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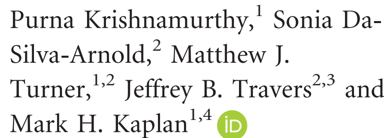
Poly-ADP ribose polymerase-14 limits severity of allergic skin disease



Purna Krishnamurthy,[1] Sonia Da-Silva-Arnold,[2] Matthew J. Turner,[1,2] Jeffrey B. Travers[2,3] and Mark H. Kaplan[1,4]

[1]*Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, IN,* [2]*Department of Dermatology, and Roudebush Veterans' Administration Hospital, Indiana University School of Medicine, Indianapolis, IN,* [3]*Department of Pharmacology and Toxicology, Wright State University, Dayton, OH, and* [4]*Department of Pediatrics and Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN, USA*

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Correspondence: Mark H. Kaplan, Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, IN 46202, USA.
Email: mkaplan2@iupui.edu
Senior author: Mark H. Kaplan

Summary

Poly-ADP ribose polymerase-14 (PARP14 or ARTD8) was initially identified as a transcriptional co-activator for signal transducer and activator of transcription 6 (Stat6), where the presence of interleukin-4 (IL-4) and activated Stat6 induces the enzymatic activity of PARP14 that promotes T helper type 2 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 Stat6VT × *Parp14*$^{-/-}$ mice develop severe atopic dermatitis (AD)-like lesions, compared with about 50% of Stat6VT mice, and have increased morbidity compared with Stat6VT mice. Despite this, gene expression in the skin and the cellular infiltrates was 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. Hence, our data suggest 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.

Keywords: inflammation; skin; T helper type 2 cells; transcription factors.

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 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 T helper type 2 (Th2) cytokines and interleukin-4 (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 remodelling,[5,6] and in models of allergic skin inflammation the loss of IL-4, IL-13 or their signalling 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 signalling has emerged as beneficial in treating patients with AD.[10]

Constitutively active signal transducer and activator of transcription 6 (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 to 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 with 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 catalyses 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] Hence, 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 (Indianapolis, IN, USA). *Parp14*$^{-/-}$ mice on C57BL/6 background were generated by an insertion into the 5′ end of the first exon of PARP14 locus, and were described previously.[21,22] Stat6VT transgenic mice were previously described.[11] Transgene positive co-founders 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 (Bar Harbor, ME). Mice were kept in specific pathogen-free conditions 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 hr at 37°, with the addition of 3 μM monensin during the last 4 hr of stimulation. After 5 hr, the cells were collected and stained with a fixable viability dye (eBioscience, San Diego, CA) and CD4 for 20 min at 4°. The cells were then fixed with 4% formaldehyde for 10 min at room temperature, permeabilized with permeabilization buffer (BD Biosciences, San Jose, CA) and with fluorochrome-conjugated antibodies for IL-4, IL-13, interferon-γ (IFN-γ) 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 Biotec, Auburn, CA) according to the manufacturer's instructions. The purified CD4^{+} cells were then stimulated in media at 1×10^{6} cells/ml with 4 μg/ml anti-CD3 (clone 2C11; Bio X Cell, Lebanon, NH). After 3 days, supernatants were harvested and analysed for cytokine production by ELISA.

Adoptive transfer

Splenic CD4^{+} cells were enriched from wild-type, Stat6VT and Stat6VT×*Parp14*$^{-/-}$ mice using CD4 microbead positive selection (Miltenyi Biotec). Cells were resuspended in PBS at a concentration of $1{\cdot}5 \times 10^{6}$ cells/ml and cells were transferred by retro-orbital injections into 8- to 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 was determined. Per cent morbidity of mice that required euthanasia and those that died due to severe lesions were determined using Kaplan–Meier plots (GraphPad Prism 7; GraphPad, San Diego, CA).

Histological examination of skin sections

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

Keratinocyte cell culture

Primary human keratinocytes 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 human keratinocytes were grown in EpiLife Complete medium (Cascade Biologics, Portland, OR) with human keratinocyte

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growth supplement (containing 5 μg/ml insulin; Cascade Biologics) and 1000 U penicillin–streptomycin (Roche, Indianapolis, IN). To stimulate keratinocyte differentiation, human keratinocytes were treated with 2 mM of $CaCl_2$ every other day and stimulated with recombinant human IL-4 (R&D Systems, Minneapolis, MN) as indicated.

