Letter to the Editor

Title: Expressions of PPARs are directly influenced by permeability barrier abrogation and inflammatory cytokines and depressed PPARα modulates expressions of chemokines and epidermal differentiation-related molecules in keratinocytes

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Short title: Regulation and role of PPARα in AD

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Abstract

Previous studies have demonstrated that the activation of peroxisome proliferator-activated receptors (PPARs) not only has positive effects on permeability barrier homeostasis but also has anti-inflammatory effects by an as yet unknown mechanism. Reduced expression of PPARα in lesion of human atopic dermatitis (AD) and in epidermis of murine AD-like dermatitis has been demonstrated. The present study revealed that expression of PPARα alone among PPARs (α, β/δ and γ) was suppressed by both permeability barrier abrogation and additional existence of Th2 cytokine in cultured normal human keratinocytes. In addition, expressions of transglutaminase 1 and loricrin and those of TARC and RANTES in cultured human keratinocytes were reduced and enhanced, respectively, by transfection with siRNA for PPARα. In conclusion, depressed PPARα in keratinocytes might be involved in a relationship between permeability barrier abrogation and allergic inflammation and could be a therapeutic target which accounts for both the aspects in AD.

Key words: PPARα, barrier abrogation, IL-4, chemokines

Abbreviation: AD, atopic dermatitis; PPAR, peroxisome proliferator-activated receptor; TNF, tumor necrosis factor; IFN, interferon; IL, interleukin; TARC, human thymus and activation-related chemokine; RANTES, regulated upon activation, normal T-cell expressed and secreted.

Background

Peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors (class II receptors) and are of three subtypes: PPARα, PPARβ/δ and PPARγ (1). Activation of PPARs stimulates lipid synthesis, epidermal differentiation, and aquaporin 3 expression and accelerates recovery from permeability barrier dysfunction (1, 2). Moreover, development of the epidermal barrier is delayed in PPARα-deficient mice (3). Activators of PPARα suppress both allergic and irritant cutaneous inflammation *in vivo* by an as yet unknown mechanism (4). Activator of PPARβ/δ also suppresses cutaneous inflammation, at least partially, via repression of the upregulation of metalloproteinase 9 (5, 6). Thus, the PPARs signaling not only has positive effects on permeability barrier homeostasis but also has anti-inflammatory effects. In fact, a topical treatment with some ligands for PPARα and PPARβ/δ restored permeability barrier dysfunction and reduced cutaneous inflammation in a hapten-induced AD-like dermatitis (7).

Staumont-Salle *et al*. demonstrated the reduced expression of PPARα in lesional skin in patients with AD and, in addition, that PPARα-deficient mice developed substantially severer phenotype of AD-like dermatitis, compared with the wild type mice (8). Chiba *et al*. recently demonstrated the reduced expression of PPARα (but not PPARβ/δ and PPARγ) in epidermis on AD-like dermatitis in NC/Nga mice (9).

Question addressed

We hypothesize that depressed PPARα in epidermis might be involved in both permeability barrier dysfunction and allergic inflammation in the pathogenesis of AD.

Experimental design

First, the effects of permeability barrier abrogation and proinflammatory cytokines such as tumor necrosis factor (TNF) α, interferon (IFN) γ and interleukin-4 (IL-4) on the expressions of PPARs in normal human keratinocytes were examined. Next, we examined the effects of reduced expression of PPARα in keratinocytes on the expression of regulated on activation normal T cell expressed (RANTES) and thymus and activation-related chemokine (TARC), which are important chemokines produced by keratinocytes in the aspect of cutaneous inflammation of AD (10, 11). In addition, effect of the reduced expression of PPARα on expression of epidermal differentiation-related molecules, such as transglutaminase 1 and loricrin was also examined to reconfirm the role of PPARα in the keratinocyte differentiation which is strongly associated with permeability barrier homeostasis. All procedures were performed as shown in the supplementary materials and methods.

