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Peptidoglycan Recognition Protein Pglyrp2 Protects Mice from Psoriasis-like Skin Inflammation by Promoting Regulatory T Cells and Limiting Th17 Responses

Shin Yong Park,* Dipika Gupta,* Risa Hurwich,* Chang H. Kim,† and Roman Dziarski*

Skin protects the body from the environment and is an important component of the innate and adaptive immune systems. Psoriasis is a frequent inflammatory skin disease of unknown cause determined by multigenic predisposition, environmental factors, and aberrant immune response. Peptidoglycan recognition proteins (Pglyrps) are expressed in the skin, and we report in this article that they modulate sensitivity in an experimentally induced mouse model of psoriasis. We demonstrate that Pglyrp2−/− mice (but not Pglyrp3−/− and Pglyrp4−/− mice) are more sensitive to the development of 12-O-tetradecanoylphorbol 13-acetate–induced psoriasis-like inflammation, whereas Pglyrp1−/− mice are less sensitive. The mechanism underlying this increased sensitivity of Pglyrp2−/− mice to 12-O-tetradecanoylphorbol 13-acetate–induced psoriasis-like inflammation is reduced recruitment of regulatory T cells to the skin and enhanced production and activation of Th17 cells in the skin in Pglyrp2−/− mice, which results in more severe inflammation and keratinocyte proliferation. Thus, in wild type mice, Pglyrp2 limits overactivation of Th17 cells by promoting accumulation of regulatory T cells at the site of inflammation, which protects the skin from the exaggerated inflammatory response. The Journal of Immunology, 2011, 187: 000–000.

The innate immune system not only protects the host from infections, but it also responds to tissue damage and environmental chemicals (including allergens), and it initiates adaptive immune responses. Skin protects the body from the environment and is the largest organ in mammals. Besides forming a mechanical barrier, skin is an important component of the innate and adaptive immune systems rich in antimicrobial peptides and Ag-sensing cells, and it maintains the proper homeostatic balance between proinflammatory and anti-inflammatory responses. Psoriasis is one of the most frequent inflammatory skin diseases in Western societies. Psoriasis has a prevalence rate of 2–4% and is manifested by areas of epidermal overgrowth and scaling. The cause of psoriasis is unknown, and it is determined by multigenic predisposition, environmental factors, and aberrant immune response (1, 2). Psoriasis does not develop spontaneously in animals; thus, a need exists for experimental animal models of psoriasis that can be used to dissect its causative factors and pathogenesis. Skin protects the body from the environment and is the largest organ in mammals. Besides forming a mechanical barrier, skin is an important component of the innate and adaptive immune systems rich in antimicrobial peptides and Ag-sensing cells, and it maintains the proper homeostatic balance between proinflammatory and anti-inflammatory responses. Psoriasis is one of the most frequent inflammatory skin diseases in Western societies. Psoriasis has a prevalence rate of 2–4% and is manifested by areas of epidermal overgrowth and scaling. The cause of psoriasis is unknown, and it is determined by multigenic predisposition, environmental factors, and aberrant immune response (1, 2). Psoriasis does not develop spontaneously in animals; thus, a need exists for experimental animal models of psoriasis that can be used to dissect its causative factors and pathogenesis, and to develop and evaluate new therapies.

One class of innate immunity proteins expressed in the skin is peptidoglycan recognition proteins (PGRPs or Pglyrps). PGRPs are conserved from insects to mammals, recognize bacterial peptidoglycan, and function in antibacterial immunity. Mammals have four PGRPs, Pglyrp1, Pglyrp2, Pglyrp3, and Pglyrp4, which were initially named PGRP-S, PGRP-L, PGRP-Io, and PGRP-Ib, respectively (3, 4). Pglyrp1, Pglyrp3, and Pglyrp4 are directly bactericidal (5–8), whereas Pglyrp2 is an N-acetylmuramoyl-L-alanine amidase that hydrolyzes peptidoglycan (9, 10). Pglyrp2 is highly expressed in polymorphonuclear leukocyte’s (PMN) granules and to a much lower extent in other cells (4, 11, 12). Pglyrp2 is constitutively expressed in the liver, from which it is secreted into blood, and Pglyrp2 expression is induced in keratinocytes and other epithelial cells (9, 10, 13–17). Different transcription factors control constitutive and inducible expression of Pglyrp2 (17). Pglyrp3 and Pglyrp4 are highly expressed in the skin and are also expressed in the salivary glands, throat, tongue, esophagus, stomach, intestine, and eyes (5, 18, 19).

In vivo, mammalian PGRPs protect mice against experimental colitis (19), but at least one PGRP (Pglyrp2) has an opposite proinflammatory effect in a model of experimental arthritis (20). We hypothesized that PGRPs play a role in the development of psoriasis because of the following factors: 1) the prominent expression of PGRPs in the skin, 2) their ability to modulate the sensitivity to collitis and arthritis (19, 20), 3) the location of Pglyrp3 and Pglyrp4 genes in the epidermal differentiation gene cluster in the psoriasis-sensitivity psors4 locus, 4) coordinated expression of Pglyrp3 and Pglyrp4 with other genes in the psors4 locus, and 5) previous evidence of genetic association of Pglyrp3 and Pglyrp4 variants with psoriasis (21, 22). In this study, we tested this hypothesis using PGRP-deficient mice and a mouse model of chemically induced, psoriasis-like inflammation.

We demonstrate that Pglyrp2−/− mice (but not Pglyrp3−/− and Pglyrp4−/− mice) are more sensitive to the development of experimental psoriasis-like inflammation, whereas Pglyrp1−/− mice are less sensitive than wild type (WT) mice. The mechanism underlying this increased sensitivity of Pglyrp2−/− mice is reduced recruitment of regulatory T cells (Tregs) to the skin and enhanced production and activation of Th17 cells in Pglyrp2−/− mice, which results in more severe inflammation and keratinocyte proliferation. Thus, in WT mice, Pglyrp2 limits overactivation of
Th17 cells by promoting accumulation of Tregs at the site of inflammation, which protects the skin from the exaggerated inflammatory response.

