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Note

Clonal deletion of T cell repertoires with specific T cell receptor Vβ chains by two endogenous superantigens in NC/Nga mice

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Superantigens (SAgs) are powerful T-cell stimulatory proteins. Because an atopic dermatitis (AD) model NC/Nga mice had two endogenous SAgs, namely *minor lymphocyte-stimulating locus-1^a (Mls-1^a)* and *mouse mammary tumor virus (MMTV)(SHN)*, SAg-responsive T-cells bearing Vβ5.1, Vβ6, Vβ8.1, Vβ8.2, Vβ8.3, Vβ9, and Vβ11 should be endogenously deleted. Here, we discuss that the endogenous SAgs-expression may be involved in AD-sensitivity in NC/Nga mice.

Key words: Superantigen; TCR Vβ; NC/Nga mice; *Mls-1a*; *MMTV(SHN)*

Superantigens (SAgs) bind to major histocompatibility complex (MHC) class II molecules and activate both CD4$^+$ and CD8$^+$ T-cells bearing variable regions of specific T-cell receptor β chains (TCR Vβ) domain.[1)] Endogenous SAg of *mouse mammary tumor virus* (MMTV or *Mtv*) causes tolerance or the clonal deletion of T cells bearing a specific Vβ element, thus allowing proliferation of infected B cells and transmission of the virus to offspring.[1)] Of the murine endogenous SAgs encoded by *Mtv*, the *minor lymphocyte-stimulating locus-1^a* (*Mls-1^a*), encoded by an open reading frame in the 3′ long terminal repeat of the *Mtv-7* provirus, is expressed in AKR, CBA/J, C58, DBA/2, and NZB mice, where it induces tolerance or clonal deletion of T-cells bearing TCR Vβ6, Vβ8.1, and Vβ9.[1)] TCR Vβ7 is also weakly stimulated by *Mls-1^a*.[2)] In contrast, *human endogenous retroviruses type K* (*HERV-K*), *HIV*, and *human T cell leukemia virus (HTLV)* are complex retroviruses with features similar to *MMTV*.[3)] The *HERV-K* family also contains endogenous SAgs, suggesting their potential involvement in autoimmunity.[3,4)] *HERV-K* family members (particularly *HERV-K10* and *HERV-K18*), *HTLV-1-related endogenous sequence (HRES)-1*, *endogenous retrovirus (ERV)-3*, and *HERV-E4-1* may also function in the pathogenesis of systemic lupus erythematosus.[5)] Although *HERV-K18* is recognized by human T-cells bearing TCR Vβ7 and Vβ13,[3,4)] it is unknown whether other *HERV* are recognized by other T-cells bearing a specific Vβ element.

NC/Nga mice were established as an inbred strain by Dr K. Kondo in 1957 based on the Japanese fancy mice (Nishiki-Nezumi).[6)] This strain has been reported to have the following biological characteristics: liver and kidney esterase isozyme patterns similar to those in DBA/2 mice and high susceptibility to X ray-irradiation and to anaphylactic shock from ovalbumin (OVA).[6)] NC/Nga mice spontaneously develop atopic dermatitis (AD)-like disease under conventional conditions, but not under specific pathogen-free conditions.[7)] Here, we show that the clonal deletion of T-cell repertoire with a specific TCR Vβ is due to the expression of two endogenous SAgs in NC/Nga mice, and discuss the role of endogenous SAgs in developing spontaneous AD-like disease in NC/Nga mice.

Animal; NC/Nga (H-2^{nc}, previously unknown),[8)] BALB/c (H-2^d, *Mls-1^b*), DBA/2 (H-2^d, *Mls-1^a*), and B10.BR (H-2^k, *Mls-1^b*) (male) (Japan SLC, Hamamatsu, Japan) were maintained in SPF conditions, and used at 8–10 weeks of age. All mice were maintained in our full-barrier animal facility under controlled temperature, humidity, and a 12-hour light/dark regimen. All experiments were approved by the Institutional Review Board (IRB) for Animal Studies of the Nippon Veterinary and Life-science University (NVLU), and were performed following the guidelines provided by the committee.

