Mechanisms of Itch in Stasis Dermatitis: Significant Role of IL-31 from Macrophages

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Stasis dermatitis (SD) is a common disease in the elderly population, with pruritus being one of the troublesome symptoms. However, there are few therapeutic modalities available for SD-associated itch because little is known about its pathophysiological mechanism. Therefore, we sought to investigate the mediators of itch in SD using an immunofluorescence study on patient lesions focusing on IL-31. Ex vivo stimulation studies using murine peritoneal macrophages were also used to elucidate the pathological mechanisms of the generation of IL-31. In SD lesions, dermal infiltrating IL-31(+) cells were increased in number compared with the healthy controls, and the majority of IL-31(+) cells were CD68(+) macrophages. The presence of itch in SD was significantly associated with the amount of CD68(+)IL-31(+) macrophages and CD68(+)CD163(+) M2 macrophages. The number of CD68(+)IL-31(+) macrophages was correlated with the number of dermal C-C chemokine receptor type 4(+) T helper type 2 cells, IL-17(+) cells, basophils, substance P(+) cells, and dermal deposition of periostin and hemosiderin. Furthermore, murine peritoneal macrophages expressed an M2 marker arginase-1 and generated IL-31 when stimulated with a combination of substance P, periostin, and red blood cell lysate (representing hemosiderin). IL-31 from macrophages may play a role in itch in SD.

INTRODUCTION

Stasis dermatitis (SD) is a common disease that predominantly affects the lower legs of elderly patients, with a prevalence of 6.2% among those older than 65 years (Yalcın et al., 2006). The main causes of SD are chronic venous insufficiency and venous hypertension. Venous hypertension promotes the cellular accumulation of inflammatory cells (e.g., T cells and macrophages) and extravasation of red blood cells (RBCs) in the affected skin (Saharay et al., 1997; Thomas et al., 1988). Extravasation and disruption of the RBCs are followed by decomposition of hemoglobin, which results in excessive tissue iron stored as hemosiderin (Caggiati et al., 2010). Hemoglobin and hemosiderin can further induce monocyte/macrophage-recruitment through hemoglobin scavenger receptor CD163 (Rubio-Navarro et al., 2015). Accumulating macrophages and other cells promote inflammation and induce the abnormal histological features of SD (e.g., epidermal spongiotic changes, papillary structure alternation, and capillary proliferation) partially through the secretion of proteolytic enzymes (Sundaresan et al., 2017; Wenk et al., 2001).

One of the troublesome symptoms reported in SD is pruritus. In an elderly population, the dermatosis that was most commonly reported to cause the complaint of itch was SD (Valdes-Rodriguez et al., 2015). Itch in SD not only impairs the patients’ quality of life but also induces scratching, which aggravates wounds and increases the risk of skin infection. Unfortunately, there are few therapeutic modalities available for itch because little is known about the pathophysiological mechanisms of SD-associated itch.

The phenomenon of itch is divided into two subgroups: histaminergic and non-histaminergic (Yosipovitch et al., 2018). Itch in SD appears to be non-histaminergic, as anti-histamines do not necessarily improve SD-associated itch. Non-histaminergic itch involves various itch mediators, including cytokines/chemokines (e.g., interleukin [IL]-31), amines, proteases and their associated receptors (e.g., protease-activated receptor-2), neuropeptides and receptors (e.g., substance P [SP] and its receptor neurokinin-1 receptor [NK1R]), ion channels (e.g., transient receptor potential ankyrin-1 and vanilloid-1), and immune cells (e.g., T cells, mast cells, eosinophils, and basophils).

IL-31, a T helper type (Th)2-related pruritogenic cytokine, has recently gained attention as a potential therapeutic target for inflammatory skin conditions with itch (Furue et al., 2018). IL-31 is involved in itch in various diseases, such as atopic dermatitis (Nattkemper et al., 2018; Sonkoly et al., 2006), prurigo nodularis (Sonkoly et al., 2006), psoriasis
IL-31 exerts its function through its receptor complex, comprising IL-31RA and oncostatin M receptor β (OSMRβ) (Sonkoly et al., 2006). Blocking IL-31RA has been shown to improve itch in atopic dermatitis (Kabashima et al., 2018). However, the involvement of IL-31 and its receptor complex in SD-associated itch is unknown. Thus, we sought to investigate the pathophysiological mechanisms of SD-associated itch focusing on IL-31 and other major itch mediators through an immunofluorescence study of patient lesions. In addition, we sought to elucidate the pathological mechanisms of IL-31 generation through ex vivo stimulation studies using murine peritoneal macrophages.