**Materials and Methods**

**Mice**

We generated Pglyrp1<sup>−/−</sup>, Pglyrp2<sup>−/−</sup>, Pglyrp3<sup>−/−</sup>, and Pglyrp4<sup>−/−</sup> mice as described previously (12, 19, 20). We generated Pglyrp1<sup>−/−</sup> Pglyrp2<sup>−/−</sup>, Pglyrp1<sup>−/−</sup>Pglyrp3<sup>−/−</sup>, and Pglyrp2<sup>−/−</sup>Pglyrp3<sup>−/−</sup> double-knockout mice and Pglyrp1<sup>−/−</sup>Pglyrp2<sup>−/−</sup>Pglyrp3<sup>−/−</sup> triple-knockout mice by breeding single- and double-knockout mice (all on BALB/c background) and screening for homozygous deletion of each Pglyrp gene by PCR analysis of genomic DNA as previously described (12, 19, 20). The lack of expression of the Pglyrp genes was confirmed by quantitative real-time RT-PCR (qRT-PCR) in mRNA from the ears. Double- and triple-homozygous Pglyrp knockout mice were viable and fertile, bred normally, and yielded the expected male-to-female ratios and similar litter size as the WT and heterozygous mice. They had similar weight as the WT and single Pglyrp knockout mice and developed normally with no obvious defects. Their major internal organs had normal macroscopic appearance and normal histological appearance on H&E-stained sections.

All mice used in experiments were 8–10 wk old and on BALB/c background. The original colony founder WT BALB/c breeder mice were obtained from Harlan-Sprague-Dawley. All knockout mice were backcrossed to the same WT BALB/c mice from our breeding colony, and all WT and knockout mice were bred and kept under conventional pathogen-free conditions in the same room in our facility to minimize the influence of differences in the environment. For each experiment, mice from several different cages and breeder pairs were used. The BALB/c background of Pglyrp-deficient mice and their negative status for all common viral and bacterial pathogens and parasites were confirmed as previously described (19). All experiments on mice were approved by the Indiana University School of Medicine-Northwest Institutional Animal Care and Use Committee.

**12-O-tetradecanoylphorbol 13-acetate–induced psoriasis-like inflammation model**

A total of 20 µl 0.01% 12-O-tetradecanoylphorbol 13-acetate (TPA [Sigma] in 2% DMSO, 98% acetone) was applied to each ear (10 µl to each side) of male mice on days 0, 2, 4, 6, and 8 (23). Ear thickness was measured each time before TPA application with Digimatic Micrometer (Mitutoyo, Japan) under constant pressure at the lowest setting. Ear swelling was determined by subtracting the untreated ear thickness (day 0).

The significance of differences in ear swelling was determined using t test.

**Histology**

For histological analysis, ears were fixed in Bouin’s fixative, postfixed in 70% ethanol, and embedded in paraffin; cross sections were stained with H&E and evaluated microscopically. Histological changes (acanthosis, formation of rete pegs, parakeratosis, parakeratotic scaling, thickening of the subepidermal layer, cellular infiltration) were evaluated semiquantitatively on a scale of 0–6 (0 = no change from untreated ears; 6 = maximum greater if calculated per ear, rather than per micrograms RNA). The genomic DNA contamination controls, reverse transcription controls, and positive PCR controls were included in each array and were all passed. Additional control to assure amplification from RNA, but not from possible contaminating DNA, included parallel reaction sets from which reverse transcriptase was omitted and which showed no amplification. ∆Ct was calculated from untreated PGRP-deficient mice and ΔCt2 was from untreated WT mice to compare baseline gene expression in untreated mice.

The results were reported as mean fold increases over untreated (treated/ununtreated) for WT mice or ratios of fold increases in Pglyrp-deficient to WT mice, calculated as follows: [(Pglyrp<sup>−/−</sup> treated)/(Pglyrp<sup>−/−</sup> untreated)]/[(WT treated)/(WT untreated)] and presented as heat maps or histograms. The latter fold differences (ratios of >1 or <1 reflect higher or lower expression levels, respectively, in Pglyrp-deficient mice than in WT mice. Heat maps were generated using Java TreeView after converting <1 ratios to negative fold difference using the formula: (−1)/ratio).

The significance of differences in gene activation between groups of mice was determined using the two-sample one-tailed t test, and typically the differences of >2-fold were significant at p < 0.05.

Expression of mRNA for PGRPs was similarly measured by qRT-PCR using Qiagen/SA Biosciences First Strand Kit (with random primers) and the TaqMan gene expression master mix. The lack of expression of the gene of interest was confirmed by including a no-RT control and which showed no amplification.

**Neutralization of IL-17, IL-22, and IL-1β**

IL-17, IL-22, or IL-1β were neutralized by i.v. injections of anti-IL-17 mAb (specific for IL-17A and not reactive with IL-1F, rat clone 50104, from R&D Systems), or anti–IL-22 mAb (rat clone IL22DOP from eBioscience), or anti-IL-1β (rat clone 30311 from R&D Systems), 100 µg on day 0 and 50 µg on days 3 and 6 of TPA treatment. These mAbs had equivalent neutralizing capacity for their respective cytokines based on the results provided by the manufacturers. Control mice were similarly treated with isotype control rat IgG2a or IgG1 mAb (clone 16-4321 from eBioscience or clone 43414 from R&D Systems).
Results

Pglyrp2<sup>−/−</sup> mice have an enhanced inflammatory response to TPA in the skin

