

A GPR40 Agonist GW9508 Suppresses CCL5, CCL17, and CXCL10 Induction in Keratinocytes and Attenuates Cutaneous Immune Inflammation

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G-protein coupled receptors (GPCR) exert diverse physiological functions, many of which are exploited therapeutically. The roles of GPCR in keratinocytes in immune response in the skin, however, remain poorly defined. In this study, we focused on Gi-coupled GPCR in keratinocytes and defined their actions in immunoactivation of cultured keratinocytes *in vitro* and immune reaction in the skin *in vivo*. We first activated HaCaT cells by tumor necrosis factor (TNF)- α and IFN- γ and examined effects of various ligands for GPCR on production of CCL17 and CCL5. Agonists for Gi-coupled receptors, particularly GW9508 for GPR40, inhibited CCL17 and CCL5 expression in a pertussis toxin-sensitive manner. The inhibitory effect by GW9508 was abrogated by depletion of GPR40 with RNA interference. GW9508 further suppressed expression of IL-11, IL-24, and IL-33 induced in HaCaT cells by TNF- α and IFN- γ . GW9508 also inhibited CCL5 and CXCL10 production by normal human epidermal keratinocytes. Administration of GW9508 topically to the skin in the challenging phase suppressed ear swelling in a repeated hapten application model and contact hypersensitivity with downregulation of CCL5 and CXCL10, respectively. Thus, in the skin, stimulation of Gi-coupled receptors attenuates induction of critical cytokines and chemokines by proinflammatory cytokines in keratinocytes and suppresses allergic inflammation in the skin.

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INTRODUCTION

G-proteins consisting of α , β , and γ subunits are signal transducers and mediate actions of G-protein coupled receptors (GPCR) with seven transmembrane domains in the plasma membrane (Neves *et al.*, 2002). Each GPCR is coupled to either a different class of G-proteins, Gs, Gi, Gq, or G_{12/13}, or a combination thereof. Gs α activates adenylate cyclase, and raises the intracellular concentration of cAMP. On the other hand, Gi α inhibits adenylate cyclase and decreases intracellular cAMP. Among more than 1,000 GPCR encoded by mammalian genomes, up to 400–500 GPCR recognize neurotransmitters, hormones, and paracrine factors, whereas others are involved in sensory functions

such as taste or odor perception. Notably, about half of all drugs currently used clinically target GPCR in various systems from the central nervous system to the cardiovascular system. However, the role of GPCR in the immune system remains obscure and their therapeutic application in this area has been limited to those for chemokines and other chemotactic substances such as sphingosine-1-phosphate (Rosen *et al.*, 2007). GPCR have been generally viewed as mediating immunosuppression through elevation of intracellular cAMP in cells such as macrophages and T cells (Nance and Sanders, 2007). However, contrary to this oversimplified view, recent studies have indicated that GPCR have diverse roles in immunity depending on the context of immune response. For example, we have recently shown that stimulation of the cAMP pathway by prostaglandin E receptors EP2 and EP4 exerts significant facilitation of Th17 expansion by regulating IL-23 expression by dendritic cells and enhancing the IL-23-induced Th17 amplification (Yao *et al.*, 2009). These findings have questioned the simple paradigm of the immunosuppression by cAMP and suggest that the action of a GPCR in the immune system has to be defined in the contexts of target cells and tissues, timing of stimulation, types of other combined stimuli, and output of the response at various levels.

The incidence and severity of allergic skin diseases such as contact hypersensitivity (CHS) and atopic dermatitis (AD)

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Abbreviations: AD, atopic dermatitis; CHS, contact hypersensitivity; GPCR, G-protein coupled receptors; NHEK, normal human epidermal keratinocyte; PTX, pertussis toxin; QRT-PCR, quantitative RT-PCR; TNF, tumor necrosis factor; TNF- α + IFN- γ , TNF- α and IFN- γ in combination

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have increased extensively over the last 20 years in developed countries, and drugs targeted to these diseases are urgently demanded. In these allergic diseases, keratinocytes have a crucial role in disease progression (Girolomoni *et al.*, 2004; Pastore *et al.*, 2006). We previously reported that administration of an agonist of prostaglandin E receptor EP3, a Gi-coupled receptor, attenuates allergic asthma (Kunikata *et al.*, 2005), CHS (Honda *et al.*, 2009), and allergic conjunctivitis (Ueta *et al.*, 2009). Stimulation of cannabinoid CB1 and CB2 receptors, both coupled to Gi, has also been shown to induce attenuation of DNFB-induced CHS (Karsak *et al.*, 2007). Although EP3 is expressed on epithelial cells in the airway and conjunctiva and keratinocytes in the skin and EP3 in these cells is suggested to mediate the above therapeutic action, a direct proof of the involvement of Gi-coupled receptors in keratinocytes in such action is lacking. In this study, we first sought to define the actions of Gi-coupled receptors in cultured keratinocytes and then to analyze the effects of such pathway in elicitation of immune inflammation in the skin. Here, we used HaCaT, a human epidermal keratinocyte cell line, normal human epidermal

keratinocytes (NHEKs), and mouse models of AD and CHS, and addressed these issues.

RESULTS

GPR40 agonist GW9508 suppresses CCL17 production through pertussis toxin-sensitive Gi protein

We first used HaCaT human epidermal keratinocyte cell line. Previous reports showed that HaCaT cells produce CCL17 in response to stimulation with tumor necrosis factor (TNF)- α or IFN- γ alone, or TNF- α and IFN- γ in combination (TNF- α + IFN- γ ; Saeki and Tamaki, 2006). We exploited this finding and reproduced synergistic production of CCL17 by TNF- α + IFN- γ in HaCaT cells, both at mRNA and protein levels (Figure 1a). We also found that TNF- α and IFN- γ synergistically enhance CCL5 production by HaCaT cells (Figure 1b). CCL17 and CCL5 are produced by keratinocytes in inflammatory states and have crucial roles in Th2-type immune responses such as AD (Pastore *et al.*, 2006; Saeki and Tamaki, 2006). To screen GPCR ligands that inhibit the TNF- α + IFN- γ -induced chemokine expression, we examined the activity of compounds in the Tocriscree Mini library in

