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Mast Cells Regulate Epidermal Barrier Function and the Development of Allergic Skin Inflammation

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Abstract

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by infiltration of eosinophils, T helper cells and mast cells. The role of mast cells in AD is not completely understood. To define the effects of mast cells on skin biology, we observed that mast cells regulate the homeostatic expression of Epidermal Differentiation Complex (EDC) and other skin genes. Decreased EDC gene expression in mice that genetically lack mast cells ($Kit^{W\text{-}sh/W\text{-}sh}$ mice) is associated with increased uptake of protein antigens painted on the skin by dendritic cells, compared to similarly treated wild-type mice, suggesting a protective role for mast cells in exposure to nominal environmental allergens. To test this further, we crossed $Kit^{W\text{-}sh/W\text{-}sh}$ mice with Stat6VT transgenic mice that develop spontaneous AD-like disease that is dependent on Th2 cytokines and associated with high serum concentrations of IgE. We observed that Stat6VT x $Kit^{W\text{-}sh/W\text{-}sh}$ mice developed more frequent and more severe allergic skin inflammation than Stat6VT transgenic mice that had mast cells. Together, these studies suggest that mast cells regulate epidermal barrier function and have a potential protective role in the development of AD-like disease.

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Conflict of Interest

The authors state no conflicts of interest.

INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease (Schmitt *et al.*, 2013) that affects 1–3% among adults and up to 15–20% of children worldwide (Asher *et al.*, 2006). The incidence of AD has increased by 2- to 3- fold over the past several decades, especially in industrialized countries. The pathogenesis of AD is multifactorial and likely results from complex interactions between environmental and genetic factors, barrier defects and immune dysregulation resulting in epidermal hyperplasia and increased penetration of allergens and microbial pathogens (Gittler *et al.*, 2012; Guttman-Yassky *et al.*, 2011; Moniaga *et al.*, 2010). Recent studies have implicated a strong association between a defect in the skin barrier and the pathogenesis of AD (Irvine *et al.*, 2011). The defect is caused by genetic loss-of-function mutations in the *FLG* gene encoding filaggrin, a key protein for formation of the skin barrier. These mutations are found in a substantial proportion of AD patients (Palmer *et al.*, 2006; Sandilands *et al.*, 2007). Parallel mutations in the mouse *Flg* cause the phenotype observed in flaky tail mutant mice (Fallon *et al.*, 2009).

Multiple cell types are involved in the development of AD, including antigen presenting cells, T cells and polymorphonuclear leukocytes (Boguniewicz and Leung, 2011; Leung and Guttman-Yassky, 2014; Sehra *et al.*, 2008b). Mast cells mediate many functions in the skin including pathogen sensing and the release of antimicrobial peptides that contribute to host defense (Di Nardo *et al.*, 2007; Kumar and Sharma, 2010; Tete *et al.*, 2012; Wang *et al.*, 2012). In skin inflammation, mast cells can have distinct effects depending on the type of inflammation. Two studies have demonstrated that mast cells have a protective effect in sensitization models of allergic contact dermatitis through secretion of cytokines that include IL-10 and IL-2 (Grimbaldeston *et al.*, 2007; Hershko *et al.*, 2011). However, several reports suggest that mast cells promote inflammation in models of induced allergic skin inflammation where there is clearly an activation of Th2-mediated immune responses. In a model of ovalbumin-induced skin inflammation using W/W^{v} mice, mast cells regulated cytokine mRNA in the skin, and serum IgE concentrations, although pathology was not clearly different (Alenius *et al.*, 2002). In a subsequent model, mast cells were required for skin inflammation following epicutaneous sensitization with house dust mite extract and staphylococcal enterotoxin B, demonstrated using both the $Kit^{W\text{-}sh/W\text{-}sh}$ model and the *Cpa3*-Cre (Cre-Master) model (Ando *et al.*, 2013). Similarly, mast cells were required for Japanese Cedar Pollen-induced skin inflammation, demonstrated using the W/W^{v} model (Oiwa *et al.*, 2008). Together, these reports suggest context-dependent roles for mast cells in skin inflammation. The mechanisms of mast cell-dependent inflammatory regulation are not entirely clear.

Although many AD models require overexpression of genes in the skin or experimental exposure of irritants to the skin, we developed a model of AD that primes a Th2 response and leads to the spontaneous development of allergic skin inflammation. These mice, termed Stat6VT transgenic mice, have T cell-specific expression of a constitutively active Stat6 protein derived by mutating V-547 and T-548 to alanine (Bruns *et al.*, 2003; Daniel *et al.*, 2000). Transgenic mice have increased Th2 cell development, increased serum IgE concentrations, and develop spontaneous allergic inflammation in tissues around the eye, in the lung, and in the skin (Sehra *et al.*, 2008a; Sehra *et al.*, 2010; Turner *et al.*, 2014). The

skin of Stat6VT mice has slower recovery from injuries including treatment with retinoic acid, detergent and vitamin D analogs (DaSilva *et al.*, 2012; Sehra *et al.*, 2010; Turner *et al.*, 2013), and is more susceptible to infection (Howell *et al.*, 2011). This correlates with diminished expression of epidermal differentiation complex (EDC) genes and decreased barrier function (Sehra *et al.*, 2010).

