



Low expression of a D^{dm7}/L^{dm7} -hybrid mutant (D/L^{dm7}) in the novel haplotype $H-2^{nc}$ identified in atopic dermatitis model NC/Nga mice

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Abstract

Environmental factors and the major histocompatibility complex (MHC) are involved in the pathogenesis of atopic dermatitis (AD). However, MHC type (*H2* haplotype) of AD model mice NC/Nga is poorly understood. Alloreactive $CD8^+$ or $CD4^+$ T cells in NC/Nga strongly responded to each antigen-presenting cells (A/J: $H-2^a$, C57BL/6: $H-2^b$, BALB/c: $H-2^d$, or C3H/HeJ: $H-2^k$), suggesting that NC/Nga has other *H2* haplotype. Polymorphic microsatellite (CA)ⁿ repeats in TNF- α gene differ based on the *H2* haplotype at present. NC/Nga's (CA)ⁿ repeats ($n = 19$) were different from other examined strains, A/J ($n = 14$), BALB/c ($n = 14$), C3H/HeJ ($n = 16$), and C57BL/6 ($n = 20$). Using flow cytometry and genotyping, we demonstrated the NC/Nga *H2* haplotype had a unique phenotype (K^d , $I-A^k$, and $I-E^k$) in which D^d and L^d lacked as protein despite sensitive mRNA detection. The loss of D^d and L^d was caused by forming a unique D^{dm7}/L^{dm7} -hybrid mutant (D/L^{dm7}). We propose to call this novel *H2* haplotype the " $H-2^{nc}$," and provide the important information regarding the AD research using NC/Nga mice.

Introduction

Human major histocompatibility complex (MHC) genes are the most potent genetic determinants of susceptibility to autoimmune and immunopathological conditions, such as atopic dermatitis (AD), which is associated with certain MHC genotypes [1, 2]. In humans, single-nucleotide polymorphisms (SNPs) in the human leukocyte antigen (HLA)-C region are strongly associated with total serum IgE levels in the Asian population [3]. The HLA-DRB1*01 allele is

associated with high serum IgE concentrations [4], and the HLA-DRB1*1101 allele is involved in the development of AD [5]. HLA-DPB1*0401-restricted cat allergen Fel d 1-specific $CD4^+$ T cells had a greater Th2 effector function in AD patients compared with healthy subjects [6]. In contrast, HLA-A32 allelic frequency was significantly increased in healthy individuals compared with AD patients [7]. In mice, to date, the *H2* haplotype of AD model NC/Nga mice is poorly understood.

Several studies have examined the role of tumor necrosis factor (TNF)- α in the pathogenesis of AD. The human TNF- α gene is located at 6p21.31 in the HLA class III region and it is a polymorphic locus [8]. In addition, polymorphic microsatellite (CA)ⁿ repeats in the TNF- α gene promoter have different lengths in various mouse strains depending on their *H2* haplotype [9]. Specific SNPs related to the transcription start site of TNF- α have direct effects on TNF- α regulation and transcriptional efficiency. These polymorphisms might affect TNF- α regulation and thus be associated with susceptibility to AD [10, 11].

NC/Nga mice spontaneously develop an AD-like disease under *conventional* conditions, but not under specific pathogen-free (SPF) conditions [12]. Because the *H2* haplotype of NC/Nga mice was thought to be $H-2^d$ [13], BALB/c mice have been used as control mice by many researchers. When conducting basic immunological

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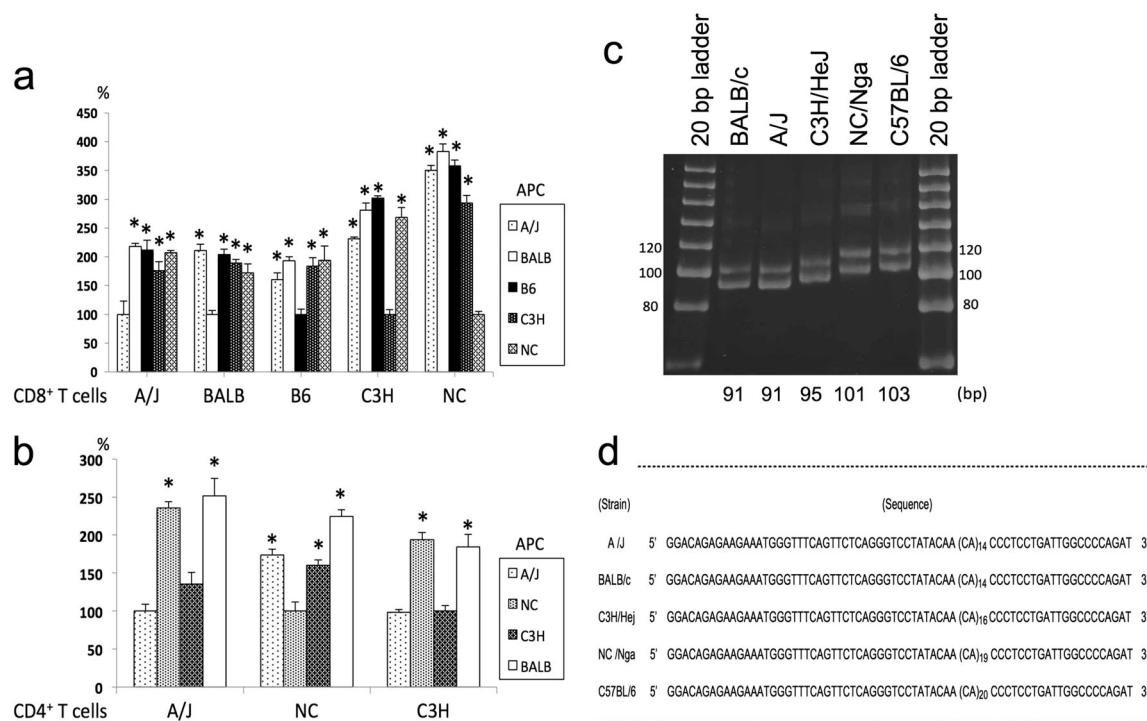


Fig. 1 *H2* haplotype in NC/Nga mice is different to *H2*^a, *H2*^b, and *H2*^k. The alloresponses of CD8⁺ and CD4⁺ T cells in NC/Nga mice were analyzed by MLR using APCs from A/J (*H2*^a), BALB/c (*H2*^d), C57BL/6 (*H2*^b), and C3H/HeJ (*H2*^k) mice. **a** The alloresponse to APCs of the indicated strains is shown as a percentage against the autoreponse of CD8⁺ T cells. **b** The alloresponse to APCs of the indicated strains is indicated as a percentage against the autoreponse of CD4⁺ T cells. Data are representative of three independent

experiments. **p* < 0.05, ANOVA. **c** The TNF- α promoter microsatellite region in genomic DNA purified from PBMCs was amplified by PCR. The electrophoresis of PCR products are shown for five mouse strains: A/J (*H2*^a), BALB/c (*H2*^d), C3H/HeJ (*H2*^k), NC/Nga (unknown haplotype), and C57BL/6 (*H2*^b). PCR product sizes are shown in the lower section. **d** Sequences of polymorphic microsatellite (CA)ⁿ repeats are shown for each mouse strain. Data are representative of three independent experiments

research with NC/Nga mice, it is important to characterize the cell surface molecules on their antigen-presenting cells (APCs). Here, we report that NC/Nga mice have a novel *H2* haplotype (hereinafter referred to as *H2*^{nc}); *K*^d, *D*^{dm7}/*L*^{dm7}-hybrid mutant (*D/L*^{dm7}), *I-A*^k, *I-E*^k, and it is discussed that the expression of the *H2* haplotype might be involved in an AD-like disease in NC/Nga mice.

