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Deletion of PPARγ in Alveolar Macrophages Is Associated with a Th-1 Pulmonary Inflammatory Response

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Peroxisome proliferator-activated receptor γ (PPARγ) is constitutively expressed at high levels in healthy alveolar macrophages, in contrast to other tissue macrophages and blood monocytes. PPARγ ligands have been shown to down-regulate IFN-γ-stimulated inducible NO synthase (iNOS) in macrophages. Because NO is an important inflammatory mediator in the lung, we hypothesized that deletion of alveolar macrophage PPARγ in vivo would result in up-regulation of iNOS and other inflammatory mediators. The loss of PPARγ in macrophages was achieved by crossing floxed (+/+) PPARγ mice and a transgenic mouse containing the CRE recombinase gene under the control of the murine M lysosome promotor (PPARγKO). Alveolar macrophages were harvested by bronchoalveolar lavage (BAL). Lymphocytes (CD8:CD4 ratio = 2.8) were increased in BAL of PPARγKO vs wild-type C57BL6; p ≤ 0.0001. Both iNOS and IFN-γ expression were significantly elevated (p ≤ 0.05) in BAL cells. Th-1 associated cytokines including IL-12 (p40), MIP-1α (CCL3), and IFN inducible protein-10 (IP-10, CXCL10) were also elevated. IL-4 and IL-17A were not detected. To test whether these alterations were due to the lack of PPARγ, PPARγ KO mice were intratracheally inoculated with a PPARγ lentivirus construct. PPARγ transduction resulted in significantly decreased iNOS and IFN-γ mRNA expression, as well as reduced BAL lymphocytes. These results suggest that lack of PPARγ in alveolar macrophages disrupts lung homeostasis and results in a Th1-like inflammatory response. The Journal of Immunology, 2009, 182: 5816–5822.

The lung is continuously exposed to environmental particulates, allergens, and microorganisms and yet does not become inflamed or infected (1). Investigations of mechanisms responsible for maintenance of lung homeostasis have centered around the alveolar macrophage (2). Peroxisome proliferator-activated receptor-γ (PPARγ)3 is a ligand activated nuclear receptor that regulates lipid and glucose metabolism (3, 4) and recently has been implicated as a negative regulator of inflammation (5–8). This negative regulation of proinflammatory genes by PPARγ is termed transrepression and occurs in a ligand-dependent manner by antagonizing the activities of other transcription factors e.g., NF-κB and AP-1 (reviewed in Ref. 9). Although weakly expressed in monocytes, PPARγ protein increases with macrophage differentiation and is predominantly nuclear in location (10). We were the first to observe marked constitutive expression in healthy human alveolar macrophages (11). The role of PPARγ in alveolar macrophages is unclear.

Absence of PPARγ is lethal, but several conditional knockout mouse models have been developed using a homozygous floxed PPARγ mice (12), such as the IFN-inducible CRE recombinase (12) or CRE under the control of the M lysosome promotor (13). PPARγ conditional knockout (KO) peritoneal macrophages have markedly reduced expression of ABCG1, reduced cholesterol efflux, and more atherosclerosis when crossed to the proatherogenic mouse models, e.g., low density lipoprotein receptor KO (12, 13). To date these PPARKO KO models have not been used to study the role of alveolar macrophage PPARγ in maintaining lung homeostasis in vivo.

PPARγ deficiencies in the lung have been observed in inflammatory conditions such as asthma, pulmonary alveolar proteinosis, and sarcoidosis, as well as several animal models of lung inflammation (14–19). Murine macrophages are prominent producers of inducible NO synthase (iNOS). PPARγ ligands have been shown to down-regulate IFN-γ-stimulated iNOS in peritoneal macrophages (5). Because of the high constitutive expression of PPARγ in lung alveolar macrophages, we hypothesized that deletion of alveolar macrophage PPARγ would result in the in vivo up-regulation of iNOS and other inflammatory mediators. To test this hypothesis, we investigated alveolar macrophages from PPARγ KO mice with regard to: 1) inflammatory mediator production and 2) the effects of in vivo replacement of PPARγ via transduction with a lenti-PPARγ virus.