Although mast cells have a putative role in skin pathogenesis, their role in homeostatic control of skin barrier function is not clear. Moreover, the function of mast cells during the development of spontaneous AD-like disease has not been examined. In this report we demonstrate that mast cells promote EDC gene expression and have a protective role in a model of AD dependent on increased Th2 immune responses.

RESULTS

Absence of mast cells results in decreased expression of EDC genes

To determine the contribution of mast cells to skin barrier integrity, we examined the expression of EDC genes in mice genetically deficient in mast cells. To this end, we characterized the expression of the EDC genes *Flg*, *Flg2*, *Ivl* and *Lor* in mast cell deficient *Kit* $^{W\text{-}sh/W\text{-}sh}$ and WT mice. Our results demonstrated a significant decrease in the expression of epidermal barrier genes in the absence of mast cells (Fig. 1, A). We observed a similar decrease in the *Klk7*, although in contrast, *Krt14* was not significantly changed and *Krt10* was increased (Fig. 1, A), suggesting that it is the later stages of keratinocyte differentiation that are affected. Further, mast cell reconstitution of *Kit* $^{W\text{-}sh/W\text{-}sh}$ mice with WT mast cells after 6 weeks resulted in a significantly increased expression of *Flg*, *Lor* and *Klk7* (Fig 1, A) but not *Ivl* or *Flg2*. The increased gene expression following mast cell transfer was not due to EDC gene expression in cultured mast cells, and the number of tissue mast cells after reconstitution was not significantly different than in WT mice (data not shown). To further demonstrate this effect, we tested expression of pro-filaggrin and involucrin in protein extracts from the skin of *Kit* $^{W\text{-}sh/W\text{-}sh}$ and WT mice. Consistent with the gene expression analysis, protein levels of pro-fillagrin and involucrin were decreased (Fig. 1, B–C). Moreover, decreased EDC gene expression correlated with increased transepidermal water loss (Fig. 1, E).

To investigate whether decreased EDC gene expression is unique to mast cell deficiency resulting from Kit deficiency, we assessed the expression of the EDC genes in a Kit-independent mast cell deficient mouse model using *Cpa3-Cre* mice that have the Cre gene encoded by the *Cpa3* locus (Feyerabend *et al.*, 2011). Our results demonstrate an impaired expression of skin barrier genes *Flg2*, *Ivl* and *Lor* in *Cpa3-Cre* transgenic mice (Fig 1, D) consistent with the observations in *Kit* $^{W\text{-}sh/W\text{-}sh}$ mice. However, we did not observe any effect on the expression of *Flg*.

Having shown that mast cell reconstitution in *Kit* $^{W\text{-}sh/W\text{-}sh}$ mice restored the expression of skin barrier genes *Flg* and *Lor*, we tested the effect of adding mast cell supernatants on the skin of WT mice to determine if secreted products from mast cells altered EDC gene expression. Incubation of mouse ear tissue with mast cell supernatants resulted in significantly increased expression of *Flg* and *Lor* but no significant increase in *Ivl* in

comparison to mouse ears incubated with media alone (Fig 2, A). These data indicate that mediators released from mast cells have an important role in regulating skin barrier integrity. Moreover, the pattern of induction in EDC gene expression in the tissue explant assay parallels the restricted induction of EDC genes in transplanted *Kit* $^{W\text{-}sh/W\text{-}sh}$ mice.

We then tested whether mast cells were also required for this response. We performed the same experiment, comparing tissue from *Kit* $^{W\text{-}sh/W\text{-}sh}$ and WT mice and observed that although *Flg* was increased in WT tissue, the mast cell supernatant did not induce *Flg* in the tissue from *Kit* $^{W\text{-}sh/W\text{-}sh}$ mice (Fig. 2, B), suggesting that mast cells were required for this in vitro tissue response. To further define the mechanism of this ex vivo response, we examined potential mediators and based on publications that histamine can regulate EDC genes (Gschwandtner *et al.*, 2013), we compared the ability of supernatants from WT or histadine decarboxylase-deficient mast cells, the latter that do not produce histamine, to mediate the effect. We observed that although supernatants from WT mast cells increased *Flg* expression, supernatants from *Hdc*−/− mast cells did not (Fig. 2, C). Moreover, histamine added directly to the culture increased *Flg* expression (Fig. 2, C). Together, these results suggest that mast cells promote gene expression in keratinocytes and that histamine is at least one mediator of the response.

Decreased barrier function in the absence of mast cells

To determine if the decreased expression of epidermal barrier genes in mast cell-deficient mice had functional consequences, we tested the ability of the protein antigen to cross the skin and be taken up by dendritic cells. We have previously used this assay and shown that allergen uptake is inversely correlated to EDC gene expression (Sehra *et al.*, 2010). Twenty-four hours after painting the shaved backs of WT and *Kit* $^{W\text{-}sh/W\text{-}sh}$ mice with Alexa647-labeled OVA, the dendritic cells in the draining lymph nodes were examined for the uptake of labeled OVA. Significantly increased percentages of Alexa647^{+} CD11c^{+} cells were observed in *Kit* $^{W\text{-}sh/W\text{-}sh}$ mice than in WT mice (Fig 3, A and B). These results demonstrate that in the absence of mast cells there is increased protein translocation across the skin barrier.