Results and discussion

Alloresponse of CD8⁺ or CD4⁺ T cells in NC/Nga mice

To test the function of the *H2* haplotype in NC/Nga mice, mixed-lymphocyte reaction (MLR) analysis was performed using BALB/c (*H2*^d), C57BL/6 (*H2*^b), C3H/HeJ (*H2*^k), A/J (*H2*^a), and NC/Nga mice (unknown haplotype). Alloresponses of CD8⁺ or CD4⁺ T cells purified from NC/Nga mice (NC/Nga CD8⁺ T cells or NC/Nga CD4⁺ T cells) were strongly induced in combination with irradiated APCs from all strains tested (Fig. 1a, b). It was suggested that *H2* haplotype of NC/Nga was not in accordance with the *H2*^a, *H2*^b, *H2*^d, and *H2*^k at least.

Unique length and (CA)ⁿ repeats of a microsatellite in the TNF- α gene promoter of NC/Nga mice

We evaluated the length variations of microsatellites located in the TNF- α gene promoter in five mouse strains. Polymorphic microsatellite (CA)ⁿ repeats in the TNF- α gene promoter have different lengths depending on the *H2* haplotype of each mouse strain [9]. Genomic DNA isolated from peripheral blood mononuclear cells (PBMCs) was analyzed to determine the specific microsatellite lengths for each mouse strain. PCR product lengths were 91 base pairs (bp) in BALB/c mice (*H2*^d), 91 bp in A/J mice (*H2*^a), 95 bp in C3H/HeJ mice (*H2*^k), 101 bp in NC/Nga mice, and 103 bp in C57BL/6 mice (*H2*^b; Fig. 1c). The upper band was considered an artifact. The length of the microsatellite in NC/Nga mice (101 bp) was different compared to NZW mice (*H2*^z; 97 bp) and NZW.PL mice (*H2*^u; 93 bp), but was the same length as in DBA/1J mice (*H2*^q) and SWR/J mice (*H2*^q; 101 bp) [14]. The number of (CA)ⁿ repeats in BALB/c, A/J, C3H/HeJ, NC/Nga, and C57BL/6 mice were 14, 14, 16, 19, and 20, respectively (Fig. 1d, Supplementary Fig. 1). Therefore, the number of (CA)ⁿ repeats in NC/Nga mice was different from the other mouse strains examined.

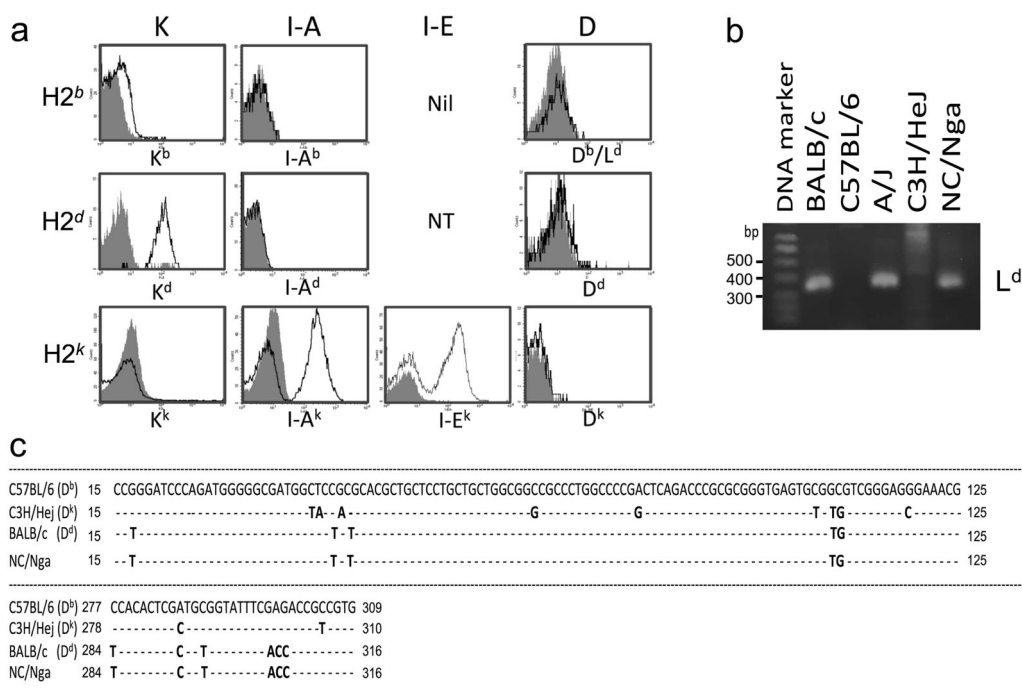


Fig. 2 NC/Nga mice have a unique *H2* haplotype. **a** The *H2* haplotype of fresh PBMCs from NC/Nga mice (no fill) was analyzed by FACS. C3H/HeJ PBMCs to *H2^b* (K^b, I-A^b, L^d/D^b)-specific each mAbs and C57BL/6 PBMCs to *H2^d* (K^d, I-A^d, D^d)- and *H2^k* (K^k, I-A^k, I-E^k, D^k)-specific each mAbs were used as the negative controls (gray fill). The image data were analyzed by fluorescence histogram overlay of

between negative control cells data and NC/Nga cells data. *NT* not tested. **b** The electrophoresis of PCR products for genome *L^d* are shown for five mouse strains: A/J, BALB/c, C3H/HeJ, NC/Nga, and C57BL/6. **c** Sequences for the typing of genome *H2-D* were analyzed in four strains: C57BL/6, C3H/HeJ, BALB/c, and NC/Nga. Data are representative of two independent experiments

NC/Nga mice have a unique phenotype (K^d, I-A^k, I-E^k) and lack the expression of D^d and L^d

H2 haplotype in NC/Nga mice was analyzed using flow cytometry. K^d was expressed as reported previously [13]; however, D^d, L^d, I-A^d, and I-E^d were not detected. Conversely, I-A^k and I-E^k were detected, but not K^k nor D^k (Fig. 2a). A PCR product for the L^d gene-specific region (intron 1–exon 2) was detected in BALB/c, A/J, and NC/Nga mice, but not in C3H/HeJ and C57BL/6 mice (Fig. 2b), and the product gene corresponded mainly to L^d by DNA-sequencing analysis. The genomic *H2* haplotype of the D region in NC/Nga mice corresponded mainly to “D^{dm}” in BALB/c mice as determined by DNA sequencing (Fig. 2c). This suggested that NC/Nga mice have a unique haplotype (K^d, D^d, L^d, A^k, and E^k) as a genotype and show the phenotype: K^d, I-A^k, and I-E^k.