Materials and Methods

All studies were conducted in conformity with Public Health Service Policy on humane care and use of laboratory animals and were approved by the institutional animal care committee. PPARγ KO conditional mice

Homozygous floxed (+/+) PPARγ mice were provided by Dr. F. J. Gonzalez (National Institutes of Health, Bethesda, MD) (12). To achieve loss of PPARγ in macrophages, homozygous floxed PPARγ mice were crossed into a transgenic mouse containing the CRE gene.
FIGURE 1. Lymphocytes are increased in BAL fluid from PPARγ KO mice. A, Cytospin preparations from BAL fluid demonstrated an increase in percentage of lymphocytes. B–D, Flow cytometric analysis of BAL fluid cells is depicted; blank (B); CD3/CD4 (C); CD3/CD8 (D). BAL lymphocytes were predominantly CD8. Representative plots of one of three experiments are shown (mean of three separate evaluations CD8 = 2.5 ± 0.3; CD4 = 0.9 ± 0.3%).

under the control of the murine M lysozyme promoter (13) from The Jackson Laboratory. These heterozygous mice were genotyped (Transnetyx) and mated accordingly to generate flox/flox animals used in experiments revealed >95% as determined by trypan blue dye exclusion. GW 9662 (an irreversible PPARγ antagonist which binds to the ligand binding domain (region E/F)) was obtained from Sigma-Aldrich and used at 10 μM in the generation of a stable cell line expressing the human parainfluenza virus type 3 C protein was used for these experiments (20, 21). cDNA corresponding to the human PPARγ was cloned into the multiple cloning sites downstream of a CMV promoter using standard techniques as described (20). The recombinant lentiviral plasmid thus obtained was then transfected into 293FT cells along with plasmids encoding the gag, pol, rev genes and a plasmid possessing the vesicular stomatitis glycoprotein using Lipofectamine 2000. At 48 h post transfection, cell culture supernatant containing the lentivirus-PPARγ (Lenti-PPARγ) was purified by centrifugation at 27,000 rpm at 4°C for 3.5 h. The Lenti-PPARγ virus pellet was resuspended in Tris-EDTA buffer and aliquots of 100 μl were stored at −70°C. The concentration of Lenti-PPARγ virus was determined by a p24 ELISA (Cell Biolabs). A lentivirus expressing the enhanced GFP was obtained using a similar protocol and used as a control in experiments for the determination of transduction efficiency. PPARγ KO received 50 μg of lenti-PPARγ in 50 μl of PBS, lentivirus expressing the enhanced GFP, or PBS alone ( sham) by intratracheal instillation. After 30 days, five animals per group were lavaged, cell differentials were performed, and RNA was extracted.

RNA purification and analysis
Total RNA was extracted from mouse cells by RNeasy protocol (Qiagen). Expression of mRNA was determined by real time RT-PCR using the ABI Prism 7300 Detection System (TaQMan; Applied Biosystems) according to the manufacturer’s instructions. RNA specimens were analyzed in duplicate using primer sets for mouse iNOS (Mm00440485), IL-4 (Mm00445259), IP-10 (Mm00445235), MIP-1α (Mm00441258), IFN-γ (Mm00801778), IL-12(p40) (Mm00434165), and IL17A (Mm00439618)

Flow cytometry
Single cell suspensions of BAL cells were stained for 30 min. at 4°C with the following anti-mouse Abs: CD3 (clone 17A2), CD4-L3T4 (clone RM4-5), CD8b Ly-3 (clone H-35-17.2), CD19 (clone 1D3), and CD45R/B220 (clone RA3-6B2) (BD Biosciences). After 30 min, the cells were washed with cold PBS containing 0.1% BSA, and fixed with 2% paraformaldehyde. FACs analysis was performed with the FACScan instrument (BD Biosciences).

Lentivirus plasmid and transduction
A self inactivating lentivirus expression vector that was previously used in the generation of a stable cell line expressing the human parainfluenza

Bronchoalveolar lavage (BAL)
BAL cells were obtained from 8- to 12-wk-old PPARγ conditional KO mice and age and gender matched WT C57BL/6 controls. For BAL cell harvest, mice received ketamine (90 mg/kg) and xylazine (10 mg/kg) i.p. The thoracic cavity was opened and the lungs were exposed. After cannulating, the trachea a tube was inserted and BAL was conducted with warmed (37°C) PBS in 1 ml aliquots × 5. The lavage was centrifuged and the cell pellet was resuspended in medium (RPMI 1640 supplemented with BCS and PS) or PBS. Differential cell counts were obtained from cytospins stained with a modified Wright’s stain. BAL cell differentials from all animals used in experiments revealed >90% macrophages. Mean viability of lavage cells was >95% as determined by trypan blue dye exclusion. GW 9662 (an irreversible PPARγ antagonist which binds to the ligand binding domain (region E/F)) was obtained from Sigma-Aldrich and used at 1 μM in vitro. To block the effects of IFN-γ, neutralizing anti-IFN-γ (80 μg/ml) or control rat IgG (80 μg/ml) were purchased from BD Biosciences and added to alveolar macrophage cultures for 24 h and subsequently RNA was harvested. For experiments at least three sets of pooled BAL cells from three to five mice were used except where indicated.
Threshold cycle values for genes of interest were normalized to a housekeeping gene (GAPDH 4352339E) and used to calculate the relative quantity of mRNA expression in PPARγ/H9253 conditional KO alveolar macrophages, compared with WT murine controls. Data were expressed as a fold change in mRNA expression relative to control values (22).