Increased numbers of mast cells in Stat6VT mice

Constitutively active Stat6 transgenic mice are prone to the development of spontaneous allergic skin inflammation with increased infiltration of eosinophils and lymphocytes. In order to determine if mast cells are present and increased in the skin of Stat6VT mice, compared to wild type mice, we analyzed the toluidine blue stained sections of ear tissue from each strain. As shown in Fig 4A–B, we observed a 2-fold increase in the numbers of mast cells in skin from Stat6VT mice, compared to WT skin. Interestingly, increased mast cells were only observed in the skin and not in the lung or peri-ocular mucosal tissues from Stat6VT transgenic mice (data not shown). Increased mast cells correlate with increased IL-9 secretion from Stat6VT transgenic T cells (Fig. 4, C). Thus, the Stat6VT model of allergic skin inflammation is characterized by increased mast cell accumulation in the skin.

Increased incidence and severity of allergic skin inflammation in Stat6VT x *Kit* $^{W\text{-}sh/W\text{-}sh}$ transgenic mice

Based on the ability of mast cells to alter EDC gene expression and barrier function, and the increased numbers of mast cells in the skin of Stat6VT mice, we next wanted to define the function of mast cells in this model of allergic skin inflammation. In order to determine if mast cells have a protective or pathogenic role in AD, we mated Stat6VT mice to *Kit* $^{W\text{-}sh/W\text{-}sh}$ mice to generate mast cell-deficient Stat6VT mice. We monitored WT, Stat6VT, Stat6VT x *Kit* $^{+/W\text{-}sh}$ and Stat6VT x *Kit* $^{W\text{-}sh/W\text{-}sh}$ for the incidence and severity of disease. Our data demonstrate that while 30% of Stat6VT mice develop severe disease (Fig 5, A), the percentage of mice that develop severe disease on the mast cell-deficient background was considerably higher, even in heterozygous mice (80% for Stat6VT x *Kit* $^{W\text{-}sh/W\text{-}sh}$ mice and 50% for Stat6VT x *Kit* $^{+/W\text{-}sh}$). Further, Stat6VT x *Kit* $^{W\text{-}sh/W\text{-}sh}$ mice developed severe AD lesions at 5–6 months, earlier than Stat6VT mice that developed severe disease at 6–7 months, indicating an earlier onset of severe disease in Stat6VT x *Kit* $^{W\text{-}sh/W\text{-}sh}$ (Fig 5B).

Next, we compared the percent of mice that required euthanasia or died due to severe AD lesions using Kaplan-Meier morbidity analysis. We observed a significantly higher percentage of Stat6VT and Stat6VT x *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice requiring euthanasia due to severe disease as compared to WT mice (Fig 5, C), but the difference between Stat6VT and Stat6VT x *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice was not significant. Overall, our results indicate that mast cell-deficiency exacerbated the development of AD in Stat6VT mice.

To further characterize the histopathology associated with increased disease in the absence of mast cells, we performed histological analysis of ear skin tissue from WT, Stat6VT and Stat6VT x *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice aged 4–7 months. Thickening of the dermis and epidermis with cellular infiltration of eosinophils, lymphocytes and mast cells was observed in Stat6VT mice. Stat6VT x *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice demonstrated a marked increase in the thickening of the dermis and epidermis and a dramatic increase in the inflammatory cell infiltrate predominated by eosinophils and lymphocytes compared to Stat6VT or WT mice (Fig 6, A). To quantitate the differences in pathology we scored inflammation and dermal thickness, and observed the expected increased score in Stat6VT samples, compared to WT samples. These scores were further increased in Stat6VT x *Kit* $^{W\text{-}sh/W\text{-}sh}$ mice (Fig 6, B & C). Increased inflammation was not due to cumulative effects of the STAT6VT transgene and the Kit mutation on EDC gene expression (Fig. 6, D).

To determine if the type of inflammation was altered in the skin of Stat6VT mice versus Stat6VT x *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, we performed qPCR for genes associated with specific cell lineages. Although not all comparisons were statistically different, changes in gene expression were consistent with a trend towards increased macrophage infiltration (*Mertk*) and decreased eosinophils (*Rnase2*), neutrophils (*Mpo*) and T cells (*Cd3*) (Fig. 6, E). Although the qPCR analysis does not distinguish between altered cell numbers in the tissue and altered gene expression with cells in the tissue, the decrease in eosinophils was confirmed by quantitation of cells in histological analysis (Fig. 6, F&G). Increased *Mertk* expression was associated with significantly increased expression of *Nos2* but not *Arg*, suggesting increased M1 macrophages in the tissue (Fig. 6, E). Together, these data indicate

that mast cell-deficiency in Stat6VT mice leads to worse disease with altered tissue pathology.

DISCUSSION

Mast cells are multi-functional cells that participate in innate immune responses and are effectors for adaptive responses, particularly in mediating immediate hypersensitivity responses. Yet, it is still not clear what role mast cells play in the development of allergic skin inflammation. In this report we have identified a function for mast cells in regulating the homeostatic expression of EDC genes. In mast cell-deficient mice there is diminished expression of multiple EDC genes, and evidence of increased barrier permeability to protein antigens. This correlated with increased incidence and severity of allergic skin inflammation in mice that lacked mast cells. Together, these data support a role for mast cells as promoting skin homeostasis.