Repeated application of TPA for 8 d (every other day) to the ears of WT BALB/c mice induced progressive moderate inflammation manifested by redness (Fig. 1A) and swelling (Fig. 1B). By contrast, similar application of TPA to Pglyrp2<sup>−/−</sup> mice induced significantly enhanced inflammation, manifested by increased redness (Fig. 1A) and significantly increased swelling, accompanied by severe scaling (Fig. 1A, 1B). This enhanced response was unique for Pglyrp2<sup>−/−</sup> mice, because it was not observed in Pglyrp1<sup>−/−</sup>, Pglyrp3<sup>−/−</sup>, and Pglyrp4<sup>−/−</sup> mice, and was dominant, because it was still observed in Pglyrp1<sup>−/−</sup>Pglyrp2<sup>−/−</sup> and in Pglyrp2<sup>−/−</sup>Pglyrp3<sup>−/−</sup> double-knockout mice (Fig. 1B). Deletion of Pglyrp1 had an opposite effect on the early response to TPA, because Pglyrp1<sup>−/−</sup>Pglyrp2<sup>−/−</sup> single-knockout, Pglyrp1<sup>−/−</sup>Pglyrp3<sup>−/−</sup> double-knockout, and Pglyrp1<sup>−/−</sup>Pglyrp2<sup>−/−</sup>Pglyrp3<sup>−/−</sup> triple-knockout mice all showed reduced ear swelling on days 2 and 4 of TPA application, but not later (Fig. 1B). Deletion of Pglyrp3 or Pglyrp4 had little effect on the response to TPA. These results indicate that Pglyrp2 has a protective effect against severe TPA-induced, psoriatic-like inflammation in WT mice, whereas Pglyrp1 has an enhancing proinflammatory effect in the early stages of the response.

We then compared the histology of TPA-induced skin lesions in WT and Pglyrp-deficient mice to determine the pathological basis of this higher sensitivity of Pglyrp2<sup>−/−</sup> mice to TPA. Ears in untreated WT mice have one- to two-cell-thick epidermis and a few cells thick subepidermal layer with blood vessels, sebaceous glands, hair follicles, muscle bundles, and central fat and connective tissue layer, with total thickness of ~200 μm. Histology of all untreated Pglyrp-deficient mice was similar to WT mice (Fig. 2A). Five TPA applications to the ears (every other day) induced strong inflammatory response that was very severe in Pglyrp2<sup>−/−</sup> mice. Cross sections of the TPA-treated ears revealed severe acanthosis (thickening of the epidermis because of proliferation of keratinocytes), formation of rete pegs (downward papillary projections of epidermis), parakeratosis (retention of keratinocytes’ nuclei in stratum corneum), parakeratotic scaling, formation of numerous epidermal microabscesses (primarily neutrophilic), and

**FIGURE 1.** Pglyrp2<sup>−/−</sup> mice have enhanced response to TPA in the skin. A. Application of TPA to ears for 9 d (every other day) induces mild inflammation in WT mice and severe inflammation in Pglyrp1<sup>−/−</sup>Pglyrp2<sup>−/−</sup> mice with increased redness, swelling, and extensive scaling. B. Ear swelling in WT mice (black triangles) and Pglyrp<sup>−/−</sup> mice (colored triangles) after TPA application to the ears on days 0, 2, 4, 6, and 8. Data are means ± SEM (SEM were often smaller than the symbols in this and other figures). n = 9–14 mice/group. Significance of differences between Pglyrp<sup>−/−</sup> and WT mice: *p < 0.02, **p < 0.0001.
marked thickening of the subepidermal layer with dense cellular infiltrates (containing many PMNs and some mononuclear cells) that were all highly prominent in Pglyrp2−/−, Pglyrp1−/−, and all other double- and triple-knockout mice deficient in Pglyrp2 (not shown). All these changes are highly characteristic of psoriatic lesions. WT (Fig. 2A), Pglyrp1−/−, and Pglyrp3−/−, and Pglyrp4−/− mice (not shown) all showed significantly less severe acanthosis, rete pegs, parakeratosis, and parakeratotic scaling, less thickening of the subepidermal layer, and fewer cellular infiltrations, as judged by semiquantitative evaluation of tissue sections (Fig. 2B). Measurements of the size of the microabscesses revealed significantly larger microabscesses in Pglyrp2−/− and Pglyrp1−/− Pglyrp2−/− mice than in WT mice (Fig. 2B).

Our results demonstrate that deletion of Pglyrp2−/− highly predisposes mice to psoriatic-like lesions in response to TPA, and the skin lesions in these mice have all main histological characteristics of human psoriatic lesions. Thus, Pglyrp2−/− mice can serve as a convenient new mouse model of psoriasis-like inflammation. These results also further demonstrate that, in WT mice, Pglyrp2 protects the skin from excessive TPA-induced, psoriasis-like inflammation.
Expression of PGRPs is increased in inflamed ears

We then compared expression of Pglyrp2 and other PGRPs in the ears in untreated and TPA-treated mice to gain further insight into how Pglyrp2 influences sensitivity to TPA-induced inflammation. Treatment with TPA induced increased Pglyrp1 expression in the ears in all strains of mice (except Pglyrp1/−/− mice) that was significantly higher than in untreated mice (Fig. 3). The expression of Pglyrp1 in the ears was significantly higher in all Pglyrp2/−/− mice than in WT mice at all time points after TPA treatment, which correlates with their higher inflammatory response and is likely due to increased infiltration with PMNs, which highly express Pglyrp1. The expression of Pglyrp2 in the ears significantly increased 6 h after the first TPA treatment and subsequently declined on days 3–9 (Fig. 3), which inversely correlates with the extent of inflammatory response and is consistent with the anti-inflammatory effect of Pglyrp2 in the TPA-induced inflammation model. Pglyrp3 has high constitutive expression in untreated skin. After TPA treatment, Pglyrp3 expression initially increased in the ears and then declined. Pglyrp4 has much lower constitutive expression in untreated skin than Pglyrp3. After TPA treatment, expression of Pglyrp4 was highly increased and was especially high in Pglyrp2/−/−Pglyrp3/−/− mice (Fig. 3), indicating a compensatory expression of Pglyrp4 in mice deficient in Pglyrp3, because Pglyrp3 and Pglyrp4 genes are tightly linked in the psoriasis sensitivity locus on chromosome 3 in mice, and their expression is correlated with the expression of keratinocyte differentiation genes (18). Thus, a decrease in Pglyrp3 expression is a consequence of the increased proliferation and dedifferentiation of keratinocytes in the psoriasis model. These changes in Pglyrp3 and Pglyrp4 expression, however, had no detectable effect on the TPA-induced inflammation, which was similar in WT and Pglyrp3/−/− and Pglyrp4/−/− mice (Fig. 1B).