D^d and L^d expressions under maturation are regulated at the protein level

To investigate whether D^d and L^d expression was upregulated under dendritic cell (DC) maturation, CD11b⁺ monocytes purified from PBMCs or CD11b⁺ myeloid cells purified from lymph nodes co-cultured for 3 days with IL-4 and GM-CSF were analyzed by FACS. DC formation after

maturation from CD11b⁺ cells was unchanged between BALB/c and NC/Nga mice by microscopy (data not shown). Only K^d expression was slightly elevated on DCs from NC/Nga mice against the upregulation of K^d, D^d, and L^d on DCs from BALB/c mice (Fig. 3a). L^d and D^d expressions on the cell surface or membrane-containing intracellular proteins (both cell surface proteins and intracellular proteins which mainly accumulated in the endoplasmic reticulum (ER)) were not elevated after DC maturation in NC/Nga mice (Fig. 3b). L^d, D^d, and K^d proteins (44–46 kDa) in the membrane fraction of BALB/c DCs were detected using commercial mAbs for western blotting assays, but the D^d in NC/Nga mice was barely detectable. K^d and L^d detected in NC/Nga mice were not elevated after DC maturation compared with BALB/c mice (Fig. 3b, c). D^d and L^d mRNA expressions before and after DC maturation in NC/Nga mice were detected, but they were not upregulated after maturation (Fig. 3d). Then, D^d and L^d mRNA expression in NC/Nga myeloid cells after culture only for 3 days tended to be lost by the effect of cell viability compared with BALB/c myeloid cells (Fig. 3d). These results indicated that the loss of L^d and D^d in NC/Nga myeloid cells occurred at the protein expression level but not at the cell surface regulation level as related to Golgi apparatus stops at ER exit sites.

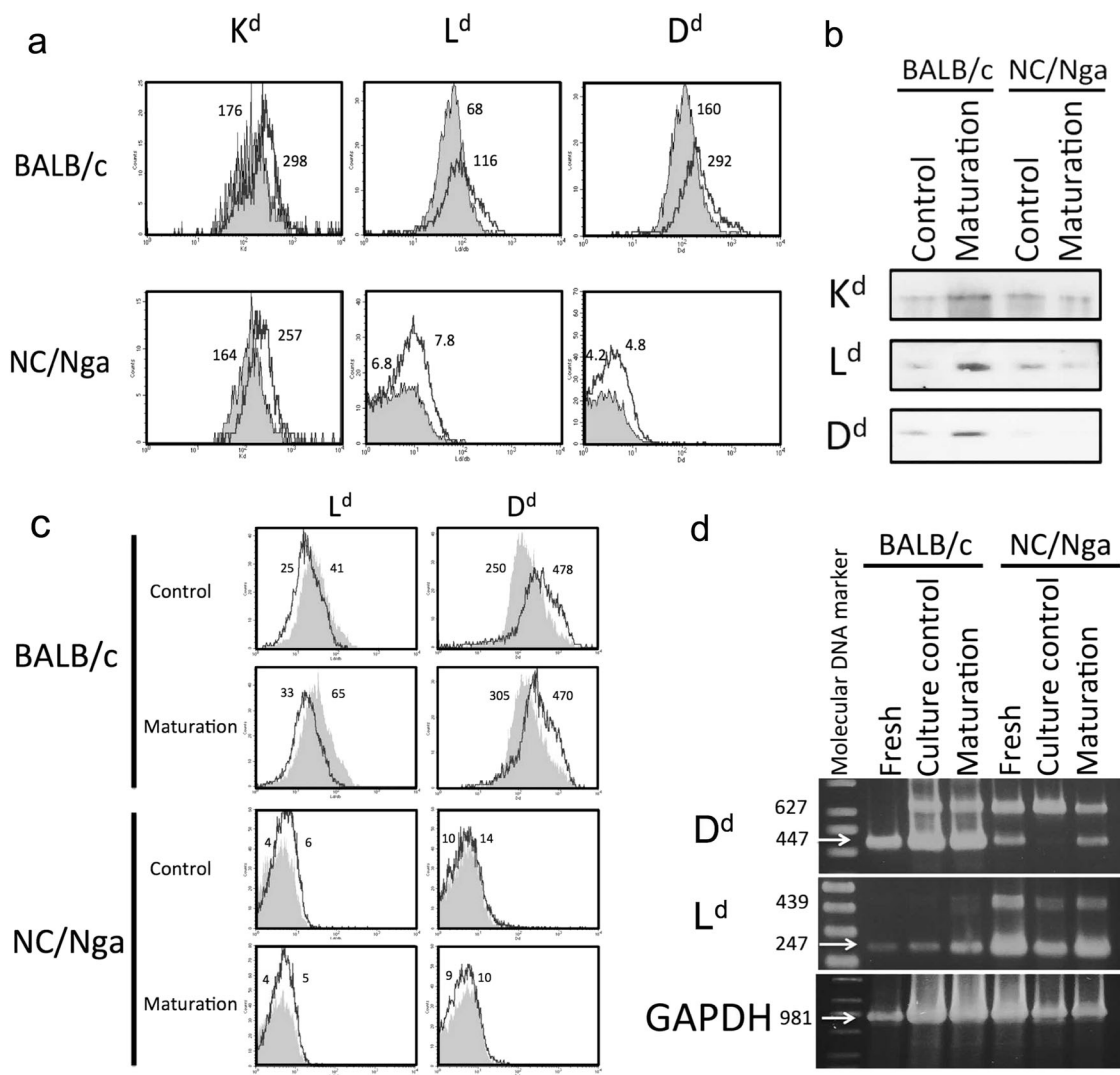


Fig. 3 D^d and L^d expressions in NC/Nga mice are not upregulated in DC maturation. **a** K^d , D^d , and L^d expressions on BALB/c or NC/Nga CD11b⁺ monocytes after control culture (gray fill) and maturation (no fill) with IL-4 and GM-CSF for 3 days were analyzed by FACS. The numbers in the panel indicate the mean fluorescence intensity (MFI). **b** The protein detection of K^d (46 kDa), D^d (45 kDa), and L^d (44 kDa) in lysed membrane fractions after control culture or maturation of CD11b⁺ macrophages was analyzed by western blotting assay. **c** D^d

and L^d expressions on the cell surface (gray fill) or membrane containing intracellular proteins (no fill) after control culture or maturation of CD11b⁺ macrophages were analyzed by FACS. The numbers in the panel indicate the MFI. **d** RT-PCR analysis of fresh, cultured control, and matured macrophages. PCR products of the region sandwiching the intron were separated into genome size (upper band) and cDNA size (lower band, rightwards arrow) by electrophoresis. Data are representative of three independent experiments