We observed decreased expression of epidermal barrier genes in two strains of mast cell-deficient mice. Interestingly, transplantation of bone marrow-derived mast cells only compensated for expression of a subset of these genes. This is possibly explained by the observations that there are distinct populations of mast cells and that transplant of BMMC does not effectively reconstitute all populations (Cyphert *et al.*, 2011). Although this was demonstrated in the lung, it is possible that this is a common limitation of the BMMC transplant procedure. A differing ability of different subtypes of mast cells to regulate EDC genes would fit with the inability of the transplanted BMMC to increase expression of *Flg2* or *Ivl* above levels observed in mast cell-deficient mice, while there was recovery of expression of the other EDC genes. Another interesting point from this analysis was that *Flg* expression was reduced in the *Kit* $^{W\text{-}sh/W\text{-}sh}$ mice but not in the *Cpa3-Cre* mice. Although the exact mechanism for this difference is unknown, it could be linked to differences in the genetic background of the mice. *Flg* and *Cpa3* are only 73 Mb apart on mouse chromosome 3, and if the *Flg* gene in the 129 genetic background DNA were less responsive to cytokines, and were retained in the backcrossed mice, it might explain the differential responsiveness.

The mechanism of mast cell-dependent regulation of EDC gene expression is still not entirely clear. The low number of mast cells in the skin, as well as the ability of mast cell supernatants in our assays to regulate EDC gene expression, suggests that the mechanism is not cell contact-dependent. Our data suggest that histamine is at least one mediator that could be impacting gene expression. However, our results are different from a recent report suggesting that histamine decreases barrier function in keratinocyte cultures (Gschwandtner *et al.*, 2013). The experimental systems were different, with Gschwandtner et al examining human skin equivalents, and our study using mouse ex vivo tissue culture. In the latter, many more cell types are present in the explant, including mast cells themselves that our data suggests are important for the effects observed in this report. Thus, there may be conflicting effects of histamine on various cell types in the skin. Moreover, histamine may just be one mediator among many and the direct effects of histamine on keratinocytes are likely only one component of the tissue milieu.

The *Kit* $^{W\text{-}sh/W\text{-}sh}$ mice obviously have defects in immune responses other than mast cell deficiency. Thus in our experiments, it is possible that non-mast cell effects might be impacting some of the assays. For example, in the allergen uptake experiment, Kit signaling can have direct and indirect effects on dendritic cell function. One report (Otsuka *et al.*, 2011) found that mast cells could impact DC migration. Yet in our study, we did not observe significant differences in CD11c+ MHC II-hi cells in the draining lymph node, arguing against this being a major factor. Another report (Reuter *et al.*, 2010) found decreased uptake of labeled OVA by DC from mast cell-deficient mice, but this was performed in a sensitization model where effects were potentially IgE-mediated, and a mechanism that is likely not relevant for our short term assay. Finally, a report examining dendritic cell-T cell interactions found that Kit signaling in dendritic cells affected T helper cell polarization, and this was caused by altered cytokine production, and not by other functional defects (Krishnamoorthy *et al.*, 2008). Ultimately, the strongest support for our conclusions comes from the parallel usage of the *Cpa3* ^{Cre} mice that have mast cell-deficiency through a Kit-independent mutation.

The skin inflammation in the Stat6VT x *Kit* $^{W\text{-}sh/W\text{-}sh}$ mice was more severe than in Stat6VT transgenic mice by several measures in this study including visual scoring of lesions and histological examination. We also determined through histological examination and gene expression analysis that the type of inflammation is altered in the absence of mast cells. Our observations suggest that in the absence of mast cells, M1 macrophages are increased in the tissue concomitant with a trend towards decreased infiltration of eosinophils and T cells. The basis for this skewing is not clear. It is possible that the increased barrier permeability, that we have shown is associated with mast cell-deficiency, results in greater environmental exposure and responses. As the environmental agent that triggers the development of allergic skin inflammation in the Stat6VT mouse model has not been defined, how barrier function changes that exposure is difficult to determine.

Our results showing a protective role of mast cells in a spontaneous model of AD differ from previously published results using inducible models of AD where mast cells were required for pathology (Ando *et al.*, 2013; Oiwa *et al.*, 2008). There are many potential reasons for these differences including that inducible models utilize skin irritation (shaving, extensive tape stripping) or adjuvants (alum, staphylococcal enterotoxin B) to catalyze the epicutaneous immune response (Alenius *et al.*, 2002; Ando *et al.*, 2014; Oiwa *et al.*, 2008). Our model also takes advantage of an immune response that is hyper-polarized to the Th2 phenotype, coincident with high concentrations of serum IgE (Bruns *et al.*, 2003). In this respect, the Stat6VT model might be more related to immune responses in patients with extrinsic AD, characterized by correlations between Th2 indicators and IgE concentrations with SCORAD (Gittler *et al.*, 2013; Suarez-Farinas *et al.*, 2013). Many of the inducible models of AD, in the absence of Th2 responses, will still generate skin inflammation, characterized by immune responses directed by other Th subsets, perhaps likening them to intrinsic AD (Czarnowicki *et al.*, 2014; Sehra *et al.*, 2008b). These results suggest that the role of mast cells in regulating skin inflammation might be dependent on the type of T helper response that is directing the allergen/irritant-specific immune response.