Results

Permeability barrier abrogation influenced the expression of PPARα, PPARβ/δ and PPARγ in epidermis in human living-skin equivalents

The expressions of PPARα and PPARγ in epidermis of human living-skin equivalents were reduced 24h after the permeability barrier abrogation (Figure 1). On the other hands, those of PPARβ/δ were up-regulated (Figure 1).

IL-4 influenced the expression of PPARα, PPARβ/δ and PPARγ in cultured normal human keratinocytes

Expressions of PPARα and PPARβ/δ in cultured normal human keratinocytes were elevated by TNFα and IFNγ (Figure 1 and S1) as reported previously (12). In AD lesion, Th2 cytokines are involved in the formation of cutaneous inflammation (13). Therefore, effects of an additional prototypic Th2 cytokine, IL-4, on those of TNFα and IFNγ were examined. The elevation of PPARα and PPARβ/δ by TNFα and IFNγ was inhibited by IL-4 (Figure 1 and 1S). IL-4 alone did not alter the expression of PPARα and PPARβ/δ (data not shown). IL-4 stimulated the expression of PPARγ which was not influenced by TNFα and IFNγ (Figure 1 and 1S). IL-4 alone also upregulated the expression of PPARγ (data not shown)

Knockdown of PPARα augmented expressions of RANTES and TARC and depressed those of transglutaminase 1 and loricrin in cultured keratinocytes

Expressions of RANTES and TARC were up-regulated in keratinocytes transfected with siRNAs specific for PPARα (Figure 2). The expressions of transglutaminase 1 and loricrin were downregulated by the knockdown of PPARα (Figure S2).

Conclusion

Interestingly, the expression of PPARα alone (but not PPARβ/δ or PPARγ) was downregulated by both permeability barrier abrogation and IL-4, a prototypic Th2 cytokine. These results suggest that a reduction in the levels of PPARα, which has been observed in epidermis of human AD and murine AD model (supplementary Table S1), might be a consequence of permeability barrier abrogation and of Th2 cytokine, both of which play an important role in the pathogenesis of AD (14).

Our present study using living-skin equivalents demonstrates for the first time that the expressions of PPARs in keratinocytes might be directly regulated by permeability barrier abrogation, although a previous study has reported the similar results using whole skin of normal human subjects *in vivo* (15). The mechanism by which permeability barrier abrogation affects the expressions of PPARs in keratinocytes

remains unclear.

In the present study, the previously reported enhanced expression of PPARα and PPARβ/δ in cultured normal human keratinocytes induced by TNFα and IFNγ (12) were inhibited by a prototypic Th2 cytokine, IL-4. In view of the positive effects of PPARα and PPARβ/δ on epidermal differentiation and permeability barrier homeostasis (1, 3, 16), the inhibitory effects of IL-4 on the upregulation of PPARα and PPARβ/δ might reflect the negative effects of IL-4 for both epidermal differentiation and permeability barrier homeostasis (14).

Expressions of RANTES and TARC were upregulated and those of transglutaminase 1 and loricrin were downregulated in keratinocytes transfected with siRNAs specific for PPARα. These results suggest that depressed PPARα in keratinocytes might be involved in both augmentation of inflammation and abnormal permeability barrier homeostasis which are principal pathogenic features of AD, although the mechanism for those remains obscure.

The present study suggests that reduced expression of PPARα in keratinocytes, at least partially, might be involved in a vicious cycle between permeability barrier abrogation and allergic inflammation in the pathogenesis of AD (supplementary Figure S3). Further examination of the involvement of PPARα in such a vicious cycle might

help to develop new and better strategies for treating both barrier dysfunction and allergic inflammation simultaneously.

Acknowledgement

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Author contribution

Y.A. and Y.H. designed the experiment, analyzed the data and wrote the manuscript. T.S. contributed to the experiments using cultured keratinocytes. S.F. contributed to the design of the experiments.

Conflict of interest

The authors have declared no conflicting interests.

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Figure legends

Figure 1: Effects of permeability barrier abrogation, TNFα, IFNγ and IL-4 on the expression of PPARα, PPARβ/δ, and PPARγ in human keratinocytes.