**RESULTS**

**CD68(+) macrophages express IL-31 and correlate with the presence of itch in SD**

In SD lesions, the number of dermal infiltrating IL-31(+) cells was significantly increased compared to healthy subjects (t-test, P = 0.001), while epidermal expression was not (t-test, P = 0.80). In addition, SD with severe itch, which was defined as a level 4 itch using the Likert scale from zero (no itch) to 4 (severe itch), showed greater infiltration of IL-31(+) cells in the dermis compared to SD without severe itch, but no statistical significance was found (t-test, P = 0.16) (Figure 1a).

Next, we investigated the cellular sources of IL-31. We detected a massive dermal infiltrate comprising various types of immune cells (Figures 1a). Most common among them...
were CD68(+) macrophages, with the number of these cells significantly increased in SD lesions than healthy controls (t-test, \( P = 0.0016 \)) (Figure 1a). Therefore, we then examined IL-31 expression by macrophages. As expected, the majority of IL-31(+) cells were CD68(+) cells. In addition, the number of IL-31(+) / CD68(+) cells was significantly associated with the presence of severe itch, whereas the number of CD68(+) cells was approximately the same in patients with and without severe itch (average ratio of IL-31(+) / CD68(+) cells to CD68(+) cells was 88.2% in patients with severe itch vs 46.5% in patients without severe itch; t-test, \( P = 0.0096 \)) (Figure 1b).

Macrophages are generally divided into two groups: M1 and M2 (Wang et al., 2014). Our group previously reported that M2 macrophages are capable of generating IL-31 (Hashimoto et al., 2019b). Thus, we examined the expression of an M2 marker, CD163, by CD68(+) cells. The number of CD163(+) / CD68(+) cells was increased in SD with severe itch compared with SD without severe itch (t-test, \( P = 0.004 \)) (Figure 1c).

**Lesional expression of IL-31RA and OSMRβ**

Both the epidermal and dermal expression of IL-31RA was increased in SD lesions compared with healthy controls (t-test, \( P < 0.0005 \) and \( P = 0.005 \), respectively) but were not associated with the presence of severe itch (t-test, \( P = 0.74 \) and 0.58, respectively). OSMRβ expression in the epidermis and the dermis was not enhanced in SD lesions compared with healthy skin (t-test, \( P = 0.95 \) and 0.086, respectively) and was not associated with the presence of severe itch (t-test, \( P = 0.18 \) and 0.78, respectively) (Figure 2).

**Th2 and Th17 immune responses are predominant in SD lesions and correlated with IL-31 expression by macrophages**

M2-macrophage skewing is closely related with Th2 immunity (Wang et al., 2014). The number of dermal cells expressing C-C chemokine receptor type 4, which is preferentially expressed by Th2 cells, increased in SD lesions compared with healthy controls (t-test, \( P = 0.0026 \) and correlated with the number of CD68(+)/IL-31(+) cells (Spearman’s correlation, \( r = 0.453 \), \( P = 0.020 \)). The number of IL-17(+) cells was also increased in the SD lesions compared with healthy skin (t-test, \( P = 0.0045 \)) and correlated with the number of CD68(+)/IL-31(+) cells (Spearman’s correlation, \( r = 0.405 \), \( P = 0.404 \)). In contrast, the number of cells that expressed C-X-C chemokine receptor type 3, a preferential marker for Th1 cells, did not change between SD and the healthy controls (t-test, \( P = 0.698 \)) and was not correlated with the number of CD68(+)/IL-31(+) cells (Spearman’s correlation, \( r = 0.326 \), \( P = 0.104 \)), even though there was a difference in the number of C-X-C chemokine receptor type 3(+) cells between the SD lesions with severe itch and those without severe itch (t-test, \( P = 0.036 \)).

