Epicutaneous Application of Toll-like Receptor 7 Agonists Leads to Systemic Autoimmunity in Wild-Type Mice

A New Model of Systemic Lupus Erythematosus

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Objective. To examine whether topical treatment of wild-type mice with Toll-like receptor 7 (TLR-7) agonists leads to lupus-like autoimmunity.

Methods. Wild-type FVB/N, BALB/c, and C57BL/6 mice were treated with the topical TLR-7 agonist imiquimod or R848 administered to the ear 3 times weekly. During treatment, the mice were monitored for serum autoantibody and creatinine levels as well as histopathology of the kidneys, spleens, livers, hearts, and skin. Immunologic abnormalities were analyzed by immunohistochemistry, quantitative reverse transcription–polymerase chain reaction, and fluorescence-activated cell sorting. The role of plasma-cytoid dendritic cells (PDCs) in the development of autoimmune disease was validated by in vivo treatment with a specific antibody. Diseased mice underwent ultraviolet B irradiation to evaluate skin photosensitivity. The disease-causing effect of topical application of imiquimod was compared with that of systemic (intraperitoneal) administration. TLR-7– and TLR-9–deficient mice were used to validate the role of TLR-7.

Results. Wild-type mice of different genetic backgrounds developed systemic autoimmune disease following 4 weeks of topical treatment with imiquimod or R848, with elevated levels of autoantibodies to double-stranded DNA and multiple organ involvement, including glomerulonephritis, hepatitis, carditis, and photosensitivity. Expression of IFna and Mx1, the interferon-α-stimulated gene, was up-regulated in the organs of imiquimod-treated mice. However, disease caused by intraperitoneal injection of imiquimod was less severe than that induced by topical application. In vivo depletion of PDCs by a specific antibody protected mice against the autoimmunity induced by topical administration of imiquimod, suggesting a role of PDCs. Furthermore, TLR-7–deficient mice, but not TLR-9–deficient mice, were protected against autoimmunity.

Conclusion. This protocol provides a novel model of inducible systemic lupus erythematosus in wild-type mice and underscores the skin as the primary organ that allows TLR-7 agonists to induce SLE.

Systemic lupus erythematosus (SLE) is the prototypical human autoimmune disease and is characterized by the production of autoantibodies and the subsequent development of inflammatory disorders such as glomerulonephritis (1). Several studies have demonstrated that altered Toll-like receptor (TLR) signaling contributes to the initiation and/or exacerbation of lupus in humans and in murine models (2,3). In recent years, it has become apparent that TLR-7 and TLR-9, which sense single-stranded RNA and unmethylated DNA, respectively, contribute to the development of autoimmune diseases such as rheumatoid arthritis, SLE, and psoriasis (3,4).

TLR-7 and TLR-9 activation in dendritic cells (DCs) induces the production of inflammatory cytokines such as interleukin-6, tumor necrosis factor α, and type I interferons (IFNs) (5). In addition, autoreactive B cells, in which TLR-7/TLR-9 activation occurs in response to RNA- and DNA-containing antigens, respec-
tively, in synergy with B cell receptor, undergo proliferation, isotype switching, and plasma cell differentiation, leading to the production of autoantibodies (6,7). In the last several years, accumulating evidence has shown that the role of TLR-7 may predominate over that of TLR-9 in human SLE and in mouse models of lupus. The lupus-like phenotype in the BXSB mouse strain has been linked to the Yaa locus, a translocation of the telomeric end of the X chromosome, which contains Tlr7, to the Y chromosome (8,9). The resulting duplication of Tlr7 appears to be responsible for the production of autoantibodies and the induction of lupus nephritis, because introduction of the TLR-7–null mutation on the BXSB background significantly reduces serum levels of autoantibodies as well as the incidence of lupus nephritis (10,11).

