IL-31–generating network in atopic dermatitis comprising macrophages, basophils, thymic stromal lymphopoietin, and periostin

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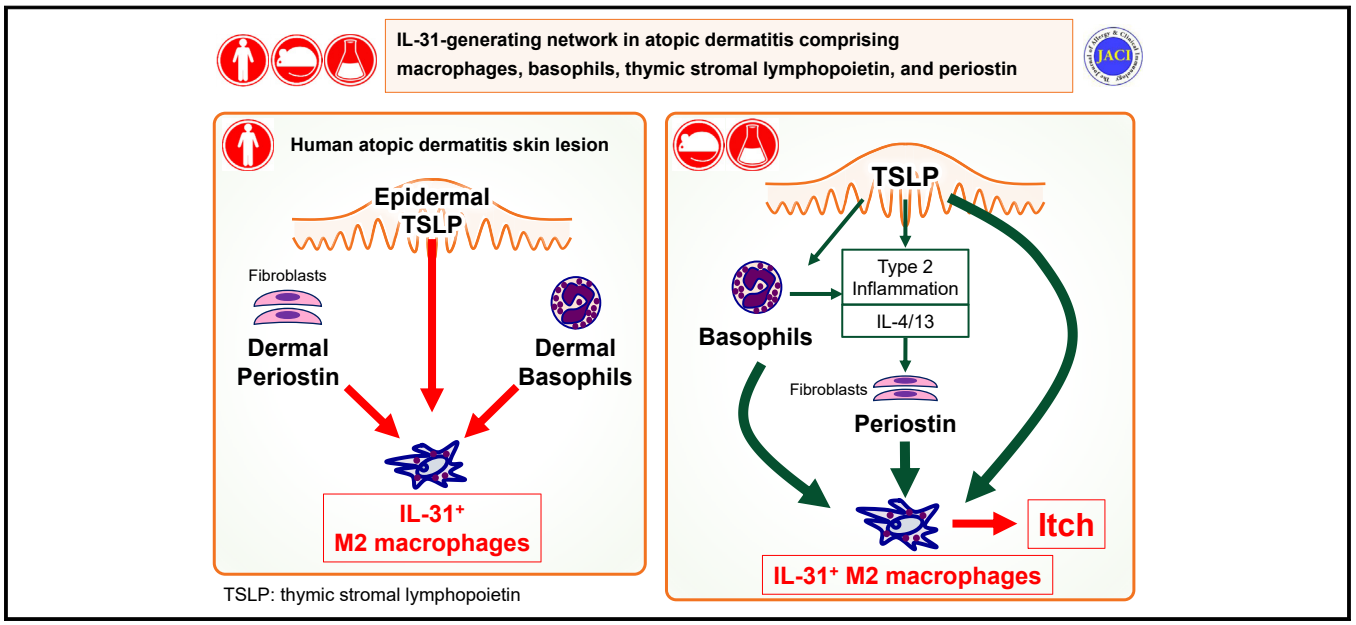
GRAPHICAL ABSTRACT



Background: IL-31 is a type 2 cytokine involved in the itch sensation in atopic dermatitis (AD). The cellular origins of IL-31 are generally considered to be T_H2 cells. Macrophages have also been implicated as cellular sources of IL-31.

Objective: We sought to determine the expression of IL-31 by macrophages and to elucidate the productive mechanisms and contributions to itch in AD skin lesions.

Methods: Expression of IL-31 by macrophages, expressions of thymic stromal lymphopoietin (TSLP) and periostin, and presence of infiltrating basophils in human AD lesions were examined through immunofluorescent staining, and correlations were assessed. Furthermore, mechanisms of inducing IL-31–expressing macrophages were analyzed in an MC903-induced murine model for AD *in vivo* and in mouse peritoneal macrophages *ex vivo*.

Results: A significant population of IL-31$^+$ cells in human AD lesions was that of CD68$^+$ cells expressing CD163, an M2 macrophage marker. The number of IL-31$^+$/CD68$^+$ cells correlated with epidermal TSLP, dermal periostin, and the number of dermal-infiltrating basophils. In the MC903-induced murine AD model, significant scratching behaviors with enhanced expressions of TSLP and periostin were observed, accompanied by massive infiltration of basophils and IL-31$^+$/MOMA-2$^+$/Arg-1$^+$ cells. Blockade of IL-31 signaling with anti–IL-31RA antibody or direct depletion of macrophages by clodronate resulted in attenuation of scratching behaviors. To

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This study was partially supported by Japan Society for the Promotion of Science (JSPS) KAKENHI Grant-in-Aid for Scientific Research (C) (grant numbers 17K16328, 19K08743, and 22K08395 to T.H. and 19K08805 and 22K08444 to T.S.).

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

Received for publication July 18, 2022; revised October 22, 2022; accepted for publication November 11, 2022.

Available online November 19, 2022.

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0091-6749/$36.00

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https://doi.org/10.1016/j.jaci.2022.11.009

effectively reduce lesional IL-31$^+$ macrophages and itch, basophil depletion was essential in combination with TSLP- and periostin-signal blocking. Murine peritoneal macrophages produced IL-31 when stimulated with TSLP, periostin, and basophils.

Conclusions: A network comprising IL-31–expressing macrophages, TSLP, periostin, and basophils plays a significant role in AD itch. (J Allergy Clin Immunol 2023;151:737-46.)

Key words: *Atopic dermatitis, basophil, IL-31, itch, macrophage, periostin, thymic stromal lymphopoietin*

Chronic itch is one of the most characteristic symptoms of atopic dermatitis (AD).[1] The itch in AD not only impairs quality of life for patients but also induces persistent scratching, which further exacerbates barrier dysfunction and type 2 inflammation, forming an "itch-scratch cycle."[2] Thus, one of the primary aims of treatment in AD is to reduce itch, but this presents a significant challenge.[3]

In the last decade, IL-31, a type 2–associated cytokine, has gained attention as a representative pruritogen in AD.[4] IL-31 exerts its pruritogenic function via the receptor complex comprising IL-31RA and oncostatin M receptor beta.[5,6] IL-31 evokes itch sensation by stimulating peripheral sensory nerve fibers in the skin[7,8] and/or by promoting other cellular types (eg, epidermal keratinocytes) to secrete other pruritogens.[9,10] In patients with AD, lesional expression of IL-31 is significantly enhanced compared with that in healthy individuals.[8,11] Nemolizumab, a humanized mAb against human IL-31RA, inhibits IL-31 signaling and significantly reduces numerical rating scale scores for itch in patients with AD.[12] Furthermore, intradermal injection of IL-31 provokes long-lasting itch in such patients.[13] Collectively, IL-31 appears to represent a major pruritogenic player contributing to itch in AD.

The major cellular source of IL-31 has been considered to be activated T_H2 cells.[6,11] However, other cellular types are also reportedly capable of secreting IL-31, including mast cells,[14] eosinophils,[15,16] and basophils.[17] Our group has demonstrated that macrophages skewed toward an M2 phenotype produce IL-31 in response to thymic stromal lymphopoietin (TSLP) and periostin, both of which are type 2 inflammation–associated proteins.[18]

Macrophages are generally divided into 2 subgroups: classically activated proinflammatory M1 and alternatively activated anti-inflammatory M2.[19] The development of M1 macrophages is promoted by infection/injury. They secrete proinflammatory cytokines including IL-1β, IL-6, and IL-12. M2 macrophages are generally considered to differentiate at the wounds during the healing stage. M2 macrophages produce anti-inflammatory cytokines including IL-10, TGF-β1, and vascular endothelial growth factor, contributing to tissue remodeling.[19] Recent findings also demonstrated the involvement of M2 macrophages in postinflammatory hyperpigmentation[20] and tumorigenesis.[21] Furthermore, M2 macrophages reportedly infiltrate into the lesional skin of human AD.[22] A murine AD model shows lesional infiltrates of M2-like macrophages, which promote efferocytosis, remodeling, and tissue repair.[23] However, the involvement of macrophages in AD itch is still to be defined.

Basophils represent less than 1% of peripheral blood leukocytes. Several lines of evidence have revealed that basophils play an important role in allergic reactions and/or immune responses.[24] Basophils initiate IgE-mediated, chronic, allergic skin inflammation.[25] These cells also elicit IgG-mediated anaphylaxis, but not IgE-mediated anaphylaxis.[26] Basophils can mediate protective immunity against helminths and ticks.[27] Basophils are regarded as major players in type 2 allergy, secreting a significant amount of IL-4 via IgE-dependent and/or IgE-independent manners, including TSLP-dependent activation.[28] Basophils also serve as antigen-presenting cells through the newly discovered mechanism of trogocytosis, further promoting T_H2 differentiation from naive helper T cells.[29] Skin lesions in murine models of AD and human AD feature dermal basophilic infiltrate.[28,30-32] In addition, basophils have recently been implicated as one of the players involved in provoking acute itch flares during skin inflammation through the release of lipid mediators.[31]

In this study, we hypothesized that M2 macrophages could be one of the major cellular sources of IL-31. To this end, IL-31 expression by M2 macrophages and its association with expressions of TSLP and periostin and dermal basophils in human AD lesions were assessed. In addition, using a murine model of AD, we attempted to elucidate the details of a complex network comprising M2 macrophages, TSLP, periostin, and basophils.

Abbreviations used
AD: Atopic dermatitis
DT: Diphtheria toxin
TSLP: Thymic stromal lymphopoietin

METHODS

Samples

Lesional skin-biopsy samples and clinical data from patients with AD and control skin samples from healthy individuals were collected at Tokyo Medical and Dental University Hospital. Clinical symptoms and laboratory findings from the enrolled patients met the criteria for AD proposed by the Japanese Dermatological Association.[33] This study was approved by the institutional review boards at Tokyo Medical and Dental University (approval number M2019-063) and National Defense Medical College (approval number 4376).

Mice

C57BL/6 mice were purchased from Sankyo Lab Service (Tokyo, Japan) and Japan SLC (Hamamatsu, Japan). Mast cell protease 8-diphtheria toxin receptor (*Mcpt8*^{DTR}) mice (C57BL/6 background) were generated as described previously.[27] Mice were maintained under specific pathogen-free conditions in our animal facility. All animal experiments were approved by the institutional animal care and use committees of Tokyo Medical and Dental University (approval numbers A2018-299A, A2018-199A, A2018-298A, and A2018-237A) and the National Defense Medical College (approval numbers 17062 and 21043).

Antibodies

For the antibodies used in this study, please see this article's Methods section in the Online Repository at www.jacionline.org.

MC903-induced murine model of AD

MC903 (calcipotriol; 2 nmol/25 μL ethanol; MedChem Express, Monmouth Junction, NJ) was applied to the earlobes once per day for 9 consecutive days.[34,35] On day 10, scratching behavior was recorded as described previously,[36] and then the animals were euthanized and ear samples were collected.

