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Peroxisome proliferator-activated receptor gamma (PPAR γ) overexpression and knockdown: Impact on human B-cell lymphoma proliferation and survival

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Abstract

PPAR- γ is a multifunctional transcription factor that regulates adipogenesis, immunity and inflammation. Our laboratory previously demonstrated that PPAR γ ligands induce apoptosis in malignant B cells. While malignant B lineage cells such as B cell lymphoma express PPAR γ , its physiological function remains unknown. Herein, we demonstrate that silencing PPAR γ expression by RNAi in human Burkitt's type B lymphoma cells increased basal and mitogen-induced proliferation and survival, which was accompanied by enhanced NF- κ B activity and increased expression of Bcl-2. These cells also had increased survival upon exposure to PPAR γ -ligand and exhibited a less differentiated phenotype. In contrast, PPAR γ overexpression in B lymphoma cells inhibited cell growth and decreased their proliferative response to mitogenic stimuli. These cells were also more sensitive to PPAR γ -ligand induced growth arrest and displayed a more differentiated phenotype. Collectively, these findings support a regulatory role for PPAR γ in the proliferation, survival and differentiation of malignant B cells. These findings further suggest the potential of PPAR γ as a therapeutic target for B cell malignancy.

Keywords

PPARgamma; B-cell lymphoma; proliferation; siRNA; overexpression; differentiation

Introduction

Burkitt's lymphoma (BL) is an aggressive non-Hodgkin B cell lymphoma usually diagnosed in children and young adults. In the United States and Western Europe, it constitutes about 1–2% of all adult lymphomas and 30–50% of pediatric lymphomas [14,28]. BL has also

been associated with Epstein-Barr virus (EBV) latent infection, which results in a lymphoproliferative phenotype and increased resistance to apoptosis [21]. Intensive chemotherapeutic regimens have greatly increase prognosis, but can have significant toxicity, including treatment-related deaths [14]. New courses of therapy are aimed at minimizing toxicity without compromising outcome, and include monoclonal antibody (Rituximab) and steroid therapies. Recent molecular evidence of the role of transcription factors in BL are yielding promise as therapeutic targets [41].

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of transcription factors that regulate lipid metabolism and adipose differentiation [6]. There are three known PPAR isoforms: PPAR α , PPAR β/δ and PPAR γ . The human PPAR γ gene is located on chromosome 3 band 3p25 [3]. This gene gives rise to three mRNA isoforms (PPAR γ 1, γ 2 and γ 3) through alternate promoter usage and splicing [18]. Both PPAR γ 1 and PPAR γ 3 mRNA translate into PPAR γ 1 protein and PPAR γ 2 mRNA gives rise the PPAR γ 2 isoform that contains 28 extra amino acids [18]. All PPAR isoforms heterodimerize with members of the retinoid X receptor (RXR) subfamily of nuclear hormone receptors. These complexes then bind to the peroxisome proliferator response element (PPRE) in the promoter regions of target genes. PPAR γ is activated by natural ligands such as 15-deoxy- $\Delta^{12,14}$ prostaglandin J₂ (15d-PGJ₂) and by certain polyunsaturated fatty acids. PPAR γ can also be activated by synthetic ligands such as the thiazolidinediones (TZDs) class of anti-diabetic drugs. PPAR γ also has anti-proliferative, anti-inflammatory and pro-differentiating properties in immune cells [20]. Importantly, PPAR γ regulates B lymphocyte function. B lymphocytes from PPAR γ -haploinsufficient mice exhibit increased proliferation and survival [54]. Our laboratory (and others) have demonstrated that both normal and malignant B lymphocytes express PPAR γ and that exposure to certain PPAR γ ligands inhibits B cell proliferation and induces apoptosis [42,43,49]. Some studies have shown that PPAR γ ligands induce differentiation of malignant cells [11,39]. Moreover, PPAR γ expression increases during differentiation of monocytes to macrophages [40]. In hematological malignancies, PPAR γ ligands can induce monocytic differentiation in myeloid leukemia cells and help sensitize malignant cells to the pro-differentiation effects of all-trans-retinoic acid [16,24,34,35,57]. These studies support the concept that PPAR γ is an important transcription factor in B cells and serves as a pro-differentiation factor for malignant cells.

The ability of PPAR γ to alter B cell proliferation and apoptosis may relate to its ability to repress other transcription factors, such as nuclear factor kappa-B (NF- κ B) [20]. NF- κ B controls B cell proliferation and survival [19,30] and is constitutively active in several human cancers, including B cell lymphomas [44,51]. Moreover, EBV activates NF- κ B in the process of B cell transformation to malignancy [27]. Straus *et al.* reported that the natural PPAR γ ligand 15d-PGJ₂ inhibits multiple steps in the NF- κ B signaling pathway [56]. PPAR γ may also regulate NF- κ B by obstructing its transcription [9]. A recent report by Pascual *et al.*, demonstrated that PPAR γ -ligand-dependent sumoylation of PPAR γ leads to the recruitment of PPAR γ to the repressor complexes on the promoter regions of genes regulated by NF- κ B, ultimately suppressing NF- κ B-driven gene expression [45].

