

# IL-33/ST2 signaling excites sensory neurons and mediates itch response in a mouse model of poison ivy contact allergy

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**Poison ivy-induced allergic contact dermatitis (ACD) is the most common environmental allergic condition in the United States. Case numbers of poison ivy ACD are increasing due to growing biomass and geographical expansion of poison ivy and increasing content of the allergen, urushiol, likely attributable to rising atmospheric CO<sub>2</sub>. Severe and treatment-resistant itch is the major complaint of affected patients. However, because of limited clinical data and poorly characterized models, the pruritic mechanisms in poison ivy ACD remain unknown. Here, we aim to identify the mechanisms of itch in a mouse model of poison ivy ACD by transcriptomics, neuronal imaging, and behavioral analysis. Using transcriptome microarray analysis, we identified IL-33 as a key cytokine up-regulated in the inflamed skin of urushiol-challenged mice. We further found that the IL-33 receptor, ST2, is expressed in small to medium-sized dorsal root ganglion (DRG) neurons, including neurons that innervate the skin. IL-33 induces Ca<sup>2+</sup> influx into a subset of DRG neurons through neuronal ST2. Neutralizing antibodies against IL-33 or ST2 reduced scratching behavior and skin inflammation in urushiol-challenged mice. Injection of IL-33 into urushiol-challenged skin rapidly exacerbated itch-related scratching via ST2, in a histamine-independent manner. Targeted silencing of neuronal ST2 expression by intrathecal ST2 siRNA delivery significantly attenuated pruritic responses caused by urushiol-induced ACD. These results indicate that IL-33/ST2 signaling is functionally present in primary sensory neurons and contributes to pruritus in poison ivy ACD. Blocking IL-33/ST2 signaling may represent a therapeutic approach to ameliorate itch and skin inflammation related to poison ivy ACD.**

itch | pain | cytokine | IL-33 | allergic contact dermatitis

Allergic contact dermatitis (ACD) is a common allergic skin condition caused by environmental or occupational allergens (1). In the United States, the most common cause of ACD is contact with poison ivy, which affects >10 million Americans per year (2, 3). Poison ivy ACD is also a serious occupational hazard, particularly among firefighters, forestry workers, and farmers, accounting for 10% of total U.S. Forest Services lost-time injuries, and it often torments outdoor enthusiasts as well (3, 4). The major allergen in poison ivy is urushiol, contained in the oleoresinous sap of the plant and of related plants (e.g., poison oak and poison sumac) (5). An estimated 50–75% of Americans are sensitized to urushiol (6). Elevated atmospheric carbon dioxide and warming temperatures have increased the biomass of poison ivy and related plants, widened their geographic distribution, and increased plant urushiol content (7). These factors will likely increase allergenicity and result in even larger case numbers of poison ivy ACD in the future (8).

The clinical manifestations of poison ivy-induced ACD are intense and persistent itch (pruritus), burning sensation, skin rashes, and swelling, followed by the appearance of vesicles in severe cases (2, 3, 9). Skin inflammation and pruritus last for weeks. The severe

itch usually triggers scratching that is hard to control, especially among children, and further injures the skin (10). Scratching exacerbates the inflammation and stimulates nerve fibers, leading to even more itch and scratching. This itch–scratch cycle can cause skin infections that require antibiotic treatment. Antihistamines are usually ineffective for treating pruritus associated with poison ivy ACD, although they are still commonly used (2, 11). Patients with severe symptoms are treated with high-dose corticosteroid regimens, which frequently have side effects and are effective only if administered shortly after exposure.

The pruritic mechanisms in poison ivy ACD remain largely unknown because of very limited clinical data and poorly characterized animal models. Itch signals are generated by a subset of primary afferent sensory neurons that innervate the skin (12). Recent studies using rodent models of pruritic conditions identified a range of non-histaminergic endogenous itch mediators acting on sensory neurons and neuronal receptor systems signaling itch. These include cytokines such as IL-31, CXCL-10, or TSLP (thymic stromal lymphopoietin); transmitters such as serotonin; and their cognate receptors and coupled ion channels (13–15). These studies used either chemicals eliciting acute itch (such as chloroquine) or ACD rodent models induced by synthetic allergens not present in the environment [2,4-dinitrofluorobenzene (DNFB) oxazolone, and others] (14, 16–18).

We contributed to these efforts, identifying the ion channel TRPA1 (transient receptor potential ankyrin 1) as a key target to

## Significance

**In the United States, the most common cause of allergic contact dermatitis (ACD) is contact with poison ivy. Severe itch and skin inflammation are the major manifestations of poison ivy-induced ACD. In this study, we have established a critical role of IL-33/ST2 (interleukin 33/growth stimulation expressed gene 2) signaling in both itch and skin inflammation of poison ivy-induced ACD and revealed a previously unidentified interaction of IL-33/ST2 signaling with primary sensory neurons that may underlie the pruritic mechanisms of poison ivy-induced ACD. Blocking IL-33/ST2 signaling may represent a therapeutic approach to ameliorate itch and skin inflammation related to poison ivy dermatitis and, possibly, other chronic itch conditions in which IL-33/ST2 signaling may participate.**

