

Phenotyping acute and chronic atopic dermatitis-like lesions in Stat6VT mice identifies a role for IL-33 in disease pathogenesis

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Keywords: Atopic dermatitis, Eczema, Stat6VT transgenic mouse, Interleukin 33 (IL-33), Keratinocytes

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Acknowledgements

This work was supported by the Dermatology Foundation (Physician-Scientist CDA) to MJT, The Department of Veterans Affairs VA CDA2 (IK2 CX001019) to MJT and VA Merit (510BX000853) to JBT, the Ralph W. and Grace M. Showalter Research Trust to MJT and NIH grants to MHK (R01 AI095282), DFS (AG048946 and ES020866), JBT (HL062996, AG048946 and ES020866). MRO and AK were supported by T32 AR062495.

Abstract

The Stat6VT mouse model of atopic dermatitis (AD) is induced by T cell specific expression of a constitutively active form of the protein signal transducer and activator of transcription 6 (STAT6). Although AD-like lesions are known to develop in Stat6VT mice, this study was designed to determine if these mice develop acute and chronic phases of disease similar to humans. To address this, AD-like lesions from Stat6VT mice were harvested at two different time points relative to their onset. Lesions harvested within 1 week after development were defined as acute lesions, and those present for 1 month or more were defined as chronic lesions. Acute and chronic AD-like lesions from Stat6VT mice exhibited histologic findings and cytokine expression patterns similar to acute and chronic AD lesions in humans. Further analysis revealed increased levels of interleukin (IL)-33 transcripts in AD-like lesions compared to Stat6VT nonlesional and wild type skin controls. Immunofluorescence also revealed increased numbers of IL-33⁺ keratinocytes in Stat6VT lesional skin and localized IL-33⁺ keratinocytes to a keratin 5⁺ subset. Furthermore, AD-like disease was more severe in IL-33-deficient Stat6VT mice compared to IL-33 sufficient Stat6VT mice. These studies suggest Stat6VT mice can serve as a model of acute and chronic AD and that IL-33 may attenuate inflammation in this system.

This is the author's manuscript of the article published in final edited form as:

DaSilva-Arnold, S. C., Thyagarajan, A., Seymour, L. J., Yi, Q., Bradish, J. R., Al-Hassani, M., ... Turner, M. J. (2018). Phenotyping acute and chronic atopic dermatitis-like lesions in Stat6VT mice identifies a role for IL-33 in disease pathogenesis. *Archives of Dermatological Research*, 310(3), 197–207. <https://doi.org/10.1007/s00403-018-1807-y>

Introduction

Atopic dermatitis (AD) is a complex and common inflammatory disease characterized and driven by skin barrier disruption and immune dysregulation [5]. Type II inflammation in the skin, which can occur via various modes, is a characteristic feature of AD [17] and can serve as a therapeutic target for the disease. For example, treatment of patients with moderate to severe AD with a neutralizing antibody to the IL-4/IL-13 receptor subunit IL-4R α led to significant reductions in disease severity in clinical trials [4]. These results suggest that targeting other type II cytokine signaling pathways may be beneficial in AD.

Helper T (Th) cell subsets, most notably Th2 cells, as well as Th1, Th9, Th17 and Th22 subsets, have been implicated in AD pathogenesis [50]. While there are a variety of animal models of AD, few are driven by defined monogenic defects in the T cell compartment. The Stat6VT transgenic mouse is one such model [43, 44]. The transgene in these mice includes the human CD2 locus control region upstream of the coding sequence for a mutant STAT6 protein, STAT6VT, which contains two alanine substitutions in the Src homology 2 domain [7, 11]. This transgene construct confers expression of a constitutively active mutant STAT6 protein in the lymphocyte compartment, favoring Th2 cell development from naïve T cells [7]. As a result of transgene expression and Th2-polarized inflammation, these mice develop an AD-like phenotype characterized clinically by scaly erythematous papules and plaques that can involve the head, neck, trunk, tail and extremities. Ocular and periocular (i.e. blepharitis, keratitis and uveitis) AD-like disease phenotypes also occur in these mice [43, 44, 52]. Histologic features of AD-like lesions include acanthosis and spongiosis and a perivascular and interstitial lymphocyte- and eosinophil-rich inflammatory cell infiltrate.

In addition to exhibiting the aforementioned clinical and histologic characteristics similar to AD in humans, there are several other features that make the Stat6VT mouse a unique and useful model to study pathogenesis. The Stat6VT model also mirrors several other aspects of human AD including the following: 1) disease develops spontaneously (i.e. it does not require exposure to environmental allergens, irritants or other physical insults); 2) nonlesional skin exhibits baseline defects in epidermal barrier function; 3) recovery of epidermal barrier function following exposure to irritants is impaired; 4) disease is critically dependent on IL-4 and 5) disease is exacerbated by loss of function mutations in epidermal differentiation complex genes like *Flg* [12, 43-45, 47, 51, 52]. In contrast to many mouse models of AD that develop disease secondary to environmental exposures or genetic defects in keratinocytes, pathogenesis in the Stat6VT model is driven by a monogenic defect in the T cell compartment thus providing a model in which to study the ability of T cells to initiate AD pathogenesis [7, 43].

In humans, AD skin lesions can be categorized as acute and chronic types [5]. In addition to exhibiting distinct clinical morphologies and histopathologic features, there are significant differences between acute and chronic AD with respect to composition of the inflammatory cell infiltrate and cytokine milieu in lesional skin [49]. In acute lesions, Th2 cells and IL-4 production predominate; in chronic lesions, while Th2 cells and IL-4 production persist, the inflammatory infiltrate and cytokine milieu become more heterogeneous with accumulation of other T cell subsets (e.g. Th1) and cytokines (e.g. IFN γ). While Th2-mediated inflammatory pathways appear to be critical for AD pathogenesis, the role for other cytokines and T cell subsets in AD is less well defined and still under investigation.