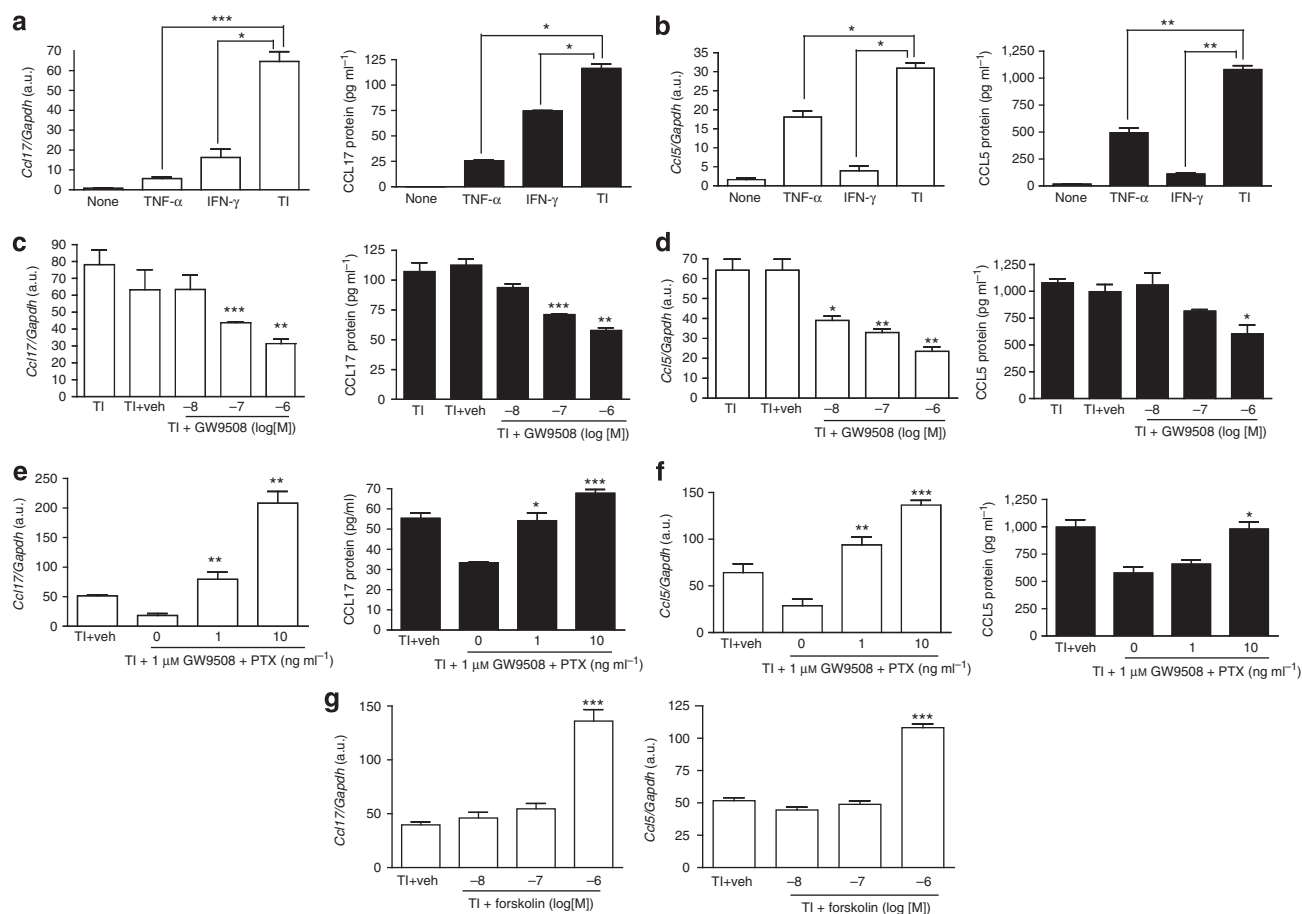


Figure 1. Inhibition by GW9508 of tumor necrosis factor (TNF)- α + IFN- γ -induced production of CCL17 and CCL5 in HaCaT cells. CCL17 (a) and CCL5 (b) production by TNF- α and/or IFN- γ . Inhibitory effect of GW9508 on TNF- α + IFN- γ -induced CCL17 (c) and CCL5 (d) production. Effect of pertussis toxin (PTX) (e and f) and forskolin (g) on TNF- α + IFN- γ -induced CCL17 and CCL5 production. TI, TNF- α + IFN- γ ; veh, vehicle. Quantitative real-time RT-PCR data are shown as arbitrary units (a.u.) where the value for unstimulated sample is set at 1. Results show a representative of three independent experiments. Data show mean $\pm$ SEM ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus TI + veh in (c–f).

this system with CCL17 as a parameter. This screening revealed that several agonists for putative Gi-coupled receptors, such as ST91 for α_2 adrenergic receptor, GP1a and GP2a for CB2, 3'-fluorobenzyl-spiperone for dopamine D2, and GW9508 for GPR40 potentially suppressed the TNF- α + IFN- γ -induced CCL17 production (Supplementary Table S1 online). The presence of these receptors in HaCaT cells was confirmed by GPCR array analysis and indicated as ΔC_t (Supplementary Table S1 online). We chose GW9508 for further analysis, because it most potently suppressed CCL17 expression. To verify the finding in the screening, we examined the dose dependency of the inhibitory effect of this compound on CCL17 and CCL5 by quantitative real-time RT-PCR (QRT-PCR) and ELISA. We found the concentration-dependent suppression of both CCL17 and CCL5 mRNA and protein production by GW9508 in HaCaT cells (Figure 1c and d) without any significant effect on their viability (Supplementary Figure S1 online). This inhibitory action of GW9508 was reversed by low doses of pertussis toxin (PTX; Figure 1e.), verifying that GPR40 stimulation exerts such effects through Gi. Conversely, forskolin, an adenylate cyclase activator, enhanced TNF- α + IFN- γ -induced production of CCL17 and CCL5 (Figure 1g). On the other hand, inhibitors of AKT, PI3 kinase, p38 MAPK, MEK, and Src did not reverse the effect of GW9508 (data not shown). These results collectively suggest that PTX-sensitive Gi signaling suppresses CCL17 and CCL5 production induced by TNF- α + IFN- γ through decrease in cAMP.

Although GW9508 is an agonist of GPR40, it also acts on GPR120 at higher concentrations ($pEC_{50} = 7.32 \pm 0.03$ and $pEC_{50} = 5.46 \pm 0.09$, respectively; Briscoe *et al.*, 2006). Reverse transcription PCR analysis detected expression of both GPR40 and GPR120 mRNA in HaCaT cells (Figure 2a). To verify that the immunosuppressive effect is mediated by GPR40, we knocked down GPR40 with RNA interference. Small interfering RNA for GPR40 significantly decreased the amount of GPR40 mRNA compared with control small interfering RNA after 72 hours (Figure 2b). Depletion of GPR40 by this procedure reversed the suppressive effect of $1 \mu M$ GW9508 on CCL17 and CCL5 production (Figure 2c), corroborating that GW9508 exerts its inhibitory effect through GPR40 and not GPR120 in HaCaT cells.