Blockade of signaling pathways for IL-31, TSLP, and periostin

For IL-31–signaling inhibition, anti–IL-31RA antibody (AF2107, 4 μg/ear; R&D Systems, Minneapolis, Minn) or control goat IgG was intradermally administered into earlobes on day 8. For blockade of TSLP and periostin signaling, anti-TSLP antibody (104108, 5 μg/ear; BioLegend, San Diego, Calif) and anti–periostin receptor CD51 antibody (MAB555, 5 μg/ear; R&D Systems) or control rat IgG was intradermally injected into earlobes on day 7.

Basophil and macrophage depletion

Mcpt8^{DTR} mice were intraperitoneally administered diphtheria toxin (DT) (750 ng/20 g body weight; Sigma-Aldrich, St Louis, Mo) on days 0, 4, and 8.[27,35] Basophil depletion was confirmed on day 10 through immunofluorescence staining using an antimurine mMCP-8 antibody (TUG8; BioLegend) that can specifically react with murine basophils, but not with mast cells.[37] Macrophages were depleted by lesional intradermal injection of clodronate liposome (Macrokiller V100, 20 μL/ear; Cosmo Bio, Tokyo, Japan) on day 8.

Immunofluorescence staining

Formalin-fixed paraffin-embedded samples (thickness, 5 μm) were deparaffinized and pretreated with Dako target retrieval solution (Dako, Glostrup, Denmark) at 60°C overnight. For TSLP and periostin staining in murine samples, formalin-fixed frozen sections (thickness, 20 μm) were used. Sections were treated with protein-blocking solution containing 0.25% casein (Dako) for 10 minutes at room temperature. Sections were then incubated with indicated primary antibodies at 4°C overnight, followed by reaction with Alexa Fluor 488– or 568–conjugated secondary antibodies. Samples were mounted with Fluoroshield with 4'-6-diamidino-2-phenylindole (GeneTex, Irvine, Calif).

Quantification

Photomicrographs were captured using a BZ-X710 microscope (Keyence, Osaka, Japan). Three different sections from each subject were analyzed. Epidermal expression of targeted proteins was measured as fluorescence intensity in arbitrary units, normalized by area and background fluorescence using Image J software (National Institutes of Health, Bethesda, Md). The number of cells indicated was manually quantified.[38]

Measurement of cytokines and chemokines

Punch samples of skin, with a diameter of 8 mm, were homogenized with 200 μL of PBS with 1% proteinase inhibitor cocktail (Sigma-Aldrich) and then centrifuged at 10,000*g* for 10 minutes. TSLP and periostin levels in the supernatants of homogenates were quantified by sandwich ELISA. ELISA kits for murine TSLP and periostin were purchased from R&D Systems.

Preparation of the CD49b$^+$ basophil-enriched cell fraction from splenocytes

Splenocytes were collected from 6- to 12-week-old naive C57BL/c mice, and CD49b$^+$ cells were isolated with biotinylated anti-CD49b antibody and streptavidin microbeads (Miltenyi Biotec, Auburn, Calif).[35] The CD49b$^+$ fraction was preincubated with recombinant TSLP (400 ng/mL; eBioscience, San Diego, Calif) before coculture with peritoneal macrophages.

Preparation of murine peritoneal macrophages

Peritoneal cells were collected from C57BL/6 mice and seeded at a concentration of 10×10^6/mL in plates in RPMI-1640 complete medium supplemented with 10% FBS and 100 IU/mL penicillin-streptomycin. Cells were incubated for 2 hours at 37°C and 5% CO_2, and then nonadherent cells were washed out.[18,38] The remaining adherent cells (>80% of macrophages) were incubated with recombinant periostin (20 ng/mL; eBioscience) plus recombinant TSLP (20 ng/mL), with or without a CD49b$^+$ basophil-enriched splenocyte cell fraction (4×10^6 cells with 15×10^6 macrophages in 1.5 mL of medium). After 24 hours, cells were subjected to flow-cytometric analysis.

Flow-cytometric analysis

Single-cell suspensions from skin samples were prepared by treating excised earlobes with collagenase type III (125 U/mL; Worthington Biochemical, Lakewood, NJ) in RPMI-1640 complete medium, followed by red blood cell lysis. Single-cell suspensions of cultured peritoneal macrophages were obtained using cell scrapers after fixation with an Intracellular Fix & Perm set (eBioscience). These suspensions were pretreated with anti-CD16/32 antibody (BioLegend) on ice for 15 minutes, followed by incubation with the indicated combinations of antibodies. Analysis was performed using a FACSCalibur cell analyzer (BD Biosciences, San Jose, Calif).

Real-time PCR

Total RNA was extracted using ISOGEN II (Nippon Gene Co., Tokyo, Japan) and reverse-transcribed with SuperScript IV VILO Master Mix (Thermo Fisher Scientific, Waltham, Mass). Quantitative RT-PCR was then performed by real-time monitoring of the increase in fluorescence of SYBR Green dye (PowerUp SYBR Green Master; MixApplied Biosystems, Austin, Tex) using an AriaMx Real-Time PCR system (Agilent Technologies Japan, Tokyo, Japan). For the primers used for PCR, please see this article's Methods section in the Online Repository at www.jacionline.org.

Statistical analysis

All data are reported as mean + SEM unless otherwise indicated. The Mann-Whitney *U* test was used to compare differences between groups. For comparisons of mRNA expressions, 2-tailed unpaired *t* tests were used. For detecting correlations, we calculated the Spearman rank correlation coefficient (*r*) using EZR statistical software (available at http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmed.html).[39] Statistical significance was set at the level of *P* less than .05.

RESULTS

Infiltration of IL-31$^+$/CD68$^+$ M2 macrophages and correlations with epidermal TSLP, dermal periostin, and basophils in human AD skin lesions

To begin to assess M2 macrophages as cellular sources of IL-31, dermal IL-31–expressing cells in human AD skin lesions were immunohistochemically observed. We collected 30 lesional skin-biopsy samples from 27 patients with AD (age, 23-79 years; 23 men, 4 women) and 6 samples from 6 healthy individuals without AD (age, 25-62 years; 4 men, 2 women). All of the enrolled patients were treated with topical corticosteroids/calcineurin inhibitors, emollients/moisturizers, and oral antihistamines, but no systemic therapies were used for their treatments. Severity scores for itch and skin inflammation and serum samples were not available at our repository.

A significant number of dermal-infiltrating IL-31$^+$ cells and CD68$^+$ macrophages were detected in lesional skin compared with healthy controls (Mann-Whitney *U* test, $P < .005$ each) (Fig 1, *A* and *B*). In addition, a certain population of IL-31$^+$ cells (mean ± SD, 38.1% ± 22.0%; median, 33.3%; range, 5.3%-79.3%) expressed CD68, indicating that these cells were macrophages (Fig 1, *A* and *B*). Macrophages are generally divided into M1 and M2 phenotypes.[19] Most CD68$^+$ macrophages in lesional skin expressed CD163, an M2 macrophage marker (Fig 1, *C*). This indicated that IL-31$^+$/CD68$^+$ macrophages showed an M2 phenotype.

Cellular sources of TSLP and periostin in AD are epidermal keratinocytes and dermal fibroblasts, respectively.[18,40-42] Epidermal expression of TSLP and dermal deposition of periostin were detected in skin lesions, and expression levels correlated

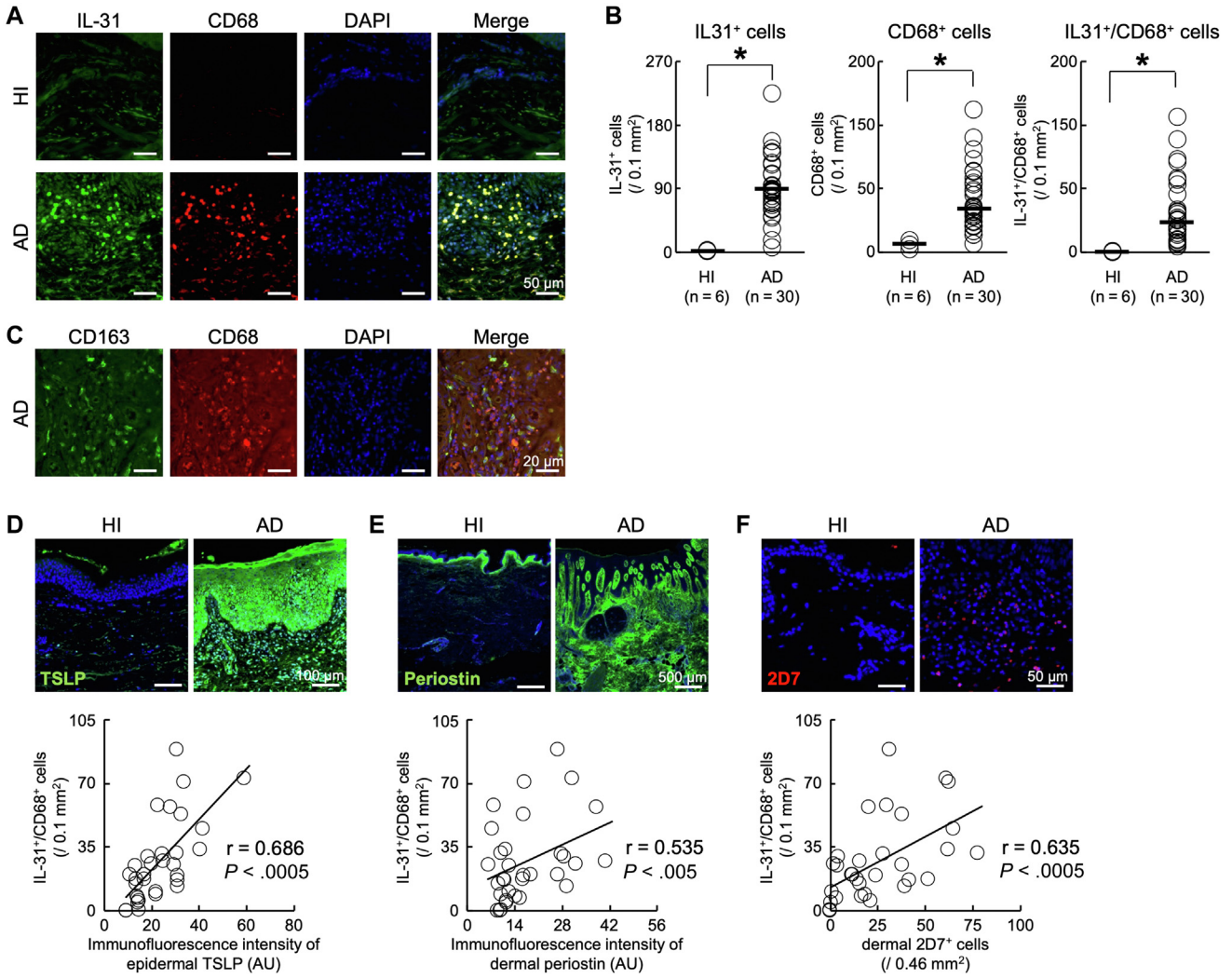

FIG 1. IL-31–expressing macrophages, epidermal TSLP, dermal periostin, and dermal-infiltrating basophils in human AD. **A** and **B,** Representative images of AD lesion and normal skin from healthy individuals (Fig 1, *A*) with quantification of staining (Fig 1, *B*). A significant number of IL-31$^+$ cells (*green*), CD68$^+$ macrophages (*red*), and IL-31$^+$/CD68$^+$ cells (*merged in yellow*) are detected in AD lesions. Horizontal bars indicate the median. **C,** CD68$^+$ cells (*red*) mostly express CD163 (*green*), an M2 macrophage marker. **D-F,** Immunofluorescence intensities of epidermal TSLP (*green*) (Fig 1, *D*), dermal periostin (*green*) (Fig 1, *E*), and the number of dermal-infiltrating basophils (*red*) (Fig 1, *F*). *AU,* Arbitrary units; *DAPI,* 4′-6-diamidino-2-phenylindole; *HI,* healthy individuals; *r,* Spearman rank correlation coefficient. Blue indicates nuclei. *P < .05, Mann-Whitney *U* test.