Analysis of gene expression

For quantitative PCR analysis of gene expression, wild-type, lesional and non-lesional skin from Stat6VT and Stat6VT×*Parp14*$^{-/-}$ mice were homogenized using a tissue lyser (Qiagen, Valencia, CA) and RNA isolated from the RNeasy fibrous tissue kit (Qiagen) was used to synthesize cDNA using a First-strand cloned AMV kit (Invitrogen, Carlsbad, CA, Portland, OR). 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 samples from mice were split and placed in a six-well dish containing 1·5 ml RPMI-1640 with Liberase (2 mg/ml; Roche, Indianapolis, IN, USA) with the dermis facing down. Skin samples were incubated at 37°, 5% CO_2 for 1·5 hr. At the end of 1·5 hr, liberase was inactivated with RPMI-1640 containing fetal bovine serum 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 (Stat6VT×*Parp14*$^{-/-}$). We monitored Stat6VT and Stat6VT×*Parp14*$^{-/-}$ mice for the development of AD-like skin lesions, disease incidence and severity. Stat6VT×*Parp14*$^{-/-}$ mice developed severe allergic skin inflammation characterized by erythema, excoriation and lichenification (Fig. 1a). A greater percentage of Stat6VT×*Parp14*$^{-/-}$ mice developed severe allergic skin inflammation compared with Stat6VT mice (Fig. 1b). On comparing the percentage of mice that required euthanasia or that died due to complications associated with severe AD lesions using a Kaplan–Meier morbidity analysis, we observed a higher percentage of Stat6VT×*Parp14*$^{-/-}$ mice requiring euthanasia because of severe disease compared with Stat6VT mice (Fig. 1c).

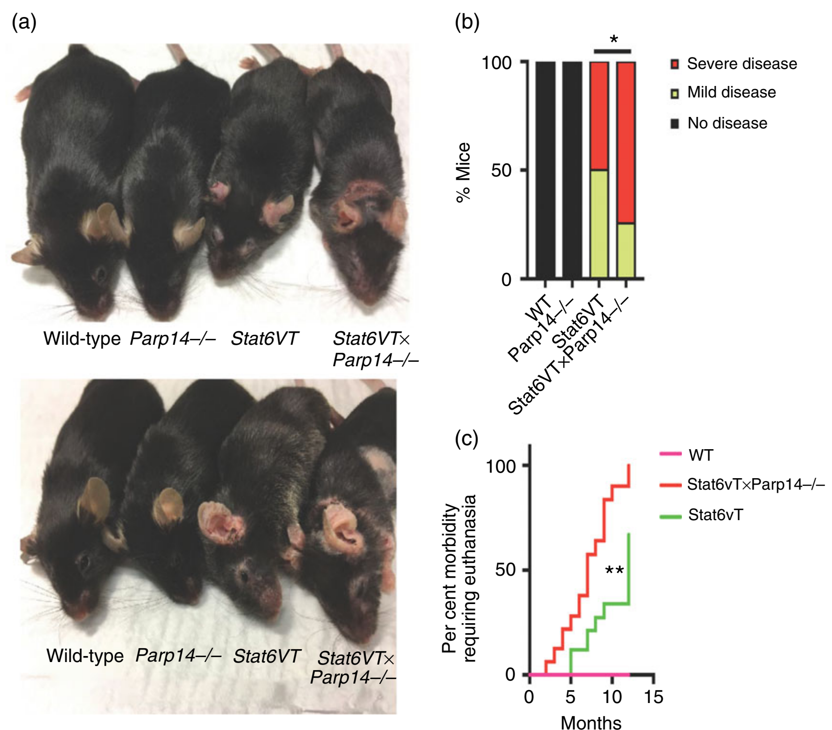

Figure 1. Poly-ADP ribose polymerase-14 (PARP14) deficiency in Stat6VT mice increases disease severity and morbidity. (a) Photographs of wild-type, *Parp14*$^{-/-}$, Stat6VT and Stat6VT×*Parp14*$^{-/-}$ mice (b) Percentage of mice with no disease, mild disease and severe disease (atopic dermatitis-like lesions), $n = 50$, *$P < 0{\cdot}05$, Fisher's exact test. (c) Percentage morbidity graphed using Kaplan–Meier analysis, **$P < 0{\cdot}001$, Mantel–Cox test.

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

We next wanted to determine if there was 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 analysed 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 with those stimulated with IL-4 alone (Fig. 2a). This is in contrast to studies from our laboratory and others that demonstrate the requirement for PARP activity for IL-4 responses in T cells and oesophageal epithelial cells (Fig. 2b).[18,22,26,27] Hence, the phenotype observed in Stat6VT×*Parp14*$^{-/-}$ mice is not likely to be the result of altered IL-4 responsiveness in keratinocytes.