Mutant formation of a D^d/L^d hybrid molecule in NC/Nga mice

MHC class I molecules consist of a heavy chain (α chain) and a common microglobulin β_2 chain (β_2m chain). The heavy chain has three extracellular domains designated $\alpha 1$, $\alpha 2$, and $\alpha 3$ (exons 2–4), a transmembrane (TM) region (exons 5–6), and a cytoplasmic (CYT) region (exons 7–8). The full lengths of heavy chain D^d and L^d in NC/Nga mice were read by cDNA sequencing analysis. Interestingly, a D^d/L^d -hybrid mutant (termed D/L^{dm7} ; GenBank: LC278397) consisting of a fusion protein containing 1/4 of

the N terminal of D^d and 3/4 of the C terminal of L^d was formed in NC/Nga mice (Fig. 4, Supplementary Fig. 2). The D/L^{dm7} was partially detected by an L^d -specific mAb (clone; 28-14-8 or 27-11-13S) but not by a D^d -specific mAb (clone; 34-5-8S or 34-2-12). There were 22 original mutations at the amino-acid (aa) level in the D/L^{dm7} of NC/Nga mice (Fig. 4, Supplementary Fig. 2). A part of D^d and L^d identified by genomic typing (Fig. 2) was the D/L^{dm7} , and not wild-type D^d and L^d (Fig. 4).

On the basis of our findings, we propose the novel $H2$ haplotype of NC/Nga mice should be named “H-2^{nc},” because NC/Nga mice are an inbred strain but not congenic

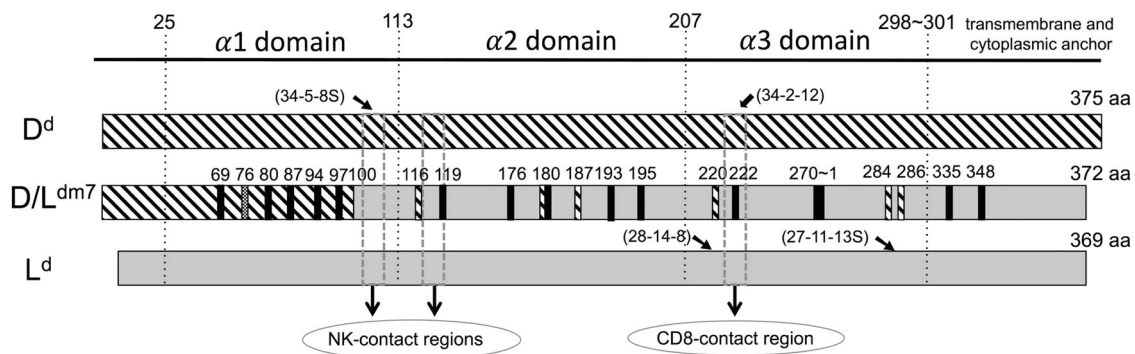


Fig. 4 Schematic diagram of the D^d/L^d-hybrid mutant (D/L^{dm7}) in NC/Nga mice. The schematic diagrams of L^d (369 aa), D/L^{dm7} (372 aa), and D^d (375 aa) are indicated. The same amino-acid (aa) sequence as D^d (hatched area) and as L^d (gray area) is indicated. The original

mutant aa (black area) with changes at aa positions 69, 76, 80, 87, 94, 97, 116, 119, 176, 179, 180, 187, 193, 195, 220, 222, 270, 271, 284, 286, 335, and 348 is shown. Regions recognized by commercial mAbs (clone names) are indicated

mice. The H-2^{nc} named after the NC of NC/Nga mice is considered to be specific to NC/Nga mice. The *H2* haplotypes of inbred strains of laboratory mice have been summarized [15, 16], but H-2^{nc} is not present in any of the following inbred mouse strains: H-2^a, H-2^b, H-2^{bc}, H-2^d, H-2^{d2}, H-2^f, H-2^{g7}, H-2^{g8}, H-2^j, H-2^k, H-2^{k2}, H-2^m, H-2^{nb1}, H-2^p, H-2^q, H-2^{q2}, H-2^r, H-2^s, H-2^{s2}, H-2^u, H-2^v, and H-2^z.

The D^d/L^d-hybrid mutant in the NC/Nga *H2* haplotype was expressed at low levels on the cell surface in DCs or macrophages. It was thought that levels of the D^d/L^d-hybrid protein would be decreased by ubiquitin-independent degradation because standard 20S proteasome activity was detected in NC/Nga mice than BALB/c mice (data not shown). A D^d/L^d-hybrid mutant molecule derived from mutant B10.D2 mice, termed “dm1” or “D/L^{dm1}” consists of the α1 and 2/3 of the α2 region identical to the D^d, and 1/3 of the α2 and α3-TM/CYT region identical to the L^d [17]. In addition, a mutant L^d molecule derived from BALB/c mice, termed “dm2” or “L^{dm2}” has a short length (p40) because of a deletion of part of L^d [18]. Moreover, two K^d mutants (K^{dm4} and K^{dm5}) and a D^d mutant (D^{dm6}) also were reported [19–21]. On the other hand, D^f shared the common motifs with both D^d and L^d molecules consisted of 95% of the D/L^{dm7} in NC/Nga mice (Supplementary Fig. 2) [22]. However, the novel D/L^{dm7} in NC/Nga mice is different to D/L^{dm1}, L^{dm2}, K^{dm4}, K^{dm5}, D^{dm6}, or D^f. Consequently, we propose to term the novel NC/Nga *H2* haplotype, H-2^{nc} (K^d, D/L^{dm7}, I-A^k, and I-E^k).

There are many common features between A/J mice H-2^a (K^k, D^d, L^d, I-A^k, and I-E^k) and NC/Nga mice H-2^{nc}, as both mouse strain *H2* haplotypes have unique mixed “k” and “d” alleles. A/J mice have a higher response to house dust mite allergens compared with BALB/c, C57BL/6, and C3H/HeN mice [23]. Therefore, A/J mice have been proposed to be a good model for genetic predisposition to AD [24]. In contrast, NC/Nga mice are also sensitive to house dust mite allergens under SPF conditions and develop a more intense

AD-like disease [25]. A thymectomy-induced autoimmune disease model was developed by the loss of peripheral tolerance mediated by natural regulatory T cells (*n*Tregs) in A/J and (C57BL/6 × A/J) F₁ mice but not in C57BL/6 mice [26]. Recent reports indicated that a decreased function and infiltration of Treg cells was related to AD development in NC/Nga mice [27, 28]. Although the role of CD4⁺ T cell (Th) has a great effect on immunoglobulin class switching of B cells in allergic diseases and autoimmune diseases, the MHC class I haplotype also is important for allergen epitope-specific CD8⁺ T-cell responses as well as MHC class II-restricted allergen-specific Th responses [29]. Recent work has highlighted interactions in the MHC region between known HLA risk haplotypes for various autoimmune diseases or allergy in humans [1, 2]. The characteristics of haplotype H-2^{nc} in NC/Nga mice might be associated with the development of AD. Hereafter, we are going to research the role of haplotype H-2^{nc} by establishing H-2^{nc}-congenic BALB/c mice or H-2^d-congenic NC/Nga mice.

