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# Protective Role of STAT6 in Basophil-Dependent Prurigo-like Allergic Skin Inflammation

Takashi Hashimoto,<sup>\*,†</sup> Takahiro Satoh,<sup>\*</sup> and Hiroo Yokozeki<sup>†</sup>

Prurigo is a common, but treatment-resistant, skin disease characterized by persistent papules/nodules and severe itching. Prurigo occurs in association with various underlying diseases, such as diabetes, chronic renal failure, and internal malignancies. Atopic dermatitis is occasionally complicated by prurigo lesions. However, the pathology of prurigo is completely undefined. We demonstrate that repeated intradermal administration of Ag to IgE-transgenic mice causes persistent and pruritic papulonodular skin lesions mimicking prurigo. Skin lesions were histopathologically characterized by irregular acanthosis and dermal cellular infiltrates comprising eosinophils, mononuclear cells, and basophils, with epidermal nerve fiber sprouting. In vivo depletion of basophils alleviated skin reactions, indicating that the inflammation is basophil dependent. Unexpectedly, STAT6 signaling was unnecessary for skin lesion development if IgE was present. Moreover, the absence of STAT6 signaling exacerbated the inflammation, apparently as the result of impaired generation of an M2-type anti-inflammatory macrophage response. These results provide novel insights into the pathologic mechanisms underlying prurigo. Although basophils are indispensable for prurigo-like inflammation, Th2 immunity mediated by STAT6 appears to play a protective role, and therapies targeting Th2-type cytokines may risk aggravating the inflammation. *The Journal of Immunology*, 2015, 194: 000–000.

Prurigo is a reactive inflammatory skin disease characterized by papulonodular lesions and severe itching. Several diagnostic names, such as papular dermatitis, urticarial papulosis, urticarial dermatitis, and “itchy red bump” disease have been ascribed to the spectrum of prurigo reactions (1–6), although the cutaneous symptoms associated with these reactions are not necessarily identical, and their morphology and pathoetiology appear to be heterogenous. In general, prurigo, particularly its subacute and chronic forms, is difficult to treat with conventional therapies, including H1 receptor antagonists and topical corticosteroids; thus, recurrence is common.

The actual factors that precipitate prurigo remain unclear. However, the acute form of prurigo can be induced by arthropod bites and is designated “papular urticaria” (5, 6). The subacute and chronic forms of prurigo may occur in patients with several diseases, such as diabetes, chronic renal failure, chronic hepatitis C (7), internal malignancy, *Helicobacter pylori* infection (8), and focal infections (9, 10). Prurigo lesions are also commonly observed in atopic dermatitis (11–13). Although certain types of prurigo, such as prurigo nodularis (Hyde), may be just a secondary skin change resulting from severe scratching due to persistent itch

(14), prurigo is also considered a subtype of dermal hypersensitivity reactions (3–5). Prurigo lesions are histopathologically characterized by lymphocyte and eosinophil infiltration, with dermal edema and/or exudates. In addition, our recent findings revealed that prurigo is a disease involving prominent basophil infiltration (15). Basophils principally reside in the blood rather than peripheral tissues. However, in some inflammatory conditions, basophils are recruited to the skin (15). Although they share morphological and functional similarities with mast cells, basophils have unique functions; for example, in addition to producing IL-4 and IL-13 (16–18), they play important roles in acquired immunity against helminths and ticks and in IgG-dependent anaphylactic reactions (19–21). Thus, certain allergic events, in particular Th2-type immune responses, are likely involved in the pathogenesis of prurigo, although the details of the underlying mechanism have not been clearly defined.

Some prurigo reactions, including those associated with arthropod bites, frequently start as urticarial lesions (immediate-type-like response) or urticarial papules, followed by the formation of persistent papulonodular lesions. Occasionally, long-lasting urticarial lesions or urticarial erythema are concomitantly observed with papulonodular changes. This morphological and pathological relationship between immediate-type-like inflammation and persistent papulonodular inflammation in prurigo is an important, but unclarified, issue. In this regard, recent murine skin inflammation model studies provided a hint regarding the nature of the immunological processes underlying prurigo. Exogenous introduction of IgE or the gene encoding IgE induces both immediate-type and late-phase responses, followed by a third-phase response several days after challenge involving an IgE-mediated very late-phase response (IgE-vLPR) or IgE-mediated chronic allergic inflammation (IgE-CAI) (22, 23). The IgE-vLPR is unique in that tissue basophils are essential for the development of inflammation. Thus, the immunological process in this mouse model appears to represent an inflammation with a continuum between immediate-type responses and chronic inflammation, similar to human prurigo. However, the IgE-vLPR is not necessarily the same as prurigo, in that it declines within 1 wk and does not involve papule/nodule formation.

<sup>\*</sup>Department of Dermatology, National Defense Medical College, Tokorozawa 359-8513, Japan; and <sup>†</sup>Department of Dermatology, Graduate School, Tokyo Medical and Dental University, Tokyo 113-8519, Japan

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Address correspondence and reprint requests to Prof. Takahiro Satoh, Department of Dermatology, National Defense Medical College, 3-2 Namiki, Tokorozawa 359-8513, Japan. E-mail address: tasaderm@ndmc.ac.jp

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Abbreviations used in this article: IgE-CAI, IgE-mediated chronic allergic inflammation; IgE-vLPR, IgE-mediated very late phase response; iNOS, inducible NO synthase; NGF, nerve growth factor; siRNA, small interfering RNA; TNP, trinitrophenyl; TNP-IgE, TNP-specific IgE; TNP-IgE-Tg, TNP-IgE transgenic; TSLP, thymic stromal lymphopoietin; WT, wild-type.

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To further understand the immunological events underlying prurigo, we developed a novel mouse model characterized by itchy persistent papulonodular skin lesions induced by IgE. We found that Th2-type immunity mediated by STAT6 signaling was unnecessary and, instead, negatively regulated prurigo-like reactions, at least in part through M2-type macrophages, whereas basophils were essential for the initiation and maintenance of papulonodular skin inflammation.

## Materials and Methods

### Mice

BALB/c and C57BL/6 mice were purchased from Sankyo Labo Service (Tokyo, Japan). Trinitrophenyl (TNP)-specific IgE (TNP-IgE)-transgenic (TNP-IgE-Tg) mice (21) (BALB/c background) were kindly provided by Dr. Hajime Karasuyama (Department of Immune Regulation, Tokyo Medical and Dental University). *STAT6*-deficient mice (*STAT6*<sup>-/-</sup>) (C57BL/6 background) were described previously (23, 24). Mice were maintained under specific pathogen-free conditions in our animal facility. The use of animals was in full compliance with the Committee for Animal Experiments of Tokyo Medical and Dental University and National Defense Medical College.

### Abs

Anti-CD3 $\epsilon$ , anti-phospho-STAT6 (Tyr 641), anti-CD163 (M-96), anti-CD206 (C-20), anti-amphiregulin, semaphorin 3A (N-15), anti-MOMA-2, and nerve growth factor (NGF) Abs were purchased from Santa Cruz Biotechnology (Dallas, TX). Allophycocyanin-conjugated anti-inducible NO synthase (iNOS; CXNFT) and biotinylated anti-CD115 (c-fms) Abs were obtained from eBioscience (San Jose, CA). Anti-phospho-STAT3 (Tyr 705) Ab was obtained from Cell Signaling Technology (Danvers, MA). Anti-PGP 9.5 goat polyclonal Ab was obtained from Enzo Life Science (Farmingdale, NY). Alexa Fluor-conjugated anti-MOMA-2 Ab was purchased from Bio-Rad (Hercules, CA). Allophycocyanin-conjugated CD11c and PE-conjugated CD206 (MMR) Abs were obtained from BioLegend (San Diego, CA). PE-conjugated Arg1 Ab was obtained from R&D Systems (Minneapolis, MN).

Anti-mouse basophil-specific Ab (TUG8) (25) and anti-CD200R3 (Ba103) Ab (26) were kindly provided by Dr. Hajime Karasuyama.

### Preparation of TNP-IgE

TNP-IgE was obtained from ascites of BALB/c-*nu/nu* mice by i.p. injection of the IGEL b4 B cell hybridoma (American Type Culture Collection, Rockville, MD; TIB141) (27). Supernatants were precipitated with 55% saturated ammonium sulfate and dialyzed in PBS. Monomeric IgE was prepared by removing aggregates by ultracentrifugation at 60,000  $\times g$  for 1 h at 4°C (28).

### Induction of IgE-mediated prurigo-like responses

TNP-IgE-Tg mice were injected s.c. into each ear lobe or the dorsal skin on days 1, 4, and 7 with TNP<sup>12</sup>-OVA (100  $\mu$ g/site; Biosearch Technologies, Novato, CA). Ear thickness was measured using a dial thickness gauge (Ozaki, Tokyo, Japan) before and after challenges. In some experiments, wild-type (WT) mice were passively and repeatedly immunized with TNP-IgE (150  $\mu$ g/mouse, i.p.) 1 d before challenge with TNP-OVA.

