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Auto-reactive B cells against peripheral antigen, desmoglein 3, escape from tolerance mechanism

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Abstract

To examine the mechanism of B cell tolerance against natural peripheral self-antigen, we generated transgenic mice expressing IgM specific for desmoglein 3 (Dsg3) from AK7 monoclonal antibody which itself does not induce blisters. Dsg3 is mainly expressed on stratified squamous epithelium and is the target antigen of an autoimmune bullous disease, pemphigus vulgaris. Transgenic B cells reactive to Dsg3 were observed in the spleen and lymph node. Although these B cells are autoreactive, they did not develop into B1 B cells. These B cells were functionally competent and anti-Dsg3 IgM was detected in the serum and on the keratinocyte cell surface. These results indicate that auto-reactive B cells against peripheral antigen (Dsg3) are able to develop in the presence of Dsg3 but are ignored by the immune system.

Introduction

Rearrangement of immunoglobulin (Ig) genes during B lymphocyte development leads to the generation of a B cell repertoire containing self-reactive B cells. Controlling such self-reactive B cells is critical in order to avoid detrimental autoimmunity. It is believed that self-reactive B cells are either eliminated or inactivated during lymphocyte development, resulting in self-tolerance (1,2). Mechanisms of B cell tolerance have been examined mostly using Ig transgenic mouse models in which both heavy (H) and light (L) chains of an Ig specific for a known antigen are expressed on the majority of B cells. Such Ig transgenic mice have been further crossed with other transgenic mice expressing the specific antigen systemically or in a tissue-restricted manner.

When immature B cells interact with ubiquitous self-antigens expressed on cell surfaces, they are either eliminated by clonal deletion (3–6) or rescued by receptor editing, which involves further gene rearrangement of the L chain loci, thus replacing the self-reactive receptor with a new receptor (7–11). Immature B cells that encounter circulating self-antigens of low valency, such as small soluble proteins, are inactivated and enter a state of anergy (12–17). In contrast to conventional B cells, $CD5^{+}B220^{low}$ B1 B cells in the peritoneal cavity are often self-reactive as a result of escaping deletion (18,19). Interestingly, evidence has accumulated that these cells are positively selected by self-antigens (20–24).

In contrast to the systemic B cell tolerance mechanisms mentioned above, how tolerance is established against antigen expressed in restricted tissues and organs is still unclear. Peripheral B cell tolerance has also been examined using several transgenic models. B cells expressing IgM specific for $H\text{-}2K^{b}$ MHC class I molecules in 3–83μδ transgenic mice are deleted even when the expression of the $H\text{-}2K^{b}$ molecule is restricted to the liver (25). Similarly, when $H\text{-}2K^{b}$ molecules are expressed in the skin under the control of the keratin 4 promoter, transgene positive B cells are also deleted, and reduced numbers of B cells are present in the lymph node and spleen (26). There are additional reports supporting the presence of peripheral deletion mechanisms (27,28). In a different model, transgenic B cells expressing anti-hen egg lysozyme (HEL) specific IgM are neither deleted nor inactivated when antigen is expressed on the surface of thyroid cells (29). Differences in the amount of the antigen or distinct anatomical sites of expression may account for the varied results obtained in distinct model systems, although deletion has been observed for some antigen receptors of extremely low affinity (30,31).

Here we examined the mechanism of B cell tolerance against a native peripheral antigen, desmoglein 3 (Dsg3), a target antigen of pemphigus vulgaris (PV), an IgG-mediated autoimmune blistering disease (32). Dsg3 is a member of the

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cadherin family expressed on stratified squamous epithelium, and mediates keratinocyte cell adhesion as a component of desmosome (33). Although antibody against tissue-specific antigen can be induced relatively easily by immunization of antigen with potent adjuvant (34-36), repetitive immunization of wild-type mice on several different genetic backgrounds with recombinant Dsg3 (rDsg3) emulsified in complete Freund's adjuvant (CFA) failed to induce autoantibody production, implying the presence of systemic tolerance against Dsg3 *in vivo*. We thus used $Dsg3^{-/-}$ mice in which tolerance against Dsg3 is not established, and developed a PV mouse model by adaptive transfer of splenocytes derived from $Dsg3^{-/-}$ mice into $Rag2^{-/-}$ mice (37,38). The mice show erosions in their oral mucous membranes with typical histological findings of PV. From this PV mouse model, we have established several mAbs reactive to native Dsg3 on keratinocytes (39). Using variable regions of both the H and L chains of one of these mAbs, AK7, we developed anti-Dsg3-IgM transgenic mice. These transgenic mice showed neither deletion nor inactivation of Dsg3-reactive B cells, and IgM deposition was observed on the keratinocyte cell surfaces. Our results indicate that self-reactive cells against peripheral antigens are present at some frequency in a normal repertoire.

Methods

Mice

$Dsg3^{-/-}$ mice (40) were obtained by mating female $Dsg3^{+/-}$ and male $Dsg3^{-/-}$ mice (Jackson Laboratory, Bar Harbor, ME). These mice have a mixed genetic background of 129/Sv ($H\text{-}2^b$) and C57BL/6J ($H\text{-}2^b$). In some experiments, we used $Dsg3^{-/-}$ mice on a 129/Sv background that we obtained by backcrossing $Dsg3^{+/-}$ mice to 129/Sv for at least seven generations. C57BL/6 mice were purchased form Taconic Farms (Germantown, NY). All mice were maintained under SPF conditions in our animal facility. All animal experiments were performed in accordance with our Institutional Guidelines.

Construction of transgenic mice

cDNA fragments corresponding to variable regions of the H and L chains (V_H and V_L) of AK7 were cloned from an anti-Dsg3 hybridoma, AK7, by RT-PCR. In brief, total RNA was prepared from AK7 cells and cDNAs for H and L chains were amplified with one step RT-PCR (Qiagen, Hilden, Germany). Subsequently, H and L chains were linked by PCR and subcloned into the pCANTAB5E modified vector (Amersham, Piscataway, NJ). The resulting plasmid was transformed into XL1-Blue (Stratagene, La Jolla, CA) and single chain Fvs (scFvs) were expressed as phage surface molecules. With rDsg3 ELISA, positive clones were obtained (AK7-12) and the sequences determined. The H chain signal sequence and variable region sequence were assembled by PCR. The signal sequence and 3′ region were amplified from an anti-TNP H chain *Eco*RI–*Eco*RI fragment kindly provided by H. Karasuyama with M76-M77 and M80-M81 as shown (41): (M76) 5′-TCTAGATGGACTAGGTTCTTATGGA-3′; (M77) 5′-AGCTTCACCTCAGAGTGGACACCTGTGAAGAGAAAG-3′; (M80) 5′-CCGTTTCCTCAGGTAAGAATGGCTTCTCCAGGT-3′; (M81) 5′-CTAGTCTAGACAGCAACTACCCTTTTGAGA-3′. AK7 H chain variable region was amplified from AK7-12 with M78 and M79 as shown: (M78) 5′-GGTGTCCACTCTGAGGTGAAGCTGGTGGAGTCTG-3′; (M79) 5′-GCCATTCTTACCTGAGGAAACGGTGACCGTGGTGCC-3′.

Each amplified fragment was purified and re-amplified with M76 and M77. The 1 kb fragment obtained was digested with *Xba*I and subcloned into pBluescriptII SK+ vector (Stratagene). The anti-TNP H chain *Eco*RI–*Eco*RI fragment was digested with *Xba*I and replaced with the AK7 H chain *Xba*I fragment. The ~10 kbp constant region of the μ chain (*Eco*RI–*Eco*RI) was obtained from pVH167μ (42) and fused to obtain the plasmid AK7-H.

The L chain signal sequence and variable region sequence were also assembled by PCR. The signal sequence and 3′ region were amplified from the anti-TNP κ chain *Hin*dIII–*Hin*dIII fragment with M86-M87-M88 and M91-M92 as shown (41): (M86) 5′-CCGGAATTCAAGCTCAGCTGTCTTGTTTCAGTG-3′; (M87) 5′-AGGGTGGCTGGAGATTGGGTCAGCACAATATCACTGCTGGAAGCTGAAACAATG-3′; (M88) 5′-GCAAGAAAGACTGACGCTATCTCCTGGAGTCACAGACAGGGTGGCTGGAGATTGGGTCAGC-3′; (M91) 5′-CCAAGCTGGAGCTGAAACGTAAG-3′; (M92) 5′-CCGGAATTCTGCAGTCAGACCCAGATCTCAATAAC-3′.

The AK7 L chain variable region of κ isotype was amplified from AK7-12 with M89 and M90 as shown: (M89) 5′-GAGATAGCGTCAGTCTTTCCTGC-3′; (M90) 5′-ACGTTTCAGCTCCAGCTTGGTCCCCCCTCCGAACGTGTGAGGCCAGCTGTCACT-3′.

The L chain 3′ region fragments (M86-M87-M88 and M91-M92) and AK7 L chain variable region were fused by amplification with M86 and M90. The 1 kb fragment obtained was digested with *Eco*RI and subcloned into the pBleuescriptII SK+ vector. The pMM222 derived 5′ upstream region containing a promoter region of an L chain gene (*Pvu*II 3.5 kbp fragment), 1 kb L chain fragment, pMM222 derived 3′ region containing an intronic enhancer, κ constant region and a 3′ enhancer of an L chain gene (*Pst*I 12 kbp fragment) were fused to obtain the plasmid AK7-L (43).

Generation of transgenic mice

The AK7 IgM H and L chain constructs were mixed at equimolar concentrations and microinjected into fertilized eggs of C57BL/6 mice, followed by transfer of viable eggs into the oviducts of pseudo-pregnant C57BL/6 mice. Alternatively, only the H chain construct was injected. For genotyping, tail DNA was amplified with M78 and M81 (H chain) or M89 and M91 (L chain). One H and L chain founder line (AK7-LH1) and five H chain-only founder lines of transgenic mice were established. For the highest expression level of IgM^a, we selected AK7-H18 from the H chain-only founder lines. Mice were used for experiments at 8–16 weeks of age.