Twenty-four h (a) and 48 h (b) after barrier abrogation [Abrogation (+)], epidermal sheets were harvested from human living-skin equivalents. Twenty-four h after the initiation of cytokine stimulation, cultured normal human keratinocytes were harvested (c-e). The expression of PPARα, PPARβ/δ, and PPARγ was examined by semiquantitative RT-PCR (a) and Western blotting (b-e), as described in "Supplementary Materials and Methods". Levels of expression in the Abrogation (+) group are given relative to levels in control groups [i.e. Abrogation (-)]. Levels of expression in the cytokine stimulation groups are given relative to levels in control groups. a, b: n=8. c-e: n=6. Similar results were obtained when the experiment was repeated (more than twice) using different cell preparations.

Figure 2: Effects of siRNA knockdown of PPARα on expression of RANTES and TARC in cultured keratinocytes.

Twenty-four h after stimulation with TNFα and IFNγ, subsequent to transfection with

PPARα-specific siRNA (siRNA) or non-targeting siRNA (Control), cultured normal human keratinocytes (a, b), HaCaT cells (d, e) and culture supernatants (c, f) were harvested. Expression of PPARα (a, d), RANTES (b, c), and TARC (e, f) was examined by semiquantitative RT-PCR (a, b, d, e) or ELISA (c, f) as described in “Supplementary Materials and methods”. n=4. Similar results were obtained when the experiment was repeated (more than twice) using different cell preparations.

Supplementary Materials and Methods

Although PPARα is expressed in a variety of cells including inflammatory cells (1), in the present study, expression of PPARα in keratinocytes was focused, because keratinocytes are directly involved in both cutaneous inflammation and permeability barrier function.

Acute barrier abrogation on human living-skin equivalents

Acute barrier abrogation on human living-skin equivalents (TESTSKIN-LSE high®; Toyobo Co., Ltd., Osaka, Japan), which provide a model of reconstructed skin, was performed as previously reported (2). Then the surface of each sample of living-skin equivalents was maintained under air-exposed conditions. The surface (24 mm in diameter) of each sample was wounded by the application of 1 mL of acetone for 5 min and then the acetone was removed. Wounding was repeated once immediately after the first wounding. Levels of transepidermal water loss (TEWL), which was measured with a skin evaporative water recorder (Tewameter® TM210, Courage & Khazawa, Germany), were enhanced by about 5 g/h m^2. The similar survival of acetone-treated living-skin equivalents (LSE) to that of non-treatment LSE was confirmed by a standard

MTT assay, according to the manufacturer's protocol (Toyobo, Osaka, Japan; data not shown). The images of representative LSEs before and after the acetone-treatment are shown in supplementary Figure S4. Twenty-four h after barrier abrogation, epidermal sheets were harvested.

Preparation of epidermal sheets

Epidermal sheets were separated from each sample of living-skin equivalents by penetration from dermal site with 1,000 IU/mL dispase (Godo Shusei Co., Ltd., Tokyo, Japan) for approximately 30 min at 37 ˚C and were subjected to analysis of levels of expression of specific mRNAs and proteins.

Treatment of cultured normal human keratinocytes and HaCaT cells with cytokines

Culture of normal human keratinocytes and of a line of human epidermal keratinocytes (HaCaT cells) and treatment of cells with cytokines were performed as previously reported (3, 4). For assessment of TARC, HaCaT cells were used since a previous study demonstrated that HaCaT cells but not cultured normal human keratinocytes produced TARC (5). Twenty-four h after stimulation with rhTNF-α (2 ng per mL; R&D

Systems, Minneapolis, MN, USA), rh IFN-γ (0.5 ng per mL; R&D Systems), and rh IL-4 (20 ng per mL; R&D Systems), supernatants and cells in each well were subjected to analysis of levels of expression of specific mRNAs and proteins.

Transfection of cultured normal human keratinocytes and HaCaT cells with PPARα-specific siRNA

Normal human keratinocytes and HaCaT cells were cultured to 70% confluence and then transfected with 25 nM siRNA specific for PPARα (D-003434-01-0005 and D-003434-02-0005; Thermo Scientific Dharmacon, Lafayette, CO, USA) or non-targeting siRNA (D-001810-01-05; Thermo Scientific Dharmacon) in combination with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in OPTI-MEM (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Stimulation with cytokines, as described above, and harvest of cultured keratinocytes for the analysis of the expression of transglutaminase 1 and loricrin were performed 36 h after transfection.