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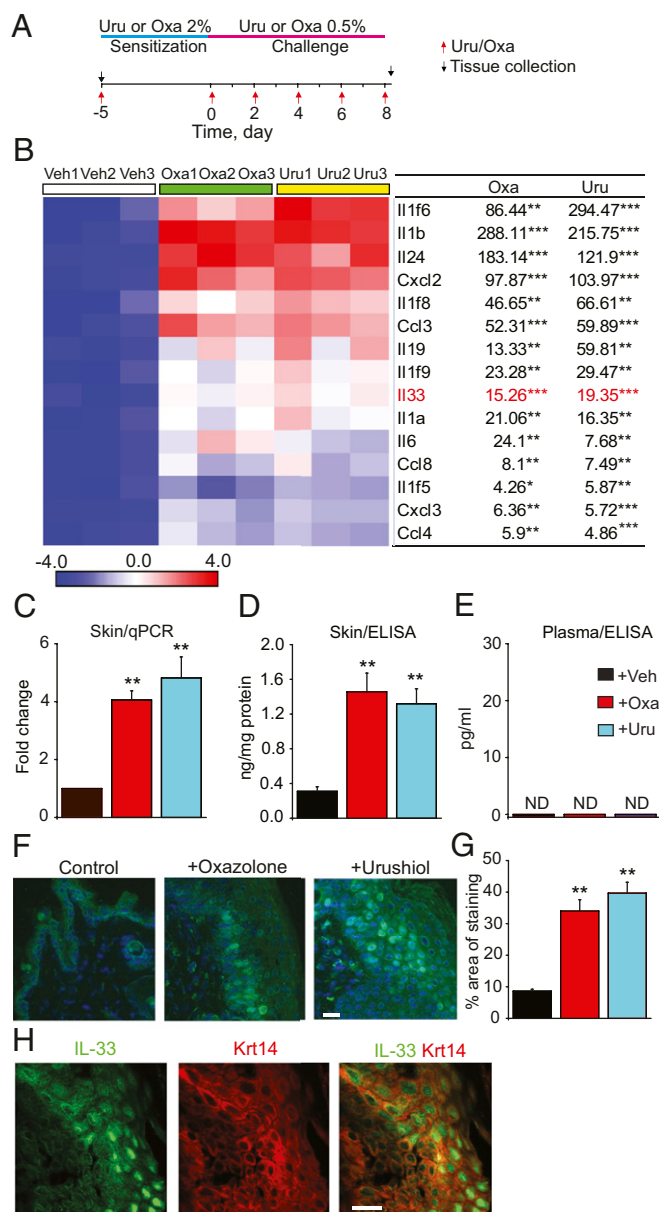
suppress itch in a mouse ACD model induced by the synthetic allergen oxazolone (16). To examine whether these pathways also contribute to itch in poison ivy ACD, we established a mouse model induced by cutaneous sensitization and challenge with urushiol. This model mimics many key clinical features of poison ivy-induced ACD, including skin inflammation and severe itch (16). TRPA1 inhibition was less efficacious in this model, suggesting that poison ivy ACD engages as-yet-unknown inflammatory and pruritic pathways (16).

The aim of the present study is to reveal these pathways through an unbiased approach by using transcriptome microarray analysis of urushiol-induced genes in the mouse skin, validation by quantitative PCR (qPCR) and biochemistry, neuronal functional imaging, and pharmacological and behavioral testing.

## Results

**Urushiol-Induced ACD Triggers the Release of IL-33 from the Inflamed Skin.** To search for potential endogenous pruritogens involved in the pruritus caused by urushiol-induced ACD, we carried out a mouse transcriptome microarray analysis to study gene expression profiles of the skin isolated from urushiol-challenged and unchallenged (acetone-treated) mice. For comparison, we included the well-established oxazolone-induced ACD mouse model. Mice were sensitized with 2.0% (wt/vol) oxazolone or urushiol on the abdominal skin, followed by challenges on the nape of neck 5 d later with 0.5% oxazolone or urushiol, a concentration known to elicit ACD in sensitized humans (Fig. 1A) (19). We found that, during the third and fifth urushiol challenge, mice usually developed a stable dermatitis condition and long-lasting scratching toward the neck. Therefore, mouse neck skin samples were collected after the fifth challenge. Total RNA from neck skins of unchallenged mice and from the mice that had received urushiol or oxazolone treatment were analyzed by using hybridization with a mouse transcriptome microarray. A total of 3,612 genes, which represents 5.5% of total genes (65,956), was identified to be significantly up- or down-regulated (greater than twofold;  $P < 0.05$ ) in mouse skin upon urushiol treatment (Tables S1 and S2). Among these differentially regulated genes, we were especially interested in inflammatory cytokines and chemokines that are abundantly up-regulated. Fig. 1B illustrates the top 15 inflammatory cytokines and chemokines that are significantly up-regulated. Among these genes, some well-established inflammatory markers, such as IL-1 $\beta$  and CXCL-2, were highly up-regulated in both the oxazolone and urushiol groups. Of particular interest was cytokine IL-33, which was significantly up-regulated in both oxazolone- and urushiol-treated groups. IL-33 has not previously been implicated in itch, but neutralizing antibodies against IL-33 were shown to attenuate skin swelling in a mouse ACD model and in humans (20, 21). IL-33 expression is also enhanced in human ACD skin (21). More importantly, skin-specific expression of IL-33 can elicit atopic dermatitis-like inflammation and scratching behavior in mice (22). In the present study, we therefore investigated the possible involvement of this cytokine in itch caused by poison ivy ACD.

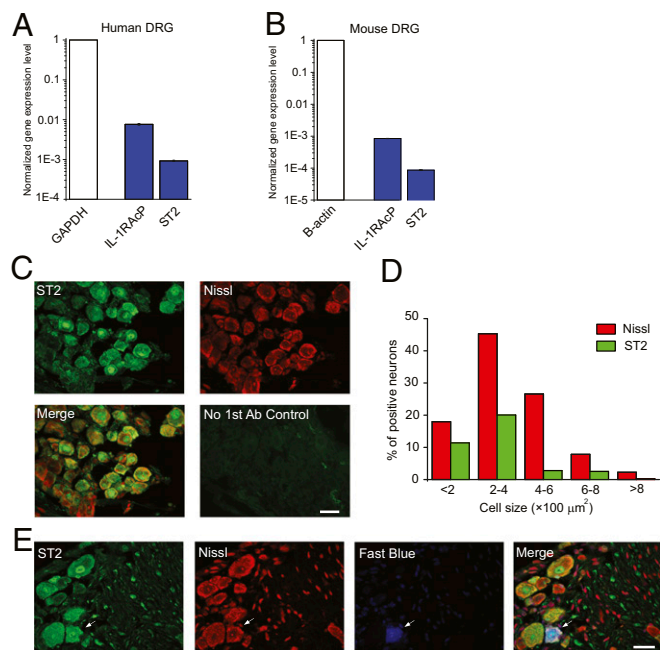
qPCR analysis confirmed that IL-33 transcription was significantly up-regulated in both oxazolone- and urushiol-treated groups compared with acetone (Fig. 1C). ELISA confirmed a corresponding increase in IL-33 protein levels in the inflamed skin, but not in plasma (Fig. 1D and E). Finally, increased levels of IL-33 were detected in mouse skin sections from both oxazolone- and urushiol-treated groups by immunofluorescence staining (Fig. 1F and G). Immunofluorescence further revealed that IL-33 was extensively expressed in cells localized in the epidermis of skin (Fig. 1F). Double immunofluorescence staining showed that IL-33-positive cells closely overlap with cells stained with keratin 14, a specific marker for keratinocytes (Fig. 1H). We conclude that IL-33 is significantly increased in the inflamed skin in urushiol-induced ACD mice because of increased production and release from keratinocytes.



