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

J Immunol published online 2 November 2011
http://www.jimmunol.org/content/early/2011/11/02/jimmunol.1101068

Supplementary Material
http://www.jimmunol.org/content/suppl/2011/11/02/jimmunol.1101068.DC1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2011 by The American Association of
Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
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.
Th17 cells by promoting accumulation of Tregs at the site of inflammation, which protects the skin from the exasperated inflammatory response.

**Materials and Methods**

**Mice**

We generated Pglyrp1−/−, Pglyrp2−/−, Pglyrp3−/−, and Pglyrp4−/− mice as described previously (12, 19, 20). We generated Pglyrp1−/− Pglyrp2+/−, Pglyrp1−/− Pglyrp3−/−, and Pglyrp2−/− Pglyrp3−/− double-knockout mice and Pglyrp1−/− Pglyrp2−/− Pglyrp3−/− 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. 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 litters 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-Department of Medicine–Nestle Institutional Animal Care and Use Committee.

**12-O-tetradecanoylphorol 13-acetate–induced inflammation model**

A total of 20 μl 0.01% 12-O-tetradecanoylphorol 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 10% formalin, and embedded in paraffin; cross sections were stained with hematoxylin and eosin (H&E) or tartrazine for evaluation of parakeratotic hyperkeratosis. Sections were evaluated using ImageJ software from the National Institutes of Health.

**RNA and qRT-PCR**

RNA was isolated from the entire untreated or treated ears or cervical lymph nodes using the TRIzol method (Invitrogen), followed by digestion with RNase-free DNase (Qiagen) and purification on RNeasy spin columns using RNeasy Minikit (Qiagen). qRT-PCR was used to quantify the amounts of mRNA in the ears or lymph nodes using custom RT2 Profiler PCR Arrays designed by us and manufactured by Qiagen/SA Biosciences, as previously described (19). RT2 Profiler PCR Arrays typically contain 30–54 assays for housekeeping genes, and reverse transcription efficiency and DNA contamination controls. All primer sets were from Qiagen/SA Biosciences, except the following primers that we designed: Pglyrp1, exon 1 and 2 primers, 5'-GTGGTGTACTTCACACAGC-3' and 5'-GGTGGTAGCCTTGTAGTGT-3'; Pglyrp2, exons 3 and 4 primers, 5'-ACAGAGGTTGCGAAGTGG-3' and 5'-AGTGCCACGATGATGTGCA-3'; and Pglyrp4, exons 4 and 5 primers, 5'-CACACCGGCTTACACAGGA-3' and 5'-CCAGCCGCTTCTTCATCCTTT-3'. cDNA was synthesized from 2 μg RNA using RT2 PCR Array First Strand Kit (Qiagen/SA Biosciences), and the arrays were performed according to the manufacturer’s instructions using Qiagen/SA Biosciences Master Mix. The lists of genes are provided in the figures. The experiments were performed on RNA pooled from 4–5 mice/group and repeated 3 times, usually with another set of 4–5 mice/group (usually total of 8–10 mice/treatment).

For each gene, ΔCt (cycle threshold) was calculated using the same threshold (0.2) for all genes, and Ct ≤ 35 was considered as no expression, followed by normalization to five housekeeping genes (Hsp90ab1, Gusb, Hprt1, Gapdh, and Actb) included in each array, followed by calculation of ΔΔCt: ΔΔCt = ΔCt2 − ΔCt1, where ΔCt1 is the TPA-treated mice and ΔCt2 is the untreated mice, using the program provided by Qiagen/SA Biosciences. This calculation gives the fold increase in expression of each gene in the treated mice versus untreated mice per microgram RNA and does not include in the calculation the increased amount of RNA obtained from the ears of TPA-treated mice compared with untreated mice (and thus the increases in gene expression would be smaller 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. ΔCt was 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 for each gene from two arrays for each group (usually total of 4–5 mice/group and repeated 3 times, usually with another set of 4–5 mice/group). The results were reported as mean fold increases for each gene from two arrays for each group (usually total of 4–5 mice/group and repeated 3 times, usually with another set of 4–5 mice/group). The results were reported as mean fold increases for each gene from two arrays for each group (usually total of 4–5 mice/group and repeated 3 times, usually with another set of 4–5 mice/group). The results were reported as mean fold increases for each gene from two arrays for each group (usually total of 4–5 mice/group and repeated 3 times, usually with another set of 4–5 mice/group).
Results

Pglyrp2−/− 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−/− 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−/− mice, because it was not observed in Pglyrp1−/−, Pglyrp3−/−, and Pglyrp4−/− mice, and was dominant, because it was still observed in Pglyrp1−/− Pglyrp2−/− and in Pglyrp2−/− Pglyrp3−/− double-knockout mice (Fig. 1B). Deletion of Pglyrp1 had an opposite effect on the early response to TPA, because Pglyrp1−/− single-knockout, Pglyrp1−/− Pglyrp3−/− double-knockout, and Pglyrp1−/− Pglyrp2−/− Pglyrp3−/− 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−/− 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−/− 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...
marked thickening of the subepidermal layer with dense cellular infiltrates (containing many PMNs and some mononuclear cells) that were all highly prominent in \textit{Pglyrp2}^{−/−} mice, \textit{Pglyrp1}^{−/−}\textit{Pglyrp2}^{−/−} mice (Fig. 2A), and all other double- and triple-knockout mice deficient in \textit{Pglyrp2} (not shown). All these changes are highly characteristic of psoriatic lesions. WT (Fig. 2), \textit{Pglyrp1}^{−/−}, \textit{Pglyrp3}^{−/−}, and \textit{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 \textit{Pglyrp2}^{−/−} and \textit{Pglyrp1}^{−/−}\textit{Pglyrp2}^{−/−} mice than in WT mice (Fig. 2B).

