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Langerhans Cells Maintain Local Tissue Tolerance in a Model of Systemic Autoimmune Disease

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Systemic autoimmune diseases such as lupus affect multiple organs, usually in a diverse fashion where only certain organs are affected in individual patients. It is unclear whether the “local” immune cells play a role in regulating tissue specificity in relation to disease heterogeneity in systemic autoimmune diseases. In this study, we used skin as a model to determine the role of tissue-resident dendritic cells (DCs) in local and systemic involvement within a systemic lupus disease model. Skin-resident DCs, namely, Langerhans cells (LCs), have been implicated in regulating tolerance or autoimmunity using elegant transgenic models, however, their role in local versus systemic immune regulation is unknown. We demonstrate that although lymphocytes from skin-draining lymph nodes of autoimmune-prone MRL/MpJ-Faslpr/lpr (MRL-lpr) mice react spontaneously to a physiological skin self-Ag desmoglein-3, epiphenomenal applications of desmoglein-3 induced tolerance that is dependent on LCs. Inducible ablation of LCs in adult preclinical MRL-lpr and MRL/MpJ-Faslpr/+/+ mice resulted in increased autoantibodies against skin Ags and markedly accelerated lupus dermatitis with increased local macrophage infiltration, but had no effect on systemic autoantibodies such as anti-dsDNA Abs or disease in other organs such as kidneys, lungs, and liver. Furthermore, skin-draining lymph nodes of LC-ablated MRL-lpr mice had significantly fewer CD4+ T cells producing anti-inflammatory cytokine IL-10 than LC-intact controls. These results indicate that a skin-resident DC population regulates local tolerance in systemic lupus and emphasize the importance of the local immune milieu in preventing tissue-specific autoimmunity, yet have no effect on systemic autoimmunity. The Journal of Immunology, 2015, 195: 000–000.

Systemic autoimmune diseases, such as lupus erythematosus (SLE), involve various combinations of multiple organ autoimmunity and tissue injury. Thus, patients present with a wide variety of clinical manifestations. Dysregulation of immune cells, such as dendritic cells (DCs) and B and T cells, has been implicated in systemic autoimmunity best reflected by the presence of autoantibodies against systemic autoantigens, such as dsDNA (1). However, because specific organ involvement varies from patient to patient, organ-specific tolerance may also play a role in regulating tissue-specific injury. Evidence supports a pivotal role of DCs in the pathogenesis of systemic autoimmune diseases. In fact, a constitutive deletion of all DCs (CD11c+ cells) in autoimmune-prone MRL/MpJ-Faslpr/lpr (MRL-lpr) mice ameliorates lupus (2). However, DCs comprise heterogeneous subsets of cells with different origins, organ of residence, and potentially differential roles. Although many studies implicate myeloid and plasmacytoid DC populations in the pathogenesis of SLE (3), the role of tissue-resident DCs in the maintenance of organ-specific tolerance in autoimmunity and development of disease is not well defined.

Langerhans cells (LCs) are epidermis-resident DCs that express langerin (Lang) and migrate through dermal lymphatics to skin-draining lymph nodes (dLNs). LCs are the prototype tissue-resident DCs studied to gain insights into tissue-specific DC function. LCs act as sentinels to orchestrate the immune response against foreign Ags including pathogens in skin. In the steady-state, and the Joseph B. Gould Foundation (to D.J.L.). Confocal microscopy was performed at the UCLA California NanoSystems Institute Advanced Microscopy Facility, which was supported by NIH-National Center for Research Resources Grants CJX1-443835-WS-29646 and NSF CHE-0722519. Flow cytometry was performed in the UCLA Flow Cytometry Core Facility, which was supported by NIH Grants CA-16042 and AI-28697. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Abbreviations used in this article: B6, C57BL/6; B6-lpr, C57BL/6 Faslpr/+; C3H, C3H/HeJ; ECL, enhanced chemiluminescence; DC, dendritic cell; dLN, skin-draining lymph node; DN, double-negative; Dog3, desmoglein-3; DTR, diphtheria toxin receptor; HEL, hen egg lysozyme; Lang, langerin; Lang’dDC, Lang* DC that inhabit the dermis; Lang-DTR, Lang-diphtheria toxin receptor–enhanced GFP; LC, Langerhans cell; MRL/lpr, MRL/MpJ-Faslpr/lpr; MRL/lpr, MRL/MpJ-Faslpr/+; PBS-T, PBS-Tween 20; SI, stimulation index; SLE, systemic lupus erythematosus; Treg, regulatory T cell.

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LCs have been implicated to induce peripheral tolerance to skin self-Ags (4, 5). However, transgenically expressed epidermal Ag can be cross-presented by migratory DCs to CD8+ T cells in dLNs under steady-state conditions resulting in autoimmunity (6, 7). Such immune-stimulatory versus regulatory roles of LCs have been well elucidated using elegant transgenic models; however, little is known about the role of LCs in chronic autoimmune conditions such as SLE. Analyses of LCs in skin biopsies from humans and mice with SLE have revealed conflicting information. For example, skin biopsies from patients with SLE show increased numbers (8) or an irregular distribution (9) of LCs in some studies, but reduced numbers of LCs in skin lesions in other studies (10). Another study reported the migration of LCs into lupus skin lesions preceding the infiltration with lymphocytes (11). To explore the contribution of LCs in the maintenance of skin tolerance before the onset of clinical disease, we used murine strains that spontaneously develop an autoimmune disease resembling human SLE.

MRL-lpr and MRL/MpFas+/– (MRL+/+) mice spontaneously develop a syndrome with anti-dsDNA Abs, nephritis, vasculitis, and dermatitis that histopathologically mimics chronic cutaneous lupus in humans (12). As in a human autoimmune bullous disease, pemphigus vulgaris (13), MRL-lpr mice develop autoantibodies to the cadherin-type desmosomal glycoprotein, desmoglein-3 (Dsg3) (14), that is expressed in stratified squamous epithelium in skin, oral mucosa, and esophagus, but not in other tissues such as kidney, liver, intestine, and muscle (14, 15). Furthermore, immunization with Dsg3 induces lupus-like skin disease in mice (14), and the adoptive transfer of Dsg3-reactive CD4+ T cell clones into immune-deficient mice induces interface dermatitis that is a feature of cutaneous lupus and a few other inflammatory skin diseases (15). Importantly, the adoptively transferred Dsg3-reactive T cells infiltrate skin and mucous membranes, but not other organs that do not express Dsg3 (15). Taken together, these observations suggest a role for tissue-specific autoimmunity in the development of lupus skin disease, which may be regulated differently from the systemic disease process. Hence we used Dsg3 as a model skin autoantigen to investigate local tolerance induction in the murine lupus model. Previously we demonstrated that lupus-prone MRL-lpr mice have impaired migration of Lang-expressing skin-DCs from skin to dLNs, which is detected early in life and correlates with the onset and severity of autoimmune skin disease (16). This migration defect leading to the paucity of Lang-expressing DCs in dLNs might jeopardize the maintenance of tolerance to skin Ags, thus contributing to the development of lupus dermatitis. Using an endogenous skin self-Ag and an inducible LC ablation model, we specifically examined the role of LCs in maintaining local skin tolerance by ablating LCs preclinically to exacerbate the LC deficiency in MRL mice. Although several Lang+ DC ablative models exist (17–19), we chose an inducible model (20) to ablate LCs in young lupus mice to obviate any potential compensatory pathways arising as a result of constitutive LC ablation from early development, as well as to mimic the loss of peripheral tolerance observed clinically in young human adults who experience development of lupus. Using this model, we, for the first time to our knowledge, demonstrate a role of LCs in protecting against the breakdown of skin tolerance in genetically autoimmune-prone mice and preventing the development of autoimmune dermatitis in murine disease.