Materials and methods

Mice

NC/Nga (unknownn, Mls-1^a) [30], C57BL/6 (H-2^b, Mls-1^b), BALB/c (H-2^d, Mls-1^b), C3H/HeJ (H-2^k, Mls-1^b), and A/J mice (H-2^a, Mls-1^b) were maintained in SPF conditions, and used at 8–10 weeks of age (male; Japan SLC, Hamamatsu, Japan). All mice were maintained in our full-barrier animal facility under controlled temperature, humidity, and a 12-h light/dark regimen. All experiments were approved by the Institutional Review Board (IRB) for Animal Studies of the Nippon Veterinary and Life science University, and were performed following the guidelines provided by the committee.

Flow cytometry

PBMCs were isolated from mouse blood using Lymphoprep (ProGen, Heidelberg, Germany). For analysis, PBMCs suspended in PBS were stained with a mixture of FITC-conjugated K^b (clone; AF6-88.5), K^k (clone; 36-7-5), D^b/L^d (clone; 28-14-8), K^d (clone; SF1-1.1), I-A^b (clone; KH74), I-A^k (clone; 11-5.2), and I-E^k (clone; 14-4-45)-specific mAbs (BioLegend, St. Louis, MO), PE-conjugated D^d (clone; 34-2-12), D^k (clone; 15-5-5), and I-A^d (clone; 39-10-8)-specific mAbs (BioLegend), PE-conjugated D^b/L^d (clone; 27-11-13S; Southern Biotechnology Associates Inc., Birmingham, AL), and biotin-conjugated D^d (clone; 34-5-8S)-specific mAbs (CedarLane, Ontario, Canada). One-color or two-color analysis was conducted by FACS (FACSCalibur, BD, Japan).

Intracellular staining assay

Cells fixed with BD Cytotfix/Cytoperm (BD) were washed with BD Perm/Wash solution (BD), and intracellular proteins and cell surface molecules were stained using a mixture of FITC-conjugated anti-L^d/D^b mAbs and PE-conjugated anti-D^d mAbs (BioLegend). Samples were analyzed by FACS (BD).

Microsatellite DNA polymorphism analysis

Primers for the promoter site of TNF- α (20 nM) were sense (5'-GGACAGAGAAGAAATGGGTTTC-3') and antisense (5'-TCGAATCTGGGGCCAATCAGGAGGG-3') [9]. The PCR products were electrophoresed on 12% acrylamide gels for 2 h at 150 V. Then, the PCR products were cloned into pGEM-T vectors according to the manufacturer's instructions (Promega, Madison, WI).

Western blotting analysis

Western blotting was performed with a SDS-PAGE Electrophoresis System (ATTO, Tokyo, Japan). Samples (CD11b⁺ cells, 1×10^6) were re-suspended in 50 μ L lysis buffer, and the membrane fraction was extracted after centrifugation. Lysates were boiled for 10 min, size-fractionated by 12.5% SDS-PAGE, and electrotransferred to a polyvinylidene difluoride membrane. The membrane was incubated for 1 h at RT in blocking buffer (5% skim milk/TBS), and reacted with primary biotin-conjugated mAbs specific to K^d (clone; SF1-1.1), D^d (clone; 34-2-12, 34-5-8S), and D^b/L^d (clone; 28-14-8, 27-11-13S), followed by horseradish peroxidase (HRP)-conjugated streptavidin (Dako). Proteins were visualized by the detection of HRP activity using an enhanced chemiluminescence kit (GE Healthcare Life Sciences).

MLR assay

The culture medium was RPMI 1640 containing 10% heat-inactivated FCS, L-glutamine, non-essential amino acids, sodium pyruvate, 2-ME, and penicillin-streptomycin (Wako, Tokyo, Japan). CD4⁺ or CD8⁺ T cells (3×10^5 /well) enriched from LNs using anti-mouse CD4 or anti-mouse CD8 Magnetic Particles-DM (BD Biosciences) were co-cultured for 72 h at 37 °C in 5% CO₂ with plate-adherent cells (3×10^5 /well) irradiated with 30 Gy X-ray (CP160, Faxitron, Wheeling, IL). Cell proliferation was analyzed by MTT assay based on WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt] uptake (CCK-8; Dojindo Molecular Technologies, Kumamoto, Japan). Briefly, 10 μ L CCK-8 was added for 4 h prior to the end of culture, and absorbance was measured at 450 nm with a microplate reader (Bio-RAD Labs, Hercules, CA, USA). The mean auto-response was calculated as 100%.

Cell culture

CD11b⁺ monocytes or myeloid cells (1×10^6 /well; >92%) enriched from PBMCs or LN using anti-mouse CD11b Magnetic Particles-DM (BD Biosciences) were cultured for 3 days with 20 ng/ml recombinant (r) IL-4 (PeproTech, Rocky Hill, NJ), and 20 ng/ml rGM-CSF (PeproTech) in a 96-well flat-plate. The culture medium was RPMI 1640 containing 10% heat-inactivated FCS, L-glutamine, non-essential amino acids, sodium pyruvate, 2-ME, and penicillin-streptomycin (Wako).

Genotyping

Genomic DNA (10 ng) purified (PureLink Genomic DNA Mini Kits, Invitrogen, Carlsbad, CA) from PBMCs was amplified in a 25 μ L PCR reaction using *Taq* polymerase (New England BioLabs, UK), according to the manufacturer's specifications. Primers were as follows: sense (5'-GCGTCGCCGCGGACGCTGGT-3') and antisense (5'-GCTCCTCGAGGCCGGGCCG-3') for genotyping the *H2-D* gene (exon 1–intron 1–exon 2; 300 bp; 20 nM), and sense (5'-CCTGCCCAGATCCCGCTCGCCTGGCCAG-3') and antisense (5'-GCCCCGCGCTCTGGTTGTAGTAGCCGAGC-3') for the *H2-L^d*-specific site of the genome (GenBank: V00752.1; intron 1–exon 2; 342 bp). PCR amplifications were conducted using a DNA thermal cycler (C1000, Bio-Rad Labs, Hercules, CA). The products were diluted fivefold with loading buffer consisting of xylene cyanol and bromophenol blue dyes, and were then electrophoresed on a 2% agarose gel. The genomic haplotype for the *H2-D* region (GenBank: L29190.1) was analyzed in specific polymorphic sites after DNA sequencing.