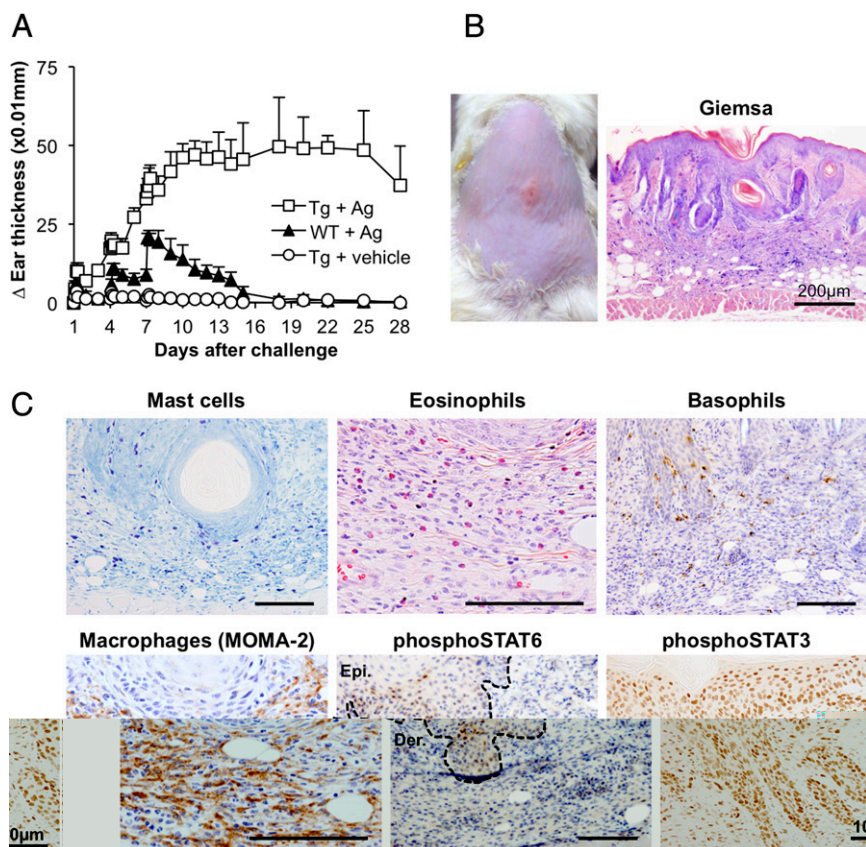
### Measurement of scratching behavior

Observation of scratching behavior was performed as previously described (29, 30). Briefly, mice were placed into individual plastic cages (10  $\times$  12.5  $\times$  15 cm) to acclimatize for 1 h. Subsequently, an unmanned video camera (Sony Handycam HDR-XR500) was set to record their behavior. The number of times that mice engaged in scratching of their ear lobes with the hind paw was counted. Mice usually scratched their ears several times over a period  $\sim$ 1 s. Each such series of movements was counted as a single scratching bout (30).

### Measurement of cytokines and chemokines

Punched skin samples (8 mm in diameter) were homogenized in PBS containing 1% protease inhibitor mixture (Sigma-Aldrich, St. Louis, MO; 250  $\mu$ l/tissue) and then centrifuged at 10,000  $\times g$  for 10 min. Cytokine and chemokine levels in the supernatants of homogenates were determined by sandwich ELISA. ELISA kits for murine IL-4, IL-5, IL-13, IL-17A, IL-22, IL-33, TGF- $\beta$ , IFN- $\gamma$ , thymic stromal lymphopoietin (TSLP), and eotaxin-1 (CCL11) were purchased from R&D Systems. The ELISA kit for IL-18 was obtained from Medical and Biological Laboratories (Nagoya, Japan).

**FIGURE 1.** Repeated Ag challenge induces prurigo-like skin lesions. **(A)** TNP-IgE-Tg mice (Tg) were challenged with injection of TNP-OVA into the ear lobes on days 1, 4, and 7. They showed persistent and long-lasting ear swelling responses, whereas the responses of WT BALB/c mice were transient. Each group consisted of at least four mice. Vertical lines represent SD. Representative results of five independent experiments are shown. **(B)** Prurigo-like papulonodular lesions induced on the dorsal skin (day 14). Histopathologically, irregular acanthosis with occasional follicular plugging and dense dermal cellular infiltration were observed. **(C)** Cellular infiltrates consisted of increased dermal mast cells (toluidine blue staining), eosinophils (Luna staining), basophils (TUG8), and macrophages (MOMA-2). Phospho-STAT6 and phospho-STAT3 also were detected in nuclei of epidermal cells and some dermal cells.



A Mouse Type I Collagen Detection Kit was obtained from Chondrex (Redmond, WA). The ELISA kits for eotaxin-2 (CCL24) and NGF were purchased from Abcam (Cambridge, U.K.) and Millipore (Billerica, MA), respectively.

#### *In vivo transfection with STAT6 siRNA*

Small interfering RNA (siRNA) was prepared for transfection using cationic liposomes (Lipofectamine 2000; Life Technologies, Carlsbad, CA), according to the manufacturer's instructions. Mice were transfected with siRNA by injection into each ear lobe to a final concentration of 0.5 nmol/30  $\mu$ l/ear diluted with Life Technologies Opti-MEM I (Invitrogen)/Lipofectamine 2000 at a ratio of 50:1 (31). The nucleotide sequences of the sense and antisense strands of mouse STAT6 siRNA were 5'-CGAAUGUGAUACAACUGUAUC-3' and 5'-UACAGUUGUAUCACAUUCGAG-3', respectively. The following siRNA was used as a scramble siRNA negative control: 5'-AUCGCGCGGAUAGUACGUATT-3' (sense strand) and 5'-UACGUACUAUCGCGCGGAUTT-3' (antisense strand).

#### *Real-time PCR*

Total cellular RNA was isolated from excised skin specimens using an RNeasy Fibrous Mini Kit (QIAGEN, Valencia, CA). Quantitative RT-PCR was performed with reverse-transcribed RNA by real-time monitoring of the increase in fluorescence of SYBR Green dye (Brilliant SYBR Green QPCR Master Mix) using an Mx3000P Real-Time PCR System (both from Stratagene, La Jolla, CA). The primers for PCR were 5'-TCGGTCATC-ATAGCATCTGGAG-3' and 5'-GCACAGTCCCTTTGGAGTTAAG-TC-3' for mouse IL-31, 5'-CTCCAAGCCAAAGTCCTTAGAG-3' and 5'-AGGAGCTGTCATTAGGGACATC-3' for mouse Arg1, 5'-GTTC-TCAGCCCAACAATAACAAGA-3' and 5'-GTGGACGGGTCGATGTC-AC-3' for iNOS, and 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TC-CACCACCTGTTGCTGTA-3' for mouse GAPDH.

#### *Immunohistochemistry*

Sections (5  $\mu$ m) from paraffin-embedded tissues were subjected to Ag retrieval with citrate buffer pH 6.0 (for TUG8, pSTAT3, amphiregulin, semaphorin 3A, and CD163 staining), target retrieval solution pH 9.0 (Dako, Carpinteria, CA; for pSTAT6, NGF, and CD206 staining), or proteinase K (Dako; for MOMA-2 staining), and incubated with methanol containing 0.3% H<sub>2</sub>O<sub>2</sub> to inhibit endogenous peroxidases and with a protein-blocking solution containing 0.25% casein (Dako) to prevent the nonspecific binding of Abs. They were incubated with the indicated Abs at 4°C overnight followed by HRP-conjugated anti-IgG Ab, and visualized with 3'-diaminobenzidine tetrahydrochloride solution (Dako).

#### *Immunofluorescence staining*

Paraffin-embedded specimens (5  $\mu$ m) were pretreated with target retrieval solution pH 9.0 (Dako), incubated with the indicated combination of Abs followed by a reaction with Alexa Fluor 488- or Alexa Fluor 596-conjugated second Abs (Life Technologies, Carlsbad, CA), and nuclear-counterstained with DAPI (Roche Diagnostics, Mannheim, Germany). For PGP 9.5 staining, cryosections (30  $\mu$ m) were incubated with anti-PGP 9.5 Ab, followed by the incubation with Alexa Fluor 488-labeled second Ab (Life Technologies). Photomicrographs were produced using fluorescence microscope Biozero BZ-8000 (Keyence, Osaka, Japan) or Fluoview FV10i confocal microscope (Olympus, Tokyo, Japan).

#### *Flow cytometric analyses*

Single-cell suspensions were prepared from the skin by treating excised ear samples with collagenase type III (125 U/ml; Worthington Biochemical, Lakewood, NJ) in RPMI 1640 complete medium for 2 h at 37°C, followed by depletion of RBCs. After preincubation with anti-CD16/32 Ab (BD Biosciences, San Jose, CA) on ice for 30 min to prevent the nonspecific binding of irrelevant Abs, cells were labeled with the indicated combinations of Abs using IC Fixation Buffer and 10 $\times$  permeabilization buffer (eBioscience) and analyzed using a FACSCalibur cell sorter (BD Biosciences).