Materials and Methods

**Methods**

**Mice**

MRL-lpr, MRL+/+, C3H/HeOul (C3H), C57BL/6 (B6), and C57BL/6 Faspro/lpr (B6-lpr) mice were purchased (The Jackson Laboratory) and/or bred locally. B6 Lang–diptheria toxin receptor (DTR)–enhanced GFP knock-in (Lang–DTR) mice that express human DTR and enhanced GFP downstream of the internal stop codon of the Cd207 gene (Lang) were provided by Bernard Malissen (20). Lang–DTR B6 mice were backcrossed onto the MRL-lpr and MRL+/+ backgrounds for >10 generations. To generate Lang–DTR B6–lpr mice, we crossed B6 knock-in mice with B6–lpr mice, and the F1 offspring intercrossed. Mice were maintained in specific pathogen-free conditions.

**Abs**

Abs against CD4 (GK1.5), CD8 (53-6.7), CD11b (M1/70), CD16/32 (93), CD11c (N418), CD19 (1D3), CD44 (IM7), CD62L (MEL-1), CD69 (H1.2F3), Foxp3 (FJK-16S), I-A/E (M5/114.15.2), IL-17 (eBio17B7), INF-γ (XMG1.2), Lang (RMUL.2), TCRβ (H57-597), and TNF-α (MP-6T22) were purchased from eBioscience (San Diego, CA). Abs for Ki-67 (B56), IL-2 (JES6-5H4), and IL-10 (JES5-16E3) were purchased from BD Biosciences (San Jose, CA).

**Flow cytometry**

Fc receptors were blocked by incubating single-cell suspensions on ice for 10 min with CD16/32 before staining. For cytokine assays, cells were cultured for 5–6 h with PMA and ionomycin (100 nM), 2 mg/ml monensin, and 1 mM EDTA at 37˚C and 5% CO2. At the last 2–3 h of culture, BD GolgiStop was added for IL-10 staining and BD GolgiPlug was added for TNF-α, INF-γ, IL-2, and IL-17 staining. Samples were acquired on FACS Calibur or LSR-II flow cytometers (BD Biosciences). Data were analyzed with FlowJo software (TreeStar, Ashland, OR).

**Epicutaneous tolerization assay**

To administer the Ag in a steady-state environment, we avoided the use of mouse strains that provide other experimental procedures that are generally used to facilitate the skin penetration of Ag. In initial experiments, we used acetone/olive oil as a carrier for Ags, followed by a patch (DuoDerm extra thin) to keep in place the emulsion. This approach led to inconsistent results, because the patch was torn by many animals soon after its application. After trials of several carriers and application methods, we selected to emulsify the Ag (500 μg) with an equal volume of IFA (Sigma-Aldrich, St. Louis, MO). Mouse ears were painted twice with emulsions with 100 μl 20% Ag in 37˚C FCS at 37% and 5% CO2. At the last 2–3 h of culture, BD GolgiStop was added for IL-10 staining and BD GolgiPlug was added for TNF-α, INF-γ, IL-2, and IL-17 staining. Samples were acquired on FACS Calibur or LSR-II flow cytometers (BD Biosciences). Data were analyzed with FlowJo software (TreeStar, Ashland, OR).

**Preparation of Dsg3**

For bacterial expression of Dsg3, the extracellular portion of Dsg3 was cloned into BamHI and NotI sites in pGEX4T1 (GE Healthcare Life Sciences, Pittsburgh, PA). Primers were chosen to PCR amplify from the signal sequence up to the transmembrane domain (5′-ggc GATCC GAATTCCTGATGAACGGCCG-3′ and 5′-ggggc GCGGCCGC TCACAGCCCTCAGGATGACT-3′) using cDNA as a template (clone 40130335, Open Biosystems). The plasmid was confirmed by sequencing. For expression, the plasmid was transformed into BL21 and grown to a density of 0.8. Protein expression was induced using 100 μM isopropylthio-β-galactoside overnight at room temperature. Inclusion body purification was from Pamela Bjornland (California Institute of Technology, www.its.caltech.edu/~bjorker/protocols.html). Cells were pelleted and resuspended in 13 ml solution buffer (50 mM Tris pH 8, 25% sucrose, 1 mM EDTA, 0.1% sodium azide, 10 mM DTT). After sonication for 30 pulses of 10 s each, 12.5 ml isosens buffer (50 mM Tris pH 8, 1% Triton X-100, 1% sodium deoxycholate, 100 mM sodium chloride, 0.1% sodium azide, 10 mM DTT) supplemented with lysozyme, DNase I, and magnesium chloride to final concentrations of 200 μg/ml, 10 μg/ml, and 1 mM, respectively. After 1-h incubation at room temperature, EDTA was added to a final concentration of 7 mM, and the lysate was snap frozen in liquid nitrogen and thawed at 37˚C for 30 min. Magnesium chloride was increased to 5 mM and incubation was allowed to continue for 1 h at room temperature. EDTA was increased to 14 mM. Incubation solutions were pelleted by centrifugation at 11,000 × g at 4˚C. The pellet was washed with wash buffer (50 mM Tris pH 8, 100 mM sodium chloride, 1 mM EDTA, 0.1% sodium azide, 1 mM DTT, 0.5% Triton X-100) at 4˚C. Another wash was performed without Triton X-100. LPS in rDsg3 preparations was measured using Limulus amebocyte lysate chromo-
genic endotoxin quantitation kit (Pierce), which showed trace amounts of LPS in most preparations. rDsg3 was preincubated with polyoxymethylene B (Sigma-Aldrich) at 10 μg/ml for 30 min at 37°C to control for endotoxin contamination before use for in vitro culture studies.

Isolating cells from skin

Ears were first split into dorsal and ventral halves with thecartilage removed from theventral sheet, to isolate DCS from skin. Ear half was floateddernal side down in PBS containing 2.5 mg/ml Dispase II (Roche, Nutley, NJ) at 4°C for 16–20 h, to split epidermis from dermis. Epidermis and dermis were separated using forceps and cut into small pieces in RPMI 1640 containing 1 mg/ml collagenase IV (Worthington, Lakewood, NJ) and 1 mg/ml DNase (Sigma-Aldrich). Tissue was incubated with the mixture for 1 h at 37°C to obtain homogenous cell suspensions. During the last 5 min of incubation, 25 mM EDTA was added.

Immunofluorescence

Ears were first split into dorsal and ventral halves using forceps. With the dermal side down, ear halves were floated on top of PBS containing 3.8% NH4SCN (Sigma-Aldrich) at 37°C for 30 min to split epidermis from dermis. After the epidermis and dermis were separated using forceps, tissue was fixed immediately in aceton at −20°C. Tissue was washed three times with PBS and blocked with 1% BSA and CD16/32 in PBS for at least 30 min. Tissues were stained with Alexa 488-Lang (eBioscience), eBioscience and Alexa 647-CD11c (Invitrogen labeling kit). Images were acquired on a Leica SP1-Inverted confocal microscope and processed using Leica imaging software.