Our report highlights a unique role for mast cells in regulating EDC gene expression and epidermal barrier function. In a model of spontaneous AD initiated by increased Th2 activity, the absence of mast cells results in more severe AD-like disease. This suggests that mast cells can play an important role in regulating AD susceptibility and severity. It will be important to extend the observations on mast cell-keratinocyte interactions from our report to other systems and in patient populations to determine if the mast cell-keratinocyte axis might be targeted for therapeutic benefit.

METHODS

Generation of Stat6VT and Stat6VT x *Kit* $^{W\text{-}sh/W\text{-}sh}$ transgenic mice

The generation of Stat6VT transgenic mice was previously described (Bruns *et al.*, 2003). Transgene-positive founders (CD2: Stat6VT [78] line), where the human Stat6 gene with V547 and T548 mutated to alanine is under transcriptional control of the CD2 locus control region, were backcrossed to C57BL/6 mice (Harlan Breeders, Indianapolis, IN). For adoptive transfer experiments, C57BL/6 *Kit*$^{W\text{-}sh/W\text{-}sh}$ were obtained from Jackson Laboratory (Bar Harbor, ME). *Hdc*−/− mice were provided by Drs. Hiroshi Ohtsu (Tohoku University) and Paul Bryce (Northwestern University) (Ohtsu *et al.*, 2001; Swartzendruber *et al.*, 2012). To obtain Stat6VT mice on a mast cell deficient background, Stat6VT mice were mated to *Kit*$^{W\text{-}sh/W\text{-}sh}$. Skin samples from *Cpa3*^{Cre} (Feyerabend *et al.*, 2011) and control mice were graciously provided by Drs. Thorsten Feyerabend and Hans-Reimer Rodewald. All mice were maintained in specific pathogen-free conditions, and experiments were approved by the Indiana University Institutional Animal Care and Use Committee.

Preparation and Adoptive Transfer of Mast Cells

The preparation of bone marrow derived mast cells was done as previously described (Kalesnikoff and Galli, 2011). The purity of MCs was >90% based on toluidine blue staining and surface expression of CD117 and FcεRI. For adoptive transfer of mast cells in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, wild type (WT) mast cells were obtained after a 4–5 week culture of bone marrow cells in medium containing 20 ng/ml recombinant mouse IL-3 (Peprotech). For MC-reconstitution studies, bone marrow derived mast cells were adoptively transferred via subcutaneous injection of 10^6 cells into eight sites in the shaved dorsal skin of 6–8 wk *Kit* $^{W\text{-}sh/W\text{-}sh}$ mice. After 6 weeks, skin was collected for analysis of EDC gene expression.

Analysis of gene and protein expression

For real-time PCR measurements, involved or uninvolved skin was homogenized in a tissue lyser (Qiagen, Valencia, CA), and RNA isolated with the RNeasy fibrous tissue kit (Qiagen) was used to synthesize cDNA with the First-Strand Cloned AMV kit (Invitrogen, Rockville, MD). Message levels of barrier function genes were determined by Taqman assay (Applied Biosystems, Foster City, CA). Cycle number of the samples was normalized to the expression of β_2- microglobulin. T cell stimulation and ELISA for IL-9 were performed as described (Chang *et al.*, 2010; Sehra *et al.*, 2008a). Immunoblot for EDC genes was performed as described (Sehra *et al.*, 2010).

Epicutaneous sensitization and skin dendritic cell migration to draining lymph nodes

WT and *Kit* $^{W\text{-}sh/W\text{-}sh}$ were epicutaneously sensitized with OVA-Alexa Fluor 647 (Invitrogen, Carlsbad, CA). Briefly, the back skin of anesthetized mice was shaven and gently tape stripped three times to remove external lipids before painting with 500 μg OVA-Alexa Fluor 647 dissolved in PBS. Twenty-four hours later, mice were sacrificed and draining lymph nodes were harvested. Cells were first incubated with anti-CD16/CD32 mAb (2.4G2; BD Biosciences, San Jose, CA) and stained with FITC anti-mouse MHC class II (MHC-II) and PE anti-mouse CD11c (BD Biosciences). The proportion of OVA-Alexa Flour 647+ cells was quantified as described previously (Sehra *et al.*, 2010).

In vitro treatment of skin with mast cell supernatants

Ear skin from WT mice was divided in quarters but incubated intact for 24h with mast cell supernatants or control media before expression of epidermal barrier genes from the treated skin was determined using Taqman assay. Mast cell supernatants were generated by culture of bone marrow derived mast cells as described above for 24 hours in the absence of any stimulation. Stimulation of mast cells in culture with antigen/IgE did not alter the results of the incubation.

Histological examination of skin sections

Skin tissues were fixed in neutral buffered Formalin. Paraffin-embedded tissue sections were stained with hematoxylin and eosin (H & E), or toluidine blue to evaluate the infiltration of inflammatory cells and mast cells, respectively, by light microscopy. The numbers of mast cells were quantified in toluidine blue stained sections by counting in 10 high power fields. The numbers of eosinophils were quantified in H&E stained sections by counting 4 high power fields and averaging among samples from separate mice.

Quantification of skin pathology

Mice were monitored for the onset and development of mild or severe AD lesions between 3–13 months. The percentage of mice that develop no disease, mild (blepharitis and/or small lesions with erythema) or severe disease (more than one lesion with skin showing signs of erythema and scaling) was determined.