#### *Statistical analyses*

The Student *t* test was used to assess the statistical significance of differences between means. Data regarding changes in skin responses over time were analyzed using the repeated-measures ANOVA test, followed by either the Student *t* test or Scheffé *F* test.

## Results

### *Repeated induction of the IgE-vLPR results in formation of persistent papulonodular skin lesions*

The IgE-vLPR (or IgE-CAI) is a chronic, but transient, skin inflammation that begins to resolve within  $\sim$ 1 wk after challenge with TNP-OVA (22). We hypothesized that a more persistent and longer-lasting chronic inflammation leading to papulonodular lesions could be induced by modifying the IgE-vLPR. To this end, we initially treated mice repeatedly with the corresponding Ag. TNP-IgE-Tg mice were injected with TNP-OVA s.c. in the ear lobes on days 1, 4, and 7. The initial challenge induced immediate responses and a weak late-phase response, which was consistent with prior reports (22, 23). Marked ear swelling was observed after the second and third challenges, and this swelling lasted  $>$ 4 wk (Fig. 1A). WT BALB/c mice treated with TNP-OVA also showed mild skin reactions, but they were transient and began to resolve within 1 wk after the last challenge.

We next asked whether repeated challenge to the dorsal skin could induce papulonodular lesions. Repeated intradermal injection of TNP-OVA resulted in apparent papulonodular changes on the dorsal skin (Fig. 1B). These lesions following immediate-type

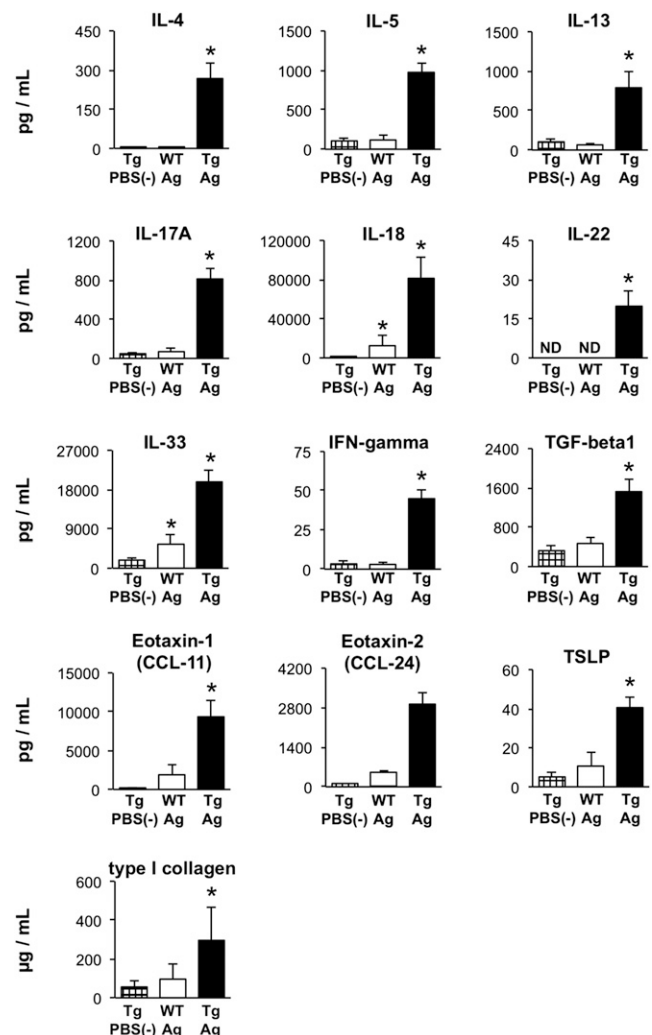


**FIGURE 2.** Cytokine profile of prurigo-like skin lesions. The dorsal skin of TNP-IgE-Tg mice (Tg) and WT mice was challenged with TNP-OVA (Ag) or PBS(-). Skin tissues were excised on day 14 and subjected to ELISA following tissue homogenization. Vertical lines represent SD. Representative results of two independent experiments are shown. \**p* < 0.05 versus PBS(-) group.

and late-phase responses were similar to human prurigo reactions with regard to phenotypic changes and time course. Histopathologically, the lesions were characterized by irregular epidermal hyperplasia, hyperkeratosis, and a dense dermal cellular infiltrate comprising eosinophils, MOMA-2<sup>+</sup> monocytes/macrophages, and increased numbers of mast cells, along with dermal fibrosis (Fig. 1B, 1C). Substantial numbers of basophils also were observed in the dermis and occasionally in the epidermis, as detected by a basophil-specific Ab (TUG8) (25). Epidermal keratinocytes and the dermal cells showed nuclear translocation of phospho-STAT6 and phospho-STAT3; these results agreed with a recent report indicating that human prurigo lesions exhibit activation of the same transcription factors (32).

#### Cytokine profiles of prurigo-like skin lesions

To elucidate the immunological mechanism of prurigo-like inflammation, the levels of cytokines and related proteins in lesion-affected skin were measured. The cytokine profile of skin lesions showed increased levels of Th2-type cytokines, such as IL-4, IL-5, and IL-13, whereas low levels of IFN- $\gamma$  also were observed (Fig. 2). In addition, IL-17A and IL-22 were detected along with a marked increase in the levels of IL-18, IL-33, and TSLP. Enhanced expression of eotaxin-1 (CCL11) and eotaxin-2 (CCL24) appeared to be associated with dermal eosinophilia. Generation of

type I collagen was promoted, together with increased expression of TGF- $\beta$ 1.

#### Scratching behavior and sprouting of nerve fibers in prurigo-like skin lesions

We also analyzed prurigo-like skin lesions with regard to pruritus. Initially, we observed significant spontaneous scratching behavior when TNP-IgE-Tg mice received repeated Ag challenge to the ear lobes (Fig. 3A). Increases in local levels of IL-31 mRNA and NGF were noted (Fig. 3B). Immunohistochemical staining with PGP 9.5 Ab revealed significant nerve fiber sprouting in the epidermis (Fig. 3C). Of note, this was more marked at the periphery than in the center of the nodules. These findings could be explained by the results of immunohistochemical staining, which showed enhanced expression of amphiregulin and NGF (factors promoting elongation of nerve fibers) and reduced expression of semaphorin 3A (an inhibitor of neurite outgrowth) in the epidermis at peripheral lesions, whereas central lesions showed the opposite expression patterns. These features were somewhat similar to those of a prior finding of increased NGF and decreased semaphorin 3A expression in the epidermis in human prurigo lesions (33). Nevertheless, intriguingly, scratching was not necessary for the induction of inflammation, because inhibition of scratching by the introduc-