The current study identified acute and chronic phases of AD-like disease in Stat6VT mice. Moreover, transcripts and protein for the cytokine, IL-33 were increased in AD-like lesions in Stat6VT mice. Functional studies performed to define the role of IL-33 in disease used IL-33 knockout Stat6VT mice. Deficiency of IL-33 was associated with exacerbated AD-like inflammation in Stat6VT mice suggesting at least some aspect(s) of IL-33 signaling could negatively regulate disease in this model.

Materials and Methods

Animals

Stat6VT mice on C57BL/6 and SKH1 backgrounds have previously been described and develop an AD-like phenotype [7, 43, 44, 51, 52]. The Stat6VT transgene includes the human CD2 locus control region directing expression of the human Stat6 coding sequence with V547 and T548 mutated to alanine such that T cells express a constitutively active Stat6 mutant protein. For these studies, Stat6VT transgenic mice on the C57BL/6 background and SKH1 hairless background were utilized along with Stat6VT x IL-33 knockout mice (C57BL/6 background). The IL-33 knockout mice (strain I133^{tm1(KOMP)Vleg}) used for these studies were obtained from the UC Davis Knockout Mouse Repository (KOMP); this line harbors a 9443 base pair

deletion on chromosome 19 that ablates the entire *I133* coding sequence (project ID VG12663). Mice used for these studies were maintained in a specific pathogen-free environment. Experimental procedures were approved by the Institutional Animal Care and Use Committee at Indiana University School of Medicine.

Disease Scoring

The disease severity scoring system used for this study is shown in [Table 1](#). This is modified version of the eczema area and severity index or EASI score [19]. The modifications to the EASI reflect anatomic differences in the distribution of the body surface area in mice as compared to humans. For example, the modified EASI for the mouse studies includes the ears as a specific subregion of the Head/Neck region. This scoring system also combines the extremities into a single region, which is further subdivided into hind limbs and forelimbs, and the tail is included as the fourth body region. The percentages of body surface area allotted to different components of each body region in the modified EASI also reflect the body surface area distribution in mice. The severity index scale is 0-3 (with intermediate scores of .5 being allowed) for each of the following criteria: erythema, infiltration, excoriations and lichenification. The severity index is the sum of the scores for these criteria. Area scores per region are tallied by determining the percentage of surface area affected by disease and converting this percentage using the following scale: 0=0%; 1=1-9%; 2=10-29%; 3=30-49%; 4=50-69%; 5=70-89%; and 6=90-100% surface area involvement. The Region Score is the product of the Severity Index, Area Score and the Multiple for a given region. The Multiples for each region are 0.15 (Head/Neck), 0.6 (Trunk), 0.15 (Extremities) and 0.1 (Tail). The total modified disease score is the sum of the 4 Region Scores; the maximal score possible is 72.

Quantitative (real time) PCR

Total RNA was isolated by placing skin biopsy tissue in RLT buffer (Qiagen, Valencia, CA) followed by homogenization with the use of stainless steel beads and a TissueLyser (Qiagen). Homogenates were then passed through a QIAshredder spin column followed by RNA isolation with an RNeasy Mini Kit and reverse transcription with the high capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad CA). Quantitative PCR (qPCR) was performed using TaqMan chemistry and a 7500 Fast Real-Time PCR System (Applied Biosystems). Primer and probe mixtures for *I14* (Mm00445259_m1), *I15* (Mm00439646_m1), *I13* (Mm00434204_m1), *I133* (Mm00505403_m1), *Ifng* (Mm01168134_m1), *Tslp* (Mm01157588_m1), *B2m* (Mm00437762_m1) and *GAPDH* (Mm99999915_g1) were purchased from Applied Biosystems. Relative expression of transcripts was determined using the $2^{-\Delta\Delta CT}$ method with *B2m* or *GAPDH* as endogenous reference controls.

Histology and quantification of eosinophils

Skin biopsy specimens were collected and fixed in 10% buffered formalin for 1 hour and subsequently transferred to 70% ethanol. Paraffin embedding, sectioning and hematoxylin and eosin (H&E) staining of samples were performed in the Department of Pathology at Indiana University School of Medicine. A pathologist (JRB) blinded to the disease state and genotype quantified eosinophil infiltration using a Nikon Eclipse E400 microscope (Nikon Corp., Melville, NY, USA) at 3200 to 3400 magnification.

Immunofluorescence

Mouse ear biopsies were fixed with 10% buffered formalin for 1 hour, and archived in 70% ethanol followed by embedding in paraffin. Five micrometer sections were obtained and subjected to deparaffinization and rehydration using the following conditions (5 minutes per incubation): xylene for 3 cycles, 100% ethanol twice, 95% ethanol once, 70% ethanol once, and finally, double distilled water once. Heat-induced antigen retrieval was performed by submerging slides in pH-adjusted retrieval solution (10 mM Citrate buffer + 75% glycerol, pH 6.0) in a pressure cooker for 15 min followed by cooling in retrieval solution at room temperature for 20 min. After washing once in PBS, samples were permeabilized with Triton-X100 (0.25% in PBS) for 5 minutes, washed three times in double distilled water and then circled with a hydrophobic pen. Sections were then treated with 3% hydrogen peroxide for 10 min at room temperature and washed three times in PBS, followed by blocking in with 0.1% BSA in CAS block (Invitrogen Cat # 008120) at room temperature in a humidifying chamber. Sections were then incubated with the following primary antibodies (10 ug/mL in CAS block), goat polyclonal anti-mouse IL-33 (R&D systems, AF3626) and rabbit polyclonal anti-cytokeratin 5 (Abcam, ab24647) overnight at 4°C. After washing three times in PBS, samples were incubated at room temperature with Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 546 (Thermo Fischer Scientific, A-11030) and Goat

anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (Thermo Fischer Scientific, A-11008). Samples were then washed three times in PBS and then in double distilled water three times followed by counterstaining with 4,6-diamidino-2-phenylindole (DAPI; 1:1000) for 15 minutes. After washing three times with PBS and double distilled water, samples were left to air dry for 10 minutes and then mounted on a cover slip with Fluoromount-G. Immunostaining patterns were visualized using an Olympus fluorescent microscope at 400x magnification.

Statistics

Statistical analyses were performed in GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA) and Microsoft® Excel® for Mac 2011 (Microsoft Corp., Redmond, Washington, USA). Data are expressed as standard error of the mean (SEM). Statistical significance for eosinophil counts and PCR data was determined using one-way ANOVA with a *P* value of less than 0.05 being considered statistically significant. Statistical significance for immunofluorescence data was determined using a one-tailed Student's *t*-test. Statistical significance for disease severity scoring data was determined using a two-way ANOVA with a *P* value of less than 0.05 being considered statistically significant.

Results

Clinical and histologic profile of AD-like lesions

Stat6VT mice develop an eczematous dermatitis resembling that seen in AD [44, 45, 47, 51, 52]. Initially developed on the C57BL/6 background, a hairless model (SKH1 background) was also generated to facilitate skin measurements (e.g. measurement of transepidermal water loss) that can be hindered by the presence of hair. This hairless Stat6VT model provided an opportunity to observe development of AD-like lesions at early time points when subtle changes could have been obscured by hair. Initial observations suggested that the clinical appearance of AD-like lesions changed from ill-defined erythematous scaly thin papules and plaques to more well-defined and indurated papules and plaques suggesting AD-like lesions might exhibit similar features to that of acute and chronic phases of AD in humans (Fig. 1a). To better understand the pathogenic mechanisms of AD-like inflammation in Stat6VT mice and how AD-like lesions evolve over time, mice were first observed for clinical evidence of AD-like lesion formation. A subset of AD-like lesions was harvested within 1 week after developing; these were classified as acute lesions. Lesions that persisted for at least 1 month were also harvested and were classified as chronic lesions. Nonlesional skin and skin from WT mice were collected as controls for these studies.

Figure 1A shows clinical images of acute and chronic AD-like lesions in Stat6VT mice in comparison to unaffected (nonlesional skin) from Stat6VT littermates and WT controls. Histologic analysis of acute and chronic lesions revealed hyperkeratosis, spongiosis, acanthosis and a lymphohistiocytic inflammatory infiltrate in the dermis (Fig. 1b). Acanthosis and the inflammatory infiltrate were more prominent in chronic AD-like lesions compared to acute AD-like lesions, similar to the distinction between chronic and acute AD lesions in humans. Furthermore, eosinophils accumulated in acute and chronic AD-like lesions but to a greater degree in chronic AD-like lesions (Fig. 1c). Taken together, these studies suggest AD-like lesions evolve over time and exhibit clinical and histologic features similar to AD lesions in humans.

Th1 and Th2 cytokine expression in AD-like lesions

Acute and chronic AD lesions in humans also exhibit differences in their cytokine profile with acute lesions being characterized by Th2 cytokine (i.e. IL-4, -5 and -13) expression and chronic lesions being characterized by a more diverse pattern of cytokine expression that includes Th2, Th1 and other cytokine families [5, 49]. To further investigate the evolution of AD-like lesions in Stat6VT mice, transcripts for signature Th2 and Th1 (i.e. *Ifng*) cytokines were quantified in acute and chronic lesions as well as nonlesional skin from Stat6VT mice and WT littermate controls. These investigations revealed increased levels of *Il4* transcripts in both acute and chronic lesional skin samples from Stat6VT mice relative to WT controls (Fig. 2). There was also a trend toward increased *Il4* transcripts in nonlesional Stat6VT samples as compared to WT samples, but this difference was not statistically significant. With respect to IL-5, transcript levels were elevated significantly in nonlesional skin and even more so in lesional skin from these mice; however *Il5* differed from *Il4* transcripts in that the former were most prominent in acute lesional skin. With respect to the last Th2 cytokine examined, IL-13, transcripts were not above WT levels in nonlesional skin and were only significantly increased in chronic lesions. Finally, transcripts for the Th1

cytokine interferon gamma (IFN γ) were significantly elevated in chronic lesional skin. Similar to the clinical and histologic studies described previously, these experiments provided evidence that the immunologic profile of AD-like lesions evolves over time. Moreover, the patterns of expression in acute AD-like lesions from Stat6VT mice are more similar to acute AD lesional skin in humans, while chronic AD-like lesions exhibit patterns more similar to chronic AD lesional skin.

Epithelial cytokine gene expression in AD-like lesions

Previously, much focus was given to T cells and other infiltrating immune cell subsets as key drivers of AD immunopathogenesis. While infiltrating immune cells are certainly important for disease, recent investigations have implicated epithelial-derived cytokines, namely IL-33 and thymic stromal lymphopoietin (TSLP) in pathogenesis [20, 48, 57]. The expression profile of these cytokines has not been reported for Stat6VT mice. To better understand the contribution of epithelial cytokines to AD-like disease in this model, *I133* and *Tslp* transcripts were quantified (Fig. 3). Expression of *I133* was significantly increased in acute lesions and more prominently than in chronic lesions relative to WT and nonlesional skin controls. In contrast, *Tslp* expression was not significantly increased in nonlesional or lesional skin compared to WT controls, though there was a trend towards increased expression in chronic lesions.

