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PARP14 limits severity of allergic skin disease

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Summary

Poly-ADP ribose polymerase-14 (PARP14 or ARTD8) was initially identified as a transcriptional co-activator for Stat6, where the presence of IL-4 and activated Stat6 induces the enzymatic activity of PARP14 that promotes Th2 differentiation and allergic airway disease. To further our understanding of PARP14 in allergic disease, we studied the function of PARP14 in allergic inflammation of skin using mice that express constitutively active Stat6 in T cells (Stat6VT) and develop spontaneous inflammation of the skin. We mated Stat6VT mice to *Parp14-/-* mice and observed that approximately 75% of the Stat6VTx*Parp14-/-* mice develop severe atopic dermatitis (AD)-like lesions, compared to about ~50% of Stat6VT mice, and have increased morbidity compared to Stat6VT mice. Despite this, gene expression in the skin and the cellular infiltrates were only modestly altered by the absence of PARP14. In contrast, we saw significant changes in systemic T cell cytokine production. Moreover, adoptive transfer experiments demonstrated that decreases in IL-4 production reflected a cell intrinsic role for PARP14 in Th2 cytokine control. Thus, our data suggests that although PARP14 has similar effects on T cell cytokine production in several allergic disease models, the outcome of those effects is distinct, depending on the target organ of disease.

Introduction

Atopic dermatitis (AD) is a chronic relapsing inflammatory condition of the skin with a characteristic phenotype that includes skin lesions, erythema, swelling, hives and cutaneous infections, and is often associated with other disorders such as allergic rhinitis and asthma [1-3]. Pathogenesis of AD likely results from the interaction between environmental factors and host factors like genetic susceptibility, immune responses and skin barrier function. Skin lesions in patients with AD have increased expression of Th2 cytokines and IL-4 is involved in the pathogenesis of AD [4]. Transgenic expression of IL-4 and IL-13 in the skin results in human AD-like characteristics and skin remodeling [5, 6] and in models of allergic skin inflammation the loss of IL-4, IL-13 or their signaling components protects from allergic skin inflammation characteristics [7, 8]. Human skin biopsies and primary keratinocytes

stimulated with IL-4 demonstrate diminished epidermal differentiation complex (EDC) gene expression, supporting at least one mechanism for the immune system altering barrier function in the skin [7, 9]. Most recently, blockade of IL-4 signaling has emerged as beneficial in treating AD patients [10].

Constitutively active STAT6 transgenic mice, designated as Stat6VT, express mutant STAT6 predominantly in T cells [11]. T cells from Stat6VT transgenic mice are predisposed to differentiate into Th2 cells both in vitro and in vivo. Stat6VT transgenic mice develop spontaneous allergic inflammation in the skin, lung and periocular mucosal tissue [5, 11-15]. The absence of IL-4 in Stat6VT transgenic mice protects them from lung and skin inflammation and helps restore barrier function and EDC gene expression, suggesting that IL-4 is a critical effector cytokine [5, 12]. In our studies on Stat6VT transgenic mice carrying mutations in filaggrin, we observed severe AD-like lesions earlier compared to Stat6VT mice, demonstrating that a defective skin barrier and a hyper Th2 environment interact in developing the pathogenesis of allergic skin inflammation [13].

To facilitate transcriptional regulation, Stat6 associates with co-factors that function as co-activators or as co-repressors. Among the co-factors that Stat6 interacts with is Poly-ADP ribose polymerase-14 (PARP14), also known as ADP-ribosyltransferase diphtheria toxin-like 8 (ARTD8). PARP14 catalyzes mono-ADP ribosylation on acceptor proteins or on PARP14 itself and contributes to diverse cellular functions [16, 17]. By interacting with Stat6 and functioning as a transcriptional co-activator, PARP14 enhances IL-4 induced gene expression in B and T cells [18, 19]. Thus, PARP14 promotes Th2 differentiation by aiding in the expression of Stat6-dependent cytokines IL-4, IL-5 and IL-13 [18] and also increases Th9 development [20]. Allergic airway disease is attenuated in PARP14 deficient mice or mice treated with the PARP inhibitor PJ34 [18]. In this report we tested whether PARP14 had a similar effect in the development of allergic skin inflammation.

Materials and methods

Mice

C57BL/6 (Wild-type) mice were purchased from Harlan Biosciences. *Parp14-/-* mice on C57BL/6 background were generated by an insertion into 5' end of the first exon of PARP14 locus, and were described previously [21, 22]. Stat6VT transgenic mice were previously described [11]. Transgene positive cofounders were (CD2:Stat6VT (78) line) carrying human Stat6 with V547 and T548 mutated to alanine under the control of CD2 locus control region (restricting expression to lymphoid populations) and backcrossed to C57BL/6 mice. Stat6VT is constitutively phosphorylated on the critical tyrosine, Y-641. This phosphorylation is important for the dimerization of Stat6VT and its ability to activate transcription. *Parp14-/-* mice were mated to Stat6VT mice to generate *Parp14-/-* deficient transgene positive mice. *Rag1-/-* mice were purchased from Jackson Laboratories. Mice were kept in specific pathogen-free condition and all studies were approved by Indiana University Institutional Animal Care and Use Committee.