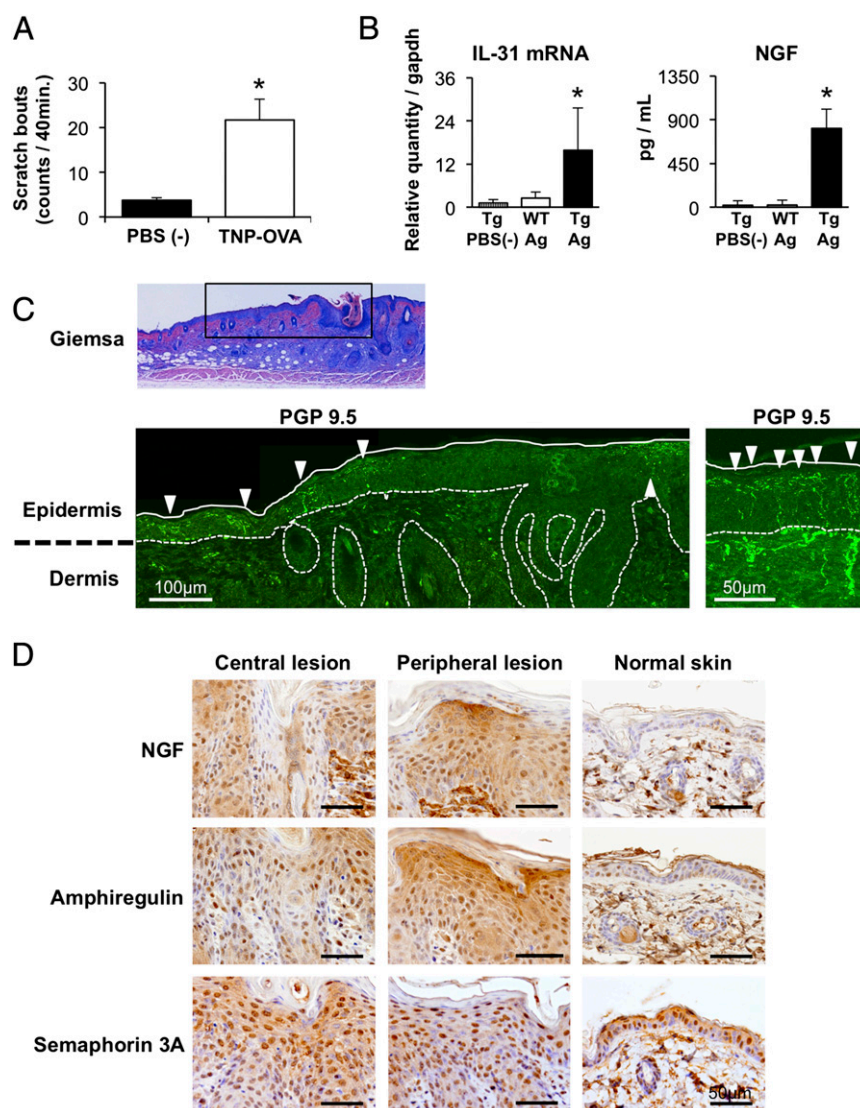


**FIGURE 3.** Prurigo-like skin lesions are pruritic. **(A)** Spontaneous scratching (day 14) of the ear lobes in TNP-IgE-Tg mice challenged with TNP-OVA or PBS(-). Each group consisted of at least four mice. **(B)** Dorsal skin lesions were excised on day 14 and homogenized, and samples were subjected to quantitative real-time PCR for IL-31 mRNA and ELISA for NGF protein. **(C)** PGP 9.5 staining of skin lesions (day 14). Sprouting of nerve fibers in the epidermis was observed at the periphery (arrow-heads) but not in the center of the lesions. **(D)** Marked expression of epidermal NGF and amphiregulin was apparent in peripheral lesions, rather than central lesions, and semaphorin 3A expression was downregulated in peripheral lesions. Visualized with 3'-diaminobenzidine tetrahydrochloride solution. Vertical lines indicate SD (A and B). Representative results of at least two independent experiments are shown. \*p < 0.05.

tion of an Elizabethan collar did not affect ear swelling responses (Supplemental Fig. 1).

*Basophils are essential for the development and maintenance of prurigo-like skin lesions*

We asked whether prurigo-like reactions were basophil dependent. To answer this question, TNP-IgE-Tg mice were treated with a basophil-depletion Ab (CD200R3; Ba103) (26) immediately after the second challenge (day 4). Administration of the basophil-depletion Ab significantly inhibited the development of skin lesions (Fig. 4A). Even when mice underwent basophil depletion after the development of skin lesions (day 8), skin inflammation was alleviated (Fig. 4B). We also observed apparent macroscopic and microscopic improvement of prurigo-like nodules on the dorsal skin when basophils were depleted (Fig. 4C).

*Th2-type immunity protects against the development of prurigo-like skin lesions*

The polarization of the cytokine profile toward Th2-type immune responses, together with basophil dependency, prompted us to examine the role of STAT6 signaling in the development of prurigo-like skin lesions. We initially induced skin inflammation in WT C57BL/6 mice through passive immunization with

TNP-IgE (days 0, 3, and 6), followed by challenge with TNP-OVA (days 1, 4, and 7). Mice showed persistent inflammation on their ears and papular lesions on the dorsal skin, although these lesions were not as pronounced and long-lasting as those observed in TNP-IgE-Tg mice. We then induced prurigo-like skin lesions in *STAT6*<sup>-/-</sup> mice. Unexpectedly, inflammation was exacerbated, rather than alleviated, in *STAT6*<sup>-/-</sup> mice compared with WT C57BL/6 mice (Fig. 5A). The negative regulatory role of STAT6 signaling in prurigo-like reactions was further confirmed by the finding that inflammation was exacerbated in WT mice treated locally with STAT6 siRNA (Fig. 5B). The prurigo-like lesions on dorsal skin were histopathologically characterized by significant irregular acanthosis and more marked cellular infiltration (including basophils) than were those from WT mice, with the exception that very few eosinophils were observed in *STAT6*<sup>-/-</sup> mice (Fig. 5C). The *STAT6*<sup>-/-</sup> mice produced higher levels of IL-4 and IL-13 than did the WT mice (Fig. 6). They also generated significant levels of IL-33 and TSLP compared with WT C57BL/6 mice, whereas the levels of eotaxin-1 (CCL11) and eotaxin-2 (CCL24) were decreased. Intriguingly, despite the exacerbated skin inflammation, spontaneous scratching behavior was less pronounced in *STAT6*<sup>-/-</sup> mice, which paralleled the decline in IL-31 mRNA levels in these mice (Supplemental Fig. 2).

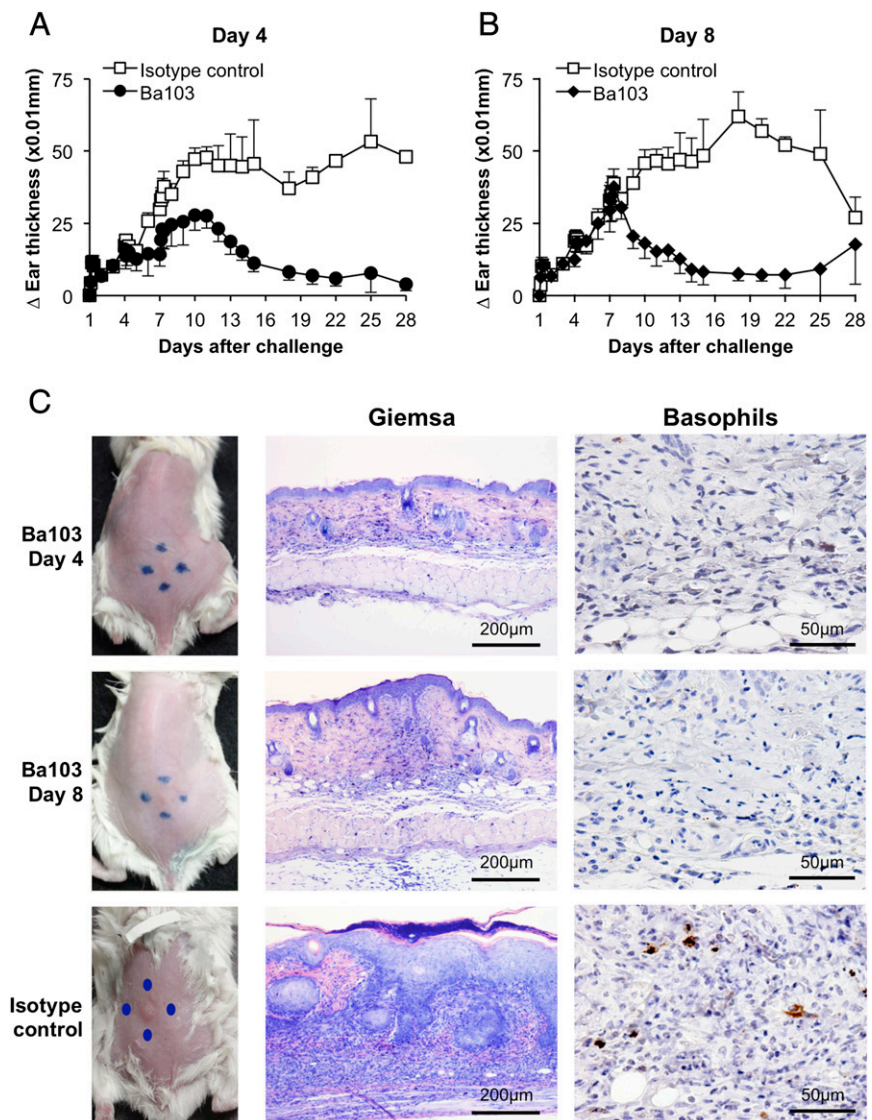


**FIGURE 4.** Basophil depletion alleviates prurigo-like inflammation. TNP-IgE-Tg mice challenged with TNP-OVA on the ear lobes on days 1, 4, and 7 were treated with basophil-depletion Ab (Ba103; CD200R3) (75  $\mu$ g/mouse, i.v.) on day 4 (**A**) or day 8 (**B**). Vertical lines indicate SD. Each group consisted of at least four mice. (**C**) Prurigo-like lesions on the dorsal skin also were improved by basophil depletion. Visualized with 3'-diaminobenzidine tetrahydrochloride solution. Representative results of three independent experiments are shown.