Recombinant Dsg3 (rDsg3)

rDsg3 was generated as described previously (37,44). We previously developed pEVmod-Dsg3-Ehis to produce recombinant baculoprotein. To stabilize the expressed protein, we introduced the hinge region of human IgG1 (EPKSCDKTHTCPPCP) before the E-tag sequence and produced pEVmod-Dsg3-EhisII. The mouse Dsg3 extracellular

domain was subcloned into pEVmod-Dsg3-EhisII digested by *BglII* and *XhoI* to replace human Dsg3 extracellular domain. Recombinant baculoviruses were obtained by cotransfection of rDsg3 with SapphireTM baculovirus DNA (Orbigen, San Diego, CA) into cultured insect Sf9 cells. High Five cells (Invitrogen, San Diego, CA) cultured in serum-free EX Cell 405 medium (JRH Bioscience, Lenexa, KS) were infected with the recombinant viruses and incubated for 3 days, and rDsg3 was produced in the culture supernatant. Baculoprotein was purified and concentrated on TALON (Clontech, Palo Alto, CA) affinity metal resin.

ELISA

Total serum IgM was measured with a mouse IgM ELISA Quantization Kit (Bethyl, Montgomery, TX). ELISA using rDsg3 was performed as previously described for human Dsg1 and Dsg3 (44). The ELISA plate was coated with 5 μg/ml of rDsg3 expressing the entire extracellular domain. For anti-Dsg3 IgM, mouse sera at 200-fold dilution were incubated for 1 h on individual ELISA plates.

Direct immunofluorescence analysis

Mice were sacrificed and specimens were taken from the hard palate and embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan) for cryostat sectioning. For standard direct immunofluorescence studies, each section was incubated with a 200-fold dilution of Alexa488 anti-mouse IgM antibody (Molecular Probes, Eugene, OR). All sections were examined using a fluorescence microscope (Eclipse E800, Nikon, Tokyo, Japan).

Flow cytometric analysis

Two- and three-color flow cytometry was performed on a FACScan or FACSCalibur (Becton-Dickinson, San Jose, CA) and analyzed using Cell Quest (Becton-Dickinson) or FlowJo (Tree Star, Ashland, OR) software. The following directly conjugated mAbs were purchased from PharMingen (San Diego, CA): phycoerythrin (PE)–anti-B220 (RA3-6B2), fluorescein isothiocyanate (FITC)–anti-IgM^a (DS-1), FITC–IgM (R6-60.2), FITC–anti-CD21 (7G6), biotin–anti-CD23 (B3B4), biotin–anti-CD5 (53–7.3). To stain AK7 idiotype, cells were incubated with 10 μg/ml rDsg3 followed by staining with Alexa488-conjugated anti-E-Tag antibody after washing (Amersham).

In vivo adoptive transfer experiments

AK7-LH1 bone marrow cells were collected and 5×10^6 cells were intravenously injected into 9 Gy irradiated wild-type C57BL/6 or 6 Gy irradiated Dsg3$^{-/-}$ mice. Since Dsg3$^{-/-}$ mice on a C57BL/6 background barely survive after birth (data not shown), we used Dsg3$^{-/-}$ mice on a mixed C57BL/6×129/Sv background. After 8 weeks, mice were analyzed.

Proliferative response

B220$^+$ B cells were purified from AK7-LH1 splenocytes with AutoMACS (Miltenyi Biotech, GmbH, Bergisch Gladbach). 2×10^5 cells were cultured with 200 μl 10% FCS–RPMI in 96-well microtiter plates. LPS (10 μg/ml), anti-mouse IgM F(ab)'$_2$ (10 μg/ml) or rDsg3 (20 μg/ml) were used as a stimulator. After 3 days, proliferation was measured by tritiated thymidine incorporation.

Calcium mobilization

Splenocytes were incubated with RPMI1640 containing 1 mM Fluo-3 (Molecular Probes) at 37°C for 30 min. Cells were washed twice with RPMI1640 and resuspended in RPMI1640 containing 10% FCS and PE–anti-B220 and incubated at 4°C for 30 min. Cells were washed twice and resuspended in RPMI1640 containing 10% FCS. Calcium mobilization was measured on a FACSCalibur.

Results

Establishment of AK7 single chain fragment variable (scFv)

In order to isolate cDNAs encoding H and L chains of anti-Dsg3 mAb, both H and L chain variable genes were amplified by mixed primers from total RNA of AK7 hybridoma cells (39) and subcloned into a pCANTAB5E (Amersham, Picataway, NJ) modified expression vector (45). Resulting scFvs expressed as fusion proteins of the inserted cDNAs were screened by ELISA for reactivity to mouse Dsg3. For positive clones, the specificity of AK7 scFv was confirmed by staining of a keratinocyte cell line, PAM 212 (Fig. 1A). Sequence analysis of positive clones revealed all clones shared an identical sequence for each V region (Fig. 1B).

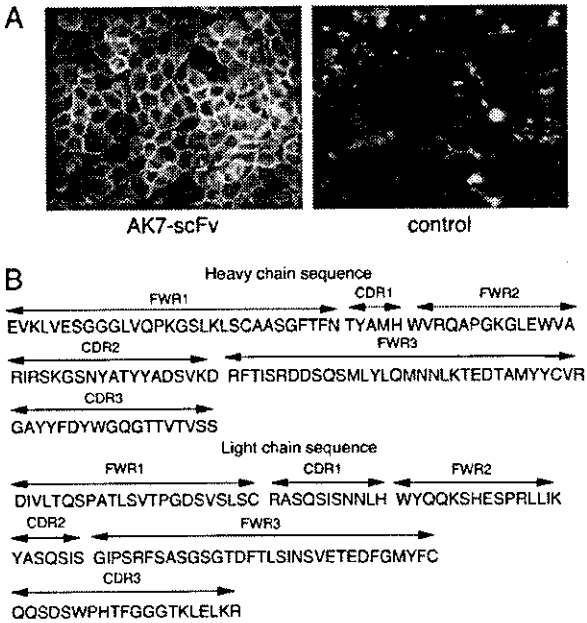


Fig. 1. Characterization of AK7-scFv. (A) AK7-scFv reacts specifically with the cell surface of PAM 212 mouse keratinocytes. PAM212 cells were incubated with AK7 scFv (left) or left untreated (right) followed by anti-E-tag antibody. Cells were then visualized by FITC-conjugated anti mouse IgG antibody. (B) Amino acid sequences of the H and L chains of AK7 were determined from the DNA sequence of AK7 scFv. FWR and CDR were annotated according to Kabat *et al.* (55).

Generation of AK7 heavy and light chain transgenic mice

Transgenic constructs encoding the H and L chains of AK7 were generated as shown in Fig. 2(A). Transcription of the transgenes is under the control of native Ig gene promoters and enhancers. The H chain transgene contains exons for both membrane-bound and secreted forms derived from an IgM^a allotype, so that both forms of IgM should be produced in mice carrying this transgene. The H chain construct alone or a combination of H and L chain constructs was injected into C57BL/6 fertilized eggs, which have an IgM^b allotype. Five founder lines for H chain transgenic mice and one founder line for H and L chain double transgenic mice were established (Fig. 2B). AK7-H18 (H chain alone) and AK7-LH1 (H and L chains) mice were used in the following studies. Quantitative PCR analysis of H chain and L chain revealed that AK7-LH1 mice contain five copies each of the H and L chain constructs and AK7-H18 contain 10 copies of the H chain construct (data not shown).

Presence of Dsg3-reactive B cells in the spleen and bone marrow

Dsg3 is primarily expressed on the cell surface of stratified squamous epithelial cells but is undetectable in the bone marrow (46) (data not shown). Thus, IgM^a positive B cells were expected to be present in the bone marrow but reduced or inactivated in the periphery. IgM^a positive B cells were readily detected in the bone marrow of AK7-LH1 mice as expected (Fig. 3A). Contrary to our expectation, however, substantial numbers of IgM^a positive B cells were detected in the spleen and lymph node of AK7-LH1 mice (Fig. 3A). It was possible

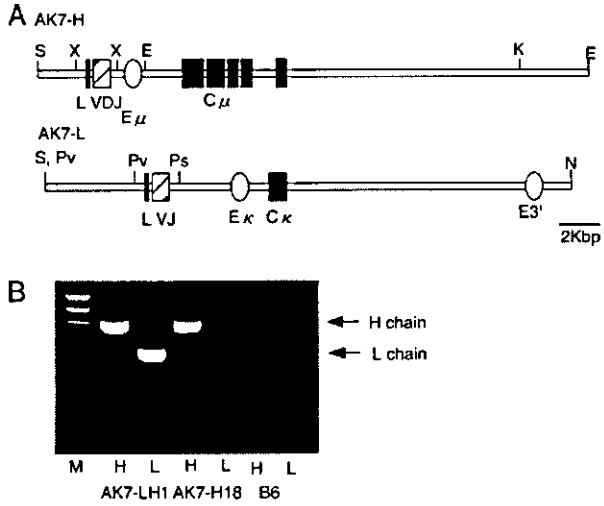


Fig. 2. Structure of AK7 H and L immunoglobulin gene constructs and generation of transgenic mice. (A) The AK7 H chain variable region contained in an *Xba*1–*Xba*1 fragment was linked with an H chain promoter and μ constant region (Cμ) including a transmembrane region. The AK7 L chain (κ isotype) variable region contained in a *Pvu*II–*Pst*I fragment was linked with a κ chain promoter and constant region. Eμ: enhancer of μ region; Eκ: enhancer of κ region; S: *Sal*I; X: *Xba*I; K: *Kpn*I; E: *Eco*RI; Pv: *Pvu*II; Ps: *Pst*I; N: *Not*I. (B) PCR analysis of AK7-LH1 and AK7-H18 tail DNAs.

that peripheral B cells utilize endogenous L chains associated with the transgenic IgM^a H chain because of incomplete allelic exclusion of the L chain loci, or that the transgenic L chain was once expressed but replaced by endogenous L chains by a receptor editing mechanism (7–11). To examine the transgenic L chain expression, we employed rDsg3 to detect the anti-Dsg3 IgM composed of the transgenic H and L chains. As shown in Fig. 3(A), >90% of IgM^a positive B cells were reactive with rDsg3, indicating that the L chain is also appropriately expressed. To further confirm the expression of transgenic H and L chains in the periphery, we bred AK7-LH1 with $Rag2^{-/-}$ mice to exclude the expression of endogenous Ig genes. B cells were also detected in the bone marrow and spleen of AK7-LH1-$Rag2^{-/-}$ mice, and such B cells expressed IgM reactive with rDsg3 on their cell surface (Fig. 3B). It was noted that while surface IgM staining of B cells from AK7-LH1 mice shows two peaks, namely IgM^{high} and IgM^{low}, B cells from AK7-LH1-$Rag2^{-/-}$ mice consist of only the IgM^{high} population.