**Fig. 1.** Mouse transcriptome microarray analysis of oxazolone- or urushiol-challenged mouse skin. (A) Scheme of treatment in urushiol- or oxazolone-induced mouse ACD model. (B) Heat map showing top 15 most up-regulated inflammatory cytokines and chemokines in oxazolone (Oxa)- and urushiol (Uru)-challenged mouse neck skin, identified by mouse transcriptome microarray analysis. Vehicle group (Veh) mice were treated with acetone.  $n = 3$  mice per group. (C) Fold changes of IL-33 gene transcript in skin samples from oxazolone- and urushiol-challenged mouse by qPCR. (D and E) IL-33 from skin and plasma of mice by ELISA.  $n = 7$  or 8 mice per group. ND, not detectable. (F) Immunofluorescence images of IL-33 staining (green) in mouse neck skin from frozen sections. Nuclei were labeled with DAPI (blue). (G) Summary of IL-33 immunostaining in  $F$ .  $n = 7$  or 8 mice per group. (H) Double immunostaining showing the overlapping of IL-33 with keratin 14 in the skin of urushiol-induced ACD mice.  $*P < 0.05$ ;  $**P < 0.01$ ;  $***P < 0.001$  vs. vehicle/control group. One-way ANOVA followed by Tukey post hoc test was used for statistical analysis. (Scale bars, 20  $\mu\text{m}$ .)

## IL-33-Specific Receptor ST2 Is Expressed in Peripheral Sensory Neurons.

Although numerous studies have shown that IL-33 acts on immune cells, IL-33 signaling in peripheral sensory neurons has not been reported. IL-33 signals through the IL-33 receptor complex, a heterodimer consisting of the accessory chain IL-1 receptor-like 1 (IL-1RAcP) and a membrane-bound IL-33-specific ST2 chain (23).



**Fig. 2.** Analysis of IL-33 receptor ST2 expression in DRG neurons. (A and B) Summary of gene expression levels of IL-33 receptor complex IL-1RAcP and ST2 in human (A) and mouse (B) DRGs. Mouse  $\beta$ -actin and human GAPDH were used as housekeeping genes. (C) Immunostaining showing the immunoreactivity of IL-33 receptor ST2 (green) in mouse cervical DRG neurons (identified by Nissl staining; red). (Magnification: 60 $\times$ .) (D) Cell size distribution frequency of ST2-positive and Nissl staining-positive neurons (636 neurons/12 inconsecutive sections/5 mice). (E) ST2 is expressed in cutaneous Fast Blue (purple) labeled DRG neurons indicated by white arrows. (Scale bars, 20  $\mu$ m.)

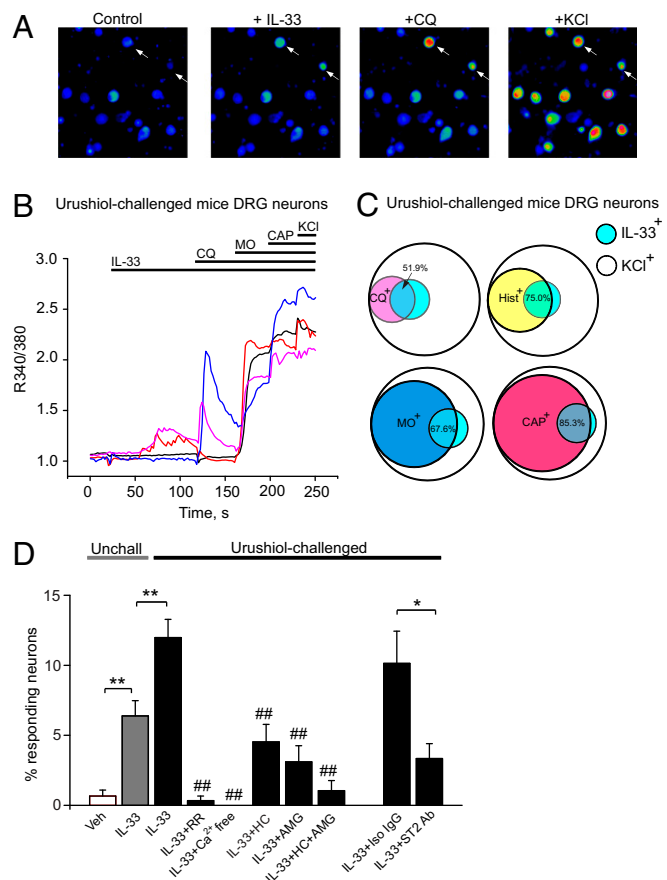
We detected both ST2 and IL-1RAcP transcripts in human and mouse dorsal root ganglia (DRGs) by using qPCR (Fig. 2A and B). Immunofluorescence staining further showed that ST2 was expressed by 34.3% of DRG neurons of mouse (218 of 636 neurons), mainly in small and medium-sized neurons (Fig. 2C and D). The specificity of ST2 antibody was confirmed by negative control staining without first antibody (Fig. 2C), and staining with first antibody preabsorbed with a blocking peptide with the ST2-derived antigenic sequence (Fig. S1A and B).

Itch is mediated by a subset of peripheral cutaneous sensory neurons (12). To study the expression of ST2 in the cutaneous sensory neurons, we used retrograde labeling dye Fast Blue to specifically label skin-innervating DRG neurons. This approach revealed that ST2 is expressed in skin-innervating DRG neurons, with 8.3% of ST2-expressing neurons labeled with Fast Blue (Fig. 2E, white arrow). Immunofluorescence analysis identified ST2 costaining with IL-1RAcP and TRPV1, but not with GS, a marker for satellite glial cells (Fig. S2A). A total of 21.5% of ST2 immunoreactive neurons showed IL-1RAcP staining and 57.1% showed TRPV1 costaining. We also identified costaining of ST2 with PGP9.5-positive free nerve endings in skin sections (Fig. S2B).