Flow cytometry; Peripheral blood mononucleated cells (PBMCs) were isolated from mouse blood using Lymphoprep (ProGen, Heidelberg, Germany). For FACS analysis, PBMCs suspended in PBS were stained with a mixture of FITC or PE-conjugated CD4 (clone; GK1.5) or CD8 (clone; 53-6.7) and FITC-conjugated Vβ8.1 8.2 (clone; KJ16-133.18), PE-conjugated Vβ5.1 5.2 (clone; MR9-4), PE-conjugated Vβ6 (clone; RR4-7)-specific mAbs, PE-conjugated Vβ9 (clone; MR10-2)-specific mAbs, or PE-conjugated Vβ11 (clone; KT11)-specific mAbs (BioLegend, St. Louis,

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Abbreviations: Sags, superantigens; TCR Vβ, variable regions of T cell receptor β chains; APCs, antigen-presenting cells; MHC, major histocompatibility complex; AD, atopic dermatitis; Mls, minor lymphocyte-stimulating locus; MMTV, mouse mammary tumor virus; HERV, human endogenous retroviruses; mAbs, monoclonal antibodies; SEB, Staphylococcal enterotoxin B.

MO). Two-color analysis was conducted by FACS (FACSCalibur, Nippon Becton Dickinson, Japan).

RT-PCR; $CD4^+$ T-cells, $CD8^+$ T-cells, B-cells (3×10^5/well), or myeloid cells were enriched from LNs using anti-mouse CD4, anti-mouse CD8, anti-mouse B220, or anti-mouse CD11b Magnetic Particles-DM (BD Biosciences). Antigen-presenting cells (APCs) were used as maturated myeloid cells cultured with treatment of 20 ng/ml recombinant (r)GM-CSF (PeproTech, Rocky Hill, NJ) and 20 ng/ml rIL-4 (PeproTech) for 72 h at 37°C in 5% CO_2. These RNA were 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′-GTCAAAGAACAGGTGCAAGGAC-3′) and antisense (5′-AAGGGATCGAAGCCAACGCG-3′) for *Mls-1*^{a} (443 bp), sense (5′-TCTGCGCACAAACGGATGAG-3′) and antisense (5′-AAGGGGGCATCTGTTGGTCT-3′) for *MMTV(SHN)* (572 bp) (GenBank: X78590.1), sense (5′-ATGGCTTCTGTGGCTACAGACC-3′) for Vβ2, sense (5′-AAGGACAAAAAGCAAAGATGAGG-3′) for Vβ3, sense (5′-AACACTGCCTTCCCTGACCC-3′) for Vβ5.1, sense (5′-CAAAAACTGACCTTGAAATGTCAA-3′) for Vβ6, sense (5′-AGAATGTTTTGCTGGAATGTGGA-3′) for Vβ7, sense (5′-GAAAGGTGACATTGAGCTGTCAC-3′) for Vβ8.1, sense (5′-GGAAAGGTGACATTGAGCTGTAAT-3′) for Vβ8.2, sense (5′-GAAAGGTGACATTGAGCTGTCAC-3′) for Vβ8.3, sense (5′-CTTCTGTCTTCTTGCAGCCACTT-3′) for Vβ9, sense (5′-TGCCTCTTGGGAATAGGCC-3′) for Vβ10, sense (5′-TGCTTCTTGAGAGCAGAACCAA-3′) for Vβ11, antisense (5′-GCAATCTCTGCTTTTGATGGCT-3′) for Cβ,[9] and sense (5′-ACCACAGTCCATGCCATCAC-3′), and antisense (5′-TCCACCACCCTGTGCTGTA-3′) for GAPDH (981 bp). The PCR conditions of *Mls-1*^{a}, *MMTV(SHN),* Vβ5.1, and Vβ6 using *Taq* DNA polymerase (BioAcademia, Osaka, Japan) were as follows: 36 cycles at 98 °C for 10 s, 56.5 °C for 30 s, and 72 °C for 70 s, then the annealing temperature for Vβ2, Vβ3, Vβ7, and Vβ8.1 are 61 °C, and the annealing temperature for Vβ8.2, Vβ8.3, Vβ9, Vβ10, and Vβ11 are 58.5 °C. The products were diluted five-fold with loading buffer consisting of xylene cyanol and bromophenol blue dyes and were electrophoresed on 2% agarose gel.