significantly with numbers of IL-31$^+$/CD68$^+$ macrophages (Spearman correlation, $r = 0.686$ and $P < .0005$ and $r = 0.535$ and $P < .005$, respectively) as assessed by immunofluorescence intensities, reflecting the amounts of the targeted proteins.[43] The number of 2D7$^+$ dermal-infiltrating basophils also correlated with the number of IL-31$^+$/CD68$^+$ macrophages (Spearman correlation, $r = 0.635$ and $P < .0005$) (Fig 1, *D-F*). TSLP, periostin, and basophils thus appear closely related to IL-31 expression by M2 macrophages in human AD.

M2 macrophages generate IL-31 and contribute to itch in the MC903-induced murine model of AD

We next assessed whether M2 macrophages contribute to itch responses via generating IL-31 in a mouse model of AD induced by MC903, a vitamin D3 analogue.[31,34,44] Topical application of MC903 on earlobes induced skin inflammation represented by overexpression of epidermal TSLP and dermal periostin, massive infiltration of TUG8$^+$ basophils (Fig 2, *A-E*), and increased mRNA expression of *IL-4* and *IL-13* (see Fig E1 in this article's Online Repository at www.jacionline.org), mirroring human AD skin lesions. These mice showed significant scratching behaviors along with lesional overexpression of *IL-31* mRNA (Fig 3, *A* and *B*). The contribution of IL-31 to itch as a major pruritogen was confirmed by the finding that local administration of an IL-31RA–antagonizing antibody significantly attenuated scratching behaviors (Mann-Whitney *U* test, $P < .05$) (Fig 3, *C*). In addition, IL-31 was found to be principally produced by macrophages, as most IL-31$^+$ cells coexpressed the monocyte/macrophage-specific marker MOMA-2 (Fig 3, *D* and *E*). Flow-cytometric analysis also revealed that more than 60% of lesional IL-31$^+$ cells expressed MOMA-2, and IL-31$^+$/MOMA-2$^+$ cells mostly

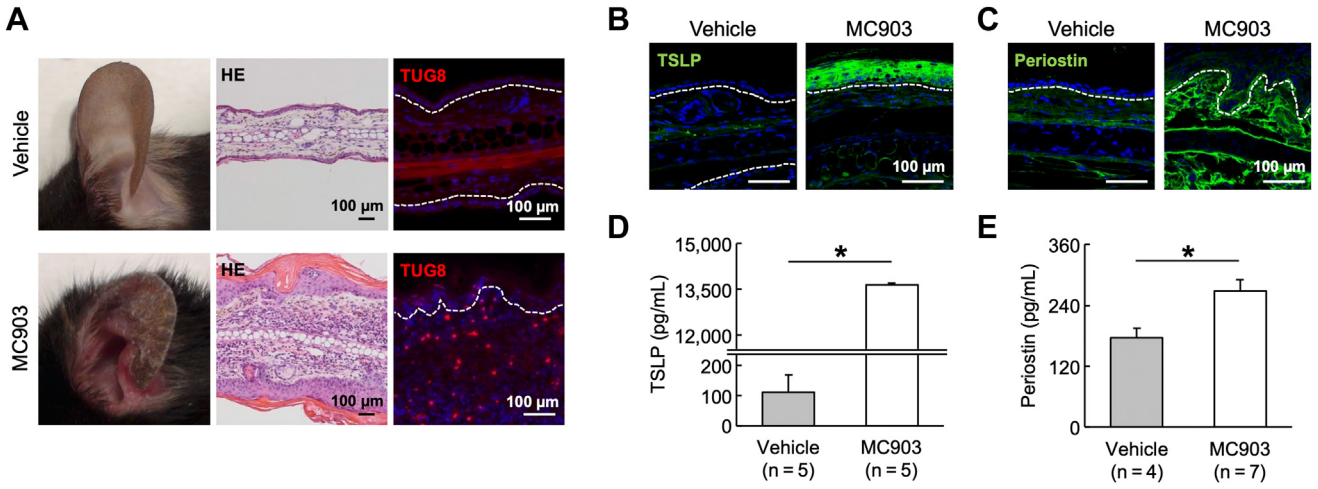

FIG 2. Basophils, TSLP, and periostin in the MC903-induced murine model of AD. **A,** Macroscopic and histopathological findings. Treatment of the earlobes with MC903 induces AD-like skin inflammation (*left panel*) with histopathological hyperkeratosis and acanthosis, accompanied by massive infiltrates in the dermis (*middle panel*) and TUG8$^+$ basophils (*red*) (*right panel*). **B** and **C,** Immunofluorescence staining. MC903-treated mice show significant positive staining reactions for TSLP (*green*) at the epidermis (Fig 2, *B*) and periostin (*green*) in the dermis (Fig 2, *C*). **D** and **E,** Lesional protein production measured with ELISA. Blue indicates nuclei. White dotted lines indicate the dermo-epidermal junctions. Values represent mean + SEM. *$P < .05$, Mann-Whitney *U* test.

expressed the M2 macrophage marker Arg-1 (Fig 3, *F*). Moreover, compared with levels in control mice, lesional mRNA expressions of *Iba1*, a macrophage-associated protein, and *Arg-1* were significantly increased (*t* test, $P < .05$ each), whereas expression of the M1 macrophage marker *iNOS* increased minimally (*t* test, $P < .05$) (Fig 3, *G*). To determine whether macrophages critically contribute to itch during inflammation, clodronate liposome was administered into inflamed skin as a macrophage-depleting agent. Local administration of clodronate liposome significantly decreased the number of IL-31$^+$ macrophages (Mann-Whitney *U* test, $P < .05$ vs free liposome-treated control mice) (Fig 3, *H* and *I*). As expected, an apparent attenuation of scratching behaviors was observed (Mann-Whitney *U* test, $P < .05$ vs control mice) (Fig 3, *J*). Of note, macrophage depletion caused an increase in mRNA expressions of *IL-4*, *IL-13*, and *Mcpt8*, a basophil specific protease, and the number of dermal TUG8$^+$ basophils (see Fig E2 in this article's Online Repository at www.jacionline.org), despite the ameliorated itch responses. Enhanced expression of TSLP and periostin, massive infiltration of basophils, and induction of IL-31$^+$ M2 macrophages in AD-like inflammation were further confirmed in another chronic itch model of dermatitis induced by repeated application of an extract from house dust mite *Dermatophagoides pteronyssinus* feces (see Fig E3 in this article's Online Repository at www.jacionline.org).

Blockade of TSLP and periostin signals is insufficient for reducing IL-31–expressing macrophages *in vivo*

To determine the functional roles of TSLP and periostin in the generation of IL-31 by macrophages *in vivo*, a TSLP-neutralizing antibody and an antagonizing antibody against CD51 (integrin αV), a periostin receptor, were administered intradermally into the earlobes of mice with MC903-induced AD-like inflammation. Unexpectedly, blocking of both TSLP and periostin signals did not decrease numbers of IL-31$^+$/MOMA-2$^+$ cells (Mann-Whitney *U* test, $P = .60$), whereas lesional expression of *IL-31* mRNA tended to be lowered (*t* test, $P = .26$) (Fig 4, *A-C*). Expressions of *Arg-1* and *iNOS* mRNA also did not significantly change (Fig 4, *C*). Nevertheless, scratching bouts were significantly reduced (Mann-Whitney *U* test, $P < .05$; Fig 4, *D*), potentially because of direct blockade of the pruritogenic functions of TSLP and periostin. Infiltrating basophils were not abolished by the blockade of TSLP and periostin signals (see Fig E4 in this article's Online Repository at www.jacionline.org).

Basophils are indispensable for promoting IL-31 generation by macrophages and induction of itch responses *in vivo*

Given the findings that blocking of TSLP and periostin signals did not affect IL-31$^+$ macrophage generation and infiltration, we hypothesized that basophils may have crucial functions in IL-31 induction from M2 macrophages. To test this hypothesis, we performed *in vivo* experiments with *Mcpt8*^{DTR} mice. Basophils of *Mcpt8*^{DTR} mice specifically express DT receptors, and DT treatment selectively and inducibly abolishes basophils in *Mcpt8*^{DTR} mice (see Fig E5 in this article's Online Repository at www.jacionline.org).[27] Basophil depletion in conjunction with neutralization of TSLP and blockade of periostin signaling resulted in a significant reduction in the number of IL-31$^+$/MOMA-2$^+$ cells (Mann-Whitney *U* test, $P < .05$) (Fig 5, *A* and *B*) and lesional *IL-31* mRNA expression (*t* test, $P < .05$) (Fig 5, *C*), whereas DT treatment alone resulted in only moderate reductions to IL-31$^+$/MOMA-2$^+$ cells and *IL-31* mRNA expression (data not shown). These were accompanied by attenuation of scratching bouts compared with vehicle-treated and control IgG-administered mice (Mann-Whitney *U* test, $P < .05$) (Fig 5, *D*). In addition, a significant decrease in lesional *Arg-1* mRNA expression and a trend toward increasing *iNOS* were observed (*t* test, $P < .05$ and $P =$