It was previously demonstrated that TLR-7 agonists induce much higher IFNα production by peripheral blood cells in women than in men (12). This might explain the remarkable prevalence of SLE among women, although no evidence for significant X-inactivation escape of the human TLR-7 gene was observed (12,13). The Yaa locus produces strikingly accelerated autoimmunity when mice with the Yaa mutation are bred to other mouse models of lupus such as Fcγ receptor IIB (FcγRIIB)–deficient mice (14,15). A study with TLR-7–transgenic mice revealed that an increased Tlr7 dose alone is essential and sufficient to promote autoreactive B cells with RNA specificities and myeloid cell proliferation (16). MLR/lpr mice bearing the TLR-7–null mutation are protected against disease, whereas those bearing the TLR-9–null mutation exhibit accelerated disease progression, including expansion of the numbers of plasmacytoid dendritic cells (PDCs) and lymphocytes, production of anti–RNP antibodies, and increased serum titers of IFNα (3,17,18). An opposing relationship between TLR-7 and TLR-9 has emerged as a potential mechanism regulating autoimmunity, and it has been suggested that TLR-9 has a regulatory role in antagonizing TLR-7 (18).

Although several studies have used lupus-prone mice to elucidate the pathogenesis of SLE, there is no murine model of inducible lupus in wild-type (WT) mice except pristane-induced lupus (19) and mice with the graft-versus-host reaction (20). Here, we show a critical role of TLR-7 activation in lupus-like autoimmune disease in WT mice elicited by epicutaneous treatment with TLR-7 agonists. In addition, our results highlight a distinct role of skin in the induction of systemic autoimmunity by TLR-7 agonists.

**MATERIALS AND METHODS**

**Mice and in vivo treatment.** FVB/N mice were purchased from Clea, and BALB/c and C57BL/6 mice were obtained from Japan SLC. All mice were 7–9-week-old females unless otherwise indicated. TLR-7– and TLR-9–null mice were on a BALB/c background (Oriental BioService). The skin on the right ears of the mice were treated topically, 3 times weekly, with either 1.25 mg of 5% imiquimod cream (Mochida Pharmaceutical) or 100 μg of resiquimod (R848; Alexis) in 100 μl of acetone. For systemic treatment, mice received intraperitoneal injections, 3 times weekly, with 125 μg of imiquimod (Tokyo Chemical Industry) in 0.5 ml 10% DMSO. To deplete PDCs, the mice were injected intraperitoneally with 500 μg of pure anti–mouse plasmacytoid dendritic cell antigen 1 (PDCA-1) antibody (Miltenyi Biotec) every 7 days during the 4-week protocol of topical treatment with imiquimod. Rat IgG2b (Miltenyi Biotec) was used as a control. For the photosensitivity experiments, 10-week-old mice treated with topical imiquimod were irradiated by ultraviolet B (UVB) using Dermaray M-DMR-1 lamp bulbs with peak emission at 311 nm, and 24 hours later the mice were killed so that histologic changes could be observed. Photosensitivity was assessed by ear thickness, based on the histologic assessments performed before and after UVB irradiation. All of the mouse experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of Kochi Medical School.

**Histopathologic assessment.** Tissue sections were fixed with neutral buffered formalin and embedded in paraffin. Three-micrometer sections of skin, liver, and heart were stained with hematoxylin and cosin. For the determination of kidney histopathology, the sections were stained with periodic acid–Schiff and reviewed by a pathologist in a blinded manner. Glomerular lesions were graded semiquantitatively on a scale of 0 to 2+ for mesangiol proliferation, endocapillary proliferation, mesangial matrix expansion, and segmental sclerosis (0 = <10%; 1 = 10–50%; 2 = >50% of the glomeruli examined). Global glomerular lesion scores were calculated for each mouse with at least 50 glomeruli.

**Immunohistochemistry and immunofluorescence.** Frozen sections of spleens were stained with anti-CD11b or anti–Gr-1 (BioLegend), followed by treatment with horseradish peroxidase (HRP)–conjugated anti-rat IgG (Dako), and were visualized using diaminobenzidine (DAB) and hematoxylin as counterstaining. For analysis of the marginal zone, frozen spleen sections were stained with antibodies to Alexa Fluor 488–conjugated anti-B220 (BioLegend) and biotinylated anti–MOMA-1 (Abcam) followed by treatment with fluorescein isothiocyanate–conjugated streptavidin (DyLight594; Vector). Thymus glands were stained with anti-B220 (RA3-6B2; Abcam) followed by treatment with Alexa Fluor 594–conjugated anti-rat IgG (Invitrogen).