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

The *Mls-1*^{a} and *MMTV(SHN)* genes were detected in mRNA of NC/Nga mice B-cells or antigen-presenting cells (APCs) using RT-PCR (Fig. 1(A) and (B)). Although the clonal deletion of TCR Vβ6 and Vβ9 by *Mls-1*^{a} expression in NC/Nga mice was assumed to be the same as in control DBA/2 mice (*Mtv-1, Mtv-6, Mtv-7, and Mtv-13*), other T-cells bearing TCR Vβ5.1, Vβ8.1, Vβ8.2, Vβ8.3, and Vβ11 should be also deleted in NC/Nga mice $CD4^+$ T-cells (Fig. 1(c)). Then, when the expression of Vβ chains on also thymocytes and

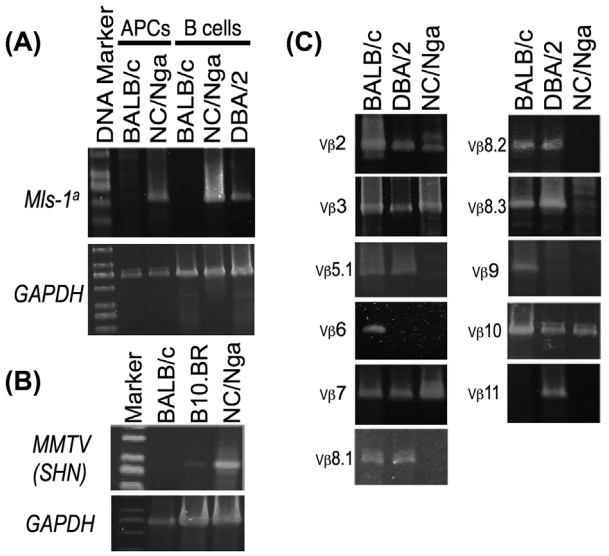

Fig. 1. The expression of TCR Vβ repertoires and endogenous superantigens in NC/Nga mice. (A) Endogenous superantigen *Mls-1*^{a}-specific expression was measured by RT-PCR using APCs purified from BALB/c and NC/Nga mice and B-cells purified from BALB/c (*Mls-1*^{b}), NC/Nga (unknown), and DBA/2 (*Mls-1*^{a}) mice, (B) The mRNA expression of *MMTV(SHN)* in B-cells purified from BALB/c, B10.BR, and NC/Nga mice were measured using RT-PCR, and (c) The mRNA expression of TCR Vβ repertoires (Vβ2, Vβ3, Vβ5.1, Vβ6, Vβ7, Vβ8.1, Vβ8.2, Vβ8.3, Vβ9, Vβ10, and Vβ11) in $CD4^+$ T-cells purified from BALB/c, DBA/2, and NC/Nga mice was measured using RT-PCR. Data are representative of two independent experiments.

