% pyruvate, 50 μM 2-ME, 100 units PSN, and 10% FBS). Both ends of individual bones were severed to expose cell-filled marrow cavity. Using a 10-ml syringe, marrow cavity was flushed with complete MLC media to expel cells. Collected cells were centrifuged for 5 min at 400 *x g*, and resuspended in a complete MLC supplemented with 10% L-929 supernatant containing macrophage-colony stimulating factor. Bone marrow-derived cells were cultured at 1×10^6 cells/well in 6-well plates, and incubated at 37 °C with 5% CO_2 atmosphere. Culture media is replaced with fresh complete MLC plus 10% L-929 supernatant every 48 h, until differentiation into mature macrophages on day 7 after start of culture.

2.5. IFN-α levels determination

Bone marrow-derived macrophages from B/6 wt and *tuara* mice were infected with JEV at MOI of 1, and supernatants were collected at 0, 12, 24, or 48 h pi and stored at 70°C before cytokine analysis. B/6 wt and tuara mice were infected sc with 10^3 PFU of JEV, serum was collected at 12, 18, and 24 h pi, and was stored at 70°C before cytokine analysis. IFN-α levels were determined using VeriKine Mouse Interferon Alpha ELISA kit following the manufacturer's recommended protocol (PBL Interferon Source).

2.6. Viral growth inhibition by IFN-α treatment

Bone marrow-derived macrophages in 6-well plates were incubated with 250, 100, 10, 1, and 0.1 Units of IFN-α3 (PBL Interferon Source) or mock-treated with media alone for 16 h. Following washing with complete MLC media plus 5% FBS, cells were incubated with 100 μl of JEV at MOI of 1, for 1 h at 37 °C, with intermittent gentle shaking. Cells were washed twice, and cultured with complete MLC media plus 10% L-929 supernatant for 48 h. Culture supernatant (100 μl) were collected at 12, 16, 24, and 48 h pi, and inhibition of virus growth by IFN-α was determined by plaque titration on Vero cell monolayers.

2.7. Allelic discrimination by Amplifluor SNPs genotyping

Allelic discrimination by Amplifluor SNPs genotyping was performed at the Australian Phenomics Facility (APF). An amplification cocktail was prepared, with each 10 μl reaction volume containing 3.15 μl distilled H20, 0.5 μl 20x amplifluor SNP FAM primer (Millipore), 0.5 μl 20x amplifluor SNP JOE primer (Millipore), 2 μl 20x SNP specific primer mix, 1 μl 10x reaction mix S plus, 0.8 μl dNTPs (2.5 mM each), 0.05 μl Platinum hotstart Taq Polymerase (Invitrogen), and 2 μl DNA samples stored in Tris-Buffered H20. DNA from B/6,

CBA, F1 mice and distilled H20 were used as controls. Sample amplification cocktails (8 µl) were placed in 96 well plates. Plates were covered with optically clear strips, and ran under cycling conditions 95 °C for 4 min for 1 cycle, 95 °C for 10 s, 60 °C for 20 s, 72 °C for 40 s for 34 cycles, and 72 °C for 3 min for 1 cycle. Plates were read with a fluorescent plate reader using the following: excitation filter 490/10 nm and emission filter 520/10 (gain 2200), and excitation filter 530/10 and emission filter 560/10 (gain 2400). Data was analyzed using Fluostar optima software. Amplifluour technology, with a newly designed SNP marker, was also utilized to distinguish the G > T nucleotide change at reference position 52,179,547 in the STAT1.

2.8. Customized sequence capture and sequencing

Customized sequence capture by hybridization (Roche NimbleGen 2.1 M capture array) was performed at the Australian Genomics Research Facility (AGRF, Melbourne). Customized sequence capture probe set was provided by Roche NimbleGen, Australia. Re-sequencing of the captured samples were done, using Illumina HiSeq sequencing system, at AGRF. Alignment was performed with Illumina CASAVA v1.8.2 and aligner module ELAND v2 (Efficient Large-Scale Alignment of Nucleotide Databases). Illumina Exome script was applied to primary alignment folder to generate CASAVA build folder and Exome coverage statistics. Functional annotation of genetic variants including single nucleotide variations, insertions, and deletions was done using the ANNOVAR software.

2.9. MHCII-Aα genotyping

MHCII-Aα genotyping by PCR was performed at APF using DNA extracted from mice ear punch samples. Briefly, a 10 µl PCR reaction volume was prepared containing 2.6 µl distilled H_20, 0.4 µl of 10 µM MCHII-Aα wt reverse primer (5′-GGTGTGAGCACGTACCATTG-3′), 0.2 µl of 10 µM MCHII-Aα common forward primer (5′-TGGAGACATTGGCCAGTACA-3′), 0.8 µl of 10 µM neomycin common primer (5′-GGATGATCTGGACGAAGAGC-3′), 5 µl 2x Mix (Qiagen Multiplex PCR kit), and 1 µl DNA sample. DNA samples from B/6 wt, F1, and MCH-II-Aα$^{-/-}$ mice were used as controls. Samples were ran under cycling conditions 95 °C for 4 min for 1 cycle, 95 °C for 15 s, 60 °C for 20 s, 72 °C for 30 s for 35 cycles, and 72 °C for 2 min for 1 cycle. Following amplification, samples were added with 30 µl of loading buffer, were loaded into 1.5% agarose gel stained with gel red. The gel was electrophoresed for 10 min at 350 V. Bottom bands indicate wt genotype, while top bands indicate presence of mutation.

2.10. Statistical analyses

Mortality data were plotted into Kaplan-Meier curves and assessed by the log-rank test for significance. The Mann-Whitney test was applied to assess differences in data gathered between two experimental groups. A P value of ≤0.05 was considered as significant.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2017.03.001.

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