For staining of PDCs in ear skin, deparaffinized skin specimens were incubated in 10 mmoles/liter sodium citrate for 5 minutes, using a microwave oven, and then were treated with H2O2 and washed with phosphate buffered saline. Slides were treated with a blocking reagent (Protein Block Serum-Free; Dako) for 1 hour at room temperature and then stained with an anti–PDC antibody (120G8.04; Dendrites), followed by treatment with HRP-conjugated anti-rat IgG (Dako), and
**Figure 1.** Fatal disease in wild-type mice following epicutaneous application of imiquimod (IMQ). A, Cumulative survival rate of FVB/N mice following topical application of imiquimod on ear skin 3 times weekly. Solid lines and broken lines indicate male (n = 7) and female (n = 7) mice, respectively. B and C, Swelling and edema (B) and marked splenomegaly (C) in imiquimod-treated mice at 10 weeks. D, Weights of spleens from FVB/N mice, BALB/c mice, and C57BL/6 mice that were untreated (unt; black circles) or treated with imiquimod for 4 weeks (blue circles) or 8 weeks (red circles). * = P < 0.05; ** = P < 0.01 by Mann-Whitney U test. E, Hematoxylin and eosin (H&E)-stained section of spleen from an FVB/N mouse treated with imiquimod for 10 weeks, showing increased cellularity compared with spleen from an untreated mouse. Bar = 400 μm. F, Increased numbers of CD11b+ cells in the spleen of imiquimod-treated BALB/c mouse compared with spleen from an untreated mouse. Images are representative of 4 independent experiments. Bar = 200 μm. G, Increased percentage of CD69+ cells among B lymphocytes (left) and T lymphocytes (right) in the spleens of BALB/c mice treated with imiquimod for 8 weeks compared with untreated mice. ** = P < 0.01 by Student’s t-test. H, H&E-stained sections of spleen from an FVB/N mouse treated with imiquimod for 8 weeks, showing ectopic megakaryocytes (arrows) and erythroblasts (arrowheads). The right panel shows a higher magnification view of the boxed area in the left panel. Bars = 40 μm and 10 μm, in the left and right panels, respectively. I, H&E-stained section of livers from mouse treated with imiquimod for 8 weeks, showing mononuclear cell infiltrates around the portal veins of FVB/N mouse (left; arrows) and the bile duct of BALB/c mouse (right; arrows). Bar = 50 μm. J, Marginal zone B cell population in spleen of FVB/N mouse that was untreated (left) or treated with imiquimod for 8 weeks, as shown by staining with anti-MOMA-1 (red) and anti-B220 (green). Arrows indicate the marginal zone B cell areas. Bar = 100 μm. K, Decreased percentage of marginal zone B cells (CD21highCD23low in gated B220+ cells) in the spleens of BALB/c mice treated with imiquimod for 8 weeks compared with untreated mice, as determined by fluorescence-activated cell sorting analysis. ** = P < 0.01 by Mann-Whitney U test. L, Anti-B220-stained sections of thymus gland from FVB/N mouse that was untreated or treated with imiquimod for 8 weeks, showing increased numbers of intrathymic B cells in diseased mouse. Broken lines indicate the boundary between the cortex (C) and the medulla (M). Bar = 100 μm. Images are representative of 4 independent experiments. In D, G, and K, symbols represent individual mice; horizontal lines show the mean.
visualized with DAB and counterstaining. Alternatively, PDCs were stained with anti–Siglec H antibody (440c; Abcam). Frozen skin sections were incubated with anti–Siglec H overnight, treated with HRP-conjugated anti-mouse IgG (Dako), followed by color development with DAB and hematoxylin.

Paraffin-embedded heart tissue specimens were stained with anti–Mx-1 antibody (ProteinTech), followed by treatment with HRP-conjugated anti-rabbit IgG (Dako) and a color development step as described above. The direct immunofluorescence technique was performed on 6-µm acetone-fixed cryostat sections of kidneys and dorsal skin, using Alexa Fluor 488–conjugated goat anti-mouse IgG, goat anti-mouse IgM (Invitrogen), and rat anti-mouse C3 (Abcam) followed by staining with Alexa Fluor 488–conjugated rabbit anti-rat IgG (Invitrogen). For detection of antinuclear antibodies (ANAs), serum was diluted 1:80 unless otherwise indicated and used for indirect immunofluorescence on Hep-2 slides (Medical and Biological Laboratories) with Alexa Fluor 488–conjugated goat anti-mouse IgG. Sections were mounted with Fluormount (Vector) and analyzed by fluorescence microscopy (Olympus).

