博士論文

論文題目　Critical roles of mast cells and their Stat5 activity in allergic skin inflammation

（マスト細胞とその Stat5 活性のアレルギー性皮膚炎症における重要性）

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Contents

Abstract

Atopic dermatitis (AD) is a chronic inflammatory skin disease. Although mast cells have been considered as a major cell type contributing to allergic reactions, no case of mast cell deficient human has been reported. Therefore, mouse models are indispensable to functionally define the mast cells' roles. Here I show that phospholipase C-β3 (PLC-β3)-deficient mice develop AD-like skin lesions spontaneously and more severe allergen-induced dermatitis than wild-type mice. Mast cells were required for both AD models and remarkably increased in the skin of $Plcb3^{-/-}$ mice due to the increased Stat5 and reduced SHP-1 activities. Mast cell-specific deletion of Stat5 gene ameliorated allergen-induced dermatitis, whereas that of Shp1 gene encoding Stat5-inactivating SHP-1 exacerbated it. PLC-β3 was regulating expressions of periostin in fibroblasts and TSLP in keratinocytes, two proteins critically involved in AD pathogenesis. Mast cells were regulating periostin levels in the allergen-induced skin lesion. Furthermore, polymorphisms in PLCB3, SHP1, STAT5A and STAT5B genes were associated with human AD, and increased mast cells with high levels of phospho-STAT5 were found in lesional skin of some AD patients. Levels of phospho-STAT5 were inversely correlated with the expression of PLC-β3. Therefore, STAT5-regulatory mechanisms in mast cells are important for the pathogenesis of AD and could be novel therapeutic targets.

List of abbreviations

AD	-	atopic dermatitis
ADEH	-	AD patients with at least one eczema herpeticum episode
ADVN	-	Atopic Dermatitis and Vaccinia Network
ANOVA	-	analysis of variance
APC	-	allophycocyanin
AU	-	arbitrary unit
BMCP	-	basophil/mast cell progenitor
BMMC	-	bone marrow-derived mast cells
BSA	-	bovine serum albumin
CD	-	cluster of differentiation
CKO	-	conditional knockout
DC	-	dendritic cell
DAPI	-	4',6-diamidino-2-phenylindole
Der f	-	*Dermatophagoides farinae*
EASI	-	eczema area and severity index
ECL	-	enhanced chemiluminescence
EDTA	-	ethylenediaminetetraacetic acid
EH	-	eczema herpeticum
ELISA	-	enzyme-linked immunosorbent assay
FACS	-	fluorescence-activated cell sorting
Fc	-	fragment crystallizable region
FcεRI	-	Fc epsilon receptor I alpha
FcγRIIb	-	Fc gamma receptor II b
FCS	-	fetal calf serum

FITC	-	fluorescein isothiocyanate
fl	-	floxed (=flanking/flanked by LoxP sites)
FLG	-	filagglin
GEO	-	Gene Expression Omnibus
GFP	-	green fluorescent protein
GM-CSF	-	granulocyte-macrophage colony-stimulating factor
GWAS	-	genome-wide association study
H&E	-	haematoxylin and eosin
HEPES	-	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPF	-	high power field
HRP	-	horseradish peroxidase
HSC	-	haematopoietic stem cell
HSV	-	Herpes simplex virus
IFN	-	interferon
IgE	-	immunoglobulin E
IgG	-	immunoglobulin G
IL	-	interleukin
IL-3R	-	interleukin 3 receptor
IRES	-	internal ribosome entry site
JAK	-	Janus kinase
KO	-	knockout
MCΔSHP-1	-	mast cell-specific deletion of SHP-1 (Mcpt5-Cre; $Shp1^{fl/fl}$)
MCΔStat5	-	mast cell-specific deletion of Stat5 (Mcpt5-Cre; $Stat5^{fl/fl}$)
MEF	-	mouse embryonic fibroblast
MCP	-	mast cell progenitor

Mcpt	-	mast cell protease
mMCP	-	mouse mast cell protease
MSCV	-	murine stem cell virus
NCBI	-	National Center for Biotechnology Information
NIH	-	National Institutes of Health
OVA	-	ovalbumin
PCR	-	polymerase chain reaction
PE	-	phycoerythrin
PLC-β3	-	phospholipase C-beta 3
PVDF	-	polyvinylidene fluoride
qPCR	-	quantitative polymerase chain reaction
RT-PCR	-	reverse transcription polymerase chain reaction
SCF	-	stem cell factor
SCORAD	-	SCORing Atopic Dermatitis
SEB	-	Staphylococcal endotoxin B
SEM	-	standard error of the mean
SHP-1	-	Src homology region 2 domain-containing phosphatase-1
SNP	-	single-nucleotide polymorphism
SPS complex	-	SHP-1-PLC-β3-Stat5 complex
Stat / STAT	-	signal transducers and activator of transcription
TCRβ	-	T cell receptor beta
TEWL	-	transepidermal water loss
T_H2	-	Type 2 helper T
TNF	-	tumor necrosis factor
TSLP	-	thymic stromal lymphopoietin

TSLPR	-	thymic stromal lymphopoietin receptor
WT	-	wild type

1. Introduction

1.1. Atopic dermatitis

Atopic dermatitis (AD) is a common, chronic or chronically relapsing skin disease characterized by the pruritic skin lesion and underlying allergic inflammation. The prevalence has increased by two to three folds during past three decades and affect 15-30% of children and 2-10% of the adult in the industrialized countries (Bieber, 2008). Clinical manifestations vary with age; in infancy, eczematous lesions usually affect cheeks and scalp. Scratching causes crusted erosions. Later in the childhood, susceptible area involves flexures, the nape, and the dorsal skin of the limbs. In adolescence and adulthood, lichenification develops in flexures, head, and neck. Throughout the clinical course, persistent itch that often causes sleep deprivation and disfiguration of the skin appearance substantially impairs the quality of a patient's life (Bieber, 2008). Pathologically, hyperkeratosis, spongiosis, and parakeratosis characterize acute lesions and marked epidermal hyperplasia and perivascular accumulation of lymphocytes and mast cells develop in chronic lesions (Leung, 2000).

The etiology of AD is still incompletely understood. However, it has been shown to be multifactorial and the disease develops through complex interactions between genetic and environmental factors (Bieber, 2008; Boguniewicz and Leung, 2011). The term “atopy” was first coined by Coca and Cooke in 1923, and it refers to the genetic tendency to develop allergic diseases such as allergic rhinitis, asthma and AD (Coca and Cooke, 1923). In fact, the concordance rate for atopic dermatitis is higher among monozygotic twins (77%) than among dizygotic twins (15%) (Schultz Larsen and Holm, 1985), suggesting the involvement of genetic factors. Later, genome-wide association studies (GWASs) have found candidate genes associated to epidermal barrier functions, innate and/or adaptive immunity, and nerve growth (Tamari and Hirota, 2014).

Atopy is typically associated with heightened immune responses to various allergens (e.g., foods, aeroallergens, microbes, and autoallergen). Immunological abmormalities in atopic dermatitis patients include elevated serum IgE level, epidermal spongiosis with apoptotic keratinocytes (Trautmann *et al*., 2001) and skin infiltration with immune cells such as CD4+ T cells, eosinophils, macrophages, and mast cells (Leung, 1999). These T cells express IL-4, IL-5, and IL-13 (Grewe *et al*., 1998), and numerous studies suggest an involvement of T helper 2 (T_H2) cell skewed immune responses in the pathogenesis of AD (Leung, 1999). However, there are reports suggesting that AD development is independent of IgE, but correlates with an increase in interferon (IFN)-γ-producing T_H1 cells (Thepen *et al*., 1996; Tsicopoulos *et al*., 1994; Werfel *et al*., 1996). In addition, transition from T_H2-type inflammation in acute skin letion to T_H1-type inflammation in chronic lesion is reported (Leung, 1999). T cells are capable of inducing keratinocyte apoptosis contributing to spongiosis formation (Trautmann *et al*., 2001). And T_H2-derived IL-4 aggravates the barrier dysfunctions (Elias *et al*., 2008) Thus, AD has been considered for a long time to be a primarily immunologically driven disease with secondary barrier defect (inside-outside hypothesis) (Irvine *et al*., 2011).

On the other hand, some investigators had hypothesized that the skin barrier dysfuntion is the primary cause of AD (outside-inside hypothesis). Various loss-of-function mutations in the FLG gene encoding filaggrin, a key protein for the skin barrier function, are recently reported in a substantial proportion of AD patients (Palmer *et al*., 2006; Sandilands *et al*., 2007). Subsequent GWAS studies could reproduce this association (Tamari and Hirota, 2014). In addition, the expressions of tight junction proteins claudin-1 and claudin-23 are reduced in AD patients (De Benedetto *et al*., 2011). Furthermore, trans-epidermal water loss, a non-invasive skin barrier function measurement, at day 2 after birth and at 2 months predicts the development of AD at 1 year (Kelleher *et al*., 2015).

Immunoregulatory functions of keratinocytes and fibroblast have also been reported. For instance, epidermal expression of thymic stromal lymphopoietin (TSLP), a T_H2-promoting cytokine (Liu, 2006; Ziegler and Artis, 2010), seems to be one of the major mechanisms for AD development (Li *et al*., 2005; Soumelis *et al*., 2002; Yoo *et al*., 2005). Periostin, an α_v integrin-interacting matricellular protein produced by fibroblasts (Hamilton, 2008; Ruan *et al*., 2009), recently emerged as another mediator for AD that induces TSLP production from keratinocytes (Masuoka *et al*., 2012).

Although these reports have validated the so-called outside-inside hypothesis (De Benedetto *et al*., 2012; Irvine *et al*., 2011), FLG mutation, for example, was not sufficient for causing AD. In addition, under a moist climate such as in Ishigaki Island, FLG mutation was not a predisposing factor for AD (Sasaki *et al*., 2014). Therefore, interplay of barrier dysfunction and other genetic and/or environmental factors likely promote the T_H2 immune response in susceptible individuals.

1.2. Mouse models of AD

A number of mouse AD models have been developed during the past twenty years, and have provided insights into the pathogenesis of human AD (Gutermuth *et al*., 2004; Jin *et al*., 2009a; Kawakami *et al*., 2009). There are three categories of models: (1) naturally occurring strains that develop skin lesions spontaneously, (2) gene-manipulated mice that either overexpress or are deficient of molecules contributing to skin inflammation/homeostasis, and develop dermatitis spontaneously, and (3) induced dermatitis by the application of allergens and/or chemicals. For instance, a naturally occurring mutant strain “flaky tail” has been shown to have mutation in FLG gene and prone to epicutaneous sensitization (Fallon *et al*., 2009; Moniaga *et al*., 2010). Another example is a keratinocyte-specific overexpression of TSLP, which caused a spontaneous

dermatitis (Li *et al*., 2005; Yoo *et al*., 2005). And an example of epicutaneous (EC) sensitization model with ovalbumin (OVA) shows epidermal and dermal thickening, infiltration of CD4+ T cells and eosinophils, and local expression of mRNAs for IL-4, IL-5 and IL-13, mimicking the skin lesion of human AD (Spergel *et al*., 1998). Dermatitis in this model required αβ T cells, but not B cells or mast cells (Alenius *et al*., 2002; Woodward *et al*., 2001). Differential roles of IL-4, IL-5, IL-10, IL-17, IFN-γ, chemokine receptors, complement components and receptors in this model were investigated using genetically modified mice (Jin *et al*., 2009a).

Kawakami et al. also developed a mouse model that mimicked human AD, in which skin inflammation was induced by repeated applications of *Dermatophagoides farinae* (house dust mite) extract (Der f) and staphylococcal enterotoxin B (SEB) (Kawakami *et al*., 2007). They chose these antigens for sensitization because there is a strong association of human AD with mite allergens (Fuiano and Incorvaia, 2012; Kimura *et al*., 1998; Scalabrin *et al*., 1999). In addition, more than 90% of AD patients are colonized with *Staphylococcus aureus*, compared to about 5% of healthy subjects. *S. aureus* infection is considered to be critical in the pathogenesis and/or aggravation of AD (Jappe, 2000; Strange *et al*., 1996). I previously validated the clinical relevance of this model by global gene expression analysis, and found a significant similarity between human AD lesion and this model. Major contributor to the similarity were genes related to epidermal growth/differention, skin barrier, lipid/energy metabolism, immune response, and extracellular matrix (Ando *et al*., 2013). In this model, mast cells and T cells, but not B cells or eosinophils were required for the full expression of skin disease. The receptor for the T_H2-promoting cytokine TSLP and the high-affinity IgE receptor, FcεRI, were contributing to the severity of the skin lesion.

1.3. Mast cells in allergic diseases

Mast cells are hematopoietic cells primarily found in the body surface tissues, such as skin, airway, and intestine. The cardinal feature of mast cells is the high affinity IgE receptor FcεRI expressed on the cell surface. Upon crosslinking of the IgEs by multivalent antigens, mast cells release various effector molecules; degranulation starts in minutes and releases histamine, proteases, and pre-formed cytokine TNF-α. Lipid mediators such as leukotriene C4, prostaglandin D2, and platelet activating factor are quickly de novo synthesized, and the production of cytokines such as IL-4, IL-5, IL-13, and TNF-α follows (Hart, 2001; Kawakami and Galli, 2002). Because of these functions, mast cells are considered as one of the most important effector cell type in allergic reactions. In fact, many of the anti-allergic drugs target mast cell-associated molecules, i.e., histamine (histamine receptor blockers), leukotriene C4 (leukotriene receptor blocker), IgE (omalizumab), and mast cell itself (disodium cromoglycate). However, there is no human lacking mast cells reported so far. To functionally test mast cells' roles in allergic diseases, mouse models are indispensable.

Investigators have utilized genetically mast cell-deficient mouse strains such as $Kit^{W/W\text{-}v}$, Sl/Sl^{d}, and $Kit^{W\text{-}sh/W\text{-}sh}$ as probes to test mast cells' functions in vivo. Stem cell factor (also known as Steel factor, Kit ligand, or mast cell growth factor) is a critical growth/differentiation factor for mast cells (Galli *et al*., 1994), and mutations in its coding loci (Sl) or receptor coding loci (Kit) leads to profound reductions in mast cell number. However, these strains have other defects as well. For example, $Kit^{W/W\text{-}v}$ mice are anemic, neutropenic and infertile. Among these three strains, $Kit^{W\text{-}sh/W\text{-}sh}$ shows the mildest phenotype: it is fertile and not anemic. However, it lacks interstitial cells of Cajal in the gut and exhibit bile reflux into the stomach (Grimbaldeston *et al*., 2005). When it gets older, myeloid cells are rather increased compared to the wild type mice (Nigrovic *et al*., 2008).

Because of these defects, one needs to perform mast cell engraftment experiment to confirm that the abnormal findings are due to lack of mast cells (Kawakami, 2009). One can culture mast cells from mouse bone marrow with IL-3 as a growth factor; these bone marrow-derived mast cells (BMMCs) can be injected to mast cell-deficient mice to replenish mast cells (Nakano *et al*., 1985; Tanzola *et al*., 2003). If the abnormality is reverted by the engraftment, one can conclude that mast cells are responsible for that phenomenon.

Another way to examine the role of specific molecule of interest in mast cells in vivo is to use mast cell-specific *Cre* recombinase expression technology. *Cre* recombinase is widely used to delete genomic fragment flanked by two loxP sequences. Mast cell-specific promoters currently available include Mcpt5 (Scholten *et al*., 2008), Cpa3 (Lilla *et al*., 2011), and a mast cell specific enhancer element for IL-4 (Sawaguchi *et al*., 2012). In combination with "floxed" gene alleles, in which a gene of interest is flanked by two loxP sequences, one can delete that gene in a mast cell-specific manner.

1.4. Phospholipase C-β3 regulates Stat5 activity

Phospholipase C (PLC) is a family of enzymes that catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate to generate diacylglycerol and inositol 1,4,5-trisphosphate (Suh *et al*., 2008). Independent of its enzymatic activity, PLC-β3 inhibits the proliferation of hematopoietic stem cells (HSCs) and myeloid cells by interacting with SHP-1 (SH2 domain-containing protein phosphatase 1) and Stat5 (signal transducer and activator of transcription 5). This SHP-1-PLC-β3-Stat5 (SPS) complex augments the dephosphorylating activity of SHP-1 towards Stat5, leading to inactivation of Stat5 (**Figure 14B**) (Xiao *et al*., 2009). As IL-3, a growth factor for mast cells, and T_H2 inducing cytokines such as TSLP and GM-CSF signal through Stat5 in the downstream of

their receptors, it is hypothesized that the deletion of PLC-β3 may increase mast cell numbers and skin allergic responses.