**Immunocytochemistry**

Immunocytochemistry was performed on the cytopsin preparations from freshly isolated alveolar macrophages. The cytopsin were stained for basal expression levels of iNOS, PPARγ, and IFN-γ (Santa Cruz Biotechnology). The slides were fixed with 4% paraformaldehyde-PBS and permeabilized with Triton X-100 and stained with the Ab at 1/500 (iNOS, PPARγ) and 1/100 IFN-γ dilution, followed by Alexa conjugated goat anti-rabbit IgG or goat anti rat IgG, respectively (Invitrogen). Slides were counter stained with 4',6-diamino-2-phenylindole (Invitrogen) or propidium iodide (Vector Laboratories) to facilitate nuclear localization.

**Statistics**

Data were analyzed by one-way ANOVA and Student’s t test using Prism software (GraphPad). Values from treated cells were compared with untreated. Significance was defined as \( p \leq 0.05 \).

**Results**

**Inflammatory cells are increased in PPARγ KO BAL**

Analysis of BAL cell population from PPARγ KO revealed a significant increase in total leukocytes (9.0 ± 0.4 × 10⁴/ml PPARγ KO vs 6.8 ± 1.5 × 10⁴/ml WT, \( p = 0.03 \)). Lymphocytes were increased as compared with WT (Fig. 1A). This lymphocyte population was further characterized by FACS and was composed of CD4⁺ and CD8⁺ cells (Fig. 1, B–D) with a predominance of CD8⁺ (2.5 ± 0.3% of total cells, \( n = 3 \)) vs CD4⁺ (0.9 ± 0.3%). CD45R/B220 cells were not detected.

IFNγ and Th1 chemokines are elevated in BAL cells

Because of the increased CD3⁺ cells, we investigated the production of the Th1 and Th2 cytokines and chemokines in the BAL cells. IFN-γ mRNA and protein expression were markedly increased in BAL cells from PPARγ KO (see Fig. 2, A and D). Additional immunocytochemical studies were conducted on cytopsin treated with Triton X to render cells permeable for examination of intracellular as well as extracellular IFN-γ. Permeabilized WT macrophages did not stain for IFN-γ (Fig. 2B); membrane

**FIGURE 3.** IL-12(p40) (A), IP10 (B), and MIP-1α (C) mRNA are increased in BAL cells from PPARγ KO mice. RNA samples from BAL cells of WT (\( n = 5 \)) and PPARγ (\( n = 5 \)) were evaluated by quantitative PCR. IL-12(p40), IP10, and MIP-1α were all significantly increased in PPARγ KO cells.

**FIGURE 4.** iNOS mRNA and protein are increased in BAL cells from PPARγ KO. A, RNA samples from BAL cells of WT (\( n = 7 \)) and PPARγ KO (\( n = 5 \)) were evaluated by quantitative PCR. B and C, Cytopsin preparations of BAL cells were stained with anti-iNOS (green) as described in the Materials and Methods section and the cell nuclei were counterstained with PI (red) and examined by confocal microscopy. WT BAL cells did not stain for iNOS (B); in contrast the macrophages of the PPARγ KO macrophages showed prominent green staining (×63) (C). D, Anti-IFN-γ treatment of cultured alveolar macrophages from PPARγ KO decreased iNOS mRNA expression as compared with Untreated (no RX) and rat IgG control (CONT Ig).
bound IFN was detectable in nonpermeabilized PPARγ KO (Fig. 2C), in contrast the cytoplasm of permeabilized PPARγ KO macrophages stained green (Fig. 2D). IL-4 and IL-17A mRNA levels were undetectable in both PPARγ KO and WT mice (data not shown). Because of the increase in the Th1 cytokine, IFN-γ, we next investigated the expression of the Th1 associated cytokine IL-12(p40) and chemokines, IP-10 (CXCL10), and MIP-1α (Fig. 3, A–C). IL-12(p40) was elevated 55.7-fold, \( p < 0.05 \); IP10 mRNA was 3.7-fold, \( p < 0.05 \); and MIP-1α was 3-fold, \( p < 0.005 \).