Semiquantitative RT-PCR

Levels of expression of mRNA for PPARα, PPARβ/δ, PPARγ, RANTES, TARC, and transglutaminase 1 were examined by semiquantitative RT-PCR, which was performed

with ^{32}P-dCTP as described previously (2-4). Each primer pair is listed in supplementary Table S2.

Western blotting

Western blotting was performed as described previously (4). Samples prepared from treated cultured human keratinocytes or epidermal sheets were fractionated by SDS-PAGE (7.5 % or 10 % polyacryl-amide) before transfer to Hybond^{TM} nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK). Membranes were probed with primary and horseradish peroxidase-conjugated (HRP-conjugated) secondary antibodies and visualized with ECL^{TM} Western Blotting Detection Reagents (GE Healthcare). Band intensities were quantified with NIH-image software. Rabbit polyclonal antibodies against PPARα, PPARβ/δ and PPARγ, and HRP-conjugated goat antibodies against rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibodies against β-actin were purchased from Biovision Research Products (Mountain View, CA, USA). Rabbit polyclonal antibodies against loricrin were purchased from Covance (Emeryville, CA, USA).

ELISA

The supernatants from cultures of normal human keratinocytes and HaCaT cells were harvested 24 h after the start of incubation with cytokines and were subjected to ELISAs to determine levels of RANTES and TARC, respectively. The ELISA quantification kits for TARC and RANTES were purchased from R&D Systems and all analyses were performed according to the manufacturer's instructions.

Statistical analysis

The statistical significance of differences was evaluated by Student's t-test or Mann-Whitney U test.