**Quantitative reverse transcription–polymerase chain reaction (qPCR).** Tissue samples were minced with scissors into small pieces on ice and disrupted by ultrasonic sonication. Total RNA was extracted using an RNA Isolation Kit (Promega) according to the manufacturer’s protocol and was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Invitrogen) with random oligonucleotide hexamers (Invitrogen). qPCRs were performed using Power SYBR Green PCR Master Mix (Applied Biosystems), and the amplification conditions were as follows: 50°C for 2 minutes, 90°C for 10 minutes for 1 cycle, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The following primers were used: for Hprt, 5'-CACAGGAATGAACACCTTC-3' (sense) and 5'-GCTGGTGAAAAAGGACCTCT-3' (antisense); for Ifa, 5'-CATCTCGAATGACCCTCAC-3' (sense) and 5'-TCAGGGGAATTTCTGCGAC-3' (antisense); for Mxi1, 5'-AAAAACCTGGATCGGAACCAA-3' (sense) and 5'-CGGGTCACTTCCATCACCAAAC-3' (antisense). Transcripts were analyzed using 7300 Fast System software (Applied Biosystems) and normalized to Hprt complementary DNA using the ΔΔCt method.

**Flow cytometric analysis.** Splenocytes and lymph node cells were analyzed with a FACSCalibur (BD Biosciences). The following monoclonal antibodies were used: anti–CD19 (BioLegend), anti–PDC (Dendritics), anti–CD11c, anti–CD3, anti–B220, anti–CD69, anti–CD21, and anti–CD23 (all from BD Biosciences), all of which were conjugated with fluorochrome. For the staining of marginal zone B cells, splenocytes were pretreated with rat anti-mouse CD16 (BD Biosciences) on ice for 10 minutes to block FcγR before staining with anti–B220, anti–CD21, and anti–CD23 monoclonal antibodies.

**SeroLogic analysis.** The following analyses were performed using commercially available assay kits, according to the manufacturers’ instructions. Serum IgG1 and IgG2a were measured by enzyme-linked immunosorbent assay (ELISA) kits (Bethyl Laboratories). Anti–double-stranded DNA (anti–dsDNA) (specific for IgG) and anti–Sm (IgG, IgA, and IgM) antibodies were detected using ELISA kits from Shibayagi and Alpha Diagnostic, respectively. Serum creatinine levels were determined using an assay kit from Cayman Chemical.

**Hematology and urinalysis.** Whole blood in EDTA was analyzed with a hematologic analyzer (MEK-3DN; Nihon Kohden). Urinary protein was determined using a Pyrogallol Red Molybdate protein assay kit (Wako).

**Statistical analysis.** Data were analyzed using Mann-Whitney 2-tailed U test and Student’s t-test. P values less than 0.05 were considered significant.

**RESULTS**

**Fatal disease in WT mice following topical treatment with imiquimod.** Wild-type FVB/N mice received topical treatment on their right ears with the TLR-7 agonist imiquimod, 3 times weekly. Strikingly, they began to die after 8 weeks of treatment, and all of the treated mice died by 15 weeks, with no sexual dimorphism (Figure 1A). At 10 weeks, the torsos of the imiquimod-treated mice appeared edematous and swollen (Figure 1B). These mice also displayed marked splenomegaly (Figure 1C), which was also observed in mice with different genetic backgrounds (i.e., BALB/c and C57BL/6 mice) (Figure 1D). An equal number of male and female mice developed edema and splenomegaly.