Thus, PPAR γ may control B cell lymphoma proliferation and survival through alterations in NF- κ B activity. We hypothesized that the level of PPAR γ plays an important role in B lymphoma cell survival. We speculated that high levels of PPAR γ would inhibit proliferation/survival and induce differentiation, while low PPAR γ levels would enhance B lymphoma survival and enhance their undifferentiated phenotype. Herein, we investigated the effects of PPAR γ expression on B cell lymphoma proliferation, survival and differentiation.

Materials and Methods

Reagents and Antibodies

Ciglitazone was purchased from Biomol (Plymouth Meeting, PA). CDDO was synthesized by Dr. T. Honda and kindly provided by Dr. Michael Sporn (Dartmouth College, Hanover, NH) [25]. MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide), DMSO and anti-Flag M2 monoclonal antibody peroxidase conjugate were from Sigma (St. Louis, MO). The anti-PAX-5 was purchased from Millipore (Billerica, MA). The anti-BLIMP-1 was purchased from Novus Biologicals (Littleton, CO). The rabbit anti-human PPAR γ antibody was purchased from Biomol. Anti-Bcl-2 (sc-7382) and anti-p65 (sc-372) antibodies were purchased from Santa Cruz (Santa Cruz, CA). Total actin (CP-01) antibody was from Oncogene (Cambridge, MA).

Construction of Lentiviral vectors

The lentiviral vector encoding the short hairpin RNA (shRNA) against PPAR γ transcript (nucleotides 1095–1113) was constructed using the oligonucleotide sequence 5'GTTTGAGTTTGCTGTGAAG3', as described by Katayama et al. [31]. The two complementary oligonucleotides were cloned downstream of the human RNA polymerase III U6 promoter and then subcloned into the FG12 lentiviral vector (gift of Dr. David Baltimore), as described earlier [48] (see also Figure 1A).

The LV-PPAR γ 1-WT and the LV-empty vector was produced as described earlier [17].

Lentiviral vector production

Human embryonic kidney 293FT cells (Invitrogen, Carlsbad, CA) were grown to 50–70% confluency in Dulbecco's modified Eagle's medium (GIBCO, Carlsbad, CA) supplemented with 10% fetal bovine serum in T-175 flasks. Subsequently, the VSVG pseudotyped HIV vector was generated by co-transfecting with 5 μ g of envelope vector (pCMV-VSVG), 14 μ g of transfer vector and 14 μ g of packaging vector pCMV- Δ 89.2, using Lipofectamine LTX (Invitrogen, Carlsbad, CA). Cells were split into two T-175 flasks 6 h post-transfection. Supernatants were collected 48 and 72 h post-transfection. Virus was harvested by ultracentrifugation at 50,000 $\times$ g for 2 h at 4 °C using a Beckman SW 28 rotor. The concentrated virus stocks were titered on 293FT cells based on GFP expression.

Cells and culture conditions

Ramos (EBV negative) and Raji (EBV positive) B lymphoma cells were cultured in RPMI 1640 tissue culture medium (Life Technologies, Grand Island, NY) supplemented with 10% Fetal Bovine Serum (FBS), 5×10^{-5} M β -mercaptoethanol (Eastman Kodak, Rochester, NY), 10 mM HEPES (US Biochemical Corp., Cleveland, OH), 2 mM L-glutamine (Life Technologies), 50 μ g/ml gentamicin (Life Technologies). Human embryonic 293FT cells were purchased from ATCC (Manassas, VA) and were grown in Dulbecco's modified Eagle's medium (GIBCO, Carlsbad, CA) supplemented with 10% fetal bovine serum.

Lentiviral infections and cell sorting

Ramos and Raji B lymphoma cells were plated at a density of 1×10^6 cells/well in a 12 well plate and infected with the different lentiviral vectors at MOIs of 1 to 5 in the presence of 6 μ g/ml of polybrene. Twenty four hours post-infection, the growth media was replaced. Lentiviral transduced cells were identified on the basis of the GFP expression. GFP-positive cells were sorted by flow cytometry using a FACS Aria (BD Bioscience, San Jose, CA).

Immunofluorescence

Ramos B lymphoma cells infected with the lentiviral constructs were incubated with mouse anti-human CD19-APC (BD Biosciences), anti-human CD38-PE (BD Biosciences), anti-human CD20-PE (BD Biosciences) or with anti-human CD40-biotin (Axxora/Ancell, Bayport, MN) in cold PBS with sodium azide (0.02%) and BSA (0.3%) for 20 min at 20°C. For CD40 surface staining, cells were washed and incubated with secondary APC-conjugated streptavidin (Caltag, Burlingame, CA).

Electrophoretic mobility shift assay (EMSA) for NF- κ B

Gel shift assay of nuclear extracts from uninfected, LV-control and LV-PPAR γ -siRNA infected Ramos cells was performed as described [49].