GW9508 inhibits CCL5 and CXCL10 production by NHEK

To further corroborate the findings in HaCaT cells, we next used primary culture of NHEKs. NHEK cells expressed GPR40 but not GPR120 (Figure 3a). Consistent with a previous report (Tsuda *et al.*, 2003), we did not find significant production of CCL17 by NHEK on stimulation with TNF- α , IFN- γ , or TNF- α + IFN- γ (data not shown). On the other hand, we noted induction of CCL5 by IFN- γ alone, and found that this induction was concentration-dependently inhibited by GW9508 (Figure 3b). Under the same conditions, we also noted production of CXCL10, which was also inhibited by GW9508 (Figure 3c). Again, GW9508 was without effect on viability of NHEK (Supplementary Figure S1 online). Therefore, GW9508 suppresses

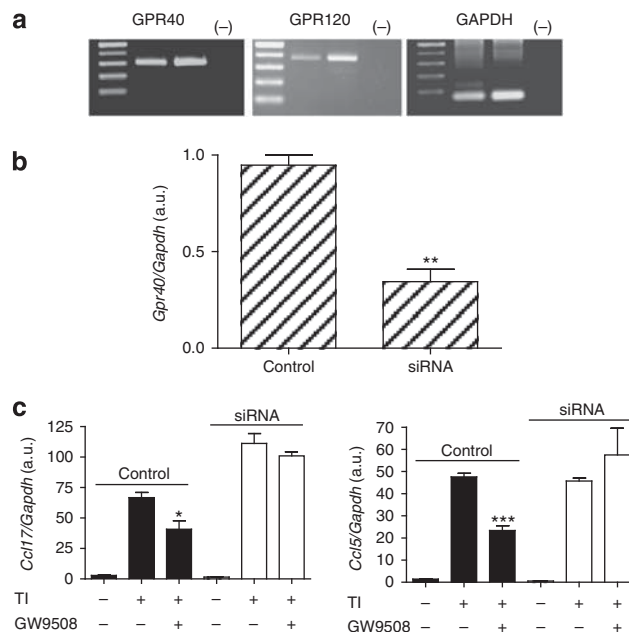


Figure 2. GPR40 expression in HaCaT cells and GPR40 knockdown experiments. (a) GPR40 and GPR120 mRNA. Data of two different batches of HaCaT cells are shown. (–), Control without template. (b) Reduction of GPR40 mRNA by small interfering RNA (siRNA) treatment. (c) Reversal of GW9508-mediated inhibition of CCL17 (left) and CCL5 (right) mRNA expression by GPR40 depletion. Control, control siRNA. Bars = 50 μm . Results show a representative of three independent experiments. Data show mean $\pm$ SEM ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control RNA interference (b) or TI + veh (c).

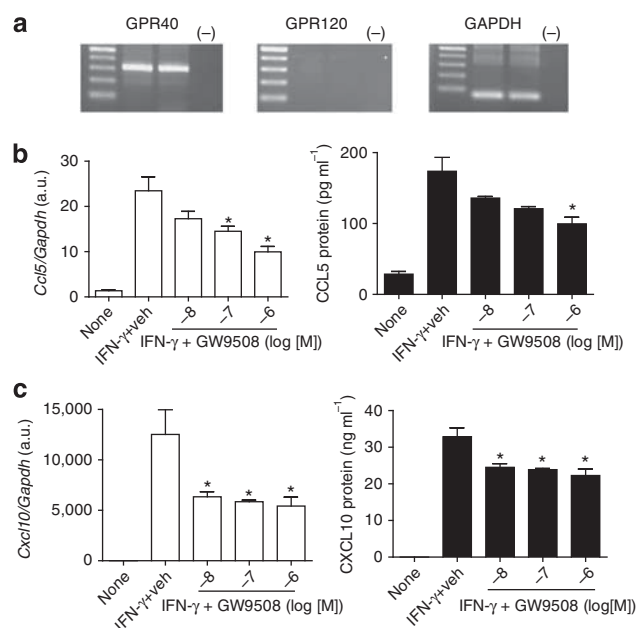


Figure 3. Effects of GW9508 on CCL5 and CXCL10 production in NHEK. Reverse transcription PCR analysis of GPR40 and GPR120 mRNA (a) and inhibition by GW9508 of CCL5 (b) and CXCL10 (c) production in normal human epidermal keratinocyte (NHEK). In (a), data of two different batches of NHEK are shown. (–), Control without template. QRT-PCR data are shown as arbitrary units (a.u.) where the value for unstimulated sample is set at 1 (b and c, left). Veh, vehicle. Results show a representative of three independent experiments. Data show mean $\pm$ SEM ($n = 3$). * $P < 0.05$ versus IFN- γ + veh.

IFN- γ -induced CCL5 and CXCL10 production through GPR40.

Changes in gene expression pattern after GW9508 treatment

Previous studies reported that besides CCL17, many chemokines and cytokines are also markedly elevated in HaCaT cells after combined stimulation with TNF- α + IFN- γ (Xiao *et al.*, 2003; Lee *et al.*, 2009; Lo *et al.*, 2010). Therefore, we speculated that many other proinflammatory proteins are upregulated by TNF- α + IFN- γ and that their expression might be differentially regulated by GPR40 stimulation. We conducted comparative microarray analyses for gene expression and noted that 16 cytokines and 13 chemokines were upregulated more than 2-fold 24 hours after the TNF- α + IFN- γ stimulation (Supplementary Table S2 online). GW9508 treatment resulted in no further upregulation of any genes but induced reduction of expression of five genes, CCL17, CCL5, IL-11, IL-24, and IL-33 (Supplementary Table S3 online). IL-33 is a Th2-related cytokine, which activates basophils and acts on dendritic cells to promote Th2 immunity (Kakkar and Lee, 2008) and its expression is elevated in lesional skin of AD patients (Pushparaj *et al.*, 2009).

Suppression by GW9508 of ear inflammation by repeated DNFB treatment

The above results demonstrate that stimulation of GPR40 suppresses the cytokine-induced expression of CCL17 and CCL5 in HaCaT cells and that of CCL5 and CXCL10 in NHEK. To assess the *in vivo* effects of GPR40 stimulation, we examined whether treatment with GW9508 could exert effects expected from the *in vitro* experiments in cutaneous immune inflammation models. We first examined GPR40 expression in keratinocytes of the mouse ear. Reverse transcription PCR analysis of the whole ear showed a similar extent of GPR40 mRNA expression in this tissue of BALB/c and C57BL/6 mice (Figure 4a). *In situ* hybridization for GPR40 mRNA further showed that GPR40 was expressed in the epidermis of BALB/c mice (Figure 4b) and in the epidermis and the dermis of C57BL/6 mice (Figure 4c). Reverse transcription PCR did not detect GPR120 mRNA expression in mouse epidermis (Figure 4a).

To examine effects of GW9508 on a Th2-type inflammation, we adopted repeated DNFB treatment in BALB/c mice. Repeated elicitation with hapten results in a shift from Th1-mediated to Th2-mediated inflammation (Kabashima *et al.*, 2003; Inagaki *et al.*, 2006), which mimics AD. From the fifth to the ninth elicitation, we applied 20 μ l of 200 μ M GW9508 or vehicle topically to the ear 1 h before each DNFB application. Treatment with GW9508 resulted in significant reduction of ear swelling compared with the control mice (Figure 5a). Histological examination revealed diminished epidermal thickening and attenuated cell infiltration in the dermis of the GW9508-treated group compared with the vehicle-treated group (Figure 5b). We next examined the expression of CCL17 or CCL5 in the epidermis. QRT-PCR analysis revealed that, although expression of CCL17 showed no elevation in either group compared with the