In AK7-H18 mice, the transgenic H chain would assemble with endogenous L chains, resulting in the expression of IgM^a on the cell surface. As expected, IgM^a positive cells were detected in the bone marrow and spleen, among which 20% of IgM^a positive cells were able to bind rDsg3 (Fig. 3A). These results collectively indicate that B cells expressing Dsg3-reactive IgM are not deleted in the bone marrow or periphery.

Lack of Dsg3-reactive B1 B cells in the peritoneal cavity

The peritoneal cavity is considered to be isolated from many self-antigens, and $CD5^+B220^{low}$ B1 B cells in the peritoneal cavity are often self-reactive as a result of escaping deletion (20–22). In addition, B1 B cells are known to be resistant to Fas-FasL induced apoptosis (46). It was thus expected that B1 B cells expressing transgenic H and L chains be abundant in the peritoneal cavity of AK7-LH1 mice. However, most of the $CD5^+B220^{low}$ B1 B cells expressing IgM^a showed little affinity to rDsg3 (Fig. 3C). No B1 B cells were detected in the peritoneal cavity of AK7-LH1-$Rag2^{-/-}$ mice. These results indicate that AK7-LH1 transgenic B cells are unable to differentiate into B1 B cells.

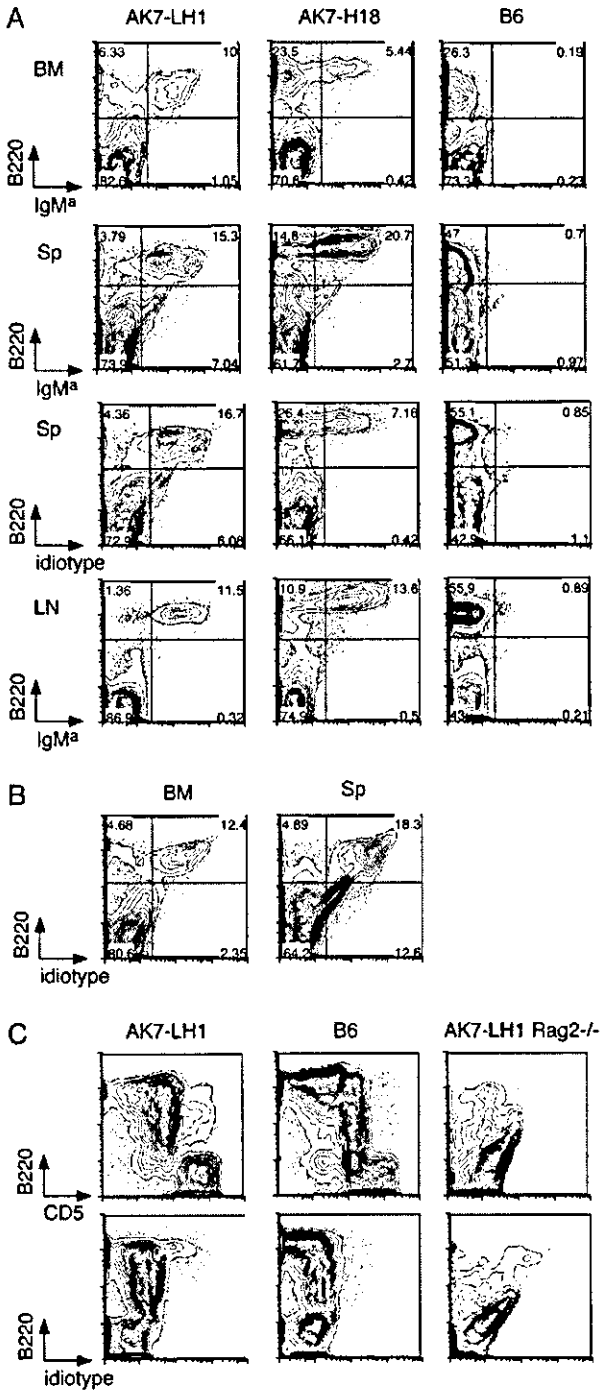
Transgenic B cells in the absence of Dsg3

To examine whether endogenous Dsg3 has any effect on the function of AK7-LH1 transgenic B cells, we transferred bone marrow cells of AK7-LH1 transgenic mice into lethally irradiated $Dsg3^{-/-}$ mice or wild-type mice and analyzed splenocytes of recipient mice 8 weeks after bone marrow transfer. IgM^a positive Dsg3-reactive B cells were detected in both $Dsg3^{-/-}$ and wild-type mice as expected. There was little difference in the number of IgM^a positive cells and the amount of surface IgM between wild-type and $Dsg3^{-/-}$ recipient mice (Fig. 4). Serum anti-Dsg3 IgM production was comparable between these recipient mice (data not shown). These data further confirm that there is little developmental block of AK7-LH1 B cells in the bone marrow and spleen in the presence of Dsg3.

AK7-LH1 transgenic B cells are not anergic

Although Dsg3-reactive B cells are present in the periphery, such B cells may be inactivated and in an anergic state. To

examine the activity of Dsg3-reactive peripheral B cells, we first checked whether anti-Dsg3 IgM is produced in the sera. As shown in Fig. 5(A), anti-Dsg3 IgM was readily detected in the sera of AK7-LH1 transgenic mice, while only a basal amount of anti-Dsg3 IgM was detected in the sera of AK7-H18 transgenic mice. Anti-Dsg3 IgM was also detected in the sera of AK7-LH1-Rag2$^{-/-}$ mice, although the amount of total IgM was much lower than AK7-LH1 mice. Approximately 20% of AK7-LH1 serum IgM were specific for Dsg3. Although not shown, anti-Dsg3 IgM was also detected in the sera of the bone marrow chimeras shown in Fig. 4. In AK7-LH1 mice, a large proportion of CD21^{high}CD23^{low} marginal zone B cells were observed in the spleen compared to wild-type mice (Fig. 5B). Direct immunofluorescence analysis showed clear deposition of IgM on the hard palates of both AK7-LH1 and AK7-LH1-Rag2$^{-/-}$ mice, indicating that the IgM indeed reacts with native antigen (Fig. 5C; data not shown). Interestingly, however, there was no obvious C3 deposition (data not shown).



We next examined the cellular function of transgenic B cells. AK7-LH1 transgenic B cells were purified with anti-B220 conjugated magnetic beads and stimulated with LPS, anti-IgM or rDsg3. Since rDsg3 used here contains the human IgG hinge sequence at the C-terminus and forms a divalent structure, rDsg3 is able to crosslink surface IgM to some extent. As shown in Fig. 6(A) (left panel), stimulation with anti-IgM as well as rDsg3 induced proliferation of transgenic B cells. We also examined anti-Dsg3 IgM production by AK7-LH1 B cells. We noted that AK7-LH1 splenic B cells secreted anti-Dsg3 IgM in culture supernatant without stimulation. When AK7-LH1 splenic B cells were stimulated with LPS, such stimulation greatly enhanced production of anti-Dsg3 IgM by AK7-LH1 B cells (Fig. 6A, right panel). In addition, marked calcium mobilization was observed upon crosslinking of surface IgM (Fig. 6B). We further examined whether the presence of Dsg3 alters functions of AK7-LH1 B cells. To this end, we crossed AK7-LH1 mice and Dsg3$^{-/-}$ mice on a 129/Sv background to generate Dsg3$^{-/-}$- and Dsg3$^{+/-}$-AK7-LH1 mice. We then measured calcium mobilization of AK7-LH1 B cells from Dsg3$^{-/-}$ and Dsg3$^{+/-}$ mice. As shown in Fig 6(C), we did not observe much difference between AK7-LH1 B cells from Dsg3$^{-/-}$ and Dsg3$^{+/-}$ mice. These results collectively indicate that the Dsg3-reactive B cells in the periphery of AK7-LH1 transgenic mice are not anergic but functionally competent.

Discussion

It is likely that B cells reactive with peripheral antigens are generated but inactivated or simply ignored by native antigen. Such B cell inactivation may be reversed by strong inflammation induced by adjuvant. In fact, it has been demonstrated that, in contrast to systemic antigen, autoimmunity against tissue-specific antigens can be induced relatively easily by immunization with potent adjuvant (34–36). In order to develop a PV mouse model, we have tried extensively to induce an

Fig. 3. The analysis of AK7-LH1, AK7-H18 transgenic mice. (A) Cells from bone marrow, spleen and lymph node of AK7-LH1 and AK7-H18 transgenic mice were analyzed by flow cytometry. In AK7-LH1 transgenic mice, IgM^a (transgene) positive B cells are detected in the bone marrow, spleen and lymph nodes. In AK7-H18 transgenic mice, ~20% of splenic B cells showed reactivity against rDsg3. (B) Bone marrow cells and splenocytes were examined for the expression of transgenic IgM^a in AK7-LH1-Rag2$^{-/-}$ mice. (C) Analysis of peritoneal cells. Most of the peritoneal cells detected in AK7-LH1 mice were CD5$^+$B220^{low} B1 B cells but they showed little affinity to rDsg3. Few B cells were detected in AK7-LH1-Rag2$^{-/-}$ mice.

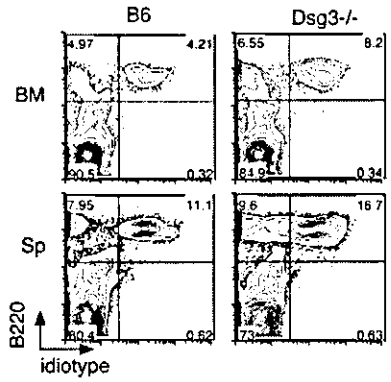


Fig. 4. Development of transgenic B cells in the absence of autoantigen. AK7-LH1 bone marrow cells were transferred into irradiated wild-type C57BL/6 or Dsg3$^{-/-}$ mice. After 8 weeks, expression of idiotype on bone marrow and spleen cells was analyzed using rDsg3 for each recipient.