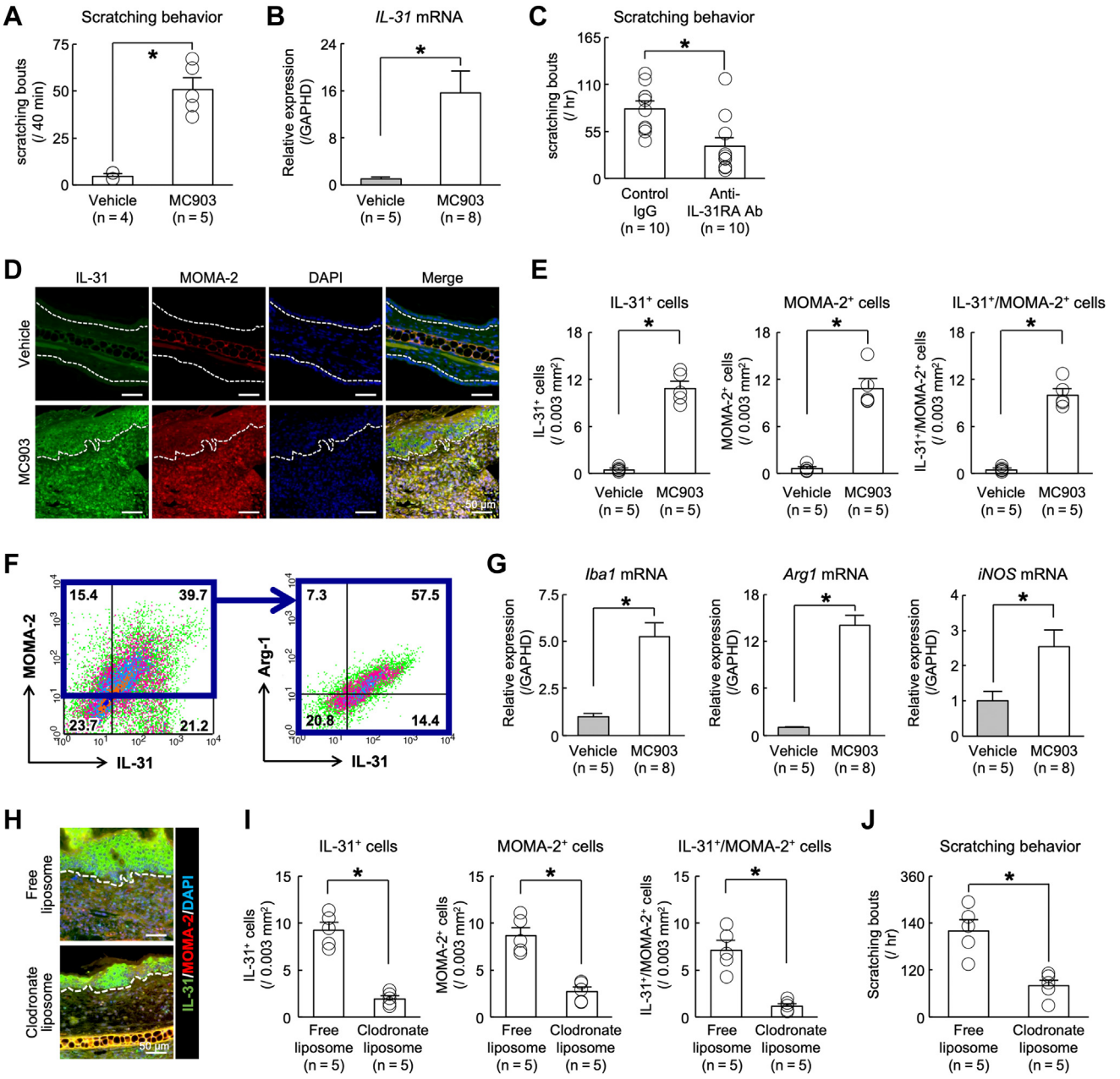

FIG 3. IL-31 is largely produced by M2-skewed macrophages and is involved in scratching behaviors in the MC903-induced murine model for AD. **A,** MC903-treated mice show significant scratching behaviors compared with vehicle-treated control mice. **B,** Lesional mRNA expression of *IL-31*. **C,** Blockade of IL-31 signaling with anti–IL-31RA Ab significantly attenuates scratching behavior. **D** and **E,** Immunofluorescence imaging with quantification. A significant number of infiltrating cells express both IL-31 (*green*) and MOMA-2 (*red*) in MC903-treated mice. **F,** Flow-cytometric analysis reveals that most IL-31$^+$ cells (>60%) are MOMA-2$^+$ cells. IL-31$^+$/MOMA-2$^+$ cells mostly express Arg-1, an M2 macrophage marker. **G,** Increased mRNA expression of *Iba1* (a macrophage-associated protein) and *Arg1* (an M2 marker) with minimally increased expression of *iNOS* (an M1 marker) are detected in the lesions of MC903-treated mice compared with those of control mice. **H-J,** Macrophage depletion with local injection of clodronate liposome decreases IL-31$^+$/MOMA-2$^+$ cells (Fig 3, *H* and *I*) and attenuates scratching behavior (Fig 3, *J*) compared with free liposome-treated control mice. *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase; *DAPI*, 4′-6-Diamidino-2-phenylindole. Blue indicates nuclei. White dotted lines indicate the dermo-epidermal junctions. Values represent mean + SEM. Representative results of at least 3 independent experiments are shown. *$P < .05$, Mann-Whitney *U* test (Fig 3, *A-C*, *E*, *I*, and *K*) and 2-tailed, unpaired *t* tests (Fig 3, *G*).

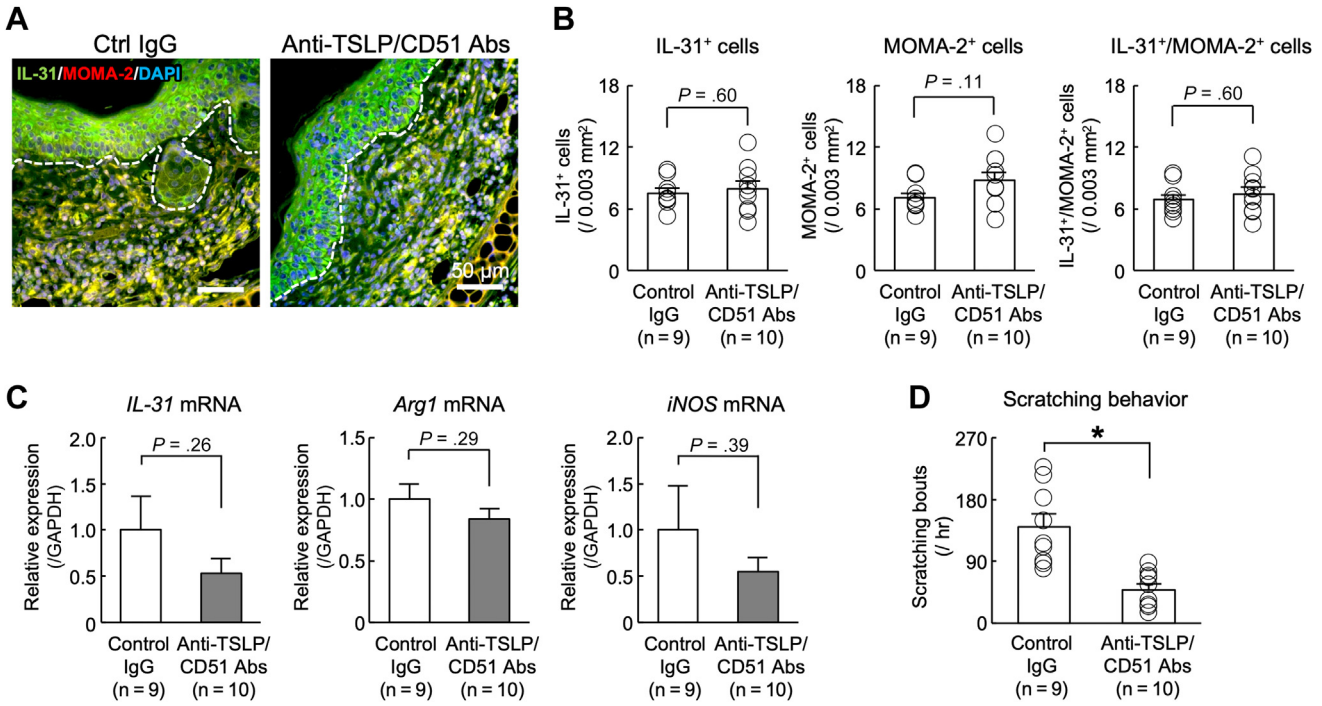

FIG 4. Neutralization of TSLP and blockade of periostin signaling does not decrease lesional macrophages or IL-31. **A** and **B,** Immunofluorescent staining images with quantification. Significant changes are not observed in the number of IL-31$^+$ cells (*green*), MOMA-2$^+$ cells (*red*), or IL-31$^+$/MOMA-2$^+$ cells (*merged in yellow*) following administration of a TSLP-neutralizing antibody and an antibody against CD51 (integrin αV), a periostin receptor, compared with control IgG-treated mice. **C,** Expressions of *IL31*, *Arg1*, and *iNOS* mRNAs in antibody-treated mice. **D,** Scratching behavior is attenuated in antibody-treated mice compared with control mice. *Ab*, Antibody; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase. Blue indicates nuclei. White dotted lines indicate the dermo-epidermal junctions. Values represent mean + SEM. *P < .05, Mann-Whitney *U* test (Fig 4, *B* and *D*) and 2-tailed, unpaired *t* tests (Fig 4, *C*).

.086, respectively) (Fig 5, *C*), indicating a reduction in M2 macrophages.

Murine basophils in conjunction with TSLP and periostin enhance IL-31 expression from macrophages *ex vivo*

To further confirm the roles of basophils in IL-31 production from M2 macrophages in the presence of TSLP and periostin, we used *ex vivo* flow-cytometric analysis with murine peritoneal macrophages. Coculture of peritoneal macrophages with TSLP-primed basophil-enriched CD49b$^+$ splenocytes (primary basophils) and mouse recombinant TSLP and periostin increased intracellular IL-31 and Arg-1 in MOMA-2$^+$ peritoneal macrophages (Fig 6, *A-C*). This finding indicates the role of basophils as an amplifier for IL-31 secretion from macrophages. We did not examine the effects of basophils on IL-31 secretion in the absence of TSLP and periostin, because basophils cannot survive without TSLP and/or IL-3 *ex vivo*.[45]

DISCUSSION

This study revealed a novel IL-31–generating network comprising M2 macrophages, TSLP, periostin, and basophils in human AD and MC903-induced AD-like skin lesions in mice.