Histological slides were scored in a blinded fashion with light microscopy and a semi-quantitative scoring scale: 0, no inflammation; 1, minimal inflammation of dermal or epidermal layers; 3, extensive inflammation in dermal and epidermal layers. Dermal thickness was scored in a similar manner.

TEWL was performed as previously described (DaSilva *et al.*, 2012).

Statistical analyses

Kaplan-Meier morbidity estimates were used to evaluate the mice that required euthanasia or those that died due to severe lesions and analysis performed with GraphPad Prism 6. For other experiments, data were expressed as means of 3 independent experiments and analyzed with the Student *t*-test or Chi-square test. A p value of less than 0.05 was considered statistically significant.

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Abbreviations

AD	atopic dermatitis
BMMC	bone marrow-derived mast cells
EDC	epidermal differentiation complex

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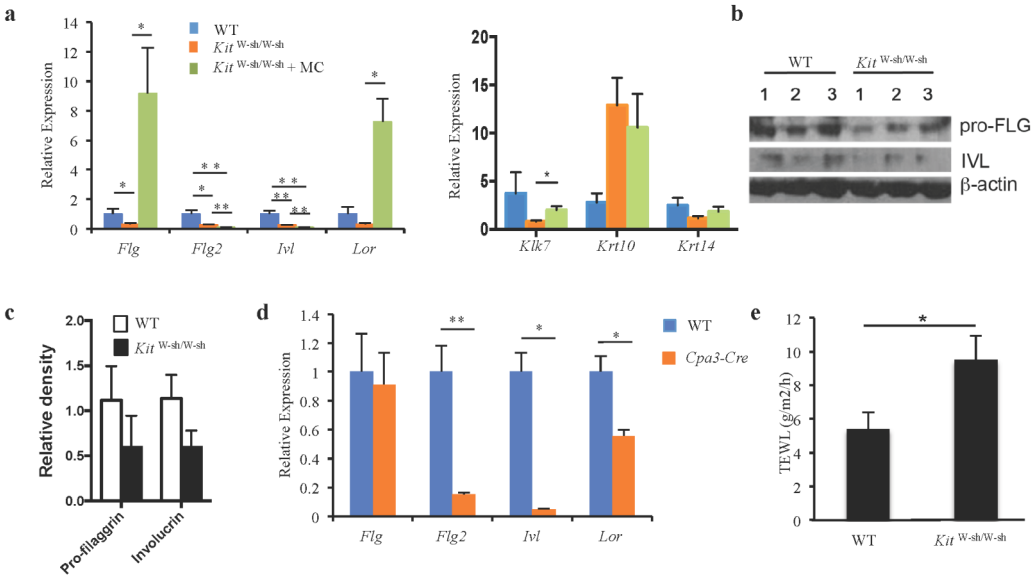

Figure 1.

Expression of EDC genes in Mast cell-deficient mice. A, RNA was isolated from the ear skin of WT and *Kit* $^{W\text{-}sh/W\text{-}sh}$ mice. Expression of the indicated genes was determined by quantitative PCR analysis of skin RNA. In mast cell reconstitution experiments, bone marrow derived mast cells were adoptively transferred via subcutaneous injection of 10^6 cells in the shaved dorsal skin of *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. After 6 weeks, skin was collected for analysis of EDC gene expression. B, Transepidermal water loss (TEWL) of shaved skin from WT and *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. C, RNA was isolated from the ear skin of WT and *Cpa3-Cre* mutant mice. D, Immunoblot analysis of ear tissue from WT and *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice for the indicated proteins. E, Densitometry of immunoblot analysis in (D). Quantitative PCR analysis was performed for gene expression from skin tissues. Data shown represent the mean ± SEM of 3 to 7 mice per group. *$p < 0.05$.

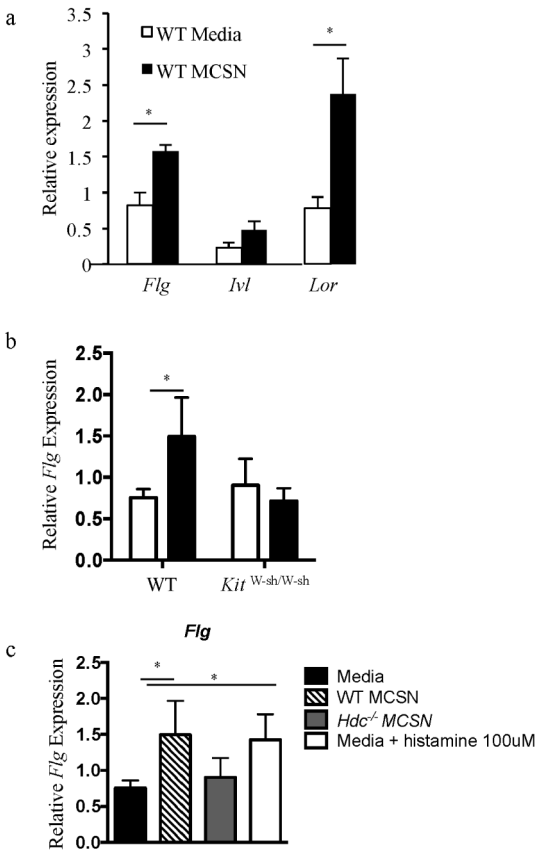

Figure 2.