Surface and intracellular staining

For splenocytes, cells were stimulated with PMA and Ionomycin or anti-CD3 (2 μg/ml) for 5 hours at 37°C, with the addition of 3 μM monensin during the last 4 hours of stimulation. After 5 hours, the cells were collected and stained with a fixable viability dye (eBioscience) and CD4 for 20 min at 4°C. The cells were then fixed with 4% formaldehyde for 10 min at room temperature, permeabilized with permeabilization buffer (BD Biosciences) and with fluorochrome conjugated antibodies for IL-4, IL-13, IFNγ and IL-17A. Stimulation and antibodies were used as previously described [23].

In vitro T cell stimulation

Splenic CD4+ T cells were purified using CD4 microbeads (Miltenyi) according to manufacturers' instructions. The purified CD4+ cells were the stimulated in media at $1x10^6$ cells/ml with 4 μg/ml anti-CD3 (clone 2C11, Bio X Cell). After 3 days, supernatants were harvested and analyzed for cytokine production by ELISA

Adoptive transfer

Splenic CD4+ cells were enriched from wild-type, Stat6VT and Stat6VTx*Parp14-/-* mice using CD4 microbead positive selection (Miltenyi Biotec). Cells were resuspended in PBS at a concentration of $1.5x10^6$ cell/ml and cells were transferred by retro-orbital injections into 8-10 week-old *Rag1-/-* mice. Mice were monitored for 10-20 weeks for the development of skin inflammation and spleens and skin were harvested.

Quantification of incidence and morbidity

Mice were monitored for the development of AD-like skin lesions and the percentage of mice that develop no disease, mild disease and severe disease were determined. Percent morbidity of mice that required euthanasia and those that died due to severe lesions were determined using Kaplan-Meier plots (GraphPad Prism7).

Histological examination of skin sections

Skin tissues were fixed in neutral buffered formalin. Paraffin embedded tissue sections were stained with hematoxylin and eosin to evaluate the infiltration of inflammatory cells by light microscopy.

Keratinocyte cell culture

Primary human keratinocytes (HK) were isolated from excised foreskin tissue as previously described [24] and washed with antibiotics. The tissue was minced and the individual cells were released from the tissue using trypsin digestion. Keratinocytes and fibroblasts were separated by differential resistance to treatment with EDTA. Isolated HK were grown in EpiLife Complete media (Cascade Biologics) with human keratinocyte growth supplement (containing 5 μg/ml insulin; Cascade Biologics) and 1000 U penicillin–streptomycin (Roche, Indianapolis, IN, USA). To stimulate keratinocyte differentiation, HK were treated with 2 mM of $CaCl_2$ every other day and stimulated with recombinant human IL-4 (R&D Systems) as indicated

Analysis of gene expression

For quantitative PCR analysis of gene expression, wild-type, lesional and non-lesional skin from Stat6VT and Stat6VTx*Parp14-/-* mice were homogenized using a tissue lyser (Qiagen, CA) and RNA isolated from the RNeasy fibrous tissue kit (Qiagen) was used to synthesize cDNA using First-strand cloned AMV kit (Invitrogen). Message levels were determined by Taqman assay and samples were normalized to β_2 microglobulin mRNA and relative expression was calculated using the change-in-threshold method.

Cell isolation from the skin

Ear skin from mice were split and placed in a 6-well dish containing 1.5 ml RPMI with Liberase (2 mg/ml; Roche) with the dermis facing down. Skin samples were incubated at 37°C, 5% CO2 for 1.5 hours. At the end of 1.5 hours, liberase was inactivated with RPMI containing FBS and the samples were disrupted to dissociate the cells using gentleMACS Dissociator (Miltenyi Biotec). Cells were gated on CD45+ cells and specific surface markers based on the cell type. Basophils – CD19-CD117-NK1.1-CD3-CD11c-CD49b+FcεRIα+, Eosinophils – CD11c-CD11b+Gr1-SiglecF+, Neutrophils –

CD11b+Gr1+, Mast cells – CD3-CD117+FcεRIα+, T-cells - CD3+, Macrophages – CD11b+Gr1- and Dendritic cells – CD11c+. Flow cytometric analysis was performed using standard protocols.

Results

PARP14 deficiency in Stat6VT mice cause disease severity and increased morbidity

To study the role of PARP14 in allergic skin inflammation we mated Stat6VT mice with *Parp14-/-* mice to generate Stat6VT transgenic mice (Stat6VTx*Parp14-/-*). We monitored Stat6VT and Stat6VTx*Parp14-/-* mice for the development of AD like skin lesions, disease incidence and severity. Stat6VTx*Parp14-/-* mice developed severe allergic skin inflammation characterized by erythema, excoriation and lichenification (Figure 1A). We observed a greater percentage of Stat6VTx*Parp14-/-* mice developed severe allergic skin inflammation compared to Stat6VT mice (Figure 1B). On comparing the percentage of mice that required euthanasia or died due to complications associated with severe AD lesions using a Kaplan-Meier morbidity analysis, we observed a higher percentage of Stat6VTx*Parp14-/-* mice requiring euthanasia because of severe disease compared to Stat6VT mice (Figure 1C).