Histologic examination of the spleens of treated mice revealed increased cellularity compared with that in untreated control mice (Figure 1E). A marked expansion in the number of myeloid cells in the spleens was observed, as indicated by staining with anti–CD11b (Figure 1F) and anti–Gr-1 (data not shown). In addition, imiquimod treatment led to increases in the numbers of both splenic B cells and splenic T cells. The mean ± SD numbers of B220+ cells from untreated mice (n = 4) and 8-week-old imiquimod-treated mice (n = 4) were 4.6 ± 0.3 × 10^7/spleen and 10.1 ± 3.4 × 10^7/spleen, respectively (P < 0.01); the mean ± SD numbers of CD3+ cells were 6.25 ± 0.65 and 15.9 ± 2.1, respectively (P < 0.05). Both B cells and T cells from imiquimod-treated mice also showed spontaneous activation, as indicated by an increase in the frequency of CD69 expression (Figure 1G), similar to previous observations in transgenic mice that overexpress TLR-7 (16).

Thus, topical imiquimod treatment led to the expansion and activation of both myeloid cells and lymphoid cells. Megakaryocytes and erythroblasts were observed (Figure 1H), indicating splenic extramedullary hematopoiesis. The splenic hematopoiesis might be a compensatory condition for thrombocytopenia and anemia. In fact, hematologic analysis revealed a significant reduction in the erythrocyte count, hemoglobin concentration, hematocrit, and platelet count in imiquimod-treated FVB/N mice (9%, 19%, 16%, and 64% reduc-
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These hematologic abnormalities are similar to those observed in lupus-prone mice with multiple Tlr7 transgenes (16) or in mice lacking A20 (Tnfaip3) (21). Livers from the diseased mice demonstrated mononuclear cell infiltrates (Figure 1I). Thus, epicutaneous application of imiquimod resulted in systemic inflammation.

**Effect of topical treatment with imiquimod on B cell alteration.** Spleen sections from imiquimod-treated mice demonstrated a marked reduction of the marginal zone, a characteristic rim of marginal zone B cells at the periphery of the follicles that is separated by MOMA-1–positive macrophages (Figure 1J). Notably, the numbers of MOMA-1–positive macrophages were increased by imiquimod treatment. MOMA-1–positive macrophages appear to play a role in the initial response to systemic infection (22), suggesting the effect of TLR-7 triggering. Flow cytometric analysis revealed a marked decrease in the percentage of marginal zone B cells identified as CD21^high^CD23^low^ cells (Figure 1K). The impaired development of marginal zone B cells was previously observed in BXSB-Yaa mice, FcγRIIB^-/-^ Yaa mice, and transgenic mice bearing increased copy numbers of Tlr7 (9,14,16), all of which developed systemic autoimmune disease. Also, the numbers of intrathymic B cells in diseased mice were increased (Fig-
ure 1L), as previously observed in lupus-prone (NZB ×
NBW)F1 mice (23).

Topical imiquimod–induced lupus-like glomeru-
lonephritis in WT mice. Beginning 4 weeks after topical
imiquimod treatment, histopathologic assessment of the
kidneys showed enlarged hypercellular glomeruli, an
increase in the mesangial matrix, and mild peritubular
mononuclear cell infiltrates (Figures 2A–D). A signifi-
cant increase in the renal histopathologic score was
observed beginning 4 weeks after imiquimod treat-
ment (Figure 2E). Simultaneously, elevated serum creatinine
levels (Figure 2F) and increased proteinuria (Figure 2G)
were observed, indicating renal dysfunction. Immunoflu-
orescence analysis detected IgG, IgM, and C3 deposits
within the glomeruli beginning 4 weeks after imiquimod
treatment (Figure 2H), suggesting glomerulonephritis
with immune complex deposits.

Autoantibody production induced by topical imi-
quimod treatment. Among diseased mice treated with
imiquimod, serum IgG1 levels remained unchanged,
while serum IgG2a levels were significantly elevated,
beginning at week 4 (Figures 3A and B). IgG2a is the
most prominent IgG subclass involved in inducing auto-
immunity, while IgG1 displays the poorest pathogenicity.
Therefore, repetitive TLR-7 activation in the skin might promote pathogenic B cell activation leading to a class-switch recombination of the γ2a locus. Furthermore, sera from imiquimod-treated mice showed anti-dsDNA, the concentration of which increased over time (Figure 3C). The expression of anti-Sm autoanti-
body, an anti-RNP antibody, was also elevated in FVB/N mice but not in BALB/c mice (Figure 3D). Indirect immunofluorescence using HEP-2 slides revealed that sera from imiquimod-treated FVB/N mice (1:80 dilution) showed anticytoplasmic antibodies only (43.5% penetrance) or ANAs with a speckled pattern (34.8%) (Figure 3E), whereas those from BALB/c mice showed anticytoplasmic antibodies only (44.4% penetrance) or homogeneous/speckled ANAs (55.5% penetrance) (Figure 3F).