Increased EDC gene expression in WT skin tissue treated with *in vitro* derived mast cell supernatants. Ear tissues from WT mice were incubated in the presence of mast cell supernatants or mast cell media for 24h. A, Expression of EDC genes in RNA isolated from ear tissue was determined by qPCR analysis. B, *Flg* expression was assessed in tissue from WT or $Kit^{W\text{-}sh/W\text{-}sh}$ mice incubated in mast cell supernatants as above. C, *Flg* expression was assessed in tissue from WT mice incubated in mast cell supernatants from WT or *Hdc*−/− mast cells, or with histamine, as indicated. Data are shown for 3–5 mice per group and representative of 2–4 experiments that yielded similar results. $*p < 0.05$.

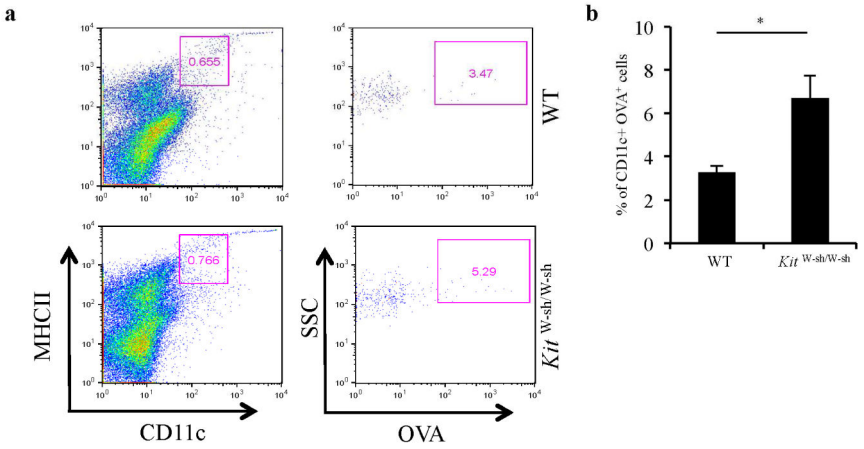

Figure 3.

Mast cell deficiency decreases skin barrier function. The shaved backs of WT and *Kit* $^{W\text{-}sh/W\text{-}sh}$ mice were treated with OVA-Alexa 647 for 24h. Uptake of OVA was assessed by measuring the percentage of DCs (CD11c+ MHC-II^{hi}) that stained positive for Alexa 647 fluorescence. Dot plots (A) are representative from 2 independent experiments with similar results. Bar graph (B) represents the mean ± SEM of the percent positive cells. *Significantly different from WT, $p < 0.05$.

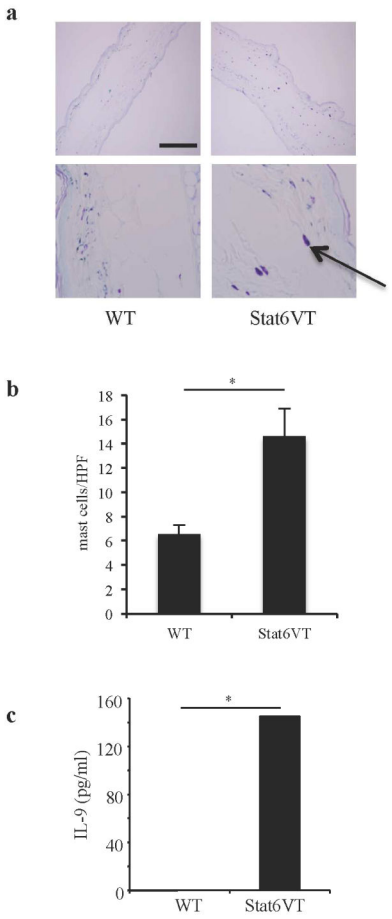

Figure 4.

Increased mast cell numbers in skin from Stat6VT mice. A, Mast cell numbers were evaluated in skin tissue sections stained with toluidine blue. Scale bar indicates 0.4 mm for the top panels and 0.07 mm for the bottom panels. An arrow pointing towards a mast cell is shown. B, Graph represents the mean ± SEM of 5–7 mice per group. C, CD4+ T cells from WT and Stat6VT transgenic mice were stimulated with anti-CD3 and supernatants were tested for IL-9 concentration using ELISA. *$p < 0.05$.