Pglyrp1 and Pglyrp2 were also constitutively expressed in the cervical lymph nodes at a similar level as in the ears, but after TPA treatment, their expression in the cervical lymph nodes did not significantly change (data not shown). Pglyrp3 and Pglyrp4 were not constitutively expressed in cervical lymph nodes, and their expression there was not induced after TPA treatment (data not shown). The above results indicate that the expression of Pglyrp1 and Pglyrp2 in the ear tissue cells, but not in immune cells (cervical lymph nodes, which contain lymphocytes and APCs), correlates with the changes in inflammatory skin responses. Pglyrp2/−/− mice have increased Th17 cells and Th17 responses in the skin

We next studied the types of inflammatory cells in the ears in TPA-treated mice to determine the cellular basis for the differences in the inflammatory response in Pglyrp2/−/− mice. We then determined which cell types significantly differed in Pglyrp2/−/− mice compared with WT mice. This was first accomplished by measuring the amounts of mRNA for several marker genes characteristic of various immune and inflammatory cell types in the untreated and the affected ears. To determine which marker genes (and thus cell types) are increased or decreased in Pglyrp2/−/− mice compared with WT mice, we calculated how many times higher or lower they were induced in Pglyrp2/−/− mice than in WT mice (fold induction in Pglyrp2/−/− mice/fold induction in WT mice).

Treatment with TPA for 9 d highly increased the numbers of monocytes and PMNs in the affected ears in WT mice (Fig. 4A, WT panel; Supplemental Fig. 1). Keratinocyte differentiation marker, loricrin (Lor), was decreased, as was Ccr10 (the receptor for keratinocyte-specific chemokine, Ccl27), whereas antimicrobial peptides, β-defensin-3 (Defb3), calgranulin A (s100a8), and calgranulin B (s100a9) were greatly increased (Fig. 4A, WT panel; Supplemental Fig. 1), reflecting dedifferentiated and activated state of keratinocytes, consistent with TPA-induced proliferation of keratinocytes. These are all expected changes consistent with the psoriasis model. Pglyrp2-deficient mice that had enhanced response to TPA (Pglyrp2−/−, Pglyrp1−/−Pglyrp2−/−, and Pglyrp2−/−Pglyrp3−/− mice) had significantly higher increases in PMNs and monocytes compared with WT mice, as well as increased B and T cells, and especially Rort-expressing cells (Fig. 4A, Pglyrp−/−/WT panel; Supplemental Fig. 1), which are characteristically (but not exclusively) Th17 cells (24). These results suggest that the common cell type preferentially increased in Pglyrp2-deficient mice that are more sensitive to TPA-induced, psoriasis-like inflammation compared with WT mice may be the Rort-expressing Th17 cells.

We then measured the expression of an extended panel of cytokines, chemokines, and other marker genes characteristic of Th1, Th2, Th17, Treg, NK, and other cell types to further define the cell types responsible for the increased sensitivity of Pglyrp2−/− mice to TPA-induced skin inflammation, and to determine which of these genes were differentially induced in the affected skin in Pglyrp2-deficient mice compared with WT mice. We included these cell types, because in addition to Th1 and Th2 cells, Th17 cells and other cell types may also be involved in skin inflammation.

TPA is a diacylglycerol analog that activates many immune and nonimmune cell types through the activation of protein kinase C. In our model, a single exposure to TPA highly activated multiple genes in many cell types in WT mice: 6 h after a single TPA treatment, 17 of 49 genes were induced 15-fold (Fig. 4B, WT 6-h panel; Supplemental Fig. 2). In Pglyrp2−/−, Pglyrp1−/−Pglyrp2−/−, and Pglyrp2−/−Pglyrp3−/− mice, a single exposure to TPA in 6 h induced moderately higher activation of genes characteristic of Th17 cells and some genes characteristic of multiple cell types (Fig. 4B, Pglyrp−/−/WT 6-h panel; Supplemental Fig. 2). TPA

![FIGURE 3. Pglyrp1, Pglyrp2, Pglyrp3, and Pglyrp4 expression is increased in the TPA-treated skin. The amounts of each PGRP mRNA in WT mice and in the indicated Pglyrp-deficient mice treated with TPA every other day were measured by qRT-PCR. Results are means of 3–4 mice ± SEM. *p < 0.04, **p < 0.001, treated versus untreated; *p < 0.04, **p < 0.001, Pglyrp/−/− versus WT.](http://www.jimmunol.org/content/ji/199/4/5.f.html)
treatment for 9 d induced prolonged inflammation in all mice, but WT mice were able to reduce the number of highly activated (>15-fold) genes to only 5 genes, namely, Cxcl2 and Cxcl5 (characteristic of Th17 cells), and Ccl3, Ccl4, and Il1b (characteristic of multiple cell types; Fig. 4B, WT 9-d panel; Supplemental Fig. 3), compared with 17 genes highly induced 6 h after application of TPA to the ears on days 0, 2, 4, 6, and 8 measured by qRT-PCR. For WT mice (left panels), the ratio of the amount of mRNA in TPA-treated to untreated mice for each gene (fold induction by TPA) is shown; for Pglyrp2+/- mice, the results are the ratios of fold induction of each gene by TPA in Pglyrp2+/- mice to fold induction of each gene by TPA in WT mice (which represents the fold difference in the response to TPA in Pglyrp2+/- versus WT mice). The results are means of three arrays from four to five mice per group in heat map format. The means ± SEM bar graphs for these results are shown in Supplemental Figs. 1–3.