Severity of disease caused by intraperitoneal administration of imiquimod. The disease-causing effects of topical and intraperitoneal administration of imiquimod were compared. Compared with topical treatment, intraperitoneal administration resulted in marginal histologic changes in the kidney and liver (data not shown). Correspondingly, the ANA titers in the sera of mice treated intraperitoneally (1:40) were much lower than those in the sera of mice treated topically (1:160). In addition, anti-dsDNA concentrations were not increased by intraperitoneal imiquimod treatment (Figure 3G). This suggests that the skin is the predominant site where TLR-7 agonists initiate lupus-like systemic autoimmune disease. However, it is possible that this was attributable to a lower dose of drug received by intraperitoneal administration.

Accumulation of PDCs in skin and decreased number of PDCs in lymph nodes following topical imiquimod treatment. PDCs constitutively express TLR-7 and TLR-9 and play critical roles in the pathogenesis of SLE through the production of IFNα following TLR-7/TLR-9 activation (5,26). Immunohistochemical analysis using anti–PDCA-1 demonstrated a focal accumulation of PDCs in the dermis of ear skin where imiquimod was applied (Figure 4B), while there were no PDCs in untreated skin (Figure 4A). Staining for Siglec H, a more specific marker for PDCs, confirmed their accumulation at the epidermal–dermal junction (Figures 4C and D). As previously described (27), imiquimod-treated skin showed psoriasis-like changes such as epidermal hyperplasia (Figures 4B and D). In contrast, the numbers of PDCs in skin-draining lymph nodes were significantly decreased following imiquimod treatment (Figures 4E and F). This observation suggested a redistribution of PDCs from lymphoid tissue to the skin where the TLR-7 agonist was applied. This result is similar to the finding that in patients with SLE, the number of circulating PDCs is decreased due to migration to the skin (28,29). A significant increase in Ifna expression was observed in the spleen, and increased expression of Mx1, the IFNa-stimulated gene (30), was observed in the kidney (Figure 4G), suggesting the role of PDCs in the systemic involvement induced by imiquimod treatment.

Inhibition of autoimmune disease development in vivo treatment with an anti-PDC antibody. In vivo treatment with an anti-PDC antibody to deplete PDCs significantly reduced the imiquimod-induced elevation of anti-dsDNA titers, while control IgG had no effect (Figure 4H). Furthermore, anti-PDC–treated mice were protected against glomerulonephritis (data not shown). These results strongly suggested that PDCs are essential for the development of the systemic autoimmunity induced by topical imiquimod treatment.

Cutaneous manifestations of murine lupus similar to those of human SLE. Because imiquimod was applied onto the ear skin, the dorsal skin was not inflamed (data not shown). However, the dorsal skin of diseased mice showed linear deposits of IgG and IgM autoantibodies along the basement membrane at the epidermal–dermal junction (Figure 4J) resembling the “lupus band,” which is characteristic of SLE. Dermal fibroblasts also showed immunoglobulin deposits, whereas untreated skin was negative for immunoglobulin deposits (Figure 4I). We next examined whether diseased mice showed photosensitivity, which is a common manifestation of SLE. Twenty-four hours after UVB irradiation, imiquimod-treated mice showed acute inflammatory changes such as edema and dermal cell infiltrates not only in the right ear skin where imiquimod was applied but also in the left ear skin, whereas such inflammatory changes were not observed in untreated mice (Figures 4J and K). Thus, the cutaneous manifestations in this lupus model, such as lupus band and photosensitivity, are similar to the cutaneous manifestations of human SLE.