IL-33 expression in AD-like lesional keratinocytes

Based on the observation that *I133* transcripts were more abundant in lesional skin of Stat6VT mice, immunofluorescence studies were undertaken to determine if and where IL-33 protein is expressed in AD-like lesions. Previous studies of IL-33 expression in keratinocytes and other cell types established that IL-33 localizes to the nucleus [8, 22]. Consistent with this, nuclear staining for IL-33 was seen in immunofluorescence studies and was most more prominent in keratinocytes from AD-like lesional skin of Stat6VT mice than WT controls (Fig. 4a). Moreover, nuclear staining for IL-33 was localized to a suprabasal keratin 5 (K5)-positive subset of keratinocytes, and the proportion of K5/IL-33 double positive keratinocytes in AD-like skin lesions was increased approximately 5 fold over WT skin ($P < 0.05$), equating to a 15-fold increase in the total number of IL-33⁺ keratinocytes in Stat6VT lesional skin compared to WT controls (Fig. 4b).

Increased AD-like disease severity in IL-33 deficient Stat6VT mice

Having demonstrated increased expression of IL-33 in AD-like lesions, the next issue investigated was how IL-33 might contribute to pathogenesis in this model. To address this, IL-33 knockout mice were crossed to Stat6VT mice to generate Stat6VTx IL-33^{-/-} mice; these mice and IL-33 sufficient (IL-33⁺) Stat6VT mice were monitored weekly from 10-20 weeks of age for development of the AD-like phenotype. Disease scoring was performed using a modified form of the eczema area and severity index (EASI). Wild type and IL-33^{-/-} mice did not develop any signs of skin inflammation. In contrast, both Stat6VT and Stat6VTx IL-33^{-/-} mice developed the AD-like phenotype. Furthermore, disease severity scores were significantly higher or trended higher for Stat6VTx IL-33^{-/-} mice at all time points compared to IL-33 sufficient Stat6VT mice (Fig. 5).

Discussion

Examining clinical, histologic and cytokine gene expression profiles of acute and chronic AD-like lesions from Stat6VT mice suggest allergic skin inflammation in this model is a dynamic process. Based on the parameters investigated in our animal model, acute AD-like lesions were similar to acute AD lesions in humans, while chronic AD-like lesions were more like chronic AD lesions in humans. Furthermore, analysis of epithelial cytokine expression revealed increased levels of IL-33 in AD-like lesions and localized this expression to a subset of keratinocytes. Finally, disease was exacerbated in IL-33 deficient Stat6VT mice compared to their IL-33 sufficient counterparts.

Epidermal barrier and immune dysregulation are the principal components of AD pathogenesis; however, the existence of multiple disease subtypes suggests the mechanisms leading to barrier and immune dysfunction may vary between populations and individuals. Fortunately, there are multiple mouse models of AD whose varied pathogenic mechanisms and dependencies on barrier dysfunction and different immune cell subsets (e.g. T vs. innate lymphoid type 2 cell) can provide insights to understanding AD in humans [7, 13, 15, 23, 25, 27, 43, 44]. In contrast to many mouse models of AD that develop disease secondary to environmental challenges or genetic defects in keratinocytes, the Stat6VT mouse develops

disease due to a monogenic defect in the T cell compartment and does not require immunization to an allergen or extended topical sensitization. The Stat6VT mouse model has many key features of human disease in that the AD-like disease phenotype in this model is critically dependent on IL-4 and is exacerbated in mice with *Flg* and *Tmem79* mutations [45]. The current studies provide evidence that acute and chronic AD-like lesions that develop in Stat6VT mice bear similarities to acute and chronic disease in humans and thus suggest this model could be a useful system in which to investigate the mechanisms involved in the evolution of AD lesions.

Interleukin 33 is an IL-1 family member cytokine produced by multiple cell types including keratinocytes and other epithelial cells [31]. Unlike many cytokines, IL-33 does not enter the secretory pathway after synthesis; instead it translocates to the nucleus, associates with chromatin and may regulate gene expression through modulation κ B and other mechanisms [2, 8, 10, 22]. Passive exit following cellular injury, active release following activation of purinergic receptors and other mechanisms have been proposed and/or demonstrated to explain how IL-33 is released from cells [24, 58]. The cytokine functions of IL-33 are mediated via binding and activation of a heterodimeric receptor composed of the ligand-specific receptor subunit, ST2L (ST2), and the IL-1 receptor family member IL-1RAcP [1, 9, 42]. Multiple cells express the IL-33 receptor including several T cell subsets (e.g. Th2 and Treg), ILC2, mast cells and eosinophils [18]. There is also evidence that keratinocytes can express an IL-33 receptor and that as a cytokine, IL-33 can impair keratinocyte maturation [38, 46]. While the immunomodulatory effects of IL-33 receptor activation vary by cell type and are numerous, final common pathways include augmentation of Th2 inflammation (e.g. through effects of Th2 and ILC2 cells), promotion of Th1 responses (e.g. promotion of IFN γ production by T and NK cells) and immunosuppression via activation of regulatory T cells [26, 35].