Macrophages have been implicated as one of the cellular sources of IL-31 in several pruritic diseases, including ordinary scabies,[18] stasis dermatitis,[38] polymorphic light eruption,[46] prurigo nodularis,[47] and AD.[7] Our current study revealed that IL-31 is principally generated by M2 macrophages in human AD skin lesions. In addition, infiltrating IL-31$^+$ macrophages correlated with expression levels of TSLP and periostin. These findings are somewhat consistent with a previous report with mouse experiments that TSLP in conjunction with periostin, but not IL-4/IL-13, effectively promoted IL-31 production from peritoneal macrophages that skewed toward M2 *in vitro*.[18]

In human AD, peripheral basophils are reportedly in an activated state,[30,48] and basophils infiltrate into skin lesions.[30] In this study, a close correlation was detected between the number of IL-31$^+$/CD68$^+$ cells and dermal basophils in human AD lesions (Fig 1). In murine AD models, basophils promoted the allergic skin inflammation induced by MC903 and oxazolone.[28,35] Furthermore, recent reports suggest that basophils are also involved in itch sensation.[31,49] We demonstrated here the novel mode of action for basophils in AD-like skin inflammation. Basophil depletion plus blockade of the TSLP and periostin signaling resulted in significantly decreased numbers of IL-31$^+$/MOMA-2$^+$ cells and *IL-31* mRNA expression, accompanying amelioration of itch responses. Importantly, these changes were not achieved by single blockade of the TSLP and periostin signaling pathways, despite the fact that TSLP and periostin have been demonstrated to promote IL-31 production in murine peritoneal macrophages *in vitro*.[18] Lesional expression of *Arg1*, a marker of M2 macrophages, was also significantly decreased

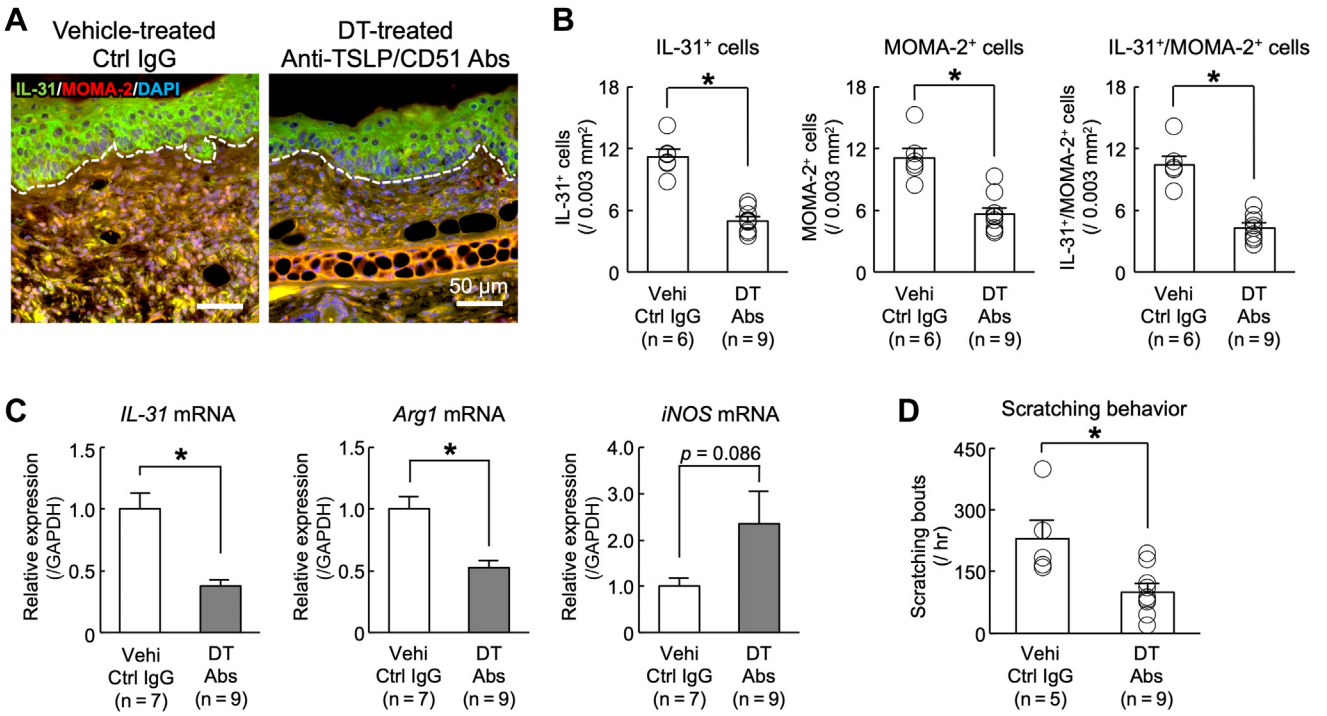

FIG 5. Basophil depletion plus neutralization of TSLP and blockade of periostin signaling significantly decreases lesional IL-31 and scratching behavior. **A** and **B,** Immunofluorescent images with quantification. The number of IL-31$^+$ cells (*green*), MOMA-2$^+$ cells (*red*), and IL-31$^+$/MOMA-2$^+$ cells (*merged in yellow*) decrease following basophil depletion with DT and administration of a TSLP-neutralizing antibody and an antibody against CD51 compared with control IgG-treated mice. **C,** Expressions of *IL31* and *Arg1* mRNAs are decreased, whereas *iNOS* expression tends to increase in DT-treated and antibody-treated mice. **D,** Scratching behaviors are attenuated in DT-treated and antibody-treated mice compared with control mice. *Ab,* Antibody; *GAPDH,* glyceraldehyde-3-phosphate dehydrogenase. Blue indicates nuclei. Values represent mean + SEM. *$P < .05$, Mann-Whitney *U* test (Fig 5, *B* and *D*) and 2-tailed, unpaired *t* tests (Fig 5, *C*).

by the depletion of basophils. This unique basophil function was further confirmed by *ex vivo* experiments, showing that peritoneal macrophages cocultured with spleen-derived primary basophils in the presence of TSLP and periostin effectively expressed IL-31 and Arg1 more than those in the presence of TSLP or periostin alone. Basophils thus appear to play essential roles in itch responses through the induction of M2 macrophages and IL-31 production.

Basophil-derived factors contributing to IL-31 generation from M2 macrophages are still to be elucidated. In MC903-induced AD-like inflammation, IL-4, M-CSF, and/or as-yet-undetermined factors released from basophils have been implicated in the induction of M2 macrophages.[23] However, IL-4 is incapable of inducing IL-31 production by macrophages.[18] We have previously demonstrated that basophil-derived amphiregulin, an epidermal growth factor receptor ligand, amplifies skin inflammation in the MC903-induced murine AD model.[35] Basophil-derived amphiregulin and/or M-CSF may be potential candidates for inducing IL-31 production by M2 macrophages, but further studies are needed to elucidate this issue.

A number of lines of evidence suggest that itch in AD is largely mediated by the cytokines IL-4/IL-13 and IL-31.[50] IL-4 and IL-13 have been proven to potentiate, or even induce, itch sensation in mice.[51,52] It was notable that macrophage depletion in AD-like skin inflammation resulted in increased IL-4 and IL-13 generation despite the reduction of scratching bouts. Thus, IL-4 and IL-13 seem not to be necessarily involved in scratching behaviors in this mouse model. Macrophage-depleted skin also showed the increased number of TUG8$^+$ basophils; this could explain the increased lesional *IL-4* mRNA expression, because infiltrative basophils seemed one of the cellular sources of IL-4 (see Fig E1, *B*, in this article's Online Repository at www.jacionline.org).[23] These data implied that, in this mouse model, basophils may not be a direct effector, but an initiator of itch through promoting IL-31 generation from macrophages. In clinical settings with human AD, dupilumab, an mAb against IL-4Rα (a shared receptor subunit in IL-4 and IL-13), attenuates itch sensation before the improvement of cutaneous inflammation.[53-55] However, dupilumab is unable to achieve full resolution of AD itch. In this regard, an intriguing recent finding was that dupilumab therapy resulted in increased *IL-31* mRNA expression as assessed by transcriptome analysis.[56] Increased IL-31 could explain the residual itch in AD during dupilumab therapy. This clinical finding also implied that macrophages, rather than IL-4-/IL-13–induced T_H2 lymphocytes, could be major cellular sources for pruritogenic IL-31.

The current study indicated that targeting the network comprising TSLP, periostin, and basophils represents a promising strategy for treating itch in AD by reducing IL-31 production by M2 macrophages. An alternative option for itch therapy in AD involves targeting the M2 macrophages themselves. Given the finding that clodronate liposome significantly reduced IL-31$^+$ macrophages and itch responses in mice (Fig 3, *I* and *J*), bisphosphonates (currently in wide use for osteoporosis) may provide another therapeutic strategy for AD itch by bringing about a systemic shift from abnormal M2 macrophages to the M1 phenotype.[57,58]