Reverse transcription (RT)-PCR

RNA from myeloid cells was isolated using BD IMag™ CD11b Magnetic Particles and extracted using ISOGEN (Nippon Gene, Tokyo, Japan). First strand cDNA was synthesized using 1 µg of isolated RNA template, *M-MLV* reverse transcriptase, Oligo (dT)₁₅ primer, RNase inhibitor, and dNTP according to the manufacturer's protocol (Nippon Gene). The primers (20 nM) for PCR were sense (5'-GCTGAGGTATTTTCGTCACCGCCGTGTC-3') and antisense (5'-TGCAGCACCAGCCTGCTCCCACTTGCGTC-3') for *H2-D^d* (exons 2–3, 447 bp), sense (5'-GGCTCCGCGCAGCTGCT-3') and antisense (5'-GTACCGGGGCTCCCCCGA-3') for *H2-L^d* (exons 1–2, 247 bp), sense (5'-ATGGCTCCGCGCACGCT-3') and antisense (5'-TCACGCTTTACAATCTCGGAGAGACATTTC-3') for *H2-D/L^{dm7}* (full-length 1140 bp), and sense (5'-ACCA-CAGTCCATGCCATCAC-3') and antisense (5'-TCCAC-CACCCTGTGCTGTA-3') for *GAPDH* (981 bp). Samples were amplified for 36 cycles with the each probes (94 °C for 30 s, 60 °C for 50 s, and 72 °C for 80 s).

Statistical analysis

Statistical analysis was performed by *ANOVA* using Excel (Microsoft) and StatPlus (AnalystSoft, Alexandria, VA). A *p* value < 0.05 was considered significant.

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Compliance with ethical standards

Competing interests The authors declare that they have no competing financial interests.