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Figure 1

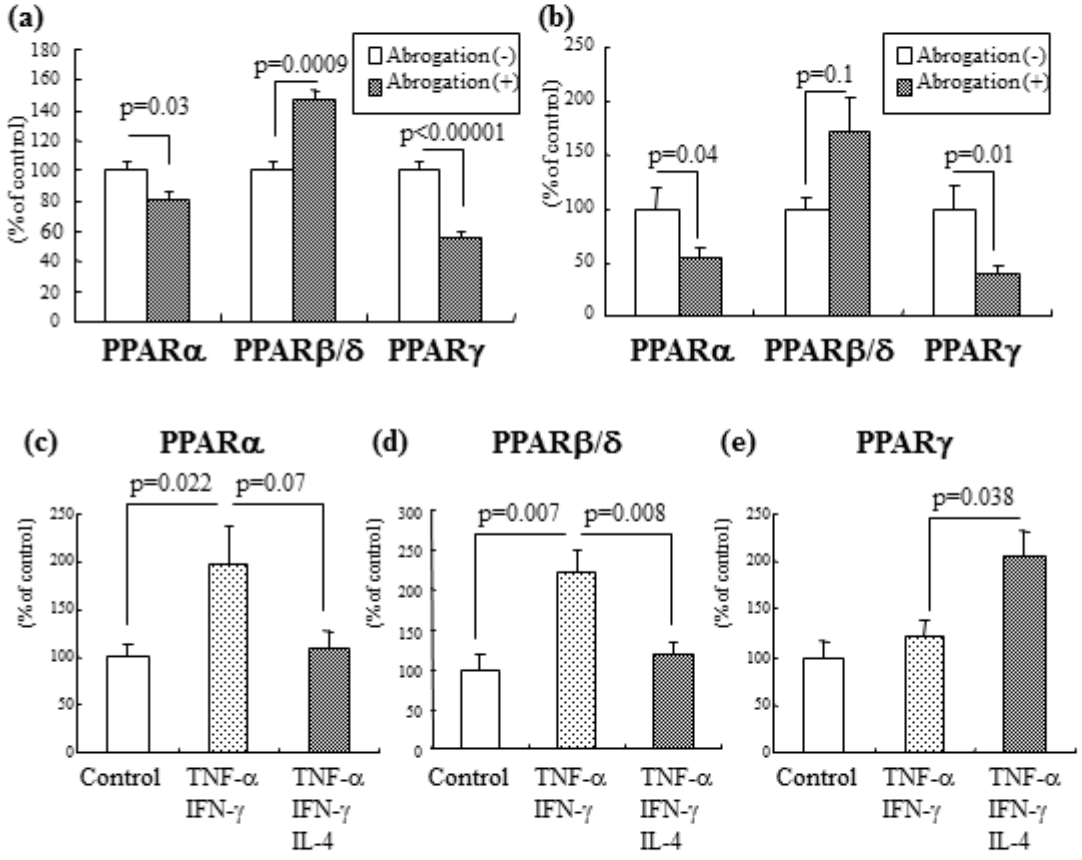

(a)
(%of control)
Abrogation(-)
Abrogation(+)
p=0.03
p=0.0009
p<0.00001
PPARα PPARβ/δ PPARγ
(b)
(%of control)
Abrogation(-)
Abrogation(+)
p=0.04
p=0.1
p=0.01
PPARα PPARβ/δ PPARγ
(c) PPARα
p=0.022 p=0.07
(%of control)
Control
TNF-α IFN-γ
TNF-α IFN-γ IL-4
(d) PPARβ/δ
p=0.007 p=0.008
(%of control)
Control
TNF-α IFN-γ
TNF-α IFN-γ IL-4
(e) PPARγ
p=0.038
(%of control)
Control
TNF-α IFN-γ
TNF-α IFN-γ IL-4

