

Systemic PPAR γ Ligation Inhibits Allergic Immune Response in the Skin

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We have shown previously that specific ligands of the peroxisome proliferator-activated receptor- γ (PPAR γ) inhibit the systemic allergic immune response. The objective of this study was to investigate the impact of PPAR γ -ligand treatment on the local allergic immune response. We established a murine model exhibiting clinical and histological features of AD-like skin lesions with high reproducibility. In this model, the PPAR γ ligand was applied in an either preventive or therapeutic manner via systemic and local routes. The affected skin areas were assessed by standardized skin score, histological analyses, and immunohistochemical examinations. Our data show that systemic application of PPAR γ ligand by a preventive protocol led to significantly reduced onset of eczematous skin lesions. This was confirmed by histology, showing decreased skin thickness accompanied by significantly reduced infiltrations of CD4+ and CD8+ lymphocytes but also mast cells. Additionally, early allergen-specific IgE and IgG1 responses were reduced (day 21/35), whereas IgG2a levels remained unchanged. In conclusion, our results demonstrate that PPAR γ -ligand treatment inhibits not only systemic allergic immune response, but also local allergen-mediated dermatitis. Our findings point to therapeutic strategies, including a PPAR γ -ligand-based treatment.

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INTRODUCTION

The peroxisome proliferator-activated receptor- γ (PPAR γ) belongs to the nuclear receptor class II superfamily, showing pleiotropic effects in inflammatory processes in their capacity as ligand-dependent transcription factor (Zingarelli and Cook, 2005). PPAR γ was found to be expressed in different cell types, involving B and T lymphocytes (Greene *et al.*, 2000), monocytes/macrophages (Tontonoz *et al.*, 1998), dendritic cells (Nencioni *et al.*, 2002), but also in epidermal differentiating keratinocytes (Rivier *et al.*, 1998). Although many nuclear receptor class II members, like the retinoid-acid (Vollberg *et al.*, 1992), vitamin D receptor (Xie *et al.*, 2002; Bikle *et al.*, 2003), as well as PPAR α and PPAR δ (Tan *et al.*, 2001; Schmuth *et al.*, 2004), are known to be involved in skin homeostasis, less is known about the impact of PPAR γ . Current studies indicate that activation of PPAR γ can improve hyperproliferative skin diseases through specific agonists, belonging to the group of thiazolidinediones (Ellis *et al.*, 2000; Demerjian *et al.*, 2006). We have shown previously that the thiazolidinedione ciglitazone inhibits systemic allergic immune response by inhibiting IgE production, but

also (pro)inflammatory cytokines of the T-cell-dependent immune response *in vitro* and *in vivo* (Ruhl *et al.*, 2003). Furthermore, a recent analysis of atopic dermatitis (AD) skin lesions show increased PPAR γ expression not only in keratinocytes, but also in infiltrating T cells and monocytes (Dahten *et al.*, 2007). In murine asthma models, ciglitazone reduced allergic airway hyperresponsiveness and characteristic parameters of T-helper cell-2 (Th2)-driven airway inflammation, like GATA-3, IL-4, IL-13 (Mueller *et al.*, 2003; Trifilieff *et al.*, 2003; Woerly *et al.*, 2003), suggesting that systemic and local effects mediated by PPAR γ ligation. Whether PPAR γ ligation affects local Th-driven inflammation in the skin besides its systemic immune modulation remains unknown.

AD is a chronic remittent skin disease, which may be associated with IgE-dependent diseases like allergic asthma, food allergy, and allergic rhinitis (Leung and Bieber, 2003; Spergel and Paller, 2003). Interactions between the local and systemic immune responses orchestrated by resident and infiltrating cells play a crucial role in the manifestation of AD. In mice, allergen-mediated dermatitis was initially demonstrated by epicutaneous (e.c.) application of proteins (Wang *et al.*, 1996, 2007; Spergel *et al.*, 1998). In these models, the sensitized skin areas exhibit significant histological signs such as skin thickening and increased mononuclear infiltrates, which are accompanied by elevated allergen-specific IgE and IgG. However, until now, data on reproducibility and demonstration of the clinical outcome were missing in these mouse models.

In this study, we investigated the severity of eczematous skin lesions after preventive and therapeutic PPAR γ -ligand

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Abbreviations: AD, atopic dermatitis; e.c., epicutaneous; i.p., intraperitoneal; OVA, ovalbumin; PPAR γ , peroxisome proliferator-activated receptor- γ

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treatment in a murine allergic dermatitis model. We demonstrate that repeated systemic application of the PPAR γ ligand results in a decrease of clinical features of allergic dermatitis, like erythema, edema, and oozing, after allergen exposure. These observations were confirmed by histological examinations showing reduced epidermal and dermal hyperplasia, accompanied by significantly reduced infiltrations of CD4+ and CD8+ lymphocytes and mast cells in sensitized skin areas of PPAR γ -ligand-treated mice. The analysis of allergen-specific humoral immunity points to a PPAR γ -mediated inhibition of allergen-specific IgE and IgG1 responses during sensitization.