LN cells in NC/Nga strain was compared, the results indicated before and after clonal deletion of periferal T cells in NC/Nga mice. (Supplemental Fig. S1). Because *MMTV(SHN)* mRNA was expressed in B10.BR or NC/Nga mice but not BALB/c mice (Fig. 1(B)), the clonal deletion of a specific TCR Vβ by *MMTV(SHN)* should also be induced. Besides, the PCR products of *Mls-1*^{a} and *MMTV(SHN)* were confirmed by the sequence analysis (Supplemental Fig. S2). Furthermore, TCR Vβ11, but not Vβ5.1, was deleted in BALB/c mice (*Mtv-6, Mtv-8, and Mtv-9*), although it is reported that the expression of *Mls*^{f} in BALB/c mice induced the deletion or tolerance of T-cells bearing TCR Vβ5.1, Vβ11, and Vβ12.[10]

RT-PCR results were then confirmed using FACS analysis. T-cell repertoires of TCR Vβ6, Vβ8.1, Vβ8.2, Vβ9, and Vβ11 were clearly deleted in NC/Nga mice. However the deletion of Vβ5.1 in NC/Nga mice was not confirmed by FACS analysis because the population of $CD4^+$ T cells was detected by mAb specific to Vβ5.1 5.2 (data not shown). Vβ11 was deleted in also BALB/c mice ($p < 0.05$, *ANOVA*). Vβ6 and Vβ9 were deleted in DBA/2 mice having *Mls-1*^{a} ($p < 0.05$, *ANOVA*; Fig. 2(A) and (B)).

Because physiological barrier dysfunctions of the skin, high numbers of *Staphylococcus aureus* among the skin surface bacterial flora, and an increased hypersensitivity to itch were reported in AD patients,[11] the effects of applying exogenous SAg *Staphylococcal enterotoxin B* (*SEB*) to the skin of AD model NC/Nga mice have been investigated.[12] However, the effect of