FIGURE 4. TPA-treated Pglyrp2+/- mice have increased PMNs, monocytes, B cells, and Th17 cells, and a Th17-associated gene expression profile in the affected skin. Expression of a panel of (A) marker genes characteristic of various inflammatory cell types and (B) cytokines, chemokines, and other marker genes characteristic of Th1, Th2, Th17, Treg, NK, and other cell types in the ears of mice 6 h or 9 d after application of TPA to the ears on days 0, 2, 4, 6, and 8 measured by qRT-PCR. For WT mice (left panels), the ratio of the amount of mRNA in TPA-treated to untreated mice for each gene (fold induction by TPA) is shown; for Pglyrp2+/- mice, the results are the ratios of fold induction of each gene by TPA in Pglyrp2+/- mice to fold induction of each gene by TPA in WT mice (which represents the fold difference in the response to TPA in Pglyrp2+/- versus WT mice). The results are means of three arrays from four to five mice per group in heat map format. The means ± SEM bar graphs for these results are shown in Supplemental Figs. 1–3.

The results described above indicate that the Pglyrp2+/- mice that have higher skin responsiveness to TPA have increased activity of Th17 cells in the affected skin, compared with WT mice. We then used flow cytometry to directly measure Th cell types in
the ears, draining lymph nodes, and the spleen, to further investigate the role of Th17 cells (and other Th cell types) in increased sensitivity of Pglyrp-deficient mice to TPA-induced, psoriasis-like skin inflammation (Fig. 5A, 5B).

TPA treatment for 9 d induced a substantial accumulation of CD4+ cells in the TPA-treated skin. Untreated ears in WT and Pglyrp2−/− mice had ~400 CD4+ cells/ear, whereas on day 9 after 5 TPA treatments, the numbers of CD4+ cells/ear increased to ~15,000/ear in WT mice and ~27,000/ear in Pglyrp2−/− mice, which is significantly higher than in WT mice (Fig. 5B). A moderate increase in the amount of CD4 mRNA detected by qRT-PCR (Fig. 4A, Supplemental Fig. 1) is an underestimation due to undetectable amount of CD4 mRNA in the untreated ears.

Th17 cells (CD4+IL-17+) were undetectable in the ears of untreated mice (~10 Th17 cells/ear, data not shown). TPA treatment for 9 d induced accumulation of Th17 cells in the ears, and their percentages were significantly higher in the affected ears in Pglyrp2−/− and Pglyrp1−/−Pglyrp2−/− mice compared with WT mice.

**FIGURE 5.** TPA-treated Pglyrp2−/− mice have high numbers of Th17 cells and low numbers of Tregs in the affected skin, and IL-17 and IL-22 are required for the enhanced response to TPA in Pglyrp2−/− mice. A. Percentages of Th1 cells, Th2 cells, Th17 cells, and Tregs in the ears, cervical lymph nodes, and spleen in WT, Pglyrp2−/−, and Pglyrp1−/−Pglyrp2−/− mice on day 9 after application of TPA to the ears on days 0, 2, 4, 6, and 8 measured by flow cytometry; means ± SEM of 5–9 mice/group (*p < 0.05, **p < 0.005, Pglyrp2−/− versus WT) or representative dot plots for Th17 cells and Tregs are shown; representative dot plots for Th1 and Th2 cells and isotype controls are shown in Supplemental Fig. 4. B. Numbers of CD4+ cells in the untreated and TPA-treated (days 0, 2, 4, 6, and 8) ears of WT and Pglyrp1−/−Pglyrp2−/− mice. Data are means ± SEM. n = 4 mice/group. Significance of differences between WT and Pglyrp1−/−Pglyrp2−/− mice: **p < 0.005. C. Ear swelling in Pglyrp1−/−Pglyrp2−/− mice treated with TPA on days 0, 2, 4, and 6, and also treated with either isotype control IgG or neutralizing anti–IL-17 or anti–IL-22 mAbs. Data are means ± SEM. n = 7 mice/group. Significance of differences between IgG control and anti–IL-17 or anti–IL-22 mAb-treated mice: *p < 0.05, **p < 0.005. D. Expression of receptors for chemokines that attract Tregs in cervical lymph nodes of WT and Pglyrp1−/−Pglyrp2−/− mice on day 9 after application of TPA to the ears on days 0, 2, 4, 6, and 8 measured by qRT-PCR; the ratio of the amount of mRNA in TPA-treated to untreated mice for each gene (fold induction by TPA) is shown as means ± SEM of three arrays from five mice per group (*p < 0.05, **p < 0.005, Pglyrp2−/− versus WT).
mice (Fig. 5A). If the numbers of Th17 cells are calculated based on the total numbers of CD4+ cells in the TPA-treated ears (Fig. 5B), the total numbers of Th17 cells increased from <10 Th17 cells/ear in untreated mice to ~375 Th17 cells/ear in WT mice and >1600 Th17 cells/ear in Pglyrp1−/−Pglyrp2−/− mice (which is >4 times more than in WT mice). By contrast, there was no significant difference in the percentages of Th1 (CD4+IFN-γ+) and Th2 (CD4+IL-4+) cells in the ears of WT and Pglyrp2−/− and Pglyrp1−/−Pglyrp2−/− mice (Fig. 5A). Virtually all detectable IL-17+ cells in the TPA-treated ears were CD4+ (Th17 cells), and there were few (<5/ear, data not shown) other IL-17+ cells in the inflamed skin (such as CD8+, γδ T, or NKT cells); therefore, the observed increases in IL-17+ cells mostly represent increases in Th17 cells (CD4+IL-17+).