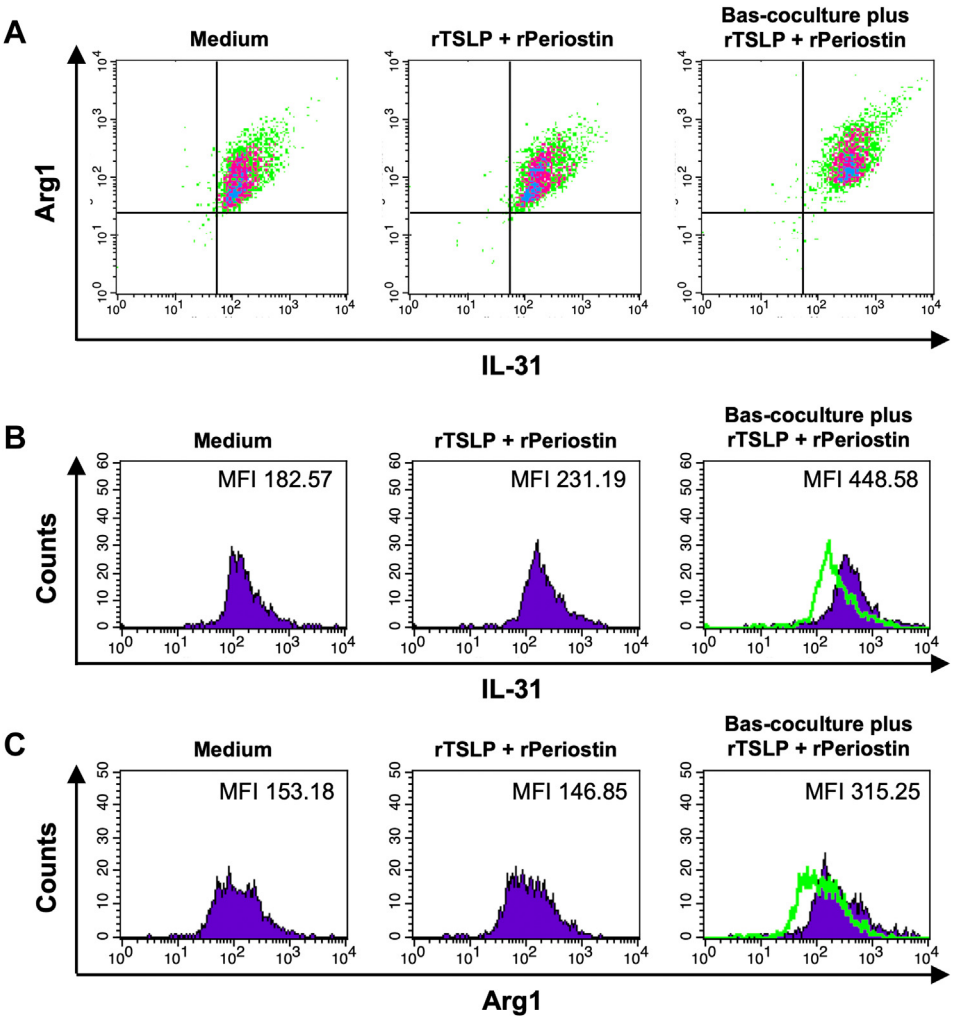

FIG 6. Murine peritoneal macrophages generate IL-31 in response to a combination of TSLP, periostin, and primary basophils *ex vivo*. **A-C,** Murine peritoneal macrophages are stimulated with mouse rTSLP, mouse rPeriostin, and TSLP-primed primary basophils (basophil-enriched CD49b$^+$ splenocytes) (Bas-coculture) for 24 hours, followed by flow-cytometric analysis with MOMA-2 gating. Stimulation with rTSLP and rPeriostin plus Bas-coculture promotes enhanced expression of IL-31 and Arg1 by MOMA-2$^+$ macrophages, compared with no stimulation or stimulation with rTSLP and rPeriostin alone. *MFI*, Mean fluorescence intensity; *rPeriostin*, recombinant periostin; *rTSLP*, recombinant thymic stromal lymphopoietin. Green lines in the right panels in Fig 6, *B* and *C*, indicate histograms of the rTSLP- and rPeriostin-stimulation groups. Representative results of at least 3 independent experiments are shown.

Key limitations of this study were the relatively small sample sizes and the examination of protein expressions exclusively through immunofluorescent analyses in human AD. In addition, we did not have numerical rating scale scores from patients for itch and clinical severity scores. We were thus unable to clarify the correlations between the number of IL-31$^+$ macrophages and itch/clinical severity.

Conclusion

The current study provides novel insights into the pathomechanisms of IL-31 generation in AD. Targeting the network comprising macrophages, TSLP, periostin, basophils, and/or IL-31 may prove beneficial for treating intractable itch in AD.

Clinical implications: A network comprising IL-31–expressing M2 macrophages, TSLP, periostin, and basophils may represent a therapeutic target for itch in AD.

We are grateful to Drs Satoshi Okuno and Yuki Yamazaki for their technical assistance.

REFERENCES

1. Ständer S. Atopic dermatitis. N Engl J Med 2021;384:1136-43.
2. Mack MR, Kim BS. The itch-scratch cycle: a neuroimmune perspective. Trends Immunol 2018;39:980-91.
3. Mollanazar NK, Smith PK, Yosipovitch G. Mediators of chronic pruritus in atopic dermatitis: getting the itch out? Clin Rev Allergy Immunol 2016;51:263-92.
4. Bağci IS, Ruzicka T. IL-31: a new key player in dermatology and beyond. J Allergy Clin Immunol 2018;141:858-66.
5. Nakashima C, Otsuka A, Kabashima K. Interleukin-31 and interleukin-31 receptor: new therapeutic targets for atopic dermatitis. Exp Dermatol 2018;27:327-31.
6. Furue M, Yamamura K, Kido-Nakahara M, Nakahara T, Fukui Y. Emerging role of interleukin-31 and interleukin-31 receptor in pruritus in atopic dermatitis. Allergy 2018;73:29-36.

7. Kato A, Fujii E, Watanabe T, Takashima Y, Matsushita H, Furuhashi T, et al. Distribution of IL-31 and its receptor expressing cells in skin of atopic dermatitis. J Dermatol Sci 2014;74:229-35.
8. Cevikbas F, Wang X, Akiyama T, Kempkes C, Savinko T, Antal A, et al. A sensory neuron-expressed IL-31 receptor mediates T helper cell-dependent itch: involvement of TRPV1 and TRPA1. J Allergy Clin Immunol 2014;133:448-60.
9. Andoh T, Harada A, Kuraishi Y. Involvement of leukotriene B4 released from keratinocytes in itch-associated response to intradermal interleukin-31 in mice. Acta Derm Venereol 2017;97:922-7.
10. Andoh T, Li S, Uta D. Involvement of thromboxane A 2 in interleukin-31-induced itch-associated response in mice. Pharmacol Rep 2018;70:251-7.
11. Sonkoly E, Muller A, Lauerma AI, Pivarcsi A, Soto H, Kemeny L, et al. IL-31: a new link between T cells and pruritus in atopic skin inflammation. J Allergy Clin Immunol 2006;117:411-7.
12. Kabashima K, Matsumura T, Komazaki H, Kawashima M. Trial of nemolizumab and topical agents for atopic dermatitis with pruritus. N Engl J Med 2020;383:141-50.
13. Hawro T, Saluja R, Weller K, Altrichter S, Metz M, Maurer M. IL-31 does not induce immediate itch in atopic dermatitis patients and healthy controls after skin challenge. Allergy 2014;69:113-7.
14. Niyonsaba F, Ushio H, Hara M, Yokoi H, Tominaga M, Takamori K, et al. Antimicrobial peptides human beta-defensins and cathelicidin LL-37 induce the secretion of a pruritogenic cytokine IL-31 by human mast cells. J Immunol 2010;184:3526-34.
15. Kunsleben N, Rüdrich U, Gehring M, Novak N, Kapp A, Raap U. IL-31 induces chemotaxis, calcium mobilization, release of reactive oxygen species, and CCL26 in eosinophils, which are capable to release IL-31. J Invest Dermatol 2015;135:1908-11.
16. Hashimoto T, Kursewicz CD, Fayne RA, Nanda S, Shah SM, Nattkemper L, et al. Pathophysiological mechanisms of itch in bullous pemphigoid. J Am Acad Dermatol 2020;83:53-62.
17. Raap U, Gehring M, Kleiner S, Rüdrich U, Eiz-Vesper B, Haas H, et al. Human basophils are a source of—and are differentially activated by—IL-31. Clin Exp Allergy 2017;47:499-508.
18. Hashimoto T, Satoh T, Yokozeki H. Pruritus in ordinary scabies: IL-31 from macrophages induced by overexpression of thymic stromal lymphopoietin and periostin. Allergy 2019;74:1727-37.
19. Yunna C, Mengru H, Lei W, Weidong C. Macrophage M1/M2 polarization. Eur J Pharmacol 2020;877:173090.
20. Han H, Kim Y, Mo H, Choi SH, Lee K, Rim YA, et al. Preferential stimulation of melanocytes by M2 macrophages to produce melanin through vascular endothelial growth factor. Sci Rep 2022;12:6416.
21. Gao J, Liang Y, Wang L. Shaping polarization of tumor-associated macrophages in cancer immunotherapy. Front Immunol 2022;13:888713.
22. Nakai K, He YY, Nishiyama F, Naruse F, Haba R, Kushida Y, et al. IL-17A induces heterogeneous macrophages, and it does not alter the effects of lipopolysaccharides on macrophage activation in the skin of mice. Sci Rep 2017;7:12473.
23. Pellefigues C, Naidoo K, Mehta P, Schmidt AJ, Jagot F, Roussel E, et al. Basophils promote barrier dysfunction and resolution in the atopic skin. J Allergy Clin Immunol 2021;148:799-812.e10.
24. Karasuyama H, Shibata S, Yoshikawa S, Miyake K. Basophils, a neglected minority in the immune system, have come into the limelight at last. Int Immunol 2021;33:809-13.
25. Obata K, Mukai K, Tsujimura Y, Ishiwata K, Kawano Y, Minegishi Y, et al. Basophils are essential initiators of a novel type of chronic allergic inflammation. Blood 2007;110:913-20.
26. Tsujimura Y, Obata K, Mukai K, Shindou H, Yoshida M, Nishikado H, et al. Basophils play a pivotal role in immunoglobulin-G-mediated but not immunoglobulin-E-mediated systemic anaphylaxis. Immunity 2008;28:581-9.
27. Wada T, Ishiwata K, Koseki H, Ishikura T, Ugajin T, Ohnuma N, et al. Selective ablation of basophils in mice reveals their nonredundant role in acquired immunity against ticks. J Clin Invest 2010;120:2867-75.
28. Yamanishi Y, Mogi K, Takahashi K, Miyake K, Yoshikawa S, Karasuyama H. Skin-infiltrating basophils promote atopic dermatitis-like inflammation via IL-4 production in mice. Allergy 2020;75:2613-22.
29. Miyake K, Shiozawa N, Nagao T, Yoshikawa S, Yamanishi Y, Karasuyama H. Trogocytosis of peptide-MHC class II complexes from dendritic cells confers antigen-presenting ability on basophils. Proc Natl Acad Sci U S A 2017;114:1111-6.
30. Ito Y, Satoh T, Takayama K, Miyagishi C, Walls AF, Yokozeki H. Basophil recruitment and activation in inflammatory skin diseases. Allergy 2011;66:1107-13.
31. Wang F, Trier AM, Li F, Kim S, Chen Z, Chai JN, et al. A basophil-neuronal axis promotes itch. Cell 2021;184:422-40.e17.
32. Leyva-Castillo JM, Sun L, Wu SY, Rockowitz S, Sliz R, Geha RS. Single-cell transcriptome profile of mouse skin undergoing antigen-driven allergic inflammation recapitulates findings in atopic dermatitis skin lesions. J Allergy Clin Immunol 2022;150:373-84.
33. Katoh N, Ohya Y, Ikeda M, Ebihara T, Katayama I, Saeki H, et al. Japanese guidelines for atopic dermatitis 2020. Allergol Int 2020;69:356-69.
34. Li M, Hener P, Zhang Z, Kato S, Metzger D, Chambon P. Topical vitamin D3 and low-calcemic analogs induce thymic stromal lymphopoietin in mouse keratinocytes and trigger an atopic dermatitis. Proc Natl Acad Sci U S A 2006;103:11736-41.
35. Hashimoto T, Satoh T, Karasuyama H, Yokozeki H. Amphiregulin from basophils amplifies basophil-mediated chronic skin inflammation. J Invest Dermatol 2019;139:1834-7.e2.
36. Hashimoto T, Satoh T, Yokozeki H. Protective role of STAT6 in basophil-dependent prurigo-like allergic skin inflammation. J Immunol 2015;194:4631-40.
37. Ugajin T, Kojima T, Mukai K, Obata K, Kawano Y, Minegishi Y, et al. Basophils preferentially express mouse Mast Cell Protease 11 among the mast cell tryptase family in contrast to mast cells. J Leukoc Biol 2009;86:1417-25.
38. Hashimoto T, Kursewicz CD, Fayne RA, Nanda S, Shah SM, Nattkemper L, et al. Mechanisms of itch in stasis dermatitis: significant role of IL-31 from macrophages. J Invest Dermatol 2020;140:850-9.
39. Kanda Y. Investigation of the freely available easy-to-use software "EZR" for medical statistics. Bone Marrow Transplant 2013;48:452-8.
40. Izuhara K, Nunomura S, Nanri Y, Ogawa M, Ono J, Mitamura Y, et al. Periostin in inflammation and allergy. Cell Mol Life Sci 2017;74:4293-303.
41. Hashimoto T, Mishra SK, Olivry T, Yosipovitch G. Periostin, an emerging player in itch sensation. J Invest Dermatol 2021;141:2338-43.
42. Hashimoto T, Nattkemper L, Kim H, Kursewicz CD, Fowler E, Shah SM, et al. Dermal periostin: a new player in itch of prurigo nodularis. Acta Derm Venereol 2021;101:adv00375.
43. Nattkemper LA, Tey HL, Valdes-Rodriguez R, Lee H, Mollanazar NK, Albornoz C, et al. The genetics of chronic itch: gene expression in the skin of patients with atopic dermatitis and psoriasis with severe itch. J Invest Dermatol 2018;138:1311-7.
44. Mishra SK, Wheeler JJ, Pitake S, Ding H, Jiang H, Fukuyama T, et al. Periostin activation of integrin receptors on sensory neurons induces allergic itch. Cell Rep 2020;31:107472.
45. Oetjen LK, Noti M, Kim BS. New insights into basophil heterogeneity. Semin Immunopathol 2016;38:549-61.
46. Patra V, Strobl J, Gruber-Wackernagel A, Vieyra-Galcia P, Stary G, Wolf P. CD 11b+ cells markedly express the itch cytokine interleukin 31 in polymorphic light eruption. Br J Dermatol 2019;181:1065-6.
47. Hashimoto T, Nattkemper L, Kim H, Kursewicz CD, Fowler E, Shah SM, et al. Itch intensity in prurigo nodularis is closely related with dermal interleukin-31, oncostatin M, IL-31 receptor alpha, and oncostatin M receptor beta. Exp Dermatol 2021;30:804-10.
48. Imamura S, Washio K, Mizuno M, Oda Y, Fukunaga A, Nishigori C. Activated steady status and distinctive FcεRI-mediated responsiveness in basophils of atopic dermatitis. Allergol Int 2021;70:327-34.
49. Hashimoto T, Rosen JD, Sanders KM, Yosipovitch G. Possible roles of basophils in chronic itch. Exp Dermatol 2019;28:1373-9.
50. Lipman ZM, Labib A, Yosipovitch G. Current clinical options for the management of itch in atopic dermatitis. Clin Cosmet Investig Dermatol 2021;14:959-69.
51. Campion M, Smith L, Gatault S, Métais C, Buddenkotte J, Steinhoff M. Interleukin-4 and interleukin-13 evoke scratching behaviour in mice. Exp Dermatol 2019;28:1501-4.
52. Oetjen LK, Mack MR, Feng J, Whelan TM, Niu H, Guo CJ, et al. Sensory neurons co-opt classical immune signaling pathways to mediate chronic itch. Cell 2017;171:217-28.e13.
53. Silverberg JI, Yosipovitch G, Simpson EL, Kim BS, Wu JJ, Eckert L, et al. Dupilumab treatment results in early and sustained improvements in itch in adolescents and adults with moderate to severe atopic dermatitis: analysis of the randomized phase 3 studies SOLO 1 and SOLO 2, AD ADOL, and CHRONOS. J Am Acad Dermatol 2020;82:1328-36.
54. Simpson EL, Bieber T, Guttman-Yassky E, Beck LA, Blauvelt A, Cork MJ, et al. Two phase 3 trials of dupilumab versus placebo in atopic dermatitis. N Engl J Med 2016;375:2335-48.
55. Yosipovitch G, Rosen JD, Hashimoto T. Itch: from mechanism to (novel) therapeutic approaches. J Allergy Clin Immunol 2018;142:1375-90.
56. Möbus L, Rodriguez E, Harder I, Stölzl D, Boraczynski N, Gerdes S, et al. Atopic dermatitis displays stable and dynamic skin transcriptome signatures. J Allergy Clin Immunol 2021;147:213-23.
57. Kaps L, Huppertsberg A, Choteschovsky N, Klefenz A, Durak F, Schrörs B, et al. pH-degradable, bisphosphonate-loaded nanogels attenuate liver fibrosis by repolarization of M2-type macrophages. Proc Natl Acad Sci U S A 2022;119:e2122310119.
58. Weber M, Homm A, Müller S, Frey S, Amann K, Ries J, et al. Zoledronate causes a systemic shift of macrophage polarization towards M1 in vivo. Int J Mol Sci 2021;22:1-19.