PPAR γ activity assay

Nuclear extracts from LV-Empty infected or LV-PPAR γ -WT infected Ramos cells were collected using a nuclear extract kit (Active Motif, Carlsbad CA). To determine PPAR γ activity, a TransAM PPAR γ activity assay kit was used (Active Motif, Carlsbad CA) as described [1].

Viability and proliferation assays

Ramos B lymphoma cells transduced with LV-PPAR γ -siRNA and LV-control-GFP (1×10^5 cells per well) were plated in a 96-well flat bottom microtiter plate. MTT was performed to assess cell viability. The tetrazolium salt MTT is taken up by viable cells and reduced to a formazan residue by functional mitochondria of living cells [4]. Cells were treated with increasing concentrations of the PPAR γ ligand CDDO and 10 μ l per well of a 5 mg/ml of MTT (in 1X PBS) was added for the last 4 h of incubation. After incubation, the plate was centrifuged, the media removed, and DMSO was added to each well to dissolve the precipitate. The plate was read at 510 nm on a Benchmark microplate reader (BioRad, Hercules, CA).

For the proliferation assay, cells were cultured as described above, and were left untreated or were treated with CD40L [29], 10 μ g/ml of rabbit anti-human F(ab')₂ anti-IgM Ab (Jackson ImmunoResearch Laboratories) and 1/1000 dilution of Pansorbin (*Staphylococcus aureus* Cowen I strain; Sigma-Aldrich) or different combinations of these mitogens. One μ Ci/well of ³H-thymidine was added for the last 18 h of culture. The cells were harvested onto a 96-well filter plate and the ³H-thymidine incorporation was detected as counts per minute (cpm) using a Topcount Luminometer (PerkinElmer, Boston, MA).

Cell cycle analysis

Cell cycle analysis of human Burkitt's lymphoma cells was performed using the APC Bromodeoxyuridine (BrdU) Flow kit (BD Pharmingen, San Diego, CA) according to the manufacturer's instructions. In the set of experiments using ciglitazone, cells were treated with ciglitazone (5 μ M) for 48 hr. Cells were then pulsed for 30 minutes with 10 nM BrdU and stained the APC-BrdU flow kit.

Western blots

Whole cell extracts were collected using ELB buffer (50 mM HEPES (pH 7), 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 10 mM NaF, 0.1 mM Na₃VO₄, 50 μ M ZnCl₂, supplemented with 0.1 mM PMSF, 1 mM DTT, and a mixture of protease and phosphatase inhibitors) and total protein was quantified using bicinchoninic acid protein assay (BCA assay kit) (Pierce, Rockford, IL). Twenty five micrograms of protein was electrophoresed on 8–16 % Precise™ protein gels (Pierce, Rockford, IL) and transferred to a polyvinylidene

fluoride (PVDF) membrane (Millopore, Billerica, MA). The membranes were analyzed for immunoreactivity with the indicated primary antibody, washed and then incubated with an appropriate horseradish peroxidase-conjugated secondary antibody. The membranes were visualized by chemiluminescence using an ECL kit (Pierce, Rockford, IL).

Statistical analysis

Results are expressed as the mean $\pm$ standard deviation (SD). Two-tailed student t test was performed and p values less than 0.05 were considered significant. All experiments were repeated at least 3 times.

Results

Construction of a lentiviral-based vector for delivery of PPAR γ siRNA

Our laboratory previously demonstrated that both normal and malignant B cells express PPAR γ and that certain PPAR γ ligands induce apoptosis [42,43,49,50]. To determine the physiological role of PPAR γ expression in certain human B cell lymphomas, we used a lentivirus-mediated short hairpin RNA expression system to knockdown PPAR γ expression in Burkitt's B lymphoma cells. To construct the siRNA expression cassette, a target sequence of siRNA for PPAR γ was selected to knockdown both PPAR γ 1 and PPAR γ 2 [31]. The 19bp PPAR γ target sequence was cloned downstream of the human RNA polymerase III U6 promoter and then subcloned into the FG12 lentiviral vector, which also expresses GFP under the Ubiquitin C (UbiC) promoter (Figure 1A) [48]. First, to determine whether the siRNA sequence effectively targets human PPAR γ mRNA, the DNA plasmid vector containing the siRNA sequence was tested in human embryonic kidney (HEK) 293 cells. HEK 293 cells were mock transfected (Figure 1B, pc-DNA3.1, lanes 1 and 2) or were co-transfected with a FLAG-tagged PPAR γ -wild type vector (Figure 1B, Flag-PPAR γ , lanes 2–6) and with either an empty DNA vector (Figure 1B, pcDNA 3.1, lane 2), increasing DNA concentrations of the parental empty vector (FG12-empty, lanes 3 and 4) or increasing concentrations of the PPAR γ siRNA expression vector (FG12-siRNA-PPAR γ , lanes 5 and 6). Exogenous PPAR γ expression was tested using an anti-FLAG antibody and endogenous PPAR γ expression was tested using an anti-PPAR γ antibody. Both exogenous and endogenous PPAR γ protein levels were dramatically reduced in HEK293 cells that co-expressed the PPAR γ -Flag vector and the FG12-siRNA-PPAR γ vector (Figure 1B, compare lane 2 with lanes 5 and 6), but not in cells co-transfected with PPAR γ -Flag and the FG12-empty vector (Figure 1B, compare lane 2 with 3 and 4). This indicates that the siRNA sequence against PPAR γ was successful at knocking down PPAR γ expression. Next, the pseudotyped lentiviral vectors were generated as described in Materials and Methods and designated LV-control and LV-PPAR γ -siRNA. Both vectors also express GFP. Ramos B lymphoma cells were infected at an MOI of 5. Approximately 30% of the Ramos B cells were transduced as determined by GFP expression (Figure 1C, left panel). These GFP-positive cells were sorted and five days post-sorting, the cells were analyzed by flow cytometry to determine the purity. More than 98% of the cells were GFP-positive (Figure 1C, right panel). Western Blot analysis confirmed that LV-PPAR γ -siRNA infected Ramos B cells have dramatically reduced levels of PPAR γ protein (Figure 1D).