Figure 2

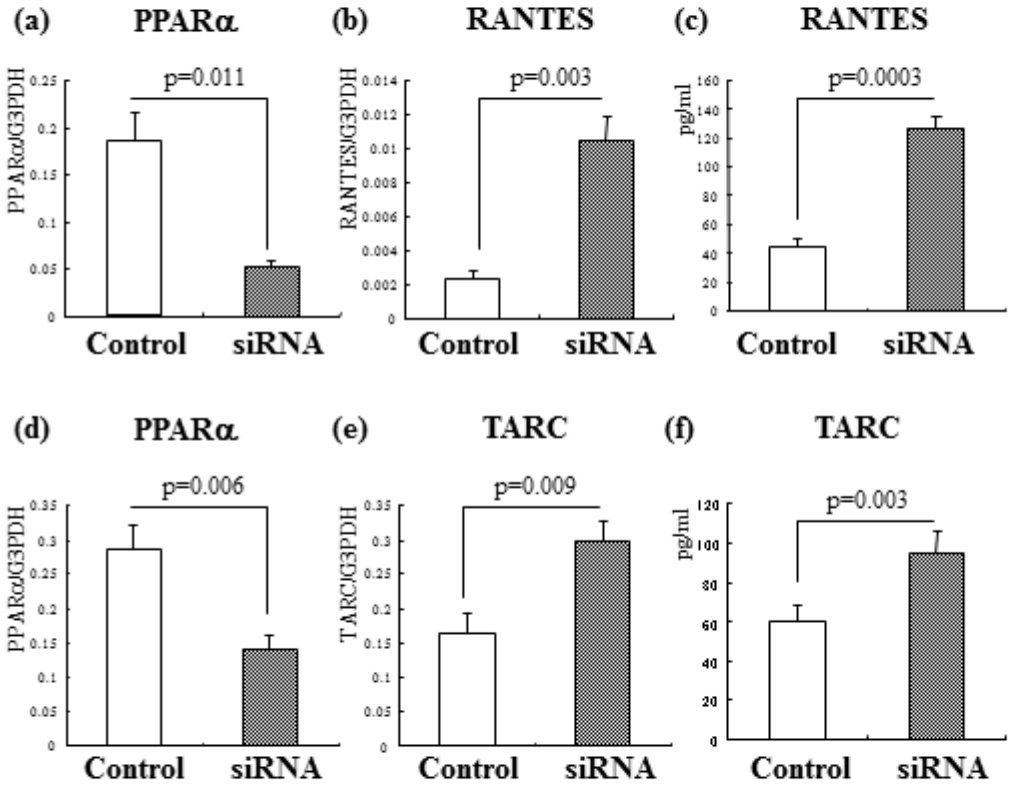

(a) PPARα
p=0.011
PPARα/G3PDH
Control
siRNA
(b) RANTES
p=0.003
RANTES/G3PDH
Control
siRNA
(c) RANTES
p=0.0003
pg/ml
Control
siRNA
(d) PPARα
p=0.006
PPARα/G3PDH
Control
siRNA
(e) TARC
p=0.009
TARC/G3PDH
Control
siRNA
(f) TARC
p=0.003
pg/ml
Control
siRNA

Figure S1

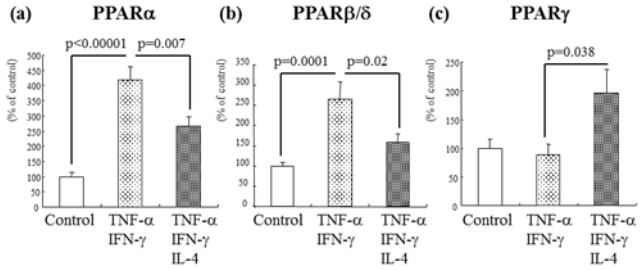


Figure 1S: Effects of TNF-α, IFN-γ and IL-4 on the expression of PPARα, PPARβ/δ, and PPARγ in human keratinocytes.

Twenty-four h after the initiation of cytokine stimulation, cultured normal human keratinocytes were harvested. The expression of PPARα (a), PPARβ/δ (b), and PPARγ (c) was examined by semiquantitative RT-PCR as described in "Supplementary Materials and methods". Levels of expression in the cytokine stimulation groups are given relative to levels in control groups. n=12.

Figure S2

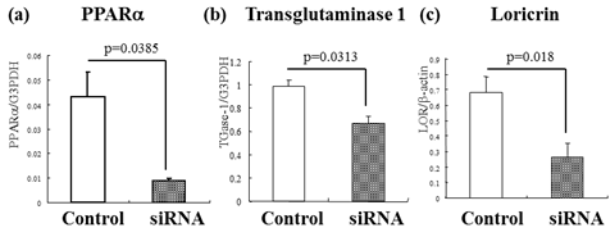


Figure S2: Effects of siRNA knockdown of PPARα on expression of transglutaminase 1 and loricrin in cultured keratinocytes.

Thirty-six h after transfection with PPARα-specific siRNA (siRNA) or non-targeting siRNA (Control), cultured normal human keratinocytes were harvested. Expression of PPARα (a), transglutaminase 1 (b) and loricrin (c) was examined by semiquantitative RT-PCR (a, b) or Western blotting (c) as described in "Supplementary Materials and methods". n=3.

Figure S3

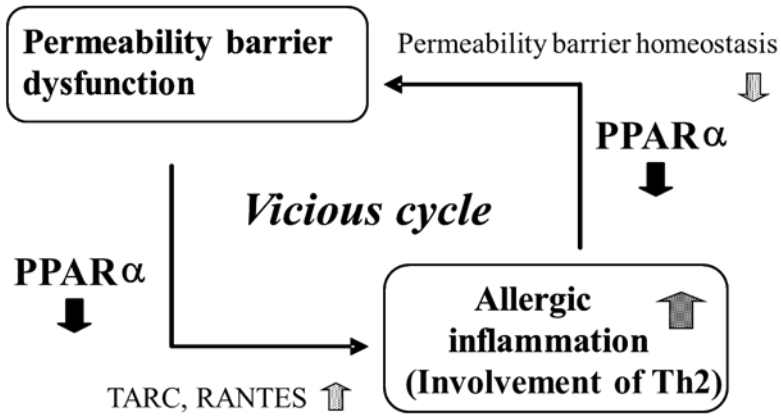
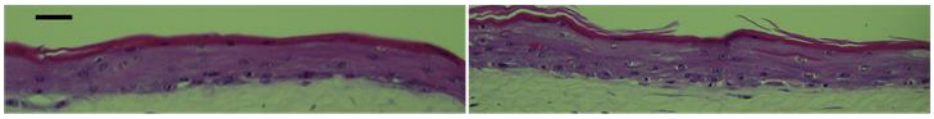