PARP inhibition in keratinocytes does not alter IL-4-induced gene expression

We next wanted to determine if there were any evidence for PARP activity being required for IL-4 stimulated gene regulation in keratinocytes. We cultured and differentiated human immortalized keratinocytes in the presence or absence of IL-4 and the PARP inhibitor PJ34 and analyzed gene expression using quantitative PCR of genes that were induced by IL-4 such as *CCL26*, *CA2*, *CISH*, *HAS3* and *SERPINB3*/*SERPINB4* [25]. Keratinocytes that were stimulated with IL-4 and treated with PARP inhibitor PJ34 did not show any difference in gene expression compared to those stimulated with IL-4 alone (Figure 2A). This is in contrast to studies from our lab and others that demonstrate the

requirement for PARP activity for IL-4 responses in T cells and esophageal epithelial cells (Figure 2B) [18, 22, 26, 27]. Thus, the phenotype observed in Stat6VTx*Parp14-/-* mice is not likely to be due to altered IL-4 responsiveness in keratinocytes.

Gene expression in the skin

As Stat6VTx*Parp14-/-* mice have more severe skin inflammation than Stat6VT mice, we tested the expression of cytokines and chemokines in the skin. *Il4* expression was significantly higher in lesional skin from Stat6VTx*Parp14-/-* mice compared to wild-type skin (Figure 3). There was a trend towards increased *Il4* in lesional skin from Stat6VTx*Parp14-/-* mice compared to lesional skin from Stat6VT mice, and if one outlier value among the Stat6VT samples were excluded, the difference would be significant. There was a significant difference in *Il13* transcripts between lesional skin from Stat6VTx*Parp14-/-* mice compared to lesional skin from Stat6VT mice (Figure 3). However, there was no significant difference in *Il17* transcripts in lesional skin from Stat6VTx*Parp14-/-* mice compared to lesional skin from Stat6VT mice. The expression of *Il1b* was higher in lesional skin compared to wild type and non-lesional skin from both Stat6VTand Stat6VTx*Parp14-/-* mice (Figure 3). *Tslp* was not detected in skin tissue from mice of any of the genotypes, and *Il33* was not different between Stat6VT and Stat6VTx*Parp14-/-* mice in lesion or non-lesion tissue (data not shown), suggesting that PARP14-deficiency did not alter the expression of early pro-allergic cytokines. We also tested for the expression of chemokines in the skin but found no significant differences among the groups for *Ccl11* or *Ccl24* expression (data not shown). Together, these data suggest an increased inflammatory response in lesional tissues, and an increase in Th2 cytokine production in lesional skin from Stat6VTx*Parp14-/-* mice compared to lesional skin from Stat6VT mice.

Skin infiltrating cells in Stat6VT and Stat6VTx*Parp14*-/- mice

To further characterize the histopathology of skin inflammation in Stat6VT and Stat6VTx*Parp14-/-* mice, we performed histological analysis of skin tissue from wild-type, Stat6VT and Stat6VTx*Parp14-/-* mice. Increased dermal and epidermal thickening along with an increase in cellular infiltration was observed in the skin of both Stat6VT and Stat6VTx*Parp14-/-* mice compared to wild-type mice, but there were not any obvious differences in the microscopic pathology caused by the absence of PARP14 (Figure 4A).

To determine if there was a difference in cellular infiltrate in the skin of Stat6VT versus Stat6VTx*Parp14-/-* mice, we determined the cell types that infiltrate the skin of Stat6VT and Stat6VTx*Parp14-/-* mice. We digested the ear skin of wild-type, *Parp14-/-*, Stat6VT and Stat6VTx*Parp14-/-* mice and analyzed the cells infiltrating the skin by flow cytometry. In skin from wild-type mice, T cells form the majority of the infiltrating cells; basophils, eosinophils, neutrophils, mast cells, dendritic cells and macrophages constitute smaller percentages of the total population (Figure 4B). The composition of cells infiltrating the skin of *Parp14-/-* mice was similar to wild-type mice.

Infiltrates from mice transgenic for Stat6VT had altered infiltrates and increased overall cell numbers recovered from the skin (Fig. 4B and C). Skin from Stat6VT mice had lower percentages of T cells and an increase in the percentage of infiltrating eosinophils, neutrophils and macrophages compared to wild-type and *Parp14-/-* skin. Similar to what was observed in Stat6VT skin, Stat6VTx*Parp14-/-* mice had a decrease in the percentage of T cells infiltrating the ear skin tissue compared to wild-type and *Parp14-/-* skin. The percentage of neutrophils was decreased, and the percentage of eosinophils and macrophages in Stat6VTx*Parp14-/-* skin were increased compared to Stat6VT skin. Basophil and mast cell frequency were also reduced in comparison to Stat6VT mice. The skin was also infiltrated by CD45+ cells that were not identified as T cells, basophils, eosinophils, neutrophils, mast cells,

dendritic cells, or macrophages. The frequency of these cells was decreased in Stat6VTx*Parp14-/-* skin compared to wild-type, *Parp14-/-* and Stat6VT skin (24% versus 30-35%). The total cell numbers of eosinophils and macrophages in Stat6VTx*Parp14-/-* skin was also increased compared to cells in Stat6VT skin (Figure 4C). Taken together, these data suggest that there are modest alterations in infiltrating cell populations between Stat6VTx*Parp14-/-* and Stat6VT transgenic mice. These alterations suggest both the quality and quantity of infiltrates changes in the absence of PARP14.