Reduction of PPAR γ expression in Ramos B lymphoma cells results in enhanced proliferation and reduced sensitivity to PPAR γ agonist-induced cell death

We first investigated whether PPAR γ plays a role in B lymphoma proliferation. Proliferation was measured by ^3H -thymidine incorporation in PPAR γ -knockdown Ramos cells. Control (LV-Control) and PPAR γ -knockdown (LV-PPAR γ -siRNA) Ramos cells were left untreated or were stimulated with Pansorbin (fixed *S. aureus*), human CD40L, anti-IgM, CD40L+anti-IgM or CD40L+ Pansorbin for 24 hr. Basal proliferation (Figure 2A, untreated) was

significantly increased following lentiviral delivery of PPAR γ -siRNA. There was also a significant increase in the proliferative response to mitogenic stimuli in PPAR γ -knockdown cells in comparison to control Ramos cells (Figure 2A). This indicates that the presence of PPAR γ dampens B lymphoma cell proliferation.

We next evaluated whether PPAR γ -knockdown Ramos B cells would have increased survival upon exposure to the synthetic PPAR γ ligand, CDDO (Figure 2B). Control and PPAR γ -knockdown Ramos cells were exposed to increasing concentrations of CDDO for 24hr and viability measured by MTT. PPAR γ -knockdown Ramos cells have increased survival when exposed to lethal doses of PPAR γ -ligand CDDO. This increase in survival was also confirmed using 7-AAD staining (data not shown). PPAR γ -knockdown cells also had better survival following serum deprivation compared to control cells (data not shown). Taken together, these results indicate that PPAR γ regulates cell proliferation and survival in B lymphoma cells.

PPAR γ knockdown B lymphoma cells display a less differentiated phenotype

To explore the effect of PPAR γ on B cell differentiation and activation, we examined the effect of PPAR γ knockdown on several important B cell markers. During B cell differentiation, CD38 expression increases while CD19 and CD20 levels are downregulated [2,7]. Therefore, we compared the levels of CD20, CD19 and CD38 of control Ramos cells versus PPAR γ -knockdown Ramos cells by flow cytometry. The surface expression of CD38 in PPAR γ -siRNA Ramos cells was slightly lower than PPAR γ -expressing Ramos cells (LV-control) (Figure 3, panel 3). Conversely, surface expression of CD20 and CD19 in PPAR γ -siRNA Ramos cells was increased (Figure 3, panels 1 and 2). These results suggest that PPAR γ participates in B cell differentiation.

We also investigated the effects of PPAR γ knockdown on the expression of two transcription factors crucial for B cell differentiation: Paired box protein 5 (PAX5) and B-lymphocyte-induced maturation protein 1 (BLIMP-1)[33]. During B cell differentiation PAX-5 levels are downregulated and/or inactivated, while BLIMP-1 is upregulated [33]. We observed a marked reduction in BLIMP-1 expression in PPAR γ -knockdown cells compared to control cells, while the levels of PAX-5 remain relatively unchanged (Figure 3B). Together, these results, in conjunction with the changes seen in B cell differentiation surface markers (Figure 3A), suggests that the reduction of BLIMP-1 expression is the result of transcriptional regulation by PPAR γ and correlates with a less differentiated phenotype.