As the immunoregulatory functions of IL-33 are multiple and complex, as are the potential mechanisms of initiating allergic skin inflammation in different model systems, it is perhaps not surprising that the contributions of IL-33 to models of AD-like disease vary widely. In some models, IL-33 can promote disease via activation of T and ILC2 cells; in others IL-33 is not required, which may be related to the ability of other epithelial cytokines like TSLP and IL-25 to compensate for loss of IL-33 [20, 23, 34, 39]. There is also evidence that IL-33 can exert immunosuppressive effects and that loss of IL-33 can attenuate inflammatory diseases including AD-like inflammation [40]. Savinko et al. used an epicutaneous ovalbumin/staphylococcus enterotoxin B (SEB)-induced model of disease to investigate the function of the IL-33 receptor in AD-like inflammation. In comparison to WT controls, IL-33 receptor deficient mice, exhibited more pronounced skin inflammation by several measures including increased skin infiltrating neutrophils and increased numbers of T cells (i.e. CD3⁺) and F4/80⁺ macrophages in the epidermis. In addition, transcript levels for *Il6*, *Ifng* and *Il5* were significant higher in lesional skin from IL-33 receptor deficient mice compared to WT control. The report by Savinko et al. is thus compatible with our finding that IL-33 signaling can attenuate AD-like inflammation [40].

One of the mechanisms by which IL-33 can attenuate pathologic inflammation, is its ability to promote wound healing by several mechanisms. For instance, Monticelli et al. discovered that IL-33 limits gut inflammation and promotes wound healing in the dextran sodium sulfate-induce mouse model of colitis via IL-33-mediated stimulation of amphiregulin production by gut ILC2 cells [30]. Interestingly, ILC2 cells isolated from human skin express amphiregulin, and IL-33 can upregulate amphiregulin production by ILC2 cells [39]. In addition to ILC2, IL-33 can stimulate regulatory T cells to produce amphiregulin and promote wound healing [3]. Thus, it is conceivable, that IL-33 could promote wound healing in AD and models of allergic skin inflammation. The ability of IL-33 to polarize macrophage differentiation to an M2 phenotype may also enhance wound healing in the skin [37, 55, 56]. Recent studies by Oshio and co-workers also suggest nuclear expression of IL-33 in keratinocytes promotes wound healing and that this may be mediated by nuclear IL-33 inhibiting NF- κ B activity and promoting keratinocyte proliferation at the wound edge [33].

Another pathway by which IL-33 can attenuate inflammatory disease is via effects on regulatory T cells. In healthy (naïve) mice, approximately 10% of CD4⁺CD25⁺Foxp3⁺ splenocytes express the ligand specific subunit of the IL-33 receptor, ST2 [28]. Regulatory T cell proliferation, differentiation and suppressor function can all be promoted by IL-33 [41, 54]. Early reports suggesting IL-33 could suppress inflammation via stimulation of Tregs came from the transplant literature and demonstrated that intraperitoneal administration of IL-33 prolonged cardiac allograft survival and expanded numbers of graft-associated and splenic regulatory T cells [6, 53]. Similar effects were observed in other mouse allograft (e.g. skin and allogeneic hematopoietic stem cell) rejection models [16, 29]. In addition to attenuating

allograft rejection, IL-33 can directly and indirectly stimulate Treg proliferation and function and suppress pathologic inflammation in the colon, liver, central nervous system and other sites [14, 21, 32, 36, 41].

While additional work is needed to understand the role of IL-33 in Stat6VT mice, a working model to explain how IL-33 may attenuate AD-like inflammation is presented in [Figure 6](#). Based on the aforementioned discussion, it is conceivable that IL-33 could directly (e.g. via activation of the IL-33 receptor on keratinocytes) or indirectly (e.g. via amphiregulin production by ILC2 and regulatory T cells) promote wound healing in Stat6VT mice. Alternatively, it is possible that IL-33 restrains T cell-mediated allergic inflammation in Stat6VT mice through its effects on regulatory T cells. Studies are ongoing to investigate these potential mechanisms.

In conclusion, the data presented here indicate AD-like lesions evolve over time in Stat6VT mice. Lesion evolution manifests as changes in the epidermis, the composition of the inflammatory cell infiltrate and the cytokine expression profile. The dichotomy between acute and chronic lesions in Stat6VT mice is similar to that in humans; thus investigations of this model might provide insights into the biology of acute and chronic AD lesion in humans. The disparate contributions of IL-33 to different mouse models of AD highlight the complex biology of this cytokine and the concept that seemingly similar clinical phenotypes can arise via divergent molecular and cellular mechanisms.

Conflict of interest: The authors declare that they have no conflicts of interest.

Compliance with Ethical Standards/Ethical Approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Figure Legends

Fig. 1 Clinical and histologic features of acute and chronic AD-like lesions in Stat6VT mouse model. (a) Shown from left to right are the clinical appearances of skin from a WT mouse, an unaffected Stat6VT mouse (Nonlesional), a Stat6VT mouse with an acute AD-like lesion and a Stat6VT mouse with a chronic AD-like lesion. (b) Histologic appearances (H&E) of WT skin and nonlesional, acute and chronic AD-like lesional skin from Stat6VT mice (200x magnification; scale bar 100 μ M). (c) Dermal eosinophil counts in skin from WT skin and nonlesional, acute and chronic AD-like lesional skin from Stat6VT mice. Error bars represent SEM. *** $p < 0.01$; **** $p < 0.001$

Fig. 2 Relative expression of transcripts for Th2 cytokines *Il4*, *Il5* and *Il13* and the Th1 cytokine *Ifng* in WT skin and nonlesional, acute lesional and chronic lesional skin from Stat6VT mice as determined by quantitative PCR. Error bars represent SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.01$; **** $p < 0.001$