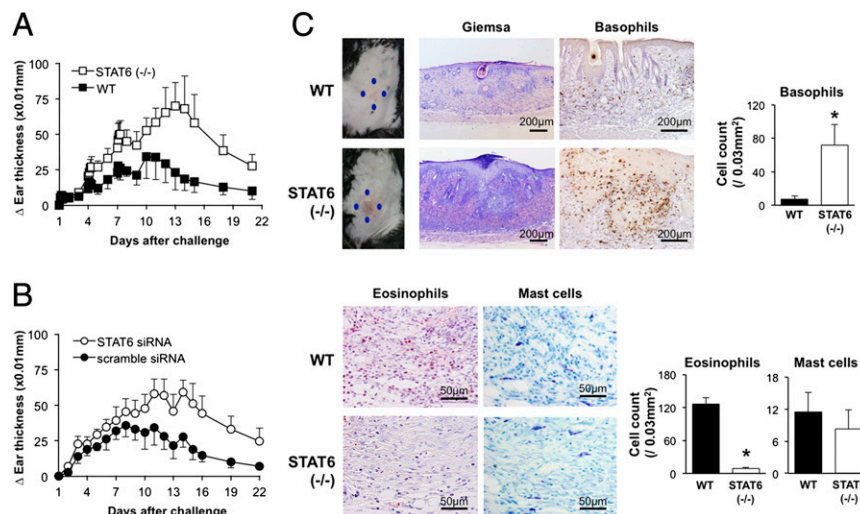


**FIGURE 5.** Exacerbated inflammation in *STAT6*<sup>-/-</sup> mice. **(A)** WT mice and *STAT6*<sup>-/-</sup> mice received repeated passive immunization with TNP-IgE (i.p.) and challenge with TNP-OVA (s.c.) on the ear lobes. WT mice showed substantial ear swelling, a response that was significantly exacerbated in *STAT6*<sup>-/-</sup> mice. **(B)** WT mice that received repeated passive immunization (days 0, 3, and 6) and challenge (days 1, 4, and 7) on the ear lobes were treated with either *STAT6* siRNA or scramble RNA (s.c.; days -1, 2, and 5). **(C)** Nodular lesions on the dorsal skin of *STAT6*<sup>-/-</sup> mice are more prominent than in WT mice. Histopathologically, *STAT6*<sup>-/-</sup> mice showed marked acanthosis and dermal cellular infiltration, including infiltration of basophils. In contrast, eosinophil infiltration was almost completely abolished in *STAT6*<sup>-/-</sup> mice. Visualized with 3'-diaminobenzidine tetrahydrochloride solution. Vertical lines represent SD. Each group consisted of at least four mice. Representative results of three independent experiments are shown. \**p* < 0.05.

#### Impaired induction of M2-type macrophages is responsible for the exacerbation of inflammation in *STAT6*<sup>-/-</sup> mice

In general, macrophages can be classified into two subtypes: the classically activated M1 type and the alternatively activated M2 type (34). Although M1-type macrophage responses are generated by the Th1 cytokine IFN- $\gamma$ , Th2 cytokines, such as IL-4 and IL-13, elicit M2-type macrophage responses. The *in vivo* role of M2-type macrophages appears to depend on the type of inflammation (26, 34, 35). Given the results from a recent study of IgE-vLPR (or IgE-CAI) that demonstrated M2-type macrophages have an anti-inflammatory function (36), we focused on determining the type of infiltrative macrophages observed in *STAT6*<sup>-/-</sup> mice in an effort to explain the unexpected observation of exacerbated prurigo-like inflammation in the absence of *STAT6* signaling. In skin

lesions of WT mice, we observed a remarkable number of cells expressing CD163, CD206, and/or Arg1, which are cell markers preferentially (but not exclusively) expressed by M2-type macrophages (36–41). In contrast, considerably fewer cells expressing these markers were observed in *STAT6*<sup>-/-</sup> mice (Fig. 7A, Supplemental Fig. 3). Impaired generation of M2-type macrophages in *STAT6*<sup>-/-</sup> mice was further confirmed by the results of flow cytometric analyses, which showed a decrease in the number of MOMA-2<sup>+</sup> cells expressing CD206 or Arg1 (Fig. 7B). In addition, local levels of Arg1 mRNA decreased in *STAT6*<sup>-/-</sup> mice, whereas an increase in iNOS mRNA, a marker of M1-type macrophages (40), was observed (Fig. 7C). In parallel with these observations, nuclear translocation of phospho-STAT6 in MOMA-2<sup>+</sup> and CD206<sup>+</sup> cells was abolished in *STAT6*<sup>-/-</sup> mice but not in WT mice (Fig. 7D).

We next examined the functional consequences of reconstituting *STAT6*<sup>-/-</sup> mice with WT monocytes. To this end, we initially attempted to adoptively transfer freshly isolated CD115<sup>+</sup> bone marrow monocytes from WT mice into the ear lobes ( $1 \times 10^6$  cells/ear) (36) of *STAT6*<sup>-/-</sup> mice, based on the assumption that this would allow the transferred monocytes to acquire an M2 phenotype in response to IL-4/IL-13 produced locally in *STAT6*<sup>-/-</sup> mice. As expected, CD115<sup>+</sup> cells (but not CD115<sup>-</sup> cells) from WT mice partially, but significantly, attenuated the inflammation in *STAT6*<sup>-/-</sup> mice (Fig. 8A). This attenuation was abolished when *STAT6*<sup>-/-</sup> mice were reconstituted with CD115<sup>+</sup> cells from *STAT6*<sup>-/-</sup> mice (Fig. 8B). Therefore, it is plausible that the exacerbated prurigo-like inflammation associated with the lack of *STAT6* signaling is due, in part, to impaired local generation of an anti-inflammatory M2-type macrophage response.

Basophils are capable of generating IL-4 and IL-13 (42). Accordingly, the observation that M2-type macrophage responses depend on *STAT6* signaling prompted us to assess the basophil dependency of these macrophages. As expected, basophil depletion with the Ba103 Ab abolished the induction of the M2-type macrophage response in skin lesions of TNP-IgE-Tg mice (Supplemental Fig. 4). This result agrees with a recent report indicating that, in ordinary IgE-vLPRs, generation of an M2-type macrophage response is dependent on basophil-derived IL-4 (36).

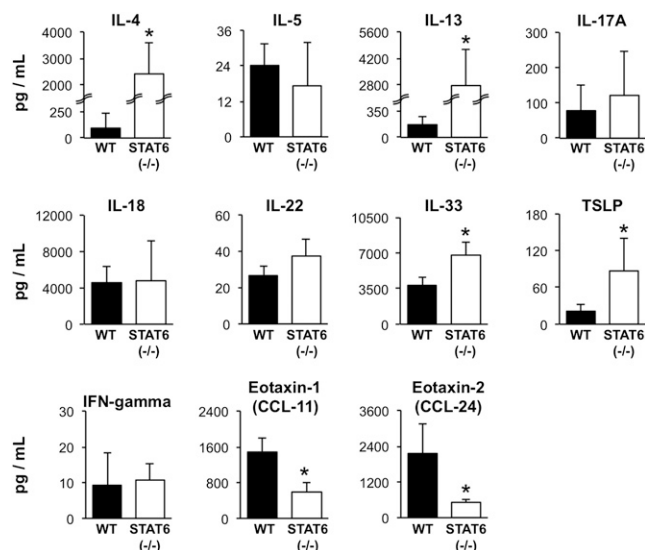


**FIGURE 6.** Local cytokine profiles of dorsal skin prepared on day 10 from WT mice and *STAT6*<sup>-/-</sup> mice. Vertical lines represent SD. Each group consisted of at least four mice. Representative results of three independent experiments are shown. \**p* < 0.05.