**IL-33 Produces  $Ca^{2+}$  Responses in DRG Neurons Through Its Receptor ST2.** We proceeded to explore whether ST2 receptors are functional in DRG neurons by testing the effects of IL-33 on intracellular  $Ca^{2+}$  mobilization of cultured cervical DRG neurons (C1-T1). In our initial experiments, we observed that IL-33 produced  $Ca^{2+}$  responses in neurons dissociated from both control and urushiol-challenged mice, with larger numbers of neurons responding in the latter amounting to  $12.0 \pm 1.3\%$  of total KCl-responsive ( $KCl^+$ ) neurons (Fig. 3A–D). Chloroquine (CQ), a well-established pruritogen that causes itch via MrgA3 in DRG

neurons (17), was applied after IL-33 to determine whether these populations overlap. Representative patterns of  $Ca^{2+}$  responses of these neurons are shown in Fig. 3B. Venn diagram analysis revealed that 51.9% of IL-33-responsive neurons also responded to CQ (Fig. 3C). In addition, 75.0% of IL-33-responsive neurons also responded to histamine (Fig. 3C). We found that 67.6% and 85.3% of IL-33-responsive neurons are activated by mustard oil (MO) and capsaicin (Cap), respectively (Fig. 3B and C). In summary,  $Ca^{2+}$  imaging revealed that IL-33 induces robust  $Ca^{2+}$  responses in a subset of DRG neurons that also mediate pain and/or itch responses.

Removal of extracellular  $Ca^{2+}$  or addition of the broad spectrum TRP channel blocker ruthenium red almost totally abolished



**Fig. 3.** IL-33 induces  $Ca^{2+}$  mobilization in cultured DRG neurons isolated from urushiol-challenged mice. (A) Pseudocolor Fura-2 ratiometric images of DRG neurons isolated from C1-T1 DRGs of mice challenged with urushiol. Images show  $Ca^{2+}$  responses of DRG neurons in control condition and upon IL-33 (1  $\mu$ g/mL), CQ (300  $\mu$ M), and KCl (40 mM) application. White arrows indicate neurons that responded positively to IL-33. (Magnification: 10 $\times$ .) (B) Time course traces illustrate the different types of  $Ca^{2+}$  responses upon IL-33 and CQ application: cell responding to both IL-33 and CQ, pink; cell responding to IL-33 only, red; cell responding to CQ only, blue; cell responding to neither IL-33 nor CQ, black. (C) Venn diagram showing the overlapping of IL-33-positive ( $IL-33^+$ ) with CQ-positive, histamine (Hist)-positive, mustard oil (MO)-positive, and capsaicin (Cap)-positive neuronal populations. Each Venn diagram contains 200–300 DRG neurons. A neuron was considered  $IL-33^+$  if the peak  $Ca^{2+}$  response was  $>20\%$  of baseline. (D) Summary of percentages of DRG neurons responding to vehicle (Veh; 0.1% BSA) and IL-33 in control,  $Ca^{2+}$ -free extracellular solution, ruthenium red (RR; 10  $\mu$ M), HC-030031 (HC; 100  $\mu$ M), AMG9810 (AMG; 6  $\mu$ M), isotype control IgG (Iso IgG; 0.5 mg/mL), and ST2-neutralizing antibody (0.5 mg/mL)-treated conditions. A total of 6–12 fields of observation were included in each group (each group contains 300–800 neurons from three to five mice). \* $P < 0.05$ ; \*\* $P < 0.01$ ; ## $P < 0.01$  compared to IL-33, urushiol-challenged. Student's *t* test or one-way ANOVA followed by Tukey post hoc test was used for statistical analysis.



the percentage of IL-33-responsive neurons, suggesting that TRP ion channels may be acting downstream of IL-33 pathways (Fig. 3D). A TRPA1-specific blocker, HC-030031, and a TRPV1-specific blocker, AMG9810, both significantly reduced IL-33 responses (Fig. 3D). Coadministration of HC-030031 and AMG9810 further reduced the percentage of IL-33-responsive neurons (Fig. 3D). Pretreating neurons with a ST2-specific monoclonal neutralizing antibody also significantly reduced the percentage of IL-33-responsive neurons (Fig. 3D). Together, these results demonstrate that the IL-33-induced  $Ca^{2+}$  response in DRG neurons is largely mediated by TRP-like channels, possibly TRPA1 and TRPV1, downstream of neuronal ST2 receptors.

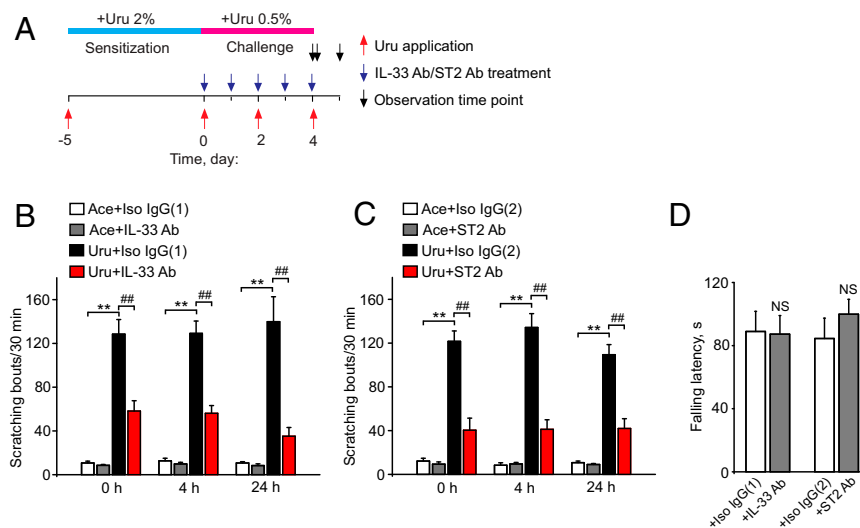
**IL-33/ST2 Signaling Mediates Chronic Scratching Behavior and Skin Inflammation in Urushiol-Challenged Mice.** To determine whether IL-33 was involved in the chronic scratching behavior associated with urushiol-induced ACD, we examined the effects of an IL-33-neutralizing antibody on scratching behavior of mice after the third urushiol challenge. Urushiol-challenged mice were administered i.p. either the IL-33-neutralizing antibody (15  $\mu$ g per mouse, i.p.) or an isotype control IgG (goat IgG = Iso IgG(1), 15  $\mu$ g per mouse, i.p.) daily, starting from day 0 until day 4 (Fig. 4A). Unchallenged mice (acetone-treated) received isotype control IgGs (i.p.) only. IL-33-neutralizing antibody significantly reduced the scratching behavior of mice at the 0-, 4-, and 24-h time points (Fig. 4B). We continued to examine whether ST2 was also involved in the scratching behaviors of mice in urushiol-induced ACD. ST2-neutralizing antibody (50  $\mu$ g per mouse, i.p.) or isotype control IgG (rat IgG = Iso IgG(2), 50  $\mu$ g per mouse, i.p.) were administered i.p. to urushiol-challenged mice in a similar treatment regimen (Fig. 4A). ST2-neutralizing antibody significantly reduced the scratching behavior of mice at the 0-, 4-, and 24-h time points (Fig. 4C). In addition, IL-33- and ST2-neutralizing antibodies did not affect motor coordination behavior in the mouse rotarod assay (Fig. 4D).