Expression of Stat6VT in T cells of PARP14-deficient mice alters lymphocyte homeostasis

The gross pathology of the Stat6VTx*Parp14-/-* mice was clearly more severe than observed in Stat6VT mice (Fig. 1), despite only modest changes in the observed tissue inflammation. Thus we next assessed whether there were systemic changes that might be linked to disease. As Stat6VT mice have altered lymphocyte homeostasis and the lymphocyte populations are normal in *Parp14-/-* mice, we first determined if absence of PARP14 had effects on lymphocyte populations in Stat6VTx*Parp14-/-* mice. To that end, we analyzed the spleens of wild-type (WT), *Parp14-/-*, Stat6VT and Stat6VTx*Parp14-/-* mice. To minimize the effects of inflammation causing secondary changes, we used younger mice (8-16 weeks old; age matched among the groups) and mice that had no obvious disease. There were no significant differences in splenic total cell number between Stat6VT and *Parp14-/-* Stat6VT mice (data not shown). As seen in previous studies with Stat6VT mice, we observed similar decreases in the frequency and cell numbers of splenic CD4+ cells in both Stat6VT and Stat6VTx*Parp14-/-* mice compared to wild-type (Figure 5A). Though the frequencies of CD4+ cells are similar in Stat6VT and Stat6VTx*Parp14-/-* mice, the number of CD4+ cells in Stat6VTx*Parp14-/-* mice is significantly lower compared to Stat6VT mice (Figure 5A). We observed a similar increase in the percentage of CD19+ B cells in Stat6VT and Stat6VTx*Parp14-/-* mice but the numbers of CD19+ B cells in the Stat6VTx*Parp14-/-* mice are significantly lower compared to Stat6VT mice (Figure 5B). These observations suggest that PARP14 deficiency has modest but significant effects on the changes in lymphocyte populations in Stat6VT transgenic mice.

PARP14 promotes differentiation of Th cells to a Th2 phenotype, promotes Th17 development and has no effect on Th1 differentiation and IFNγ production [18, 23]. To study how the absence of PARP14 affects cytokine production in Stat6VT mice, we analyzed CD4 T cell production of IL-4, IL-13, IFNγ and IL-17A by intracellular cytokine staining and ELISA (Fig. 5C-D). Although Stat6VT expression raised production of all effector cytokines, PARP14-deficiency diminished production of IL-4 and modestly affected IL-13 (Fig. 5C-D). IL-17 production was increased by PARP14-deficiency (Fig. 5C-D) contrasting decreases in IFNγ production (Fig. 5D).

CD4+ cells from Stat6VTxParp14-/- mice have decreased Th2 cytokines in the absence of inflammation

Since Stat6VT mice have increased cytokine production, we wanted to test whether the effects of PARP14 deficiency were intrinsic to T cells or an effect of inflammation. To examine the effects of PARP14-deficiency in Stat6VT T cells in isolation, we transferred total CD4+ cells from Stat6VT or Stat6VTx*Parp14-/-* mice to *Rag1-/-* mice. Despite effective transfer of T cells, we did not observe the development of skin inflammation in recipients of either Stat6VT or Stat6VTx*Parp14-/-* T cells. This suggested that T cells are not sufficient to cause disease, but also provided the opportunity to examine T cell function in the absence of inflammation. There were no significant differences among the groups in any parameter studied at the 10-week time point. We observed that 20 weeks post-transfer, Stat6VT CD4+ cell recipient mice have a significantly higher frequency of IL-4- and IL-13-producing cells compared to Stat6VTx*Parp14-/-* recipients (Figure 6A), though the frequency of IFNγ- and IL-17A-producing cells was similar between both groups of mice (Figure 6B). Splenocytes isolated from the *Rag1-/-* recipient mice were also re-stimulated with anti-CD3 for 72 hours and the levels of secreted cytokines were measured. At 20 weeks, IL-4 levels were significantly lower in Stat6VTx*Parp14-/-* recipient mice, IFNγ levels were also reduced (Figure 6C) compared to Stat6VT recipient mice. The ratio of IFNγ to IL-4, a measure of the balance of the Th1/Th2 inflammatory milieu, is significantly higher in Stat6VTx*Parp14-/-* recipients at 10 weeks compared to Stat6VT

recipients and the ratio further increased at 20 weeks compared to 10-week post-transfer Stat6VTx*Parp14-/-* recipient cells (Figure 6D). Taken together, these data suggest that the decreases in IL-4 caused by PARP14-deficiency are intrinsic to the T cells, but the effects on IL-17 observed in Figure 5 are an effect of the inflammatory environment. Moreover, these results suggest that there is a systemic shift in cytokine production from T cells in the absence of PARP14, and that the systemic changes in the inflammatory milieu, rather than local changes in the skin, might be responsible for the increased disease pathology.