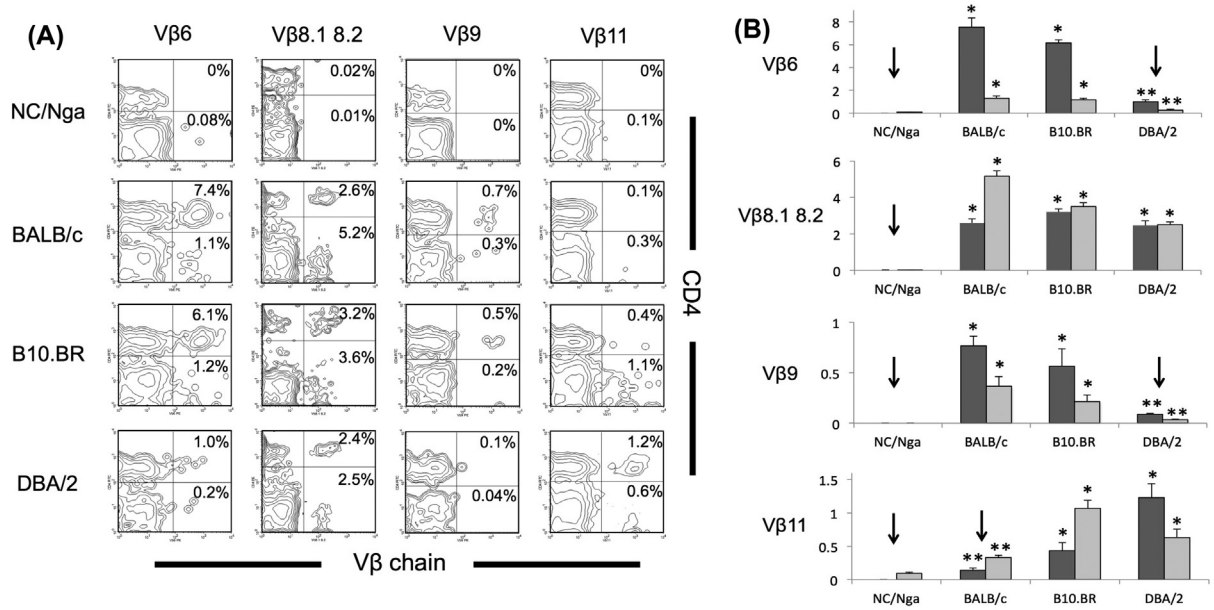

Fig. 2. FACS analysis of the expression of TCR Vβ6, Vβ8.1–8.2, Vβ9, and Vβ11. (A) Samples were stained using a mixture of mAbs specific to CD4 and TCR Vβ6, Vβ8.1–8.2, Vβ9, or Vβ11 in PBMCs of NC/Nga, BALB/c, B10.BR, and DBA/2 mice and analyzed using FACS. An example of a two-color panel is shown, (B) The percentages of TCR Vβ6, Vβ8.1–8.2, Vβ9, or Vβ11-positive populations in $CD4^+$ cells (dark gray) or $CD8^+$ cells (light gray) of NC/Nga, BALB/c, B10.BR, and DBA/2 mice ($n = 4$) are shown. Significant downregulation (down arrow) by clonal deletion is indicated.

Notes: *$p < 0.02$; *ANOVA,* when compared with the respective NC/Nga mice. **$p < 0.05$, *ANOVA*, when compared with respective *-marks. Bars represent mean ± SE of data. Data are representative of two independent experiments.

SEB on NC/Nga mice may be reviewed because it was identified that endogenous SAgs *Mls-1*^{a} and *MMTV (SHN)* were expressed in these mice. Although it was reported that TCR $V\beta8^+$ T-cells, but not $V\beta2.1^+$and $V\beta7.1^+$ T-cells, were absent in NC/Nga mice,[12] it is considered to be caused by the endogenous clonal deletion by *Mls-1*^{a}. The expression of the *Mtv-7 provirus* product, *Mls-1*^{a}, induces the deletion or tolerance of TCR Vβ6, Vβ8.1, and Vβ9.[1] Our results indicated NC/Nga mice lack T-cells bearing TCR Vβ5.1, Vβ6, Vβ8.1, Vβ8.2, Vβ8.3, Vβ9, and Vβ11 and contain *MMTV(SHN)* in addition to *Mls-1*^{a}. Because the expression of *Mtv-8, Mtv-9,* and *Mtv-11 proviruses* product, *Mls*^{f}, induces the deletion or tolerance of TCR Vβ5.1, Vβ11, and Vβ12,[10] and the expression of *Mtv-4 provirus* product, *MMTV(SHN),* induces the deletion or tolerance of TCR Vβ7, Vβ8.1, Vβ8.2, and Vβ8.3,[13] we suggest that NC/Nga mice also contain *Mls*^{f} in addition to *Mls-1*^{a} and *MMTV(SHN)*. In contrast, *SEB* stimulates $V\beta3^+$, $V\beta7^+$, $V\beta8.1^+$, $V\beta8.2^+$, $V\beta8.3^+$, or $V\beta17^+$ T-cells in mice.[12] Interestingly, *SEB*-induced autoreactive T-cell responses were blocked by IL-10 production from tolerized $V\beta8^+$ T-cells in CBA/J mice (*Mls-1*^{a}).[14] A similar phenomenon may also occur in NC/Nga mice. *SEB*-induced Th1 dominant state mediated by IL-12 or IL-18 was inhibited by the absence of $V\beta8^+$ T-cells in NC/Nga mice, resulting in a Th2 dominant state.[12] Recent studies conducted on the pathogenesis, biomarkers, and itch in AD have reported the potential role of *Staphylococcus aureus*, IL-18, nerve growth factor (NGF), and semaphorin 3A in the pathogenesis and treatment of AD.[15] To investigate this, NC/Nga mice have been used as an AD model. Therefore, the correct characterization of NC/Nga mice is important when interpreting the *in vivo* responses.

Author contribution

K. O-T. designed the experiments, and performed the experiments. The manuscript was written by K. O-T. All authors participated in the discussion of the data and in the production of the final version of the manuscript.

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Disclosure statement

No potential conflict of interest was reported by the authors.

Supplemental material

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