TAPA-treated mice had substantially swollen cervical lymph nodes (>3 mm in diameter compared with <0.5 mm in untreated mice), and the numbers of all Th cell types were significantly higher in Pglyrp2−/− and Pglyrp1−/−Pglyrp2−/− mice compared with WT mice in the cervical lymph nodes and, to a lesser extent, in the spleen (Fig. 5A). These results indicate preferential recruitment and retention of Th17 cells in the TPA-treated ears in Pglyrp2−/− and Pglyrp1−/−Pglyrp2−/− mice compared with WT mice, consistent with our mRNA gene expression data.

**IL-17 is required for enhanced response to TPA in Pglyrp2−/− mice**

We then compared the severity of ear inflammation in TPA-treated Pglyrp1−/−Pglyrp2−/− mice in which IL-17 activity was inhibited with neutralizing anti–IL-17 mAb to determine whether IL-17 (Th17 cytokine) is required in vivo for the high sensitivity of Pglyrp2−/− mice to TPA-induced, psoriasis-like inflammation. In vivo neutralization of IL-17 activity in Pglyrp1−/−Pglyrp2−/− mice significantly decreased TPA-induced ear inflammation compared with mice treated with an isotype control IgG (Fig. 5C), reducing it back to the level of inflammation seen in WT mice (compare 35–40% decrease in ear swelling in Pglyrp1−/−Pglyrp2−/− mice treated with anti–IL-17 mAb relative to isotype control IgG in Fig. 5C with 35–40% increase in ear swelling in Pglyrp1−/−Pglyrp2−/− mice relative to WT mice in Fig. 1B).

One of the main Th17 effector cytokines induced by IL-17 is IL-22 (which, in turn, induces production of PMN-attracting chemokines in keratinocytes). We thus also evaluated the role of Th17 cells in the TPA-induced skin inflammation, because PMNs were the most prominent infiltrating inflammatory cells in this model. Treatment of Pglyrp1−/−Pglyrp2−/− mice with neutralizing anti–IL-22 mAbs significantly reduced ear inflammation in this model compared with mice treated with an isotype control IgG (Fig. 5C), causing ~75% reversal of enhanced swelling in Pglyrp1−/−Pglyrp2−/− mice relative to WT mice (compare differences in swelling in Figs. 5C and 1B). These results show that IL-22 is one of the main IL-17–induced cytokines, but not the only one, responsible for the higher skin inflammatory responsiveness of Pglyrp2−/− mice compared with WT mice.

Our results demonstrate that IL-17 and IL-22 are required for manifestation of the enhanced skin inflammation in Pglyrp2−/− mice in the TPA-induced, psoriasis-like inflammation model. Pglyrp2−/− mice have decreased numbers of Tregs in the skin

We then tested whether the ability of WT mice to limit TPA-induced skin inflammation more effectively than Pglyrp2−/− mice is due to impaired generation or function of Tregs (CD4+Foxp3+ Treg) in Pglyrp2−/− mice. TPA-treated WT mice efficiently recruited Tregs into the affected skin, as evidenced by an increase in Foxp3-expressing cells in the affected skin shown both by the qRT-PCR (Fig. 4) and by flow cytometry, which revealed high numbers of CD4+Foxp3+ cells in the affected skin in WT mice (Fig. 5A). By contrast, Pglyrp-deficient mice that had enhanced inflammatory responses (Pglyrp2−/−, Pglyrp1−/−Pglyrp2−/−, and Pglyrp2−/−Pglyrp3−/− mice) all had lower expression of Foxp3 mRNA in the affected ears compared with WT mice (Fig. 4A, Supplemental Fig. 1). TPA-treated Pglyrp2−/− and Pglyrp1−/−Pglyrp2−/− mice also had significantly lower numbers of CD4+Foxp3+ Tregs in the affected skin compared with WT mice measured by flow cytometry (Fig. 5A). These results suggest impaired recruitment and/or maintenance of Tregs in the inflammation in Pglyrp2−/− mice.

We also compared the numbers of Tregs in the draining cervical lymph nodes and in the spleen of TPA-treated WT and Pglyrp2−/− mice to further investigate whether Pglyrp2−/− mice have less efficient generation of induced Tregs in lymphoid tissues in general or less efficient recruitment and/or maintenance of these cells in the inflamed skin. Pglyrp2−/− and Pglyrp1−/−Pglyrp2−/− mice treated with TPA had significantly higher numbers of Tregs in the cervical lymph nodes and in the spleen than TPA-treated WT mice (Fig. 5A). These results indicate that Pglyrp2−/− and Pglyrp1−/−Pglyrp2−/− mice are able to generate induced Tregs in lymphoid organs and suggest that they are less efficient than WT mice in recruiting these cells to the inflamed skin and/or retaining them in the skin.

There could be at least two reasons for this less efficient recruitment of Tregs to the skin in Pglyrp2−/− mice: insufficient production of Treg-attracting chemokines in the skin and/or insufficient expression of receptors for these chemokines in Tregs in Pglyrp2−/− mice. Our results show lower levels of mRNA for Treg-attracting chemokines (Ccl11, Ccl17, and Ccl27a) in Pglyrp2−/− mice compared with WT mice (Fig. 4B, Pglyrp1−/−Pglyrp2−/− WT 9-d panel; Supplemental Fig. 3), indicating insufficient production of Treg-attracting chemokines in the skin in Pglyrp2−/− mice. To investigate the second of the abovementioned possibilities, we determined whether Tregs in the draining cervical lymph nodes in Pglyrp2−/− mice had sufficient expression of receptors for Treg-attracting chemokines (Ccr4, Ccr8, and Ccr10). Treatment of the ears with TPA for 9 d induced significantly higher levels of mRNA for Ccr4, Ccr8, and Ccr10 in the draining cervical lymph nodes in Pglyrp1−/−Pglyrp2−/− mice compared with WT mice (Fig. 5D, consistent with higher numbers of Tregs in the cervical lymph nodes of Pglyrp1−/−Pglyrp2−/− mice compared with WT mice (Fig. 5A). These results indicate that Tregs in the draining lymph nodes in TPA-treated Pglyrp2−/− mice have sufficient expression of receptors for Treg-attracting chemokines, but that these cells are not recruited to the inflamed skin, likely because of the insufficient production of Treg-attracting chemokines in the skin, as shown above (Fig. 4B, Supplemental Fig. 3). Our results thus indicate that, in WT mice, Pglyrp2 promotes efficient population of the skin with Tregs in the TPA-induced, psoriasis-like inflammation.