Basophils and eosinophils are involved in Th2 immunity (Hashimoto and Satoh, 2018). The number of dermal basophils was increased in SD lesions compared with healthy controls (t-test, \( P < 0.0005 \)) and correlated with the number of CD68(+)/IL-31(+) cells (Spearman’s correlation, \( r = 0.603 \), \( P = 0.001 \)). The number of dermal eosinophils was neither increased in SD compared with healthy controls (t-test, \( P = 0.106 \)) nor correlated with the number of CD68(+)/IL-31(+) cells (Spearman’s correlation, \( r = 0.145 \), \( P = 0.481 \)).

**Periostin, substance P, and hemosiderin, but not thymic stromal lymphopoietin, are correlated with IL-31 expression by macrophages**

Thymic stromal lymphopoietin and periostin promote M2 skewing (Furudate et al., 2016; Han et al., 2013) and IL-31 generation from M2 macrophages (Hashimoto et al., 2019b). Epidermal expression of thymic stromal lymphopoietin was not enhanced in the SD lesions compared with healthy controls (t-test, \( P = 0.182 \)). It was also not associated with the presence of severe itch (t-test, \( P = 0.355 \)) or with the number of CD68(+)/IL-31(+) cells (Spearman’s correlation, \( r = 0.213 \), \( P = 0.295 \)). In contrast, the dermal deposition of periostin was increased in SD lesions compared with healthy controls (t-test, \( P < 0.001 \)) and correlated with the number of CD68(+)/IL-31(+) cells (Spearman’s correlation, \( r = 0.552 \), \( P = 0.003 \)) but was not directly related to the presence of severe itch (t-test, \( P = 0.803 \)).

SP and hemosiderin are also capable of promoting M2 skewing (Leal et al., 2015; Lim et al., 2017; Rubio-Navarro et al., 2015). The number of dermal SP(+) cells and dermal deposition of hemosiderin were increased in the SD lesions compared with healthy controls (t-test, \( P < 0.001 \) and \( P = 0.005 \), respectively) and correlated with the number of CD68(+)/IL-31(+) cells (Spearman’s correlation, \( r = 0.463 \), \( P = 0.017 \) and \( r = 0.506 \), \( P = 0.008 \), respectively) but was not related to the presence of severe itch (t-test, \( P = 0.802 \) and 0.859, respectively) (Figure 4).

**CD68(+) macrophages express NK1R**

SP exerts its function mainly, but not exclusively, through its receptor NK1R (Azimi et al., 2017; Ständer and Yosipovitch, 2019). SP stimulates NK1R expressing cells, resulting in the release of additional itch mediators and evoking itch (Yosipovitch et al., 2018). Epidermal and dermal expression of NK1R was enhanced in SD compared with healthy controls (t-test, \( P = 0.001 \) and \( P < 0.001 \), respectively) but was not associated with the presence of severe itch (t-test, \( P = 0.483 \) and 0.559, respectively) (Figure 5a). Of note, almost all the CD68(+) macrophages expressed NK1R (Figure 5b).

**Skin innervation and other itch mediators**

Intraepidermal nerve fibers (IENF) are involved in itch (Pereira et al., 2016). We found reduced IENF density in the SD lesions compared with healthy controls (t-test, \( P = 0.010 \)) but no association between IENF density and the presence of severe itch (t-test, \( P = 0.28 \)).

Mast cells can release various itch mediators, including histamine, prostaglandins, and proteases (Steinhoff et al., 2018). The number of dermal mast cells did not change between SD and healthy controls, or between SD with severe itch and SD without severe itch.

Protease-activated receptor 2 is a representative receptor for non-histaminergic itch (Steinhoff et al., 2003). The epidermal expression of protease-activated receptor 2 was not enhanced in SD compared with healthy controls (t-test, \( P = 0.734 \)) and was reduced in SD without severe itch compared to SD with severe itch (t-test, \( P = 0.022 \)).
Transient receptor potential ankyin-1 and transient receptor potential vanilloid-1 are ion channels that are involved in itch (Kittaka and Tominaga, 2017). The epidermal expression of transient receptor potential ankyin-1 and transient receptor potential vanilloid-1 was neither enhanced in SD compared with healthy controls (t-test, $P_{\text{0.071}}$ and 0.283, respectively) nor related to the presence of severe itch (t-test, $P_{\text{0.375}}$ and 0.139, respectively) (Supplementary Figure S1).