RESULTS

Repeated systemic and local sensitizations with OVA induced reproducible skin inflammation

To establish a murine model showing local skin inflammation with high response rates, we initiated a panel of different sensitization protocols (Figure 1), following the previous protocol of e.c. ovalbumin (OVA) sensitization (Spergel *et al.*, 1998; Wang *et al.*, 2007). Our data show that only e.c. OVA sensitization, in combination with an initial systemic sensitization, lead to significant response rate with visible AD-like skin lesions in comparison with age- and weight-matched mice without any OVA sensitization. As shown in Figure 2a, these sensitized mice (OVA) developed local dermatitis with visible clinical symptoms, like dryness, extension, erythema, edema, papules, oozing, and/or crusts, which were similar to resemble clinical skin features of human AD. The observed clinical symptoms were graded by the total clinical skin score (CSS) up to 13 points (with a mean of 9 ± 1), whereas non-sensitized mice reached a mean of

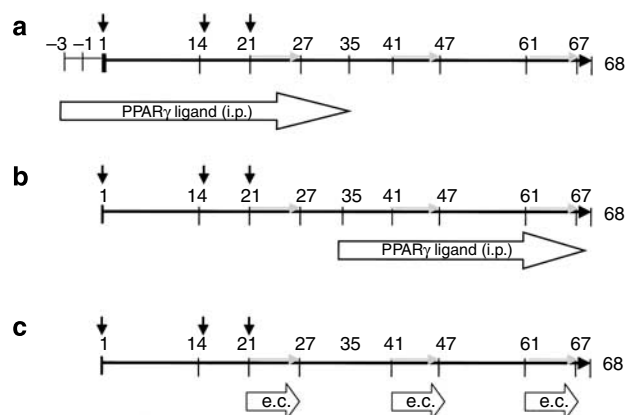


Figure 1. Protocol of OVA sensitization and PPAR γ -ligand treatment in mice. Mice were sensitized intraperitoneally with 10 μ g OVA on days 1, 14, and 21 (black arrows), and epicutaneously by patches with 100 μ g OVA, twice a week (gray arrows). E.c. sensitization comprised three 1-week exposures. (a) Preventive treatment with PPAR γ -ligand started 2 days before the first sensitization and ended with day 35. (b) Therapeutic exposure with PPAR γ -ligand occurred from day 35 until 69. In both application groups, the ligand was applied at 4 mg kg $^{-1}$ body weight via i.p. injection, every second day. (c) PPAR γ ligand was applied epicutaneously in combination with OVA sensitization via patch. On day 70 mice were killed and skin and blood samples were collected.

4 ± 1 . Furthermore, affected skin areas showed histopathological features of eczema, such as epidermal and dermal skin thickening (twofold), parakeratosis, spongiosis, and a dense perivascular infiltrate (Figure 2b), which was accompanied by increased mast cell infiltration and degranulation (Figure 2c). Immunohistochemical analysis revealed significant dermal increase up to sixfold of dermal CD4+ (Figure 2d), threefold of CD8+ (Figure 2e), and twofold of CD11+ cells (Figure 2f). The cytokine profile in local skin lesions was dominated by Th2 cytokines, like IL-4 and IL-10, as determined by PCR analysis (Table 1).

Skin characteristics of mice with systemic and local sensitization (model III) in comparison with mice, which were sensitized only by e.c. allergen application (model II) indicate that the combined sensitization model led more to formation of AD-like skin lesions (Table 1). First, the clinical signs and symptoms of AD, graded by the CSS, were strong, clear visible (Figure 2a), and exhibited high response rates ($P < 0.01$) only in mice sensitized by the intraperitoneal (i.p.) e.c. method, whereas e.c. sensitization alone did not induce significant reproducible skin features. Accordingly, histological changes regarding skin thickness and numbers of infiltrating CD4+ lymphocytes and mast cells were less prominent in model II.

Next, we used this dermatitis model to study the effect of PPAR γ -ligand treatment on the local inflammatory immune response in eczematous skin lesions. Therefore, severity of inflammatory skin lesions was analyzed in mice after repeated PPAR γ -ligand applications at three different time points of (Figure 1).

Repeated exposure of PPAR γ -ligand improved severity of clinical AD symptoms in the skin

Macroscopic examination of dermatitis severity (Figure 3) shows that OVA-sensitized mice reached a mean CSS of 9 ± 1 points. The non-sensitized mice group developed due to the patched application also a slight inflammatory response, which was significantly lower than in the OVA group (CSS of 4 ± 1). The PPAR γ -ligand application groups show that the group with i.p. exposures of ciglitazone clearly exhibited less inflammation of lesional skin, regarding erythema, oozing, and crusts. By contrast, local application of ciglitazone did not alter the clinical outcome of AD-like skin lesions at all.