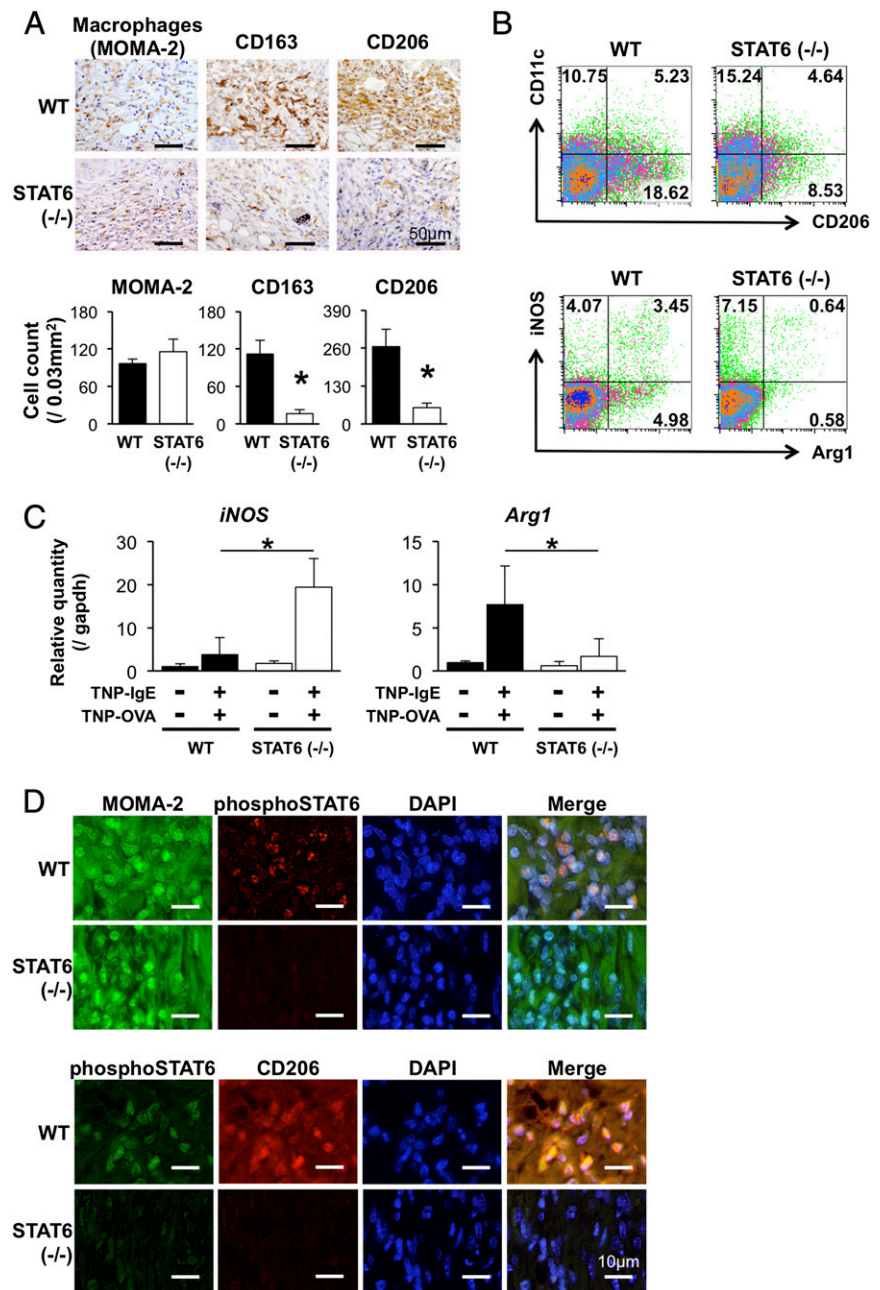


**FIGURE 7.** M2-type macrophages in prurigo-like skin lesions. **(A)** Immunohistochemical staining for macrophages. Very few macrophages positive for CD163 and/or CD206 were observed in *STAT6*<sup>-/-</sup> mice. **(B)** Flow cytometric analysis of dermal cells from inflamed skin on day 10. MOMA-2<sup>+</sup> cells were gated, followed by analysis of their expression of M2-type macrophage markers (CD206 and Arg1) and M1-type macrophage markers (CD11c and iNOS). **(C)** Arg1 and iNOS mRNA levels in inflamed skin excised on day 10. **(D)** Nuclear translocation of phospho-STAT6 in MOMA-2<sup>+</sup> and CD206<sup>+</sup> cells was observed in WT mice but not in *STAT6*<sup>-/-</sup> mice. \**p* < 0.05.

## Discussion

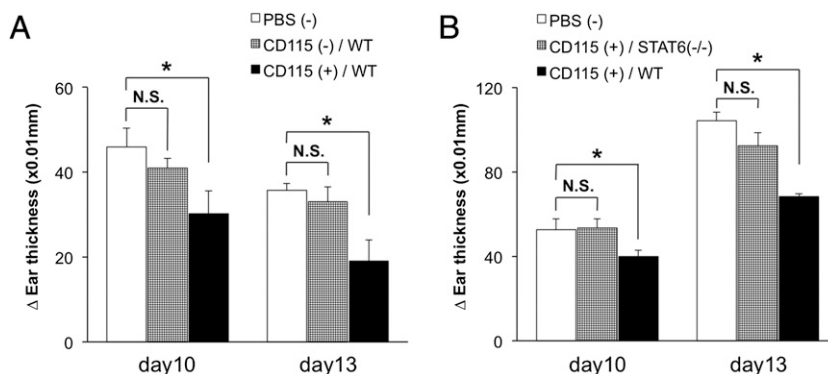
Prurigo is a pruritic skin disorder of unknown etiology. In this study, we showed that IgE-mediated skin inflammation develops into prurigo-like skin lesions in mice. Repeated administration of Ag into the skin of TNP-IgE-Tg mice induced immediate-type (urticarial) and late-phase responses, followed by persistent inflammation that led to the formation of papulonodular skin lesions lasting >1 mo; this, in principle, resembled some types of prurigo lesions in humans. This study also provided evidence that basophils are indispensable key players that link urticarial and papulonodular skin reactions. The fact that a number of basophils are present in long-lasting urticarial lesions and prurigo in humans (15) suggests that basophils, but not mast cells or T cells, play a major role in the pathogenesis of these diseases.

One of the major and troublesome symptoms of prurigo is itching. In the current study, prurigo-like lesions showed increased generation of IL-31 [a cytokine that induces itching (43)] and nerve fiber sprouting locally in the lesion, in association with enhanced

epidermal NGF and amphiregulin expression. As a consequence, we observed spontaneous scratching behavior due to itching in our prurigo-like mouse model. In addition, it was intriguing to note that intraepidermal nerve fiber sprouting was most marked at the periphery, rather than at the center, of nodules. In human prurigo lesions, contradictory results were reported regarding nerve fiber innervation in the epidermis (33, 44). These inconsistent results may be explained by the present mouse model demonstrating transitional changes in the intraepidermal nerve fiber density within lesions.

In the current study, generation of IL-4 and IL-13 in prurigo-like lesions was accompanied by increased production of IL-17A and IL-22. These data agree with a prior finding that the levels of IL-4, IL-17A, and IL-22 mRNA are increased in human prurigo lesions (45). Enhanced TGF-β1 generation could be derived, at least in part, from infiltrative eosinophils induced by eotaxin-1 (CCL11) and eotaxin-2 (CCL24), reflecting tissue remodeling as mirrored by increased type I collagen synthesis.

**FIGURE 8.** STAT6-dependent anti-inflammatory function of bone marrow monocytes. **(A)** *STAT6*<sup>-/-</sup> mice received transfer of either CD115<sup>+</sup> or CD115<sup>-</sup> bone marrow cells from WT mice into the ear lobes on days 1, 4, and 7. **(B)** *STAT6*<sup>-/-</sup> mice received transfer of CD115<sup>+</sup> bone marrow cells from either WT or *STAT6*<sup>-/-</sup> mice. Vertical lines represent SD. Each group consisted of at least four mice. Representative results of at least two independent experiments are shown. \**p* < 0.05.



Of note, local levels of IL-18 and IL-33 were markedly elevated in our experiments. Both IL-18 and IL-33 were shown to activate basophils and mast cells (42, 46). IL-33 also induces the generation of IL-5, IL-6, and IL-13 from type 2 innate lymphoid cells (47, 48), and skin-specific IL-33 expression elicits atopic dermatitis-like inflammation (49). Additional studies are needed to elucidate whether and how innate immune responses mediated by IL-18 and IL-33 contribute to the pathogenesis of prurigo-like reactions.

Th2-type immunity is considered an important and actual pathogenic process in several types of allergic inflammation. Previously, we demonstrated amelioration of contact hypersensitivity and allergic rhinitis by local administration of STAT6 siRNA and in *STAT6*-deficient mice (24, 31). We also reported the clinical benefits of *STAT6*-decoy oligodeoxynucleotide ointment in treating atopic dermatitis (50). Based on the observed bias of the cytokine profile toward Th2-type responses characterized by increased expression of IL-4 and IL-13, both of which can be produced by basophils, we expected to find that Th2-type immunity contributes significantly to the development of prurigo-like skin lesions and, therefore, that STAT6 would be a promising therapeutic target. In sharp contrast to our expectations, prurigo-like skin lesions in mice were exacerbated in the absence of STAT6 signaling. In parallel with these findings, histopathologically, basophil infiltration was enhanced, which might be associated with increased IL-33 and TSLP expression (46, 51) in *STAT6*<sup>-/-</sup> mice. In this regard, we observed that generation of an M2-type macrophage response was impaired in *STAT6*<sup>-/-</sup> mice. A recent study demonstrated that, in the IgE-vLPR (or IgE-CAI), circulating (but not resident) CD115<sup>+</sup> inflammatory monocytes acquire an anti-inflammatory M2 phenotype in response to basophil-derived IL-4. Consistent with this, in the current study, adoptive transfer of CD115<sup>+</sup> monocytes from WT mice, but not from *STAT6*<sup>-/-</sup> mice, resulted in the amelioration of inflammation in *STAT6*<sup>-/-</sup> mice, producing significant amounts of IL-4 in skin lesions. Hence, it is plausible that, in prurigo-like reactions, STAT6 signaling is involved in the generation of an M2-type anti-inflammatory monocyte response, thereby terminating or inhibiting excessive inflammation, as is the case in ordinary IgE-vLPRs (36).

Eosinophil infiltration in the dermis is a characteristic feature of human prurigo reactions, but the pathogenic role of eosinophils in allergic inflammation is still a matter of disagreement. We observed that *STAT6*<sup>-/-</sup> mice showed significantly diminished dermal eosinophilia, potentially due to a lack of eosinophil chemoattractant generation, in particular, generation of eotaxin-1 (CCL11) and/or eotaxin-2 (CCL24), which are principally secreted in response to IL-4 and/or IL-13 (52, 53). The observed exacerbation of inflammation, despite the absence of

dermal eosinophilia, suggests that eosinophils were unable to augment the prurigo-like inflammation and might have down-modulating functions.