Preparation of whole-tissue skin lysate

Nair was applied to MRL-lpr mouse skin to remove hair and then washed off thoroughly. Skin was harvested and homogenized on ice with an electric homogenizer in lysis buffer (150 mM NaCl, 20 mM Tris-HCl, 0.5% Triton X-100) containing protease inhibitor mixture (Sigma-Aldrich). Homogenized skin was transferred to Eppendorf tubes and centrifuged at 200,000 × g at 4°C. Supernatants were collected and stored at −20°C. Protein concentration was determined by BCA assay (Pierce; Rockford, IL).

Detection of autoantibodies

Autoantibodies were detected by Western blot or ELISA, as described previously (21, 22). To detect skin-reactive autoantibodies in mouse serum, we incubated skin lysate (50 μg/lane) with NuPAGE LDS Sample Buffer (Invitrogen) and DTT at 70°C for 10 min and then run in a 4–12% gradient bis/tris polyacrylamide gel (Invitrogen, Grand Island, NY) in MOPS buffer. Precision Plus Protein Kaleidoscope Standards (Bio-Rad, Hercules, CA) were run in every third lane. Gels were blotted to 0.45-μm polyvinylidene difluoride membranes (Invitrogen). Blots were then incubated with 1:200 dilution secondary Ab (Mouse TrueBlot ULTRA: anti-mouse IgG HRP; eBioscience) for 1 h at room temperature. Blots were visualized using Super Signal West Femto Maximum Sensitivity Substrate Kit (Thermo Scientific, Waltham, MA). Band densities were calculated using Image software. The bands visualized by Ponceau S staining was used to adjust for loading volume variances.

To detect autoantibodies to dsDNA by ELISA, we plated 5 μg/ml calf thymus DNA (Sigma-Aldrich) at 50 μl/well in 96-well enzyme immunoassay plates and incubated them overnight at 4°C. After washing plates three times with 0.05% PBS-T, we blocked wells with 2% BSA in PBS for 2 h at room temperature. The plates were then washed thrice with PBS-T, incubated with 50 μl of 100× diluted mouse serum for 2 h at room temperature, washed three times again, and incubated with goat anti-mouse IgG conjugated to HRP (Santa Cruz Biotechnology, Dallas, TX) for 1 h at room temperature. After a final triple wash, plates were developed using the TMB Substrate Reagent Kit (Biolegend, San Diego, CA). Plates were read at 450 nm absorbance. Images were acquired on a Leica SP1-Inverted confocal microscope and analyzed using Leica imaging software.

Evaluation of disease severity

To assess clinical skin disease, blinded observers scored skin lesions on a scale of 0 (none) to 3 (severe), as previously described (23, 24). A score ≥2 (marked alopecia with or without hemorrhage) reflected mice with skin disease, as shown in Figs. 4 and 5. Histopathological scoring of H&E-stained sections of ear skin, nape skin, lung, liver, and kidney was performed by two independent reviewers trained by a dermatopathologist, using criteria described in Supplemental Table I. Tissues were fixed in 10% formalin for 24 h and then stored in 70% ethanol until processed by paraffin embedding, sectioning, and standard H&E staining techniques. Proteinuria was measured using Siemens Alustix reagent strips.

Detection of immune cell infiltrates in skin by immunohistochemistry

Five-micrometer frozen tissue sections were fixed in 4°C acetone for 10 min, blocked with 0.3% hydrogen peroxide to block endogenous peroxidase activity, rinsed with PBS, and blocked with PBS containing 1% BSA and 5% normal rabbit serum for 30 min at room temperature. Avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA) was used to block endogenous avidin and biotin. Sections were then incubated at 4°C with primary mAbs CD4 (1:20 dilution; BD Pharmingen), CD8 (1:20 dilution; eBioscience), CD68 (1:200 dilution; Serotec), and CD11c (1:20 dilution; BD Pharmingen), rinsed with PBS, and then incubated with biotinylated secondary Ab anti-rat IgG (1:100; Vector Laboratories) for 1 h at room temperature. Sections were then incubated with avidin-peroxidase complex (Vestacstain ABC kit; Vector Laboratories) for 30 min at room temperature, rinsed, and then exposed to 3,3′-diaminobenzidine for a chromogenic reaction and counterstained with hematoxylin. Negative control slide sections were stained in the same way without the primary Ab. Images were viewed on an Olympus BX51 microscope and acquired using MicroSuite (V) software. All images were prepared using Adobe Photoshop software.

Statistics

The cumulative prevalence of animals without skin lesions was analyzed using log-rank tests. For MRL-lpr mice, first an overall log-rank test was used and then pairwise log-rank tests to compare two curves individually. The Fisher’s exact test was conducted to compare the frequency of mice with skin disease at 20 wk of age. Data on anti-dsDNA Ab levels, proteinuria, histology scores, and Western blot band density were analyzed using Wilcoxon’s rank sum test. Data on immune cell subsets, cytokine-producing cells, and skin infiltrates were analyzed using two-tailed Student t test. Results of the epicutaneous tolerance assay were analyzed using two-way ANOVA for dose-response assays, and one-way ANOVA with a Tukey posttest for single-dose assays.

Results

Spontaneous loss of tolerance to skin autoantigens in mice prone to develop lupus dermatitis

MRL-lpr mice have a marked reduction in Lang-expressing DCs in their dLNs at an early age (16). Such deficiency of skin DCs in dLNs might lead to the loss of tolerance to skin. To test this, we first analyzed sera from MRL-lpr mice against skin lysate (Supplemental Fig. 1), which revealed Abs against multiple Ags, including a 110-kDa band that is consistent with the m.w. of Dsg3, a known skin autoantigen (14). To determine whether the reactivity to skin-autoantigens arises before disease onset, we cultured dLN cells from naive preclinical MRL-lpr and age-matched MRL+/- and C3H mice with Dsg3 or irrelevant foreign Ags, namely, HEL and OVA. Whereas the proliferative response of dLN cells to Con A was equivalent in the three strains of mice, dLN cells from naive preclinical MRL-lpr mice showed a significant proliferative response to Dsg3 in MRL-lpr mice as compared with age-matched control animals (Fig. 1A, 1B). There was no difference in Ki67+ cells in dLN cells to Con A was equivalent in the three strains of mice, and skin infiltrates were analyzed using three-way ANOVA for dose-response assays, and one-way ANOVA with a Tukey posttest for single-dose assays.

The Fisher’s exact test was conducted to compare the frequency of mice with skin disease at 20 wk of age. Data on anti-dsDNA Ab levels, proteinuria, histology scores, and Western blot band density were analyzed using Wilcoxon’s rank sum test. Data on immune cell subsets, cytokine-producing cells, and skin infiltrates were analyzed using two-tailed Student t test. Results of the epicutaneous tolerance assay were analyzed using two-way ANOVA for dose-response assays, and one-way ANOVA with a Tukey posttest for single-dose assays.
of tolerance to skin autoantigens in MRL-lpr mice before the onset of disease.