We proceeded to examine the effects of IL-33- and ST2-neutralizing antibody treatment on skin inflammation. Consistent with our previous observation (16), sensitization and challenge of the mouse neck skin with urushiol produced a strong increase in skin

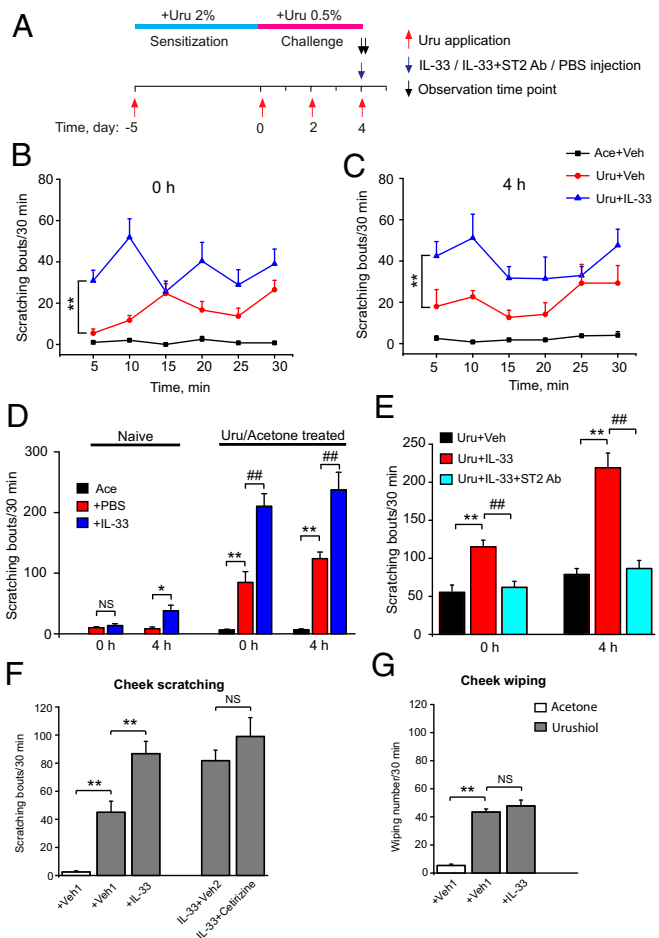
bifold thickness, transepidermal water loss (TEWL), and dermatitis score (Fig. S3). Daily treatment with IL-33- or ST2-neutralizing antibodies produced a modest, although significant, reduction in skin bifold thickness (Fig. S3A and B). In addition, antibody treatment significantly reduced the TEWL and dermatitis score associated with ACD (Fig. S3C and D). Collectively, these findings support the hypothesis that IL-33/ST2 signaling is involved in chronic scratching behavior and skin inflammation of urushiol-induced mouse ACD.

**Exogenous IL-33 Exacerbates Itch-Related Scratching Behaviors and Skin Inflammation in Urushiol-Induced ACD Mice.** To gain further insights into the role of IL-33 in chronic itch, we examined the potential behavioral effects of IL-33 in urushiol-induced mouse ACD. Right after the third urushiol challenge of the neck skin, a single dose of IL-33 [300 ng per site, intradermally (i.d.)] or PBS was injected into the nape of the neck (Fig. 5A). As expected, urushiol-challenged mice showed scratching behavior compared with acetone-treated controls (Fig. 5B and C). Notably, we observed that IL-33 injection robustly enhanced the scratching behaviors in the urushiol-challenged mice at both the 0- and 4-h time points (Fig. 5B–D). Two-way ANOVA analysis indicated significant differences between the IL-33- and vehicle-injected groups (Fig. 5B and C). IL-33 began to take effect within 5 min after the injection, indicating a rapid action of IL-33 in eliciting scratching behavior in urushiol-challenged mice (Fig. 5B). In contrast, IL-33 injection into naïve mice did not elicit significant scratching during the first 30 min (Fig. 5D). These data indicate that IL-33 is more potent in inducing scratching behaviors in mice with fully established urushiol ACD.

We continued by determining whether the potentiating effect of IL-33 on scratching behavior in urushiol-induced ACD mice is mediated through the ST2 receptor. IL-33 together with ST2-neutralizing antibody or isotype control IgG were coinjected s.c. into the neck skin of urushiol-induced ACD mice. The ST2-neutralizing antibody almost completely abolished the potentiating effect of IL-33 on scratching at both the 0- and 4-h time points (Fig. 5E). Thus, we have established a pivotal role of



**Fig. 4.** Effects of inhibition of IL-33/ST2 signaling on chronic itch in urushiol-challenged mice. (A) Treatment of urushiol-induced mouse ACD model. Mice were treated (i.p.) with isotype control IgGs (Iso IgGs) or IL-33- or ST2-neutralizing antibody (Ab) every day for a total of five times. Scratching behaviors were monitored at 0-, 4-, and 24-h time points as indicated. (B and C) Summarized scratching behaviors of unchallenged mice (Ace) and urushiol-challenged mice (Uru) after the treatment with isotype control IgGs, IL-33-neutralizing (15  $\mu$ g per mouse), or ST2-neutralizing (50  $\mu$ g per mouse) antibody. (D) Motor coordination behavior measured by rotarod. Mice were treated with isotype control IgGs or IL-33, ST2 antibody.  $n = 7$  or 8 mice per group.  $**P < 0.01$ ;  $##P < 0.01$ ; NS, no significance. One-way ANOVA followed by Tukey post hoc test or Student's  $t$  test was used for statistical analysis.