**IL-1β is not responsible for the enhanced response to TPA in Pglyrp2−/− mice**

IL-1β is an important proinflammatory cytokine produced by many cell types, and in our model of TPA-induced ear inflammation, IL-1β mRNA was highly induced by TPA in WT mice and also was induced higher in the TPA-sensitive Pglyrp2−/− mice than in WT mice (Fig. 4B, Supplemental Fig. 3). To determine whether IL-1β is required in vivo for the high sensitivity of Pglyrp2−/− mice to TPA-induced skin inflammation, we determined the severity of ear inflammation in TPA-treated Pglyrp1−/−Pglyrp2−/− mice in which IL-1β activity was inhibited with neutralizing anti–IL-1β mAb. In vivo neutralization of IL-1β activity in Pglyrp1−/−Pglyrp2−/− mice had no effect on TPA-
induced ear inflammation compared with mice treated with an isotype control IgG (Fig. 6A). Both groups of mice (anti–IL-1β mAb and control IgG-treated Pglyrp1−/− Pglyrp2−/− mice) also had similar numbers of Th17 cells and Tregs in TPA-treated skin (Fig. 6B, 6C). These results indicate that IL-1β is not responsible for the increased inflammatory response to TPA in Pglyrp2−/− mice or for the decreased numbers of Tregs and increased numbers of Th17 cells in the skin of these mice after TPA treatment; thus, increased IL-1β production in TPA-treated Pglyrp2−/− mice is a consequence but not the cause of increased skin inflammation in Pglyrp2−/− mice.

Discussion

We demonstrate that Pglyrp2−/− mice experience development of more severe TPA-induced, psoriasis-like inflammation than WT mice. Thus, in WT mice, Pglyrp2 protects mice from the development of TPA-induced, psoriasis-like inflammation. The mechanism underlying increased sensitivity of Pglyrp2−/− mice to TPA-induced inflammation is decreased recruitment and activity of Tregs, and enhanced production and activation of Th17 cells in the affected skin, which results in more severe inflammation and keratinocyte proliferation. Thus, in WT mice, Pglyrp2 promotes recruitment and retention of Tregs in the inflamed skin, which limits overactivation of Th17 cells and protects the skin from exaggerated inflammatory response.

Th17 cells were originally thought to play a role in some autoimmune diseases and in recruitment of PMNs to the sites of inflammation (25–28), but they have many other functions. They play a role in inflammatory bowel diseases, skin diseases, asthma, graft rejection, atherosclerosis, periodontal disease, and arthritis (29–31). We extend these findings by showing that Th17 cells exacerbate skin inflammation in the experimental model of TPA-induced, psoriasis-like inflammation in a Pglyrp2-dependent manner. Psoriasis was originally thought to have Th1 bias, but it involves complex interactions of many cell types and lately is considered to have Th17 bias (2, 32–37). Our results support this view and indicate that Pglyrp2 is involved in the control of Th17 cells in the inflamed skin, because increased Th17 activity and their increased recruitment to the skin are associated with more severe psoriasis-like inflammatory phenotype in Pglyrp2−/− mice.

Our results show that Pglyrp2, a member of a family of innate immunity proteins, affects the functions of both innate and adaptive immune cells with an outcome of enhancing the recruitment and activity of Tregs and inhibiting the activity of Th17 cells. Pglyrp2−/− mice have decreased numbers of Tregs and increased numbers of Th17 cells in the inflamed skin compared with WT mice. Proinflammatory stimuli (such as TPA) in WT mice initially induce vigorous cytokine and chemokine production. However, upon chronic exposure, WT mice are able to recruit and maintain large numbers of Tregs in the inflamed skin and are able to limit the proinflammatory response by both drastically reducing the number of proinflammatory genes that are activated and reducing the level of their activation. By contrast, Pglyrp2−/− mice have fewer Tregs and higher numbers of Th17 cells in the affected skin and are unable to limit inflammatory responses.

Human skin is also enriched in Tregs compared with peripheral blood and lymphoid organs; up to 80% of CD4+ T cells could be Foxp3+ Tregs in the skin of healthy control individuals (38). In contrast with these healthy controls, patients with psoriasis (similar to Pglyrp2−/− mice with psoriasis-like skin inflammation) have lower percentages of Tregs in the affected skin, for example, 33 (38), 45 (39), or 50% (40) of total CD4+ cells. Thus, deficient recruitment, generation, or maintenance of Tregs also seems to be a feature of human psoriasis and may be a significant factor contributing to the sensitivity to psoriasis in humans, although this aspect has not been sufficiently studied in human psoriasis.

There may be multiple reasons for the imbalance in Treg/Th17 cells that we observed in Pglyrp2−/− mice. It could come from reduced recruitment of Tregs and increased recruitment of Th17 cells to the affected skin, and/or from enhanced local differentiation of T cells into Th17 cells (including conversion of Tregs into Th17 cells) under the influence of locally produced chemokines and cytokines. T cell populations are dynamic and have considerable plasticity based on local cytokine milieu, as, for example, Tregs can differentiate into Th17 cells under the influence of locally produced proinflammatory cytokines (41, 42). The enhanced recruitment and differentiation of Th17 cells is supported by higher production of Th17 cell-promoting cytokines (IL-17, IL-22, IL-23) in the inflamed skin in Pglyrp2−/− mice. Decreased recruitment of Tregs to the inflamed skin is supported by the