Tolerance to a skin autoantigen can be induced by epicutaneous applications of the autoantigen in lupus-prone mice

To directly test the role of Lang-expressing DCs in the breakdown of tolerance to skin autoantigens in MRL-lpr mice in vivo, we used an inducible tolerization model using high-dose autoantigen Dsg3 in young, predematitis mice. On days 0 and 1, high-dose Dsg3 (500 μg) or PBS mixed with equal proportions of vehicle emulsion was applied epicutaneously onto mouse ears and dried thoroughly with fan to ensure efficient application. After 7 d, dLN cells were harvested to assess a recall proliferative response to Dsg3 (Fig. 2A). Consistent with data in Fig. 1, LC-intact MRL-lpr mice applied with only vehicle emulsion had a robust in vitro proliferative response to Dsg3 autoantigen in a dose-dependent manner. In contrast, MRL-lpr mice that received the tolerizing regimen of Dsg3 administered epicutaneously on ear skin showed a significant reduction in the proliferation of dLN cells to Dsg3 (p = 0.003; Fig. 2B). The stimulation index (SI; ratio of cpm in cultures containing Dsg3 to cpm in wells containing media alone) at the highest recall concentration was 8-fold lower in Dsg3-painted animals than in control vehicle-painted animals (mean SI of 3 and 24, respectively). As a control for the in vivo immunogenicity of Dsg3, a s.c. injection of Dsg3 emulsified in CFA at the hock site induced a strong in vitro recall proliferative response (Supplemental Fig. 2). To highlight that Dsg3-induced epicutaneous tolerance is self-Ag specific, epicutaneous applications of foreign Ags (HEL or OVA) did not elicit an in vitro proliferative response in spontaneous or tolerizing conditions (Fig. 2C [HEL] and data not shown [OVA]). The epicutaneous applications of the self-Ag Dsg3 also did not affect the in vitro response to the control foreign Ag (Fig. 2E, left two columns) and vice versa (Fig. 2F). The SI in Dsg3 recall response in vehicle-painted animals was comparable between Fig. 2B and 2F (mean SI of 24 and 27, respectively). Thus, although MRL-lpr mice develop spontaneous loss of tolerance to skin autoantigens, they are still amenable to tolerance induction by the topical administration of high doses of a tissue autoantigen which induced tolerance in an Ag-specific manner in MRL-lpr mice.

LCs are necessary for the epicutaneous tolerance induction to a skin autoantigen

To evaluate the in vivo contribution of LCs in the autoantigen-specific tolerance in autoimmune background, we generated Lang-DTR MRL-lpr mice by introgressing the knock-in mutation from the stock B6 (20) onto the MRL-lpr background for >10 generations. In this model, a single i.p. injection of diphtheria toxin (DT) temporally deletes all Lang+ DCs that comprise LCs and a recently discovered subset of Lang+ DCs that inhabit the dermis (Lang+dDCs) (25–27). Recent studies accounting for both Lang+ subsets suggest that LCs and Lang+dDCs appear to elicit diverse roles in regulating immunity and tolerance (5, 18, 28). In the Lang-DTR model, Lang+dDCs begin to repopulate by day 3, but epidermal LCs remain depleted for >10 d. In contrast, MRL-lpr mice that received the tolerizing regimen of Dsg3 administered epicutaneously on ear skin showed any reduction in the recall proliferative response of dLN cells to Dsg3 when pretreated with Dsg3 as compared with animals receiving vehicle emulsion application (p = 0.59; Fig. 2D). There was no difference in proliferation between vehicle-treated
LC-intact versus LC-depleted mice (p = 0.13; Fig. 2B [vehicle] versus 2D [vehicle]). However, the recall proliferative responses of dLN cells to Dsg3 after epicutaneous tolerization to Dsg3 were different (p = 0.007) between LC-intact (Fig. 2B [Dsg3]) and LC-depleted mice (Fig. 2D [Dsg3]). As a control, the in vitro proliferation of dLN cells to an irrelevant Ag (HEL) after its epicutaneous applications in PBS-injected mice (LC-intact). (D) In vitro recall response to Dsg3 was assessed in DT-injected mice (LC-depleted). (E) Epicutaneous application of a self-Ag does not affect the in vitro responsiveness to a control foreign Ag. LC-intact or LC-depleted mice were epicutaneously (ECI) treated with Dsg3 or vehicle, and dLN cells cultured with control Ag. Mean ± SD triplicate cpm values from cultures containing HEL (500 μg/ml) are shown. (F) Epicutaneous application of a foreign Ag does not affect the in vitro responsiveness to a self-Ag. LC-intact mice were epicutaneously treated with a foreign Ag (HEL) or vehicle, and dLN cells cultured with increasing doses of Dsg3. Results are shown as the mean ± SD triplicate cpm values. (G) Representative FACS plots show frequency of Tregs in LC-intact and LC-depleted mice 7 d after the epicutaneous application of vehicle or Dsg3. Plots were gated from TCRβ+, CD4+ lymphocytes. Gate frequencies indicate mean ± SD of three independent experiments (**p < 0.01, one-way ANOVA with a Tukey posttest, n = 3 mice). Results shown are representative of three independent experiments, each using three to five mice per condition.
in increased autoantibody responses to skin Ags. To test this in vivo in a chronic autoimmune disease model, we used a long-term LC ablation strategy by administering repeated injections of DT in young Lang-DTR MRL-lpr mice and measured serum autoantibody responses. Specifically, young 6- to 8-wk-old female mice were given DT injections every 10–14 d for up to eight total injections. This injection regimen allowed for continual depletion of LCs during the time before dermatitis onset, whereas Lang+ dDCs have repopulated and are present (Fig. 3A, 3B) during this period. Results show that serum autoantibodies against homogenized whole-skin lysate were increased in LC-depleted mice (Fig. 3C) compared with LC-intact mice. Specifically, the 26-, 40-, 73-kDa, and cumulative (indicated as “All”) band densities were comparatively higher in LC-depleted mice compared with LC-intact controls ($p = 0.05$ to 0.02). Although not statistically significant, 24-, 48-, 53-, 63-, and 110-kDa (representing Dsg3) bands also showed trends of increased densities in LC-depleted mice compared with controls (Fig. 3D). In contrast, serum autoantibody levels to the systemic autoantigen dsDNA were similar between LC-depleted and LC-intact mice (Fig. 3E), thus demonstrating tissue specificity with depletion of tissue-specific DCs. Therefore, the depletion of the local DCs, the LCs in skin, led to increased autoantibodies to skin but had no impact on Abs to a systemic autoantigen.

**LC depletion in adult preclinical MRL-lpr mice accelerates dermatitis, but not other systemic manifestations of lupus**

Because LC ablation in adult preclinical MRL-lpr mice results in impaired epicutaneous tolerance induction to skin autoantigen (Fig. 2) and increased skin autoantibody production (Fig. 3), we surmised that continual LC ablation in preclinical mice will accelerate autoimmune dermatitis. Indeed, LC-depleted mice had a marked acceleration of clinically relevant lupus dermatitis lesions (score $\geq 2$) compared with their littermate controls (Fig. 4A), with a median onset of 18 versus 28 wk of age, respectively ($p < 0.001$; Fig. 4B).

To confirm that the acceleration of clinical manifestations of dermatitis in LC-ablated mice is consistent with lupus skin histopathology, we scored skin sections for acanthosis, vacuolar changes, lymphocytic infiltrates, apoptotic keratinocytes, and fibrosis, as described in Supplemental Table I. LC-ablated mice had markedly increased histology scores in both ear and nape skin (Fig. 4C, 4D). However, LC-ablated mice had no increased pathology in noncutaneous organs such as the lungs, liver, or kidneys.