Gene expression in the skin

As Stat6VT×*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 Stat6VT×*Parp14*$^{-/-}$ mice compared with wild-type skin (Fig. 3). There was a trend towards increased *Il4* in lesional skin from Stat6VT×*Parp14*$^{-/-}$ mice compared with lesional skin from Stat6VT mice, and if one outlier value among the Stat6VT samples was excluded, the difference would be significant. There was a significant difference in *Il13* transcripts between lesional skin from Stat6VT×*Parp14*$^{-/-}$ mice compared with lesional skin from Stat6VT mice (Fig. 3). However, there was no significant difference in *Il17* transcripts in lesional skin from Stat6VT×*Parp14*$^{-/-}$ mice compared with lesional skin from Stat6VT mice. The expression of *Il1b* was higher in lesional skin compared with wild-type and non-lesional skin from both Stat6VT and Stat6VT×*Parp14*$^{-/-}$ mice (Fig. 3). *Tslp* was not detected in skin tissue from mice of any of the genotypes, and *Il33* was not different between Stat6VT and Stat6VT×*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 Stat6VT×*Parp14*$^{-/-}$ mice compared with lesional skin from Stat6VT mice.

Skin infiltrating cells in Stat6VT and Stat6VT×*Parp14*$^{-/-}$ mice

To further characterize the histopathology of skin inflammation in Stat6VT and Stat6VT×*Parp14*$^{-/-}$ mice, we performed histological analysis of skin tissue from wild-type, Stat6VT and Stat6VT×*Parp14*$^{-/-}$ mice. Increased

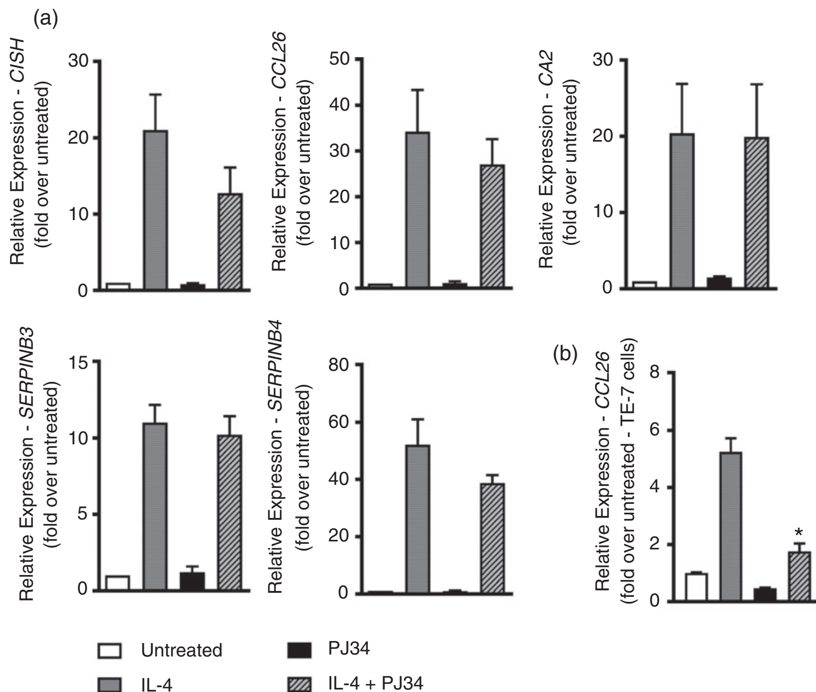

Figure 2. Poly-ADP ribose polymerase-14 (PARP14) is not required for interleukin-4 (IL-4) -induced gene expression in keratinocytes. Human primary keratinocytes (a) or the human oesophageal epithelial cell line TE-7 (b) were differentiated and stimulated with human IL-4 and treated with the PARP inhibitor, PJ34. Gene expression was measured by RT-PCR. Results are an average of at least three experiments.

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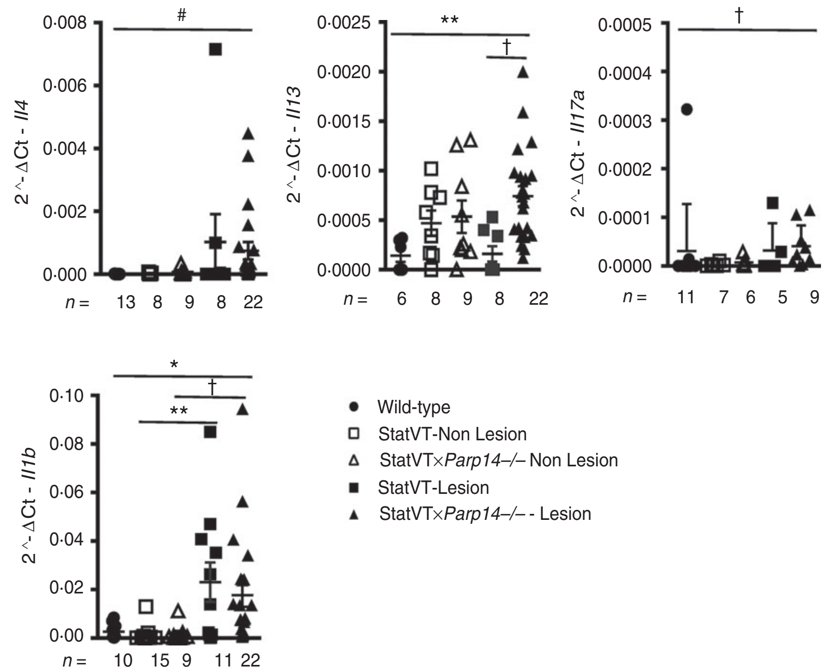