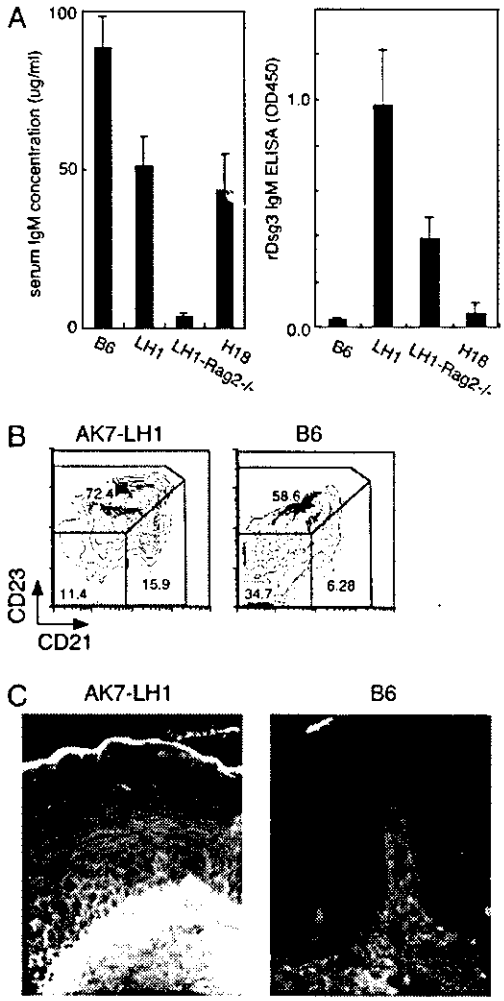


Fig. 5. Anti-Dsg3 IgM was detected in AK7-LH1 transgenic mice. (A) Serum concentrations of total IgM and anti-Dsg3 IgM were measured by ELISA. Although amount of total IgM of AK7-LH1-Rag2$^{-/-}$ mice was low, they have anti-Dsg3 IgM. The serum of AK7-H18 mice showed little affinity to Dsg3. Serial dilutions of AK7-LH1 and AK7-LH1-Rag2$^{-/-}$ mice sera were measured the reactivity against Dsg3 by ELISA. It was revealed that AK7-LH1 mice sera contained 2.5-fold more anti-Dsg3 IgM than AK7-LH1-Rag2$^{-/-}$ mice (data not shown). Due to the deficiency of Rag2 gene, all of the IgM in AK7-LH1-Rag2$^{-/-}$ sera should be Dsg3 specific. From the IgM concentration data, we calculated that ~20% of AK7-LH1 mice sera were Dsg3 specific. (B) Analysis of marginal zone B cells. Splenocytes were stained with FITC-anti-CD21, PE-anti-B220 and biotin-anti-CD23 followed by Red670-streptavidin. B220^{+} B cells were gated and staining patterns for CD21 and CD23 are shown. (C) Deposition of IgM in the hard palate of the AK7-LH1 mice. IgM deposition was examined by direct immunofluorescence as described in Methods.

immune response to Dsg3 in various strains of wild-type mouse without success. Repetitive immunization with rDsg3 emulsified in CFA has failed to induce autoimmunity in wild-type mice, whereas autoantibody is easily produced in Dsg3$^{-/-}$ mice (37). It was thus expected that self-tolerance against Dsg3 is established in wild-type mice. As shown here, however, we observed neither deletion nor inactivation of self-reactive B cells in Dsg3-specific IgM transgenic mice. In AK7-LH1 transgenic mice, a considerable amount of anti-Dsg3 IgM was detected in the sera, and deposition of IgM on the surface of keratinocytes was readily observed without immunization. These results indicate that the self-reactive B cells develop normally in AK7-LH1 mice and the presence of Dsg3 on keratinocyte cell surfaces has little effect on the maintenance of functional B cells reactive to Dsg3. Analysis of AK7-H18 also supports this notion.

In 3-83μδ×albumin promoter-K^{b} double transgenic mice in which the target antigen, K^{b}, is expressed mainly in the liver but not in the bone marrow, peripheral B cells are deleted and no autoantibody is detected in the serum (47). In 3-83μδ×keratin 4-K^{b} double transgenic mice, deletion is incomplete and a low amount of antibody is detected in the serum. In the HEL-IgM×thyroid mHEL double transgenic mouse model, in which the specific antigen is expressed on cell surfaces in the thyroid gland (29), normal numbers of anti-HEL IgM positive B cells are detected in the spleen.

The lack of deletion or inactivation of Dsg3-reactive B cells may be due to the physical isolation of Dsg3 from B cells, since expression of Dsg3 is mainly restricted to the surface of stratified squamous epithelium in peripheral tissues, as in the case of HEL-Ig×thyroid-mHEL transgenic mice (29). However, it is unlikely that B cells never encounter skin-specific antigen, because it has been demonstrated that self-antigens in the epidermis and dermis are continuously transported to the draining lymph nodes by DCs (48). Such continuous transportation of Dsg3 to the peripheral lymphoid organs would result in elimination or inactivation of Dsg3-reactive B cells. In fact, peripheral deletion has also been observed in 3-83μδ×keratin 4-K^{b} double transgenic mice where cognate antigen is expressed mainly in the skin (26). As shown here, however, Dsg3-reactive B cells are readily observed in the AK7-LH1 transgenic mice. It is theoretically possible that the affinity between transgenic IgM and Dsg3 is too weak to induce deletion or inactivation. However, this is unlikely to be the case, since AK7 was originally established from PV model

mice where most of the Dsg3-reactive autoantibodies are of a high affinity IgG isotype with somatic hypermutations and affinity maturation (38). In fact, transgenic B cells are able to bind rDsg3 in solution, confirming sufficient affinity between IgM and Dsg3. We noted that while both IgM^{high} and IgM^{low} populations were detected in AH7-LH1 mice, only the IgM^{high} population was detected in AK7-LH1 $Rag2^{-/-}$ mice. Since $Rag2^{-/-}$ DCs are reported to be defective in presenting antigen (49), it is possible that antigen-induced down-regulation of surface IgM occurs in AK7-LH1 mice, but such down-regulation is impaired in AK7-LH1-$Rag2^{-/-}$ mice. The IgM^{low} population was detected when T cells were transferred into AK7-LH1 $Rag2^{-/-}$ mice (data not shown), supporting the above notion. In addition, surface IgM level on B cells from $Dsg3^{-/-}$ mice after bone marrow transfer from AK7-LH1 mice was higher than that of wild-type mice after bone marrow transfer from AK7-LH1 mice, suggesting that the presence of the cognate antigen affects the surface IgM levels but the expression level is insufficient to induce deletion or anergy induction.

In AK7-LH1 as well as AK7-LH1-$Rag2^{-/-}$ mice, anti-Dsg3 IgM was produced in the sera and deposition of IgM on the keratinocyte cell surface was observed, indicating that autoantibody is produced without active immunization. The peritoneal cavity is considered to be isolated from most self-antigens, and the majority of IgM class autoantibodies in normal serum are derived from B1 B cells in the peritoneal cavity (20,21). Recent studies suggest that B cells need to have affinity against self-antigen to develop into B1 B cells (22–24). It was thus possible that the anti-Dsg3 IgM in the sera of AK7-LH1 mice was derived from B1 B cells. In AK7-LH1 mice, however, peritoneal cells contained no $CD5^{+}B220^{low}$ B1 B cells expressing anti-Dsg3 IgM. Similarly, AK7-LH1-$Rag2^{-/-}$ mice had no $CD5^{+}B220^{low}$ B1 B cells in the peritoneal cavity. It is likely from these results that the AK7 antibody reacts only with Dsg3 and no other self-antigen, and B cells expressing the AK7 antibody are unable to differentiate to peritoneal $CD5^{+}B220^{low}$ B1 B cells. Similar results were obtained in ATAμκ×Thy-$1^{-/-}$ mice where B cells expressing anti-Thy-1 antibody cannot differentiate into $CD5^{+}B220^{low}$ B1 B cells in the absence of Thy-1 (22). Our results indicate that anti-Dsg3 IgM is produced by conventional B cells, not by peritoneal B1 B cells.

It has been shown using VSV-G transgenic mice that B cells do not produce antibodies in response to monomeric antigens without T cell help (50). It is possible that the presence of T cells capable of recognizing Dsg3 is involved in the production of anti-Dsg3 IgM in AK7-LH1 mice. However, anti-Dsg3 IgM was also produced in $Dsg3^{-/-}$ mice after bone marrow transfer from AK7-LH1 mice at a concentration similar to, or less than, that in AK7-LH1 mice, indicating that anti-Dsg3 IgM production does not require Dsg3-reactive T cells generated in $Dsg3^{-/-}$ mice where tolerance against Dsg3 is not established. It has

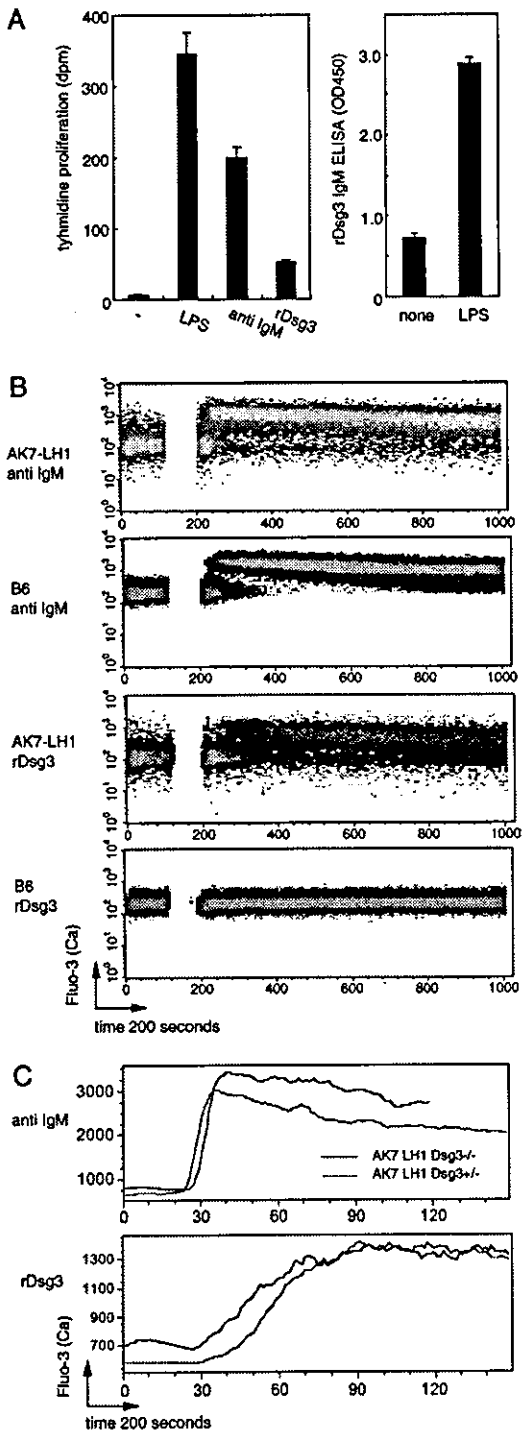


Fig. 6. Proliferative response and calcium mobilization. (A) (Left panel) Proliferative responses of AK7-LH1 B cells in response to LPS, anti-IgM F(ab)'$_2$ and rDsg3 were analyzed by thymidine incorporation. (Right panel) Production of anti-Dsg3 IgM from AK7-LH1 splenic B cells. Purified AK7-LH1 splenic B cells were cultured for 3 days with or without LPS. (B) Calcium mobilization assay. AK7-LH1 B cells were labeled with Fluo-3 and calcium level measured after stimulation by anti-IgM F (ab)'$_2$ or rDsg3. Splenic B cells from C57BL/6 mice were used as a control. (C) Calcium mobilization assay of AK7-LH1 B cells from $Dsg3^{+/-}$ and $Dsg3^{-/-}$ mice after stimulation with anti-IgM F(ab)'$_2$ (upper panel) and rDsg3 (lower panel).

also been reported that repetitive, polymeric determinants are able to activate B cells to produce antibodies without T cell help (50). Since Dsg3 is expressed on keratinocyte cell surfaces and thus can form repetitive, polymeric determinants, it is possible that cell surface Dsg3 triggers anti-Dsg3 IgM production. However, anti-Dsg3 IgM was also produced in $Dsg3^{-/-}$ mice reconstituted with AK7-LH1 bone marrow cells in amounts similar to wild-type recipients. Therefore, Dsg3 expressed on keratinocyte cell surface is unlikely to play a role in triggering anti-Dsg3 IgM production.