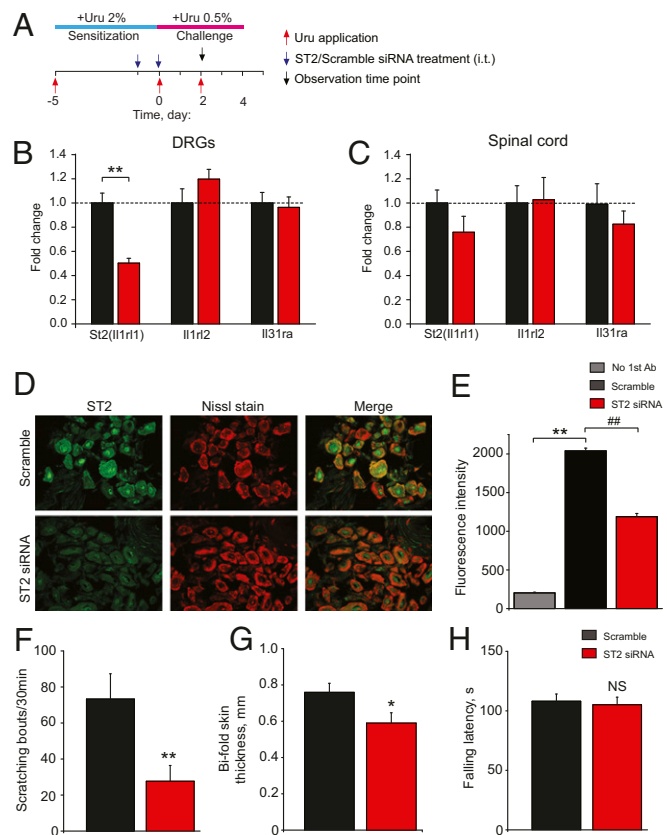
**Fig. 5.** IL-33 promotes itch-related scratching in urushiol-induced ACD mice through ST2. (A) Experimental scheme of urushiol-induced ACD on mouse neck. (B and C) Scratching behavior at 0 h (B) and 4 h (C) in unchallenged (acetone-treated; Ace) and urushiol-challenged (Uru) mice in 5-min intervals during a 30-min period after vehicle (0.1% BSA) or IL-33 (+IL-33; 300 ng per site) injection at the nape of the neck. (D) Summarized scratching behavior at 0- and 4-h time point for the entire 30-min recording period. (E) Scratching behaviors of urushiol-challenged mice upon vehicle (0.1% BSA), IL-33-neutralizing (300 ng per site), and IL-33+ST2-neutralizing antibody (ST2 Ab; 50  $\mu$ g per site) injection at the nape of neck at 0- and 4-h time points. (F) Effects of IL-33 or IL-33/cetirizine coadministration on cheek-scratching behavior recorded within 30 min after the third urushiol challenge of the cheek. Acetone group was challenged with acetone only. Cetirizine (10 mg/kg) or PBS was administered (i.p.) 30 min before IL-33 injection. Vehicle 1, 0.1% BSA in PBS (Veh1); Vehicle2, PBS (Veh2). (G) Cheek-wiping behavior in the same first three groups of mice shown in F.  $n = 7$  or 8 mice per group. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . NS, no significance. One- or two-way ANOVA followed by Tukey post hoc test was used for statistical analysis.

IL-33 in mediating the chronic scratching in urushiol-induced ACD mouse.

Behavioral responses to itch and pain are difficult to distinguish. We therefore used a recently introduced rodent model in which pruritogenic (or algogenic) stimuli are applied to the cheek (24). After the third urushiol challenge on the cheek, urushiol-challenged mice showed more cheek-directed scratching and wiping behavior than acetone-treated mice (Fig. 5 F and G). Injection of IL-33 into the cheek of urushiol-challenged mice significantly increased scratching, but not wiping, compared with vehicle-injected mice (Fig. 5 F and G). The histamine H<sub>1</sub> receptor antagonist, cetirizine, had no effect on IL-33-induced cheek-scratching behavior (Fig. 5F). These results demonstrate that IL-33 can evoke

itch-related behavior in the inflamed skin of urushiol-induced ACD mice via a histamine-independent mechanism.

**DRG-Expressed ST2 Is Essential for the Itch Response of Urushiol-Induced ACD Mice.** To examine the contribution of neuronal ST2 to the itch response of urushiol-induced ACD mouse, we intrathecally (i.t.) administered ST2-targeted siRNA to knock down its neuronal expression. Scrambled control siRNA was used as a control (Fig. 6A). qPCR confirmed that repeated i.t. delivery of ST2 siRNA significantly reduced *St2* gene expression in DRG neurons, without altering *Il1rl2* or *Il31ra* gene expression (Fig. 6B). Spinal cord *St2* gene expression was not significantly changed by siRNA treatment (Fig. 6C). ST2 protein expression in DRG neurons was also significantly reduced by siRNA treatment (Fig. 6D and E). Notably, i.t. ST2 siRNA significantly attenuated the itch response and skin inflammation of urushiol-induced ACD mice, without altering locomotion activity of the mice (Fig. 6F–H). Therefore, these results established a crucial role of ST2 expressed in DRG neurons in mediating the itch response of urushiol-induced ACD mice.



**Fig. 6.** Effects of DRG-specific knockdown of ST2 on the itch response of urushiol-induced ACD mice. (A) Protocol for i.t. delivery of ST2 siRNA or scrambled control siRNA to mice. (B and C) qPCR analysis of transcript levels of *St2* (*Il1rl1*), *Il1rl2*, and *Il31ra* in DRGs (B) or spinal cords (C) of ST2 siRNA or scrambled siRNA treatment groups. (D) Immunostaining of DRG sections showing analyzing expression of ST2 protein of ST2 siRNA- or scrambled siRNA-treated group. (Magnification: 60 $\times$ .) (E) Summary of ST2 staining fluorescence intensities of neurons from ST2 siRNA or scrambled siRNA-treated mice. A total of 100–120 neurons pooled from five mice from each group were compared. (F) Analysis of scratching behavior of urushiol-induced ACD mice after i.t. injection of ST2 siRNA. (G) Bifold skin thickness of urushiol-induced ACD mice treated with ST2 siRNA or scrambled siRNA. (H) Comparison of motor coordination activity of siRNA-treated mice tested with rotarod.  $n = 6$  or 7 mice per group. \* $P < 0.05$ ; \*\*\* $P < 0.001$ ; ### $P < 0.01$ . NS, no significance. Student's *t* test or one-way ANOVA followed by Tukey post hoc test was used for statistical analysis.