1.5. Aims of this study

It is well known that AD often serves as an initial manifestation of the so-called "allergy march"(Baba *et al*., 1988) or "atopic march", where children suffering from AD and/or food allergy tend to develop allergic rhinitis and/or asthma later in their life (Almqvist *et al*., 2007; Bantz *et al*., 2014; Ricci *et al*., 2006). Furthermore, AD and its disease severity is shown to be the main risk factors for food allergen sensitization independently of *FLG* mutation in exclusively breastfed infants (Flohr *et al*., 2014). Therefore, it is suggested that there may be a window of opportunity in which appropriate intervention targeting skin barrier or AD may prevent subsequent progression of allergic disorders (Bantz *et al*., 2014).

The first-line treatment for AD is the topical corticosteroids (Akdis *et al*., 2006; Katayama *et al*., 2014). However, steroid phobia is still widespread in Japan and other countries because of confusion and misinformation (Bewley and Dermatology Working, 2008; Kojima *et al*., 2013). Although topical steroids are proven to be safe when appropriate class of steroids are used (Bewley and Dermatology Working, 2008), misuse of the potent steroids causes side effects such as skin atrophy, hypothalamic–pituitary–adrenal axis suppression and growth retardation (Morley and Dinulos, 2012). In addition, calcineurin inhibitors, which are good alternatives for especially AD lesions on the face, are not very strong and often cause a side effect of annoying burning sensation (Eichenfield *et al*., 2002; Reitamo *et al*., 2000). Therefore, there is a demand for new therapeutics for AD.

In this study, I aimed to investigate whether PLC-β3 and its associated molecules can be new therapeutic targets for the treatment of AD. To this end, I first examined

whether PLC-β3-deficient mice spontaneously develop AD-like skin lesions. Since this was the case, I further investigated the cellular and molecular mechanisms for spontaneous and allergen-induced AD-like dermatitis in Plcb3$^{-/-}$ mice. Then I revealed that mast cell and its Stat5 play a critical role in the development of dermatitis in mouse models. Finally, by examining AD patient samples, I found evidences that the PLC-β3-STAT5 axis may be operational in mast cells of a subset of AD patients. Therefore, STAT5-regulatory mechanisms in mast cells are important for the pathogenesis of AD and could be novel therapeutic targets.

2. Materials and methods

2.1. Mice

Plcb3$^{-/-}$ mice (Li *et al*., 2000) were backcrossed to C57BL/6 mice for 12 generations. μMT (Kitamura *et al*., 1991), TCRβ$^{-/-}$ (Mombaerts *et al*., 1991), C57BL/6-Kit$^{W\text{-}sh/W\text{-}sh}$ (Grimbaldeston *et al*., 2005), FcεRIα$^{-/-}$ (Dombrowicz *et al*., 1993), TNF-α$^{-/-}$ (Pasparakis *et al*., 1996), TSLPR$^{-/-}$ (Al-Shami *et al*., 2004), and GM-CSF$^{-/-}$ (Stanley *et al*., 1994) mice were kind gifts from original developers and bred to Plcb3$^{-/-}$ mice. Their F1 mice were intercrossed to generate double mutant mice. Stat5$^{fl/fl}$ (Cui *et al*., 2004), SHP-1$^{fl/fl}$ (Pao *et al*., 2007), and Mcpt5-Cre transgenic mice (Scholten *et al*., 2008) were also kind gifts from original developers. I crossed Stat5$^{fl/fl}$ or SHP-1$^{fl/fl}$ mice with Mcpt5-Cre mice to develop mast cell-specific Stat5 deleted mice (MCΔStat5 or Mcpt5-Cre; Stat5$^{fl/fl}$) or mast cell-specific SHP-1 deleted mice (MCΔSHP-1 or Mcpt5-Cre; Shp1$^{fl/fl}$).

Dermatitis in both male and female mice under SPF conditions was scored by gross appearance and confirmed by histology. Animal experiments were approved by the Animal Use and Care Committee of the La Jolla Institute for Allergy and Immunology (LJI) and carried out in the LJI animal facility.

2.2. Der f/SEB-induced dermatitis

AD-like skin lesions were induced by two rounds of epicutaneous treatment of shaved back skin of male C57BL/6 (B6) mice or mutant mice with a C57BL/6 genetic background as previously described (Kawakami *et al*., 2007). Briefly, solutions of 500 ng of SEB (Sigma-Aldrich, St. Louis, MO) in sterile water and 10 μg of Der f extract (100 μg/ml, Greer Laboratories, Lenoir, NC) in PBS were pipetted onto a 1 cm x 1 cm square gauze pad placed on the shaved back. This portion was occluded with a Tegaderm™ Transparent Dressing (3M HealthCare, St. Paul, MN) using bandages. Three days later, the

dressings were replaced with a new one containing fresh Der f extract and SEB. After additional 4 days, the dressings were removed and the mice were kept without treatment for the next week. The one-week Der f/SEB treatment was repeated once more. A scientist who did not know the identities of mice scored the clinical severity 2 days after removing the dressings in the last cycle. Clinical AD scores were based on the severity (0, no symptoms; 1, mild; 2, intermediate; 3, severe) of four possible symptoms (redness, bleeding, eruption, and scaling). Der f/SEB experiments were performed using 3-6 mice per group and cumulative data from 2-5 experiments are presented.

2.3. Histological analysis

Skin biopsies were fixed with 10% formaldehyde and embedded in paraffin. Sections were stained with H&E, Toluidine blue, Congo-red or Masson trichrome. For immunohistochemistry, skin biopsies were immediately embedded in O.C.T. compound (Sakura Tissue-Tek) and stored at -80°C. Cryosections were fixed, and endogenous peroxidase was quenched with 3% H_2O_2 in cold methanol. ABC Staining Systems (Santa Cruz Biotechnology) were used to visualize stainings with anti-CD4 (Santa Cruz biotechnology), anti-CD8 (BD Biosciences) or anti-Mac-1 (BD Biosciences) antibodies as primary antibodies. For immunofluorescence, biopsies were fixed in 4% paraformadehyde at 4°C, washed in 10-20% sucrose in phosphate buffered saline, embedded in O.C.T. compound and stored at -80°C. Cryosections were dried, rehydrated and incubated with anti-CD4, anti-CD8, anti-F4/80 (Abcam) or anti-TSLP (Amgen) at 4°C overnight. After washing, sections were incubated with Texas-red conjugated goat anti-rat IgG (Southern Biotech), and mounted with ProLong Gold antifade reagent with DAPI (Invitrogen). For the detection of periostin and phospho-Stat5 in mast cells, 10% formalin-fixed sections were subjected to heat-induced antigen retrieval after deparaffinization. Anti-periostin

(Masuoka *et al*., 2012), anti-phospho-Stat5 (Abcam), anti-mast cell tryptase (Abcam), or anti-mMCP4 and anti-mMCP6 (Shin *et al*., 2006) was used as a primary antibody, in combination with Alexa Fluor 488, 568 or 647-conjugated goat anti-mouse, rat or rabbit antibody (Invitrogen) as a secondary antibody. Fluorescent images were observed at the magnification of 400 under either Nikon Eclipse 80i fluorescence microscope or Olympus FluoView FV10i confocal laser microscope. Human skin mast cells were counted (>100 cells per sample) in rectangular regions covering the entire skin layers from the epidermis to subcutaneous adipose tissues. Fluorescence intensity was measured by ImageJ software version 1.47 (National Institutes of Health, NIH).

2.4. Serum IgE, IgG1 and IgG2a

Serum IgE, IgG1 and IgG2a levels were measured using an enzyme-linked immunoassay kit purchased from BD Biosciences Pharmingen.

2.5. Determination of trans-epidermal water loss (TEWL)

TEWL was measured on lesional skin and the shaved dorsal skin of normal mice by using a TewameterTM 300 (Courage-Khazaka Electronics, Cologne, Germany) according to the manufacturer's instruction as described (Li *et al*., 2010).

2.6. Flow cytometry

Expression of c-Kit, FcεRI, and IL-3 receptor on mast cells was analyzed using FACSCalibur (BD Biosciences) after staining with APC-conjugated anti-c-Kit (2B8, BD Biosciences), FITC-conjugated anti-FcεRI (MAR-1, BioLegend), or PE-conjugated anti-IL-3 receptor (5B11, BD Biosciences).

2.7. Microarray analysis of gene expression

Total RNA was extracted from skin using Trizol One Step RNA Reagent (BioPioneer Inc., San Diego, CA). The same amount of RNA from 2-6 mice were mixed and cleaned by RNeasy Total RNA Mini Kit (Qiagen). A microarray analysis was performed using 200 ng of total RNA from each cohort and SurePrint G3 Mouse Gene Expression 8x60K arrays (Agilent Technologies) according to the manufacturer's instructions. The microarray data is deposited in Gene Expression Omnibus (GSE53132). Data analysis was performed with GeneSpring software (version 11.5.1). Signal intensity was normalized by 75 percentile shift and reduced difference in the levels of beta-actin and 18S ribosomal subunit was confirmed. To exclude genes containing only a background signal, genes were selected only when the raw values of expression signal were more than 100.

Detection of similarity in expression pattern between different species was performed as described previously (Ando *et al*., 2013; Hoffmann *et al*., 2007; Lottaz *et al*., 2006; Yang *et al*., 2006). Briefly, human microarray data were obtained from Gene Expression Omnibus (National Center for Biotechnology Information, NCBI). Orthologs were collected from human and mouse microarray data sets according to the HomoloGene database build 65 (NCBI). These genes were sorted in the order of fold changes, and the sorted gene lists for mice and human were compared using OrderedList package on R software version 2.14.1. OrderedList package calculates a similarity score for paired ranked gene lists G_1 (mouse) and G_2 (human), based on the number of common genes found in top and bottom ranks of both lists. If an ortholog is ranked high at either top (upregulated) or bottom (downregulated) end in both human and mouse gene expression lists, this ortholog contributes highly to the similarity score. By calculating the random distribution of the scores with 1000 permutations, p-value for the significance of similarity was

obtained. Computation was performed at alpha=0.01151 (max rank=1000).

2.8. Real-time RT-PCR

RNA was extracted from skin or cultured keratinocyte samples. cDNA was prepared with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) and random hexamer (Invitrogen, Carlsbad, CA). Primers for mouse periostin and TSLP (sequences to be provided upon request) were tested for the amplification efficiency along with 18S ribosomal RNA as an internal control. Real-time RT-PCR was performed using LightCycler® 480 System (Roche Applied Science). Expression levels were determined as relative expression to 18S internal control.

2.9. Cultures of mast cells and retroviral transduction

Bone marrow cells or mast cell progenitors (MCPs) purified by fluorescence activated cell sorting (FACS) (Chen *et al*., 2005) were cultured in RPMI1640 medium supplemented with 10% fetal calf serum (FCS) and IL-3 conditioned medium or IL-3 from PeproTech (10 ng/mL). IL-3 conditioned medium was obtained from D11 IL-3-producing cell line. Live cells were counted during weekly media changes. Recombinant retroviruses were generated by transfection of Plat-E cells (Morita *et al*., 2000) with pMSCV-IRES-GFP vectors. pMSCV-IRES-GFP vector was a kind gift from Daniel Link (Washington University). Bone marrow cells or MCPs were infected with the retroviruses, and GFP+ cells were FACS-purified and cultured. Expression of c-Kit and FcεRI (>95% of the cells) was confirmed by flow cytometry before use.

2.10. Mast cell engraftment

BMMCs derived from Plcb3$^{-/-}$ mice were transferred by intradermal injection (4 x

10^6 cells in 50 μl DMEM per site, totally 20 sites distributed in five rows down the length of shaved back skin) into 4-week-old Plcb3$^{-/-}$;Kit$^{W\text{-}sh/W\text{-}sh}$ mice as described (Nakano *et al*., 1985).

2.11. Neonatal skin-derived mast cells

Whole newborn skin was incubated with 0.5% trypsin-EDTA (Invitrogen) for 1 h, and epidermis was removed. Separated dermis was minced and incubated in DMEM containing 2 U/mL of Liberase TL (Roche Applied Science) and 1.6 mg/mL of hyaluronidase (Sigma-Aldrich). Dispersed cells were cultured in RPMI1640 supplemented with 10% FCS, IL-3 and stem cell factor (SCF) for four to six weeks. FcεRIα+ c-Kit+ cells were sorted and subject to immunoblot analysis.

2.12. In vitro migration assay

Migration of BMMC was assayed using 24-well Transwell chambers (Corning, NY) as described previously (Kitaura *et al*., 2005). Briefly, the lower surface of 5-μm polycarbonate filters were coated overnight with 20 μg/mL fibronectin followed by blocking with 4% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) at 37°C for 1 hour. 10^6 cells/well of BMMCs were seeded onto upper wells in 0.2 mL of RPMI medium containing 1% BSA and 20 mM HEPES [N-2-hydroxyethylpiperazine-N′ -2-ethanesulfonic acid] (pH 7.4). In lower wells, 0.6 mL medium containing various concentrations of IL-3 or SCF was applied. After 8 hours of incubation, cells migrated into lower wells were counted using a hemocytometer.

2.13. Immunoblotting

Mast cells were stimulated with IL-3 and lysed in 1% NP-40 lysis buffer. Lysates

were analyzed by SDS-PAGE followed by electroblotting to PVDF membranes (Millipore). Membranes were incubated with primary antibody, and then with an HRP-conjugated secondary antibody. Antibody-bound proteins were revealed by ECL reagent (PerkinElmer Life Sciences). Anti-Stat5, anti-SHP-1, anti-PLC-β3, and anti-Actin antibodies were obtained from Santa Cruz Biotechnology. Anti-phospho-Stat5 antibody was from Upstate Biotechnology. Anti-phospho-SHP-1 was from ECM Bioscience. Anti-periostin antibody was described previously (Masuoka *et al*., 2012).

2.14. Human subjects

Two independent populations were used for genetic association analysis, including 414 European American subjects and 323 African American subjects. Subjects were recruited as part of the National Institute of Allergy and Infectious Diseases (NIAID)-supported Atopic Dermatitis and Vaccinia Network (ADVN). Demographic characteristics are presented in **Table 1**. AD patients and healthy individuals were also recruited at University of California San Diego. Skin biopsies using a 4 mm punch biopsy were performed on a lesional target site of atopic skin and a target non-lesional site at least 4 cm apart from the lesional site. Normal matched control samples were obtained from healthy donors in similar locations.

2.15. AD diagnosis and phenotypes

AD was diagnosed using the US consensus conference criteria (Eichenfield *et al*., 2003). ADEH was defined as AD patients with at least one eczema herpeticum episode documented either by an ADVN investigator or by another physician confirmed by polymerase chain reaction (PCR) detection of herpes simplex virus (HSV) infection, tissue immunofluorescence, Tzanck smear and/or culture. AD severity was defined according to

the ‘eczema area and severity index’ (EASI), a standardized grading system (Hanifin *et al*., 2001), and total serum IgE was measured using the UniCap 250 system (Pharmacia and Upjohn). Clinical characteristics are presented in **Table 1**. The study was approved by the institutional review boards at National Jewish Health in Denver, Johns Hopkins University, Oregon Health and Science University, University of California San Diego, Children’s Hospital of Boston, and University of Rochester. All subjects gave written informed consent prior to participation.

2.16. Genotyping and quality control

A total of 22 single-nucleotide polymorphisms (SNPs) for the four candidate genes were selected from the HapMap (http://www.hapmap.org/) using a tagging approach: 8 PLCb3 SNPs, 6 STAT5A SNPs, 4 STAT5B SNPs and 4 SHP1 SNPs. The four SNPs in STAT5B were genotyped using the custom-designed Illumina OPA for the BeadXpress Reader System and the GoldenGate Assay with VeraCode Bead technology according to the manufacturer’s protocol. The rest of SNPs were genotyped and analyzed on a 7900HT Sequence Detection System (Applied Biosystems) with Applied Biosystems Genotyper software (SDS system, version 2.2). As part of quality control, additional 74 SNPs identified as ancestry informative markers selected for maximal difference between African and European populations were genotyped, and potential confounding factors due to population substructure were assessed using genotype data and the STRUCTURE program (v2.2; http://pritch.bsd.uchicago.edu/software).

2.17. Statistical analysis

For genetic analysis, logistic regression, adjusting for the first two principal components, was used to test for association between each individual marker (under an

additive model) and disease status using PLINK software (http://pngu.mgh.harford.edu/~purcell/plink/to). To test for association between genetic markers and total serum levels of log-adjusted IgE and log-adjusted EASI score, linear regression models were used adjusting for confounding variables, age and gender as well as the first two principal components. Departures from Hardy-Weinberg equilibrium at each locus were tested with chi-squared tests separately for cases and controls using PLINK. Other statistical analyses were performed by Student's t-test, One-way ANOVA with Tukey's post-hoc test. Differences were considered statistically significant at P values < 0.05.