**FIGURE 3.** Inducible LC ablation in adult preclinical MRL-lpr mice promotes autoantibody response to skin autoantigens but does not increase systemic autoantibody levels. (A) LC ablation schema. Lang-DTR knock-in MRL-lpr mice are given repeated DT injections (initial dose 1 mg starting at 6–8 wk of age, followed by subsequent 100 ng every 10–14 d) to continuously deplete epidermal LCs in preclinical period. White cells represent absent DCs. (B) LCs and Lang+dDCs in the skin of MRL-lpr mice after eight repeated weekly injections. FACS plots of epidermal cells were gated off large cells, and dermal cells were gated off MCHII+ cells. Gate frequencies represent mean ± SD, with dermal migratory LC (group I) and Lang+dDC (group II) gate frequencies located under their corresponding FACS plot ($n = 3–6$ mice/plot, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$). (C) Western blots were used to assess levels of serum IgG autoantibodies against whole-skin lysate in LC-intact and LC-depleted Lang-DTR (DTR) MRL-lpr mice ($n = 8–11$ mice/group). Serum samples from BALB/c and B6 mice were used as negative controls, and a pooled serum sample from inbred MRL-lpr mice was used as a reference serum between different Western blot experiments. Representative blots are shown. Numbers indicate molecular sizes in kilodaltons. Ponceau S staining (PR) of skin lysate was performed for loading controls, with the corresponding 63-kDa PR band shown. (D) Band densities of the indicated protein sizes were calculated relative to the corresponding band in the reference serum, as well as normalized against Ponceau S staining (all bands: *$p = 0.033$, 26 kDa: *$p = 0.041$, 40 kDa: *$p = 0.050$, 63 kDa: *$p = 0.061$, 73 kDa: *$p = 0.012$, $n = 8–11$ mice/group). (E) Serum anti-dsDNA autoantibodies were measured using ELISA. Results are expressed as the U/ml from individual mice. Results are pooled from three independent experiments.
compared with LC-intact mice (Fig. 4E, 4F). Thus, LC ablation exacerbates lupus dermatitis, without affecting other organ systems.

**Acceleration of dermatitis by LC ablation occurs in the autoimmune MRL background and is independent of the Faslpr mutation**

To determine whether the long-term LC depletion regimen that accelerates dermatitis in young MRL-lpr mice will induce skin autoimmune disease in nonautoimmune mouse strains, we depleted LCs in Lang-DTR B6 mice. There was no incidence of skin disease up to >1 y of follow-up in these mice (Fig. 5A). This suggests a requirement for an autoimmune genetic background or the lpr mutation for LC ablation-induced trigger in skin inflammation.

To assess whether the dermatitis exacerbating effect of LC ablation in MRL-lpr mice is because of the lpr mutation, we generated Lang-DTR B6-Fasl<sup>lpr/lpr</sup> (B6-lpr) and Lang-DTR MRL<sup>+/+</sup> mice by introgressing the knock-in mutation in these backgrounds. Whereas LC ablation did not elicit autoimmune dermatitis in B6-lpr mice up to 1 y of follow-up (Fig. 5A), LC-ablated MRL<sup>+/+</sup> mice experienced a significant acceleration of autoimmune skin disease compared with littermate LC-intact controls (Fig. 5A, 5B). LC-ablated MRL<sup>+/+</sup> mice had a median onset of skin disease at 41 wk compared with only 17% of LC-intact mice that developed skin disease at this age (p = 0.04). Thus, LC ablation accelerates dermatitis in a genetically autoimmune-prone background, regardless of the presence of the lpr mutation.

**FIGURE 4.** Inducible LC ablation in adult preclinical MRL-lpr mice accelerates and exacerbates autoimmune skin disease but does not affect disease in other organ systems. (A and B) Six- to 8-wk-old female wild-type or knock-in (DTR) MRL-lpr littermates received six to eight i.p. injections of DT (Depleted) or PBS (Intact) every 10–14 d, and animals were monitored for lupus skin disease. (A) Representative images of clinical lesions of LC-intact and LC-depleted mice taken at 20 wk of age. (B) Cumulative prevalence of animals without skin lesions (red versus green, p = 0.004 for red versus blue, p = 0.002 for red versus black, p = 0.001 for red versus green, p = 0.004 for red versus black, p = 0.002 for red versus blue). Control groups were not significantly different among themselves (p > 0.2). The median age of skin disease was 18 wk in the LC-depleted mice and 28–33 wk in the control groups. (C and D) Ear and nape skin tissues were harvested at 20 wk of age, and skin sections were stained with H&E. Representative photomicrographs are shown in (C). Scale bars, 200 μm. (D) H&E-stained skin sections were scored, as described in Supplemental Table I. Results are expressed as the mean ± SEM composite histological scores (***p < 0.001, n = 10 and 11). (E) H&E-stained sections of lung, liver, and kidney from littermate LC-intact and LC-depleted MRL-lpr mice were scored, as described in Supplemental Table I. Results are expressed as composite histology scores from individual mice (n = 9–12 mice/group). (F) Proteinuria was estimated using dipstick urinalysis, and read on a scale of trace (0.5) and 1 to 3 (10–11 mice/group).
LC depletion in adult preclinical MRL-lpr mice reduces IL-10–producing CD4+ T cells in dLNs and increases macrophage infiltration in skin

To understand the mechanism underlying accelerated skin disease in LC-depleted mice, we first asked whether the depletion of LCs chronically results in an impaired tolerance induction to a skin autoantigen. To address this, we epitopically applied high doses of Dsg3 or vehicle to MRL-lpr mice after six DT or PBS injections. Similar to results in mice with acute LC depletion after one DT injection (Fig. 2D), chronically LC-depleted MRL-lpr mice did not develop high-dose tolerance to Dsg3 and exhibited significantly increased recall proliferative responses to Dsg3 as compared with their age-matched LC-intact control littermates (data not shown). Such lack of local skin-specific immune tolerance in LC-depleted mice may account for the accelerated skin disease in autoimmune-prone mice.

To test the hypothesis that the loss of tolerance with increased skin autoimmunity in chronically LC-ablated MRL-lpr mice results from an altered homeostasis of effector versus regulatory cells in the dLNs, we continuously depleted LCs during the preclinical period in young mice (as in Fig. 3A) and examined its effect on immune cell subsets and cytokine production in the dLNs (Fig. 6A–F). There was no difference in the frequency of CD4+, CD8+, and DN T cells (Fig. 6A), or naive, memory, effector, and activated CD4+ T cells (Fig. 6B) in the dLNs of LC-ablated mice compared with LC-intact mice. There was a trend toward increased B cell numbers in the dLNs of LC-ablated mice, but this difference was not statistically significant (p = 0.06; Fig. 6C). Interestingly, in contrast with the acute LC depletion that prevented the Foxp3+ Treg expansion that is observed upon epidermal tolerance induction to Dsg3 in LC-intact mice (Fig. 2G), we found an increase in the frequency of Foxp3+ Tregs in chronically LC-depleted mice compared with LC-intact mice (p = 0.02; Fig. 6D).