Figure 3. Expression of epidermal differentiation complex (EDC) genes and cytokines in skin. RNA was isolated from wild-type, lesional and non-lesional Stat6VT and Stat6VT×*Parp14*$^{-/-}$ skin tissue. Expression of the indicated genes were measured by quantitative RT-PCR, samples were normalized to the expression of β_2-microglobulin mRNA. $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $\dagger P < 0{\cdot}005$; $^{\#}P < 0{\cdot}0001$; post hoc analysis after one-way analysis of variance.

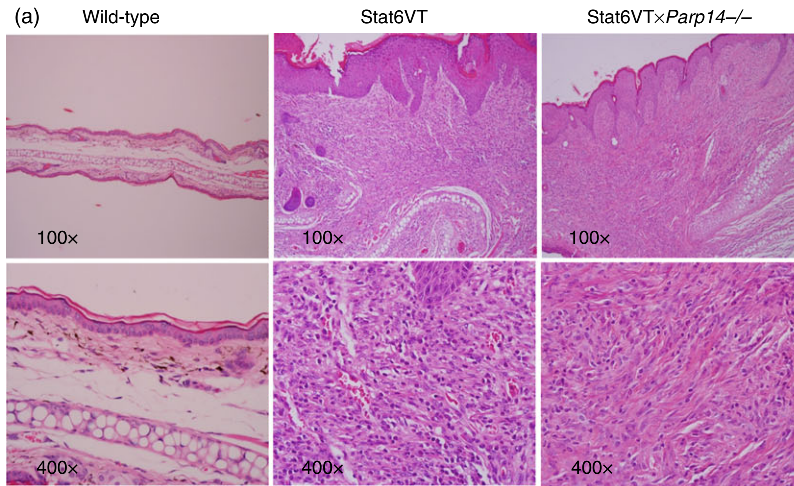
dermal and epidermal thickening along with an increase in cellular infiltration was observed in the skin of both Stat6VT and Stat6VT×*Parp14*$^{-/-}$ mice compared with wild-type mice, but there were no obvious differences in the microscopic pathology caused by the absence of PARP14 (Fig. 4a).