3. Results

3.1. PLC-β3-deficient mice spontaneously develop mast cell-dependent AD-like dermatitis

PLC-β3 interacts with Stat5 and SHP-1 to form the multi-molecular SPS complex, and juxtaposition of SHP-1 and Stat5 via interactions with PLC-β3 enhances the catalytic activity of SHP-1 to dephosphorylate Stat5 (Xiao *et al*., 2009; Yasudo *et al*., 2011). Stat5 is utilized in the downstream signaling of cytokine receptors such as TSLPR, IL-3R, and GM-CSF receptor. Young (4-10 week-old) *Plcb3*$^{-/-}$ mice displayed no obvious abnormalities in their phenotype. By contrast, a majority of older mice developed eczematous skin lesions and hair loss in their periocular areas, cheeks, ears, neck and trunk (**Figure 1A-B**). The lesions showed hyperkeratosis, thickened epidermis and dermis, and infiltration of T cells, mast cells, macrophages, eosinophils and neutrophils in the dermis (**Figure 1C-D**). Eczematous *Plcb3*$^{-/-}$ mice had high levels of serum IgE and IgG1, whereas dermatitis-free young *Plcb3*$^{-/-}$ mice had low IgE levels (**Figure 1E** and **Figure 2A**). There was a good correlation between IgE levels and numbers of the involved body parts (**Figure 1F**). Transepidermal water loss (TEWL) increased only after dermatitis development (**Figure 2B**), suggesting that skin barrier function was not primarily impaired in *Plcb3*$^{-/-}$ mice.

No *Plcb3*$^{-/-}$*;Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (n=24) deficient in mast cells developed skin lesions during an observation period of 12 months (**Figure 1G**). By contrast, skin lesions were observed in the majority of αβ T cell-deficient *Plcb3*$^{-/-}$ (*Plcb3*$^{-/-}$*;TCRb*$^{-/-}$) mice and B cell-deficient *Plcb3*$^{-/-}$*;μMT/μMT* mice, although skin inflammation in *Plcb3*$^{-/-}$*;TCRb*$^{-/-}$ mice was less prominent than that in *Plcb3*$^{-/-}$ mice (**Figure 1H**). These results suggest that mast

cells, but not αβ T or B cells, are indispensable for the spontaneous development of skin lesions in *Plcb3*$^{-/-}$ mice.

3.2. *Plcb3*$^{-/-}$ mice develop severe allergen-induced dermatitis

Der f/SEB-induced dermatitis is dependent on mast cells and T cells, but not B cells or eosinophils (Ando *et al*., 2013). Epicutaneous treatment with Der f and SEB of young (5-11 week-old) *Plcb3*$^{-/-}$ mice, which did not show any skin lesions before experiment, induced more severe skin lesions with thicker epidermis and dermis and higher levels of mast cell and neutrophil infiltration, compared to WT mice (**Figure 3A-E**). Although Der f/SEB treatment increased serum levels of IgE and IgG1, some of which recognized Der f antigens, their levels were comparable in WT and *Plcb3*$^{-/-}$ mice (**Figure 4A-B**). Mast cell deficiency also resulted in less severe skin lesions in Der f/SEB-treated *Plcb3*$^{-/-}$*;Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, compared to *Plcb3*$^{-/-}$ mice (**Figure 3F-G**). Moreover, engraftment of *Plcb3*$^{-/-}$ BMMCs into the back skin of *Plcb3*$^{-/-}$*;Kit*$^{W\text{-}sh/W\text{-}sh}$ mice restored the severity of Der f/SEB-induced dermatitis to levels in *Plcb3*$^{-/-}$ mice (**Figure 3F-H**). Therefore, similar to spontaneous dermatitis in *Plcb3*$^{-/-}$ mice, mast cells contribute substantially to the development of Der f/SEB-induced dermatitis in these mice. Consistent with increased Der f-specific IgE levels in WT and *Plcb3*$^{-/-}$ mice, FcεRI-deficient mice exhibited less severe skin lesions in *FcεRIα*$^{-/-}$ and *FcεRIα*$^{-/-}$*;Plcb3*$^{-/-}$ mice than the respective control FcεRI-sufficient mice (**Figure 4C**). These results indicate that FcεRI is required for full-blown allergen-induced dermatitis.

3.3. *Plcb3*$^{-/-}$ mast cells are hypersensitive to IL-3

Mast cells are derived from HSCs via bipotent basophil/mast cell progenitors (BMCPs) and mast cell progenitors (MCPs) (Arinobu *et al*., 2005; Chen *et al*., 2005).

Consistent with the increase in mast cells in the skin, the numbers of BMCPs in spleen and MCPs in bone marrow were increased in young (6-10 week-old) *Plcb3*$^{-/-}$ mice (**Figure 5A**). Consistent with these *in vivo* results, 10-fold or more mast cells were generated from bone marrow cells of *Plcb3*$^{-/-}$ mice in IL-3-containing medium, compared to WT cells (**Figure 6A**), although expression levels of FcεRI and c-Kit were similar in both BMMCs (**Figure 6B**). IL-3-induced proliferation was 2- to 3-fold higher in *Plcb3*$^{-/-}$ than in WT BMMCs (**Figure 6C**), whereas SCF-induced proliferation was similar in the two genotypes. *Plcb3*$^{-/-}$ BMMCs migrated more vigorously towards IL-3 than WT cells (**Figure 6D**). These results suggest that the increased proliferation and chemotaxis in response to IL-3 could be the major mechanisms for the increased mast cells in *Plcb3*$^{-/-}$ mice. This IL-3 hyper-responsiveness seems due to increased IL-3 receptor (IL-3R) signaling downstream of the receptor, as expression of IL-3R in mast cells and that of IL-3 mRNA in skin tissues were comparable between WT and *Plcb3*$^{-/-}$ mice (**Figure 5B-C**).

3.4. PLC-β3 inhibits Stat5 activity in mast cells by interacting with SHP-1 and Stat5

As shown for *Plcb3*$^{-/-}$ HSCs (Xiao *et al*., 2009), IL-3-induced phosphorylation of Stat5 at Tyr^{694}, the phosphorylation site critical for its activation (Gouilleux *et al*., 1994), was enhanced in *Plcb3*$^{-/-}$ BMMCs (**Figure 6E**). Furthermore, phosphorylation of SHP-1 at Tyr^{536}, which is necessary for efficient interaction with Stat5 (Xiao *et al*., 2010), was abolished in *Plcb3*$^{-/-}$ cells.

Introduction of dominant-negative Stat5 drastically inhibited proliferation of *Plcb3*$^{-/-}$ BMMCs in vitro (**Figure 6F**). Transduction with WT SHP-1 restored SHP-1 phosphorylation and inhibited IL-3-induced Stat5 phosphorylation (**Figure 6G**) and mast cell proliferation (**Figure 6F**). These results show that IL-3 hyper-responsiveness caused by PLC-β3 deficiency depends on increased Stat5 activity and reduced SHP-1 activity. Given

the comparable expression of IL-3 and IL-3R between WT and *Plcb3*$^{-/-}$ mice (**Figure 5B-C**), not only IL-3 but also other factors that lead to Stat5 activation would contribute to the increased mast cells in *Plcb3*$^{-/-}$ mice.

3.5. Stat5 in mast cells is critical for full expression of allergen-induced dermatitis

The above results imply that Stat5 and SHP-1 might contribute to dermatitis development by regulating the mast cell responsiveness to IL-3 (and other stimuli). To investigate this possibility more directly, I generated mice with mast cell-specific deletion of *Stat5* (*A* and *B*) or *Shp1* (coding SHP-1) loci, termed *MCΔStat5* and *MCΔSHP-1* mice, respectively by crossing Stat5$^{fl/fl}$ or SHP-1$^{fl/fl}$ mice with mast cell-specific Mcpt5 promoter driven Cre recombinase transgenic mice. Loss of expression of the targeted loci was confirmed by immunoblotting of neonatal skin-derived mast cells (**Figures 7A**) and/or immunofluorescence microscopy of skin mast cells (**Figures 8A**). Der f/SEB treatment induced significantly lower skin scores in *MCΔStat5* mice (**Figure 7B**) with lower infiltration of neutrophils and eosinophils (**Figure 7D**), but with WT levels of skin thickness (**Figure 7C**), compared to control mice. By contrast, higher skin scores were observed in *MCΔSHP-1* mice than in control mice (**Figure 8B**). There were similar numbers of mast cells in the ears of WT, *MCΔStat5* (**Figure 7D**) and *MCΔSHP-1* mice (**Figure 8C**). Mast cells reactive with anti-phospho-Stat5 antibody were abundant in the inflammatory dermis from Der f/SEB-induced dermatitis (**Figure 7E-F**), although phospho-Stat5 positive cells were not restricted to mast cells. Further confirming the role of Stat5 in skin inflammation, TG101348, an inhibitor of Jak2 kinase that activates Stat5 activity (Pardanani *et al*., 2011), ameliorated Der f/SEB-induced dermatitis with reduced skin thickness and reduced numbers of mast cells, neutrophils and eosinophils (**Figure**

8D-F). These results collectively demonstrate that Stat5 activity in mast cells is required for full expression of allergen-induced skin inflammation.

3.6. TSLP is highly expressed in the epidermis and critical for skin inflammation in *Plcb3*$^{-/-}$ mice

Overexpression of TSLP in keratinocytes in transgenic mice results in an AD-like phenotype (Li *et al*., 2005; Yoo *et al*., 2005). TSLP, which activates Stat5 (Isaksen *et al*., 1999), is highly expressed by keratinocytes in human AD (Soumelis *et al*., 2002) and, along with IL-1 and TNF, induces mast cells to secrete IL-13, IL-5, and other cytokines (Allakhverdi *et al*., 2007). Therefore, I examined the role of TSLP in dermatitis in *Plcb3*$^{-/-}$ mice. Similar to a report (Bogiatzi *et al*., 2007), TSLP protein was highly expressed in the epidermis of lesional skin (**Figure 9A**). As expected, I observed TSLP expression in the epidermis in Der f/SEB-treated *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice and identically treated WT mice. However, TSLP expression was more intense in the thickened epidermis in the latter mice than in the former mice (**Figure 10A**). It was even more exaggerated in Der f/SEB-treated *Plcb3*$^{-/-}$ and *Plcb3*$^{-/-}$*; Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. I interpret that the overexpression of epidermal TSLP in *Plcb3*$^{-/-}$ and *Plcb3*$^{-/-}$*; Kit*$^{W\text{-}sh/W\text{-}sh}$ mice reflects the loss of PLC-β3-mediated suppression of TSLP expression in keratinocytes (**Figure 10B**) in these mice.

Importantly, no *Plcb3*$^{-/-}$*;TSLPR*$^{-/-}$ mice lacking both PLC-β3 and TSLPR (n=10) developed dermatitis for 12 months (**Figure 9B**). Consistent with the role of TSLPR in Der f/SEB-induced dermatitis (Ando *et al*., 2013), Der f/SEB-induced dermatitis was less severe in *Plcb3*$^{-/-}$*;TSLPR*$^{-/-}$ mice than in *Plcb3*$^{-/-}$ mice (**Figure 9C**). Lesional skin in the former mice had reduced numbers of eosinophils and neutrophils (**Figure 9D**). TSLP expression was low in Der f/SEB-treated skin of TG101348-treated mice (**Figure 8G**).

Therefore, the TSLP-TSLPR axis is critically important for both spontaneous and allergen-induced dermatitis in *Plcb3*$^{-/-}$ mice.

Proinflammatory cytokines such as TNF, in combination with T_H2 cytokines, can induce the expression of TSLP in keratinocytes (Bogiatzi *et al*., 2007). However, all *Plcb3*$^{-/-}$*;TNF*$^{-/-}$ mice (n=34) spontaneously developed dermatitis within 9 months (**Figure 9E**). GM-CSF can also activate Stat5 (Mui *et al*., 1995), promote T_H2 cell responses (Kusakabe *et al*., 2000), and might contribute to the establishment and chronicity of AD lesions (Bratton *et al*., 1995). All *Plcb3*$^{-/-}$*;GM-CSF*$^{-/-}$ mice (n=31) spontaneously developed dermatitis within 8 months (**Figure 9F**). Therefore, both TNF and GM-CSF were dispensable for spontaneous skin inflammation in *Plcb3*$^{-/-}$ mice.

3.7. PLC-β3 regulates periostin production by fibroblasts

A recent study showed that periostin is a critical mediator for a Der f-induced AD model and that periostin expression is correlated with the severity of human AD (Masuoka *et al*., 2012). Periostin was highly expressed in the lesional dermis of spontaneous dermatitis in *Plcb3*$^{-/-}$ mice (**Figure 11A**) as well as Der f/SEB-induced dermatitis in WT and *Plcb3*$^{-/-}$ mice (**Figure 11B**). By contrast, its protein and mRNA expression was reduced in Der f/SEB-induced dermatitis in mast cell-deficient mice (**Figure 11B-C**). Consistent with the known role of periostin in fibrosis, Masson trichrome staining confirmed remarkable fibrosis in skin lesions of spontaneous dermatitis (**Figure 2C**). To further analyze periostin expression, embryonic fibroblasts (MEFs) and NIH/3T3 fibroblasts were used. When fibroblasts were co-cultured with IgE/antigen-stimulated BMMC, periostin secretion was increased (**Figure 11D**). I confirmed that IL-13, a major cytokine produced by IgE/antigen-stimulated mast cells (Burd *et al*., 1995), enhances the production and

secretion of periostin in fibroblasts (**Figure 11E**). These results collectively demonstrate that mast cells can regulate periostin production in fibroblasts.

Importantly, basal levels of periostin were remarkably higher in *Plcb3*$^{-/-}$ than in WT MEFs. Therefore, constitutive expression of periostin appeared to be negatively regulated by PLC-β3. Co-stimulation of WT, but not *Plcb3*$^{-/-}$, MEFs with TSLP inhibited IL-13-induced periostin production/secretion (**Figure 11F**). This result suggests the presence of feedback inhibition in the skin for fibroblasts' periostin production by TSLP, which can be induced in keratinocytes by periostin (Masuoka *et al*., 2012), and this feedback inhibition is lost in *Plcb3*$^{-/-}$ MEFs. I also found that TSLP mRNA expression is higher in *Plcb3*$^{-/-}$ than in WT keratinocytes (**Figure 10B**), consistent with increased TSLP expression in the epidermis of lesional skin of *Plcb3*$^{-/-}$ mice (**Figure 10A**). When mast cells are stimulated with IgE plus antigen together with skin extract, cytokine production was enhanced partially dependently on TSLP receptor (**Figure 10C**). These results not only add a new cellular component, *i.e*., mast cells, to the hypothetical vicious cycle of skin inflammation consisting of T_H2 cytokines (from T_H2 cells)-periostin (from fibroblasts)-TSLP (from keratinocytes) (Masuoka *et al*., 2012), but also show that PLC-β3 intrinsically regulates periostin production in fibroblasts and TSLP expression in keratinocytes.

3.8. Association of the SPS complex genes and mast cell-expressed phospho-STAT5 with human AD

The clinical relevance of Der f/SEB-induced dermatitis was supported by similarity in gene expression profiles between this dermatitis and human AD (Ando *et al*., 2013). Gene expression profiles in spontaneous dermatitis in *Plcb3*$^{-/-}$ mice were also more similar to those in human AD than those in human psoriasis (**Table 2**). In light of the strong

clinical relevance of the two dermatitis models and functional relationship among PLC-β3, STAT5 and SHP-1 in mast cells, SNPs were genotyped for these genes (**Table 3**) in AD populations (**Table 1**). The AD patients were stratified as ADEH+ and ADEH- based on those who suffered eczema herpeticum and those who did not, respectively. Eczema herpeticum is a severe disseminated herpes virus infection that occurs at skin lesions of AD and other conditions (Wollenberg *et al*., 2003); ADEH+ patients suffer more severe dermatitis than ADEH- patients (Beck *et al*., 2009). Among the *PLCB3* SNPs tested, rs2244625 was associated with risk of ADEH in European American subjects (**Table 4**). Furthermore, a non-synonymous SNP (rs35169799) was significantly associated with decreased levels of IgE among controls but not among AD subjects (**Figure 12A**). **Table 4** shows that *STAT5B* SNP rs9900213 and *SHP1* SNP rs7310161 were significantly associated with the risk of AD among European American subjects. One *STAT5A* SNP (rs16967637) and four *SHP1* SNPs were associated with the severity of AD among African Americans. No AD-associated SNPs except for rs35169799 change protein structures.