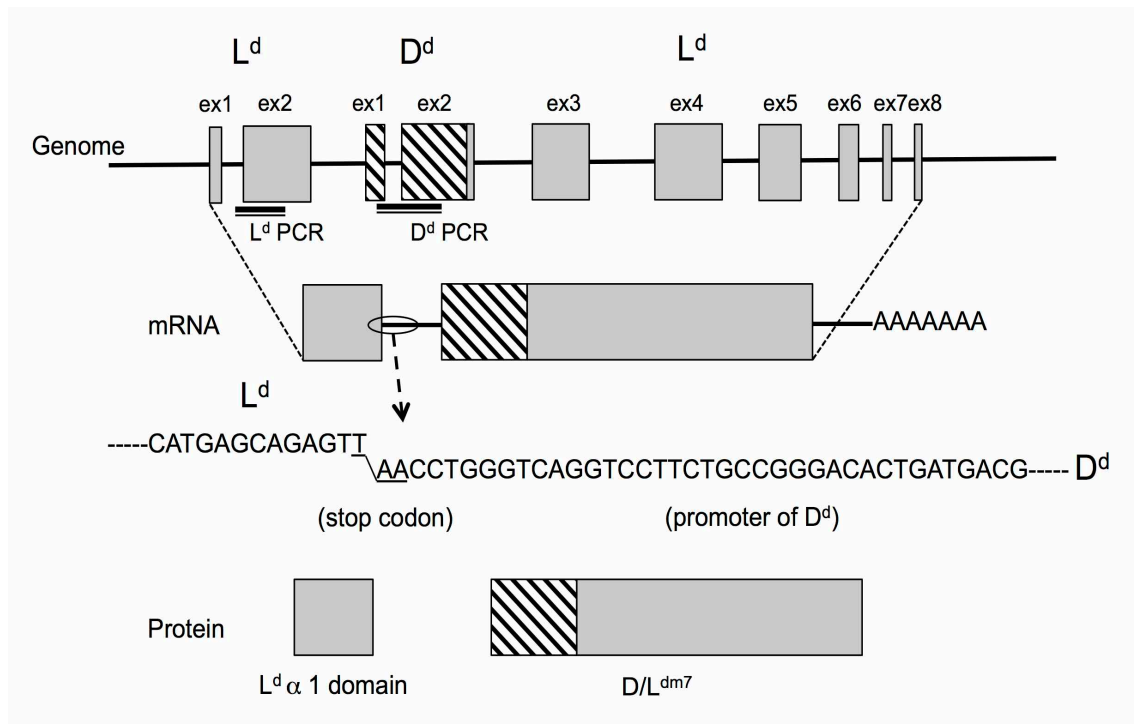
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<i>D/L^{dm7}</i> ATGGGGCGATGGCTCCGCGCACGCTGCTCTGCTGCTGGCGGCGCCCTGGGTCCGACT <i>L^d</i> -----ATGGCTCCGCGCACGCTGCTCTGCTGCTGGCGGCGCCCTGGCGCCGACT <i>D^d</i> ATGGGGCGATGGCTCCGCGCACGCTGCTCTGCTGCTGGCGGCGCCCTGGGTCCGACT <i>D^f</i> ATGGGGCGATGGCTCCGCGCACACTGCTCTGCTGCTGGCGGCGCCCTGGCGCCGACT <i>D^k</i> ATGGGGCGATGGCTCCGCGCACGCTGCTCTGCTGCTGGCGGCGCCCTGGCGCCGATT		<i>D/L^{dm7}</i> GCGACGCTGCTGCGCACAGATTCCCCAAAGGCACATGTGACCCATCACCCAGATCTGAA <i>L^d</i> GCGACGCTGCTGCGCACAGATTCCCCAAAGGCACATGTGACCCATCACCCAGATCTGAA <i>D^d</i> GGTACGCTGCTGCGCACAGATTCCCCAAAGGCACATGTGACCCATCACCCAGATCTGAA <i>D^f</i> GCGACGCTGCTGCGCACAGATTCCCCAAAGGCACATGTGACCCATCACCCAGATCTGAA <i>D^k</i> GCGACGCTGCTGCGCACAGATTCCCCAAAGGCACATGTGACCCATCACCCAGATCTGAA	60
<i>D/L^{dm7}</i> CAGACCCGCGCGGGCCACACTCGATGCGGTATTTCTGAGACCCCGGTGTCCCGGCCGGC <i>L^d</i> CAGACCCGCGCGGGCCACACTCGATGCGGTATTTCTGAGACCCCGGTGTCCCGGCCGGC <i>D^d</i> CAGACCCGCGCGGGCCACACTCGCTGAGGTATTTCTGACCCCGGTGTCCCGGCCGGC <i>D^f</i> CAGACCCGCGCGGGCCACACTCGATGAGGTATTTCTGACCCCGGTGTCCCGGCCGGC <i>D^k</i> CAGACCCGCGCGGGCCACACTCGCTGAGGTATTTCTGAGACCCCGGTGTCCCGGCCGGC		<i>D/L^{dm7}</i> GGTAAAGTACACCTGAGGTGCTGGGCCCTGGGCTTCTACCCCTGCTGACATCACCCGTACC <i>L^d</i> GGTAAAGTACACCTGAGGTGCTGGGCCCTGGGCTTCTACCCCTGCTGACATCACCCGTACC <i>D^d</i> GGTGATGACACCTGAGGTGCTGGGCCCTGGGCTTCTACCCCTGCTGACATCACCCGTACC <i>D^f</i> GGTGAAAGTACACCTGAGGTGCTGGGCCCTGGGCTTCTACCCCTGCTGACATCACCCGTACC <i>D^k</i> GTTGAAGTACACCTGAGGTGCTGGGCCCTGGGCTTCTACCCCTGCTGACATCACCCGTACC	120
<i>D/L^{dm7}</i> TCCGGGAGCCCCGGTACATGGAAGTCGGCTACGTGGACAACACGGAGTTCGTGCGCTTC <i>L^d</i> CTCGGGAGCCCCGGTACATCTCTGTCGGCTATGTGGACAACACGGAGTTCGTGCGCTTC <i>D^d</i> TCCGGGAGCCCCGGTACATGGAAGTCGGCTACGTGGACAACACGGAGTTCGTGCGCTTC <i>D^f</i> TCCGGGAGCCCCGGTACATCTCTGTCGGCTACGTGGACAACACGGAGTTCGTGCGCTTC <i>D^k</i> CTCGGGAGCCCCGGTTCATCTCTGTCGGCTACGTGGACAACACGGAGTTCGTGCGCTTC		<i>D/L^{dm7}</i> TGGCAGTTGAATGGGAGGAGCTGACCCAGGACATGGAGCTTGTGGAGACCAGGCCTGCA <i>L^d</i> TGGCAGTTGAATGGGAGGAGCTGACCCAGGACATGGAGCTTGTGGAGACCAGGCCTGCA <i>D^d</i> TGGCAGTTGAATGGGAGGAGCTGACCCAGGACATGGAGCTTGTGGAGACCAGGCCTGCA <i>D^f</i> TGGCAGTTGAATGGGAGGAGCTGACCCAGGACATGGAGCTTGTGGAGACCAGGCCTGCA <i>D^k</i> TGGCAGTTGAATGGGAGGAGCTGACCCAGGACATGGAGCTTGTGGAGACCAGGCCTGCA	180
<i>D/L^{dm7}</i> GACAGCGACGCGGAGAAATCCGAGATTTGAGCCCGCGGCGCGGTGGATGAGCAGGTGGAG <i>L^d</i> -----GAGAATCCGAGATTTGAGCCCGCGGCGCGGTGGATGAGCAGGTGGAG <i>D^d</i> GACAGCGACGCGGAGAAATCCGAGATTTGAGCCCGCGGCGCGGTGGATGAGCAGGTGGAG <i>D^f</i> GACAGCGACGCGGAGAAATCCGAGATTTGAGCCCGCGGCGCGGTGGATGAGCAGGTGGAG <i>D^k</i> GACAGCGACGCGGAGAAATCCGAGATTTGAGCCCGCGGCGCGGTGGATGAGCAGGTGGAG		<i>D/L^{dm7}</i> GGGGATGGAACCTTCCAGAAGTGGGCACTGTGGTGGTGCCTTGGGAAGGAGCAGAAT <i>L^d</i> GGGGATGGAACCTTCCAGAAGTGGGCACTGTGGTGGTGCCTTGGGAAGGAGCAGAAT <i>D^d</i> GGGGATGGAACCTTCCAGAAGTGGGCACTGTGGTGGTGCCTTGGGAAGGAGCAGAAT <i>D^f</i> GGGGATGGAACCTTCCAGAAGTGGGCACTGTGGTGGTGCCTTGGGAAGGAGCAGAAT <i>D^k</i> GGGGATGGAACCTTCCAGAAGTGGGCACTGTGGTGGTGCCTTGGGAAGGAGCAGAAT	240
<i>D/L^{dm7}</i> CCGGAGTATTGGGAGCGGACACACGGAGAGCCAGGGCATGAGCAGACTTCCGAGTG <i>L^d</i> CCGGAGTATTGGGAGCGGATCAGCAGATCCGCAAGGGCAGGAGCAGTGGTCCGAGTG <i>D^d</i> CCGGAGTATTGGGAGCGGAGACACGGAGAGCCAGGGCATGAGCAGACTTCCGAGTG <i>D^f</i> CCGGAGTATTGGGAGCGGAGACACGGATCCGCAAGGGCATGAGCAGACTTCCGAGTG <i>D^k</i> CCGGAGTATTGGGAGCGGAGACACGGATCCGCAAGGGCATGAGCAGACTTCCGAGTG		<i>D/L^{dm7}</i> TACACATGCCATGTGAAACATGAGGGGCTGCCTGAGCCCTCACCTGAGATG----- <i>L^d</i> TACACATGCCATGTGAAACATGAGGGGCTGCCTGAGCCCTCACCTGAGATG----- <i>D^d</i> TACACATGCCATGTGAAACATGAGGGGCTGCCTGAGCCCTCACCTGAGATG----- <i>D^f</i> TACACATGCCATGTGAAACATGAGGGGCTGCCTGAGCCCTCACCTGAGATG----- <i>D^k</i> TACACATGCCATGTGAAACATGAGGGGCTGCCTGAGCCCTCACCTGAGATG-----	300
<i>D/L^{dm7}</i> AACCTGAGGACCTGCTCGGCTACTACAACAGAGCGCGGGCGGCTCTCACACACTCCAG <i>L^d</i> AACCTGAGGACCTGCTCGGCTACTACAACAGAGCGCGGGCGGCTCTCACACACTCCAG <i>D^d</i> AACCTGAGGACCTGCTCGGCTACTACAACAGAGCGCGGGCGGCTCTCACACACTCCAG <i>D^f</i> AACCTGAGGACCTGCTCGGCTACTACAACAGAGCGCGGGCGGCTCTCACACACTCCAG <i>D^k</i> AACCTGAGGACCTGCTCGGCTACTACAACAGAGCGCGGGCGGCTCTCACACACTCCAG		<i>D/L^{dm7}</i> GAGGAGCCTCCTCTCATCCACCAAGACTAACACAATAATATGCTGTTCCGGTGTCTCT <i>L^d</i> GAGGAGCCTCCTCTCATCCACCAAGACTAACACAATAATATGCTGTTCCGGTGTCTCT <i>D^d</i> GAGGAGCCTCCTCTCATCCACCAAGACTAACACAATAATATGCTGTTCCGGTGTCTCT <i>D^f</i> GAGGAGCCTCCTCTCATCCACCAAGACTAACACAATAATATGCTGTTCCGGTGTCTCT <i>D^k</i> GAGGAGCCTCCTCTCATCCACCAAGACTAACACAATAATATGCTGTTCCGGTGTCTCT	360
<i>D/L^{dm7}</i> TGGATGTAAGGCTGTGACGTGGGGTCCGACGGGCGCCTCTCCCGGGGTACGAGCAGTTC <i>L^d</i> TGGATGTAAGGCTGTGACGTGGGGTCCGACGGGCGCCTCTCCCGGGGTACGAGCAGTTC <i>D^d</i> TGGATGTAAGGCTGTGACGTGGGGTCCGACGGGCGCCTCTCCCGGGGTACGAGCAGTTC <i>D^f</i> TGGATGTAAGGCTGTGACGTGGGGTCCGACGGGCGCCTCTCCCGGGGTACGAGCAGTTC <i>D^k</i> TGGATGTAAGGCTGTGACGTGGGGTCCGACGGGCGCCTCTCCCGGGGTACGAGCAGTTC		<i>D/L^{dm7}</i> GGAGCTATGGCCATCATTGGAGCTGTGGTGGCTTTTGTGATGATGAGGAGGAGAAACACA <i>L^d</i> GGAGCTATGGCCATCATTGGAGCTGTGGTGGCTTTTGTGATGATGAGGAGGAGAAACACA <i>D^d</i> GGAGCTATGGCCATCATTGGAGCTGTGGTGGCTTTTGTGATGATGAGGAGGAGAAACACA <i>D^f</i> GGAGCTATGGCCATCATTGGAGCTGTGGTGGCTTTTGTGATGATGAGGAGGAGAAACACA <i>D^k</i> GGAGCTATGGCCATCATTGGAGCTGTGGTGGCTTTTGTGATGATGAGGAGGAGAAACACA	420
<i>D/L^{dm7}</i> CCTACGACGGCTGCGATTACATCGCCCTGAACGAAGACCTGAAACGTTGGACGGCGGGC <i>L^d</i> CCTACGACGGCTGCGATTACATCGCCCTGAACGAAGACCTGAAACGTTGGACGGCGGGC <i>D^d</i> CCTACGACGGCTGCGATTACATCGCCCTGAACGAAGACCTGAAACGTTGGACGGCGGGC <i>D^f</i> CCTACGACGGCTGCGATTACATCGCCCTGAACGAAGACCTGAAACGTTGGACGGCGGGC <i>D^k</i> CCTACGACGGCTGCGATTACATCGCCCTGAACGAAGACCTGAAACGTTGGACGGCGGGC		<i>D/L^{dm7}</i> GGTGAAAAGGAGGGGACTATGCTCGGCTCCAGGCTCCAGAGCTCTGAAATGTCTCTC <i>L^d</i> GGTGAAAAGGAGGGGACTATGCTCGGCTCCAGGCTCCAGAGCTCTGAAATGTCTCTC <i>D^d</i> GGTGAAAAGGAGGGGACTATGCTCGGCTCCAGGCTCCAGAGCTCTGAAATGTCTCTC <i>D^f</i> GGTGAAAAGGAGGGGACTATGCTCGGCTCCAGGCTCCAGAGCTCTGAAATGTCTCTC <i>D^k</i> GGTGAAAAGGAGGGGACTATGCTCGGCTCCAGGCTCCAGAGCTCTGAAATGTCTCTC	480
<i>D/L^{dm7}</i> GACATGGCGGCGAGATCACCCGACGCAAGTGGGAGCAGGCTGGTATGCAGAGAGGAGT <i>L^d</i> GACATGGCGGCGAGATCACCCGACGCAAGTGGGAGCAGGCTGGTATGCAGAGAGGAGT <i>D^d</i> GACATGGCGGCGAGATCACCCGACGCAAGTGGGAGCAGGCTGGTATGCAGAGAGGAGT <i>D^f</i> GACATGGCGGCGAGATCACCCGACGCAAGTGGGAGCAGGCTGGTATGCAGAGAGGAGT <i>D^k</i> GACATGGCGGCGAGATCACCCGACGCAAGTGGGAGCAGGCTGGTATGCAGAGAGGAGT		<i>D/L^{dm7}</i> CGAGATTGTAA-----AGCGTGAAGACAGCTGCCTGGA <i>L^d</i> CGAGATTGTAA-----AGCGTGAAGACAGCTGCCTGGA <i>D^d</i> CGAGATTGTAA-----AGCGTGAAGACAGCTGCCTGGA <i>D^f</i> CGAGATTGTAA-----AGCGTGAAGACAGCTGCCTGGA <i>D^k</i> CGAGATTGTAA-----AGCGTGAAGACAGCTGCCTGGA	540
<i>D/L^{dm7}</i> AGGGCCTACCTGGAGGGCAGCTGCGTGGAGTGGCTCCGAGACACCTGAAGAACGGGAAT <i>L^d</i> AGGGCCTACCTGGAGGGCAGCTGCGTGGAGTGGCTCCGAGATACCTGAAGAACGGGAAT <i>D^d</i> AGGGCCTACCTGGAGGGCAGCTGCGTGGAGTGGCTCCGAGATACCTGAAGAACGGGAAT <i>D^f</i> AGGGCCTACCTGGAGGGCAGCTGCGTGGAGTGGCTCCGAGACACCTGAAGAACGGGAAT <i>D^k</i> AGGGCCTACCTGGAGGGCAGCTGCGTGGAGTGGCTCCGAGATACCTGGAGTCCGGGAAT		<i>D/L^{dm7}</i> GGTGAAAAGGAGGGGACTATGCTCGGCTCCAGGCTCCAGAGCTCTGAAATGTCTCTC <i>L^d</i> GGTGAAAAGGAGGGGACTATGCTCGGCTCCAGGCTCCAGAGCTCTGAAATGTCTCTC <i>D^d</i> GGTGAAAAGGAGGGGACTATGCTCGGCTCCAGGCTCCAGAGCTCTGAAATGTCTCTC <i>D^f</i> GGTGAAAAGGAGGGGACTATGCTCGGCTCCAGGCTCCAGAGCTCTGAAATGTCTCTC <i>D^k</i> GGTGAAAAGGAGGGGACTATGCTCGGCTCCAGGCTCCAGAGCTCTGAAATGTCTCTC	600