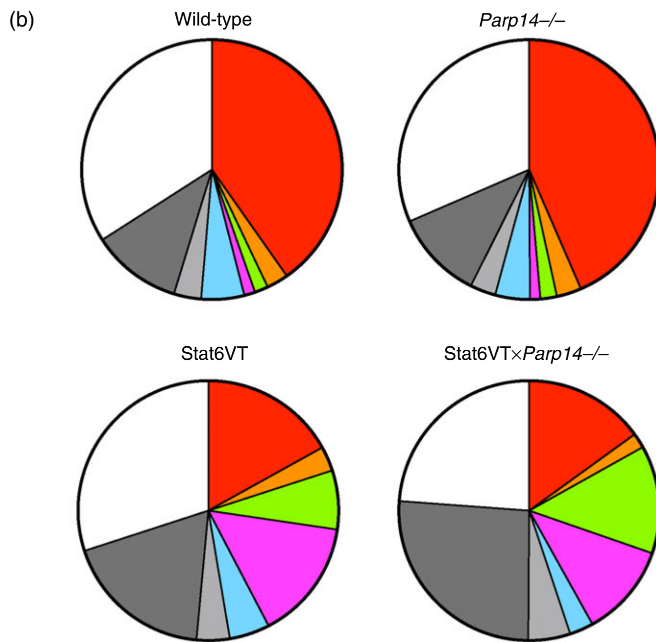
To determine if there was a difference in cellular infiltrate in the skin of Stat6VT versus Stat6VT×*Parp14*$^{-/-}$ mice, we examined the cell types that infiltrate the skin of Stat6VT and Stat6VT×*Parp14*$^{-/-}$ mice. We digested the ear skin of wild-type, *Parp14*$^{-/-}$, Stat6VT and Stat6VT×*Parp14*$^{-/-}$ mice and analysed 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 (Fig. 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,c). Skin from Stat6VT mice had lower percentages of T cells and an increase in the percentage of infiltrating eosinophils, neutrophils and macrophages compared with wild-type and *Parp14*$^{-/-}$ skin. Similar to what was observed in Stat6VT skin, Stat6VT×*Parp14*$^{-/-}$ mice had a decrease in the percentage of T cells infiltrating the ear skin tissue compared with wild-type and *Parp14*$^{-/-}$ skin. The percentage of neutrophils was decreased, and the percentage of eosinophils and macrophages in Stat6VT×*Parp14*$^{-/-}$ skin was increased compared with 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 Stat6VT×*Parp14*$^{-/-}$ skin compared with wild-type, *Parp14*$^{-/-}$ and Stat6VT skin (24% versus 30–35%). The total cell numbers of eosinophils and macrophages in Stat6VT×*Parp14*$^{-/-}$ skin was also increased compared with cells in Stat6VT skin (Fig. 4c). Taken together, these data suggest that there are modest alterations in infiltrating cell populations between Stat6VT×*Parp14*$^{-/-}$ and Stat6VT transgenic mice. These alterations suggest that 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 Stat6VT×*Parp14*$^{-/-}$ mice was clearly more severe than observed in Stat6VT mice (Fig. 1), despite only modest changes in the observed tissue inflammation. For this reason, we 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 Stat6VT×*Parp14*$^{-/-}$ mice. To that end, we analysed the spleens of wild-type, *Parp14*$^{-/-}$, Stat6VT and Stat6VT×*Parp14*$^{-/-}$ mice. To minimize the effects of inflammation causing secondary changes, we used





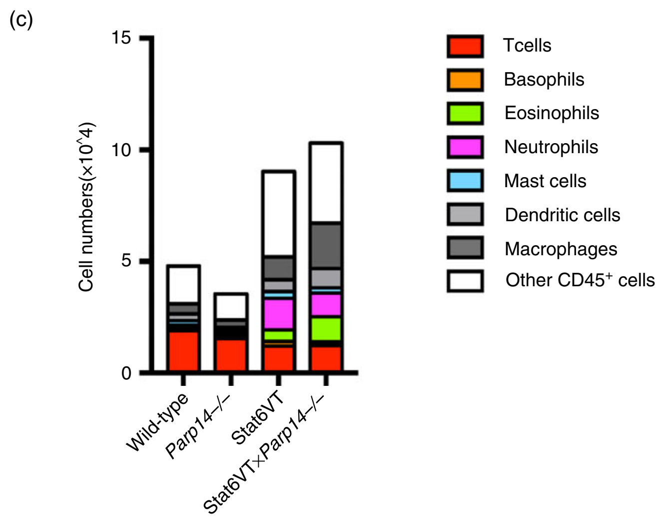


Figure 4. Analysis of skin inflammation. (a) Histological analysis of ear tissue from wild-type, Stat6VT and Stat6VT×*Parp14*$^{-/-}$ mice. Samples were fixed and stained with haematoxylin & eosin. (b) Ear skin samples isolated from wild-type, *Parp14*$^{-/-}$, Stat6VT and Stat6VT×*Parp14*$^{-/-}$ mice 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 numbers of cells infiltrating the ear skin of the indicated populations were determined. Data are mean of 6–10 mice in each genotype.

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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 Stat6VT×*Parp14*$^{-/-}$ mice compared with wild-type (Fig. 5a). Though the frequencies of CD4^{+} cells are similar in Stat6VT and Stat6VT×*Parp14*$^{-/-}$ mice, the number of CD4^{+} cells in Stat6VT×*Parp14*$^{-/-}$ mice is significantly lower compared with Stat6VT mice (Fig. 5a). We observed a similar increase in the percentage of CD19^{+} B cells in Stat6VT and Stat6VT×*Parp14*$^{-/-}$ mice but the numbers of CD19^{+} B cells in the Stat6VT×*Parp14*$^{-/-}$ mice are significantly lower compared with Stat6VT mice (Fig. 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 IFN-γ production.[18,23] To study how the absence of PARP14 affects cytokine production in Stat6VT mice, we analysed CD4 T-cell production of IL-4, IL-13, IFN-γ and IL-17A by intracellular cytokine staining and ELISA (Fig. 5c,d). Although Stat6VT expression raised the production of all effector cytokines, PARP14 deficiency diminished production of IL-4 and modestly affected IL-13 (Fig. 5c,d). Production of IL-17 was increased by PARP14 deficiency (Fig. 5c,d), contrasting with decreases in IFN-γ production (Fig. 5d).