Murine peritoneal macrophages generate IL-31 in response to the combination of substance P, peristin, and red blood cell lysate

To elucidate the mechanisms of IL-31-generation from macrophages, we employed an ex vivo stimulation test with murine peritoneal macrophages (pM) (Hashimoto et al., 2019b). pM were stimulated with substance P, peristin, and/or RBC lysate, which represents hemosiderin. Stimulation with SP alone did not significantly increase IL-31 mRNA expression in pM (Supplementary Figure S2). In contrast, stimulation with peristin alone or peristin and SP significantly increased IL-31 mRNA expression. Of note, stimulation with the combination of SP, peristin, and RBC lysate dramatically enhanced IL-31 mRNA expression (Figure 6a). The protein expression of IL-31 was confirmed with flow cytometric analysis. Flow cytometric analysis also revealed that MOMA-2(+) /IL-31(+) pM express an M2 marker arginase-1, indicating that M2 macrophages generate IL-31 in response to a combination of SP, peristin and hemosiderin (Figure 6b, c).
DISCUSSION

This data showed that dermal IL-31 appeared to play a significant role in itch in SD. Notably, the majority of IL-31-expressing cells were CD68(+) macrophages. It is generally established that IL-31 is generated mainly by activated Th2 cells (Dillon et al., 2004; Sonkoly et al., 2006). Other cellular types are also capable of generating IL-31, including macrophages (Cornelissen et al., 2011), eosinophils (Kunsleben et al., 2015), mast cells (Niyonsaba et al., 2010), basophils (Raap et al., 2017), and keratinocytes (Nattkemper et al., 2016). Among these, macrophages are reported to be the main cellular source in human skin lesions of scabies (Hashimoto et al., 2019b), polymorphic light eruption (Patra et al., 2019), and atopic dermatitis (Kato et al., 2014).

In SD lesions with severe itch, the majority of CD68(+)IL-31(+) macrophages expressed CD163, indicating they were M2 macrophages. In SD lesions without severe itch, a smaller population of CD68(+) macrophages expressed CD163. These findings are congruent with our previous study in scabies lesions with severe itch, which showed that dermal accumulating M2 macrophages are the main cellular sources of IL-31 (Hashimoto et al., 2019b).

M2-macrophage polarization is promoted by Th2 immunity (Wang et al., 2014). We found that a significant number of C-C chemokine receptor type 4(+) Th2 cells and basophils infiltrated the SD lesions. The dermal deposition of periostin, a Th2-related protein (Masuoka et al., 2012), was also enhanced. These findings indicate Th2 immunity-predominance in the SD lesions. Even though these factors were not directly associated with the presence of itch, they were significantly correlated with the number of CD68(+)IL-31(+) cells.
Interestingly, the number of IL-17(+) cells was increased in the SD lesions, indicating that Th17 immunity is also predominant in SD. The number of IL-17(+) cells was not directly related to the presence of itch, but it was significantly correlated with the number of CD68(+) cells. The dermal deposition of both peristin and hemosiderin, as well as the number of substance P(+) cells, was increased in the SD lesions and correlated with CD68(+) cells. All the aforementioned factors were not significantly higher in SD with severe itch compared to SD without severe itch. Bar = 100 μm for peristin and substance P. Bar = 200 μm for hemosiderin. *p < 0.05, unpaired t-test.

Figure 4. Peristin, hemosiderin, and substance P, but not TSLP, are correlated with the presence of IL-31(+) macrophages. Representative images of SD lesion and healthy skin with the quantification of staining. The epidermal expression of TSLP was not enhanced in the SD lesions and did not correlate with the number of CD68(+) cells. The dermal deposition of both peristin and hemosiderin, as well as the number of substance P(+) cells, was increased in the SD lesions and correlated with CD68(+) cells. All the aforementioned factors were not significantly higher in SD with severe itch compared to SD without severe itch. Bar = 100 μm for TSLP and substance P. Bar = 1 mm for peristin, and bar = 200 μm for hemosiderin. *p < 0.05, unpaired t-test. Dotted lines indicate the dermo-epidermal junction. Vertical bars indicate standard deviation. AU, arbitrary unit; NS, not significant; SD, stasis dermatitis; TSLP, thymic stromal lymphopoietin; w/, with; w/o, without.