iNOS mRNA and protein are elevated in BAL cells

IFN-γ is a known stimulator of iNOS (5, 23), and so we thus investigated iNOS mRNA and protein in BAL cells (Fig. 4A). Levels of iNOS mRNA were increased 74-fold in PPARγ KO BAL cells ex vivo (\( p = 0.006 \)) compared with WT in which iNOS levels were undetectable. Immunocytochemistry in WT (Fig. 4B) did not show iNOS expression while alveolar macrophages from PPARγ KO (Fig. 4C) demonstrated cytoplasmic localization of iNOS protein. To determine whether IFN-γ is responsible for iNOS expression, neutralizing anti-IFN-γ (BD Biosciences) or control rat IgG were added to BAL cell cultures for 24 h and iNOS mRNA was measured by RT-PCR. Anti-IFN-γ significantly decreased iNOS expression (\( p < 0.005 \)) (Fig. 4D).

FIGURE 5. The PPARγ antagonist, GW9662, up-regulates iNOS and IFN-γ in WT alveolar macrophages. A and B, RNA samples from BAL cells of WT mice (\( n = 3 \)) were evaluated by quantitative PCR. C–H, Alveolar macrophages were cultured in chamber slides for 24 h with and without GW9662 (1 \( \mu M \)). After fixation, cells were stained by immunocytochemistry for PPARγ (C and D), IFN-γ (E and F), or iNOS (G and H). Scale bar = 50 \( \mu m \).

FIGURE 6. eGFP is expressed in alveolar macrophages from lentivirus-eGFP instilled PPARγ KO mice and PPARγ expression is up-regulated in lentivirus-PPARγ instilled mice. A, Cytospin preparations from BAL fluid were quantified for the number of eGFP positive cells (at least 100 cells per preparation were evaluated (\( n = 5 \)). B, Image showing eGFP expression in BAL macrophages (\( \times 40 \)). C, Confocal image of alveolar macrophages from a nontransduced PPARγ KO (\( \times 63 \)). D, Confocal image of alveolar macrophages from lenti-PPARγ instilled mouse (\( \times 63 \)).
Antagonism of PPARγ in vitro elevates iNOS and IFN-γ in WT BAL cells

To determine whether the low levels of iNOS and IFN-γ expression in WT alveolar macrophages were due to the high constitutive expression of PPARγ, as was shown previously (21), we used GW9662, a known PPARγ antagonist (5, 23, 24). Untreated alveolar macrophages demonstrated no detectable iNOS mRNA expression. In contrast, macrophages treated with GW9662 in vitro showed elevated iNOS expression (>100-fold, n = 3, p ≤ 0.001) (Fig. 5A). Similarly, untreated cells showed no detectable IFN-γ, but displayed a significant increase with GW9662 treatment (p = 0.05) (Fig. 5B). These observations were subsequently verified by immunocytochemistry (Fig. 5, C–H). In the presence of GW9662, a reduction of PPARγ was confirmed (Fig. 5D), while IFN-γ (Fig. 5F) and iNOS (Fig. 5H) were increased. In untreated cells, PPARγ was prominent (Fig. 5C), but IFN-γ (Fig. 5E) and iNOS (Fig. 5G) were at basal levels.

Lentivirus-mediated in vivo expression of PPARγ decreases IFN-γ, iNOS, and chemokine expression

To test whether these inflammatory alterations were due to the lack of PPARγ, PPARγ KO mice were transduced by intratracheal inoculation with a PPARγ lentivirus construct and a control lentivirus-eGFP construct. Analysis of BAL cells 30 days post transduction revealed a 76 ± 4% (n = 5) transduction efficiency as measured by eGFP-positive cells (see Fig. 6, A and B). To confirm that lenti-PPARγ transduction up-regulated PPAR expression, cytopsin preparations of BAL cells were stained with anti-PPARγ. In Fig. 6C, nontransduced alveolar macrophages demonstrate no PPARγ staining, whereas in Fig. 6D, lenti-PPARγ transduced cells show marked PPARγ staining. PPARγ KO transduced mice demonstrated a 90% decrease in BAL lymphocytes (p = 0.03). We next examined mRNA expression of the BAL cells. Comparing lentivirus-eGFP to lenti-PPARγ transduced cells revealed a significant decrease in IFN-γ, p ≤ 0.05; MIP-1α, p ≤ 0.005; IP10, p ≤ 0.05; and iNOS, p ≤ 0.005 (Fig. 7, A–D). PBS inoculated sham controls were not different from lentivirus-eGFP inoculated mice (data not shown). Immunostaining of the BAL cells recovered from lentivirus PPARγ transduced mice confirmed a reduction in both IFN-γ and iNOS protein as these cell preparations were indistinguishable from WT controls (data not shown).