The contribution of scratching to the development of prurigo diseases also has been a matter of debate. Intriguingly, *STAT6*<sup>-/-</sup> mice showed diminished scratching behavior in association with a reduction in the local levels of IL-31. This seemed somewhat consistent with the observation that inhibition of scratching did not affect inflammation (Supplemental Fig. 1). Scratching appears to be dispensable for developing prurigo-like reactions once triggering factors, such as allergens, are directly introduced into the dermis.

The present results in *STAT6*<sup>-/-</sup> mice highlight another important issue: Th2-type cytokines are not necessary for the initiation and development of prurigo-like lesions if IgE and basophils are present. Thus, a question remains unanswered: What are the essential factors for triggering immunological events in basophil-dependent prurigo-like reactions? In addition to histamine, basophils are capable of releasing several chemical mediators, such as mouse mast cell protease 11 (54), which may be involved in initiating inflammation. However, further and more detailed studies are needed to determine the actual and essential processes initiated and mediated by basophils independent of Th2 immunity.

Considering the mouse model shown in this study, it can be postulated that prurigo in human atopic dermatitis may be caused by basophil activation via interaction of specific IgE with environmental allergens that are repeatedly inoculated through the epidermis by mechanical processes, such as scratching. In contrast, in general, there are many other types of human prurigo in which IgE involvement is unlikely. In this regard, we may need to consider the possibility of an alternative basophil-activation pathway that acts independently of IgE and that may involve IL-18, IL-33, TSLP (51), and protease allergen-induced activation (17).

The present study provides novel insights into the pathological and immunological events associated with prurigo, although our mouse model does not represent all types of human prurigo reactions. Basophils participate in the initiation and prolongation of inflammation, leading to the formation of papulonodular skin lesions; however, paradoxically, therapies against Th2-type immune responses possibly mediated by basophils may risk aggravating prurigo reactions.

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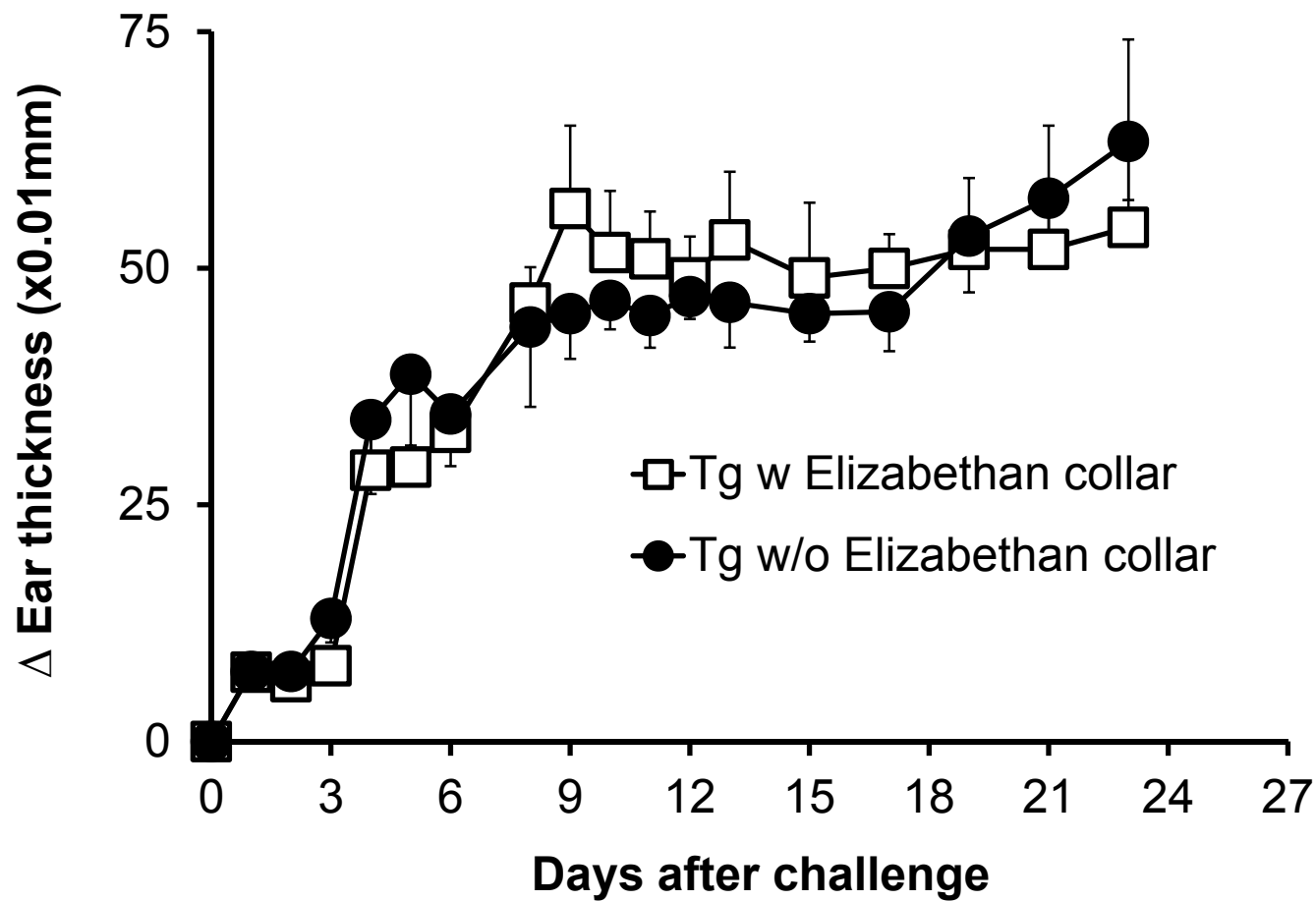
## Disclosures

The authors have no financial conflicts of interest.