CD4^{+} cells from Stat6VT×Parp14$^{-/-}$ 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 Stat6VT×*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 Stat6VT×*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 with Stat6VT×*Parp14*$^{-/-}$ recipients (Fig. 6a), though the frequency of IFN-γ- and IL-17A-producing cells was similar between both groups of mice (Fig. 6b). Splenocytes isolated from the *Rag1*$^{-/-}$ recipient mice were also re-stimulated with anti-CD3 for 72 hr and the levels of secreted cytokines were measured. At 20 weeks, IL-4 levels were significantly lower in Stat6VT×*Parp14*$^{-/-}$ recipient mice, IFN-γ levels were also reduced (Fig. 6c) compared with Stat6VT recipient mice. The ratio of IFN-γ to IL-4, a measure of the balance of the Th1/Th2 inflammatory milieu, is significantly higher in Stat6VT×*Parp14*$^{-/-}$ recipients at 10 weeks compared with Stat6VT recipients and the ratio further increased at 20 weeks compared with 10-week post-transfer Stat6VT×*Parp14*$^{-/-}$ recipient cells (Fig. 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 Fig. 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 Stat6VT×*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 Stat6VT×*Parp14*$^{-/-}$ mice compared with skin from Stat6VT mice. Together, these data suggest that PARP14 deficiency alters immune responses, so 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 that 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 Stat6VT×*Parp14*$^{-/-}$ mice. Adoptive transfer of Stat6VT×*Parp14*$^{-/-}$ CD4^{+} T cells into *Rag1*$^{-/-}$ mice resulted in an increased IFN-γ/IL-4 ratio compared with 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 Stat6VT×*Parp14*$^{-/-}$ mice compared with Stat6VT mice. Although there were differences in the cellular make-up