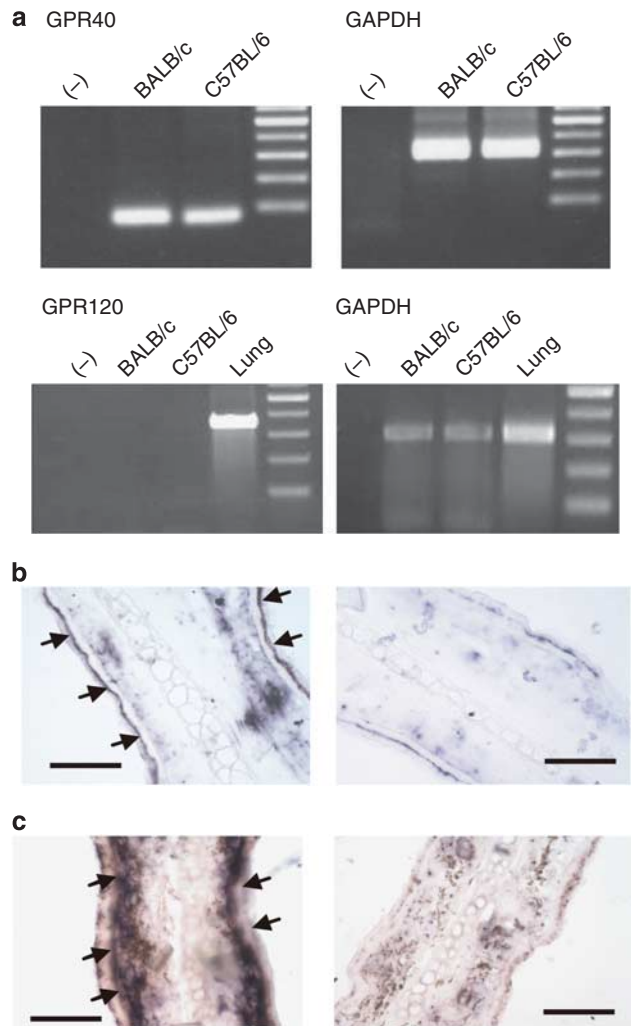


Figure 4. GPR40 and GPR120 expression in mouse epidermis. (a) Reverse transcription PCR (RT-PCR) of GPR40 mRNA expression in the ear (upper row) and GPR120 mRNA expression in the epidermis (lower row) of BALB/c and C57BL/6 mice. Mouse lung cDNA was used as a positive control for PCR of GPR120 (Hirasawa *et al.*, 2005). (-), Control without template. (b,c) *In situ* hybridization for GPR40 mRNA in unstimulated ears of BALB/c mice (b) and C57BL/6 mice (c). Left, hybridization with antisense probe; right, hybridization with sense probe. Positive signals are indicated with arrows. Bars = 100 μ m. A representative of three independent experiments is shown.

non-immunized, non-challenged group, CCL5 expression was significantly elevated in the immunized, challenged and vehicle-treated group (the vehicle-treated group) and the GW9508 treatment significantly attenuated the expression of this chemokine compared with that found in the vehicle-treated group (Figure 5c). Immunostaining for CCL5 revealed that the CCL5 signal was enriched in the cytoplasm of keratinocytes of the skin of the vehicle-treated animals, and that the GW9508 treatment markedly attenuated the signal (Figure 5d). Consistent with this suppression, the number of eosinophils infiltrating in the skin was significantly suppressed in the GW9508-treated group compared with the control, vehicle-treated group (Figure 5b and e). Attenuation of mast cell infiltration was also noted in the

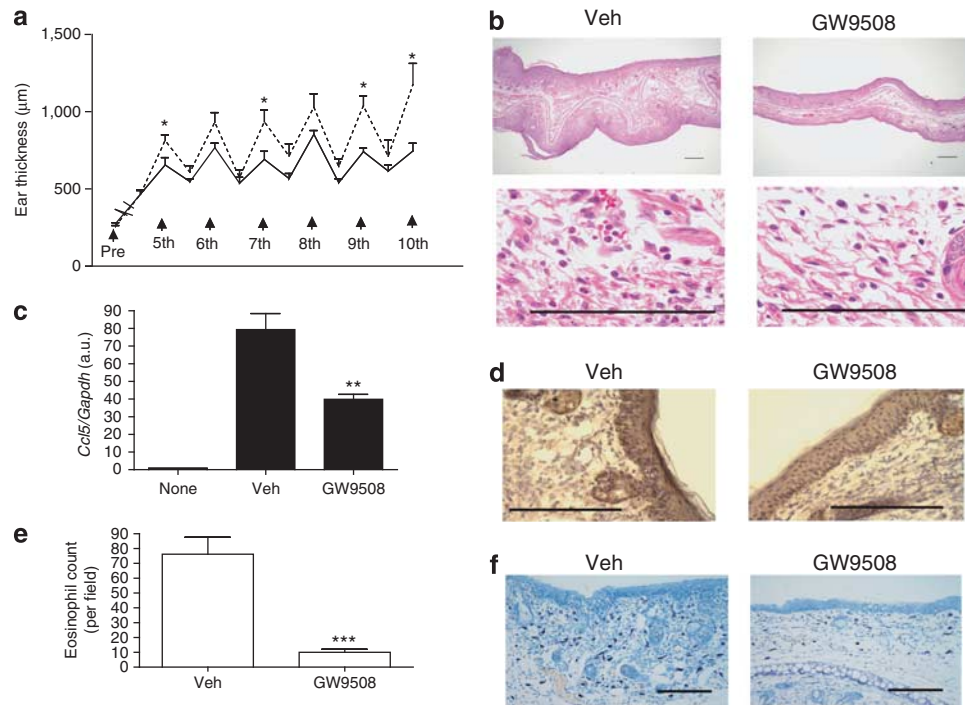


Figure 5. Inhibition by GW9508 of immune inflammation with repeated DNFB treatment, a model of atopic dermatitis. (a) Time course of ear swelling of control- (broken line) and GW9508-treated (solid line) mice. (b) Hematoxylin-eosin staining. (c) QRT-PCR analysis of CCL5 mRNA expression. Data are shown as arbitrary units (a.u.) where the value for unstimulated group is set at 1. (d) Immunohistochemistry for CCL5 in inflamed ears. (e) Eosinophil count per field. (f) Toluidine blue staining. None, non-immunized and unstimulated group; veh, vehicle-treated group; GW9508, GW9508-treated group. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ versus vehicle. Data show mean $\pm$ SEM of four to five mice. Bars = 100 μ m. A representative of three independent experiments is shown.

GW9508-treated group (Figure 5f). These data collectively suggest that GW9508 reduces CCL5 production in keratinocytes and infiltration of eosinophils and mast cells, and attenuates ear swelling in repeated DNFB treatment, a model of AD.

Suppression by GW9508 of elicitation of CHS

We next examined whether GPR40 stimulation has a suppressive effect also in Th1-type skin inflammation, because GPR40 stimulation inhibited CXCL10 production in NHEK. CXCL10 recruits Th1 cells and is known to have important roles in Th1-type immune responses (Nakae *et al.*, 2003). To this end, we subjected wild-type C57BL/6 mice to DNFB-induced CHS and treated mice with different concentrations of GW9508 topically to the ear 30 minutes before the challenge. Application of GW9508 suppressed ear swelling in a concentration-dependent manner (Figure 6a). Histological examination revealed marked attenuation of epidermal thickening and infiltration of cells in the dermis in the GW9508-treated group (Figure 6b). QRT-PCR analysis of the whole ear showed marked elevation of CXCL10 mRNA expression in mice subjected to CHS, and this increase was significantly suppressed by GW9508 treatment (Figure 6c). Changes in expression of CCL5 and CCL17 could not be detected, probably because the mouse CHS is Th1-dependent and not Th2-dependent immune inflammation. Immunostaining for CXCL10 exhibited strong accumulation of this chemokine in the epidermis of the vehicle-treated group,

which was markedly attenuated by GW9508 treatment (Figure 6d). Taken together, these results suggest that GW9508 attenuates CHS at least in part by suppressing CXCL10 expression by keratinocytes.