We also investigated other itch mediators and IENF density in SD. Decreased IENF density is reported to be involved in chronic itch in many skin diseases, such as AD, psoriasis, and prurigo nodularis (Schuhknecht et al., 2011; Tan et al., 2019). IENF density was also reduced in SD lesions. No significant changes were observed in the number of mast cells and in the expression of protease-activated receptor 2, transient...
receptor potential ankyrin-1, and transient receptor potential vanilloid-1 between SD and healthy controls. Therefore, we conclude that these factors do not significantly contribute to itch in SD.

IL-31 directly stimulates peripheral nerve fibers through IL-31RA and OSMRβ (Cevikbas et al., 2014). In addition, IL-31 stimulates IL-31RA expressing cells, including macrophages, basophils, and keratinocytes, to enhance inflammation (Nakashima et al., 2018). Our study showed the over-expression of IL-31RA, but not OSMRβ, both in the epidermis and the dermis. Targeting IL-31 itself or IL-31RA may be beneficial in treating itch in SD. Macrophages could also be potential therapeutic targets, in addition to SP and NK1R, periostin, and hemosiderin. However, these targets require further consideration. In wound sites, M2 macrophages contribute to wound healing through the promotion of fibrosis and angiogenesis (Kim and Nair, 2019; Snyder et al., 2016). SD is often accompanied by wound and chronic venous ulcers (Sundaresan et al., 2017). The inhibition of M2-skewing might result in delayed wound healing. This point should be examined in future studies.

The key limitation of this study was the lack of patients’ numerical rating scale scores for itch, and we were not able to calculate the correlation between protein expression and itch severity. However, this study provides insights into the pathophysiological mechanisms of itch in SD. IL-31 and IL-31RA can be useful therapeutic targets for itch in SD.

**MATERIALS AND METHODS**

**Samples**

Skin lesional biopsy specimens and clinical data were obtained from 20 patients with SD (age, 42-93 years old; 5 males and 15 females) and from six non-itchy healthy control subjects (age, 25-62; four males and two females) at Tokyo Medical and Dental University Hospital and University of Miami Hospital. Their diagnoses were confirmed based on clinical and histological findings. This study was approved by the ethical committees of Tokyo Medical and Dental University (#M2018-033; informed written consent was not required because the specimens were de-identified) and the University of Miami (no IRB number; informed written consent was not required as tissue was collected from a de-identified repository and considered non-human research). Based on the presence of itch according to their medical information, the subjects were divided into two groups: with severe itch (n=9; ages, 44-93; three males and six females) and without severe itch (n=11; ages, 42-85; two males and nine females). Severe itch was defined as scale 4 in the Likert scale.

![Figure 5. CD68(+) macrophages express neurokinin-1 receptor.](image-url) Representative images of SD lesions and healthy skin with quantification of staining. (a) Both epidermal expression of neurokinin-1 receptor (NK-1R) and the number of dermal NK-1R(+) cells were increased in SD lesions but did not correlate with severe itch. (b) CD68(+) cells expressed NK-1R. *P < 0.05, unpaired t-test. Dotted lines indicate the dermo-epidermal junction. Vertical bars indicate standard deviation. AU, arbitrary unit; NK-1R, neurokinin-1 receptor; NS, not significant; SD, statis dermatitis; w/, with; w/o, without. Bar = 100 μm.
from zero (no itch) to 4 (the worst), and without severe itch was defined as scales zero to three. All patients had no other pruritic skin disorders.

Mice
Seven-week-old female C57BL/6 mice were obtained from Sankyo Lab Service (Tokyo, Japan). The mice were maintained under specific-pathogen-free conditions in our animal facility. All animal experiments were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University (Protocols A2018-299A, A2018-199A, A2018-298A, and A2018-237A) and performed at the Tokyo Medical and Dental University.