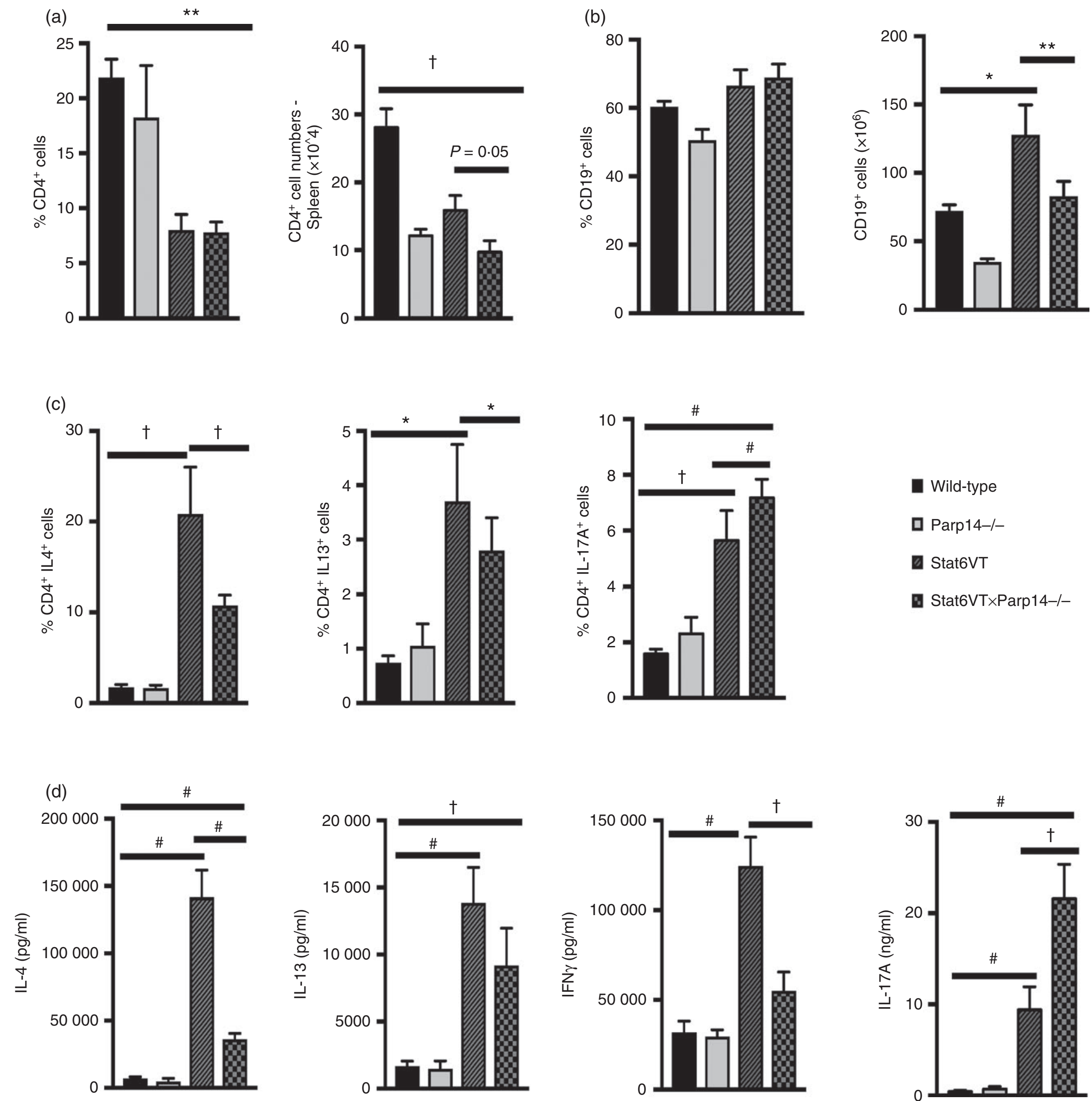

Figure 5. Altered lymphocyte homeostasis in poly-ADP ribose polymerase-14 (PARP14) -deficient Stat6VT mice. (a) Splenocytes from age-matched wild-type, *Parp14*$^{-/-}$, Stat6VT and Stat6VT×*Parp14*$^{-/-}$ mice were isolated and the percentage of CD4^{+} T cells population was determined by flow cytometry. 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 re-stimulated 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 hr, the cell-free supernatants were collected and the cytokines produced were measured by ELISA. Data are mean ± SEM of three to six mice in each genotype (a–c) and mean ± SEM of 6–10 mice in each genotype (d). Statistical significance was determined by post hoc analysis after one-way analysis of variance (a–c) and two-tailed test (d), is indicated as follows $*P < 0{\cdot}05$, $**P < 0{\cdot}01$, $\dagger P < 0{\cdot}005$, $^{\#}P < 0{\cdot}0001$.

of the infiltrate (Fig. 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.

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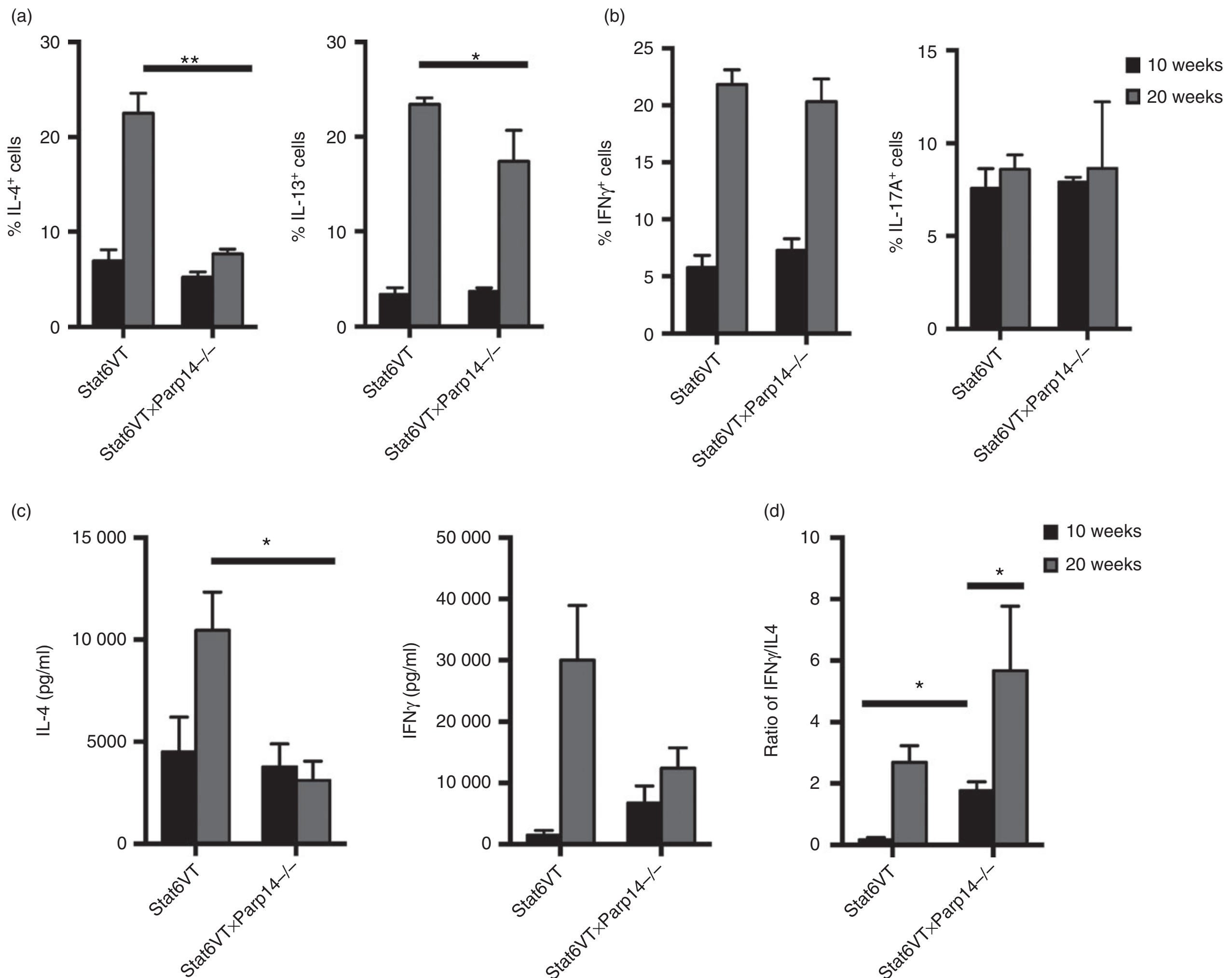