AK7 is specific to Dsg3 but is not pathogenic (39). Thus, AK7 antibody was unable to induce any blistering or inflammation when produced *in vivo*. We have recently obtained a pathogenic mAb, AK23, which is able to induce PV phenotype. The epitope of AK23 was identified as the region critical for the homophilic adhesive interaction between two Dsg3 molecules (39). Transgenic mice expressing H and L chains of AK23 may thus show a different phenotype from AK7-LH1. AK23 IgM would induce inflammation, and the development of B cells carrying this IgM may be blocked by deletion. Experiments are currently underway.

To understand the pathophysiology of autoimmune diseases, it is necessary to investigate the mechanisms of tolerance break, preceding disease onset. In the field of pemphigus, populations which have anti-desmoglein IgG autoantibodies without development of an apparent pemphigus phenotype have been described. One such example is the endemic form of pemphigus foliaceus, fogo selvagem, in which the target antigen is desmoglein 1 (Dsg1), a close relative of Dsg3 in the cadherin superfamily (51,52). Unlike sporadic pemphigus foliaceus, which is a disease of mostly middle-aged and older patients, fogo selvagem affects young adults and children of any race exposed to the local ecology in rural areas. In these areas, >50% of normal individuals have anti-Dsg1 IgG autoantibodies, and the onset of the disease is preceded by a sustained antibody response (53). Another example is that a subset of silicosis patients has anti-desmoglein IgG autoantibodies without apparent clinical phenotype (54). Our present results clearly show the presence of self-reactive B cells in the periphery at some frequency, implying that Dsg3-reactive B cells are also present in normal individuals. Together with our findings in mouse models, elucidation of the immunological mechanisms preceding onset of disease will be critical in unveiling the mystery of the tolerance break in autoimmune diseases.

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Abbreviations

Dsg	desmoglein
HEL	hen egg lysozyme
PV	pemphigus vulgaris
rDsg3	recombinant mouse Dsg3
scFv	single chain fragment variable

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Heterozygous null mutation of myelin P0 protein enhances susceptibility to autoimmune neuritis targeting P0 peptide

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Mice with a heterozygous null mutation in myelin protein zero ($P0^{+/-}$) develop late-onset clinical paralysis associated with inflammatory pathology in the peripheral nerves. Although the development of this illness is known to require T cells and macrophages, little is understood regarding the immunological defect in the mice. Here we report that young $P0^{+/-}$ mice, free from clinical manifestations, have a defect in central tolerance to P0, and are more prone to induction of experimental autoimmune neuritis (EAN) by sensitization against $P0_{180-199}$ peptide. Notably, we found that the P0 gene is transcribed in the thymus of wild-type and the $P0^{+/-}$ mice in an amount proportional to the gene dosage. We then replaced the thymus of wild-type mice with that of the P0-deficient mice and vice versa. Immunization of these mice with $P0_{180-199}$ revealed that a lower thymic P0 transcript would be associated with the higher recall T cell response to $P0_{180-199}$, thus accounting for the higher susceptibility of the $P0^{+/-}$ mice to P0-induced EAN. These results imply that a heterozygous mutation in an autoantigen could cause defective central tolerance to the autoantigen. As such, autoimmune T cells may play some role in "genetic" diseases caused by a heterozygous gene defect.

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1 Introduction

Owing to major advances in genetic analysis, a great number of molecular defects have been identified in humans as probable causes for intractable neurological diseases such as genetic peripheral neuropathies [1, 2]. However, it remains largely speculative as to how such a molecular defect leads to tissue destruction and functional alterations accounting for the disease conditions. In fact, because the cellular and molecular events caused by the genetic defects remain poorly understood, we cannot answer clearly yet why patients having the same molecular defect are so heterogeneous in age of onset, clinical manifestations, or type of the pathology [3–5]. An interesting question that has to be addressed is why genetic neurological diseases affecting the peripheral nervous system (PNS) could occasionally be associated with transient or persistent inflammatory pathology in the affected tissue [6–10]. Although previous studies have described the inflammatory conditions as "secondary reactions" to tissue damage, it is possible that the "secondary" inflammatory reactions play a role in the tissue destruction at least in some of the cases. We support this idea, because anti-inflammatory treatment with corticosteroid could temporarily control some cases of genetic PNS diseases [6–9].

Although various factors might be involved in the tissue inflammation seen in the genetic diseases, a challenging hypothesis could be that a defect in the gene coding self-antigen might somehow raise a risk for autoimmune disease targeting the gene product. We here explore this intriguing possibility using a mouse model of genetic peripheral neuropathy, with a mutation in the gene of myelin protein zero (P0) [11–13]. P0 is a transmembrane glycoprotein that constitutes a major component of PNS myelin [14]. This adhesion molecule belongs to the immunoglobulin superfamily and fulfills multiple functions during myelin development and maintenance. Studies have revealed a number of mutations in the P0 gene that appear to be causal of human genetic neuropathies, such as Charcot-Marie-Tooth disease type 1B or Dejerine-Sottas disease [1, 2, 15]. Mice with a homozygous null mutation in the gene for P0 ($P0^{-/-}$) typically develop overt signs of peripheral neuropathy within one

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Abbreviations: dGuo: Deoxyguanosine **Dsg3:** Desmoglein 3 **EAN:** Experimental autoimmune neuritis **MBP:** Myelin basic protein **MOG:** Myelin oligodendrocyte glycoprotein **PNS:** Peripheral nervous system **P0:** Myelin protein zero

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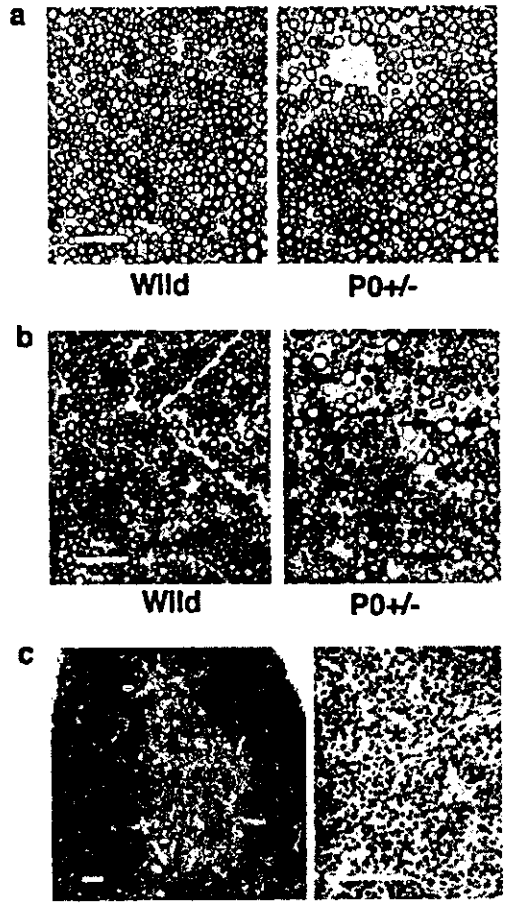
month after birth [11, 12]. In contrast, mice with a heterozygous mutation ($P0^{+/-}$) are free from symptoms for 5~ months after birth, but then start to develop a gait disorder and clinical paralysis [13]. Of note, although the pathology of $P0^{-/-}$ mice is characterized by a serious defect in myelination in the PNS, demyelinating lesions in $P0^{+/-}$ mice accompany cellular infiltration, indicative of immune pathogenesis. Recent studies by Martini, Schachner and colleagues have revealed that the full-blown development of the late-onset disease in $P0^{+/-}$ mice would require T cells and macrophages [16, 17], suggesting a pathogenic role for immune cells. The unique involvement of immune cells would allow us to use the $P0^{+/-}$ mice as a tool for studying the mechanism for the inflammatory pathology accompanying genetic diseases.

We here report that young $P0^{+/-}$ mice are more prone to induction of experimental autoimmune neuritis (EAN) mediated by T cells reactive to P0 residues 180–199 [18], and that the higher susceptibility to the P0-induced EAN of the mice results from a defective T cell tolerance to P0. Recent studies demonstrate that a number of autoantigens, including those that were believed to be secluded from the immune system, are actually expressed in the thymus [19–27]. Furthermore, experiments have proven that such a promiscuous expression of autoantigen would contribute to eliminating pathogenic autoimmune T cells from the T cell repertoire [24, 25]. We found that the P0 gene is also transcribed in the thymus, but the transcription level is reduced in $P0^{+/-}$ mice as compared to wild-type mice. Subsequent studies with thymus transplantation revealed that the reduced P0 gene dosage in the thymus of $P0^{+/-}$ mice leads to the inefficient elimination of P0-reactive T cells and defective T cell tolerance to P0, accounting for the higher susceptibility to P0-induced EAN. These results may have important implications for understanding the role of autoimmune T cells in the genetic diseases of the PNS.