Finally, I found that mast cells are more abundant in the dermis of AD lesions than in that of nonlesional skin of AD patients or healthy individuals (**Figures 13A and 13B**). More importantly, mast cells were abundantly detected with higher nuclear intensities of phospho-STAT5 staining in lesional skin of some AD patients (**Figures 13C–E**). Nuclear STAT5 phosphorylation in mast cells was correlated with skin mast cell numbers (**Figure 12B**) and inversely correlated with PLC-β3 expression in mast cells (**Figures 12C-F**). The above SNP and immunofluorescence data support the idea that dysregulation of the SPS complex leading to STAT5 activation is involved at least in some cases of human AD.

4. Discussion

In this study, I showed that mast cells, but not αβ T or B cells, are required for spontaneously occurring dermatitis in *Plcb3*-/- mice. To the best of my knowledge, this is the first mast cell-dependent naturally occurring AD model (Kawakami *et al*., 2009) despite the limitations of Kit mutant mice as a model of mast cell deficiency (Rodewald and Feyerabend, 2012). *Plcb3*$^{-/-}$ mast cells were hyper-responsive to IL-3 due to increased Stat5 activity, which could be antagonized by PLC-β3 and SHP-1. These *in vitro* results support the *in vivo* anti-inflammatory role of PLC-β3 and SHP-1 that regulate the proliferative and chemotactic behavior of mast cells by controlling Stat5 activity. Characterization of spontaneous dermatitis in *Plcb3*$^{-/-}$ mice was complemented by allergen-induced dermatitis experiments, as the particular importance of Stat5 regulation in mast cells was demonstrated by alleviated allergen-induced dermatitis in mice lacking Stat5 in mast cells and by efficient suppression of allergen-induced dermatitis by chemical inhibition of Stat5 activity. Spontaneous dermatitis in *Plcb3*$^{-/-}$ mice depended on the TSLP/TSLPR axis, which uses Stat5 as a critical signal transducer (Isaksen *et al*., 1999). Thus, Stat5 activation is required for the development of both spontaneous and allergen-induced dermatitis. Human data suggest that STAT5 activating pathways in mast cells are operational in a subset of AD patients.

Various studies suggest the role of T cells in AD pathogenesis (Jin *et al*., 2009a; Kawakami *et al*., 2009). Skin inflammation in the Der f/SEB model requires αβ T cells (Ando *et al*., 2013), in line with the widely accepted scenario for AD development (Bieber, 2008; Boguniewicz and Leung, 2011); impaired skin barrier function allows allergens easy access to the inside of skin; allergens are taken up by Langerhans cells and dermal dendritic cells, and these cells migrate and mature to present allergens to naive helper T cells in lymph nodes; activated and differentiated T_H2 cells migrate back to skin sites re-exposed to

allergens; these effector T_H2 cells recruit mast cells, eosinophils, and other granulocytes in order to cause tissue damages. Although αβ T cells were dispensable for spontaneous dermatitis in *Plcb3*$^{-/-}$ mice, αβ T cells seem to contribute to a severe phenotype in this dermatitis. Thus, skin inflammation in *Plcb3*$^{-/-}$*;TCRb*$^{-/-}$ mice was less prominent than that in *Plcb3*$^{-/-}$ mice. By contrast, B cells (the source of IgE) appeared to be dispensable for both spontaneous dermatitis and allergen-induced dermatitis, although there was a clear correlation between IgE levels and the severity of dermatitis. This apparent discrepancy seems to be related to the presence of both activating (FcεRI) and inhibitory (FcγRIIb) receptors for IgE in mast cells (and other cells), as FcεRI was required for maximal allergen-induced skin inflammation.

Masuoka et al. recently proposed a vicious cycle for allergic skin inflammation consisting of T_H2 cytokines (from T_H2 cells)-periostin (from fibroblasts)-TSLP and other proinflammatory cytokines (from keratinocytes) (Masuoka *et al*., 2012). The Der f/SEB induction model requires T cells for full-blown dermatitis, whereas spontaneous and persistent dermatitis in *Plcb3*$^{-/-}$ mice does not. The TSLP-TSLPR axis and mast cells are required for both AD models, whereas T cells are required only for allergen-induced dermatitis. Therefore, I speculate that T cells and mast cells are required for TSLP overexpression. Once sustained overexpression of TSLP is established, mast cells may play a more important role in persistent dermatitis as the cellular source of T_H2 cytokines (**Figure 14**). Related to this network, this study showed that PLC-β3 can regulate activities of the cellular elements of the network, such as proliferation in mast cells, periostin expression in fibroblasts and TSLP expression in keratinocytes. My data also suggest the presence of a feedback loop for inhibition of fibroblasts' periostin production by TSLP, and PLC-β3 in fibroblasts is required for this negative feedback. Among the cytokines induced

in keratinocytes by periostin, I found that TSLP, but not TNF or GM-CSF, is important for the development of spontaneous dermatitis in *PLCb3*$^{-/-}$ mice.

Human data shown here are consistent with mouse data by showing genetic linkage with all four genes that encode components of the SPS complex. However, the *p*-values of the association were relatively high because of the limited number of the subjects available in this study. In addition, the SNPs found to be potentially associated with the AD phonotypes are not nonsynonymous mutations. Therefore, validation with larger cohort is essential to conclude the significance of these linkages. Although IL-3 alone or IL-3 plus IL-4 cannot induce the differentiation of human mast cells (Saito *et al*., 1988), human mast cells express functional receptors for IL-3, IL-5, and GM-CSF (Dahl *et al*., 2004) and, along with SCF, IL-3 can enhance mast cell growth by decreasing mast cell apoptosis (Gebhardt *et al*., 2002). Thus, the breakdown of PLC-β3/SHP-1-dependent suppression of STAT5 activity, which is recruited by IL-3 or other cytokines in human mast cells, may represent a pathogenic mechanism for human AD. Alternatively, SPS complexes might regulate the growth properties of cell types other than mast cells. Several cytokines including IL-5, IL-21, IL-31, TSLP and GM-CSF, which are implicated in AD pathogenesis (Jin *et al*., 2009b; Sonkoly *et al*., 2006; Soumelis *et al*., 2002; Yamamoto *et al*., 2003), utilize the JAK-STAT5 pathway for their signal transduction. Reduced expression of PLC-β3, thus improper regulation of STAT5 activity in those cells, would contribute to AD development. These considerations provide additional reasoning for the JAK-STAT5 pathway as a potential target of AD treatment. In fact, topical application of a JAK inhibitor TG101348 successfully ameliorated the Der f/SEB-induced dermatitis. TG101348 (also known as Fedratinib or SAR302503) was developed as a candidate of therapeutics for myelofibrosis, a hematopoietic malignancy (Zhang *et al*., 2014a). Unfortunately, the development was terminated after phase 3 clinical trial by the severe

adverse effect of Wernicke's encephalopathy. Later, it was shown that TG101348 suppresses the uptake of thiamine (Zhang *et al*., 2014b). Importantly, this effect was not through the inhibition of JAKs. Rather, the unique structure of TG101348 seems to mimic the structure of thiamine, thereby competing two human thiamine transporters at the intestinal epithelial cells. Therefore, the window of opportunity for other JAK inhibitors as AD therapeutics is still open. In addition, because topical treatment would be enough for the treatment of AD, there remains a possibility that TG101348 can be used without severe adverse effect.

STAT5 is expressed in various cell types including hematopoietic cells (Lattin *et al*., 2008; Wu *et al*., 2013; Wu *et al*., 2009). Deletion of both Stat5a and Stat5b genes is perinatal lethal (Cui *et al*., 2004), and Jak2 knockout mice are embryonic lethal (Neubauer *et al*., 1998; Parganas *et al*., 1998). In both cases, severe anemia due to the lack of erythropoietin receptor signaling was observed. In addition, STAT5 signaling under IL-2 receptor has been shown to be important for the development of regulatory T cells in mice and humans (Cohen *et al*., 2006; Yao *et al*., 2007). Therefore, a careful observation of the adverse effects is essential for the development of the therapeutic drugs targeting JAK2 and STAT5. In fact, several, but not all JAK inhibitors exhibited myelosuppression such as anemia, thrombocytopenia and neutropenia when administered systemically to myelofibrosis patiens (Quintas-Cardama and Verstovsek, 2013). A JAK inhibitor Tofacitinib (also known as tasocitinib and CP-690,550) was approved for rheumatoid arthritis in Japan as well as in the United States. Although Tofacitinib also has an adverse effect of myelosuppression, it has been shown that it preserves regulatory T cell function while suppressing effector T cell function (Sewgobind *et al*., 2010). Furthermore, a pilot study of Tofacitinib on 6 moderate to severe AD patients who had failed standard treatment was recently reported (Levy *et al*., 2015). In all 6 patients, decreases in pruritus, sleep loss,

and Scoring Atopic Dermatitis (SCORAD) index were observed without any adverse events, warranting further investigation. In addition, the results of recently completed phase IIa clinical trial of tofacitinib ointment for the treatment of AD will soon be reported.

However, not all AD patients showed an increased expression of STAT5 phosphorylation in mast cells. This result implies that AD is not a single disease entity but has a heterogeneous background. Recently, the term 'endotype' was proposed to describe a subtype or a classification closely related a specific biologic mechanism of a disease (Anderson, 2008; Lotvall *et al*., 2011). For instance, IL-13 has been considered as a key T_H2 type cytokine that directs many of the important asthma features such as airway inflammation and remodeling (Hershey, 2003). However, humanized monoclonal antibody against IL-13 (Lebrikizumab) was effective only for asthma patients with high serum periostin level, but not for other asthma patients (Corren *et al*., 2011). Since periostin production is induced by IL-13 (Sidhu *et al*., 2010; Woodruff *et al*., 2007), periostin levels may reflect IL-13 dependency of the asthma. In other words, periostin level defines IL-13-driven/independent 'endotypes' of asthma. Therefore, defining endotypes of a complex disease or a syndrome by a certain biomarker is crucial for the success of the targeted molecular therapies. My result suggests that there are at least two endotypes in AD. Development of a standardized quantitative technique to measure the STAT5 phosphorylation and/or PLC-β3 expression directly or its upstream/downstream biomarkers would be beneficial to dissect AD endotypes. Furthermore, this stratification of AD may be important for clinical application of drugs targeting STAT5 activating pathways.

In summary, I found that STAT5-regulatory mechanisms such as JAKs and SPS complex in mast cells are important for the pathogenesis of AD and could be novel therapeutic targets.

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7. Figures

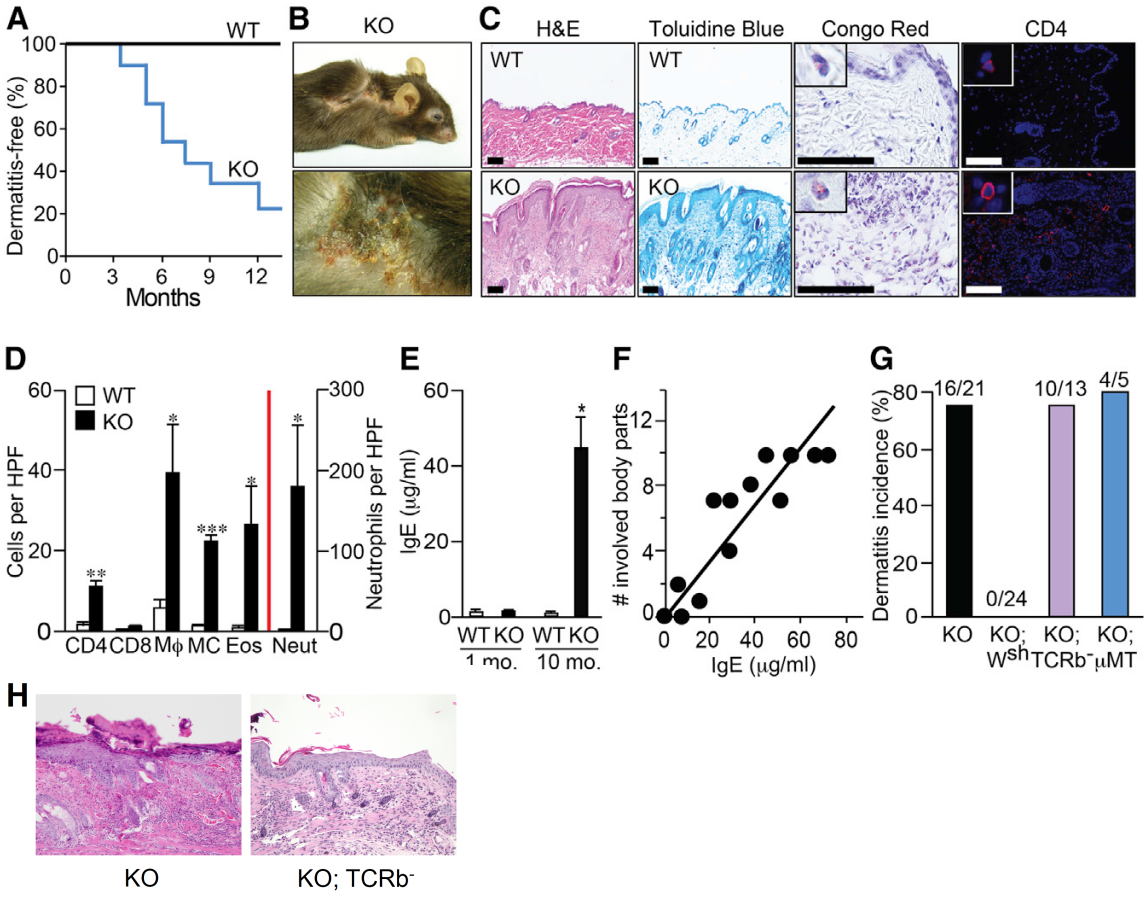

Figure 1. Plcb3$^{-/-}$ Mice spontaneously develop AD-like skin lesions in a mast cell-dependent manner.

(A) Kaplan-Meier plots for dermatitis development in *Plcb3*$^{-/-}$ mice (n=21). (B) Note the eczematous skin lesions and hair loss in periocular areas, cheeks, ears, neck and flanks in a 10 month-old *Plcb3*$^{-/-}$ mouse. (C) Histology of healthy (WT) and skin lesions (*Plcb3*$^{-/-}$) in ear. Bar, 100 μm. (D) Graphic representation of histological analysis of ear skin lesions of 8~10-month-old WT and *Plcb3*$^{-/-}$ mice. Neutrophils (Neut), eosinophils (Eos) and mast cells (MC) were enumerated in H&E-, Congo red- and Toluidine blue-stained preparations, respectively. Immunofluorescence staining was performed to detect CD4^{+}, CD8^{+} and F4/80^{+} (Mϕ) cells. Data represent mean ± SEM. *, **, ***: $p<0.05$, $p<0.01$, $p<0.001$ vs. WT mice by Student's *t*-test. Similar results were obtained in lesional skin in cheeks and neck (data not shown). HPF, high power field. (E) Serum IgE levels were increased in 8~10-month-old *Plcb3*$^{-/-}$ mice. (F) Correlation between serum IgE levels and numbers of body parts with skin lesions (see the legend for panel B for eczematous body parts). r^2 = 0.78, $p<0.0001$, Pearson's correlation. (G) Incidence of skin lesions in *Plcb3*$^{-/-}$ (KO), *Plcb3*$^{-/-}$;*Kit*$^{W\text{-}sh/W\text{-}sh}$ (KO;W^{sh}), *Plcb3*$^{-/-}$;*TCRb*$^{-/-}$ (*KO;TCRb*$^{-}$) and *Plcb3*$^{-/-}$;*μMT/μMT* (*KO;μMT*) mice

for 12 months. Results in *E* and *F* are representative of two independent experiments using 3-6 mice per group. (H) H&E staining of spontaneous skin lesions of *Plcb3*$^{-/-}$ (KO) and *Plcb3*$^{-/-}$*;TCRb*$^{-/-}$ (KO; TCRb^{-}) mice.