PPAR γ knockdown Ramos B lymphoma cells have enhanced NF- κ B activity and express higher levels of the NF- κ B-dependent pro-survival gene Bcl-2

NF- κ B is an important transcription factor for B cell proliferation and survival [22]. Because previous studies have demonstrated the direct effects of PPAR γ on NF- κ B [32,46], we investigated whether PPAR γ expression affected NF- κ B. First, the expression of the NF- κ B subunit p65 was investigated in PPAR γ -knockdown Ramos cells versus uninfected and control Ramos cells. Nuclear and cytoplasmic extracts were collected from uninfected, control and siRNA-PPAR γ transduced Ramos cells and western blot analysis for the NF- κ B p65 subunit was performed. An accumulation of p65 occurred in the nucleus ($\approx$ 3 fold induction compared to controls), with a concomitant decrease in the cytoplasm ($\approx$ 5 fold reduction compared to controls), of PPAR γ -knockdown Ramos cells in comparison to uninfected and control cells (Figure 4A). Next, an electrophoretic mobility shift assay (EMSA) was performed on nuclear extracts (described in Materials and Methods) from uninfected, LV-control and LV-PPAR γ -siRNA infected Ramos cells to measure NF- κ B DNA binding activity. Figure 4B shows that PPAR γ -knockdown Ramos cells have increased NF- κ B DNA binding activity in comparison to uninfected and control cells. Bcl-2

is an anti-apoptotic protein that has an important role in normal and malignant B-cell proliferation and survival [52]. Bcl-2 is also a key target gene of NF- κ B. Therefore, we investigated the levels of Bcl-2 in PPAR γ -siRNA Ramos cells, uninfected and control Ramos cells. PPAR γ -knockdown Ramos cells had $\approx$ 10-fold higher Bcl-2 levels compared to uninfected or control Ramos cells (Figure 4C). These findings support the idea that PPAR γ regulates NF- κ B activation.

Design of a lentiviral-based vector for PPAR γ 1 overexpression in human B lymphoma cells

To further determine the physiological role of PPAR γ in human B cell lymphoma, overexpression studies were performed using a lentiviral vector we constructed for PPAR γ gene delivery. The lentiviral vector contains a Flag-tagged PPAR γ 1 cDNA under the control of the CMV promoter and Copepod GFP (CopGFP) under the EF1- α promoter. As a control, the backbone lentiviral vector that only expresses CopGFP was used (Figure 5A). Ramos cells were infected at an MOI of 5 and GFP-positive Ramos cells were sorted by flow cytometry. Interestingly, we observed a decrease in GFP expression over time after sorting. We hypothesized that selective pressure was responsible for decreasing the levels of both PPAR γ and GFP (Figure 5B). For these reasons, cells were only cultured for a maximum of one month after sorting, in which time we evaluated the effects of PPAR γ overexpression. The presence of exogenous PPAR γ expression was determined by western blot using an anti-FLAG antibody. We found that cells stably transduced with LV-PPAR γ , but not LV-empty nor uninfected cells expressed the Flag-tagged PPAR γ protein (Figure 5C). To test whether the overexpressed PPAR γ was capable of binding DNA, an ELISA-based assay to quantify the binding of PPAR γ to its promoter response element was used. Cells overexpressing PPAR γ had a two-fold increase in PPAR γ activity compared to LV-empty infected cells (Figure 5D). These results demonstrate that the increased levels of PPAR γ were able to bind DNA and activate transcription.

Ramos B lymphoma cells transduced with LV-PPAR γ have a decreased proliferative response

We next determined the effects of PPAR γ overexpression on B lymphoma cell proliferation using 3 H-thymidine incorporation. Both basal proliferation, as well as stimulated proliferative responses were tested. Ramos B lymphoma cells stably transduced with LV-empty or LV-PPAR γ were untreated or were stimulated with CD40L, anti-IgM, Pansorbin and combination of CD40L plus anti-IgM or CD40L plus Pansorbin for 24 hr. LV-empty expressing cells were able to respond to stimuli, as seen by an increase in 3 H-thymidine incorporation (Figure 6A). However, LV-PPAR γ transduced cells poorly responded to mitogenic stimuli. The robust proliferative response to CD40L (with or without anti-IgM or Pansorbin) was dramatically reduced following PPAR γ overexpression (Figure 6A). Since PPAR γ overexpression inhibited B lymphoma cell proliferation, we next assessed cell cycle kinetics using BrdU and 7-AAD double staining. LV-PPAR γ transduced cells had a decrease in the percentage of cells in the S phase of the cell cycle when compared to those of LV-empty infected control cells (47% in LV-empty cells versus 39% in LV-PPAR γ cells) (Figure 6B). These results confirm that PPAR γ overexpression inhibited basal cell growth of B lymphoma cells. To determine whether PPAR γ overexpression had the same effect on another Burkitt's lymphoma cell line, EBV⁺ Raji B lymphoma cells were infected at an MOI of 5. GFP-positive cells were then sorted and tested for basal proliferation using BrdU and 7-AAD staining. LV-PPAR γ overexpressing Raji B lymphoma cells also showed a decrease in the percentage of cells in S-phase, while the fraction of cells with G0/G1 and G2/M DNA content was increased in comparison to LV-empty cells (Figure 6C). We next investigated whether these cells were more sensitive to PPAR γ ligand-mediated growth inhibition. To test this, we treated LV-empty and LV-PPAR γ Raji cells with a sublethal dose of the PPAR γ

ligand, Ciglitazone (5 μ M). Ciglitazone treatment had a minimal effect on proliferation of LV-empty infected cells (compare LV-empty and LV-empty+Cig). However, ciglitazone treatment resulted in further growth inhibition when PPAR γ was overexpressed (LV-PPAR γ), as seen by an even lower number of cells in the S-phase of the cycle (Figure 6C, compare LV-PPAR γ and LV-PPAR γ +Cig). These findings indicate that PPAR γ negatively regulates cell proliferation by decreasing the number of cells entering S phase and further enhanced their susceptibility to PPAR γ -ligand induced cell cycle arrest.