Fig. 3 Relative expression of transcripts for the epithelial-derived cytokines *Il33* and *Tslp* in WT skin and nonlesional, acute lesional and chronic lesional skin from Stat6VT mice as determined by quantitative PCR. Error bars represent SEM. *** $p < 0.01$; **** $p < 0.001$

Fig. 4 Increased staining for IL-33 in keratinocytes from Stat6VT AD-like lesions. (a) Immunofluorescence staining of WT and AD-like lesion skin for keratin 5 (K5; green), IL-33 (red) and nuclei (DAPI; blue). (b) Percentage of K5⁺ keratinocytes that are IL-33⁺ based on quantitation of four image fields (400x magnification; scale bar 50 μ M) per mouse (n=3 mice/group). Error bars represent SEM. *** $p < 0.01$

Fig. 5 Stat6VT mice on IL-33 deficient background exhibit more severe disease than IL-33 sufficient Stat6VT mice. Mice were monitored weekly from 10-20 weeks of age for determination of disease severity as described in the material and methods (n=11 mice/group). Error bars represent SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.01$

Fig. 6 Working model showing potential nodes at which IL-33 might act to attenuate AD-like disease in Stat6VT mice

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Table 1 Score tally sheet for modified eczema area and severity index (EASI) for AD-like disease in Stat6VT mice.

Head/Neck	SEVERITY INDEX				AREA		REGION SCORE	
	Nil 0	Mild 1	Moderate 2	Severe 3				
Erythema					Face (30%)			
Infiltration					Scalp (20%)			
Excoriations					Neck (40%)			
Lichenification					Ears (10%)			
SEVERITY SCORE					AREA SCORE			Multiple 0.15
Trunk	SEVERITY INDEX				AREA		REGION SCORE	
	Nil 0	Mild 1	Moderate 2	Severe 3				
Erythema								
Infiltration								
Excoriations					dorsal (50%)			
Lichenification					ventral (50%)			
SEVERITY SCORE					AREA SCORE			Multiple 0.6
Extremities	SEVERITY INDEX				AREA		REGION SCORE	
	Nil 0	Mild 1	Moderate 2	Severe 3				
Erythema								
Infiltration								
Excoriations					forelimbs (40%)			
Lichenification					hindlimbs (60%)			
SEVERITY SCORE					AREA SCORE			Multiple 0.15
Tail	SEVERITY INDEX				AREA		REGION SCORE	
	Nil 0	Mild 1	Moderate 2	Severe 3				
Erythema								
Infiltration								
Excoriations					dorsal (50%)			
Lichenification					ventral (50%)			
SEVERITY SCORE					AREA SCORE			Multiple 0.1
							TOTAL	