Systemic PPAR γ -ligand treatment results in decreased skin inflammation

The amelioration of skin inflammation by systemic PPAR γ ligation was also reflected by reduced skin thickness and less perivascular infiltrates. Figure 4a shows reduced epidermal and dermal thickness of mice skin, which received systemic PPAR γ -ligand treatment. This was accompanied by less infiltration of lymphocytes into the skin (Figure 4b). Detailed analysis of systemically treated mice groups revealed that preventive treatment with PPAR γ ligand was more efficient than therapeutic PPAR γ -ligand application. First, epidermal thickening was less prominent, and second, less infiltration of epidermal and dermal lymphocytes, but also mast cells, was observed. However, neither preventive nor therapeutic

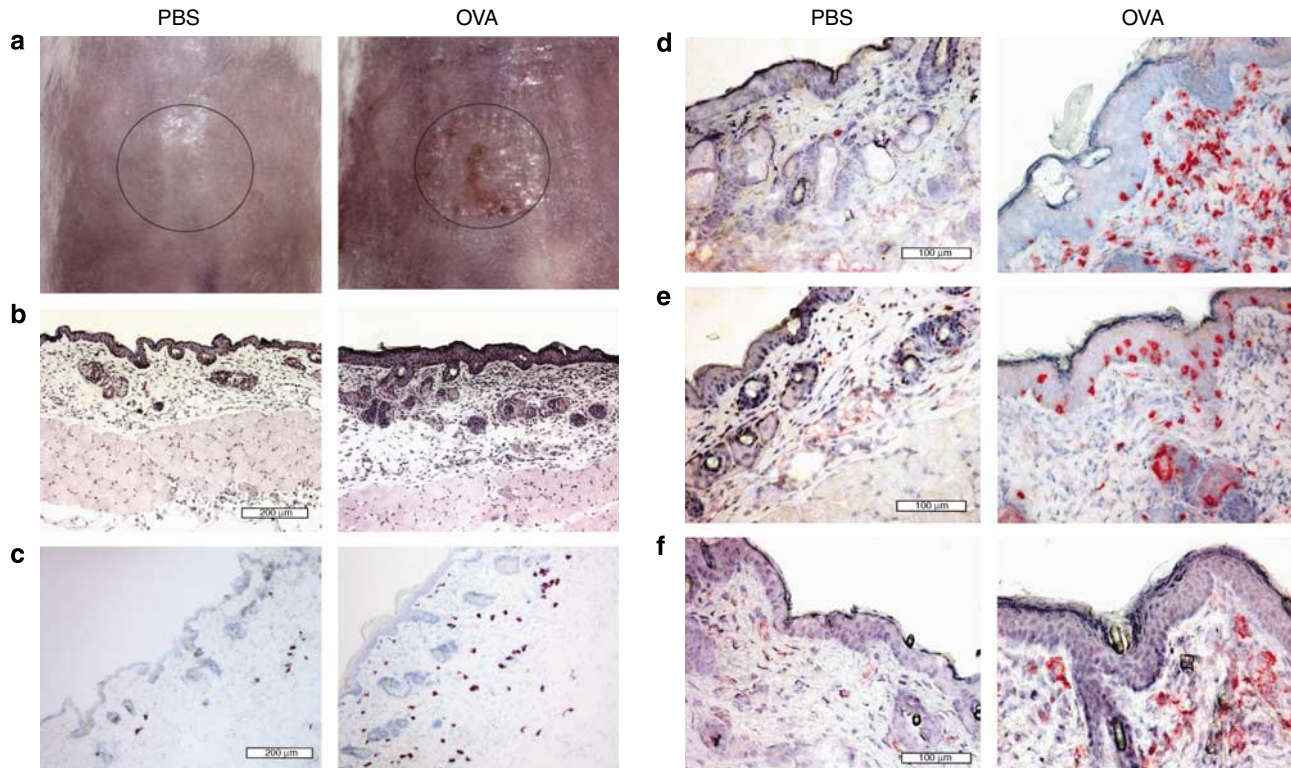


Figure 2. Histological analysis of skin biopsies from OVA-sensitized mice (right column) compared with non-sensitized mice (left column). Sensitized mice show (a) clinical skin features, like erythema, edema/papule, oozing, and dryness within the patched areas (encircled). (b) Hematoxylin- and (c) o-toluidine blue staining of 5- μ m sections revealed thickness of epidermis and dermis, accompanied by a dense dermal mononuclear infiltrate consisting of mononuclear and mast cells. Immunohistochemical staining of 4- μ m sections pointed to significantly increased numbers of CD4+ (d), CD8+ (e), and CD11c+ (f) cells after repeated OVA exposures. Images in panels (b) and (c) were taken at original magnification $\times 100$ (bar = 200 μ m) and that in panels (d)–(f) at original magnification $\times 200$ (bar = 100 μ m).

PPAR γ -ligand treatment ameliorated the numbers of CD11+ cells (data not shown).

Preventive PPAR γ ligation inhibits allergen-specific IgE immune response

As patients with AD often exhibit elevated specific IgE levels (Novak and Bieber, 2003), we examined specific IgE in the different treatment groups (Figure 5a). After OVA sensitization (black bars), specific IgE, IgG1, and IgG2a levels increased dramatically. Preventive application of PPAR γ -ligand (Figure 1a) resulted in a profound decrease of specific IgG1 on day 21 (white bars). A significant inhibition of specific IgG1 was also detectable on day 35. Specific IgE level peaked later than IgG1 level, and was also significantly reduced on day 35 in the group receiving preventive PPAR γ -ligand treatment. Therapeutic treatment with PPAR γ ligand (Figure 1b) did not bring about a significant change in the humoral immune response (data not shown). Importantly, allergen-specific IgG2a production was not reduced by PPAR γ -ligand treatment, ruling out general immunosuppression by ciglitazone at the used concentrations. These data point to PPAR γ -mediated suppression of the development, but not inhibition, of an established humoral immune response.