METHODS

Antibodies

Antibodies obtained from Abcam (Cambridge, UK) included anti-CD68 (ab955), IL-31 (ab102750), basophil (2D7) (ab155577), TSLP (ab188766), periostin (ab14041), arginase-1 (ab92274), and monocyte/macrophage (MOMA-2) (ab33451). Anti-CD163 antibody (bs-2527R) was purchased from Bioss Antibodies (Woburn, Mass). R-phycoerythrin–conjugated anti–MOMA-2 antibody (MCA519PE) was obtained from Bio-Rad Laboratories (Hercules, Calif). Antimouse basophil (TUG8) and IL-4 (504111) antibodies were purchased from BioLegend (San Diego, Calif). Alexa Fluor 488– and 568–conjugated secondary antibodies were obtained from Abcam.

Primers

The primers used for PCR were 5′-AAACAAGAGTCTCAGGATCTTTA-TAACAAC-3′ and 5′-ACGGCAGCTGTATTGATTCGT-3′ for mouse *IL-31* in Fig 3 (vehicle-treated mice vs MC903-treated mice); 5′-ATA-CAGCTGCCGTGTTTCAG-3′ and 5′-AGCCATCTTATCACCCAAGA-3′ for mouse *IL-31* in Figs 4 and 5 (control IgG group vs anti-TSLP/CD51 antibodies, and control IgG with vehicle-treated group vs anti-TSLP/CD51 antibodies with DT-treated mice, respectively); 5′-CAGACTGCCAGCC-TAAGACA-3′ and 5′-AGGAATTGCTTGTTGATCCC-3′ for mouse *Iba1*; 5′-CTCCAAGCCAAAGTCCTTAGAG-3′ and 5′-AGGAGCTGTCAT-TAGGGACATC-3′ for mouse *Arg1*; 5′-GTTCTCAGCCCAACAATA-CAAGA-3′ and 5′-GTGGACGGGTCGATCTCAC-3′ for mouse *iNOS*; 5′-AGATGGATGTGCCAAACGTCCTCA-3′ and 5′-AATATGCGAAG-CACCTTGGAAGCC-3′ for mouse *IL-4*; 5′-AGCATGGTATGGAGT GTGGA-3′ and 5′-TTGCAATTGGAGATGTTGGT-3′ for mouse *IL-13*; 5′-GTGGGAAATCCCAGTGAGAA-3′ and 5′-TCCGAATCCAAGGCA-TAAAG-3′ for murine *Mcpt8*; and 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′ for mouse *glyceraldehyde 3-phosphate dehydrogenase*. Expression levels of mRNA were calculated using the comparative ΔΔCt method relative to glyceraldehyde 3-phosphate dehydrogenase, a housekeeping gene.

Mite extract–induced murine dermatitis model

The mouse model of allergic skin inflammation was generated as previously described with modifications.[E1,E2] Briefly, both sides of the earlobes were stripped 3 times with cellophane tape (Nichiban, Tokyo, Japan) and an extract from house dust mites *Dermatophagoides pteronyssinus* feces (Wako Pure Chemical, Osaka, Japan; 10 mg/mL in PBS(−) containing 0.5% Tween 20) was applied at 25 μL on each earlobe every other day for 12 days. On day 13, scratching behavior was recorded and ear samples were collected using 8-mm biopsy punches (Kai Industries Co., Gifu, Japan).

REFERENCES

E1. Gao XK, Nakamura N, Fuseda K, Tanaka H, Inagaki N, Nagai H. Establishment of allergic dermatitis in NC/Nga mice as a model for severe atopic dermatitis. Biol Pharm Bull 2004;27:1376-81.

E2. Masuoka M, Shiraishi H, Ohta S, Suzuki S, Arima K, Aoki S, et al. Periostin promotes chronic allergic inflammation in response to Th2 cytokines. J Clin Invest 2012;122:2590-600.

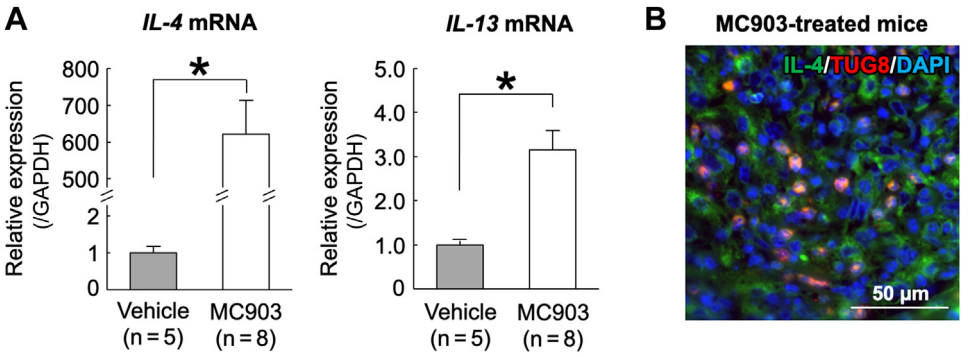

FIG E1. Lesional IL-4, IL-13, and basophils in the MC903-induced murine model of AD. **A,** Increased mRNA expressions of *IL-4* and *IL-13* in MC903-treated mice. Values represent mean + SEM. * $P < .05$ compared with vehicle-treated control mice (2-tailed, unpaired *t* tests). **B,** TUG8^{+} basophils (*red*) express IL-4 (*green; merged in yellow*) in M903-treated mice. Blue indicates nuclei. *DAPI*, 4′-6-Diamidino-2-phenylindole; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase.