Figure 1

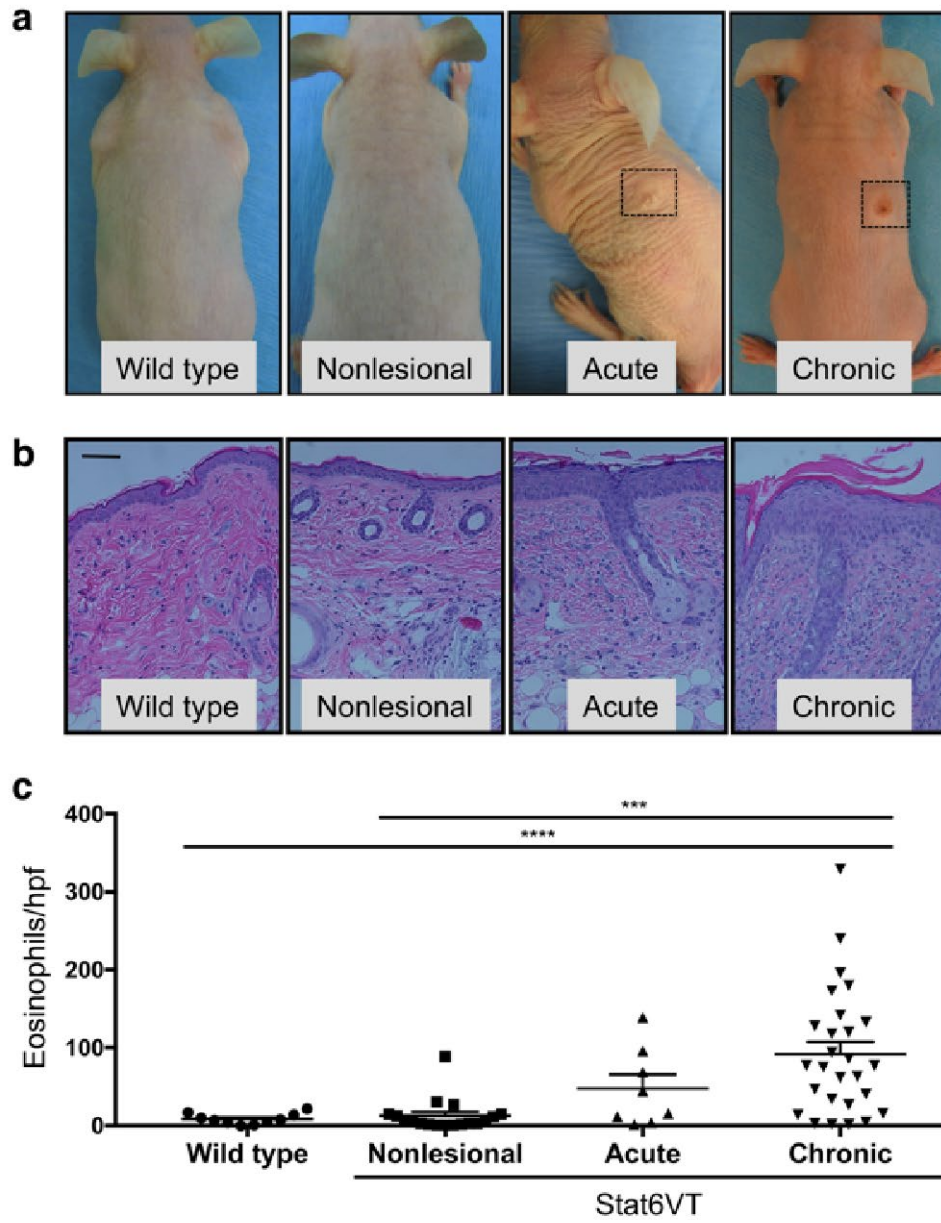


Figure 2

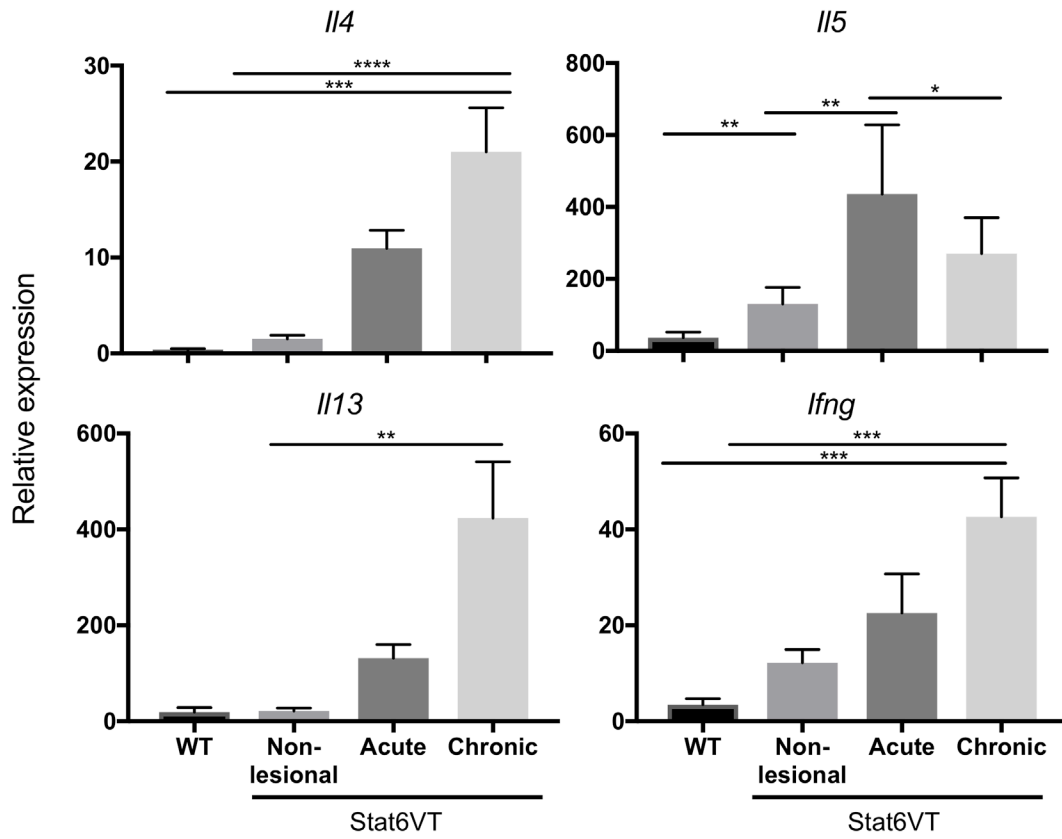


Figure 3

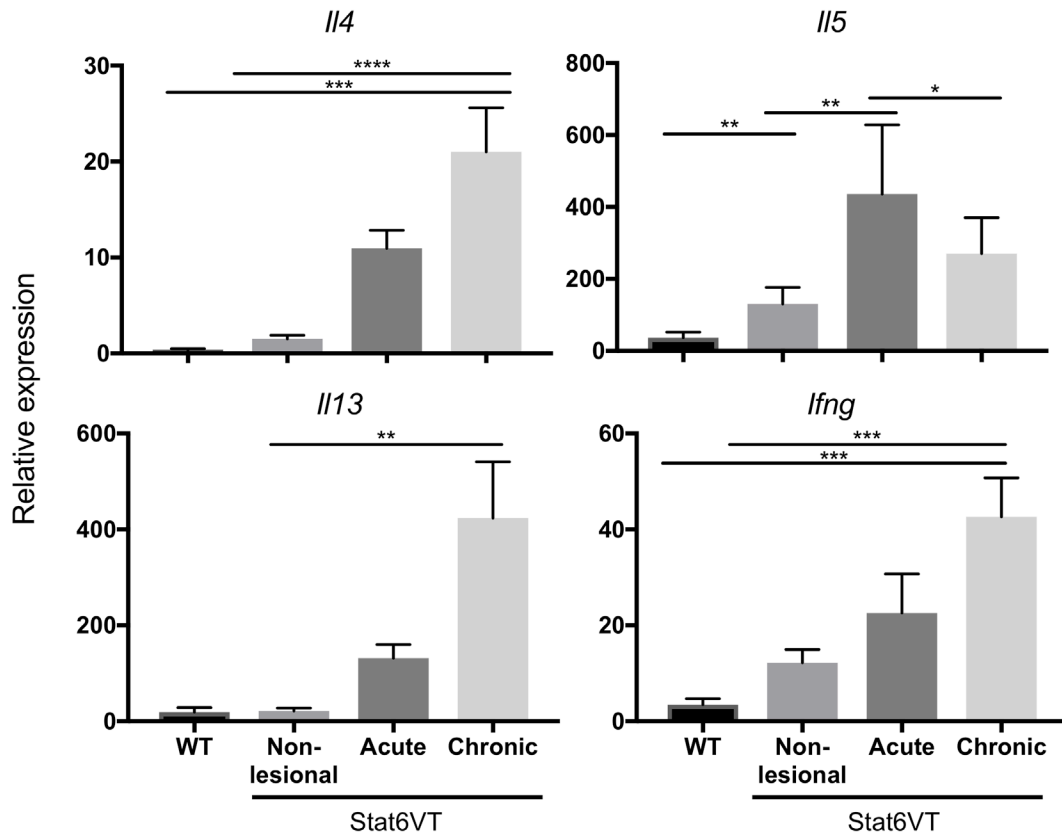