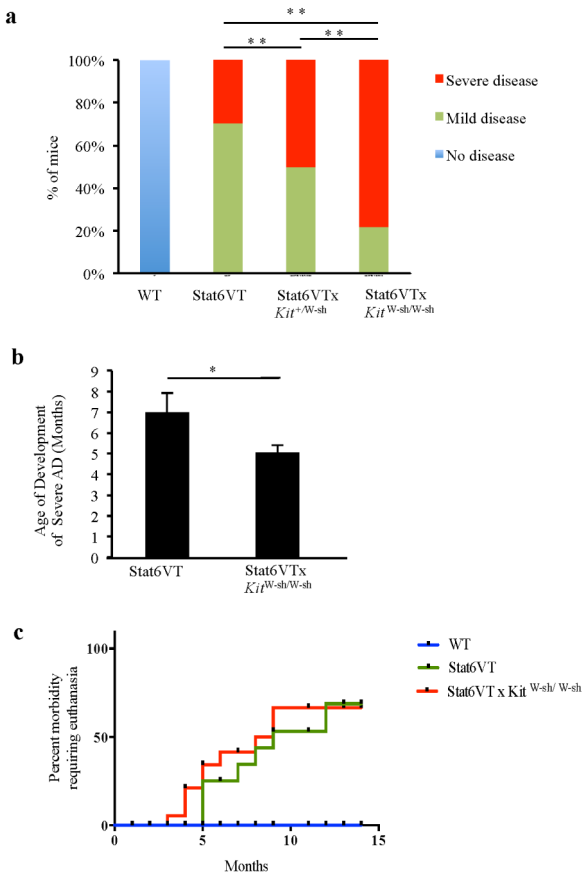

Figure 5.

Increased incidence and higher mortality in Stat6VT x *Kit* $^{+/W\text{-}sh}$ and Stat6VT x *Kit* $^{W\text{-}sh/W\text{-}sh}$ mice. A, Percent of mice that develop no disease, mild disease or severe disease in mice of the indicated genotypes. Data were analyzed by Chi-square test ** $p < 0.0001$, n=13–24. B, The average age of development of severe disease in months is indicated in mice of the respective genotypes. Data are the mean ± SEM of 22–24 mice per group and were analyzed with student's t-test. * $p < 0.05$ C, Morbidity curves of the indicated genotypes by Kaplan-Meier analysis, n=8–10/group.

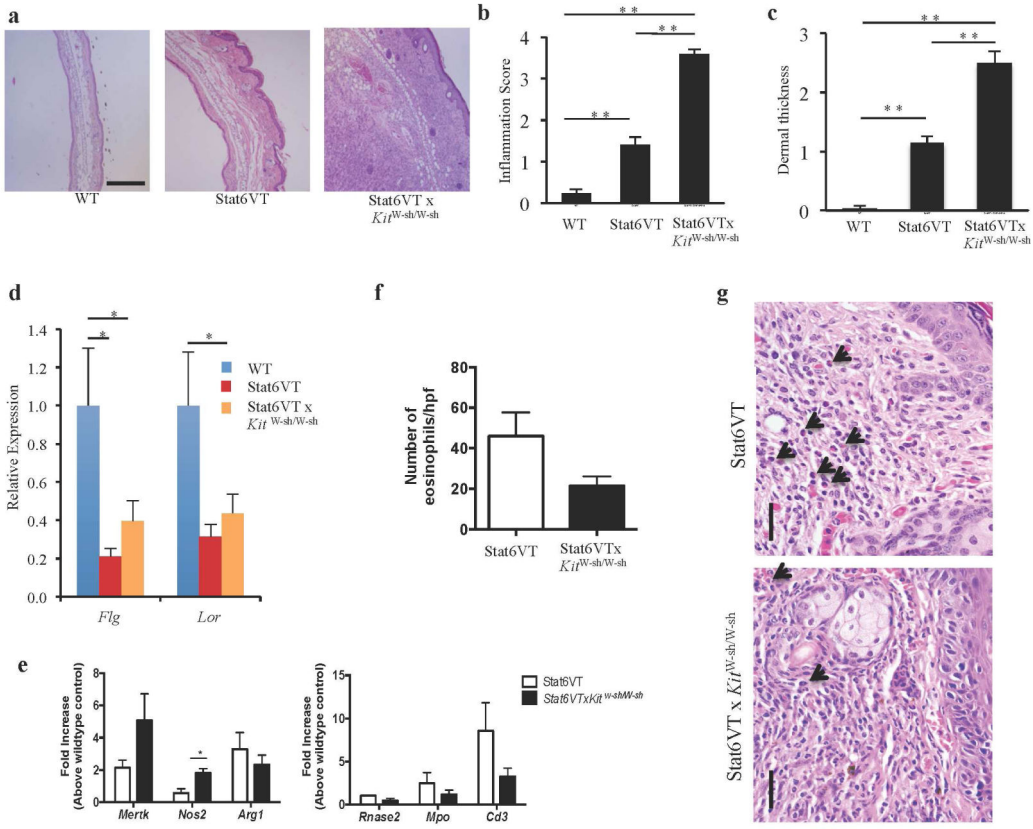

Figure 6.

Histological examination of mast cell-deficient Stat6VT transgenic mice. A, Histological analysis of ear tissue from WT, Stat6VT, and Stat6VT x *Kit* W-sh/W-sh mice. Samples were fixed and stained with H & E. Bar represents 0.4 mm. B, The inflammation in these sections was blindly scored for several pathophysiological parameters using a semi-quantitative scale of 0–3. All values are presented as the mean ± SEM. $^{**}p < 0.01$, n=10–12 per group. C, The dermal thickness scores were also evaluated using a semi-quantitative scale of 0–3. $^{**}p < 0.01$, n=10–12 per group. D, Expression of EDC genes in skin from mice of the indicated genotypes and analyzed as in Fig. 1. E, Expression of inflammatory genes in skin from mice of the indicated genotypes and analyzed as in Fig. 1. F, Quantitation of eosinophils from tissues shown in (G). G. High power photomicrographs of inflamed tissue from mice of the indicated genotypes. Eosinophils are indicated by arrows. Bars represent 0.04 mm.