Figure S3: Depressed PPARα in keratinocytes might be associated with permeability barrier dysfunction and allergic inflammation and could be a therapeutic target which accounts for the both aspects simultaneously in atopic dermatitis

Depressed PPARα in keratinocytes, at least partially, might be involved in a vicious cycle between permeability barrier abrogation and allergic inflammation in the pathogenesis of atopic dermatitis (AD). Permeability barrier abrogation might reduce the expression of PPARα. Then keratinocytes with reduced expression of PPARα might accelerate production of chemokines relevant to allergic inflammation, such as TARC and RANTES, with resultant augmentation of allergic inflammation. IL-4, a prototypic Th2 cytokine produced during allergic inflammation, inhibits the upregulation of PPARα in keratinocytes, resulting in the suppression of homeostatic reaction to restore the permeability barrier dysfunction.

Figure S4



Abrogation - Abrogation +

Figure S4: Histological finding of living-skin equivalent after the barrier abrogation with acetone treatment.

Note that even 72h after the barrier abrogation, there is no significant difference between acetone-treated (Abrogation +) and non-treated (Abrogation -) living-skin equivalent. Hematoxylin & eosin stain. Size bar: 20 μm

Supplementary Table S1: Expression profiles of PPARs

	#1) AD Human lesional skin	Barrier #2) Abrogation Human Epi	Barrier #3) Abrogation Human KC	TNFα #4) IFNγ Human KC	#5) IL-4 Human KC	AD-like #7) model Mouse Epi
PPARα	⇩	⇩	⇩	⬆	⇩ #6)	⇩
PPARβ/δ	n.d.	⬆	⬆	⬆	⇩ #6)	→
PPARγ	n.d.	⇩	⇩	→	⬆	→

⬆, Upregulation; ⇩, Downregulation; →, Not change; n.d., not determined

KC: keratinocyte, Epi: epidermis, AD:atopic dermatitis

#1, ref. 8 (Staumont-Sallé *et al*. 2008); #2, ref. 15 (Törmä *et al*. 2009); #3, the present study;
#4, ref. 12 (Tan *et al*. 2001) and the present study; #5, the present study
#6, inhibitory effect on the upregulation by TNFα and IFNγ;
#7, ref. 9 (Chiba *et al*. 2012)

Supplementary Table S2. Primers used for RT-PCR

Gene product	Primer	Sequence
PPARα	Upstream	5”-TCATCAAGAAGACGGAGTCG-3”
	Downstream	5”-CGGTTACCTACAGCTCAGAC-3”
PPARβ/δ	Upstream	5”-TCCCTCTTTCTCAGTTCCTC-3”
	Downstream	5”-CAGGAGACAGAAGTGAGGAC-3”
RRARγ	Upstream	5”-ATGACAGCGACTTGGCAATA-3”
	Downstream	5”-GCAACTGGAAGAAGGGAAAT-3”
RANTES	Upstream	5”-ATGAAGGTCTCCGCGGCACGCCT-3”
	Downstream	5”-CTAGCTCATCTCCAAAGAGTTG-3”
TARC	Upstream	5”-ATGGCCCCACTGAAGATGCT-3”
	Downstream	5”-TGAACACCAACGGTGGAGGT-3”
TGase 1	Upstream	5”-AATCCTCTGATCGCATCACC-3”
	Downstream	5”-AGCTAAGCAGGATCTCCACG-3”
G3PDH	Upstream	5”-CCCATCACCATCTTCCAG-3”
	Downstream	5”-CCTGCTTCACCACCACCTTCT-3”

PPAR: peroxisome proliferator-activated receptor;
RANTES: regulated upon activation, normal T-cell expressed and secreted;
TARC: human thymus and activation-related chemokine; TGase: transglutaminase;
G3PDH: glyceraldehyde-3-phosphate dehydrogenase