Figure 4

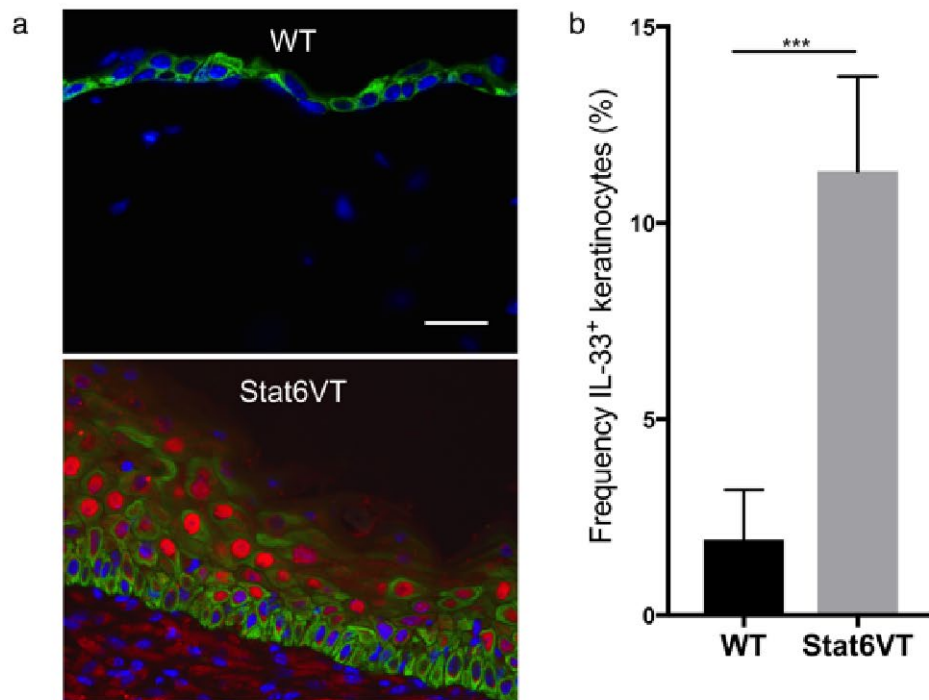


Figure 5

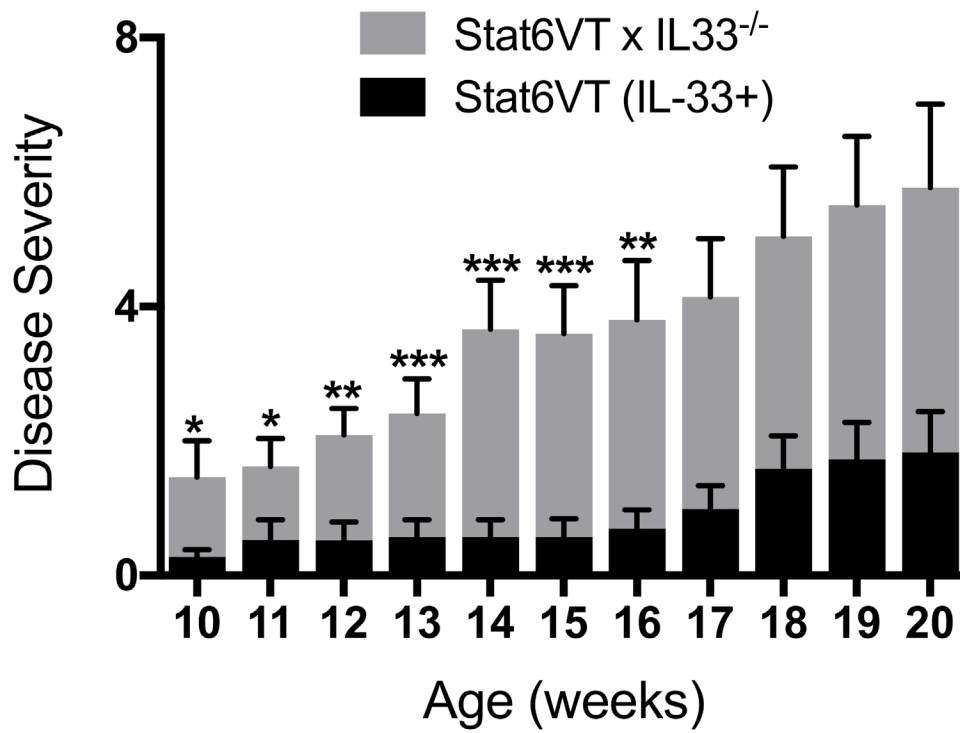


Figure 6