2 Results

2.1 Higher susceptibility of $P0^{+/-}$ mice to P0-induced neuritis through an antigen-specific mechanism

Previous studies have documented that $P0^{+/-}$ mice start to develop a "waddling" gait as a first clinical sign around 5 months of age [12, 13]. In the colony maintained in Japan, however, the mice are free from clinical paralysis until 9–12 months of age. We do not know the reason for the late onset of illness in our colony. But studies on experimental autoimmune encephalomyelitis (EAE) and experimental autoimmune orchitis [28] indicate that differences in mouse substrains used for back-crossing or levels of cleanliness in the facilities may account for the varying timing of onset in different colonies. It occurred to us that the $P0^{+/-}$ mice would be more useful as a model for genetic neuropathy if the disease develops earlier. Then we sought conditions under which the heterozygous mutant mice might develop the disease earlier. We previously found that depletion of NK and NKT cells with anti-NK1.1 monoclonal antibody enhances clinical manifestations of EAE [29]. However, treatment with this antibody did not show any effect on $P0^{+/-}$ mice (data not shown). Then we immunized young (8-week-old) $P0^{+/-}$ mice with $P0_{180-199}$, a weakly neuritogenic peptide for C57BL/6 (B6) mice [18], to explore if this challenge has a significant effect on the development of disease. At this age, there are no morphological differences in sciatic nerves between wild-type and $P0^{+/-}$ mice (Fig. 1a). Immunization of wild-type B6 mice with the P0 peptide induced a mild, transient paralysis in one third of recipients (Table 1). The paralysis was mild, but it was objectively evaluated based on tail curving or bent tail. The sign, due to the paralysis of the distal half of the tail, was evident, when the mice were kept with the head down. When $P0^{+/-}$ mice were immunized with $P0_{180-199}$, a significantly higher proportion developed the tail paralysis (Table 1, Fig. 2a). Accordingly, the maximum clinical score as well as cumulative score of clinical severity was significantly elevated in $P0^{+/-}$ mice as compared to wild-type mice ($p<0.05$). We found diffuse cellular infiltration and degenerated nerve fibers in the sciatic nerves on day 14 after immunization in both wild-type and $P0^{+/-}$ mice (Fig. 1b), but the morphological changes appeared to be milder in wild-type mice. Consistent with this impression, the number of mononuclear cells was increased in $P0^{+/-}$ mice: 154.5 cells/mm^2 in $P0^{+/-}$ versus 100.0 cells/mm^2 in wild-type ($p<0.05$). It is possible that $P0^{+/-}$ mice are more prone to all forms of EAN because of a higher vulnerability of the peripheral nerve tissue to immune-mediated injuries. Or they might be more prone to autoimmune disease, regardless of the organ/tissue affected. So we immunized young mice with another PNS peptide $P2_{53-78}$ [30] (Fig. 2b), or with an encephalitogenic peptide, myelin oligodendrocyte glycoprotein MOG_{35-55} [31] (Fig. 2c). $P2_{53-78}$ was previously identified as a neuritogenic peptide for rats, but we have found that it can induce EAN in B6 mice as well. There was no significant difference between $P0^{+/-}$ and wild-type B6 in the severity of EAN or EAE induced by these peptides (Table 1). The results indicate that the higher susceptibility of $P0^{+/-}$ mice to P0-induced EAN would involve an antigen-specific immunological mechanism, but this is not because the heterozygous P0 mutation makes the PNS of mice more vulnerable to inflammation or would disrupt antigen nonspecific immunoregulation.



◀ *Fig. 1.* Histological examination of sciatic nerves and transplanted thymus. (a) Sciatic nerves of wild-type and $P0^{+/-}$ 8-weeks-old mice. Sciatic nerves were sampled from naive, wild-type B6 mice ($P0^{+/+}$) or $P0^{+/-}$ mice at 8 weeks, and the cross sections were examined with toluidine blue staining. At this age, $P0^{+/-}$ mice were free from any clinical signs and histological abnormalities as compared with wild-type mice. (b) Sciatic nerves of the mice that were immunized with P0 peptide. Sciatic nerves were randomly sampled from wild-type B6 mice or $P0^{+/-}$ mice at age of 8 weeks that had been immunized with P0 peptide 14 days previously. One third of the wild-type and more than two-thirds of the $P0^{+/-}$ mice developed signs of tail paralysis around 10 days after immunization. Shown are representative figures of degenerated fibers (arrow) with axonal changes and cellular infiltration (arrowhead). When the infiltrating cells were enumerated, there was a tendency for the cell number to be increased in $P0^{+/-}$ mice compared with wild-type mice ($P0^{+/+}$). Bar =50 μm. (c) Histological confirmation of the transplanted thymus. We conducted thymus transplantation experiments with various combinations of donor and recipient (See legend to Fig. 5). This figure shows a representative histopathology of the engrafted thymus ($P0^{+/-}$ thymus grafted into wild-type mouse) stained with hematoxylin and eosin. In the right panel, we find the epithelial reticular cells and Hassall's corpuscles (*), that are regarded as unique components in the thymus. Bar =50 μm.

2.2 Increased reactivity of T cells to $P0_{180-199}$ in P0-deficient mice

As a possible mechanism for the higher susceptibility to P0-induced EAN, we explored if T cell reactivity to the P0 peptide may be augmented in the $P0^{+/-}$ mice. First, we evaluated the recall response to $P0_{180-199}$ by measuring the LN proliferative response to $P0_{180-199}$ in wild-type, $P0^{+/-}$ and $P0^{-/-}$ mice sensitized against this peptide. Although mice immunized with MOG_{35-55} or ovalbumin (OVA) showed strong recall responses to the immunogens, LN cells from wild-type and P0 mutant mice primed with $P0_{180-199}$ peptide showed only a marginal proliferative response to the peptide (data not shown). Because cellular proliferation and cytokine production could be dissociated in autoimmune T cells with certain specificity, we next evaluated the cytokines released into the supernatant. Using standard ELISA, we could reveal significant antigen-induced responses of the primed LN cells and found that LN cells from $P0^{+/-}$ and $P0^{-/-}$ mice would produce significantly larger amounts of IFN-γ in response to $P0_{180-199}$ than those from wild-type animals (Fig. 3a). By contrast, there was no such difference in the responses to $P2_{53-78}$, MOG_{35-55} or OVA (data not shown). ELISPOT assay also showed that the number of cells producing IFN-γ in response to $P0_{180-199}$ was significantly increased in $P0^{-/-}$ mice and marginally increased in $P0^{+/-}$ mice (Fig. 3b). Since IL-4 was not detected in any of the samples, we interpreted the results as reflecting an antigen-specific elevation of the Th1 response to P0 in the mutant mice. Taken together, we conceive that self-tolerance to P0 is defective in the P0-deficient mice and that the Th1 responses to P0 inversely correlate with the transcription levels of P0 mRNA. Thus, we postulate that endogenous P0 may inactivate or eliminate P0-reactive T cells, as endogenous myelin basic protein (MBP) does for potentially encephalitogenic T cells [32–34].

2.3 Transcription levels of P0 in the thymus inversely correlate with anti-P0 Th1 response

Studies have demonstrated that myelin proteins of the central nervous system are not sequestered antigens but could ectopically or promiscuously be expressed in the thymus or other lymphoid organs [19–21, 24, 25]. Moreover, it has been proven that a splice variant of myelin proteolipid protein (PLP), an encephalitogenic antigen

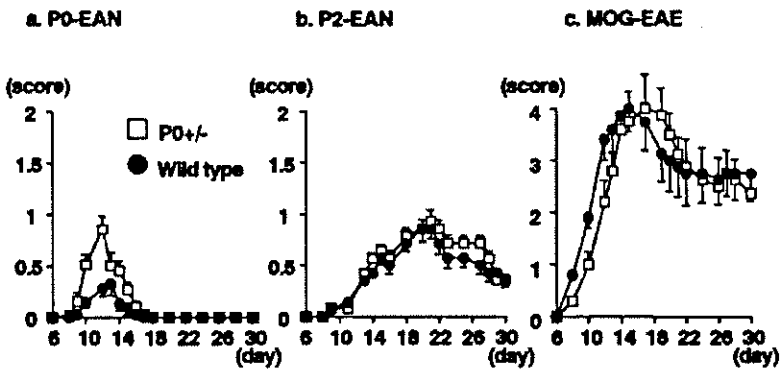


Fig. 2. EAN and EAE in $P0^{+/-}$ and wild-type B6 mice. We immunized 37 wild-type and 36 $P0^{+/-}$ mice with $P0_{180-199}$ (a), and 7 wild-type and 7 $P0^{+/-}$ mice with $P2_{53-78}$ (b) to induce EAN. We also immunized 7 wild-type and 7 $P0^{+/-}$ mice with MOG_{35-55} (c) for induction of EAE. Shown are the means ± SEM. of clinical scores at indicated time points. The clinical grade of the mice developing EAN was exclusively 0 (healthy) or 1 (paralysis of the distal half of the tail). This implies that the difference between wild-type mice and $P0^{+/-}$ mice reflects the frequency of the sign of grade 1 paralysis at each time point.

causing EAE, is promiscuously expressed in the thymus and plays a role in shaping the autoimmune T cell repertoire [24, 25]. Given this information together with our experimental data, an attractive hypothesis was that self-tolerance to P0 might be defective in P0-deficient mice owing to a lower expression of P0 in the thymus, a place for establishing self-tolerance to P0. In order to test this possibility, we then evaluated the transcription of P0 mRNA in the thymus. To avoid contamination with non-lymphoid cells, we carefully prepared single-lymphoid cell suspensions and applied these samples to RT-PCR analysis. Using sets of the primers, we could amplify the corresponding size of transcripts (whole exons; 678 bp, exons 4–6; 273 bp) from sciatic nerves and thymi of wild-type mice, but not from the cells of $P0^{-/-}$ mice. These results indicate that a gene encoding a PNS protein could also promiscuously be expressed in the thymus. Using a quantitative PCR assay, we next compared the quantity of P0 mRNA in thymocytes obtained from $P0^{-/-}$, $P0^{+/-}$ and wild-type mice. The results revealed that $P0^{+/-}$ thymocytes would express a level of P0 transcript intermediate between $P0^{-/-}$ and wild-type mice (Fig. 4). Hence, transcription levels of thymic P0 inversely correlate with the frequency of P0-reactive Th1 T cell response. We also made an attempt to demonstrate differential levels of P0 protein expression in the thymus of wild-type and $P0^{+/-}$ mice. However, the available anti-P0 mAb [35] was unable to demonstrate P0 expression in the thymus, although it did stain the PNS myelin brightly (not shown).