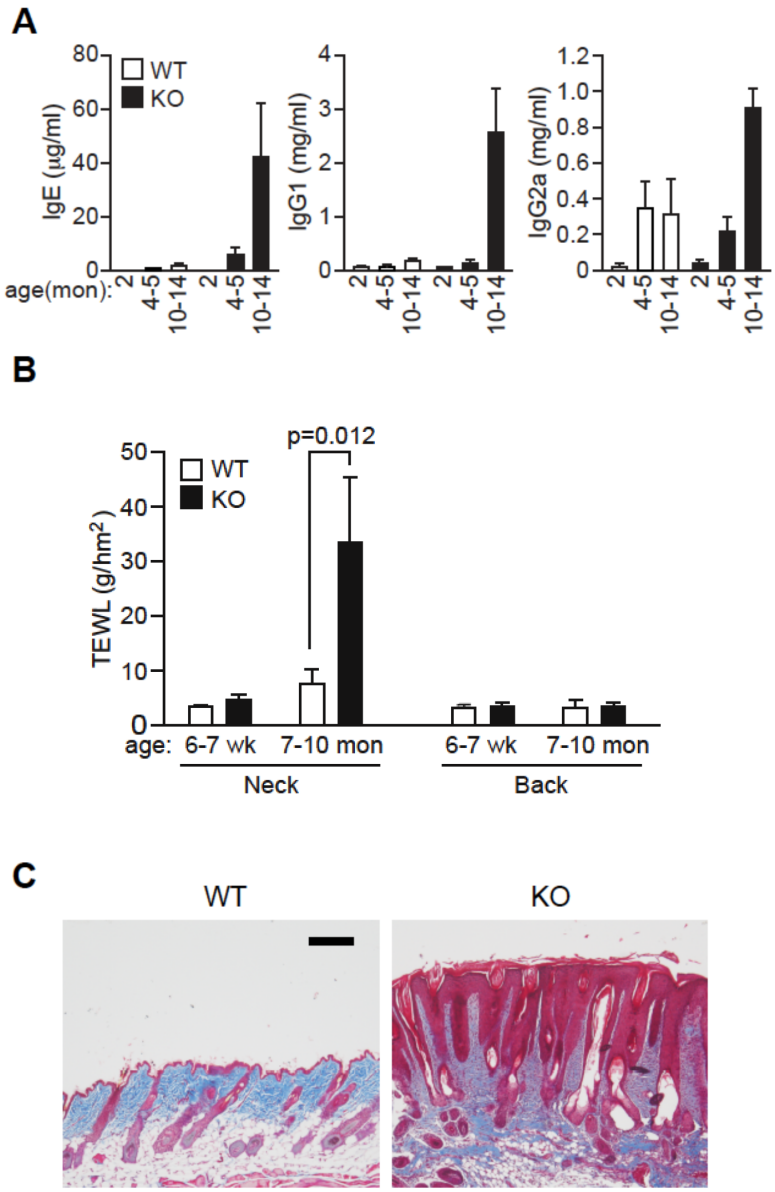

Figure 2. Eczematous *Plcb3*$^{-/-}$ mice exhibit high serum IgE, increased transepidermal water loss (TEWL) and strong fibrosis

(A) Serum antibody levels in young and old WT and *Plcb3*$^{-/-}$ mice. (B)TEWL was measured on the shaved neck skin and back of young (6-7 weeks old) and old (7-10 months old) mice using Tewameter® TM 300 (CK electronic GmbH, Cologne, Germany). AD-like skin lesions were observed in the neck, but not back, skin of the old *Plcb3*$^{-/-}$ mice. (C) Skin lesions of spontaneously occurring dermatitis in *Plcb3*$^{-/-}$ (KO) and equivalent anatomical sites in WT mice were stained by Masson Trichrome. Blue staining represents deposition of collagen. Bar, 200 μm. Results in *A-C* are representative of 2 experiments using 3-5 mice per group.

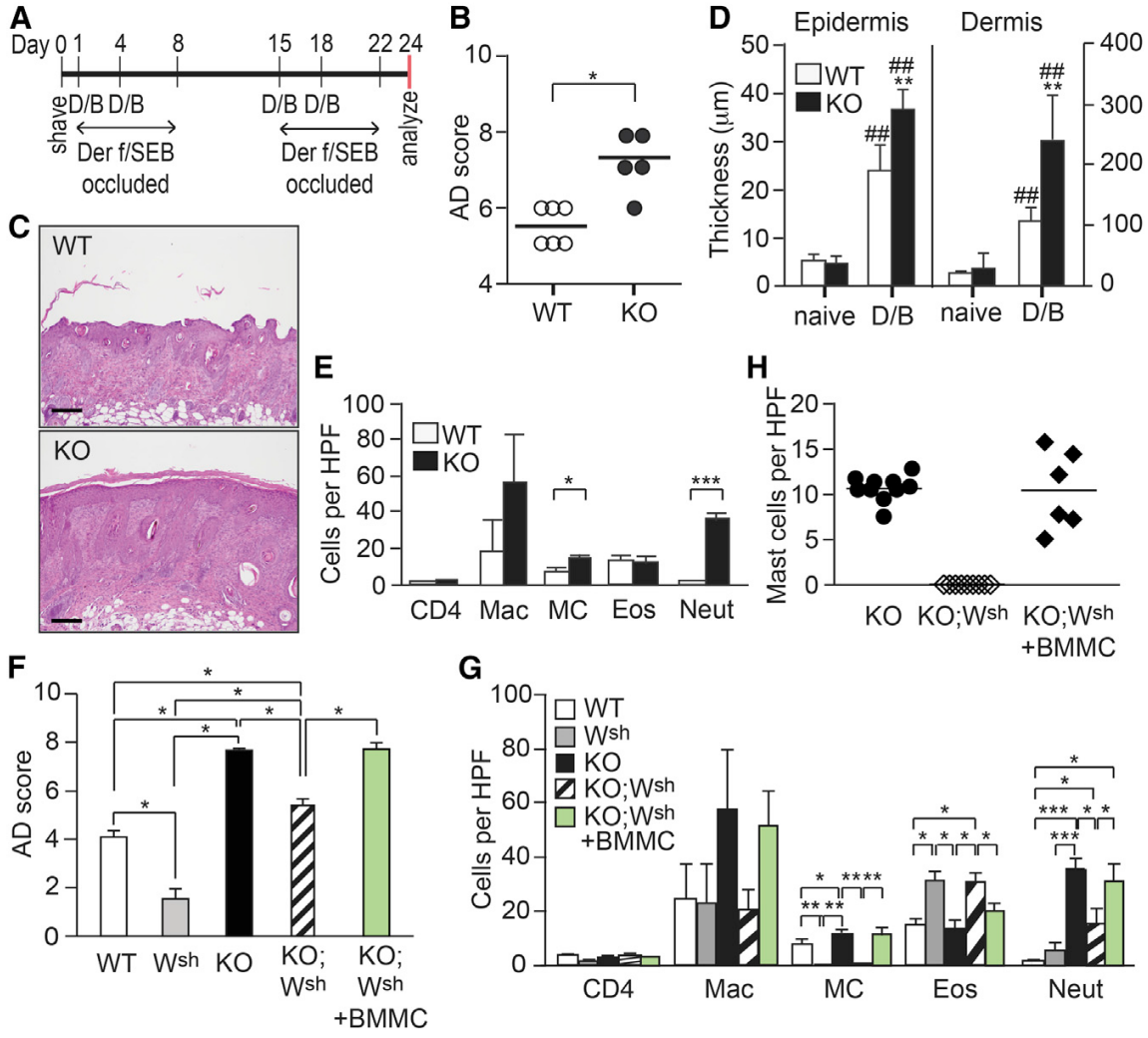

Figure 3. Mast cells significantly contribute to the increased severity of Der f/SEB-induced skin lesions in *Plcb3*$^{-/-}$ mice.

(A) AD-like skin lesions were induced as described previously (Kawakami *et al*., 2007). D/B, treatment with Der f and SEB. The periods when Der f/SEB-treated back skin is occluded with Tegaderm™ are also shown. (B) AD scores on day 24 with WT and *Plcb3*$^{-/-}$ mice. (C) H&E staining of lesional skins in WT and *Plcb3*$^{-/-}$ (KO) mice. (D) Thicknesses of epidermis and dermis at basal and Der f/SEB (D/B)-treated levels were measured on H&E-stained lesional skins. Results in *B-D* are representative of 3 independent experiments using 5-8 mice per group.

(E) Histologic analysis of Der f/SEB-induced dermatitis. Numbers of CD4^{+} T cells (CD4), macrophages (Mac), mast cells (MC), eosinophils (Eos) and neutrophils (Neut) are shown as cells per high power field (HPF). (F,G) Dermatitis was induced in WT, *Kit*$^{W\text{-}sh/W\text{-}sh}$ (W^{sh}), *Plcb3*$^{-/-}$ (KO) and *Plcb3*$^{-/-}$;*Kit*$^{W\text{-}sh/W\text{-}sh}$ (KO;W^{sh}) mice. A group of *Plcb3*$^{-/-}$;*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice received bone marrow derived mast cells (BMMCs) derived from *Plcb3*$^{-/-}$ mice 6 weeks

before Der f/SEB treatment. (F) AD scores on day 24. (G) Histologic analysis of dermatitis. Data represent mean ± SD. *, **, ***: $p<0.05$, $p<0.01$, $p<0.001$ vs. WT mice or indicated pairs by Student's *t*-test or ANOVA; ##, $p<0.01$ vs. naïve mice. (H) Mast cells were quantified on Toluidine blue-stained skin tissues. HPF, high power field. Results in *F-H* are representative of 2 independent experiments using 6 mice per group.

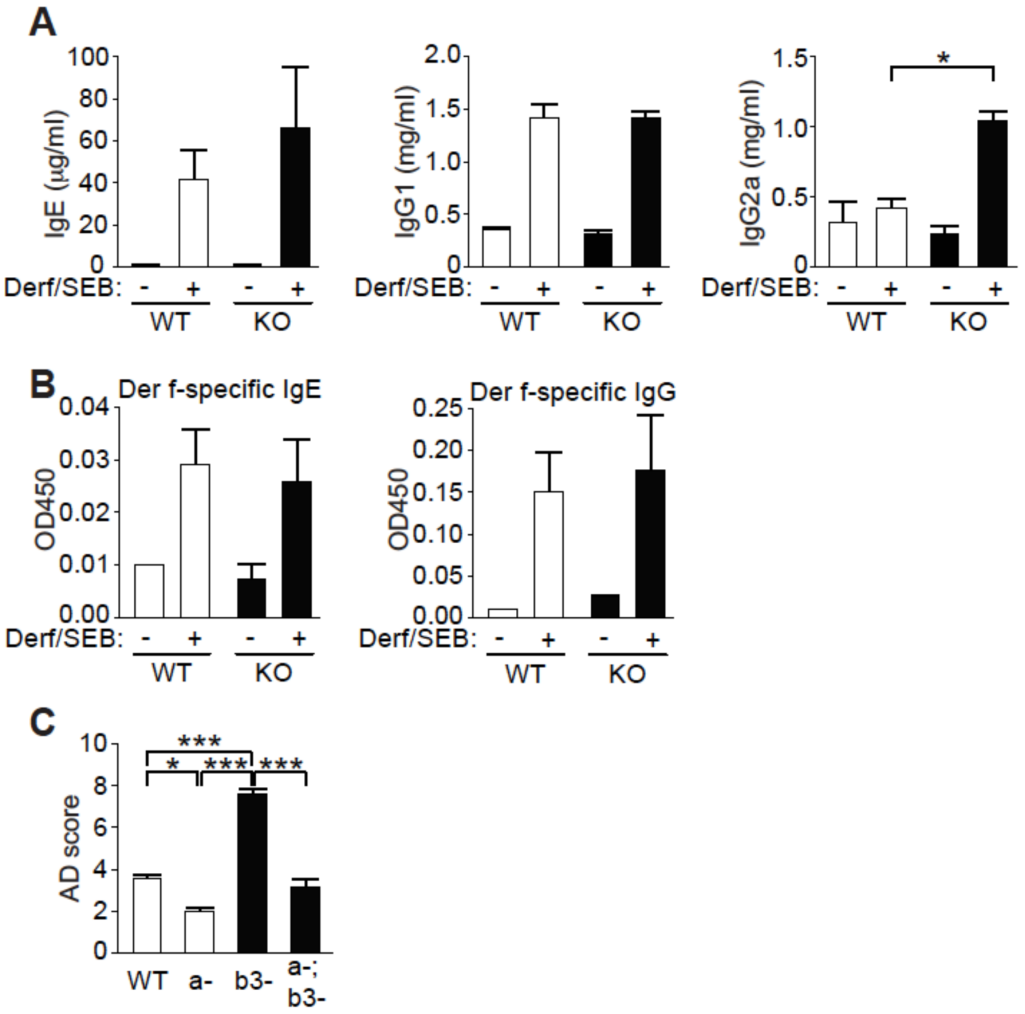

Figure 4. Der f/SEB-stimulated *Plcb3*$^{-/-}$ mice have similar levels of serum IgE and IgG1 compared to Der f/SEB-stimulated WT mice, Related to Figure 2. Total serum (A) and Der f-specific (B) immunoglobulins were quantified by ELISA on day 24 (see the Der f/SEB treatment schedule in Figure 3A). (C) AD scores with WT, *FceRIa*$^{-/-}$ (a-), *Plcb3*$^{-/-}$ (b3-), and *FceRIa*$^{-/-}$*;Plcb3*$^{-/-}$ (a-;b3-) mice. Results in *A-C* are representative of 3 experiments using 3-6 mice per group.

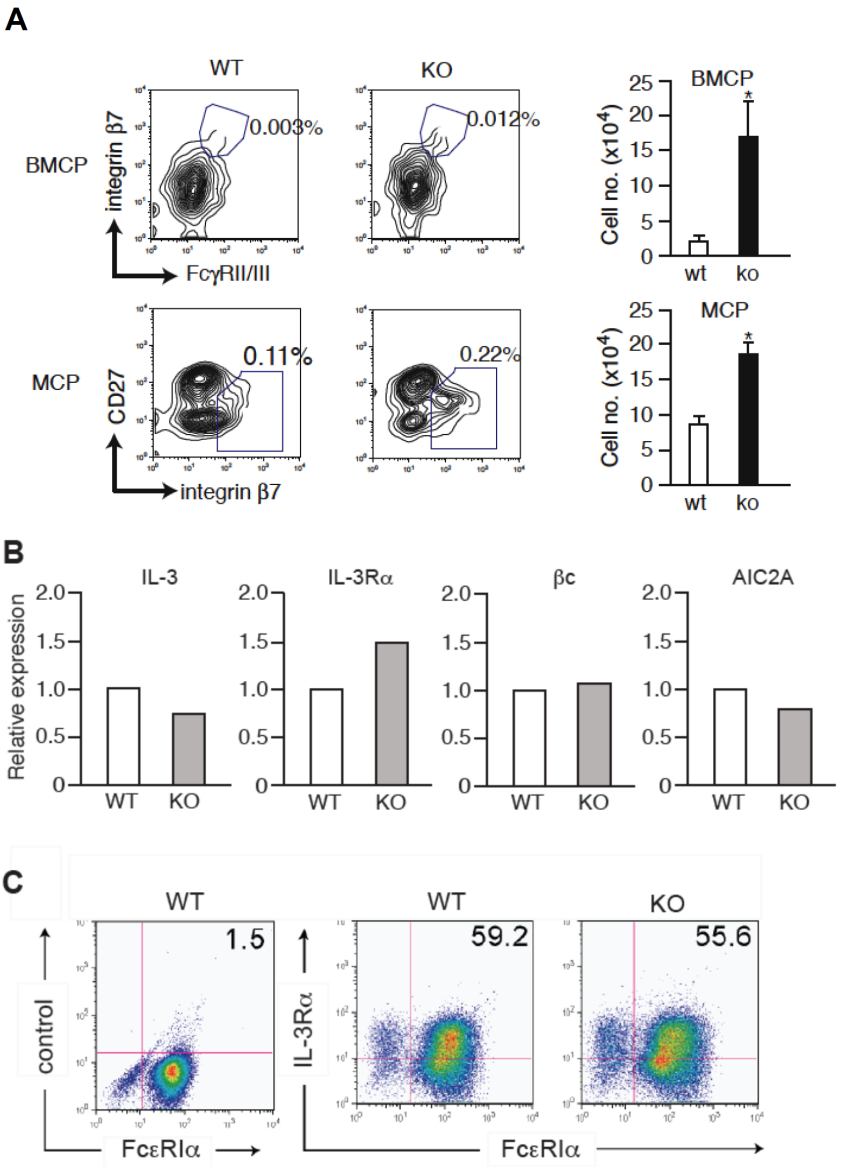

Figure 5. *Plcb3*$^{-/-}$ mice have increased basophil/mast cell progenitors (BMCPs) and mast cell progenitors (MCPs) whereas expression of IL-3 and IL-3R was comparable to WT mast cells

(A) Spleen and bone marrow cells from 10 week-old mice were analyzed by flow cytometry. Spleen cells were stained for c-Kit, FcgRII/III, integrin β7, and lineage markers, and c-Kit^{+}Lin^{-} cells were gated for detecting BMCPs. Lin^{-}Sca-1^{-}Ly6c^{-}FcεRI^{-}c-Kit^{+} bone marrow cells were gated for MCPs. *, $p<0.05$ vs. WT mice by Student's *t*-test. Results are representative of 2 experiments using 3 mice per group. (B) mRNAs for IL-3 or IL-3R components were quantified by microarray for ear skin tissues from 10 week-old WT and *Plcb3*$^{-/-}$ mice (average of 4 mice each). (C) IL-3R on WT and *Plcb3*$^{-/-}$ bone marrow derived mast cells (BMMCs) was stained for flow cytometry.