DISCUSSION

Keratinocytes are a major cellular component of the skin and have a key role in establishing local inflammatory milieu in various cutaneous diseases (Pastore *et al.*, 2006). In this study, we screened Gi-coupled GPCR expressed in HaCaT cells by chemical library screening and GPCR array, and found GPR40 as one of them. Stimulation of GPR40 suppresses expression of CCL17 and CCL5 *in vitro* in HaCaT cells stimulated with TNF- α + IFN- γ and CCL5 and CXCL10 expression in NHEK stimulated with IFN- γ . Furthermore, stimulation of GPR40 suppresses immune inflammation *in vivo* in the skin in the mouse.

CCL17 is a ligand for CCR4, which is mainly expressed on Th2 cells. CCL17 is produced by diverse cell types, including bronchial epithelial cells, fibroblasts, endothelial cells, and dendritic cells in addition to HaCaT cells (Saeki and Tamaki, 2006). Although we have not detected elevation of CCL17 in our mouse model for AD, keratinocytes in skin lesion of AD patients are stained positive for CCL17. Further, the serum concentration of CCL17 is significantly higher in AD patients than in psoriasis vulgaris subjects or normal healthy donors, and correlates well with the severity of AD (Kakinuma *et al.*, 2001). These findings suggest that CCL17

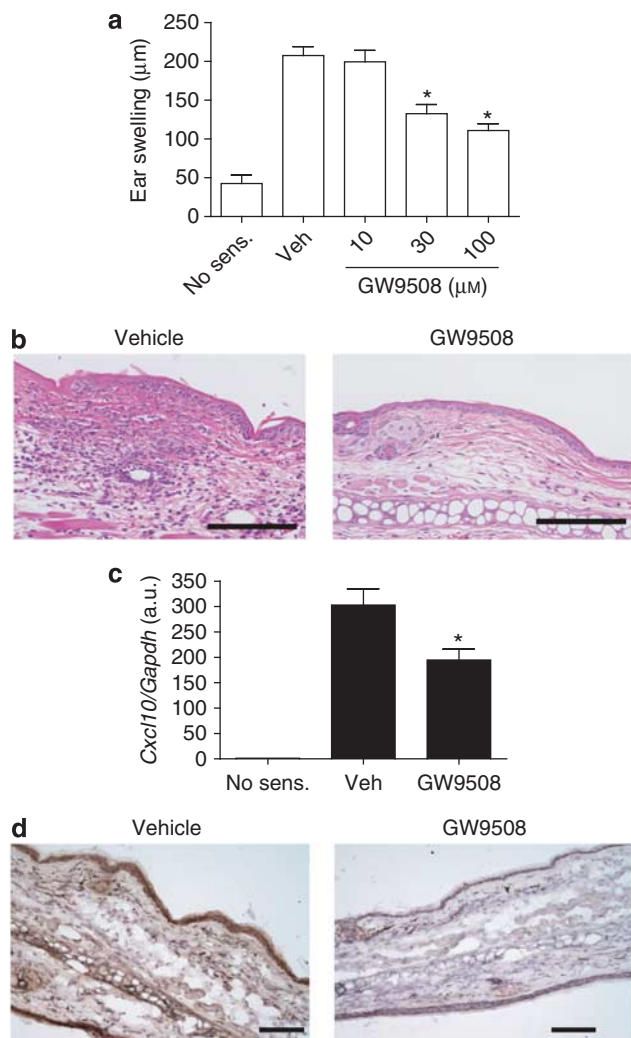


Figure 6. Inhibition of contact hypersensitivity by topical application of GW9508. (a) Effect of GW9508. No sens., group without sensitization and treated with 0.3% DNFB; veh, vehicle-treated group; GW9508, GW9508-treated group. (b) Hematoxylin-eosin staining. (c) QRT-PCR analysis of CXCL10 mRNA. Data are shown as arbitrary units (a.u.) where the value for the group without sensitization is set at 1. (d) Immunostaining for CXCL10. Bars = 100 μm . Results are representative of three independent experiments. Data show mean $\pm$ SEM of five mice. * $P < 0.05$ compared with vehicle.

has a key role in the pathogenesis of AD in humans. Given the suppression of CCL17 expression by GPR40 stimulation in HaCaT cells, it may be worthwhile to test the effect of a GPR40 agonist on CCL17 expression in the skin of AD patients.

CCL5 is a chemokine secreted by platelets, fibroblasts, airway epithelial cells (Castellani *et al.*, 2007), and epidermal keratinocytes (Yamada *et al.*, 1997; Park *et al.*, 2005). CCL5 promotes migration of T cells, dendritic cells, eosinophils, natural killer cells, mast cells, and basophils (Levy, 2009). Recently, single-nucleotide polymorphism was found in the promoter region of CCL5 gene, and significant association of the variant with enhanced promoter activity with AD was reported (Nickel *et al.*, 2000; Tanaka *et al.*, 2006). In the present work, we found the elevation of CCL5 in the

epidermis with repeated DNFB treatment in the mouse model of AD, and that GW9508 treatment attenuated ear swelling in this model with concomitant decrease of the CCL5 production in the epidermis. Suppression of infiltration of eosinophils and mast cells was also observed with GW9508 treatment. Our results are thus consistent with the above genomic study and suggest that GPR40 stimulation inhibits Th2-type skin inflammation in part by inhibiting CCL5 expression by keratinocytes.

We also examined effects of GPR40 stimulation in CHS, a Th1-dependent inflammation. In CHS, keratinocytes produce many chemokines to attract inflammatory cells to the site of inflammation. Among them, CXCL10 is one of the most important chemokines in CHS pathogenesis (Nakae *et al.*, 2003; Mori *et al.*, 2008). CXCL10 is a ligand for CXCR3, which is primarily expressed on Th1 cells. CHS response was decreased in TNF- α deficient mice, which was reversed by CXCL10 injection during the elicitation phase. Moreover, CHS was suppressed by treatment with anti-CXCL10 mAb, suggesting a critical role for CXCL10 in CHS (Nakae *et al.*, 2003). We found that GW9508 suppressed ear swelling and dermal infiltration of inflammatory cells in CHS with concomitant suppression of CXCL10 expression in keratinocytes. In conclusion, GPR40 stimulation inhibits chemokine production by keratinocytes in not only Th2-dependent but also Th1-dependent skin inflammation, and suppresses progression of the inflammation.