2.4 Thymic expression of P0 is critical in establishing self-tolerance to P0

Next we explored if the thymic P0 would truly contribute to eliminating P0-reactive T cells. To this end, we replaced thymus of wild-type mice with that of P0-

Table 1. Clinical scores of EAN and EAE in $P0^{+/-}$ and wild-type mice[a]

Disease	Mice	Maximum score	Incidence	Cumulative score
P0-EAN	Wild	0.32 ± 0.09	12/37	0.77 ± 0.26
	$P0^{+/-}$	0.78 ± 0.12[b]	28/36[b]	1.92 ± 0.40[b]
P2-EAN	Wild	0.86 ± 0.32	4/7	8.64 ± 4.09
	$P0^{+/-}$	0.93 ± 0.44	3/7	9.64 ± 4.61
MOG-EAE	Wild	4.25 ± 0.23	7/7	55.0 ± 6.45
	$P0^{+/-}$	4.30 ± 0.30	7/7	45.5 ± 4.25

[a] Data shown are the mean ± SEM. As described in the text, P0-EAN was induced with $P0_{180-199}$, P2-EAN with $P2_{53-78}$ peptide, and MOG-EAE with MOG_{35-55}.

[b] $p<0.05$ (Mann-Whitney U-test, $P0^{+/-}$ versus wild-type mice).

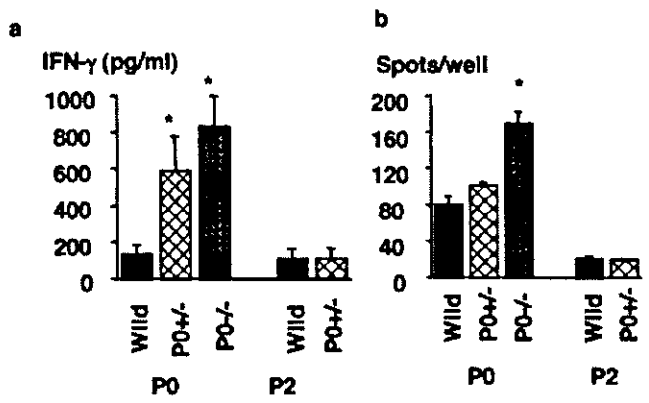


Fig. 3. Enhancement of recall responses to $P0_{180-199}$ in P0-deficient mice. We immunized wild-type, $P0^{+/-}$ and $P0^{-/-}$ mice with $P0_{180-199}$ emulsified in complete Freund's adjuvant, and removed the draining LN on day 14 after immunization. By stimulating the LN cells with $P0_{180-199}$ peptide, the recall response to the peptide was evaluated with regard to IFN-γ production. As a control, we immunized wild-type and $P0^{+/-}$ with $P2_{53-78}$ peptide and measured the recall responses to P0. (a) IFN-γ levels in the culture supernatants (ELISA). We collected the supernatant 48 h after culture and measured the concentration of IFN-γ by ELISA. Here we show means ± SEM of the data from each mouse (n=3–8 for each group). (b) The number of IFN-γ-positive spots (ELISPOT). The number of spots representing IFN-γ-secreting T cells was determined by ELISPOT. Means ± SEM of the data from each mouse (n=3 for each group).

deficient mice and vice versa (Fig. 1c, 5). Among 143 mice that underwent thymus transplantation, 62 were excluded because they were transplanted with gender-mismatched thymus. The remaining 81 were sensitized with $P0_{180-199}$ peptide, and we evaluated the recall response to the P0 peptide by measuring IFN-γ contents in the supernatant. Among these, we finally excluded 33 mice from analysis since the transplanted thymus was not detectable or looked residual upon post-mortem examination. Analysis of the remaining 47 mice revealed that the anti-P0 recall response in wild-type mice was significantly augmented by replacing the thymus with that from $P0^{+/-}$ or $P0^{-/-}$ mice ($p<0.05$) (Fig. 5a). The mice transplanted with $P0^{-/-}$ thymus showed a higher recall response to P0 than those implanted with $P0^{+/-}$ thymus. This indicates that the thymic P0 transcript would inactivate or eliminate P0-reactive T cells in a gene dosage-dependent manner. In contrast, the enhanced response to P0 in the P0-deficient mice was depressed to the level of wild-type mice by replacing their thymus with that of wild-type mice (Fig. 5b). These results indicate that the thymus is the critical site for endogenous P0 to deplete P0-reactive T cells, and that a reduced P0 expression in the thymus would account for the defective tolerance and the higher susceptibility of $P0^{+/-}$ mice to P0-induced EAN.

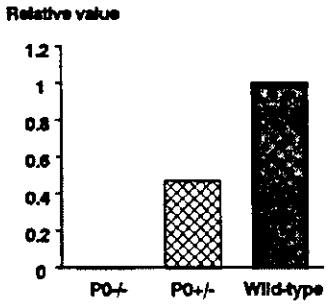


Fig. 4. Quantitative analysis of P0 mRNA in the thymus. We examined three sets of thymi for amplification of P0 mRNA by LightCycler™ PCR™. This is a representative experiment of three with similar results. The expression level in the thymus of $P0^{+/-}$ mouse is shown as a relative value, after assigning the value for $P0^{-/-}$ thymus as zero and that for wild-type ($P0^{+/+}$) as 1.0.

Next we asked which cellular component in the thymus might transcribe P0 mRNA. To this end, we treated thymic lobes obtained from newborn wild-type or $P0^{+/-}$ mice with deoxyguanosine (dGuo), which is known to cause selective depletion of the lymphoid cells of myeloid origin while allowing survival of the epithelial cells. RT-PCR examination of thymus cell suspensions showed that P0-expressing cells were still present in the thymus of both wild-type and $P0^{+/-}$ mice after dGuo treatment (Fig. 6). This result allows us to speculate that dGuo-resistant stromal cells are probably involved in the presentation of P0, without excluding the possible contribution of other cell types present in the thymus.

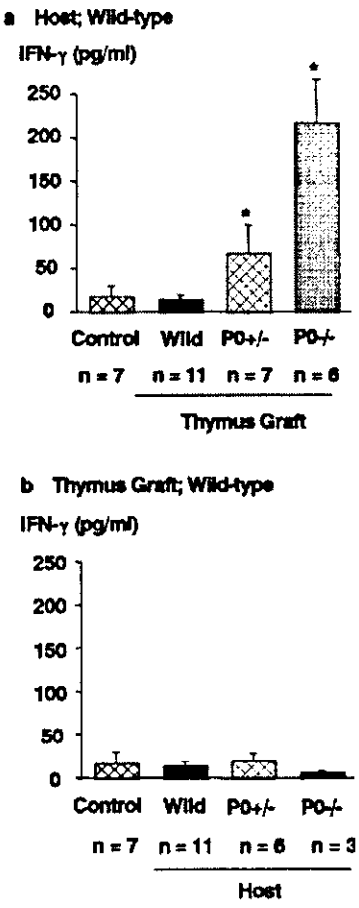


Fig. 5. Thymic P0 expression is critical in eliminating P0-reactive T cells. We sensitized mice that had undergone thymus replacement 8 weeks earlier, and estimated the recall response to $P0_{180-199}$ by measuring IFN-γ production as conducted in the experiment of Fig. 3a. First we determined the IFN-γ value (pg/ml) for each mouse after calculating the mean value for 10–20 supernatant samples collected from different wells. Here we show means ± SEM of the values in each group of mice. When we used wild-type mice as the host (a), mice engrafted with thymus from $P0^{+/-}$ or $P0^{-/-}$ showed a significantly stronger response to $P0_{180-199}$ than controls (Control; no operation) or mice engrafted with the thymus of wild-type mice. In contrast, transplantation of wild-type thymus reduced the increased anti-P0 response found in P0-deficient mice to the level of wild-type mice (b). *$p<0.05$ as compared to operated or unoperated wild-type ($P0^{+/+}$) mice (Mann-Whitney U-test)

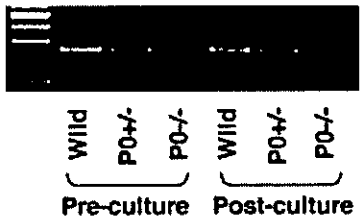


Fig. 6. Thymic expression of P0 mRNA is resistant to dGuo treatment. Using RT-PCR with primers for exon 4–6 of P0, we examined P0 expression in thymocytes freshly isolated from newborn mice (Pre-culture) and those from thymus treated with dGuo for one week (Post-culture). Note that the 273-bp band corresponding to P0 exon 4–6 can be detected in thymus of $P0^{+/-}$ and wild-type mice before and after dGuo treatment.

3 Discussion

Genetic neurological diseases could be accompanied by an inflammatory pathology in the affected tissue. However, the immunological basis for this interesting phenomenon remained unclear. Here we attempted to clarify the relationship between a genetic defect and an accompanying inflammatory pathology in $P0^{+/-}$ mice [11–13] that would spontaneously develop inflammatory neuropathy. Notably, the heterozygous mutant mice showed a significantly higher susceptibility to P0-induced EAN and mounted stronger recall responses to $P0_{180-199}$ than did wild-type mice. However, the severity of EAN was not augmented in the mutant when immunized with a peptide from another neuritogenic protein ($P2_{53-78}$). This result excludes the possibility that $P0^{+/-}$ mice may be more prone to EAN owing to greater vulnerability of the PNS tissue to inflammatory reactions. Instead, it indicates that self-tolerance to P0 is defective in $P0^{+/-}$ mice. It was previously reported that T cells isolated from $P0^{+/-}$ mice after onset of the spontaneous neuropathy would show enhanced reactivity to multiple components of PNS myelin, which include P0, P2 and MBP [16]. We interpret that the T cell responses to the multiple myelin antigens arose from the "epitope spreading" phenomenon [36] secondary to the myelin damage, and would not reflect the primary defect in the immune system of young $P0^{+/-}$ mice.

Then we addressed the questions of how T cell tolerance is disrupted in the P0 mutant. In principle, immunological tolerance to self-protein is established in the thymus by negative selection (central tolerance) for developing thymocytes showing stronger interactions with their peptide/MHC ligand. However, for lymphocytes that escaped central tolerance, peripheral tolerance mechanisms would operate to protect against destructive auto-

immunity through active suppression by regulatory cells or via a mechanism called clonal ignorance [37, 38]. Of particular interest, prior studies have revealed that central tolerance is operative for so-called "sequestered" antigens such as PLP [24, 25]. In fact, a splice variant of PLP is promiscuously expressed in the thymus and would play some role in shaping the T cell repertoire. We found that the P0 gene is transcribed in the thymus and that a recall Th1 response to $P0_{180-199}$ inversely correlates with the transcription level of P0 in the thymus. Furthermore, we showed that transplantation of a thymus from P0-deficient mice into wild-type mice led to a substantial augmentation of the T cell response to the P0 peptide in the $P0^{+/+}$ hosts. By contrast, P0-deficeint mice became tolerant to P0 when we replaced the thymus with a $P0^{+/+}$ thymus. Taken together, we conclude that the reduced P0 expression in the thymus would account for the defective tolerance to P0 in $P0^{+/-}$ mice.