To find out whether alteration of humoral immune response is T-cell cytokine dependent, we next examined

IL-4/IFN- γ production (Figure 5b). The observation of *ex vivo* measurements in cultured splenocytes showed no detectable baseline cytokine production, which increased after stimulation with OVA and anti-CD28 up to 750 ± 95 pg ml $^{-1}$ (IL-4) and 130 ± 40 pg ml $^{-1}$ (IFN- γ), respectively. Splenocytes from mice receiving therapeutic PPAR γ -ligand treatment exhibited reduced IL-4 ($60 \pm 10\%$) and IFN- γ ($85 \pm 10\%$) responses. In mice receiving preventive PPAR γ -ligand treatment, a decrease in IFN- γ production was clearly detectable.

DISCUSSION

Our recent findings point to the role of PPAR γ ligands in the regulation of systemic inflammatory immune responses (Ruhl *et al.*, 2003; Dahten *et al.*, 2007). Now we demonstrate the impact of systemic PPAR γ ligation on local inflammatory immune response in the skin.

We developed an optimized murine AD model showing local inflammatory skin lesions that were clinically, histologically, and immunologically comparable to human AD. In contrast to previously described models, our protocol leads to induction of AD-like eczema with macroscopic skin features and high response rates. It has been shown that CD4+ T cells predominate in the dermis of acute skin lesions of AD patients, whereas only a few CD8+ T cells were detected (Simon *et al.*, 2004). This is consistent with our data showing

Table 1. Systemic OVA sensitization followed by repeated e.c. OVA exposure-induced skin inflammation and exacerbates allergic dermatitis

Analysis	Route of sensitization		
	I W/o OVA	II OVA e.c.	III OVA e.c.+i.p.
Eczema versus non-eczema	0/10	5/10	9/10
Clinical skin score ¹	4 $\pm$ 1	6 $\pm$ 1 ^a	9 $\pm$ 1 ^{a,b**}
Skin thickness (μ m) ²			
Epidermis	14 $\pm$ 1	19 $\pm$ 1 ^{a**}	23 $\pm$ 1 ^{a***b**}
Dermis	73 $\pm$ 4	73 $\pm$ 4	142 $\pm$ 4 ^{a,b***}
Cell infiltrates (per HPF) ³			
Mast cells	24 $\pm$ 2	24 $\pm$ 2	33 $\pm$ 2 ^{a,b**}
CD4	7 $\pm$ 1	22 $\pm$ 3 ^{a**}	46 $\pm$ 3 ^{a,b***}
CD8	4 $\pm$ 1	5 $\pm$ 1	14 $\pm$ 2 ^{a***}
CD11c	7 $\pm$ 1	ND	18 $\pm$ 2 ^{a**}
Cytokine expression ⁴			
IL-4	1	2 $\pm$ 1 [*]	10 $\pm$ 2 ^{**}
IL-10	1	1 $\pm$ 1	2 $\pm$ 1
IFN- γ	1	-4 $\pm$ 1	1 $\pm$ 1

e.c., epicutaneous; HPF, high-power field; i.p., intraperitoneal; ND, not detected; OVA, ovalbumin; PBS, phosphate-buffered saline.

Data are shown as mean $\pm$ SEM ($n=10$). Statistical significance (P) is based on Mann-Whitney U -test, *** $P<0.001$, ** $P<0.01$, and * $P<0.05$, versus I (a), versus II (b).

¹Clinical skin score was defined as the sum of individual scores grades as 0 (none), 1 (mild), 2 (moderate), and 3 (severe) for erythema, edema/papule, oozing/crusts, dryness, and extension.

²Skin thickness was determined in 5- μ m hematoxylin-stained skin sites by digital measurement tools.

³Positively stained cells were counted in 10 HPFs and expressed as cells per HPF.

⁴Expression of cytokine mRNA was determined by reverse transcriptase-PCR.

strong CD4+ T-cell infiltration in the skin. However, our data also show that this sensitization protocol recruits significant amounts of CD8+ cells as well. Recently, some studies pointed to the role of epidermal CD8+ cells in the pathogenesis of AD (Akdis *et al.*, 1999; Savinko *et al.*, 2005). In mice depleted of CD8+, no significant signs of inflammation could be observed after allergen exposure, whereas CD4+ depletion did not abolished allergen-induced skin inflammation (Hennino *et al.*, 2007). These data suggest CD8+ T cells are important effector cells for allergen-induced skin inflammation. Whether these cells are allergen-specific or are a result of bystander activation is currently under investigation. Since PPAR γ has been shown to be expressed in CD8+ lymphocytes (Dahten *et al.*, 2007), this cell population may be involved in PPAR γ -targeted treatment of AD.