Antibodies
Antibodies (Abs) used in this study are listed in the Supplementary Material.

Immunofluorescence and iron staining
Formalin-fixed paraffin-embedded samples (5-μm) were deparaffinized and pretreated with Dako target retrieval solution (Dako, Glostrup, Denmark) at 60 °C overnight. The slides were then treated with phosphate buffer solution with 5% normal donkey serum and 0.2% Triton X-100 for 2 hours at room temperature. Next, sections were incubated with primary Abs at 4 °C overnight followed by reaction with Alexa Fluor 488- or 594-conjugated secondary Abs (Molecular Probes, Eugene, OR). The samples were then mounted with Vectashield with DAPI (Vector Laboratories, Burlingame, CA). For hemosiderin detection, Prussian blue staining was used.

Quantification
Photomicrographs were captured with a CTR6000 microscope (Leica Microsystems, Wetzlar, Germany) at original magnification ×20, and three images from each subject were analyzed. For periostin detection, whole scanning images were analyzed. The epidermal expression and dermal periostin deposition were measured as fluorescence intensity in arbitrary units normalized by area and background fluorescence using Image J software (NIH, Bethesda, MD). The number of dermal infiltrating cells was manually quantified. The IENF density was calculated by dividing the number of neuron specific marker β-tubulin III(+) nerves crossing the dermo-epidermal junction by the length of the epidermis (Sanders et al., 2019).

Preparation of murine peritoneal macrophages
Peritoneal cells were collected from C57BL/6N mice, seeded at the concentration of 5×10^5/well in plates in RPMI-1640 complete medium supplemented with 10% fetal bovine serum and 100 IU/mL penicillin-streptomycin and incubated for 2 hours at 37 °C and 5% CO₂. The non-adherent cells were washed out, and the remaining adherent cells (>80% of macrophages) were incubated with or without substance P (1 μM; Peptide institute, Osaka, Japan), recombinant periostin (20 ng/mL; eBioscience, San Diego, CA), and/or RBC lysates (5% volume of total medium volume). After 24 hours, the cells were subjected to total RNA extraction or flow cytometric analysis. The RBC lysates were prepared as follows: murine whole blood was collected and centrifuged, and serum were removed. The blood was frosted and thawed twice.

Real-time PCR
Total cellular RNA was extracted from cells using ISOGEN II (Nippon Gene Co., Tokyo, Japan), reverse-transcribed with SuperScript IV VILO Master Mix (Thermo Fisher Scientific), and quantitative reverse transcriptase-PCR was performed by real-time monitoring of the increase in fluorescence of SYBR Green dye (Brilliant SYBR Green QPCR Master Mix; Agilent Technologies Japan, Ltd., Tokyo, Japan) using the AriaMx Real-Time PCR System (Agilent Technologies). The
Flow cytometric analyses

Single cell-suspensions of cultured pM were obtained using cell scrapers after fixation. They were pretreated with anti-CD16/32 Ab (BioLegend, San Diego, CA) and labeled with monocye/macrophage-2 PE conjugated Ab (BioRad, Hercules, CA), anti-argininase-1 Ab (Abcam plc, Cambridge, UK: ab92274), and anti-IL-31 Ab (abcam: ab102750) followed by reaction with Alexa Fluor 647 anti-goat IgG- and Alexa Fluor 488 anti-rabbit IgG- secondary Abs (Abcam plc, ab150131 and ab150061, respectively) using Intracellular Fix & Perm set (eBioscience). They were then analyzed with FACSCalibur cell analyzer (BD Biosciences, San Jose, CA).

Statistical analysis

All data are reported as the mean ± Standard deviation. In order to compare the differences between two groups, two-tailed, unpaired t-tests were used. For detecting correlation, we calculated Spearman’s rank correlation coefficient (r) using a statistical software “EZR” hosted at Mendeley Data.

Data Availability Statement

Datasets related to this article can be found at https://doi.org/10.17632/bmmkj2psfz.1, an open-source online data repository hosted at Mendeley Data.