## Discussion

Environmental exposure to poison ivy is the most common cause of ACD in the United States, representing a significant public health burden. ACD is elicited by direct contact with the plant or indirectly by contact with contaminated clothing, shoes, and tools. Inhalation of smoke from burning plant material is especially hazardous and can trigger severe respiratory allergic responses (2, 5). Strong and persistent itch and skin inflammation are the major manifestations of poison ivy ACD. In contrast to other environmental allergic conditions, little is known about the specific mechanisms underlying itch and skin inflammation in poison ivy-induced ACD.

Studies modeling ACD almost exclusively used synthetic experimental allergens, such as oxazolone and DNFB, that are not present in the environment. However, different allergens cause widely divergent immune responses, making it difficult to predict mechanisms and therapeutic strategies for ACD elicited by common environmental allergens such as urushiol (25, 26). We therefore optimized and characterized a mouse model of poison ivy ACD and applied transcriptomic, biochemical, neurophysiological, and behavioral methods to identify the inflammatory and pruritic mechanisms engaged in murine poison ivy ACD. We identified a critical role of IL-33/ST2 signaling in both itch and skin inflammation in this model and revealed a previously unknown interaction of IL-33 with primary sensory neurons that may underlie the itch mechanism of urushiol-induced ACD.

The concentrations of urushiol used for sensitization (2.0%) and challenge (0.5%) in the mouse model in the present study are within the range of concentrations known to elicit contact dermatitis in humans. A patch test study observed that challenge with a 1.0% solution resulted in allergic skin responses (erythema to bullae) in 75% of U.S. volunteers, representative of the degree of sensitization estimated to be present in the U.S. population (19). Leaves of the plants belonging to the Toxicodendron family, such as poison ivy, oak, and sumac, contain up to 2.5% urushiol (wt/wt) that is concentrated in resin droplets on the leaf surface (27). The resin of the Japanese lacquer tree, also of the Toxicodendron family and known to cause occupational allergies in lacquer workers, contains between 55% and 75% urushiol (28). Thus, local skin exposures are heterogeneous, but well within the range of concentrations used in our study.

Emerging data have demonstrated that certain cytokines and chemokines can act as endogenous itch mediators (29). Some cytokines are released from skin and immune cells and contribute to the cross-talk between the immune and nervous systems. Examples include IL-31 and CXCL10, which were shown to signal through their cognate receptors on primary sensory neurons to induce itch. However, we did not observe up-regulation of IL-31 and CXCL10 transcription in the skin of urushiol-challenged mice, indicating that these cytokines do not contribute to the observed pathology, with IL-33 fulfilling this role instead (Tables S1 and S2). Nevertheless, because we observed residual itch responses in mice after IL-33/ST2-neutralizing antibody treatment, other endogenous pruritogens or mediators, in addition to IL-33, are likely involved in the itch response of urushiol-induced ACD mice as well and remain to be identified.

IL-33 is a member of the IL-1 cytokine family. In addition to the well-documented role in immune and inflammatory diseases, IL-33/ST2 signaling was also found to contribute to pain (30, 31). Although ST2 expression has been detected in spinal cord neurons and implicated in spinal pain mechanisms, expression and function of ST2 in peripheral sensory neurons have not been explored in detail.

Our study has provided several lines of evidence that suggest functional expression and a physiological signaling role of ST2 in peripheral sensory neurons. First, our qPCR data demonstrate that ST2 transcript is expressed in both mouse and human

DRGs. This finding is supported by a recent study that also demonstrated ST2 transcript expression (at a level comparable to the functional IL-5R) in Nav1.8-expressing primary nodose ganglion neurons (32). Second, our immunofluorescence-staining and retrograde-labeling experiments revealed that ST2 is expressed in a subset of small to medium-sized TRPV1-expressing DRG neurons, including neurons that innervate the skin. Immunostaining also identified the presence of ST2 in skin free nerve endings, where IL-33 produced in the skin would have direct access. Thirdly, our Ca<sup>2+</sup> imaging experiments clearly demonstrated that IL-33 can induce robust Ca<sup>2+</sup> responses in cultured DRG neurons, and this response is largely abolished by a monoclonal ST2-neutralizing antibody. IL-33 activated Ca<sup>2+</sup> influx into ~7% of cultured DRG neurons from naïve mice. Although our immunofluorescence studies detected ST2 in a larger proportion of neurons in sectioned DRGs, the essential binding partner IL-1RACp was detected in a smaller percentage of cells, suggesting that functional IL-33 receptors, consisting of ST2 and IL-1RACp, are limited to a relatively small neuronal population. IL-33-induced Ca<sup>2+</sup> responses in DRG neurons were almost completely abolished in Ca<sup>2+</sup>-free extracellular solution or by ruthenium red, a TRP channel blocker, and largely abolished by specific TRPA1 and TRPV1 antagonists. It is well established that TRPA1 and TRPV1 are involved in itch transduction (33). Therefore, it is possible that TRPA1 and TRPV1 act downstream of IL-33/ST2 signaling to initiate itch signaling. Lastly, our targeted knockdown of ST2 expression in DRG neurons by i.t. siRNA injection significantly reduced the itch response of urushiol-induced ACD mice. This result further demonstrates the importance of neuronal ST2 in mediating the itch signal under the ACD condition.

Keratinocytes are known as a source of IL-33 in the skin, often induced by proinflammatory factors such as TNF- $\alpha$  and IFN- $\gamma$  (20). We found that IL-33 expression is significantly increased in the epidermis and is exclusively expressed in keratinocytes under urushiol-induced ACD conditions. The interaction between keratinocytes and primary sensory neurons plays an important role in the development and maintenance of chronic itch conditions (34). Therefore, we propose here that keratinocytes may directly communicate with cutaneous sensory neurons via IL-33, which is released upon tissue inflammation or injury, to promote itch.