Discussion

The function of PARP14 in allergy is emerging, though its role in allergic skin inflammation and in keratinocytes is still unclear. In this study we demonstrate that decreases in Th2 cytokine production by Stat6VTx*Parp14-/-* mice are intrinsically due to PARP14 deficiency in T cells. The absence of PARP14 in Stat6VT mice escalates the severity of spontaneous allergic skin disease. In keratinocytes, PARP14 is not required for the induction of IL-4 and Stat6 responsive genes. In addition, altered systemic immune responses resulted in altered cellular infiltrate in the skin of Stat6VTx*Parp14-/-* mice compared to skin from Stat6VT mice. Together, these data suggest PARP14 deficiency alters immune responses thus altering pathology in the skin.

One of the critical questions in this study is which of the cytokines are linked to pathology. PARP14 has been shown to alter Th2 cytokine production from primary T cells[18]. Previous studies on Stat6VT mice have demonstrated Stat6VT T cells are predisposed to differentiate into Th2 cells and secrete IL-4 and IL-13 [11]. We demonstrated a decrease in IL-4 and IL-13 production in T cells from Stat6VTx*Parp14-/-* mice. Adoptive transfer of Stat6VTx*Parp14-/-* CD4+ T cells into *Rag1-/-* mice resulted in an increased IFNγ/IL-4 ratio compared to T cells from Stat6VT mice. This could be attributed to the ability of PARP14 to negatively regulate *Stat1* and positively regulate *Stat6* expression [28]. Importantly, we observed a trend towards increased *Il4* mRNA, and significantly

increased *Il13* mRNA, in the skin of Stat6VTx*Parp14-/-* mice compared to Stat6VT mice. Although there were differences in the cellular make-up of the infiltrate (Figure 4 and discussed below), this suggests that type 2 cytokine-secreting *Parp14-/-* cells might be more efficient in responding to recruiting signals or in infiltrating inflamed tissue. Theoretically, this might compensate for the diminished Th2 cytokine production per cell, and facilitate the greater disease pathology.

IL-17 might be another important effector cytokine. *In vitro*, *Parp14-/-* Th17 cells had significantly reduced frequency of IL-17A+ cells and *in vivo*, in a model of allergic airway disease, *Parp14-/-* mice had decreased numbers of IL-17A producing CD4+ cells and reduced concentration of IL-17A from antigen-stimulated splenocytes [23]. However, in Stat6VTx*Parp14-/-* mice, we observed an overall increase in IL-17A-producing CD4+ cells. IL-4 has been shown to suppress IL-23 and IL-17 [29], suggesting that the increase in IL-17A in Stat6VTx*Parp14-/-* T cells could be due to decreases in IL-4. Increases in IL-17A might also be a result of increased bacterial infections in the AD-like lesions [30]. Studies in AD patients revealed a correlation of AD severity with increased IL-17 producing T cells in the peripheral blood and in acute lesional skin [31]. Importantly, adoptive transfer of CD4+ T cells into *Rag1-/-* mice resulted in similar IL-17A production from Stat6VTx*Parp14-/-* and Stat6VT T cells, suggesting that altered IL-17A production in this model might be secondary to disease and the inflammatory environment. Moreover, as we did not observe an increase of *Il17* mRNA in the lesional tissue, it would suggest that it is not a critical cytokine that regulates inflammation in this model.

One of the questions we addressed is whether the skin infiltrating cells were altered in Stat6VTx*Parp14-/-* mice. We characterized the cells infiltrating the skin from Stat6VT and Stat6VTx*Parp14-/-* mice and observed that the cellular infiltrate had an increase in the frequency of eosinophils and macrophages in Stat6VTx*Parp14-/-* skin compared to Stat6VT skin. Other studies in human AD have also shown the involvement of macrophages in both acute and chronic lesions. In chronic lesions macrophages participate in skin remodeling and dominate the dermal mononuclear

cell infiltrate [32, 33]. Similarly, eosinophils release a broad panel of inflammatory mediators. Although there was a decrease in the frequency of mast cells, the absolute number of mast cells was similar in the skin of both Stat6VT and Stat6VTx*Parp14-/-* mice. Moreover, we observed the infiltration of hematopoietic cells (CD45+) that were not classified into any of the characterized lineages. Though the frequency of these cells are similar among all four groups of mice, the cell numbers of other CD45+ cells are over 2 fold higher in Stat6VT and Stat6VTx*Parp14-/-* skin compared to wild-type and *Parp14-/-* skin. It is possible that the lack of identification of these cells was due to a technical limitation, primarily that cells could be stripped of their cell surface markers during enzymatic digestion of the skin tissue. CD45+ cells in the skin might also constitute the mast cell progenitors that migrate to the skin [34] and could represent at least a portion of the cells examined. Some bone marrow-derived spindle shaped cells that resemble fibroblasts also express CD45, further contributing to the pool of CD45+ cells in the skin [35]. It is still not clear if these multiple modest changes in the cellular infiltrate result in biologically significant changes in inflammatory mediators in the skin. However, it is possible that the altered cellular infiltrate is linked with a more severe disease phenotype.