The local skin lesions were also characterized by sparse epidermal infiltration of dendritic cells and increased expres-

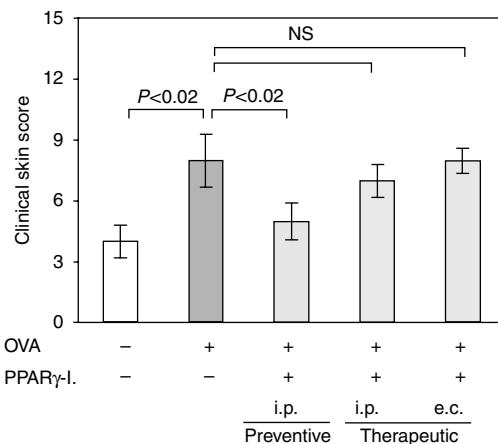


Figure 3. Improved severity of dermatitis in PPAR γ -ligand-treated mice.

In non-sensitized, vehicle-treated mice (white bar), OVA-sensitized mice (black bar), and OVA-sensitized plus PPAR γ -ligand (PPAR γ -L)-treated mice (gray bars), clinical skin conditions (erythema, edema/papule, oozing/crusts, dryness, and extension) were macroscopically scored at the sensitized belly area and grades as 0 (none), 1 (mild), 2 (moderate), and 3 (severe). The total clinical skin score for each mouse was defined as the sum of individual scores for every described sign and symptom. Data are presented as mean values $\pm$ SEM (bars). Statistical significance (P) is based on Mann-Whitney U -test; NS indicates no statistical significance.

sion of Th2 cytokine profile (IL-4, IL-10). These observations are in line with reports on acute skin lesions (Allam and Novak, 2006; Boguniewicz *et al.*, 2006). From this finding, we determined an induction of allergen-specific humoral response in sensitized mice. Elevated total and specific IgE levels, but increased expression of cytokines, like IL-4, and IL-10, have also been reported previously in the majority of AD patients (Ohmen *et al.*, 1995; Leung and Bieber, 2003; Novak and Bieber, 2003) and also in murine AD models (Nelde *et al.*, 2001; Chen *et al.*, 2004; Gutermuth *et al.*, 2004; Lewkowich *et al.*, 2004).

These data underline the importance of route and duration of allergen exposure regarding development and outcome of allergen-induced dermatitis. Our AD model provides an optimized tool to study the pathogenic processes of AD and to establish treatment approaches under well-defined conditions.

PPAR γ has been shown to exhibit anti-inflammatory activities in different models of inflammatory diseases, such as atherosclerosis, inflammatory bowel disease, or multiple sclerosis (Forman *et al.*, 1995; Su *et al.*, 1999; Natarajan *et al.*, 2003). Our own previous studies indicated that the PPAR γ -ligand ciglitazone inhibits allergic immune response by inhibiting Th2-driven IgE production and also production of (pro)inflammatory cytokines of the Th response *in vitro* and *in vivo* (Ruhl *et al.*, 2003). We now show that ciglitazone inhibits not only systemic but also local inflammatory immune response in the skin by diminishing the severity of allergen-induced dermatitis. Our data demonstrate that systemic, but not topical, applications of PPAR γ -ligand reduce the inflammatory response in the skin. Several lines of evidence support this finding. First, only mice obtaining i.p.

treatment developed less clinical signs of protein dermatitis, like erythema, oozing, and crusts. Second, histological signs of dermatitis, like skin thickness and numbers of dermal CD4+ lymphocytes, were significantly reduced. However, we cannot rule out the possibility that inefficient local penetration of PPAR γ ligand was responsible for the missing efficacy. Venkatraman *et al.* (2004) have shown anti-inflammatory effects for orally and also topically applied thiazolidinedione derivatives in an oxazolone-challenged dermatitis model. In line with our results, oral application of PPAR γ ligands acts at lower dosages than topical applications, which indicates improved systemic mechanisms for these substances. The fact that therapeutic application of systemic PPAR γ -ligand treatment seems to be less efficient than its preventive application, points in turn to the less important role of PPAR γ in the established allergic immune response. Our histological data confirm this assumption. Only by preventive PPAR γ -ligand application could skin thickening and numbers of CD8+ lymphocytes and mast cells be significantly reduced, reaching the numbers in non-sensitized mice.

IgE production is a prerequisite for type-1 allergic diseases (Geha *et al.*, 2003). In line with our previous data, analysis of the humoral immune response shows ameliorated allergen-specific IgE and IgG1 production in PPAR γ -ligand-treated mice (Ruhl *et al.*, 2003). In preventively PPAR γ -treated mice,

showing significantly reduced dermatitis, specific IgG1 and IgE levels also were significantly reduced. It has been shown that severity of AD correlates with elevated levels of IgE (Flohr *et al.*, 2004; Boguniewicz *et al.*, 2006). If so, it remains to be determined whether non-IgE-mediated (intrinsic) form of AD will be affected by PPAR γ -ligand treatment as well. Patients with extrinsic AD, besides elevated IgE levels, exhibit increased Th2 immune response. Also in mice, IgE and IgG1 responses are Th2 cell dependent (Coffman, 1993). Although IL-4 production was decreased in the therapeutic protocol, humoral immune response was not. Hahn *et al.* (2003) demonstrated previously that antagonizing the IL-4 receptor during the OVA sensitization phase leads to inhibition of OVA-specific IgE induction and development of allergic phenotype. By contrast, inhibition of IL-4 did not affect the ongoing IgE response after sensitization. Since depletion of IFN- γ results in less allergen-induced dermal hypertrophy (Spergel *et al.*, 1999), reduced IFN- γ production in splenocytes from preventively and therapeutically treated mice points to a possible mechanism for the significantly reduced dermal thickness in both treatment groups.