GPR40, a Gi- and Gq-coupled GPCR, is a recently orphanized long-chain free fatty acid receptor (Itoh *et al.*, 2003). It is abundantly expressed on pancreatic β cells and stimulates insulin secretion. The actions of GPR40 in the immune system are currently unknown. Our findings suggest that GPR40 expressed in the skin regulates cutaneous immune responses. Keratinocytes proliferate at the basal layer of the epidermis and differentiate as they migrate toward the surface. The outermost layer of the epidermis, the stratum corneum, serves as a rigid barrier, which prevents water loss and the entry of pathogens into the dermis (Proksch *et al.*, 2008). Intracellular components of the barrier function of stratum corneum consist of keratins and filaggrin, whereas the extracellular components consist of long-chain free fatty acids, cholesterol, and ceramide. Essential free fatty acid deficiency (Feingold, 2007) or defective fatty acid transport (Klar *et al.*, 2009) causes skin barrier defects characterized by red and scaly skin, severe bacterial infection, and impaired wound healing. Our present findings may indicate that long-chain free fatty acids in the skin not only form a barrier, but also suppress abnormal immune responses by stimulating GPR40 to protect terrestrial organisms from water loss and noxious stimuli. In the mouse ear, GPR40 is expressed in the epidermis. Quite recently, GPR120, a GPCR related to GPR40, functions as a receptor for ω -3 unsaturated fatty acids and exerts anti-inflammatory actions (Oh da *et al.*, 2010). Although GW9508 can act also on GPR120 at higher concentrations (Briscoe *et al.*, 2006), our small interfering RNA experiments as well as expression study suggest that GW9508 acts on GPR40 and not GPR120 to exert immunosuppressive effect on keratinocytes both *in vitro* and *in vivo*.

Our present findings that stimulation of a Gi-coupled receptor, GPR40, suppresses immune inflammation in the skin are consistent with previous reports by our laboratories and others on other Gi-coupled GPCR such as PGE receptor EP3 subtype and CB1 and CB2 cannabinoid receptors. Stimulation of EP3 inhibits experimental allergic conjunctivitis (Ueta *et al.*, 2009), allergic airway inflammation (Kunikata *et al.*, 2005), and CHS (Honda *et al.*, 2009) in mice, and stimulation of CB1 and CB2 suppresses CHS (Karsak *et al.*, 2007). These results suggest that Gi-coupled GPCR including GPR40 can be a good candidate for drug development against allergic diseases targeted to the epithelium.

MATERIALS AND METHODS

Mice

Female BALB/c mice and C57BL/6NCrSlc mice, 7–11 weeks of age, were obtained from Japan SLC (Hamamatsu, Japan). They were housed at the Institute of Laboratory Animals of Kyoto University on a 12-hours light/dark cycle under specific pathogen-free conditions. All experimental procedures were approved by the Committee on Animal Research of Kyoto University Faculty of Medicine.

Reagents and cytokines

Tocriscreen Mini library containing GW9508 was from Tocris Cookson (Avonmouth, UK). PTX was from List Biological Laboratories (Campbell, CA). Forskolin was from Sigma-Aldrich (St Louis, MO). Human TNF- α and IFN- γ were from Peprotech (Rocky Hill, NJ). DNFB was from Nacalai Tesque (Kyoto, Japan).

Cell culture

HaCaT cells (Boukamp *et al.*, 1988; Cell Lines Service, Eppelheim, Germany) were cultured in high glucose DMEM (Gibco, Carlsbad, CA) supplemented with 50 U ml⁻¹ penicillin, 50 μ g ml⁻¹ streptomycin (Nacalai Tesque) and 10% fetal bovine serum (Gibco). They were seeded at 2×10^4 cells per well in a 96-well plate in 200 μ l of the above medium containing serum. After 24 hours, the medium was replaced with the serum-free medium and the cells were cultured for another 24 hours. The medium was then changed with fresh serum-free medium, and drugs were added to the cells. After 5 minutes, 10 ng ml⁻¹ TNF- α and/or 10 ng ml⁻¹ IFN- γ were added and the cells were cultured for indicated times. After incubation, the medium was saved for ELISA and the cells were lysed for RNA preparation for QRT-PCR. NHEK (KURABO, Osaka, Japan) were cultured in HuMedia-KG2 (KURABO) supplemented with insulin, bovine pituitary extract, epidermal growth factor, hydrocortisone, kanamycin, and amphotericin B. They were seeded at 2×10^4 cells per well in 96-well plates. After 24 hours, medium was replaced with HuMedia-KG2 without supplements and cultured for 24 hours. The medium was then changed and treated with GW9508 and stimulated with cytokines as described for HaCaT cells.

Repeated DNFB treatment and CHS protocol

Repeated DNFB treatment was performed essentially as described (Inagaki *et al.*, 2006). From the fifth to the ninth elicitation, 10 μ l of 200 μ M GW9508 in 99% ethanol was topically applied to each ear on days of elicitation 1 hour before DNFB treatment. Ears were

collected 6 hours after the last elicitation. Mice treated with vehicle without DNFB were used as control. CHS was performed essentially as described (Honda *et al.*, 2009). GW9508 was administered topically to the ear 30 minutes before elicitation. Ear thickness was measured with a micrometer before and 24 hours after challenge to assess inflammation. Ears were then collected for histology and QRT-PCR analysis.

For other methods including GPCR array, QRT-PCR, RT-PCR, ELISA, microarray experiments, MTT assay, RNA interference, histological studies, and *in situ* hybridization, see Supplementary Materials and Methods online. Primers used for QRT-PCR and RT-PCR are indicated in Supplementary Table S4 online.

Statistical analysis

Data were expressed as means $\pm$ SEM, and statistical analyses were performed by means of Student's *t*-test. A *P* value <0.05 was considered to be statistically significant.

CONFLICT OF INTEREST

TF, TM, KK, and TaH are employed by the Coordination Fund from the Japan Science and Technology Agency and Astellas Pharma, and SN received a grant from this Fund. TeH declares no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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Supplemental Materials and Methods

GPCR array

HaCaT cells, 1×10^5 per well, were seeded in 24-well plates (IWAKI, Tokyo, Japan) and serum-starved for 24 hours. Total RNA was extracted with RNeasy (Qiagen, Hilden, Germany). cDNA was synthesized from 2 μ g RNA with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Human GPCR Array (Applied Biosystems) was performed using TaqMan ABI 7900T fast real-time PCR machine (Applied Biosystems) according to the manufacturer's protocol. The expression of mRNA is represented as Δ Ct, where Δ Ct = [Ct (target gene)]-[Ct (gapdh)].