Supplementary information Fig. 2. The cDNA base sequence of the *D^{dm7}/L^{dm7}*-hybrid mutant (*D/L^{dm7}*) in NC/Nga mice. The cDNA base sequence of *D/L^{dm7}* (GenBank: LC278397.1), *L^d* (GenBank: M33151.1), *D^d* (GenBank: U47326.1), *D^f* (GenBank: M34961.1), and *D^k* (GenBank: U47327.1) region of NC/Nga mice are indicated. Shadows indicate a different base compared with *D/L^{dm7}*. Underlines indicate differences at the amino acid (aa) level compared with *D/L^{dm7}*. Base sequence of *D^d* (yellow), *L^d* (green), and specific to *D/L^{dm7}* (pink) are indicated.



Supplementary information Fig. 3. The schematic diagram of D/L^{dm7} in NC/Nga mice. Genomic model of the L^d and D^d clusters are shown. It is presumed that D^d (exon 1-2) gets in intron 2 of L^d . The PCR site for genotyping of $H2-D$ and the L^d -specific PCR product in experimental results are indicated by double underline. Since a mRNA of mutant molecule consisted of L^d (exons 1-2), a part of promoter of D^d , D^d (exons 1-2), and L^d (exons 3-8), it was expected that both $L^d \alpha 1$ domain and D/L^{dm7} would be synthesized as protein, and be cleaved.