Taken together, our data suggest that PPAR γ -ligand-mediated amelioration of allergic symptoms in the skin were mediated by a modulation of the humoral and also the cellular immune response. PPAR ligands repress the secretion

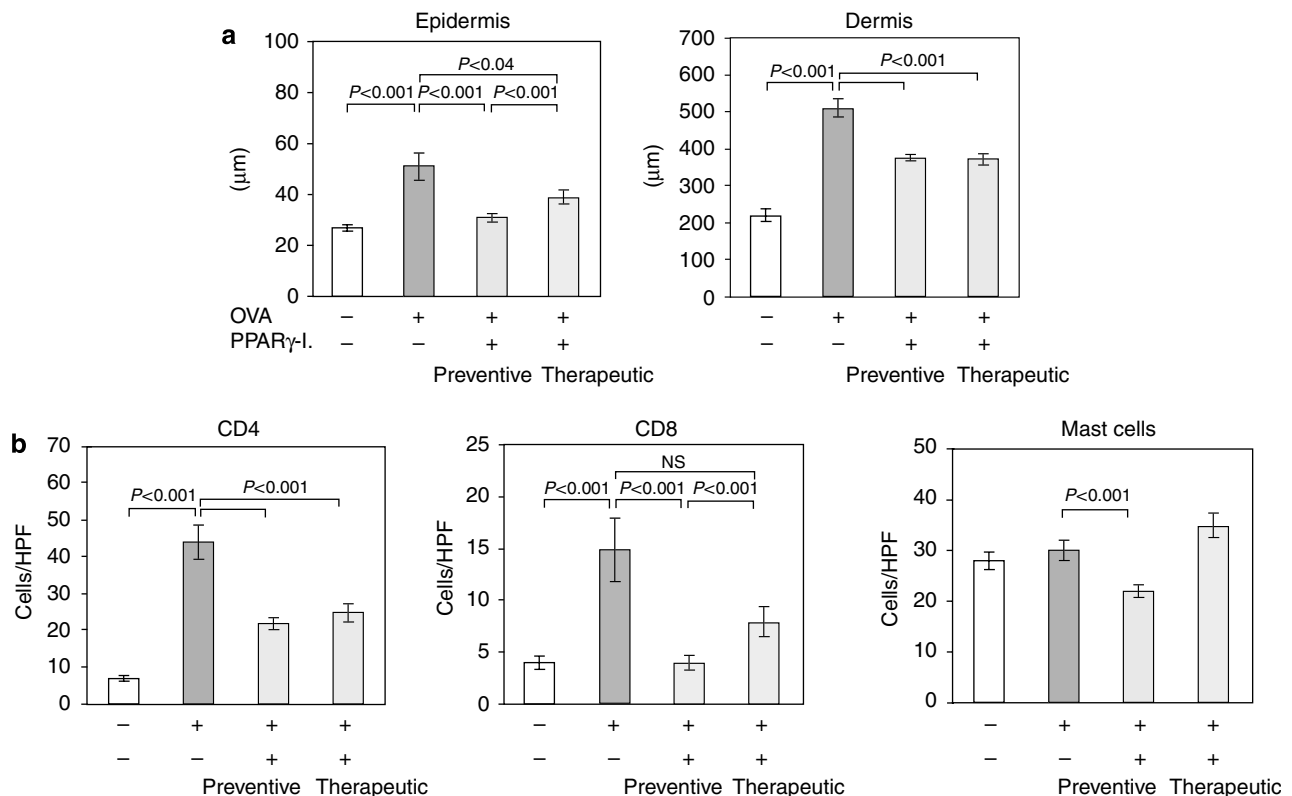


Figure 4. Histological features of dermatitis were reduced in mice with repeated systemic PPAR γ -ligand treatment. Skin thickness (a) and cell infiltrates of CD4+, CD8+, and mast cells (b) were determined in 4–5 μ m sections of frozen skin from non-sensitized, vehicle-treated mice (white bar), OVA-sensitized mice (black bar), and OVA-sensitized plus systemically (preventive, therapeutic) PPAR γ -ligand (PPAR γ -I.)-treated mice (gray bars). Skin thickness (μ m) was determined in hematoxylin-stained skin site by digital measurement tools. Cells positive for the immunohistochemical stain were counted in 10 high-power fields and expressed as cells per high-power field. Data are presented as mean values $\pm$ SEM (bars). Statistical significance (P) is based on Mann-Whitney U-test; NS indicates no statistical significance.