Development of severe autoimmune disease in WT mice following topical treatment with R848. Topical treatment with the TLR-7 agonist R848 also led to autoimmune disease that was similar to or even more severe than that caused by imiquimod and included marked splenomegaly (Figures 5A and B), serum anticytoplasmic autoantibodies and ANAs with a homogeneous pattern (100% penetrance), and high titers of anti-dsDNA (Figures 5C and D). The mice developed severe glomerulonephritis along with tubulointerstitial inflammation (Figures 5E–G). They also showed inflammation in the heart, including pericardial calcification (Figure 5H), which was reminiscent of pericarditis in patients with SLE (31), and myocarditis, as indicated by Mx1 expression (Figure 5I). In addition, histologic evaluation of the liver revealed severe mononuclear cell infiltration around the portal veins and hepatocyte necrosis (Figure 5J).
Protection against topical imiquimod–induced autoimmunity in TLR-7–deficient but not TLR-9–deficient mice. TLR-7–knockout mice were used to confirm whether TLR-7 contributes to imiquimod-induced systemic autoimmunity. Although topical imiquimod treatment for 8 weeks profoundly facilitated the expansion of splenic B cells in both WT and TLR-9–knockout mice, this was not the case with TLR-7–
knockout mice (Figure 6A). In addition, treatment with topical imiquimod did not increase the titers of anti-dsDNA antibodies in TLR-7–knockout mice, while the anti-dsDNA titers in TLR-9–knockout mice (Figure 6B) were as high as those in WT mice (Figure 3C). Correspondingly, TLR-7–knockout mice were protected against the development of glomerulonephritis (Figures 6C and 6E), which was observed in TLR-9–knockout mice (Figures 6D and 6E) as in WT mice (Figures 2D and 6E). Taken together, these results clearly indicate that TLR-7, but not TLR-9, is required for the development of imiquimod-induced systemic autoimmune disease.

**DISCUSSION**

This study is the first to demonstrate that TLR-7 activation through topical TLR-7 agonist treatment in WT mice leads to lupus-like systemic autoimmune disease. Furthermore, TLR-7 deficiency ameliorates the phenotype, indicating that TLR-7 signaling is necessary for the development of autoimmune disease. Although a prevalence of females is characteristic of human SLE, the effects of topical treatment with TLR-7 agonists, including mortality and morbidity (e.g., kidney disease and autoantibody production) were equal in male and female mice (data not shown). Both serum creatinine levels and proteinuria were increased in imiquimod-treated mice. Therefore, it is likely that renal failure was the cause of death. However, the involvement of multiple organs (including the liver and hematopoietic system) was observed in imiquimod-treated mice; thus, we cannot exclude the possibility that multiplex morbidities led to their death.

The role of TLR-7 in the pathogenesis of SLE has been suggested by several studies using lupus-prone
mice. Male BXSB mice develop lupus because of the Yaa locus, which confers a duplication of Tlr7 (9). In contrast, Tlr7 deficiency in lupus-prone MRL/lpr mice reduced B cell activation and ameliorated renal disease (17). A reduction of the TLR-7 gene dose abolished the Yaa phenotype, but increasing the dose promoted fatal acute inflammatory pathology (16). Thus, it is likely that homeostatic TLR-7 activation by repetitive treatment of WT mice with topical imiquimod or R848 leads to autoimmune disease, which might be analogous to increasing the TLR-7 gene dose.

It has been suggested that TLR-7 and TLR-9 might have opposing contributions to autoimmune pathology (17,32). A recent study demonstrated that TLR-9 protected against autoimmune disease by antagonizing TLR-7 (18). More recently, a role of UNC93B1, an endoplasmic reticulum–resident protein, was shown to control the intracellular trafficking of TLR-9 and TLR-7 (33). TLR-9 competes with TLR-7 for UNC93B1-dependent trafficking and predominates over TLR-7, thereby regulating homeostatic TLR-7 activation. Reciprocal TLR-7/TLR-9 balance, which is dependent on UNC93B1, is required for limiting systemic autoimmunity (34). However, in our experimental setting, TLR-9–knockout mice did not show an increased response to imiquimod compared with WT mice with respect to disease induction.

PDCs play a critical role in the pathogenesis of SLE through IFNα production upon TLR-7/TLR-9 ligation (5,35). IFNα released from PDCs promotes autoreactive B cell expansion, causes the differentiation of plasma cells to produce autoantibodies, and activates myeloid cells and autoreactive T cells (33,35). The essential role of PDCs in the development of autoimmune disease was clearly demonstrated by depleting them. Anti–PDCA-1, used for the depletion of PDCs, recognizes the bone marrow stromal cell marker antigen 2, which is expressed predominantly by PDCs but also by other myeloid cells following stimulation with IFNs (36). Therefore, in vivo treatment with anti–PDCA-1 might affect some population(s) of myeloid cells as well. The targeted depletion of Siglec HG–expressing cells (37), for example, will help further clarify the role of PDCs in imiquimod-induced lupus in the future.