Of interest, Amagai et al. [39] have recently reported that a homozygously null mutation of desmoglein 3 (Dsg3) would also associate defective tolerance to Dsg3, the target autoantigen for an autoimmune skin disease, pemphigus vulgaris. However, unlike our observation in $P0^{+/-}$ mice, self-tolerance to Dsg3 was not disrupted in the $Dsg3^{+/-}$ mice. We speculate that the level of thymic Dsg3 expression in $Dsg3^{+/-}$ mice is higher than that which enables elimination of the majority of high avidity autoimmune T cells, whereas the amount of P0 protein in the $P0^{+/-}$ thymus may not reach such a level. Consistent with this idea, although Dsg3 protein can be detected in the thymus of wild-type mice by immunostaining methods [39], P0 protein cannot be detected in the cortex or medulla of the thymus (unpublished results).

Goverman and colleagues [33] previously demonstrated that T cells recognizing MBP residues 121–150 were subject to central tolerance in wild-type mice, but would dominate the T cell repertoire in $MBP^{-/-}$ *shiverer* mice. This observation was striking because $MBP_{121-150}$ is not expressed by thymus components. But a recent study from the same group has indicated that the tolerance to $MBP_{121-150}$ is mediated by bone marrow-derived antigen-presenting cells presenting exogenously derived MBP peptide [34]. Notably, they also showed that $MBP^{+/-}$ mice expressing the transgenic TCR for $MBP_{121-150}$ were more prone to MBP-induced EAE [34], revealing that the central tolerance to MBP is dependent on gene dosage. We speculate that different levels of antigen expression in the thymus can explain the discrepancy between this result and the work by Amagai et al. [39] in the gene dosage effect on central tolerance. Because T cell repertoire in the TCR-transgenic mice of Goverman and colleagues [34] is highly enriched in a T cell population that is eliminated in wild-type mice, the data obtained by using the mice cannot be extrapolated without reservations to physiological or pathological conditions in humans. In this regard, the results of the present study would have more physiological implications in human disease with a heterozygous gene defect. In fact, we showed for the first time to our knowledge that susceptibility to an autoimmune disease can be enhanced in non-TCR transgenic mice that express a reduced (but not null) level of the autoantigen in the thymus.

Recent works showing central tolerance to pathogenic autoantigens have revealed that either bone marrow-derived cells or epithelial cells can endogenously synthesize ectopic autoantigens [23–25]. Using the thymus organ culture treated with dGuo, we showed that dGuo-resistant cells (represented by thymic stromal cells) might synthesize P0 in B6 mice. On the other hand, we were unable to detect P0 mRNA in the bone marrow cells of B6 mice (unpublished results). These results indicate that thymic stromal cells may synthesize P0 protein endogenously and play some role in tolerance induction. However, since the thymus is innervated by autonomic nerves, we can not exclude the possibility that the degradation product of the PNS myelin or Schwann cells in the thymus may be the source of the P0 peptide that would induce central tolerance to P0. If this is the case, the P0 protein or its fragments derived from the PNS may be processed and presented by bone marrow-derived cells to developing thymocytes. To better understand the mechanism underlying central tolerance to P0, we will need to use alternative approaches for identifying the cell population expressing P0 protein.

Collectively, we propose that a heterozygous null mutation for a self-component could raise the risk for developing autoimmune disease, if the gene product is not sufficiently expressed in the thymus. Of note, our theory is relevant not only for a null mutation, but also for a point mutation allowing expression of the mutated protein. For example, if the relevant peptide of a mutated protein (a potential target for autoimmune T cells) has a weaker affinity to the corresponding self-MHC molecule, this may lead to a lower avidity interaction between T cells and peptide/MHC during the process of central tolerance. This would allow the pathogenic autoimmune T cells to escape from negative selection and render the individual more prone to the autoimmune disease. To verify the relevance of this idea to human genetic diseases, we need to systematically investigate the expression of self-antigen in the human thymus and inflammatory pathology in the affected organs/tissues. It would also be interesting to survey patients with autoimmune diseases for the possible presence of a mutation in target antigens. Investigation of the control mechanism for thymic expression of the self-antigen may also provide

insights into the pathogenesis of autoimmune diseases. Hence, central tolerance to autoantigen is a key issue to understanding the link between genetic and autoimmune diseases.

Our work has showed that $P0^{+/-}$ mice are more prone to P0-induced EAN due to the defective central tolerance to P0, but we have not proven that the defective tolerance to P0 is causal of the development of the spontaneous inflammatory neuropathy in the $P0^{+/-}$ mice. In fact, the immunological processes in EAN and the spontaneous neuropathy would substantially differ: whereas $CD4^+$ T cells mainly mediate the induced autoimmune disease EAN, macrophages and $CD8^+$ T cells appear to dominate the pathology of the spontaneous neuropathy [16]. It still remains speculative regarding the link between a defect in central tolerance to P0 and the pathomechanism of the late-onset spontaneous neuropathy. Obviously, this interesting subject will be experimentally verified in the future.

4 Materials and methods

4.1 Mice

$P0^{-/-}$ mice were generated as previously described [11]. We back-crossed the $P0^{+/-}$ mice with B6 mice in our animal facilities under specific-pathogen-free conditions. A standard PCR analysis [12] determined genotypes of the P0 mutation.

4.2 Immunization

For induction of EAN, we immunized mice subcutaneoulsy (tail base and foot pads) with 200 µl of an inoculum, containing 400 µg of $P0_{180-199}$ (amino acids SSKRGRQTPVLYAMLDHSRS) or $P2_{53-78}$ (RTESTFKNTEISFKLGQEFEETTADN) and 1 mg of *Mycobacterium tuberculosis* H37Ra (Difco Laboratory, Detroit, MI) in incomplete Freund's adjuvant. We administered pertussis toxin (200 ng; List Biological Laboratories, INC., Campbell, CA) intravenously on days 0 and 2 after immunization. EAE was induced with MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK) as described [40]. Recall response to peptide was examined in mice that had not been given pertussis toxin.

4.3 Cell proliferation assay

We removed draining lymph nodes (LN) on day 11 after immunization. The lymphoid cells were suspended in RPMI1640 medium (Gibco, Grand Island, NY) supplemented with 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml of penicillin and streptomycin and 1% autologous mouse serum, and seeded onto 96-well U-bottom plates (2 or 8×10^5 cells/well). The cells were stimulated with peptide (200 µg/ml) for 72 h at 37°C in a humidified atmosphere with 5% CO_2. To measure cellular proliferation, [^{3}H]thymidine was added (1 µCi/well) and the uptake of the radioisotope during the final 18 h of culture was measured with a beta-1205 counter (Pharmacia, Uppsala, Sweden).

4.4 Cytokine analysis

In parallel, the LN cells were cultured with peptide for 48 h at a higher cell density (1×10^6/well). We measured the concentrations of IFN-γ and IL-4 in the supernatants by a sandwich ELISA [40] and conducted ELISPOT analysis for IFN-γ-producing cells according to a described method [41]. The cytokine values and the number of cytokine-positive spots were determined for individual mice by examining three to six wells each.

4.5 RT-PCR

We detected P0 transcript in the thymus by using RT-PCR with two sets of primers: one set for amplifying all exons (exons 1–6) of P0 [-sense, 5'-CAGGTCATTCAGATGTAGCG-3', and –antisense, 5'-GCTTTTCGAAGTCATCTCG-3'] and another set for exons 4–6 [-sense, 5'-GGCATTTTCTCA-ACGCATTACTT-3', and –antisense, 5'-TGGATCTGGAATTT-CTCTGCTACA-3']. The sequence encoding the neuritogenic P0 residues 180–199 is located at the boundary between exon 5 and 6. In brief, we extracted total RNA from single-cell suspensions of thymic lobes with RNAzol™ B (Biotecx Laboratories, Friendswood, TX), and then prepared the oligo (dT)-primed cDNA with the first-strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, GB). The cDNA samples were incubated for 5 min at 95°C and then underwent 30 cycles of PCR amplification (at 94°C for 30 s, at 60°C for 30 s, and at 72°C for 60 s). For qualitative assessment, the PCR products were analyzed on 5% acrylamide gels stained with ethidium bromide. For quantitative analysis, we used a Light Cycler™ quantitative PCR system (Roche Molecular Biochemicals, Mannheim, Germany). The PCR amplification was repeated for 40 cycles (at 95°C for 0 s, at 60°C for 5 s, and at 72°C for 10 s) using a commercial kit (Light Cycler-DNA Master SYBR Green I; Roche Molecular Biochemicals). Based on the standard values of the wild-type thymus as a control, the relative value for thymus from $P0^{+/-}$ or $P0^{-/-}$ mice was determined with the Light Cycler software.

4.6 Clinical and pathological assessment

Clinical grade for EAN and EAE was determined according to the following scale: 0, no clinical sign; 1, loss of tonicity in the distal half of the tail; 2, completely limp tail; 3, abnormal gait; 4, mild hindlimb paraparesis; 5, complete hindlimb paralysis; 6, fore- and hindlimb paralysis or a moribund state. For histopathological analysis, we randomly selected several mice from each group on day 14. We stained cross

sections of sciatic nerves (1-μm thickness) with toluidine blue as described [42], and evaluated the severity of the EAN pathology under a light microscope by calculating the total number of infiltrated cells/mm^2.

4.7 Thymus transplantation

We removed thymi from pairs of newborn mice (3 days after birth) *en bloc* and then transplanted each thymus into the thorax of the counterpart. Eight weeks later, we carefully examined the gender of the mouse pairs and selected only gender-matched pairs for further experiments. At the end of all the experiments, we routinely checked if the thymus was successfully engrafted.

4.8 dGuo treatment of thymus organ culture

We cultured the thymic lobes in the presence of dGuo, according to a previously described method with some modifications [43]. In brief, we placed Millipore filters (0.45-μm pore size) on Gelfoam™ sponge (Pharmacia Upjohn/Sumitomo) in a 90-mm petri dish, and added RPMI1640 medium supplemented with 10% FCS and 1.35 mM dGuo. Several hours later, we placed thymus lobes freshly obtained from newborn mice on the surface of the filter and cultured them for 7 days. The thymi were washed in RPMI1640 medium supplemented with 10% FCS and single-cell suspensions were processed for RT-PCR.

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