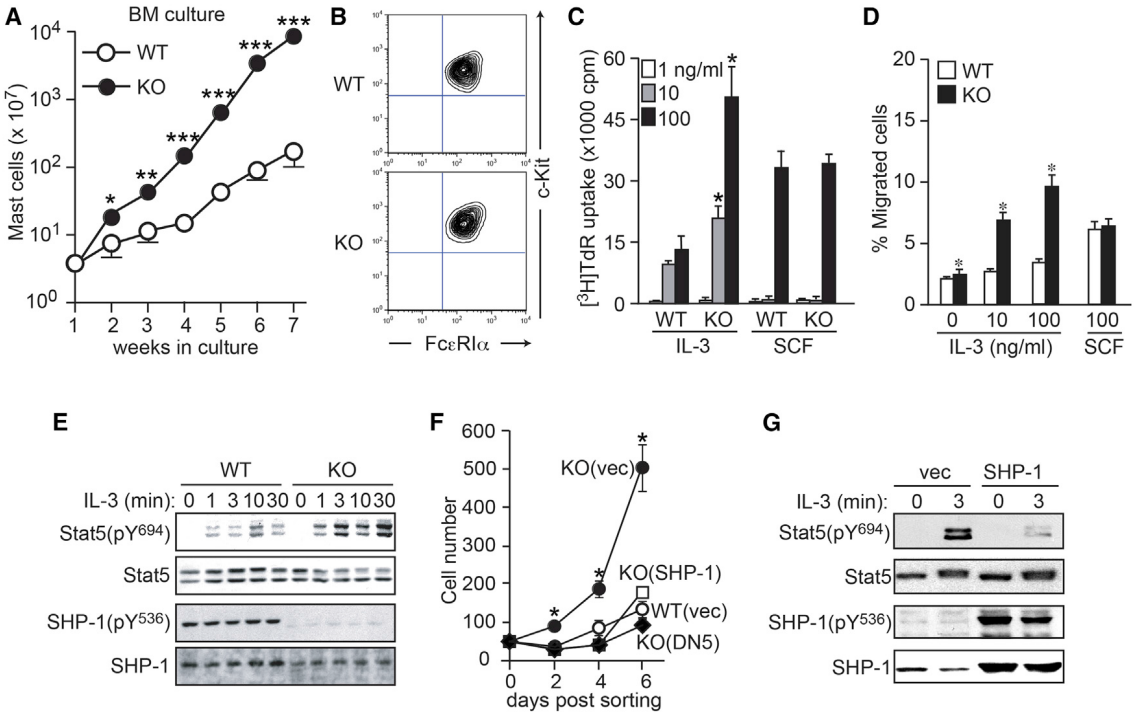

Figure 6. *Plcb3*$^{-/-}$ mast cells are hypersensitive to IL-3 stimulation. (A) Bone marrow cells derived from WT or *Plcb3*$^{-/-}$ mice were cultured in IL-3-containing medium for the indicated periods. IL-3 was derived from IL-3-producing cell line D11. Final concentration of IL-3 was 5-10 ng/mL. Live cells were counted. (B) >98% of 5-week cultured BMMCs expressed c-Kit and FcεRI. Results in *A* and *B* are representative of at least 10 experiments using 3-4 mice per group. (C) BMMCs were depleted of IL-3 for 8 h and cultured in the indicated concentrations of IL-3 or SCF for 36 h. DNA synthesis was measured by [^{3}H]thymidine incorporation ([^{3}H]TdR uptake) during the last 18 h of culture. (D) Chemotaxis of BMMCs towards IL-3 or SCF was assayed in Transwell for 8 h. Results in *C* and *D* are representative of 2 experiments using 3 mice per group. (E) WT and *Plcb3*$^{-/-}$ BMMCs were stimulated with 10 ng/ml of IL-3 for the indicated periods. Cell lysates were analyzed by SDS-PAGE followed by western blotting using antibodies for the indicated molecules. (F,G) WT and *Plcb3*$^{-/-}$ MCPs were transduced with bicistronic retroviral vectors coding for DN Stat5 (DN5), WT SHP-1 or empty vector. GFP$^+$ transduced cells were FACS-sorted and cultured in IL-3 (F). *, $p<0.05$ vs. empty vector-transduced WT cells (WT (vec)) by Student's *t*-test. Some transduced *Plcb3*$^{-/-}$ mast cells were stimulated with IL-3 and subjected to western blot analysis (G). Results in *E-G* are representative of 2 transduction experiments.

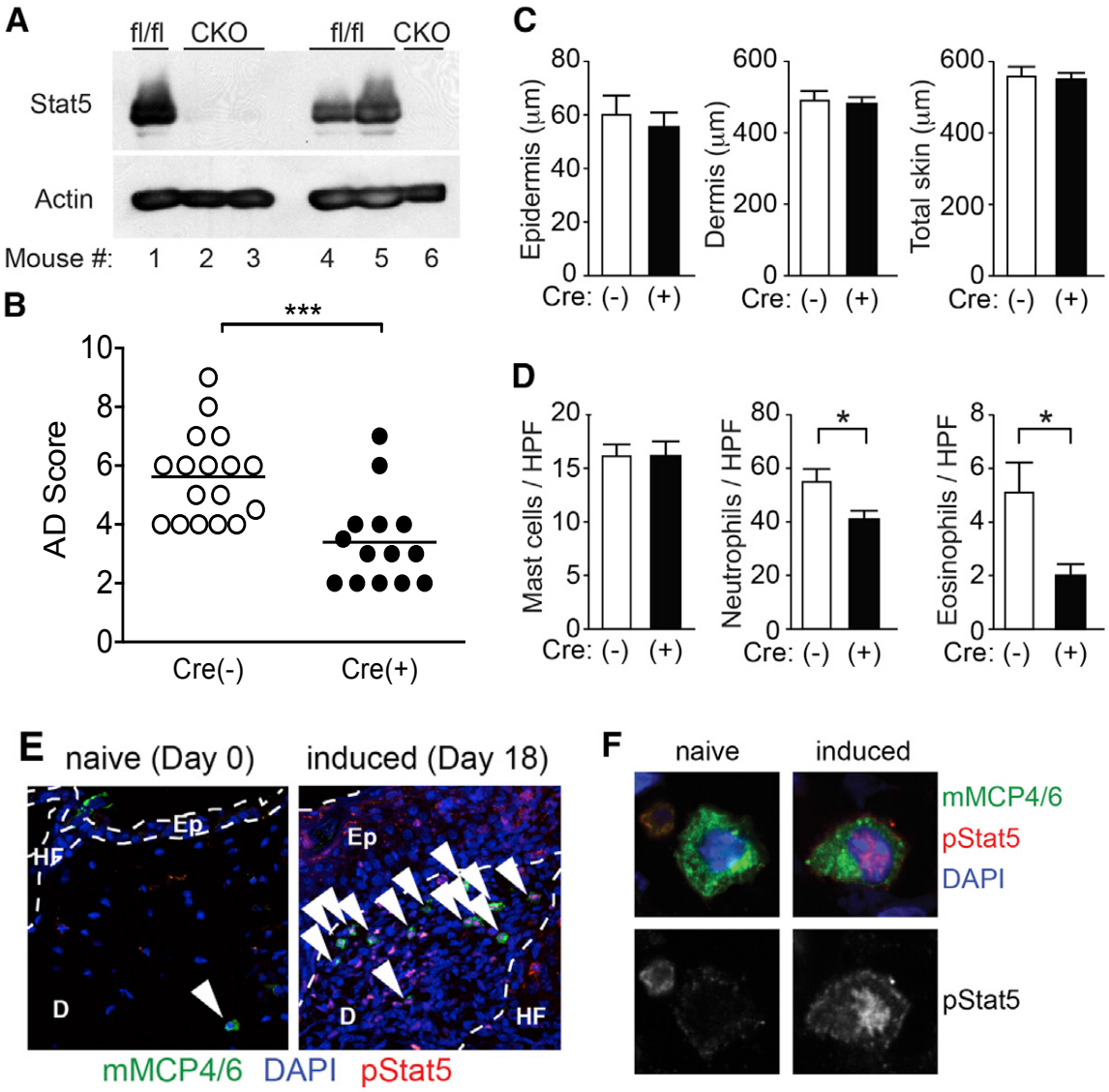

Figure 7. Stat5 in mast cells regulates Der f/SEB-induced dermatitis. Dermatitis was induced with Der f/SEB in MCDStat5 mice (CKO or Cre(+)) and their floxed control (fl/fl or Cre(-)) mice. *, ***: $p<0.05$, $p<0.001$ by Student's *t*-test. (A) Western blot analysis of Stat5 in mast cells derived from neonatal skin of MCΔStat5 (CKO) and control (fl/fl) mice. (B) AD scores accumulated from 4 separate experiments using 3-5 mice per group. (C) Thicknesses of epidermis and dermis after Der f/SEB (D/B)-treatment. (D) Histologic analysis of Der f/SEB-induced dermatitis. (E,F) Skin sections of naïve and Der f/SEB-induced (6 h after 4th induction) dermatitis in WT mice were stained for phospho-Stat5, mMCP4 and mMCP6. Arrowheads indicate pStat5-positive mast cells. Ep, epidermis; D, dermis; HF, hair follicle. Representative images of mast cells from 3 experiments are shown in *E* and *F*.

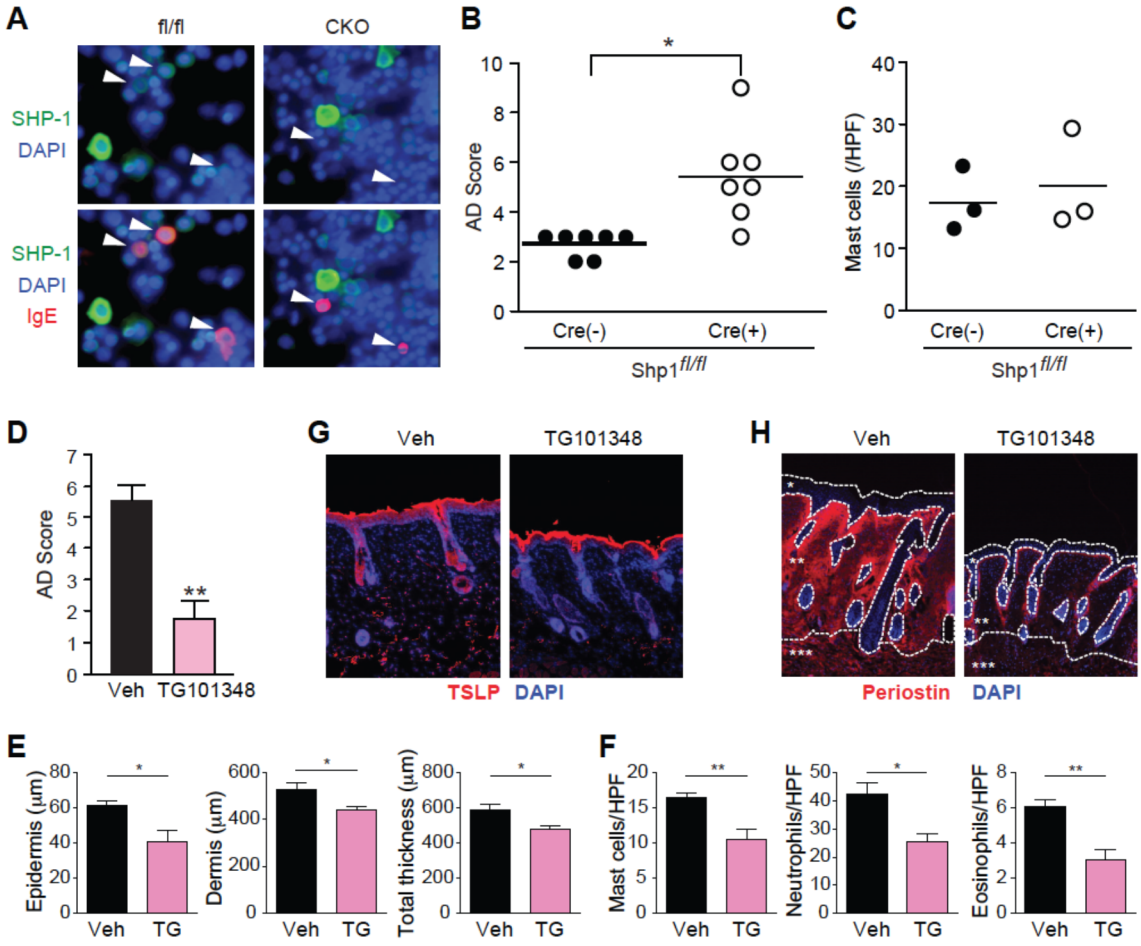

Figure 8. Der f/SEB-induced AD scores are increased in *MCΔSHP-1* mice, while JAK Inhibitor TG101348 inhibits Der f/SEB-induced dermatitis.

(A-C) Dermatitis was induced with Der f/SEB in *MCΔShp1* mice (CKO or Cre(+)) and their floxed control (fl/fl or Cre(-)) mice. (A) Loss of expression of the targeted loci was confirmed by immunofluorescence microscopy of skin mast cells of MCΔShp1 (CKO) and control (fl/fl) mice. Arrowheads indicate mast cells. (B) Accumulated AD scores from 2 experiments. *, $p<0.05$ by Student's *t*-test. (C) Mast cells in the ears were stained with Toluidine blue. HPF, high-power field. (D-H) B6 mice were co-treated epicutaneously with 100 μM TG101348 along with Der f/SEB. (D) AD scores. (E) Thicknesses of epidermis and dermis after Der f/SEB treatment. Veh, vehicle. (F) Histologic analysis of Der f/SEB-induced dermatitis. Immunofluorescence confocal microscopy was performed to detect TSLP (G) and periostin (H). Results in *D-H* are representative of 2 experiments using 3-5 mice per group.