We demonstrate here that compared with intraperitoneal administration of imiquimod, topical application of imiquimod to the skin efficiently promotes systemic autoimmune disease, although it was not determined how much imiquimod was substantially absorbed from the skin. Topical treatment with R848 also induces severe systemic autoimmune disease, including nephritis and hepatitis, whereas internal organ involvement was not observed in mice when R848 was administered intraperitoneally (38). Furthermore, a recent study in which inflammatory responses in rats treated with subcutaneous and intraperitoneal imiquimod were compared clearly indicated that more potent systemic effects were elicited by subcutaneous injection (39). Therefore, subsequent systemic effects of TLR-7 triggering might be largely dependent on the treatment site. Imiquimod has been used to treat various skin neoplasms, including genital warts, actinic keratoses, and superficial basal carcinomas (40). As far as we know from the literature, however, there has been no case in which the development of SLE resulted from imiquimod treatment. We have had some patients with actinic keratoses in whom transient ANA positivity developed during treatment with imiquimod, but SLE did not develop afterward. This suggests that therapeutic doses of imiquimod are relatively smaller than those that induce autoimmune disease in mice. In fact, the dose of imiquimod used for mice in this study (41.7 mg/kg) was 200-fold higher than the clinical dose used in humans (0.21 mg/kg).

Topical imiquimod treatment induced psoriasis-like epidermal hyperplasia and inflammatory cell infiltrates, as previously demonstrated (41). PDC accumulation was observed in the dermis of imiquimod-treated mice, similar to human psoriasis (42). Furthermore, topical application of imiquimod aggravated psoriatic lesions through PDC activation, suggesting the role of TLR-7–mediated innate immunity in the pathogenesis of psoriasis (27). In our experimental setting, however, full-blown psoriatic lesions were observed within 3 weeks of imiquimod treatment but were attenuated thereafter (data not shown). Likewise, a previous study demonstrated imiquimod-induced skin lesions within 1 week of imiquimod treatment (41). Similar to psoriasis, cutaneous lesions of lupus are frequently associated with PDC infiltrates (43,44). Furthermore, topical application of imiquimod led to a histopathologic pattern similar to that of cutaneous lupus in humans (45). Thus, it is likely that the pathogenesis of both psoriasis and lupus share roles of PDCs and type I IFN.

Potential candidates for naturally occurring TLR-7 ligands are RNA viruses such as endogenous retrovirus, because “pseudoviral” immunity is evolving as a recent concept in understanding the pathogenesis of SLE (13,46). UV radiation is a worsening factor not only for cutaneous lesions but also for systemic symptoms in patients with SLE. It was previously shown that UV light–induced injury led to an amplification cycle of cutaneous lupus through activation of autoimmune T
cells and PDCs (47). Very recently, epidermal keratinocytes damaged by UV light exposure were shown to release self-noncoding RNA by which TLR-3 was ligated, leading to an alteration in immune responses (48). In addition, self-nucleic acids released after skin injury have been shown to trigger TLR-7/TLR-9 activation and to promote PDC infiltrates and the production of type I IFN (49,50). In this regard, we speculate that the continuous release of self-RNA from apoptotic epidermal cells after injury or solar damage may trigger homeostatic TLR-7 activation, leading to systemic autoimmune disease. Patients with SLE are photosensitive, and this effect was also reproduced in imiquimod-treated mice. It is noteworthy that the skin where imiquimod was applied showed marked photosensitivity, suggesting that locally activated TLR-7 in the skin might contribute to an acute inflammatory response to UV irradiation. In conclusion, the present study not only provides a novel protocol for generating a lupus model in WT mice but also highlights the skin as the primary organ where TLR-7 signaling leads to systemic autoimmunity, as in SLE.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Sano had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Sano.

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Analysis and interpretation of data. Terada, Sano.

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