PPAR γ -overexpressing B lymphoma cells display a more differentiated phenotype

Since reduced PPAR γ expression in B lymphoma cells resulted in a less differentiated phenotype (Figure 3), we next explored whether PPAR γ overexpression had an effect on B cell differentiation markers (CD20, CD19 and CD38) and activation marker (CD40) expression (Figure 7A). Surface expression of CD38 was not changed and CD19 expression was slightly reduced by PPAR γ overexpression. Additionally, surface expression of CD20 and CD40 were dramatically reduced in PPAR γ -overexpressing Ramos cells (Figure 7A). These results suggest a role for PPAR γ in B cell differentiation and activation.

To confirm that PPAR γ regulates differentiation of Ramos cells, we next examined the effects of PPAR γ overexpression on PAX-5 and BLIMP-1. BLIMP-1 expression was upregulated in LV-PPAR γ -infected cells in comparison to LV-empty infected cells. In contrast, the levels of PAX-5 were downregulated in these cells (Figure 7B). Therefore, we propose that PPAR γ promotes differentiation of GC B cell-derived cells toward plasma cells.

Discussion

Burkitt's lymphoma is an aggressive form of Non-hodgkin lymphoma, and it is the most common childhood cancer in Central Africa [14]. Although the survival rates have increased, new innovative therapies are needed. PPAR γ , and PPAR γ ligands, have emerged as a new molecular target to treat cancer. Data from our laboratory (and others) have demonstrated that ligand-initiated activation of PPAR γ inhibits growth and induces apoptosis in B cell malignancies [34,42,43,47,49,50]. Further, PPAR γ expression correlates with patient prognosis in certain cancers [53,58]. In the present study, we demonstrated that alterations of PPAR γ expression levels in the germinal center (GC) B cell-derived cells Ramos and Raji affect cell proliferation, survival and differentiation. Interestingly, a decrease in PPAR γ expression was associated with increase B lymphoma cell proliferation and survival, both basally and after mitogenic stimuli (Figure 2A). Moreover, PPAR γ -knockdown malignant B cells survive better when exposed to increasing doses of CDDO in comparison to PPAR γ -expressing cells (Figure 2B). These data, in conjunction with our recently published results [42,43,50], further confirm that part of the cytotoxic effects of PPAR γ ligands are mediated directly through PPAR γ .

Nuclear Factor-kappa B (NF- κ B) is an important transcription factor for B cell development and survival. Activation of the NF- κ B pathway induces expression of anti-apoptotic proteins, such as the Bcl-2 family members [22]. The anti-inflammatory effects of PPAR γ have been linked to the inhibition of NF- κ B [46]. Studies performed in PPAR γ haploinsufficient mice indicate that PPAR γ is important in controlling B cell proliferation and survival. These mice exhibit enhanced B cell proliferation after LPS stimulation and increased NF- κ B activity in comparison to their wild type counterparts [54]. Here, we showed that PPAR γ knockdown human B lymphoma cells have increased NF- κ B activity and increased levels of the anti-apoptotic protein Bcl-2, which supports the mouse studies. PPAR γ has also been proposed to have pro-differentiating properties [5,16,34]. During normal B cell differentiation, the levels of CD20 and CD19 are downregulated, whereas the

levels of CD38 are upregulated. We observed that upon PPAR γ downregulation, there was a reduction of CD38 surface expression and an increase of CD20 and CD19 expression, indicative of a less differentiated phenotype. These findings demonstrate that the expression of PPAR γ , as well as its activity, are necessary to control B cell lymphoma proliferation, survival and differentiation. Although we did not observe changes in CD38 expression upon PPAR γ overexpression, we did observe a slight decrease in CD19 surface expression and a considerable decrease in CD20 expression on PPAR γ -overexpressing B lymphoma cells, which correlates with a more differentiated phenotype. Therefore, we speculate that PPAR γ overexpression would improve the outcome of patients with B cell-lymphoma, since a more differentiated phenotype correlates with a better prognosis [15].

Studies performed in thyroid carcinoma cells demonstrated that PPAR γ overexpression resulted in an induction of cell cycle arrest and cell death [37]. In concordance with these studies, when we overexpressed PPAR γ in Burkitt's lymphoma cells, and recently in multiple myeloma [17], we found that PPAR γ overexpression inhibited both basal and stimulated proliferation in B lymphoma cells. Interestingly, we observed a decrease in GFP expression in lymphoma cells over time. These results might explain a direct effect of PPAR γ overexpression on GFP transcription, or a deleterious effect of PPAR γ that confers growth disadvantage to PPAR γ overexpressing cells. We hypothesize that over time, selective pressure was responsible for decreasing the levels of both PPAR γ and GFP. In addition, we observed a decrease of CD40 expression in cells overexpressing PPAR γ . Previous studies have shown that CD40 ligation can rescue Burkitt's lymphoma from B cell receptor crosslinking-induced cell death [26]. Therefore, we speculate that reduced levels of surface expression of CD40 would render the lymphoma cells less responsive to CD40 ligand stimulation and subsequently contribute to their reduced cell growth and survival.