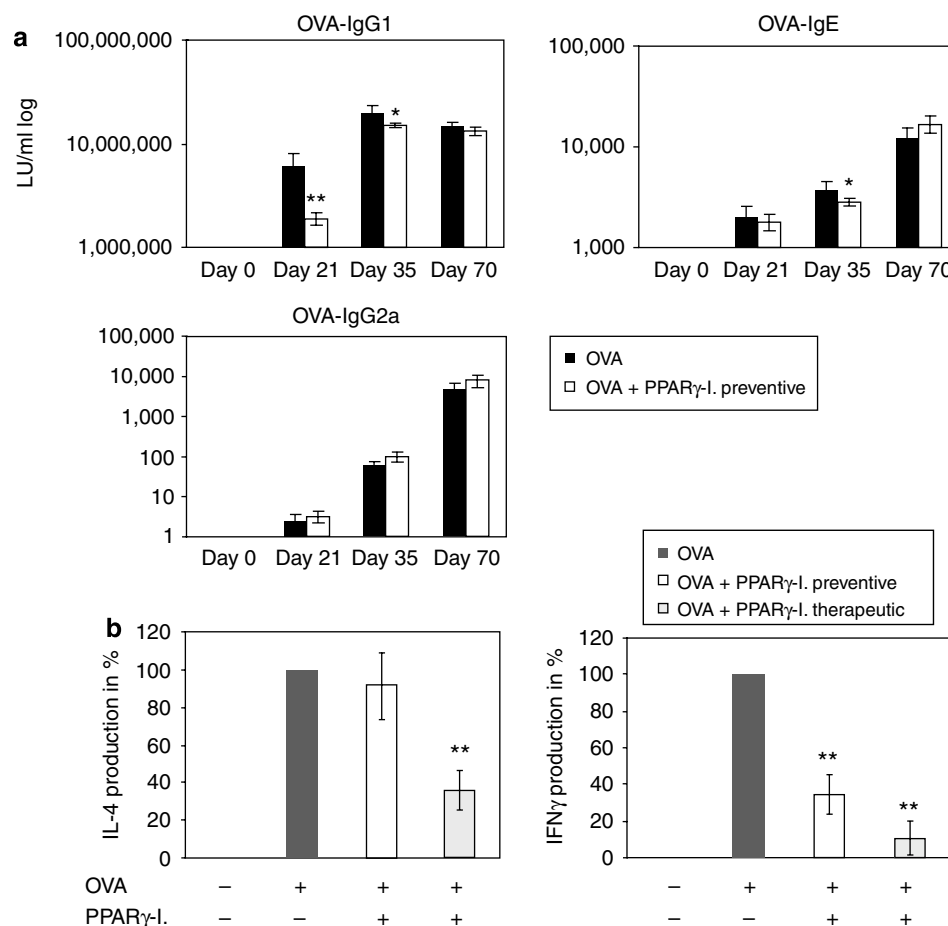


Figure 5. The systemic immune response is regulated by systemic PPAR γ ligation. (a) OVA-specific IgG1, IgE, and IgG2a levels were analyzed in the sera of OVA-sensitized (black bar) and OVA-sensitized plus preventively PPAR γ -ligand (PPAR γ -l.)-treated mice (white bar) by ELISA. Time points of analysis were days 21 (during systemic sensitization), 35 (after systemic sensitization), and 70 (after e.c. sensitization). At day 0 (before systemic sensitization) no specific Ig levels were detectable. (b) IL-4 and IFN γ production were analyzed in the supernatants of unstimulated and OVA plus anti-CD28-restimulated splenocytes of OVA-sensitized mice (black bar), OVA-sensitized plus preventively PPAR γ -ligand-treated mice (white bar), and OVA-sensitized plus therapeutically PPAR γ -ligand-treated mice (gray bar) by ELISA. The asterisks indicate statistical significance (*P*), based on Mann-Whitney *U*-test comparing OVA-sensitized mice with mice sensitized plus ligand treated; ***P* < 0.01 and **P* < 0.05.

of chemokines by epithelial and other cells, decreasing recruitment of leukocytes to the site of inflammation (Straus and Glass, 2007). As shown in an asthma model, PPAR γ ligands reduce the chemokine-mediated infiltration of inflammatory cells (Nie *et al.*, 2005). The anti-inflammatory activity of PPAR γ ligands in mouse models, and our finding that PPAR γ ligand exerts anti-inflammatory effects on the development of allergic skin inflammation, suggests a possible application in treating allergic diseases, including AD. Therefore, our data may provide the basis for development of therapeutically molecules involving a PPAR γ -targeted immune modulation.

MATERIALS AND METHODS

Mice sensitization and treatment

Eight-week-old female BALB/c mice were purchased from the Federal Institute of Risk Assessment, Berlin. All mice were housed within an accredited animal facility at the Charité-Universitätsmedizin (Berlin), and maintained under pathogen-free conditions with standard laboratory food and water *ad libitum*. Procedures were

performed in compliance with protocols approved by the local State Office of Health and Social Affairs.