AD is predominantly a Th2 mediated disease. However, Stat6VTx*Parp14-/-* mice develop severe AD like skin inflammation even with significant decreases in Th2 cytokine production from peripheral T cells, compared to Stat6VT mice. Despite this, there was evidence of increased Th2 cytokine mRNA in lesional tissue of Stat6VTx*Parp14-/-* mice, compared to Stat6VT mice. Thus, there could be at least two explanations for increased AD-like disease in the absence of PARP14. The first, as discussed above, is that in the absence of PARP14, and in contrast to the diminished Th2 cytokines in the periphery, there is increased recruitment of Th2-cytokine secreting cells in Stat6VTx*Parp14-/-* mice, compared to Stat6VT mice. The second explanation, based on the altered immune skewing, suggests pathology linked to the ratio of Th1/Th2 cytokines. This interpretation is not unprecedented. In other models of AD like Nc/Nga mice, the absence of Stat6 did not inhibit the development of AD-like lesions similar to that observed in Stat6 competent NC/Nga mice [36]. Similarly, mice that are

transgenic for IL-18 or Caspase-1 develop AD like skin lesions even on a Stat6-deficient background [37]. In both these studies, the authors observe an IFNγ-dominant skin microenvironment that is the likely cause of skin pathology. We also observed a shift in the IFNγ/IL-4 ratio that might contribute to altered disease. This change in ratio was observed systemically and in the absence of inflammation, and suggests that altered T cell responses are likely a critical component of altered allergic skin inflammation in the absence of PARP14.

One surprising outcome of these studies was that disease was exacerbated in the absence of PAPR14. This was opposite to the observations in allergic lung disease models and the correlations in eosinophilic esophagitis patients where PARP14 seemed to promote disease [18], [27] . Although PARP14 might work as a repressor at some genes, perhaps with distinct transcription factors, it is unclear how the distinct inflammatory sites would have different affects on PARP14-dependent functions. It is possible that among the genes that PARP14 regulates are genes that control potential for recruitment to inflamed sites. For example, if PARP14 regulated genes required for recruitment to the lung, inflammation would be expected to decrease, as was observed [18]. In contrast, if homing receptors for Th2 cells to migrate into the skin were independent of PARP14, even though Th2 cytokine production per cell might be decreased, cellular infiltration might proceed unimpeded. Indeed, this is what we observe in this study where overall inflammation in the Stat6VTx*Parp14-/-* skin is comparable to that in Stat6VT mice. Although the exact mechanisms are still unclear, this work suggests that there are distinctly regulated genes that impact tissue-specific inflammation at diverse anatomical locations.

It should be noted that the Stat6VTx*Parp14-/-* mice lack PARP14 expression in all cells. PARP14 function has been investigated in T cells, B cells, macrophages, airway and esophageal epithelial cells [18, 22, 26, 27] though its function in other immune cells is yet to be explored. Several genes have been identified that are PARP14-dependent and Stat6-independent [28], suggesting that PARP14 could impact other pathways in distinct cell types. Together, our findings demonstrate that PARP14 has

similar effects in cytokine production in multiple models of allergic inflammation, though the consequence of this altered cytokine response is distinct depending on the target organ.

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Author Contributions

PK performed experiments. SD-A aided in analysis of mouse pathology. MJT and JBT provided advice in experiments. PK and MHK designed the studies and wrote the paper.

Competing Interests

The authors declare there are no competing interests.

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Figure Legends

Figure 1. PARP14 deficiency in Stat6VT mice increases disease severity and morbidity. (A) Photographs of wild-type, *Parp14-/-*, Stat6VT and Stat6VTx*Parp14-/-* mice **(B)** Percent of mice with no disease, mild disease and severe disease (AD-like lesions), n = 50, * $p < 0.05$, Fisher's exact test. **(C)** Percent morbidity graphed using Kaplan-Meier analysis, n= ** $p < 0.001$, Mantel-Cox test.

Figure 2. PARP14 is not required for IL-4-induced gene expression in keratinocytes. Human primary keratinocytes (A) or the human esophageal epithelial cell line TE-7 (B) were differentiated and stimulated with human IL-4 and treated with PARP inhibitor, PJ34. Gene expression was measured by RT-PCR. Results are an average of at least 3 experiments.

Figure 3. Expression of EDC genes and cytokines in skin. RNA was isolated from wild-type, lesional and non-lesional Stat6VT and Stat6VTx*Parp14-/-* skin tissue. Expression of the indicated genes were measured by qRT-PCR, samples were normalized to the expression of β2-microglobulin mRNA. *p<0.05; **p<0.01; †p<0.005; #p<0.0001- Post hoc analysis after one-way ANOVA.