**FIGURE 6.** IL-1β is not responsible for greater inflammation, increased numbers of Th17 cells, and decreased numbers of Tregs in the skin of TPA-treated Pglyrp1−/− Pglyrp2−/− mice. A, Ear swelling in Pglyrp1−/− Pglyrp2−/− mice treated with TPA on days 0, 2, 4, and 6, and also treated with either isotype control IgG or neutralizing anti–IL-1β mAbs. Data are means ± SEM. B, Percentages of Th17 cells and Tregs in the ears of Pglyrp1−/− Pglyrp2−/− mice on day 9 after application of TPA to the ears on days 0, 2, 4, 6, and 8 measured by flow cytometry. Data are means ± SEM. C, Representative dot plots for Th17 cells and Tregs. n = 7 mice/group; all differences between IgG control and anti–IL-1β mAb-treated mice were NS (p > 0.05).
presence of higher numbers of Tregs in the draining lymph nodes and spleen in TPA-treated Pglyrp2−/− mice than in WT mice, but lower numbers in the skin. These Tregs in the draining lymph nodes in Pglyrp2−/− mice express receptors for Treg-attracting chemokines (CCR4, CCR8, and CCR10), yet do not migrate in sufficient numbers to the inflamed skin. These results suggest efficient generation of induced Tregs in lymphoid organs but defective recruitment to the inflamed skin. This mechanism is further supported by decreased production of Treg-attracting chemokines (CCL1, CCL17, CCL27) in the skin of Pglyrp2−/− mice. Thus, both increased recruitment and generation of Th17 cells, and decreased recruitment and retention of Tregs in the skin are likely responsible for increased inflammation in Pglyrp2−/− mice.

Pglyrp2 is primarily expressed in the liver, and its expression is induced in other nonimmune cells, including keratinocytes (4, 5, 15, 17). Keratinocytes are an important local source of chemokines and cytokines, and activation of keratinocytes by proinflammatory stimuli and increased expression of Pglyrp2 correlates with the ability of WT mice to reduce chronic inflammation in the skin. The lack of Pglyrp2 correlates with increased inflammation in Pglyrp2−/− mice. The effects of Pglyrp2 in the inflamed skin are likely exerted through a change in the local production of chemokines and cytokines in the skin, which modulates the recruitment and activity of these Tregs and Th17 cells. Thus, in Pglyrp2−/− mice, reduced numbers of Tregs allow dominating expansion of Th17 cells, which can increase inflammatory responses in the skin. Therefore, in WT mice compared with Pglyrp2−/− mice, the immune balance is shifted toward Tregs that control detrimental inflammation induced by proinflammatory Th17 cells. Our results suggest that defects in the Pglyrp2 gene could be predisposing to psoriasis-like skin inflammation through the aforementioned shift in immune homeostasis.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1. TPA-treated *Pglyrp*−/− mice have increased PMNs, monocytes, B cells, and Th17 cells in the affected skin. Expression of a panel of marker genes characteristic of various inflammatory cell types in the ears of mice on day 9 after application of TPA to the ears on days 0, 2, 4, 6, and 8 measured by qRT-PCR is shown. For WT mice (top panel), the ratio of the amount of mRNA in TPA-treated to untreated mice for each gene (fold induction by TPA) is shown; for *Pglyrp*−/− mice, the results are the ratios of fold induction of each gene by TPA in *Pglyrp*−/− mice to fold induction of each gene by TPA in WT mice (which represents the fold difference in the response to TPA in *Pglyrp*−/− versus WT mice). The results are means ± SEM of 3 arrays from 4–5 mice/group and are shown as heat maps in Fig. 4A in the main article.
Supplemental Figure 2. Multiple inflammatory and immune genes are induced early in TPA-treated skin. Expression of a panel of cytokines, chemokines, and other marker genes characteristic of Th1, Th2, Th17, Treg, NK, and other cell types in the ears of mice 6 hrs after a single application of TPA to the ears measured by qRT-PCR. For WT mice (top panel), the ratio of the amount of mRNA in TPA-treated to untreated mice for each gene (fold induction by TPA) is shown; for Pglyrp2−/− mice the results are the ratios of fold induction of each gene by TPA in Pglyrp2−/− mice to fold induction of each gene by TPA in WT mice (which represents the fold difference in the response to TPA in Pglyrp−/− versus WT mice). The results are means ± SEM of 3 arrays from 4–5 mice/group and are shown as heat maps in Fig. 4B in the main article.
Supplemental Figure 3. Th17 gene expression profile is preferentially induced by TPA in the affected skin of *Pglyrp2*−/− mice. Expression of a panel of cytokines, chemokines, and other marker genes characteristic of Th1, Th2, Th17, Treg, NK, and other cell types in the ears of mice on day 9 after application of TPA to the ears on days 0, 2, 4, 6, and 8 shows higher induction of several Th17 marker genes in *Pglyrp2*−/− mice compared to WT mice (in addition to genes characteristic of many cell types) measured by qRT-PCR. For WT mice (top panel), the ratio of the amount of mRNA in TPA-treated to untreated mice for each gene (fold induction by TPA) is shown; for *Pglyrp2*−/− mice, the results are the ratios of fold induction of each gene by TPA in *Pglyrp2*−/− mice to fold induction of each gene by TPA in WT mice (which represents the fold difference in the response to TPA in *Pglyrp*−/− versus WT mice). The results are means ± SEM of 3–4 arrays from 4–5 mice/group and are shown as heat maps in Fig. 4B in the main article.
Supplemental Figure 4. A. Representative dot plots of Th1 and T2 cells in the ears of WT, Pglyrp2−/− and Pglyrp1−/−Pglyrp2−/− mice on day 9 after application of TPA to the ears on days 0, 2, 4, 6, and 8 measured by flow cytometry. The means ± SEM of 5–9 mice/group are shown in Fig. 5A in the main article. B. Representative dot plots with ear CD4+ lymphocytes from Pglyrp2−/− mice after 9 days of treatment with TPA as above in A, stained with isotype control IgG for IL-17 Ab (Th17 cells), IFN-γ Ab (Th1 cells), IL-4 Ab (Th2 cells), or Foxp3 Ab (Treg cells).