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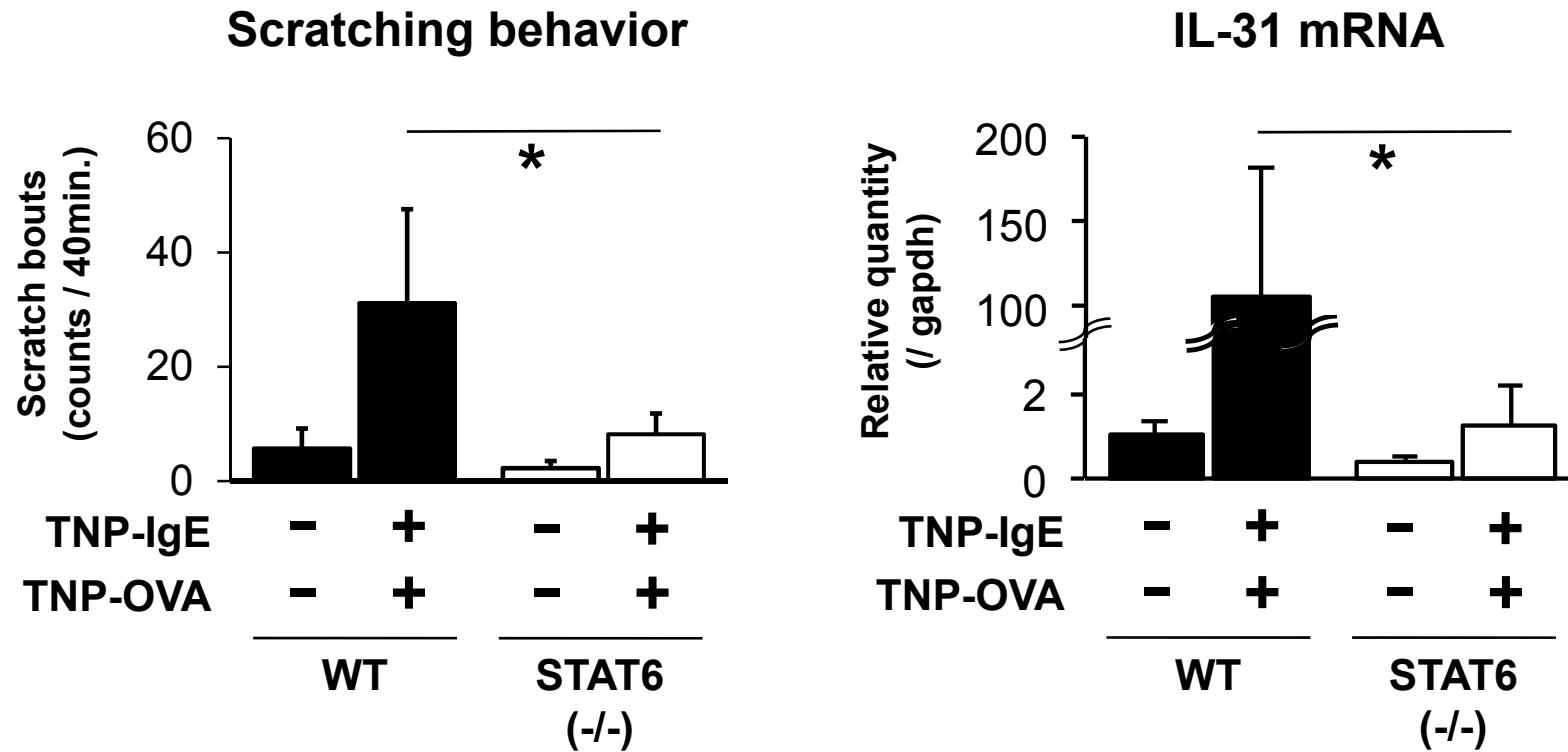
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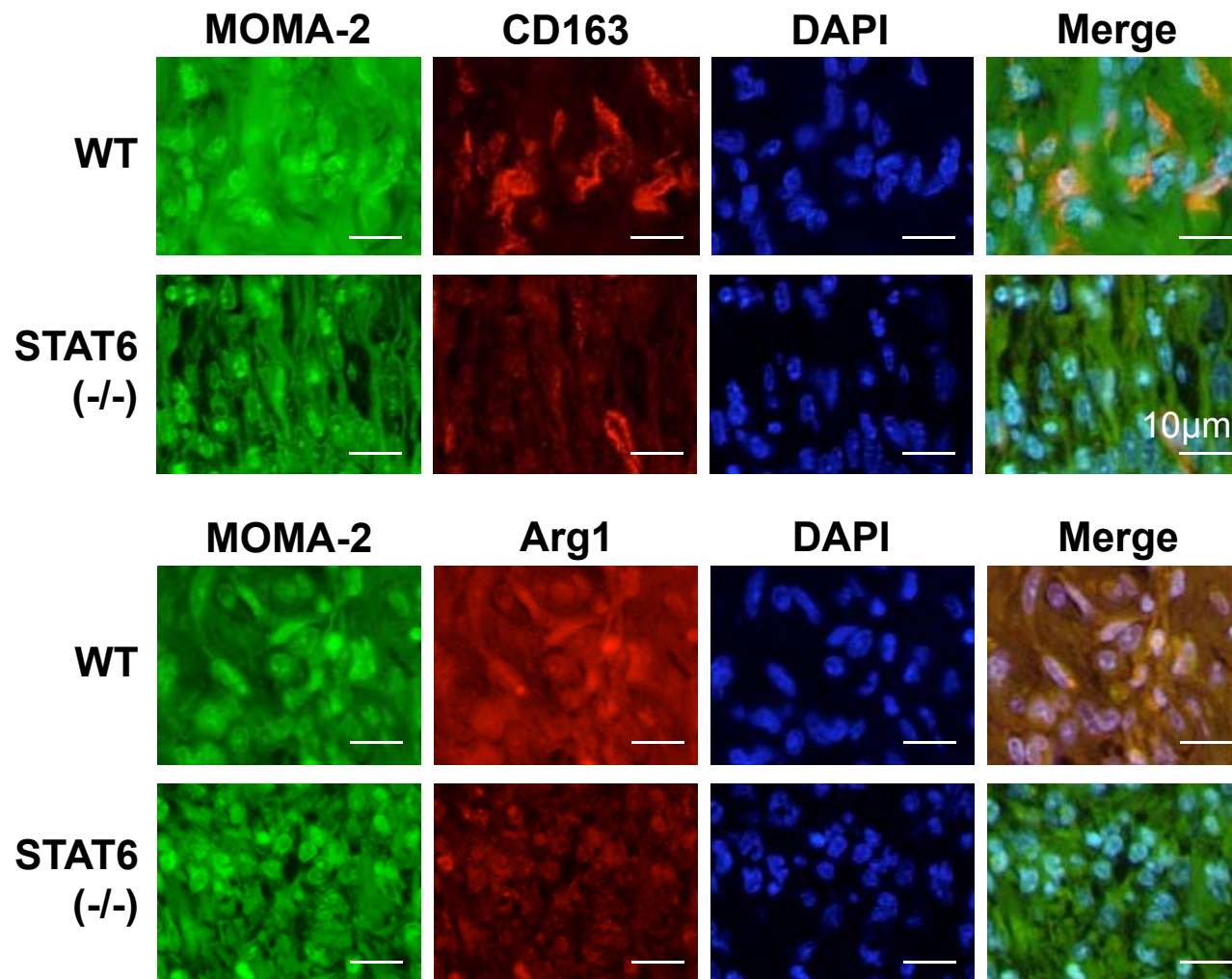
**Supplemental figure 1: Scratching was not essential for the inflammation.**

IgE transgenic mice (Tg) were repeatedly challenged on ear lobes in the presence (w) or absence (w/o) of Elizabethan collar.

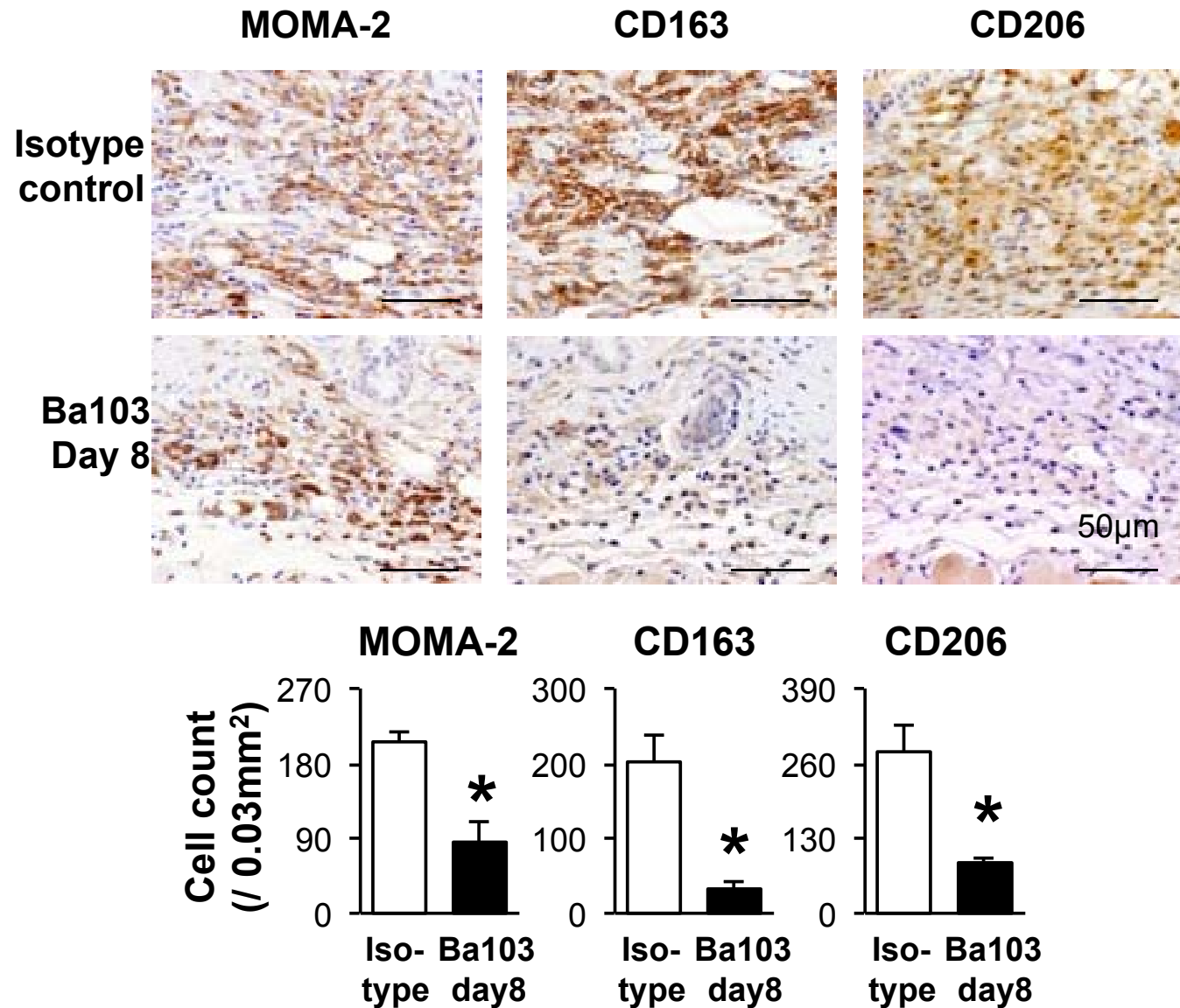


**Supplemental figure 2: Alleviation of pruritus in *STAT6* (-/-) mice.**

Scratching behavior and IL-31mRNA were reduced in the absence of STAT6.



**Supplemental figure 3: Impaired generation of M2-type macrophages in *STAT6* (-/-) mice.**



**Supplemental figure 4: Basophil-depletion Ab (Ba103) (day 8) abolished the generation of M2-type macrophages.**