QRT-PCR and RT-PCR

Total RNA were obtained from HaCaT cells and NHEK by using CellAmp Direct RNA Prep Kit for One Step RT-PCR (Real Time) (Takara, Ohtsu, Japan), and subjected to QRT-PCR using One Step SYBR PrimeScript RT-PCR Kit II (Perfect Real Time) (Takara) according to the manufacturer's protocol. QRT-PCR was performed by LightCycler480 (Roche, Basel, Switzerland). The sequences of the primers are indicated in Table S4. The expression of mRNA is represented as fold increase ($2^{-\Delta\Delta$ Ct}), where $\Delta\Delta$ Ct = [Δ Ct (sample)]-[Δ Ct (unstimulated sample)], and Δ Ct = [Ct (target gene)]-[Ct (gapdh)]. For QRT-PCR from mouse ears, samples were collected, snap-frozen in liquid nitrogen and stored at -80 °C until use. For detecting CXCL10 mRNA in CHS, whole ears were cut into pieces and put into 1 ml Trizol (Invitrogen, Carlsbad, CA) and homogenized at 3000 rpm for 30 seconds with Polytron PT2100S (KINEMATICA Inc., Lucerne, Switzerland). For repeated DNFB treatment and for the isolation of epidermis from mouse ears, ventral and dorsal sides of the ears were split off and then incubated for 1 hour at 4°C in 3 mg/ml Dispase II (Godoshusei, Matsudo, Japan) in PBS (-). Epidermis was then isolated with tweezers, washed in PBS (-) and homogenized in 1 ml Trizol. Total RNA was then isolated according to the manufacturer's instructions. One μ g of RNA was then treated with DNase (Promega, Madison, WI) and 500 ng of DNase-treated RNA was reverse-transcribed to cDNA by using the PrimeScript RT reagent kit (Perfect Real Time) (Takara). Mouse lung cDNA was a kind gift from Dr. Toru Oga (Department of Respiratory Care and Sleep Control Medicine, Kyoto University Faculty of Medicine) and was synthesized as described (Oga *et al*, 2009). QRT-PCR was then performed with SYBR Premix Ex Taq (Perfect Real Time) (Takara). The expression of mRNA is represented as fold increase ($2^{-\Delta\Delta$ Ct}), as described above.

RT-PCR for mouse and human GPR40 and GPR120 was performed as follows. For HaCaT cells and NHEK, total RNA was extracted with RNeasy Mini kit (QIAGEN). For RT-PCR from mouse ears, RNA extraction and reverse transcription were performed as described above. One μ g of total RNA was used in reverse transcription using PrimeScript RT reagent kit (Perfect Real Time) (Takara). PCR reaction was done using KOD plus (Toyobo, Osaka, Japan) and visualized in a 2 % agarose gel.

ELISA

The medium was collected from each culture and spun at 3000 ×g for 5 minutes. The supernatants were saved and stored at -80°C until use. ELISA was performed using ELISA kits for human CCL17, CCL5 and CXCL10 (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol.

Microarray experiments

HaCaT cells were seeded in 24-well plates at 1×10^5 cells/well, and stimulated in triplicate with or without TNF- α + IFN- γ as described above and with TNF- α + IFN- γ in the presence or absence of 1 μ M GW9508. After 24 hours, the cells were lysed and RNA was isolated with RNeasy columns (Qiagen). By using GeneChip 3' IVT Express Kit (Affymetrix, Santa Clara, CA), 100 ng of total RNA /sample was converted into cDNA, which was then processed into RNA. Complementary RNA was labeled with biotin probes and fragmented. Twelve point five μ g of fragmented complementary RNA was used for hybridization to the Human U-133 2.0 Plus Genome Array (Affymetrix) according to the manufacturer's protocol. Microarray expression values were generated using Affymetrix GeneChip Expression Analysis software (Microarray Suite 5.0). Data normalization and analysis were performed with Genespring GX10 software (Silicon Genetics, Redwood City, CA). For data normalization, each raw intensity value was divided by the median of the chip. Then, each value is further divided by the median value of each gene in control samples, resulting in the final value normalized to control samples. Means of normalized values thus obtained were log2-transformed and shown in Table S2 and S3. For analysis of effects of each treatment, genes exhibiting differential expressions higher than two fold or less than half in treated samples ($n = 3$) over controls ($n = 3$) and were subjected to statistical analysis using TT test (Welch). *P* values less than 0.05 were considered statistically significant. Complete microarray data are deposited in Gene Expression Omnibus with the accession number GSE20297.

MTT assay

HaCaT cells and NHEK were seeded at 2×10^4 cells / well in a 96-well plate in a total volume of 100 μ l. The next day, medium was changed to serum-free DMEM for HaCaT cells and to HuMedia without supplements for NHEK, and cultured for another 24 hours. After medium was replaced with a fresh one, different concentrations of GW9508 was added to the culture and incubated for 24 hours. Ten μ l of Cell Count Reagent SF (Nacalai Tesque, Kyoto, Japan) was then added and, after 1 hour-incubation, optical density at 450nm was measured with a microplate reader (Molecular Devices, Sunnyvale, CA).

RNA interference

siRNAs for depletion of GPR40 (FFAR1) were obtained from Invitrogen. FFAR1-HSS142273 was designed to target the nucleotide number 259-283 of human FFAR1 (GPR40) mRNA (GenBank accession no. NM_005303.2). As a negative control siRNA, Stealth RNAi Negative Control High GC Duplex 2 (Invitrogen) was used. siRNAs were transfected into HaCaT cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cells were

incubated for 72 hours with the replacement of medium 24 hours after the transfection. Cells were then harvested and subjected to QRT-PCR analysis for evaluation of GPR40 expression. For the evaluation of CCL17 and CCL5 production, cells were serum-starved 48 hours after transfection and incubated for 24 hours. One μ M GW9508 and 10 ng/ml each of human TNF- α and IFN- γ were added after replacement of medium and incubated for another 24 hours. Total RNA was then collected by CellAmp Direct RNA Prep Kit for One Step RT-PCR (Takara) and CCL17 and CCL5 expression was evaluated by One Step SYBR PrimeScript RT-PCR Kit II (Takara)

Histological studies

Ears were isolated 6 hours after the last elicitation in repeated DNFB treatment or 24 hours after the elicitation in CHS. They were then fixed in 10% formalin, and embedded in paraffin. Sections of 7 μ m in thickness were prepared and stained with hematoxylin and eosin. For eosinophil count, the slides were blinded and eosinophils were counted in at least 8 high-power fields ($\times 400$). For toluidine blue staining, samples were deparaffinized and incubated in 0.05 % toluidine for 30 minutes. They were then washed 3 times in deionized water and coverslipped with Mount-Quick (Daido Sangyo, Saitama, Japan).

For immunohistochemistry for CCL5 and CXCL10, collected ears were embedded in Tissue-Teck (Sakura Finetek, Tokyo, Japan) filled with OCT compound (Sakura Finetek). Frozen sections of 10 μ m in thickness were prepared and fixed in cold acetone. Goat anti-mouse CCL5/RANTES (AF478, R&D Systems), goat anti-mouse CXCL10/IP-10 (AF466, R&D Systems), or goat IgG (Chemicon International, Temecula, CA) was applied at 5 μ g/ml. After incubation with rabbit anti-goat IgG-biotin antibody (Jackson Laboratory, Bar harbor, Maine), signal was amplified with Vectastain (Vector Laboratories, Burlingame, CA) followed by 3,3'-diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries, Osaka, Japan) treatment. Finally, samples were counterstained with hematoxylin, dehydrated and mounted in Aquatex (Merck, Darmstadt, Germany).

***In situ* hybridization**

For detecting GPR40 mRNA, *in situ* hybridization was performed essentially as follows (Hirasawa *et al*, 2005) with modifications. Briefly, digoxigenin-labeled probes were synthesized by using digoxigenin RNA Labeling Kit (Roche Diagnostics, Basel, Switzerland). We prepared fresh frozen sections of mouse ears (10 μ m in thickness) and fixed them in ethanol/acetic acid (3:1). The sections were hybridized with the probe and the signal was detected with nitro blue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate solution (Roche Diagnostics).