Our results demonstrate that deletion of \textit{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, \textit{Pglyrp2}^{−/−} mice can serve as a convenient new mouse model of psoriasis-like inflammation. These results also further demonstrate that, in WT mice, \textit{Pglyrp2} protects the skin from excessive TPA-induced, psoriasis-like inflammation.

\textbf{FIGURE 2.} Ear histology in TPA-treated \textit{Pglyrp2}^{−/−} mice has many features of psoriasis. TPA application to the ears on days 0, 2, 4, 6, and 8 induced in \textit{Pglyrp2}^{−/−} mice acanthosis (Ac), formation of rete pegs (Rp), parakeratosis (Pk), parakeratotic scaling, formation of numerous epidermal microabscesses (M, primarily neutrophilic), and marked thickening of the subepidermal layer (Se) with dense cellular infiltrates (containing mainly PMNs and mononuclear cells), which were all highly prominent in \textit{Pglyrp2}^{−/−} and \textit{Pglyrp1}^{−/−}\textit{Pglyrp2}^{−/−} mice, and less severe in WT mice. A, H&E stained cross sections at low magnification (large panels, scale bar, 200 μm) with high-magnification insets. B, Semiquantitative evaluation of the extent of the indicated histological changes and quantitative measurements of the surface areas of microabscesses on cross sections of the ears. Data are means ± SEM. \textit{n} = 40 high-power fields for histology scores; \textit{n} = 78–123 microabscesses, all from 4 mice/group. Significance of differences between \textit{Pglyrp}^{−/−} and WT mice: *\textit{p} < 0.02, **\textit{p} < 0.001.
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 Pglyrp4−/− 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 susceptibility 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. Pglyrp-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 Rorγt-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 Pglyrp-deficient mice that are more sensitive to TPA-induced, psoriasis-like inflammation compared with WT mice may be the Rorγt-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 Pglyrp-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.
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

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, and 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).
nodes (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 Th17 cells/ear in Pglyrp1−/−Pglyrp2−/− mice 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 (<50/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+).

TPA-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 WT mice to TPA-induced skin inflammation more effectively than Pglyrp2−/− mice (Fig. 5A). This was shown 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 inflamed skin 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, 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−/− mice promote 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 it was also 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 num-
bers 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 inflam-
nation 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 devel-
opment of TPA-induced, psoriasis-like inflammation. The mech-
anism 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 au-
toimmune 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 in-
duce vigorous cytokine and chemokine production. However,
on 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 (simi-
lar 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 differenti-
ation 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 consid-
erable plasticity based on local cytokine milieu, as, for example,
Tregs can differentiate into Th17 cells under the influence of lo-
cally 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 in the skin of Pglyrp2 mice. Thus, both 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.

Acknowledgments

We are grateful to Bankston for help in interpreting histology slides and Robert Rukavina, Julie Cook, Panida Giridfonoff, and Tiffany Calaug for maintaining and breeding mice.

Disclosures

The authors have no financial conflicts of interest.

References

3. Kang, D., G. Liu, A. Lundstrom, E. Gelius, and H. Steiner. 1998. A peptidoglycan recognition protein 2 (N-acetylmuramoyl-L-Ala amidase) is induced in keratinocytes by proinflamatory cytokines and chemokines 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.

Acknowledgments

We are grateful to Bankston for help in interpreting histology slides and Robert Rukavina, Julie Cook, Panida Giridfonoff, and Tiffany Calaug for maintaining and breeding mice.

Disclosures

The authors have no financial conflicts of interest.

References

3. Kang, D., G. Liu, A. Lundstrom, E. Gelius, and H. Steiner. 1998. A peptidoglycan recognition protein 2 (N-acetylmuramoyl-L-Ala amidase) is induced in keratinocytes by proinflamatory cytokines and chemokines 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.

Acknowledgments

We are grateful to Bankston for help in interpreting histology slides and Robert Rukavina, Julie Cook, Panida Giridfonoff, and Tiffany Calaug for maintaining and breeding mice.

Disclosures

The authors have no financial conflicts of interest.

References

3. Kang, D., G. Liu, A. Lundstrom, E. Gelius, and H. Steiner. 1998. A peptidoglycan recognition protein 2 (N-acetylmuramoyl-L-Ala amidase) is induced in keratinocytes by proinflamatory cytokines and chemokines 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.

Acknowledgments

We are grateful to Bankston for help in interpreting histology slides and Robert Rukavina, Julie Cook, Panida Giridfonoff, and Tiffany Calaug for maintaining and breeding mice.

Disclosures

The authors have no financial conflicts of interest.

References

3. Kang, D., G. Liu, A. Lundstrom, E. Gelius, and H. Steiner. 1998. A peptidoglycan recognition protein 2 (N-acetylmuramoyl-L-Ala amidase) is induced in keratinocytes by proinflamatory cytokines and chemokines 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.

Acknowledgments

We are grateful to Bankston for help in interpreting histology slides and Robert Rukavina, Julie Cook, Panida Giridfonoff, and Tiffany Calaug for maintaining and breeding mice.

Disclosures

The authors have no financial conflicts of interest.