To determine the effect of LC depletion on T cell functions, we stimulated dLN cells with PMA/ionomycin and analyzed intracellular cytokine production (Fig. 6E, 6F). Although LC ablation had no effect on the frequency of CD4+ T cells producing IFN-γ, IL-2, TNF-α, and IL-17, the frequency of IL-10–producing CD4+ T cells was reduced in the dLNs of LC-ablated mice compared with LC-intact controls (p = 0.04; Fig. 6F). Because Foxp3+ Tregs and CD4+Foxp3+ IL-10–producing (Tr1) cells can suppress inflammation in an IL-10–dependent manner (30), we analyzed IL-10 production in CD4+Foxp3+ versus CD4+Foxp3− T cells (Fig. 6E). There was no difference in IL-10+CD4+Foxp3+ T cells between the groups (p = 0.28, n = 4 mice/group). Thus, LC ablation reduces the frequency of IL-10–producing CD4+ T cells in dLNs of MRL-lpr mice but had no significant effect on proinflammatory cytokine-producing CD4+ T cells.

To determine which cell types may be affected locally at the site of disease, we performed immunohistochemical analysis in skin tissues from LC-ablated versus control animals. We found that LC-ablated MRL-lpr mice had a significant increase in CD68+ macrophages in the skin as compared with LC-intact controls (Fig. 6G, 6H). The number of infiltrating CD4+ and CD8+ T cells, and CD11c+ cells were not statistically different between the groups at 20 wk of age. To detect cytokines in skin, we separated epidermis and dermis using Dispass and isolated leukocytes using collagenase IV. Isolated leukocytes from the epidermis and dermis were treated with PBS or DT every 10–14 d for six to eight injections and monitored for skin lesions (p = 0.04, log-rank test, n = 16 and 19 mice, respectively).

Discussion

In this article, we report a novel protective role of LCs in lupus dermatitis. We demonstrate that LC ablation in adult MRL-lpr mice led to inability to induce tolerance to a skin autoantigen, elevated Abs to skin Ags, and accelerated dermatisis, with no effect on disease in other organs tested or on anti-dsDNA autoantibodies. The increased lupus dermatitis in LC-ablated mice was associated with reduced IL-10–producing CD4+ T cells in dLNs and increased local dermal macrophage infiltration. Thus, the tissue-resident immune cells regulate local immune tolerance without affecting systemic responses. These observations offer a potential explanation that immune regulation at the local level may underlie the heterogeneity of multiple-organ involvement in lupus.

An important function of skin migratory DCs is to acquire and transport skin Ags to the dLNs in the steady-state to induce peripheral tolerance (4, 28). The role of tolerance in tissues has been elegantly examined using ectopic expression of transgenes encoding neoantigens in combination with mice expressing transgenic TCRs...
FIGURE 6. Effect of sustained LC ablation in adult preclinical MRL-lpr mice on immune-cell phenotype in skin and dLNs. Six- to 8-wk-old Lang-DTR MRL-lpr mice were treated with vehicle or DT every 10–14 d; dLNs and skin tissue were harvested after six to eight injections and analyzed for immune cell subsets and cytokine production. (A–C) Frequency of T cell subsets in dLNs, including CD4, CD8, and DN (TCRβ⁺B220⁺CD4⁻CD8⁻) T cells (A), CD4⁺ naive, memory, and effector activated T cells (B), and B cells (C) in LC-intact versus LC-depleted mice. Error bars show SEM (n = 5–13 mice/group). (D) dLN cells were analyzed for Tregs (CD4⁺CD25⁺Foxp3⁺). Representative flow plots depict Foxp3⁺/CD25 staining in gated CD4⁺ T cells. Results are expressed on the left as the mean ± SEM of CD25⁺Foxp3⁺ Tregs (*p = 0.02, n = 6 LC-intact and 5 LC-depleted mice). (E and F) Single-cell suspensions from dLNs were stimulated with PMA/ionomycin and analyzed for intracellular cytokines in gated TCRβ⁺CD4⁺ cells. Representative flow plots of IL-10–producing cells among Tregs (Foxp3⁺ CD4⁺) and Foxp3⁻ CD4⁺ T cells are shown in (E). Results are expressed in (F) as the mean (horizontal bars) and individual data symbols of cytokine-producing Foxp3⁻ CD4⁺ T cells (*p = 0.04, n = 9 LC-intact and 10 LC-depleted mice). (Figure legend continues)
specific to the neoantigen. For example, in mice where OVA is expressed by keratinocytes, adoptively transferred OVA-specific transgenic CD8\(^+\) T cells undergo tolerance (31). It is important, however, to understand whether peripheral tolerance is impaired in autoimmune-prone conditions, such as cutaneous SLE. To investigate the role of LCs in the local skin versus systemic organ tolerance, we reasoned to use a physiological Ag system that is specific for skin and is not cross-reactive with other systemic autoantigens. Although Abs against laminin have been reported in patients with cutaneous lupus erythematosus, these autoantibodies have also been implicated in lupus nephritis (32–34). Previous studies have also shown cross-reactivity between Abs against laminin or \(\alpha\)-actinin and dsDNA (34, 35). In animals, immunizations with laminin or a passive transfer of anti-laminin Abs either had no effect or induced limited nephritis, myositis, abortion, reproductive failure, or subepidermal splits, but no lupus dermatitis (36). A previous study showed that immunizations with a skin-associated autoantigen, Dsg3, induced lupus-like skin disease in Fc\(\gamma\)RIIb\(^{-}\) mice. Genetically lupus-prone MRL-lpr mice also spontaneously develop autoantibodies to Dsg3, which correlate with skin disease severity (14). Furthermore, IgG deposits at the dermal–epidermal junction colocalized with Dsg3 in the UV B–induced NZB/W cutaneous lupus model (37). Importantly, the adoptive transfer of Dsg3-reactive CD4\(^+\) T cells into immunodeficient mice induced skin inflammation that mimics interface dermatitis that is a distinct T cell–mediated inflammation at the dermal–epidermal junction seen in cutaneous lupus, graft-versushost disease, and other diseases (15). In this model, the adoptively transferred Dsg3-reactive T cells infiltrate the stratified squamous epithelium in skin, oral mucosa, and esophagus, which express Dsg3, but not the other organs, such as liver, small and large intestine, spleen, and muscle, which do not express Dsg3 (14, 15). Taken together, the Dsg3 provides a suitable model Ag system for our studies to investigate the local immune tolerance in skin.

Using the Dsg3 model Ag system, we demonstrate that MRL-lpr mice that have reduced numbers of LCs in their dLN exhibit spontaneous loss of tolerance to Dsg3. This spontaneous breakdown in tolerance to Dsg3 observed in MRL-lpr mice can be corrected by epicutaneous applications of high doses of Dsg3, for which LCs are necessary. This role of LCs in inducing skin tolerance is in resonance with previous bone marrow chimera studies, where only host LCs expressed the MHC class II-E that could mediate T cell tolerance (41) and to skew DCs toward a tolerogenic phenotype (42). Thus, our data suggest a role of LCs in the generation of both Foxp3\(^+\) and IL-10–producing Foxp3\(^+\) T cells in dLN. It remains to be determined whether the impaired generation of Foxp3\(^+\) T cells in response to high-dose skin Ag or the reduced IL-10 Treg population in the dLN of LC-ablated MRL-lpr mice contributes to loss of tolerance to skin Ags.