CONFLICT OF INTEREST

GY serves on the Scientific Boards of Menlo, Trevi, Siena, Sanofi, Regeneron, Galderma, Pfizer, Novartis, Bayer, Kiniksa, Eli Lilly, and Ortho. Research support was provided by Pfizer, Sun Pharma, Leo, Menlo, and Kiniksa. The other authors state no conflicts of interest.

ACKNOWLEDGMENTS

The authors thank Ms. Chiyako Miyagishi at Tokyo Medical and Dental University for technical assistance. This work was supported by a Japan Society for the Promotion of Science (JSPS) KAKENHI Grant-in-Aid for Young Scientists (B) (#17K16328) and by an unrestricted fellowship grant from Menlo Therapeutics.

AUTHOR CONTRIBUTIONS

Conceptualization: TH, GY. Data curation: TH. Formal analysis: TH. Funding acquisition: TH, HY, GY. Investigation: TH, CK, RF, SN, SS. Methodology: TH, LN. Project administration: TH, LN, HY, GY. Resources: TH, LN, HY. Supervision: LN, HY, GY. Validation: TH, GY. Visualization: TH, GY. Writing — Original Draft Preparation: TH, Writing — Review and Editing: TH, CK, RF, SN, SS, LN, HY, and GY. All the authors have read the manuscript and have approved this submission.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2019.09.012.

REFERENCES


SUPPLEMENTARY MATERIALS AND METHODS

Antibodies
Antibodies (Abs) obtained from Abcam plc (Cambridge, UK) included: Anti-mast cell tryptase (AAI; ab2378), IL-31 (ab102750), IL-31RA (ab113498), C-X-C chemokine receptor type 3 (ab64714), C-C chemokine receptor type 4 (ab1669), IL-17 (ab79056), CD68 (KP1; ab955), CD163 (ab182422), basophil (2D7; ab155577), Substance P (ab106291), thymic stromal lymphopoietin (ab188766), periostin (ab14041), transient receptor potential vanilloid 1 (ab3487), transient receptor potential ankyrin 1 (ab62053), and arginase-1 (ab92274) Abs. Anti-neurokinin 1 receptor (PA3-301), protease-activated receptor 2 (sc-5597), β-tubulin III (TuJ1; Mo15013), major basic protein (MBP) (NBP1-42140-1ml), oncostatin M receptor β (LS-B11477) Abs were obtained from ThermoFisher Scientifics (Waltham, MA), Santa Cruz Biotechnology (Dallas, TX), Neuromics (Edina, MN), Novus biologics (Centennial, CO), and LifeSpan BioSciences (Seattle, WA), respectively. R-Phycoerythrin-conjugated anti-monocyte/macrophage-2 Ab (MCA519PE) was purchased from Bio Rad Laboratories (Hercules, CA). Alexa Fluor 647 anti-goat IgG (ab150131) and Alexa Fluor 488 anti-rabbit IgG (ab150061) secondary Abs were obtained from Abcam plc.
Supplementary Figure S1. Epidermal nerve fibers, mast cells, PAR-2, and TRPA1 in lesional skin of stasis dermatitis. Representative images of SD lesions and healthy skin with the quantification of staining. Intraepidermal nerve fiber (IENF) density was reduced in the SD lesions but did not correlate with itch. The number of dermal mast cells was not increased and did not correlate with itch in the SD lesions. The epidermal expression of PAR-2, TRPA1, and TRPV1 was not enhanced in the SD lesions. Bar = 100 μm. Dotted lines indicate the dermo-epidermal junction. *p < 0.05, unpaired t-test. Vertical bars indicate standard deviation. AU, arbitrary unit; IENF, intraepidermal nerve fiber; NS, not significant; PAR-2, protease-activated receptor-2; SD, stasis dermatitis; TRPA1, transient receptor potential ankyrin-1; TRPV1, transient receptor potential vanilloid-1; w/, with; w/o, without.
Supplementary Figure S2. IL-31 mRNA expression of murine peritoneal macrophages in response to substance P. Representative results of two independent experiments are shown. Values represent mean ± SD of three samples. NS, not significant, unpaired t-test, compared with non-treated macrophages.