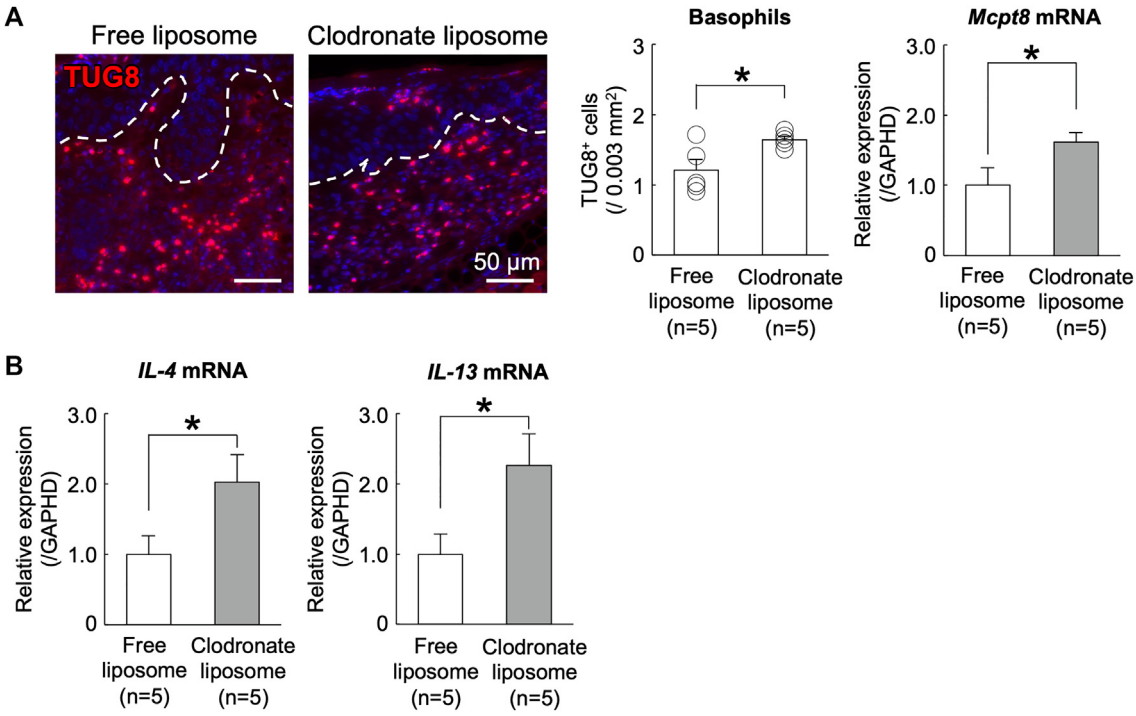

FIG E2. Macrophage depletion induces an increase in lesional IL-4 and IL-13 mRNAs and the number of basophils in the MC903-induced murine model of AD. **A,** The number of TUG8^{+} basophils (*red*) and mRNA expression of *Mcpt8*, a basophil-specific protease, increased following macrophage depletion with clodronate liposome (2-tailed, unpaired *t* tests, $P = .044$ and $P = .048$, respectively). **B,** Increased mRNA expressions of *IL-4* and *IL-13* in the macrophage-depleted lesions. Blue indicates nuclei. White dotted lines indicate the dermo-epidermal junctions. Values represent mean + SEM. *$P < .05$ compared with free liposome–treated control mice (2-tailed, unpaired *t* tests). *GAPDH*, Glyceraldehyde 3-phosphate dehydrogenase.

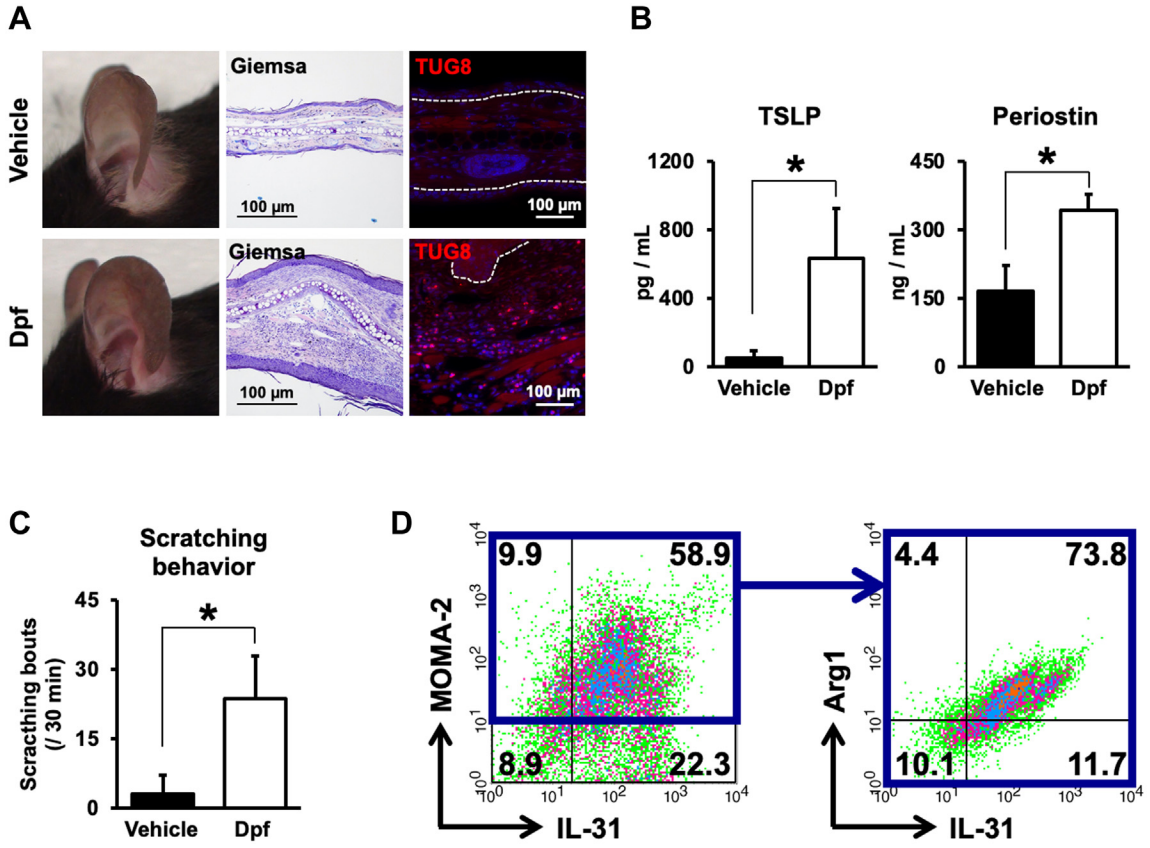

FIG E3. IL-31$^+$ monocytes/macrophages in Dpf-induced dermatitis in mice. **A,** Application of Dpf (250 μg/25 μL PBS-0.5% Tween 20) every 2 days for 12 days induced erythematous ear-swelling responses, showing histopathological features of inflamed skin (*Giemsa staining, middle panels*) with TUG8$^+$ basophilic infiltrates (*indicated in red, right panels*). **B,** TSLP and periostin production in inflamed skin. **C,** Dpf treatment induced scratching behaviors. **D,** Flow-cytometric analysis of dermal-infiltrating cells. A major population of IL-31$^+$ cells comprised MOMA-2$^+$ cells expressing Arg1. *Dpf, Dermatophagoides pteronyssinus* feces. Values represent mean + SD of at least 3 samples. **P* < .05 compared with vehicle-treated control mice (Mann-Whitney *U* test). White dotted lines indicate the dermo-epidermal junction.

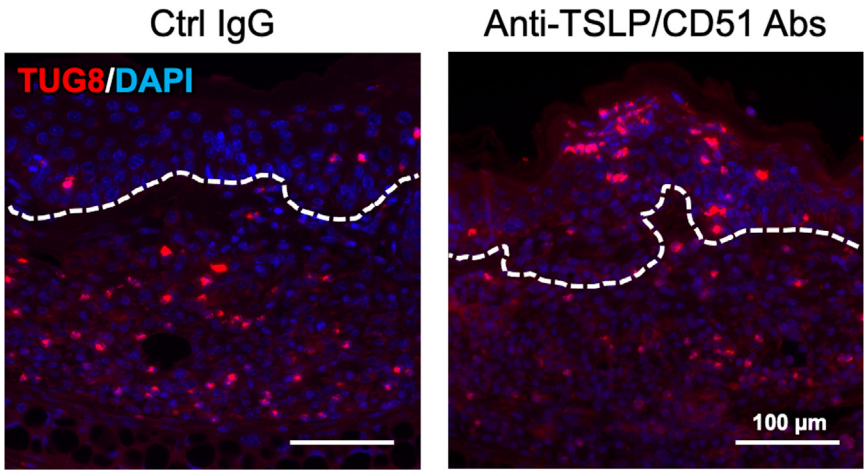

FIG E4. TUG8^{+} basophils in lesional skin of anti-TSLP and anti-CD51 antibody-treated C57BL/6 mice. *Ab*, Antibody; *DAPI*, 4′-6-diamidino-2-phenylindole. TSLP neutralization and CD51 blockade did not abolish lesional basophils. White dotted lines indicate the dermo-epidermal junction.

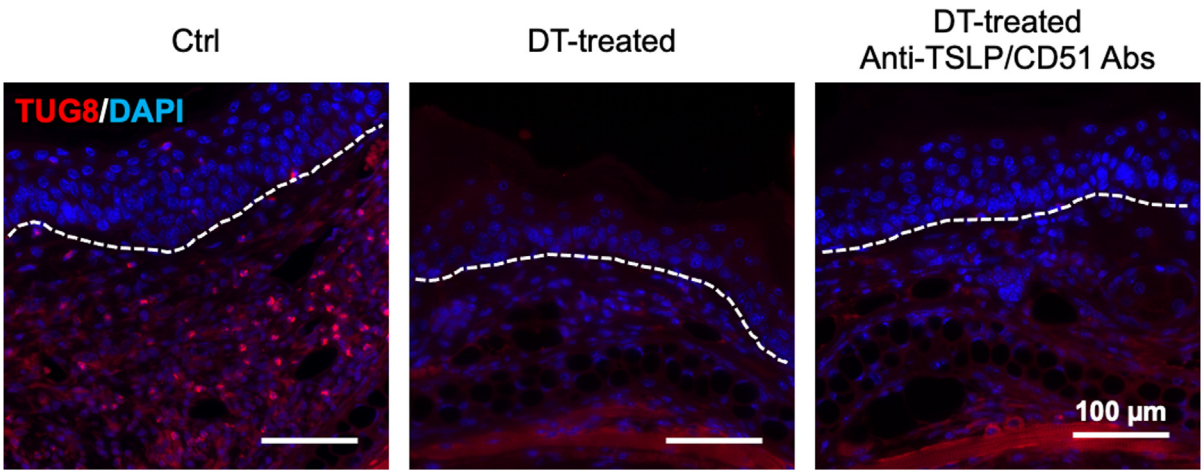

FIG E5. TUG8^{+} basophils in lesional skin of DT-treated *Mcpt8*^{DTR} mice. DT treatment with or without TSLP neutralization/CD51 blockade abolished lesional basophils. *Ab,* antibody; *DAPI,* 4′-6-diamidino-2-phenylindole. White dotted lines indicate the dermo-epidermal junction.