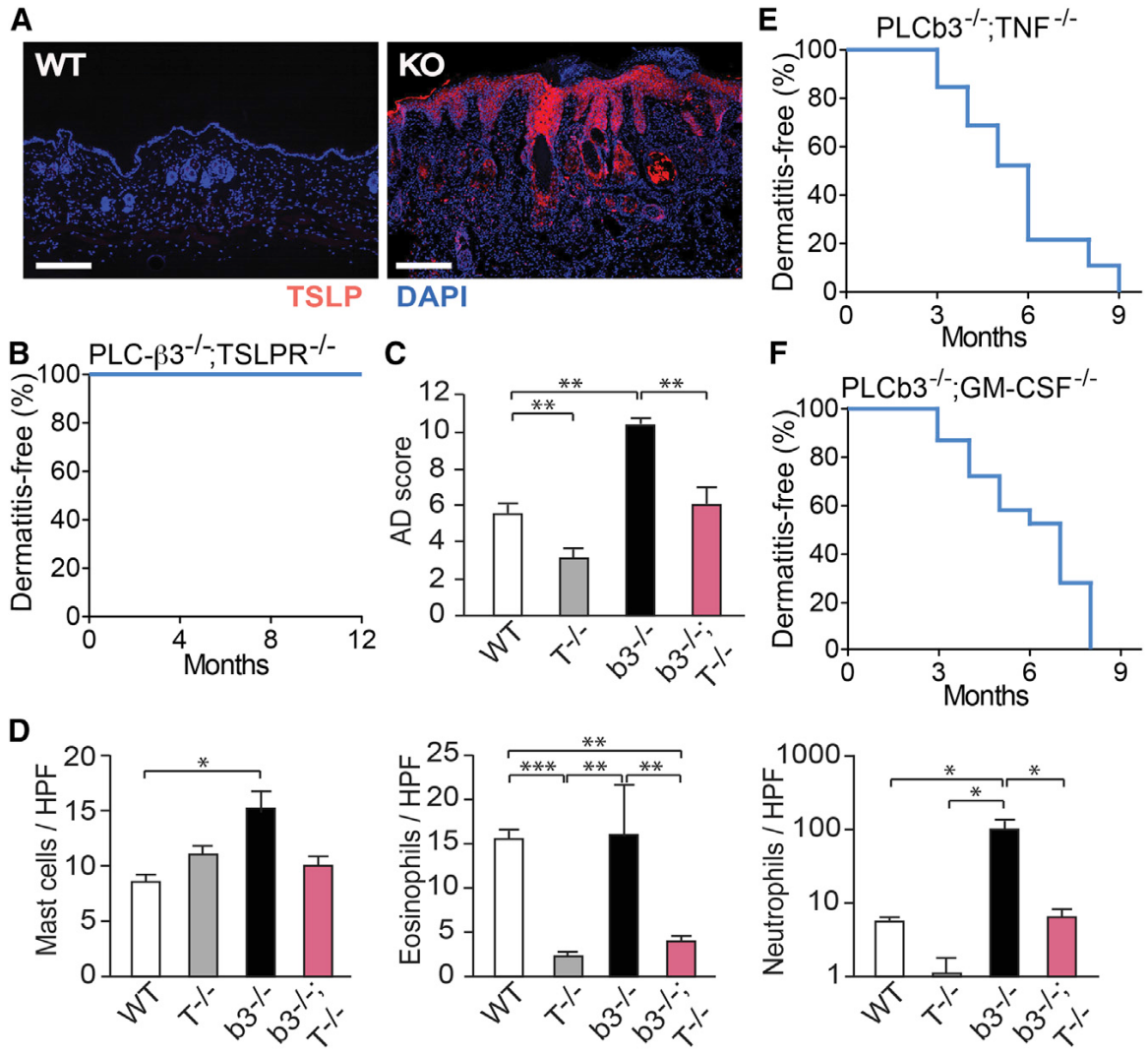

Figure 9. Role of the TSLP-TSLPR axis in Der f/SEB-induced dermatitis and spontaneous dermatitis in *Plcb3*$^{-/-}$ mice. (A) Lesional skin from 10-month-old *Plcb3*$^{-/-}$ mice and healthy control (WT) were stained for TSLP (red) and nuclei (blue). (B) Kaplan-Meier plots for dermatitis development in *Plcb3*$^{-/-}$*;TSLPR*$^{-/-}$ (n=10). (C) Dermatitis was induced with Der f/SEB in WT, *TSLPR*$^{-/-}$ (T-/-), *Plcb3*$^{-/-}$ (b3-/-) and *Plcb3*$^{-/-}$*;TSLPR*$^{-/-}$ (b3-/-;T-/-) mice. (D) Histologic analysis of Der f/SEB-induced dermatitis. Results in *C* and *D* are representative of 2 independent experiments using 3-6 mice per group. (E,F) Kaplan-Meier plots for dermatitis development in *Plcb3*$^{-/-}$*;TNF*$^{-/-}$ (n=34), and *Plcb3*$^{-/-}$*;GM-CSF*$^{-/-}$ (n=31) mice. *, **, ***: $p<0.05$, $p<0.01$, $p<0.001$ by ANOVA.

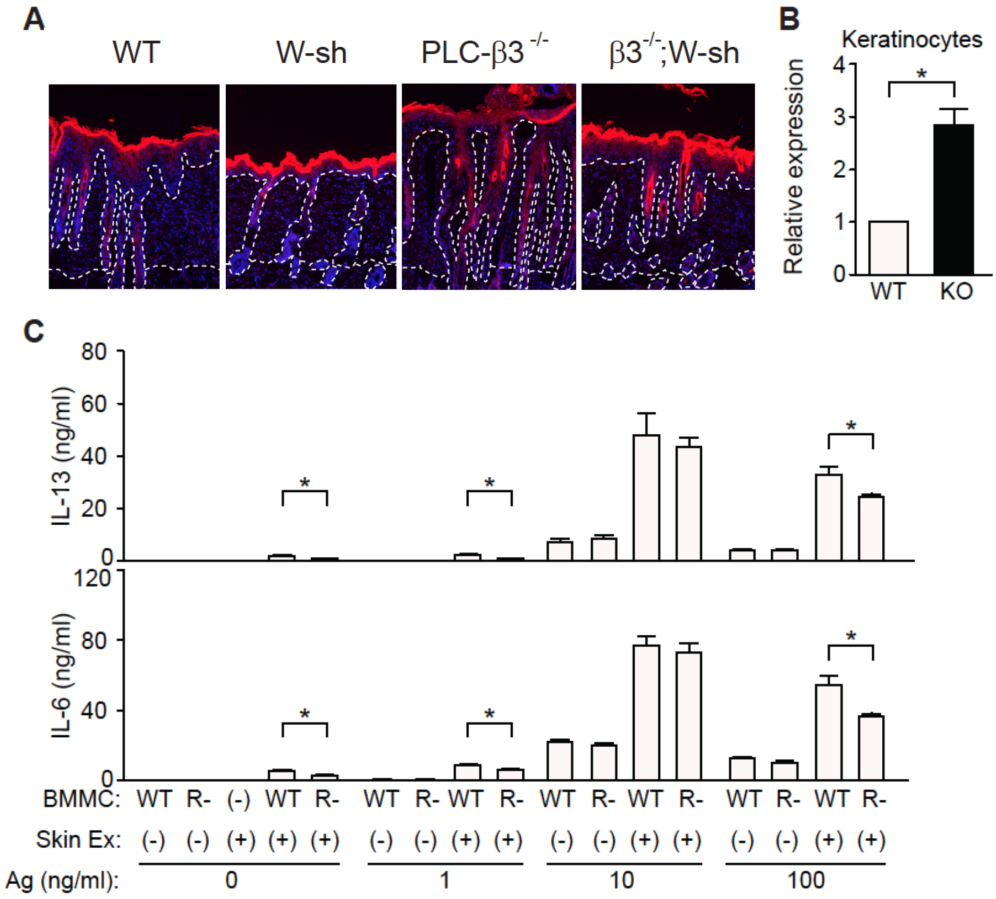

Figure 10. TSLP expression in the skin and synergistic mast cell cytokine production by TSLP and FceRI stimulation.

(A) Dermatitis was induced with Der f/SEB in WT, *Kit*$^{W\text{-}sh/W\text{-}sh}$, *Plcb3*$^{-/-}$, and *Plcb3*$^{-/-}$;*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. Lesional skin was stained for TSLP (red) and nuclei (blue). Borders of epidermis, dermis and subcutaneous fat tissues are indicated by dotted lines. (B) TSLP mRNA was quantified by qPCR with cultured keratinocytes derived from WT and *Plcb3*$^{-/-}$ mice. 18S RNA was used as a control. Results are representative of 2 experiments using 3 mice per group. (C) WT and *TSLPR*$^{-/-}$ (R-) BMMCs were sensitized with anti-DNP IgE and incubated with the indicated concentrations of antigen in the presence or absence of skin extract (Skin Ex) from WT mice. IL-13 and IL-6 secreted into culture media for 20 h were measured by ELISA. Note 1) that skin extract did not contain measurable amounts of IL-13 or IL-6, but skin extract alone induced cytokine production in BMMCs, 2) that antigen stimulation synergized with skin extract to induce cytokine production, and 3) that both IL-13 and IL-6 production induced by skin extract was lower by 20-30% in *TSLPR*$^{-/-}$ BMMCs than in WT BMMCs, except when BMMCs were stimulated with an optimal antigen concentration (dinitrophenyl-conjugated human serum albumin, 10 ng/ml).

Therefore, TSLP in skin extract enhances cytokine production by FcεRI-stimulated BMMCs. Essentially identical results were obtained using skin extract from Der f/SEB-treated skin (data not shown). *, $p<0.05$ by Student's *t*-test. Results in *C* are representative of 3 independent experiments.

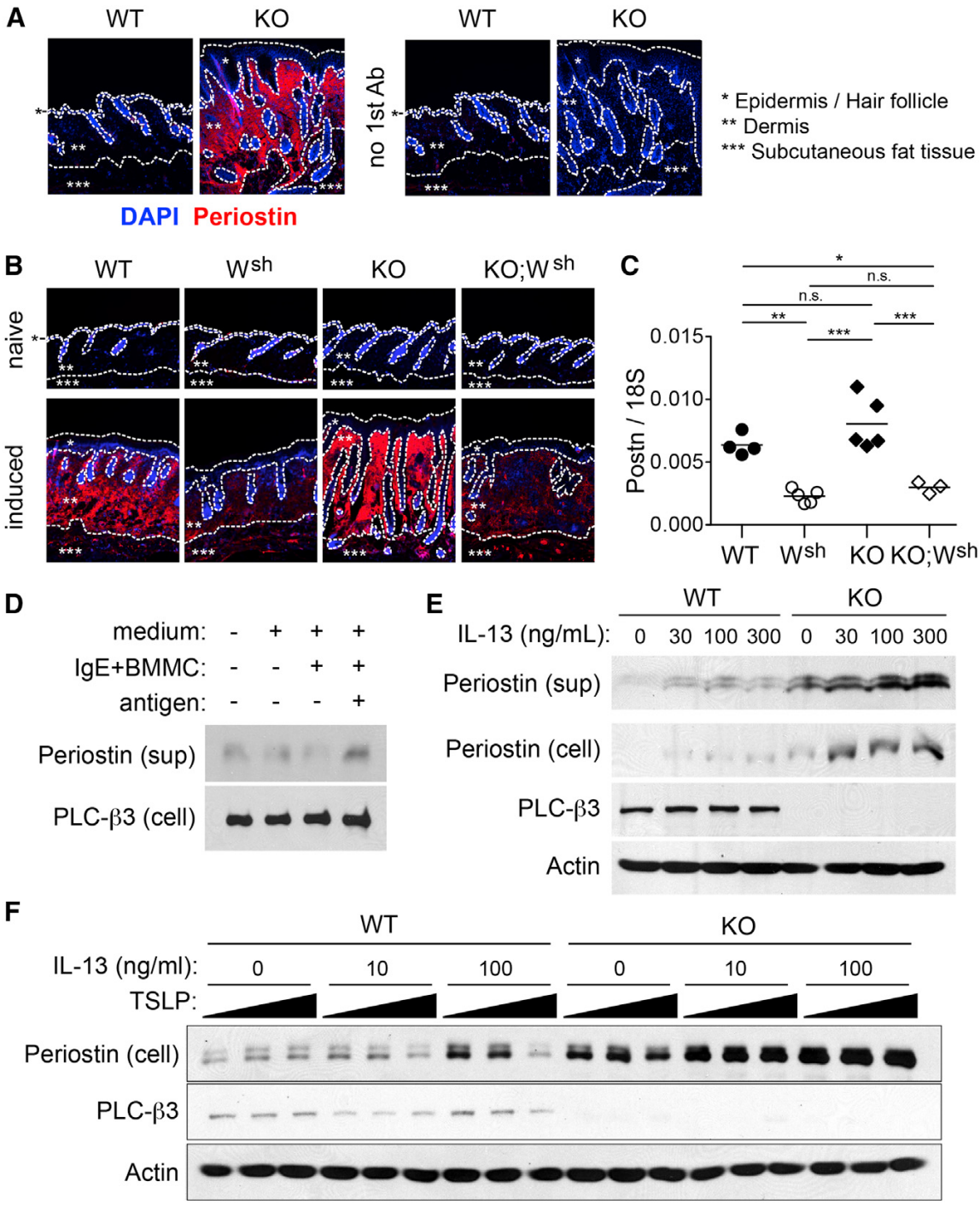

Figure 11. Regulation of periostin production in fibroblasts.

(A) Lesional skin of spontaneous dermatitis in *Plcb3*$^{-/-}$ mice (KO) and normal skin from WT mice (WT) were stained for periostin (red) and nuclei (blue). Right panels show control stainings without anti-periostin antibody. (B) Periostin was stained before (naïve) and after Der f/SEB induction of dermatitis (induced) in WT, *Kit*$^{W\text{-}sh/W\text{-}sh}$ (W^{sh}), *Plcb3*$^{-/-}$ (KO) and *Plcb3*$^{-/-}$;*Kit*$^{W\text{-}sh/W\text{-}sh}$ (KO;W^{sh}) mice. Borders of epidermis, dermis and subcutaneous fat

tissues are indicated by dotted lines. (C) Periostin mRNA expression in Der f/SEB-treated skin was quantified by qPCR. Results in *A-C* are representative of 3 experiments using 3-5 mice per group. (D) NIH/3T3 cells were incubated with or without IgE-sensitized BMMCs in the presence or absence of antigen. (E,F) WT and *Plcb3*$^{-/-}$ MEFs were stimulated by the indicated concentrations of IL-13 in the absence (E) or presence (F) of 0, 10 or 100 ng/ml of TSLP for 24 h. Periostin protein in culture supernatants and lysates of the cells was analyzed by western blotting. *, **, ***: $p<0.05$, $p<0.01$, $p<0.001$ by ANOVA. In vitro experiments with results similar to *D-F* were performed 2-4 times.

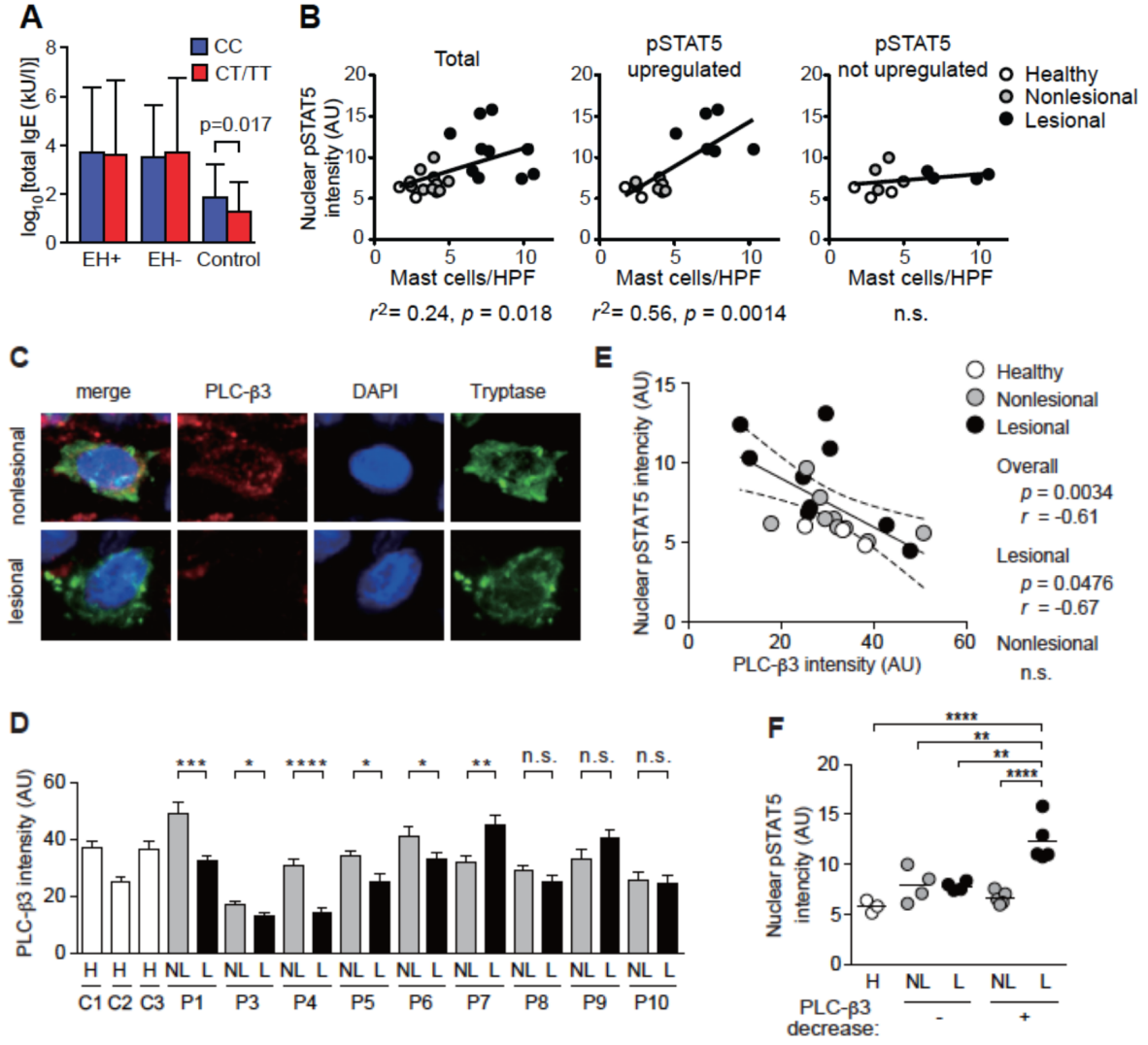

Figure 12. Correlation between mast cell numbers and nuclear phospho-STAT5 levels in mast cells among human AD and healthy individuals and inverse correlation of STAT5 phosphorylation with PLC-β3 expression in mast cells in human skin.