Figure 4. Analysis of skin inflammation. (A) Histological analysis of ear tissue from wild-type, Stat6VT and Stat6VTx*Parp14-/-* mice. Samples were fixed and stained with hematoxylin and eosin. **(B)** Ear skin isolated from wild-type, *Parp14-/-*, Stat6VT and Stat6VTx*Parp14-/-* mice were digested in Liberase to dissociate cells that were stained with antibodies to various infiltrating cells and percentages of CD45+ cells infiltrating the skin of indicated mice were determined by flow cytometry. **(C)** Total number of cells infiltrating the ear skin of the indicated populations were determined**.** Data are mean of 6 to 10 mice in each genotype.

Figure 5. Altered lymphocyte homeostasis in PARP14 deficient Stat6VT mice. (A) Splenocytes from age matched wild-type, *Parp14-/-*, Stat6VT and Stat6VTx*Parp14-/-* mice were isolated and percentage of CD4+ T cells population was determined by flow cytometry. Cell numbers of CD4+ T cells in the spleen of the indicated populations were determined **(B)** Splenocytes isolated from the indicated mice were stained with antibody to CD19 and the percentage of CD19+ cells was determined by flow cytometry. Cell numbers of CD19+ cells in the spleen of the indicated populations. **(C)** Splenocytes isolated from the indicated mice were restimulated with PMA/Ionomycin and stained for cytokines produced followed by flow cytometry analysis. **(D)** MACS

sorted CD4+ T cells from the spleens of indicated mice were stimulated with plate bound anti-CD3 for 72 hours, the cell free supernatants were collected and cytokines produced were measured by ELISA. Data are mean ± SEM of 3 to 6 mice in each genotype (A-C) and mean ± SEM of 6 to 10 mice in each genotype (D). Statistical significance was determined by post hoc analysis after one-way ANOVA (A-C) and two-tailed test (D), is indicated as follows * $p<0.05$, ** $p< 0.01$, †$p<0.005$, # $p<0.0001$

Figure 6. Decreased Th2 cytokines from Stat6VTx*Parp14-/-* CD4+ cells. (A-B) CD4+ cells from Stat6VT and Stat6VTx*Parp14-/-* mice were adoptively transferred to *Rag1-/-* mice. Splenocytes from the recipients were restimulated with PMA/Ionomycin and stained for IL-4, IL-13, IFNγ, IL-17A producing cells and analyzed by flow cytometry. **(C-D)** Cytokines produced from supernatants of anti-CD3 stimulated splenocytes were analyzed by ELISA. Data from 10-week experiment have 5-11 mice per group and 20-week experiment have 6-8 mice per group. *$p<0.05$, **$p<0.001$ – Post hoc analysis after one-way ANOVA, ***$p<0.001$ – t-test.

Figure 1.

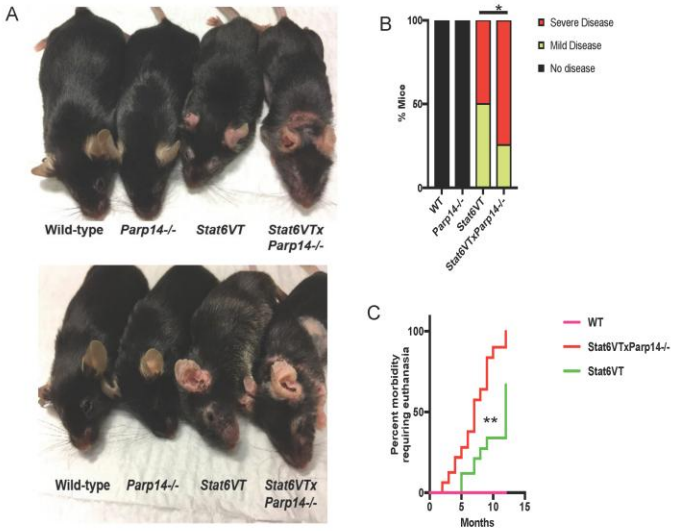
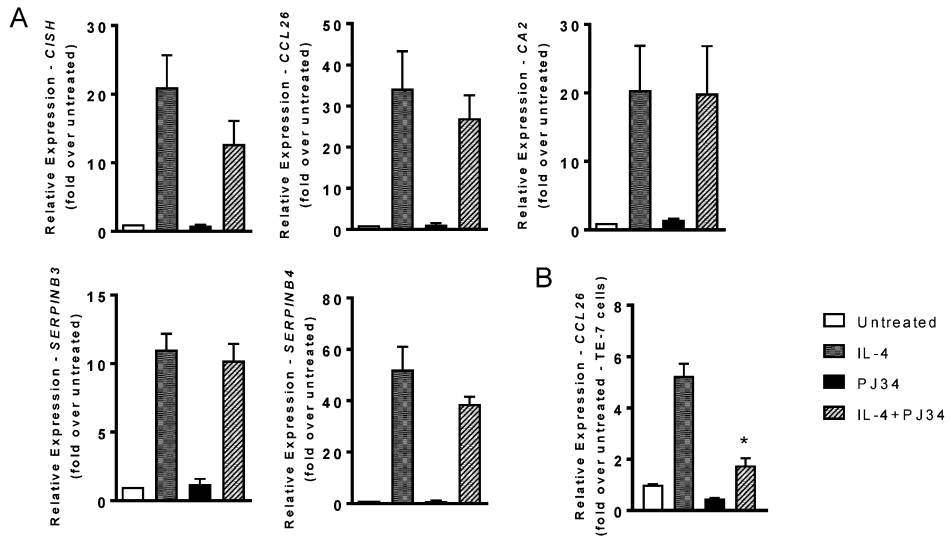