As shown in Figure 1, mice were sensitized intraperitoneally on days 1, 14, and 21 with 100 μ l of 10 μ g OVA adsorbed to 10 μ g Al(OH)₃ or with phosphate-buffered saline as control (all Sigma-Aldrich, Taufkirchen, Germany). E.c. allergen application was performed as described previously (Spergel *et al.*, 1998). Briefly, after i.p. anesthesia with 0.02% xylazine solution (Bayer, Leverkusen, Germany), the skin on the mice belly was shaved and OVA or phosphate-buffered saline was applied by the patch test method. Each mouse had a total of three 1-week exposures to the same skin site. Ciglitazone (Alexis Biochemicals, Grünberg, Germany) was dissolved at 25 mg ml⁻¹ in DMSO and further diluted in phosphate-buffered saline before application. Ciglitazone or vehicle (control) was applied either preventively (Figure 1a) or therapeutically (Figure 1b) via i.p. injection at 4 mg kg⁻¹ body weight every second day, or by e.c. application at 10 mm by the allergen-patch application method (Figure 1c). Preventive treatment with PPAR γ ligand was started 2 days before the first sensitization and was stopped at day 35. Therapeutic application with PPAR γ ligand was

performed from day 35 until 69. At day 70 mice were killed and skin, spleen, and blood samples were collected for further analysis.

Clinical skin features

To evaluate the severity of OVA-induced eczematous skin lesions, we used a skin scoring system, which considers typical skin features of AD, like erythema, edema/papules, oozing/crusts, dryness, and extension. The standardized clinical skin score (CSS) is based on the clinical features of human AD (Hanifin and Rajka, 1980; Leung *et al.*, 1990) and was previously described in a murine model with spontaneous manifestation of AD-like skin lesions (Matsuda *et al.*, 1997). For calculating total CSS, the marked lesions, visible on the patched mice area, were graded as 0 (none), 1 (mild), 2 (moderate), and 3 (severe) by two independent persons. The total score was taken as index of dermatitis severity.

Histological analysis

For histological analysis, 5- μ m sections of skin samples were stained with hematoxylin for 1 minute. Thickness of epidermis and dermis was determined by Axiovision measuring-tools on the Axioplan light microscope (Zeiss, Berlin, Germany) at $\times 100$ magnification. Ten measurements were obtained for each mouse and expressed in micrometers with mean and SEM calculated.

Immunohistochemistry

Sections measuring 4 μ m were prepared and used for staining by the streptavidin-biotin complex method. Sections were incubated with rat anti-mouse CD4 (RM4-5) and CD8 (53-6.7), followed by incubation with biotinylated goat anti-rat IgG (all BD Pharmingen, Heidelberg, Germany). CD11c-positive cells were detected using purified hamster anti-mouse antibody (HL3) and biotinylated anti-hamster IgG cocktail (BD Pharmingen). Signals were detected by Chem-Mate $\text{\textregistered}$ alkaline phosphatase/red detection kit (Dako, Hamburg, Germany) and by hematoxylin counterstaining. To detect infiltrating mast cells, 5- μ m skin sections were stained for 1 hour with *o*-toluidine blue. Positively stained cells were counted in 10 high-power fields at $\times 200$ magnification and expressed as cells per high-power field, with mean and SEM calculated.

RNA preparation and reverse transcriptase-PCR

Total RNA was extracted from 30 μ g of frozen skin samples using RNeasy mini Kit (Qiagen, Hilden, Germany). A 1–2 μ g weight of total RNA was transcribed into cDNA using TaqMan $\text{\textregistered}$ reverse transcription reagents according to the manufacturer's guidelines. The mRNA expressions of cytokines were determined using TaqMan reagents IL-4 (Mm00445259_m1), IL-10 (Mm00439616_m1), and IFN- γ (Mm00801778_m1) primer and probe. The thermal cycling conditions were set to 95 $^{\circ}$ C for 10 minute, followed by 40 cycles of amplification at 95 $^{\circ}$ C for 15 s and 60 $^{\circ}$ C for 1 minute for each cycle. Efficiency-corrected relative quantification was performed by the 2 $^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Serum antibody levels

OVA-specific IgE, IgG1, and IgG2a levels were measured by ELISA. Briefly, 96-well microtiter plates were coated overnight, at 4 $^{\circ}$ C, with 100 μ l of 0.1 M NaHCO $_3$ containing the appropriate antibody. Serum samples and standards were diluted in 1% skimmed milk and incubated for 2 hours. The amounts of bound antibody were

detected with biotin-conjugated anti-mouse isotype-specific antibody, followed by treatment with streptavidin-horseradish peroxidase (R&D Systems, Wiesbaden, Germany) and with the peroxidase substrate 3,3',5,5'-tetra-methyl-benzidine dihydrochloride (Sigma-Aldrich) at 405 nm. Serum titers were expressed as laboratory units of serum dilution. Plates were read at 450 nm.

Cytokine determination

A total of 10 ^{6} freshly isolated murine splenocytes were cultured with and without 250 μ g ml $^{-1}$ OVA and 5 μ g ml $^{-1}$ anti-CD28 (both BD Pharmingen) in RPMI 1640 culture medium supplemented with L-glutamine (2 mM), penicillin (100 U ml $^{-1}$), streptomycin (100 mg ml $^{-1}$), and 10% heat-inactivated fetal calf serum for 96 hours (all Biochrom/Seromed, Berlin, Germany). All cell cultures were carried out at 37 $^{\circ}$ C in a 5% CO $_2$ atmosphere. Cytokines were quantified in the supernatants of cultured cells by ELISA (R&D Systems), according to the manufacturer's guidelines.

Statistical analysis

Data were compared by non-parametric analysis (SPSS Software, Munich, Germany) using Mann-Whitney *U*-test for independent groups. *P*-values less than 0.05 were considered statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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