The continuous in vivo ablation of LCs in adult preclinical MRL-lpr mice led to increased serum autoantibodies to endogenous skin Ags. A previous investigation that used a patch immunization method where the dorsal mouse ears were tape-stripped five times with Scotch tape before applying a filter paper wetted with Staphylococcus aureus–derived exfoliative toxin or OVA showed reduced levels of Abs against these foreign Ags in LC-depleted mice as compared with LC-intact controls (43). It is likely that the repeated tape stripping used in this study caused local trauma leading to a marked increase in the activation and migration of Ag-loaded LCs to dLN, where they facilitated Ab production. To be able to administer the Ag in a steady-state environment, we avoided the use of tape stripping and applied the Ag in an emulsion with IFA followed by blow drying of the painted emulsion to ensure the consistent application. Use of acetone/olive oil and other carriers for Ags followed by a Duoderm patch led to inconsistent results on epicutaneous tolerance (data not shown). Nevertheless, these observations suggest that LCs may be competent to support the production of Abs against foreign Ags, whereas they regulate T–B cell tolerance to self-Ags at the steady-state in lupus. These observations also suggest a plasticity in the role of LCs on immune responses, which may depend on the nature of the Ag (e.g., foreign or self), route of Ag exposure, and presence or absence of environmental cues, including trauma, pathogens, and inflammation (44).

Skin biopsies from patients with SLE show increased numbers (8) or an irregular distribution (9) of LCs in some studies, but reduced LC numbers in skin lesions by others (10, 45). An irregular distribution with clusters of large LCs is also seen in the epidermis of preclinical MRL-lpr mice (16), which may represent activated LCs that are unable to migrate to dLN. To directly investigate the role of LCs in lupus dermatitis, investigators of a previous study generated transgenic MRL-lpr mice that use the human Lang promoter to express attenuated DT subunit A. These mice have a constitutive LC ablation from early development (2). To obviate the potential compensatory pathways arising as a result of life-long LC deficiency, we chose an inducible model and an ablation schema that continuously deletes LCs only during the preclinical young adult life of MRL-lpr and MRL\(^{-}\) mice. This LC ablation regimen resulted in a profound acceleration of dermatitis, such that MRL\(^{-}\) mice that normally do not display any

\(|\text{G and H}|\) Nape skin from LC-intact and LC-depleted MRL-lpr mice was stained for the presence of macrophages (CD68), T cell subsets (CD4 and CD8), and CD11c by immunohistochemistry. Representative sections of nape skin are shown in (G). Scale bar, 100 \(\mu\)m (original magnification \(\times 20\)). (H) Infiltrative cells were quantified using HistoQuest Software (TissueGnostics) (**p = 0.001, 4 mice/group). Differences were not statistically significant for CD4, CD8, and CD11c staining. Results represent at least three independent experiments.
clinical or pathological evidence of dermatitis until 8–10 mo of age begin to exhibit severe dermatitis upon LC depletion at an earlier age (0 versus 39% in LC-intact versus LC-depleted mice, respectively, by 26 wk of age). A similar acceleration of dermatitis was seen upon LC depletion in MRL-lpr mice (33 versus 92% in LC-intact versus LC-depleted mice, respectively, by 26 wk of age). In contrast, the constitutive ablation of LCs using the Lang-DTA transgenic model did not result in increased skin disease in MRL-lpr mice (2). Thus, it would be important to identify mechanisms that might compensate for the loss of LCs from birth in the constitutive LC ablation model. Nevertheless, our data clearly demonstrate a protective role of LCs in young adult mice prone to development of lupus dermatitis.

The ability of LCs to confer tolerance to skin Ags may underlie their protective role in lupus dermatitis. Increased dermatitis in LC-depleted mice might also be due to the reduced frequency of CD4+ T cells that produce IL-10. Previous studies have shown that IL-10 deficiency can exacerbate dermatitis in MRL-lpr mice (46), and T cell–derived IL-10 has been shown to play a role in curtailing skin inflammation in contact hypersensitivity (CHS) (47, 48). IL-10 may also exert its effect in skin inflammation via its ability to induce the apoptosis and inhibit the activation of plasmacytoid DCs (49) that infiltrate the lesions of cutaneous SLE (50). IL-10 can control DC function during the effector phase of inflammatory skin reaction (42) and to inhibit macrophage functions (51). Thus, the reduced IL-10 Tregs in dLNs of LC-ablated MRL-lpr mice might contribute to the increased macrophage infiltration in skin that we observed. Although further studies are needed to understand how LCs regulate the homeostasis of IL-10–producing cells, it is possible that LCs mediate their effects at least in part via IL-10–producing CD4+ T cells.

The elucidation of immune-stimulatory versus regulatory role of LCs has been complicated by the discovery of Lang+ dDCs (25–27), prompting re-examination of prior studies using Lang as an exclusive marker for LCs alone. Recent studies accounting for both Lang subsets suggest that LCs and Lang+dDCs appear to elicit diverse and complex roles in regulating immunity and tolerance (5, 18, 28), which are not fully elucidated yet. Like epidermal LCs, Lang+dDCs that arise from bone marrow precursors to dermal DCs (49) that infiltrate the lesions of cutaneous SLE (50). IL-10 can control DC function during the effector phase of inflammatory skin reaction (42) and to inhibit macrophage functions (51). Thus, the reduced IL-10 Tregs in dLNs of LC-ablated MRL-lpr mice might contribute to the increased macrophage infiltration in skin that we observed. Although further studies are needed to understand how LCs regulate the homeostasis of IL-10–producing cells, it is possible that LCs mediate their effects at least in part via IL-10–producing CD4+ T cells.

This differential kinetics allowed us to demonstrate that the continuous depletion of LCs, with Lang+dDCs mostly present, in preclinical Lang-DTR MRL-lpr mice injected with DT every 10–14 d exacerbates dermatitis. It is possible, however, that a functional alteration in repopulating Lang+dDCs confers the observed phenotype in this model. This issue can be addressed by using Lang-DTA mice, where Lang+dDCs are continually present. Roles of LCs and Lang+dDCs have been extensively investigated in CHS. Results have been variable under different experimental conditions. Under conditions where only LCs are ablated, LCs appear to negatively regulate CHS (17, 39, 52, 53). However, depletion of LCs and Lang+dDCs both resulted in the diminished (19) or unaffected (20) CHS. Other studies suggest a compensatory role of these two skin DCs in the sensitization phase of CHS (54). Consensus is emerging that LCs may generally regulate CHS, whereas Lang+dDCs may promote CHS (5, 18, 28). Our ongoing studies will determine the role of Lang+dDCs and other heterogeneous populations of Lang+ dermal DCs that remain intact in Lang-DTR mice in the development of lupus dermatitis.