(A) SNP analysis on AD patients (ADEH+ and ADEH-) indicates an association of rs35169799 in *PLCB3* with log-transformed mean serum levels of total IgE (KU/L) in non-atopic European American subjects. (B) Pearson's correlation coefficients were calculated on the data in Figure 13. All data points were calculated (Total) or data points were divided into two groups based on the presence or absence of phospho-STAT5 upregulation in lesional mast cells. The results suggest the presence of two subsets of AD patients with vs. without increased phospho-STAT5 levels in lesional mast cells. (C) Skin samples of AD patients were stained for PLC-β3 (red), tryptase (green), and nuclei (blue). Representative images are shown from patient P4. (D-F) PLC-β3 levels in mast cells in lesional (L) and nonlesional (NL) skin samples of AD patients and healthy skin (H) were

measured by Image J software (NIH). Skin sample from patient P2 was not available for this analysis due to sample shortage. (D) Data represent mean ± SEM. *, **, ***, ****: $p<0.05, 0.01, 0.001, 0.0001$ by Student's *t*-test. (E) Median of nuclear pSTAT5 intensity is plotted against that of PLC-β3 intensity. Linear regression curve (solid line) and the 95% confidence interval (dotted lines) are overlaid. Pearson's correlation is shown. (F) Nuclear pSTAT5 intensity in mast cells was compared between lesional and nonlesional skins. Patients are stratified by the presence or absence of a decrease of PLC-β3 expression in panel D. **, $p<0.01$; ****, $p<0.0001$ by Tukey's multiple comparison (ANOVA).

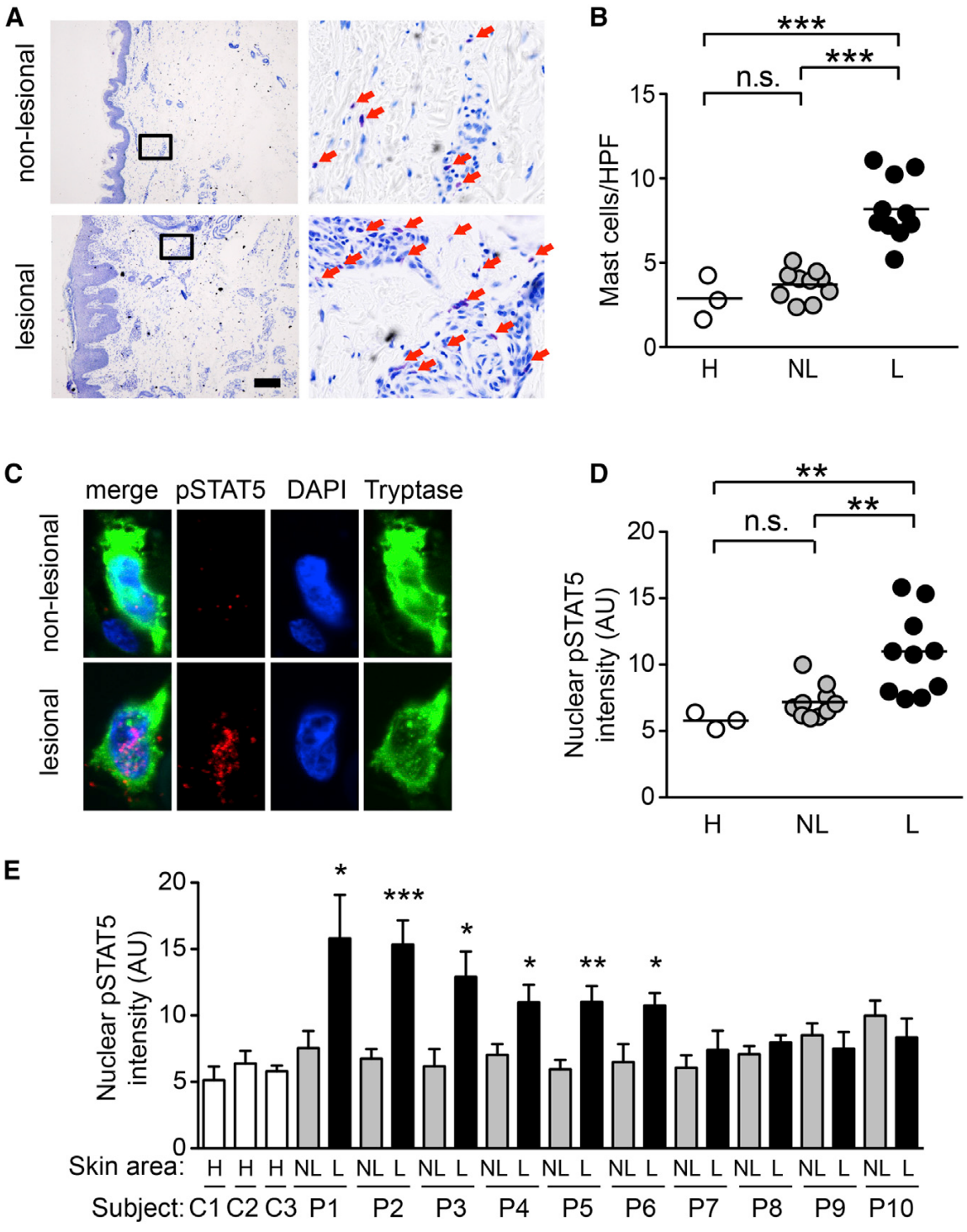

Figure 13. Increased numbers of mast cells with enhanced STAT5 phosphorylation in human AD patients. (A) Lesional and nonlesional skin samples of human AD patients were analyzed by Toluidine blue staining. Portions indicated by rectangle are enlarged on right panels. Red arrows indicate mast cells. (B) Quantification of mast cells. **, $p<0.01$; ***, $p<0.001$ by Tukey's multiple comparison test (ANOVA). (C) Skin samples of human

AD patients were stained for phospho-STAT5 (red), tryptase (green), and nuclei (blue). Representative images are shown from patient P1. (D,E) Nuclear phospho-STAT5 levels in mast cells in lesional (L) and nonlesional (NL) skin samples of human AD patients and healthy skin (H) were measured by ImageJ software (NIH). Data represent mean ± SEM. *, **, ***: $p<0.05$, $p<0.01$, $p<0.001$ vs. nonlesional (NL) skin by Student's *t*-test.

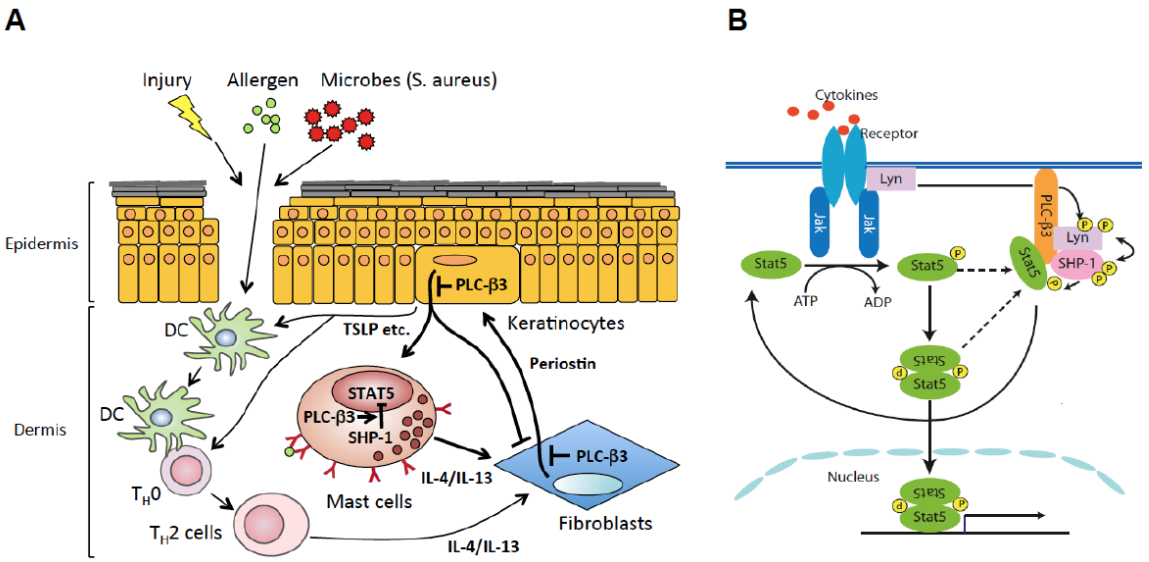

Figure 14. Hypothetical vicious cycle of allergic skin inflammation consisting of T_H2 cytokines (secreted from T_H2 cells and mast cells)-periostin (secreted from fibroblasts)-TSLP and other proinflammatory cytokines (secreted from keratinocytes).

(A) Allergen-specific T_H2 cells stimulate production/secretion of periostin from fibroblasts. Then, periostin in turn stimulates keratinocytes to produce and secrete TSLP and other inflammatory cytokines. In this scheme, T_H2 cells seem to be required for initial epidermal overexpression of TSLP. Once sustained overexpression of TSLP is established, mast cells may play a more important role in persistent dermatitis than T_H2 cells. PLC-β3 can regulate activities of the cellular elements of this network, such as proliferation of mast cells, periostin production/secretion in fibroblasts and TSLP production/secretion in keratinocytes. My data also suggest the presence of a feedback loop for inhibition of fibroblasts' periostin production by TSLP and PLC-β3 in fibroblasts is required for this feedback inhibition. DC, dendritic cell; T_H0, naïve $CD4^+$ T cells. (B) Model of SPS complex-mediated mast cell regulation. SHP-1 efficiently dephosphorylates Stat5 at Tyr^{694} in a PLC-β3-dependent manner in hematopoietic stem cells (Xiao *et al.*, 2009) and mast cells (this study). In a previous study it is shown that Lyn is another member of the SPS complex and that Lyn and SHP-1 regulate each other (Xiao *et al.*, 2010). I propose that SPS members regulate mast cell biology (*e.g.*, proliferation) and AD pathogenesis.

8. Tables

Table 1. Demographic characteristics of AD and control populations

Characteristics	European American		African American	
	AD	Healthy	AD	Healthy
No. of subjects	248	156	171	152
Males; N (%)	91 (36.7%)	63 (40.4%)	40 (23.4%)	77 (50.7%)
Age (yr); mean (SD)	33.1 (18.5)	36.6 (13.2)	35.3 (12.5)	41.1 (10.3)
Geometric mean IgE levels (95%CI)	694.1 (522-922)	59.1 (48-111)	504.8 (378-1229)	141.2 (113-299)
Geometric EASI score (95%CI)[†]	4.6 (3.9-5.4)	NA	3.7 (3.0-4.5)	NA

The following abbreviations used are: AD, atopic dermatitis; EASI, eczema area and severity index; and NA, not applicable. [†]EASI score is determined by the percentage of eczema area on a 7-point ordinal scale: 0 =<10%; 1=10%-29%; 3=30%-49%; 4=50%-69%; 5=70%-89%; and 6=90%-100%.

Table 2. Similarity of gene expression profiles between spontaneous dermatitis in Plcb3$^{-/-}$ mice, Der f/SEB-induced dermatitis in WT mice and human AD

GEO accession	Orthologs common in the lists	Comparison	*Plcb3*$^{-/-}$ spontaneous skin lesion / WT normal skin		WT Der f/SEB-induced skin lesion / WT normal skin	
			Score	p-value	Score	p-value
GSE6012	10873	AD lesional / Normal skin	1252.7	<0.001	1198.9	<0.001
GSE5667	14325	AD lesional / Normal skin	952.5	<0.001	972.3	<0.001
GSE16161	10873	AD lesional / Normal skin	915.4	0.001	994.1	<0.001
GSE5667	14325	AD lesional / AD non-lesional	961.2	<0.001	1029.0	<0.001
GSE27887	14878	AD lesional / AD non-lesional	941.9	<0.001	1156.9	<0.001
GSE5667	14325	AD non-lesional / Normal skin	350.4	0.556	277.8	0.868
GSE26952	14889	AD non-lesional / Normal epidermis	913.5	<0.001	888.8	<0.001
GSE26952	14889	PS non-lesional / Normal epidermis	600.4	0.003	577.9	0.012

Similarity scores of gene expression changes in spontaneous dermatitis in Plcb3$^{-/-}$ mice (vs. normal skin in WT mice), Der f/SEB-induced dermatitis in WT mice (vs. normal skin in WT mice) and human AD (the indicated matches in Comparison column) were computed by OrderedList algorithm. Numbers of the orthologs compared, similarity scores and p-values are shown. For comparison, data with Der f/SEB-induced skin lesions (Ando *et al*., 2013) and human psoriasis (PS) are included.

Table 3. SNP identities, location and minor allele frequency in PLCB3, STAT5A, STAT5B and SHP1

Gene (Chromosome)	dbSNP ID	Location	Type of Variant	Risk allele	Allele frequency	
					European American (N=156)	African American (N = 152)
PLCB3	rs2244625	63782720	Coding exon	G	0.328	0.772
(11q13.1)	rs915987	63784464	Intron	A	0.141	0.030
	rs35169799	63787817	Coding exon	T	0.057	0.020
	rs3815362	63790131	Intron (boundary)	T	0.340	0.093
STAT5A	RS16967637	37699948	Intron	A	0.349	0.391
(17q21.2)	RS7217728	37700927	Intron	T	0.652	0.366
	RS13380828	37701981	Intron	A	0.010	0.259
	RS9906989	37709372	Intron	T	0.181	0.173
	RS2272087	37713088	Intron (boundary)	G	0.187	0.254
	RS2293154	37714529	Intron (boundary)	T	0.186	0.171
STAT5B	rs17500235	37609886	Intron	C	0.056	0.010
(17q21.2)	rs9900213	37629407	Intron	A	0.206	0.541
	rs6503691	37647616	Intron	A	0.138	0.612
	rs17591972	37652970	Intron	G	0.103	0.185
SHP1	RS7310161	6927395	Promoter	A	0.560	0.204
(12p13.31)	RS7966756	6932652	Intron	A	0.063	0.454
	rs10744724	6935542	Intron	C	0.059	0.507
	RS759052	6939881	Intron (boundary)	T	0.122	0.431

Table 4. Association between SNPs in PLCb3, STAT5A, STAT5B and SHP1 and AD and related phenotypes

Gene and SNP	Position (Mb)	Risk allele	AD				ADEH				EASI	
			European American		African American		European American		African American		African American	
			OR (95%CI)†	P	OR (95%CI)	P	OR (95%CI)	P	OR (95%CI)	P	Beta	P
PLCB3												
rs2244625	63.783	G	1.03 (0.72, 1.48)	0.87	0.98 (0.66, 1.44)	0.904	1.62 (1.06, 2.48)	**0.027***	0.82 (0.33, 2.05)	0.678	0.028 (-0.14, 0.19)	0.738
STAT5A												
rs16967637	37.7	A	0.74 (0.53, 1.04)	0.08	0.91 (0.63, 1.31)	0.609	0.83 (0.54, 1.29)	0.411	1.31 (0.63, 2.74)	0.466	0.151 (0.00, 0.30)	**0.048**
STAT5B												
rs9900213	37.629	A	0.66 (0.44, 99)	**0.045**	0.87 (0.61, 1.24)	0.448	1.4 (0.86, 2.30)	0.18	1.74 (0.76, 3.99)	0.191	0.075 (-0.07, 0.22)	0.32
SHP1												
rs7310161	6.927	T	1.55 (1.10, 2.17)	**0.012**	1.32 (0.86, 2.03)	0.207	1.05 (0.72, 1.54)	0.694	0.85 (0.34, 2.13)	0.735	-0.226 (-0.40, -0.05)	**0.012**
rs7966756	6.933	A	0.9 (0.47, 1.73)	0.756	1 (0.71, 1.41)	0.99	0.9 (0.38, 2.11)	0.802	1.04 (0.52, 2.08)	0.922	-0.227 (-0.36, -0.10)	**0.0009**
rs10744724	6.935	C	0.95 (0.49, 1.83)	0.875	1.26 (0.90, 1.78)	0.183	0.76 (0.31, 1.87)	0.553	0.72 (0.34, 1.50)	0.376	-0.258 (-0.39, -0.12)	**0.0003**
rs759052	6.94	T	1.07 (0.66, 1.75)	0.785	1.3 (0.92, 1.84)	0.139	0.69 (0.36, 1.34)	0.272	0.42 (0.22, 1.14)	0.101	-0.241 (-0.38, -0.10)	**0.001**

*P=0.006 when analysis was done in a recessive model.

† Allelic odds ratios