The population of IL-33-responsive DRG neurons correlated to a large extent with that of CQ- or histamine-responsive neurons, indicating that IL-33 activates primary sensory neurons that mediate the sensation of itch. In addition, treatment of urushiol-induced ACD mice with IL-33- or ST2-neutralizing antibodies significantly attenuated the scratching response. Cutaneous IL-33 injection rapidly increased scratching behavior in urushiol-challenged mice. IL-33 is known to cause mast cell degranulation and release of histamine (35, 36). However, IL-33-induced itch in the present study is unlikely to be mediated by histamine because the antihistamine, cetirizine, at a dosage that effectively blocks histamine-related itch (37), had no effect on the IL-33-induced itch response. Therefore, these results suggest that IL-33/ST2 signaling may likely mediate allergic itch response through direct activation of itch-sensing primary sensory neurons.

Although our data strongly support a neuronal mechanism of ST2 in mediating itch signaling in urushiol ACD, we cannot rule out the possible participation of more indirect pathways involving nonneuronal cells. It is known that ST2 is expressed in Th2 cells, in a variety of innate immune cells as well as in skin cells (23). IL-33 can interact with keratinocytes, mast cells, and other immune cells through ST2 to produce proinflammatory mediators and cytokines, including TSLP, histamine, serotonin, and IL-13, that have known roles in the initiation of allergic diseases and itch (35, 38–40). Our *in vivo* experiments using ST2-neutralizing antibodies may have interfered with the actions of these cell types in addition to the sensory neurons. These cells may also contribute to the residual scratching behavior we observed



in mice injected with ST2-neutralizing antibodies, and the pathways involved remain to be studied.

Our study showed that IL-33 did not elicit any obvious scratching behavior in naïve mice, but did evoke significantly more itch-related scratching after urushiol challenge. In line with this finding, we observed that IL-33-induced  $Ca^{2+}$  responses are more pronounced in DRG neurons from urushiol-treated mice than from naïve mice. The above phenomenon is likely due to the sensitization of itch-signaling pathways under chronic itch conditions. Sensitization of itch-signaling pathways has been proposed as a critical mechanism underlying chronic itch (41, 42). In ACD and atopic dermatitis, nerve fiber densities are increased in the epidermis (43, 44). Extension of these nerve fibers into the epidermis may contribute to spontaneous itch, aggravating itch responses and sensitization of itch signaling pathways (42). Enhanced animal behavioral scratching and DRG neuron responses to pruritogens have been documented in a mouse chronic dry-skin model (45). Similarly, CXCL10, which is a nonpruritogenic chemokine in naïve mice, was found to turn into a potent pruritogen in the inflamed skin of a mouse model of ACD (14). Along the same lines, it is likely that urushiol-induced ACD sensitizes itch-signaling pathways, such that IL-33 becomes a pruritogen to evoke itch responses under the ACD condition.

Our findings suggest that blocking IL-33/ST2 signaling may represent a therapeutic approach to ameliorate itch and skin inflammation in poison ivy ACD and, possibly, other chronic itch conditions in which IL-33/ST2 signaling may participate. Currently, ACD patients are treated with antihistamines and corticosteroids. Antihistamines are largely ineffective to counteract itch, an observation replicated in our mouse model in which cetirizine failed to suppress the scratching behavior. Corticosteroids have known side effects and need to be administered early after exposure to be effective. Therapies targeting IL-33/ST2 signaling may be especially useful in individuals known to develop severe anaphylactic complications after allergen exposure and in individuals known to develop life-threatening respiratory allergic responses to urushiol, including forest firefighters for whom poison ivy is an occupational hazard (4).

## Materials and Methods

**Animals.** Experimental procedures were approved by the Institutional Animal Care and Use Committee of Duke University. Male C57BL/6 mice (6–8 wk old) were purchased from The Jackson Laboratory. Mice were housed at facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care in standard environmental conditions (12-h light–dark cycle and 23 °C). Food and water were provided ad libitum.

**Urushiol/Oxazolone-Induced Allergic Contact Dermatitis Model.** C57BL/6 male mice were sensitized by applying 2.0% (wt/vol) urushiol or oxazolone to the shaved abdomen. After 5 d (day 0), mice were challenged with 0.5% urushiol

or oxazolone by painting on the shaved nape of the neck. On days 2 and 4, mice were challenged with urushiol or oxazolone in the same way as day 0, for a total of three to five challenges.

**Scratching Behavior Analysis.** Behavioral experiments were performed as described (16). All behavioral tests were performed by an experimenter blinded to experimental conditions.

**DRG Neuron Culture and  $Ca^{2+}$  Imaging.** C1-T1 bilateral mouse DRGs from either acetone- or urushiol-treated mice were dissociated as described (46).  $Ca^{2+}$  imaging using Fura-2 was performed 24 h after DRG dissection.

**Mouse Transcriptome Microarray.** RNA samples were processed by Affymetrix GeneChip Mouse Transcriptome Assay 1.0. The Affymetrix Mouse Transcriptome 1.0 CEL files were imported into Affymetrix Expression Console Software and analyzed by using the Gene Level-SST RMA normalization method. The datasets of the microarray analysis are illustrated in Tables S1 and S2.

**Retrograde Labeling of Skin-Innervating DRG Neurons.** The 0.5% Fast Blue was injected (i.d., 3  $\mu$ L per site) at seven sites on the shaved nape of the neck of mice under anesthesia. At 4–5 d later, bilateral cervical DRG neurons (C1-T1) were collected.

**Immunofluorescent Staining.** Immunofluorescent staining was performed and analyzed as described (16).

**siRNA Knockdown.** Selective ST2 siRNA and scrambled ST2 control siRNA were synthesized by Dharmacon. siRNA was dissolved in 5% glucose and mixed with the in vivo transfection reagent in vivo-jetPEI (Polyplus) and incubated at room temperature for 15 min according to the manufacturer's protocol. Intrathecal injection was performed by a lumbar puncture to deliver reagents to the cerebral spinal fluid under anesthesia. A total of 3  $\mu$ g of siRNA in 10- $\mu$ L volume was injected i.t. once a day for two days to knockdown ST2 expression. Two days after the last siRNA injection, C1-T1 DRG neurons and spinal cord tissue were collected and subjected to qPCR or immunostaining to test the efficiency of ST2 knockdown.

**Statistics.** Statistical analysis were made between groups by using Student's t test (for comparison between two groups) or one- or two-way ANOVA (for comparison among three or more groups) followed by Tukey post hoc test. Comparison was considered significantly different if  $P$  was  $<0.05$ . Data in bar graphs are expressed as means  $\pm$  SE.

Details of methods and materials are described in *SI Materials and Methods*.

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