We propose that PPAR γ regulates B lymphoma cell differentiation. PAX-5 is inactivated by an unknown stimulus and is one of the first steps needed for plasma cell differentiation, while BLIMP-1 is upregulated. BLIMP-1 functions as an important regulator of plasma cell differentiation by inhibiting proliferation through inhibition of *c-myc*, an important factor for cell proliferation [36,55]. BLIMP-1 also represses PAX-5, which is crucial during B cell differentiation [55]. In addition to the changes on surface expression of B cell differentiation markers (Figure 3A and Figure 7A), there was also a reduction of BLIMP-1 in the PPAR γ knockdown cells and an upregulation with PPAR γ overexpression. While the expression of PAX-5 was relatively unchanged upon PPAR γ knockdown, the levels of PAX-5 were downregulated in cells overexpressing PPAR γ . Collectively, these findings suggest that PPAR γ may regulate B cell differentiation via direct or indirect regulation of key transcription factors, such as BLIMP-1 and PAX-5 and may contribute, at least in part, to the inhibitory effects of PPAR γ on proliferation and cell cycle progression in B cell lymphomas.

Collectively, our findings support PPAR γ as a potential new target to control malignant B lymphoma proliferation, survival and differentiation. Therapeutic efforts to alter PPAR γ levels in malignant B cells may demonstrate synergistic cytotoxicity when used in conjugation with PPAR γ ligands. One mechanism to selectively target malignant B cells *in vivo* would be to use lentiviral vectors that have a B cell lymphoma-specific promoter, thereby allowing therapeutic gene expression only in malignant B cells. A new generation of lentiviral vectors known as self-inactivating vectors (SIN) allow restriction or silencing of gene expression regulated by the viral promoter [10]. This feature makes possible the use of an internal promoter (e.g. B cell lymphoma-specific) to drive the gene of interest. In fact, this method has recently been used to introduce therapeutic genes in mammalian cells [8,12,13,38]. For example, in multiple myeloma, the use of a minimal immunoglobulin promoter as well as the Kappa light chain intronic and 3' enhancers to drive the gene of

interest was able to selectively transduce myeloma cells [12]. Therefore, using a cell or tissue-specific promoter and/or enhancer element could target a specific cell population. One possible candidate would be the promoter or enhancer regions of *c-myc*, which is highly expressed in Burkitt's lymphoma [23].

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Figure 1. Construction of a lentiviral vector for delivering human PPAR γ siRNA

A) Schematic diagram of the siRNA-expressing lentiviral vector. The short hairpin form of siRNA is expressed under the control of a human U6-RNA Pol III promoter (Pol III). The vector contains a GFP marker under the UbiC promoter for tracking transduced cells **B)** HEK 293 cells were mock transfected (lane 1) or were co-transfected with a FLAG-tagged-PPAR γ -WT vector (Flag-PPAR γ) and with either an empty DNA vector, (pcDNA3.1, lane 2), increasing DNA concentrations of empty parental FG12 vector (lane 3 and 4) or increasing DNA concentrations of the FG12 vector expressing siRNA against PPAR γ (lane 5 and 6). Numbers represent microgram amounts of plasmid DNA. All transfections included equivalent concentrations of DNA, which were normalized with the empty DNA vector, pcDNA3.1. **C)** Ramos cells were infected at an MOI of 5 and the GFP-positive cells were sorted by FACS. After 5 days, cells were analyzed by flow cytometry to determine the purity. LV-control and LV-PPAR γ -siRNA infected cells showed > 95% GFP-positive cells. **D)** Reduction of PPAR γ protein levels in Ramos cells transduced with LV-PPAR γ -siRNA (siRNA). Total actin was used as a loading control.

Figure 2. Reduction of PPAR γ expression in Ramos B lymphoma cells results in enhanced proliferation and reduced sensitivity to PPAR γ ligand-induced cell death

A) LV-control and LV-PPAR γ -siRNA transduced Ramos B lymphoma cells were untreated or treated with 1:1000 Pansorbin (fixed *S. aureus*), human CD40L, 10 μ g/ml anti-IgM, a combination of CD40L+ anti-IgM, and CD40L+ Pansorbin for 24 hr. Proliferation was measured by [3 H] thymidine incorporation. **B)** LV-Control and LV-PPAR γ -siRNA infected Ramos cells were exposed to increasing concentrations of the PPAR γ ligand CDDO. Viability was measured by MTT assay. PPAR γ -knockdown Ramos cells have increased survival in comparison to control cells when exposed to the PPAR γ agonist CDDO.

*($p < 0.05$), ns= not significant.

A. Ramos B lymphoma cells

B.