Figure 6. Decreased T helper type 2 cytokines from Stat6VT×*Parp14*$^{-/-}$ CD4^{+} cells. (a, b) CD4^{+} cells from Stat6VT and Stat6VT×*Parp14*$^{-/-}$ mice were adoptively transferred to *Rag1*$^{-/-}$ mice. Splenocytes from the recipients were re-stimulated with PMA/Ionomycin and stained for interleukin-4 (IL-4), IL-13, interferon-γ (IFNγ), IL-17A-producing cells and analysed by flow cytometry. (c, d) Cytokines produced from supernatants of anti-CD3-stimulated splenocytes were analysed by ELISA. Data from the 10-week experiment have 5–11 mice per group and 20-week experiment have six to eight mice per group. *$P < 0{\cdot}05$, **$P < 0{\cdot}001$ – Post hoc analysis after one-way analysis of variance.

Interleukin-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 a reduced concentration of IL-17A from antigen-stimulated splenocytes.[23] However, in Stat6VT×*Parp14*$^{-/-}$ mice, we observed an overall increase in IL-17A-producing CD4^{+} cells. Interleukin-4 has been shown to suppress IL-23 and IL-17,[29] suggesting that the increase in IL-17A in Stat6VT×*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 Stat6VT×*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 Stat6VT×*Parp14*$^{-/-}$ mice. We characterized the cells infiltrating the skin from Stat6VT and Stat6VT×*Parp14*$^{-/-}$ mice and observed that the cellular infiltrate had an increase in the frequency of eosinophils and macrophages in Stat6VT×*Parp14*$^{-/-}$ skin compared with 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 remodelling 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 Stat6VT×*Parp14*$^{-/-}$ mice. Moreover, we observed the infiltration of haematopoietic cells (CD45^{+}) that were not classified into any of the characterized lineages. Though the frequencies of these cells were similar among all four groups of mice, the cell numbers of other CD45^{+} cells were more than twofold higher in Stat6VT and Stat6VT×*Parp14*$^{-/-}$ skin compared with 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.

Atopic dermatitis is predominantly a Th2-mediated disease. However, Stat6VT×*Parp14*$^{-/-}$ mice develop severe AD-like skin inflammation even with significant decreases in Th2 cytokine production from peripheral T cells, compared with Stat6VT mice. Despite this, there was evidence of increased Th2 cytokine mRNA in lesional tissue of Stat6VT×*Parp14*$^{-/-}$ mice, compared with Stat6VT mice. Hence, 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 Stat6VT×*Parp14*$^{-/-}$ mice, compared with Stat6VT mice. The second explanation, based on the altered immune skewing, suggests a 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 those 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 of these studies, the authors observed an IFN-γ-dominant skin microenvironment that is the likely cause of the skin pathology. We also observed a shift in the IFN-γ/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 PARP14. This was the opposite of the observations in allergic lung disease models and the correlations in patients with eosinophilic oesophagitis 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 the potential for recruitment to inflamed sites. For example, if PARP14-regulated genes were 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 Stat6VT×*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 Stat6VT×*Parp14*$^{-/-}$ mice lack PARP14 expression in all cells. PARP14 function has been investigated in T cells, B cells, macrophages, airway and oesophageal 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.

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Disclosures

The authors declare that there are no competing interests.

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