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Table S1. Agonists for Gi-coupled GPCR with suppressive effects on CCL17 production by HaCaT cells

compound	Mechanism of Action	1 μ M	10 μ M	Δ Ct
ST91	α 2 adrenergic receptor agonist	0.2	1.14	15.11 (α 2A ¹) 9.83 (α 2B ¹)
GP2a	CB2 (cannabinoid receptor CB2) agonist	0.15	0.2	18.32
GP1a	CB2 agonist	0.26	0.11	18.32
3'-fluorobenzyl-spiperone	D2 (dopamine receptor D2) agonist	0.67	0.003	16.14
GW9508	GPR40 and GPR120 agonist	0.1	0.15	11.12

Compounds included in Tocriscreen Mini were screened for their effects on TNF- α + IFN- γ -induced CCL17 production by HaCaT cells. Agonists for Gi-coupled GPCR with suppressive effects were picked up. Δ Ct values of the target receptors of each compound were measured by GPCR array as described.

¹ α 2A and α 2B refer to α 2A and α 2B adrenergic receptor, respectively.

Table S2. Cytokines and chemokines up-regulated after TNF- α + IFN- γ treatment

Gene Symbol	Probe Set ID	none	TNF-α + IFN-γ
IL32	203828_s_at	0.15	11.44
IL8	211506_s_at	0.19	10.78
CCL8	214038_at	-0.06	8.94
CXCL3	207850_at	0.7	8.7
IL8	202859_x_at	-0.05	8.65
CCL20	205476_at	0.19	8.18
CXCL5	215101_s_at	0.01	8.08
CXCL5	214974_x_at	0.07	7.78
CCL17	207900_at	0.14	7.05
CCL2	216598_s_at	0	6.83
CXCL14	218002_s_at	0.24	6.44
CCL5	1555759_a_at	-0.07	6.44
CCL22	207861_at	0.2	6.41
IL29	1552917_at	0.39	6.38
IFNB1	208173_at	0.01	6.24
IL28A /// IL28B	1552609_s_at	0.13	6.12
IL6	205207_at	0.11	6.03
IL1B	205067_at	0.01	5.87
CCL4	204103_at	0.13	5.39
IL1B	39402_at	0.07	5.34
TNF	207113_s_at	0.25	5.13
LTB	207339_s_at	-0.57	5.09
IL1F9	220322_at	-0.7	4.99
IL33	209821_at	-0.24	4.9
CSF2	210229_s_at	0.09	4.55
CXCL1	204470_at	-0.02	4.24
IL24	206569_at	0.1	4.16
CXCL14	222484_s_at	-0.38	4.04
CCL7	208075_s_at	-0.04	3.8
IL11	206924_at	0.13	2.4
IFNE1	1553574_at	-0.05	2.16
CXCL16	223454_at	-0.06	2.11
CXCL5	207852_at	0.07	1.86
IL16	209827_s_at	-0.77	1.68

Cytokines and chemokines significantly up-regulated by TNF- α + IFN- γ treatment in HaCaT cells (with $p < 0.05$ in TT test (Welch), differential expression more than 2 fold). Given are means of log2-transformed normalized data.

Table S3. Chemokine- and cytokine-related probe sets differentially regulated by 1 μ M GW9508

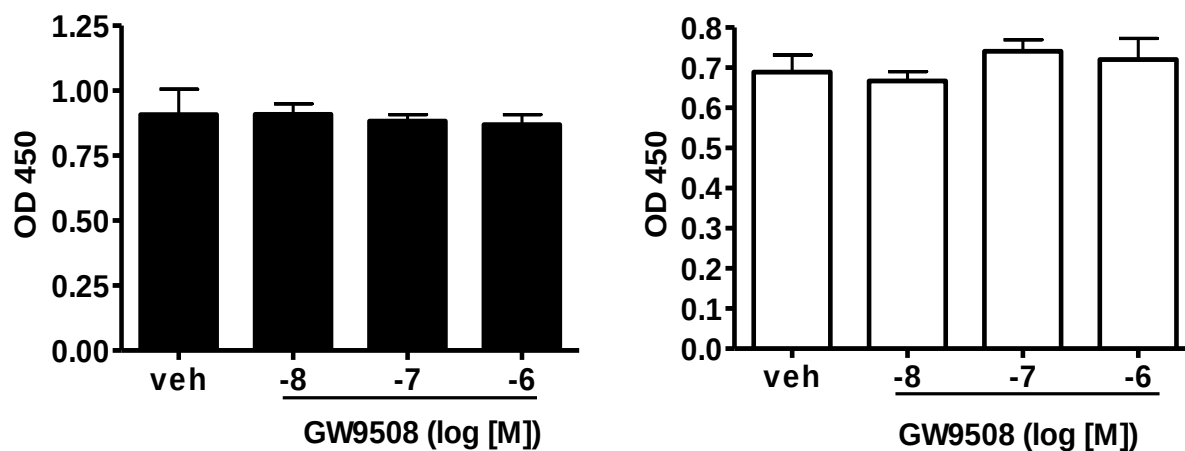
Gene Symbol	Probe Set ID	TNF-α + IFN-γ + vehicle	TNF-α + IFN-γ +GW9508
CCL17	207900_at	0	-1.2
CCL5	204655_at	-0.06	-1.16
IL11	206924_at	0.03	-1.13
CCL5	1555759_a_at	-0.07	-1.11
IL24	206569_at	0.07	-1.05
IL33	209821_at	0.25	-0.94

Chemokine- and cytokine-related genes differentially regulated in HaCaT cells by GW9508 (with $p < 0.05$ in TT test (Welch), differential expression more than 2 fold). Given are the means of log2-transformed normalized data.

Table S4. Primers used in the experiments

gene symbols		Human	Mouse
<i>Gapdh</i>	sense antisense	agccacatcgctcagacac gccaatacgaccaaacc	tgaacgggaagctcactgg tccaccaccctgttgctgta
<i>Ccl17</i>	sense antisense	ggcttctctgcagcacatc ggaatggctccctgaagta	
<i>Ccl5</i>	sense antisense	cctcattgctactgcctct ggtgtggtgtccgaggaata	gctgcttgctacctctcc tcgagtgacaaacagactgc
<i>Cxcl10</i>	sense antisense	gtggcattcaaggagtacctc gccttcgattctggattcagaca	gccgtcattttctgcctcat gcttccctatggccctcatt
<i>Ffar1</i> (GPR40)	sense antisense	ctgggtctacgccctgaacct gagcctccaacccaaagacc	cgctgggctttccattga gctgggagtgagtcgcagtt
<i>Gpr120</i>	sense antisense	gaaatttcgatttgacact gtttagggtgaattagcaa	gcataggagaaatctcatgg gagttggcaaacgtgaaggc

Figure S1. Effect of GW9508 on cell viability of HaCaT cells (left) and NHEK (right)



HaCaT cells (left) or NHEK (right) were treated with different concentrations of GW9508 for 24 hours. Cell viability was measured by MTT assay. Veh, vehicle. OD 450, optical density at 450 nm. Data are expressed as means $\pm$ SEM (n = 6).