Discussion

The present study focused on the role of PPARγ in alveolar macrophages by using a conditional KO mouse model in which PPARγ is specifically disrupted in macrophages and neutrophils. Deletion of PPARγ in murine alveolar macrophages revealed several unique alterations in lung homeostasis: 1) both iNOS and IFN-γ were up-regulated in alveolar macrophages themselves; 2) infiltration of T cells into the lung was increased; and 3) Th1-associated cytokines were produced by BAL cells.

PPARγ KO mice displayed elevated iNOS and IFN-γ expression in cells recovered from BAL fluid. Immunostaining demonstrated enhanced iNOS protein only in alveolar macrophages whereas IFN-γ protein was present in both lymphocytes and macrophages. Expression of IFN-γ has been noted previously in murine (25, 26) and human (27) macrophages (reviewed in Ref. 28). Production of IFN-γ by professional phagocytes and APCs has been suggested to play an important role in host defense against infection (29, 30). In PPARγ KO mice, however, alveolar macrophage production of IFN-γ has not been previously noted, and the consequences of IFN-γ overexpression in these animals remains to be explored. Our data suggest, however, that PPARγ restrains macrophage production of IFN-γ although the precise pathways of IFN-γ dysregulation in PPARγ KO mice have not yet been established.

The association of increased IFN-γ, iNOS activity, and inflammatory cytokines has been noted in the lungs of a number of transgenic models with disrupted lung homeostasis. GM-CSF KO mice beginning at 4–6 wk and progressing with age display lung pathology similar to human pulmonary alveolar proteinosis. GM-CSF KO alveolar macrophages have markedly deficient PPARγ (21) with elevated levels of inflammatory cytokines and mediators in BAL fluid including MCP-1, metalloproteinases-2 and -9, IFN-γ (31–33), and most recently elevated iNOS mRNA expression in alveolar macrophages (unpublished observation, MJ. Thomassen). Surfactant protein D deficient mice also exhibit an increase in inflammatory mediators in the lung including metalloproteinases, IFN-γ and reactive oxygen species accompanied by early emphysematous changes in the lung (34, 35). Selective
inhibition of iNOS activity in vivo reverses inflammatory abnormalities in Surfactant protein D deficient mice (36). Alveolar macrophage PPARγ expression has not been investigated in this animal model. The source of IFN-γ in these animal models was not defined. In the present study enhanced levels of IFN-γ were found in both lymphocytes and macrophages recovered from the lung.

Data in the literature suggest an inverse relationship between PPARγ and IFN-γ. In murine adipocytes, IFN-γ inhibits PPARγ synthesis and augments protein degradation (37, 38). IFN-γ also antagonizes PPARγ expression in murine peritoneal macrophages (39, 40). Other evidence supporting an inverse relationship between PPARγ and IFN-γ is derived from PPARγ heterozygote mice in which IFN-γ is elevated and these mice have enhanced susceptibility to autoimmune disease (41).

The elevated expression of IL-12(p40), MIP-1α, and IP-10 along with IFN-γ and lack of IL-4 and IL-17A in the BAL cells of the PPARγ KO mice appears to favor a Th1 inflammatory response. Support for this assertion is also derived from several autoimmunity disease models in which PPARγ ligands alter the orientation of the immune responses by favoring a Th2 response (42). Immune dysfunction associated with autoimmune disease is thought to be caused by an imbalance between Th1 and Th2 (43).

The present data support the concept that PPARγ plays a unique role in the lung with regard to maintenance of lung homeostasis. In summary, we have for the first time demonstrated an inverse relationship between PPARγ and IFN-γ in alveolar macrophages. IFN-γ has been shown to up-regulate iNOS expression (5). We show that with a deficiency in alveolar macrophage PPARγ levels of inflammatory mediators, increase in the lung together with an influx of lymphocytes and a shift toward production of Th1 cytokines. Finally, we show reversibility of these phenomena when PPARγ is restored by in vivo transduction with a lentivirus-PPARγ construct. Taken together these observations support a critical role for alveolar macrophage PPARγ in the maintenance of lung homeostasis and support the notion that PPARγ agonists might be useful therapeutic options for inflammatory lung diseases in which Th1 cytokines predominate.

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Disclosures

The authors have no financial conflict of interest.

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