Epicutaneous tolerance with Dsg3 led to increased CD4+CD25+ Foxp3+ Tregs in LC-intact but not in acutely LC-depleted mice, indicating a requirement of LCs in generating Tregs in response to high-dose epicutaneous Ag. These data are consistent with previous reports indicating a role of LCs in the induction of Tregs (39, 44). However, we were surprised to find an increase in the frequency of Tregs in the dLNs of chronically LC-depleted mice compared with LC-intact mice. The reason for this discrepancy in the role of LCs in Treg generation is unclear. Recent studies using the OVA transgenic model suggest that migratory Lang+dDCs, not LCs, mediated tolerance in CD4+ T cells, where adaptively transferred naïve OVA-specific transgenic CD4+ T cells were converted into Foxp3+ Tregs (28). Thus, it is possible to speculate that Lang+dDCs that remain mostly intact and repopulate rather quickly after multiple DT injections contribute to the accumulation of Tregs in the dLNs of chronically LC-ablated MRL-lpr mice. However, despite the presence of Lang+dDCs and increased Tregs, the skin autoimmunity is accelerated in chronically LC-ablated MRL-lpr mice. It is also possible that Lang+dDCs that repopulate the dermis after repeated LC ablations are inefficient in inducing functionally competent Foxp3+ Tregs, especially under autoimmune conditions. In fact, Tregs are functionally impaired in MRL-lpr mice (55). Thus, an increase in Tregs might still not be commensurate with a profound increase in activated T cells in these mice.

Our observations offer a potential explanation that immune regulation at the local level may underlie the heterogeneity of multiple-organ involvement in lupus. There are other examples of differential regulation of inflammation in different organs in a systemic autoimmune disease. For example, MRL-lpr mice rendered deficient in β2-microglobulin (56), CD1d (23), and CD40L (57) exhibit increased lupus dermatitis, but less or unaffected renal disease. In another example, nephritis is accelerated by multiple pregnancies in MRL-lpr mice, whereas skin disease is prevented (58), presumably because of opposite effects of IL-10 in skin versus kidneys. Higher renal IL-10 levels were associated with more nephritis, whereas higher IL-10 levels in skin were associated with lower skin disease. The latter observation is akin to our findings showing reduced IL-10–producing CD4+ T cells in dLNs of mice with increased dermatitis. We show a specific role for LCs in the epidermis in young adult mice to regulate tolerance in skin, but not other organs. The localized worsening of skin disease with epidermal LC ablation may be due to the unique location of this DC subset.

This study highlights the role of local immune tissue regulation as a key regulatory layer for tolerance induction in systemic autoimmune disorders. These data have implications for therapy at the local organ level, providing a target for therapy to correct a local breakdown in tolerance rather than attempting correction at a systemic level. This has potential advantages in patients with persistent cutaneous lupus when systemic symptoms are absent or have subsided. Further studies should continue to shed light on predictors of organ specificity in the context of systemic multorgan autoimmune diseases.

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We thank Dr. Bernard Malissen (Marseille, France) for providing Lang-DTR knock-in B6 mice, Miguel-Angel Gutierrez for performing Limulus amebocyte lysate assay and thymidine uptake assays, Dora Acuna for immunohistochemical staining of skin section, Dr. Robert Modlin for reading the manuscript, TissueGnostics for help with analyses of stained skin sections, Julia Pinkhasov for helpful discussions, and David Elashoff, Department of Medicine Biostatistics Core, for statistical analysis.
Disclosures
The authors have no financial conflicts of interest.

References
Supplemental Fig. S1. MRL-lpr mice exhibit humoral immune response against skin antigen desmoglein-3 (Dsg3). Western blot was used to detect reactivity of sera from 12-13 wk-old MRL-lpr mice against whole skin lysate (left lane) or Dsg3 (lanes labeled 1 and 2) or no antigen (-). Recombinant Dsg3 was prepared as described in Materials and Methods. MRL-lpr sera exhibited multiple reactivities against skin lysate, including a ~110kDa band that also represented reactivity against Dsg3. Thus, MRL-lpr mice spontaneously develop autoantibodies against various skin autoantigens including Dsg3.
Supplemental Fig. S2. Subcutaneous immunization with an emulsion containing Dsg3 in complete Freund’s adjuvant (CFA) elicits a strong in vitro recall proliferative response in MRL-lpr mice. 12-wk-old MRL-lpr mice were immunized with 100 µg of recombinant Dsg3 or vehicle-containing emulsion with CFA in each hock. Draining lymph nodes were harvested 7-9 days later, and single cell suspension cultured without or with 50 µg/ml of Dsg3 or BSA for 48 hours. Lymph node cell proliferation was assessed using $[^{3}H]$-thymidine incorporation. Results are expressed as the mean ± SEM stimulation index (SI) that was calculated by dividing cpm values in cultures containing stimulants by the background cpm (~300) in culture wells containing medium alone (**p=0.0002; n= 4 mice/group).
Supplemental Fig. S3. Kinetics of langerin\(^+\) cell depletion in epidermis and dermis of langerin-DTR-eGFP knock-in MRL-lpr mice. Fresh epidermal (A) and dermal (B) sheets from dorsal ear-halves of Lang-DTR eGFP MRL-lpr mice were stained for langerin and MHCII on indicated days after a single 1\(\mu\)g injection of DT. LCs and Lang\(^d\)dDCs are depleted after 24hrs, but eLC begin to replete at day 14, while Lang\(^d\)dDCs begin to replete by day 3-4. Scale bar indicates 50\(\mu\)m.
Supplemental Table S1. Histology Scoring Criteria. Histological examination and scoring were performed on H&E stained sections of nape and ear skin from LC-intact and LC-depleted MRL-lpr mice (see images in Fig. 4C) with B6 mice as a control (data not shown). A composite histological score was computed by summing the sub-scores, with a maximum score of 18 for nape skin (Skin Scoring) and 14 for ears (Ear Scoring).

Scores for kidney histology were determined by averaging the sub-scores for each category [glomerular activity score (GAS), tubulointerstitial activity score (TIAS), and vascular activity score (VAS)] and then summing each category to generate the composite score (maximum score of 9).

For lung and liver histology, the indicated regions were given an inflammation index (II) and distribution index (DI) on a scale of 0-4 (0-none, 1-minimal, 2-mild, 3-moderate, 4-marked), as modified from (1). Sub-scores for each region were calculated by multiplying II and DI, and then the sub-scores were summed to generate the composite score for each tissue (maximum score of 48).

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| Tubulointerstitial Activity Score (TIAS) | Scale |
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| Tubular Flatting         | 0-3   |
| Interstitial Inflammation| 0-3   |

| Vascular Lesion Score (VLS) | Scale |
| Vascuittis                  | 0-3   |
| Perivascular Infiltration   | 0-3   |

Composite Score: GAS/6 + TIAS/3 + VLS/2
Maximum Score for kidney is 9

<table>
<thead>
<tr>
<th>Lung &amp; Liver</th>
<th>Inflammation Index (II)</th>
<th>Distribution Index (DI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung Region</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peri-vascular</td>
<td>0-4</td>
<td>0-4</td>
</tr>
<tr>
<td>Alveolar</td>
<td>0-4</td>
<td>0-4</td>
</tr>
<tr>
<td>Peri-bronchiolar</td>
<td>0-4</td>
<td>0-4</td>
</tr>
<tr>
<td>Peri-bronchial</td>
<td>0-4</td>
<td>0-4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Liver Region</th>
<th>Inflammation Index (II)</th>
<th>Distribution Index (DI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centrilobular</td>
<td>0-4</td>
<td>0-4</td>
</tr>
<tr>
<td>Periportal</td>
<td>0-4</td>
<td>0-4</td>
</tr>
<tr>
<td>Random</td>
<td>0-4</td>
<td>0-4</td>
</tr>
</tbody>
</table>

Composite Score: II x DI for each region, then add all regions
Maximum Score for lung and liver is 48