Figure 2



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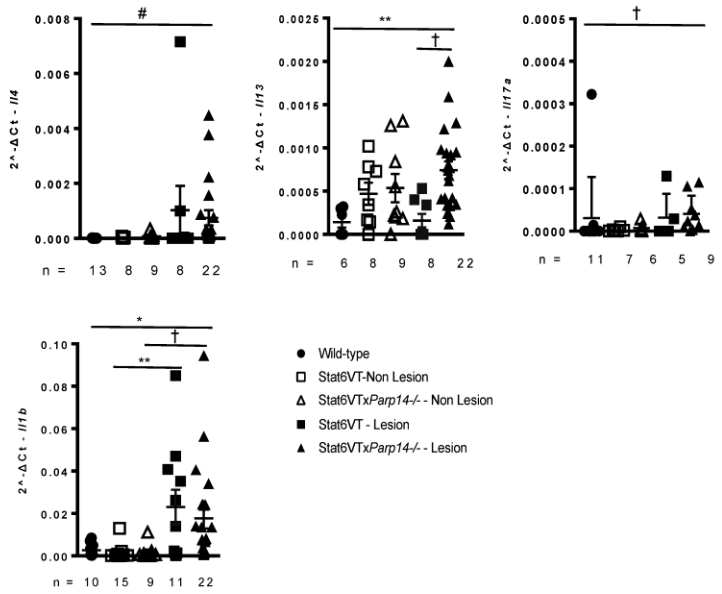

Figure 4

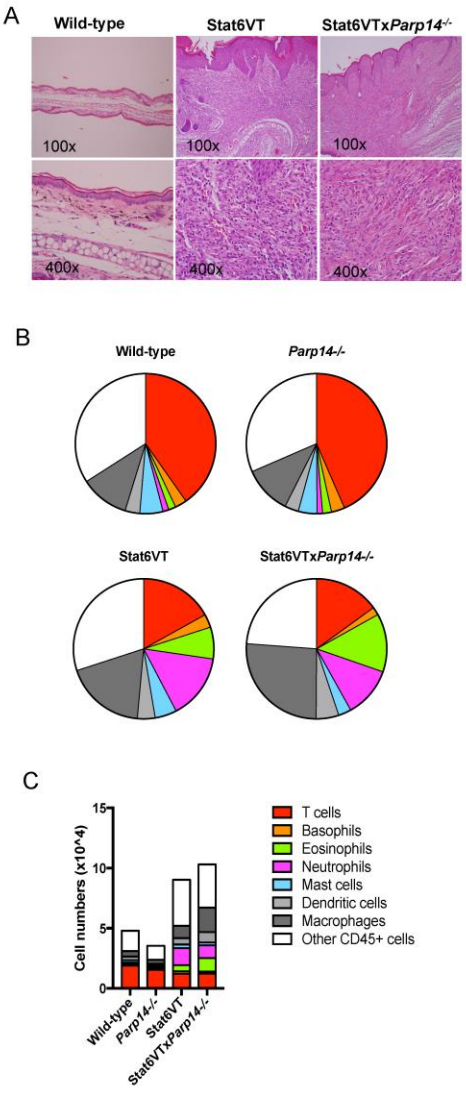

Figure 5

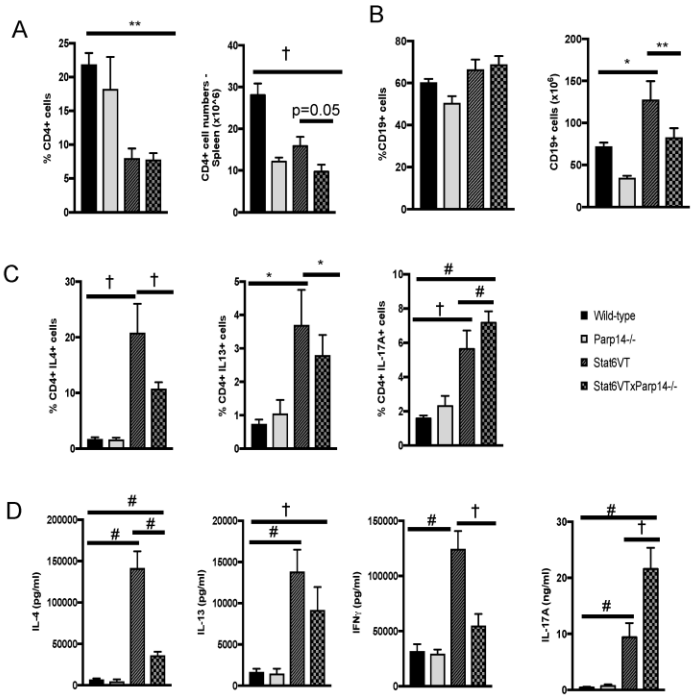

Figure 6