Figure 3. PPAR γ -knockdown Ramos human B lymphoma cells have a less differentiated phenotype

A) Ramos B lymphoma cells stably transduced with LV-control or LV-PPAR γ -siRNA were analyzed for the expression of CD20, CD19 and CD38. Cells that express PPAR γ -siRNA showed increased levels of CD20 and CD19 and decreased levels of CD38. MFI's are shown in the histogram. This experiment is representative of three separate experiments. **B)** LV-control and LV-PPAR γ -siRNA infected cells were lysed and expression of PAX-5 and BLIMP-1 were analyzed by western blot as indicated. Total actin was used to normalize protein loading.

Figure 4. PPAR γ knockdown Ramos B lymphoma cells have enhanced NF- κ B activity and express higher levels of the NF- κ B-dependent pro-survival gene Bcl-2

A. Nuclear and cytoplasmic extracts from uninfected, LV-control and LV-PPAR γ -siRNA transduced Ramos cells were collected and NF- κ B p65 levels analyzed by western blot. Total actin was used as a loading control. Graph shows representative densitometry. **B.** Nuclear extracts were incubated with a radiolabeled DNA binding sequence for NF- κ B and a gel shift assay was performed. **C.** Bcl-2 protein expression was analyzed by western blot in whole cell lysates from uninfected, LV-control and LV-PPAR γ -siRNA transduced Ramos B cells. Graph shows representative densitometry.

Figure 5. Design of a lentiviral vector for PPAR γ overexpression in human B cell lymphoma
A) Schematic diagram of the PPAR γ 1-expressing lentiviral vector. The Flag-PPAR γ 1 cDNA is expressed under the control of a CMV promoter. The vector also contains an elongated factor 1 α (EF-1 α) promoter driving the GFP marker gene for tracking transduced cells. 5' LTR, HIV-1 5' LTR; Δ 3' LTR, HIV-1 self-inactivating 3' LTR; WPRE, woodchuck hepatitis B virus RNA regulatory element. **B)** Ramos B lymphoma cells were transduced with LV-empty and LV-PPAR γ (MOI=5). At 48hr post-transduction, cells were analyzed for GFP expression using flow cytometry. GFP-positive cells were then sorted on the basis of GFP expression. GFP expression was monitored over time (one day and one week after sorting) to determine purity and the stability of gene expression **C)** Exogenous PPAR γ 1 expression

was evaluated by western blotting using an anti-FLAG antibody. After sorting, the FLAG-PPAR γ protein was detected in the cells infected with LV-PPAR γ , but not in the LV-empty infected cells. β -tubulin levels were assayed to normalize protein loading. **D)** Nuclear extracts from Ramos cells stably transduced with either LV-empty or LV-PPAR γ were collected and transactivation of PPAR γ was analyzed by ELISA using TransAm® technology (* $p < 0.05$).

Figure 6. Ramos B lymphoma cells transduced with LV-PPAR γ have decreased basal and stimulatory proliferative responses

A) Ramos B lymphoma cells stably transfected with LV-Empty and LV-PPAR γ were left untreated or were treated with human CD40L, 10 μ g/ml anti-IgM, a combination of CD40L plus anti-IgM, 1:1000 Pansorbin (fixed *S. aureus*), and CD40L plus Pansorbin for 24 hr. Proliferation was measured by [3 H] thymidine incorporation. * (p<0.05), ** (p<0.001) **B)** Cell cycle analysis: Ramos cells transduced with LV-empty and LV-PPAR γ were incubated for 30 minutes with BrdU and labelled with anti-BrdU and 7-AAD (gated on GFP-positive cells). Cells that overexpressed PPAR γ showed a reduction in the percentage of cells entering the S-phase of the cycle and a G2/M cell cycle arrest. This profile is representative

of three separate experiments. C) Raji B lymphoma cells were infected with either LV-empty or LV-PPAR γ at an MOI of 5 and sorted by GFP expression. Cells were left untreated or were treated with 5 μ M ciglitazone (+ Cig) for 48 hr. Cells were pulsed with BrdU for 30 minutes and intracellularly stained with an anti-BrdU antibody. Cells that were infected with LV-PPAR γ had a slight reduction in BrdU incorporation in comparison to LV-empty infected cells. When cells were treated with ciglitazone, a dramatic reduction in BrdU incorporation was observed in the cells that overexpress PPAR γ (LV-PPAR γ), but not in the LV-empty infected cells. These results are representative of three separate experiments.

Figure 7. Ramos B lymphoma cells overexpressing PPAR γ showed a more differentiated phenotype

A) Ramos B lymphoma cells stably transduced with LV-Empty or LV-PPAR γ were analyzed for the expression of CD20, CD19, CD38 and CD40. Cells that overexpress PPAR γ showed a slight decrease on CD19 expression and a dramatic decrease on CD20 and CD40, but no changes in CD38 expression. **B)** LV-empty and LV-PPAR γ infected cells were lysed and expression of PAX-5 and BLIMP-1 were analyzed by western blot as indicated. Total actin was used to normalize protein loading.