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Peroxisome proliferator-activated receptor gamma (PPAR γ) overexpression suppresses growth and induces apoptosis in human multiple myeloma cells

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Abstract

Purpose—Peroxisome proliferator-activated receptor gamma (PPAR- γ) is a transcription factor that regulates immune and inflammatory responses. Our laboratory has demonstrated that normal and malignant B cells, including multiple myeloma, express PPAR γ . Moreover, certain PPAR γ ligands can induce apoptosis in multiple myeloma cells. Since PPAR γ ligands can also have PPAR γ -independent effects, the role of PPAR γ in B cell malignancies remains poorly understood. To further understand the role of PPAR γ , we examined the functional consequences of its overexpression in human multiple myeloma.

Experimental design—In the present work, we developed a lentiviral vector for PPAR γ gene delivery. We transduced multiple myeloma cells with a lentivirus expressing PPAR γ and studied the involvement of this receptor on cell growth and viability.

Results—PPAR γ overexpression decreased multiple myeloma cell proliferation and induced spontaneous apoptosis, even in the absence of exogenous ligand. These PPAR γ -overexpressing cells were dramatically more sensitive to PPAR γ -ligand induced apoptosis, compared to uninfected or LV-empty infected cells. Apoptosis was associated with the downregulation of anti-apoptotic proteins XIAP and Mcl-1, as well as induction of caspase-3 activity. Importantly, PPAR γ overexpression-induced cell death was not abrogated by co-incubation with bone marrow stromal cells (BMSCs), which are known to protect multiple myeloma cells from apoptosis. Additionally, PPAR γ overexpression in multiple myeloma or BMSCs inhibited both basal and multiple myeloma-induced IL-6 production by BMSCs.

Conclusions—Our results indicate that PPAR γ negatively controls multiple myeloma growth and viability, in part through inhibition of IL-6 production by BMSCs. As such, PPAR γ is a viable therapeutic target in multiple myeloma.

Keywords

PPARgamma; multiple myeloma; apoptosis; caspase-3; ciglitazone

Introduction

Multiple myeloma is a neoplasm of differentiated plasma cells that is largely incurable, with a median survival duration of 3–5 years (1). It constitutes ~10% of hematological cancers and ranks as the second most frequent hematological malignancy in the United States (2,3). Current therapies for the disease include chemotherapy with or without stem cell transplantation, glucocorticosteroids, thalidomide, the proteasome inhibitor Bortezomib and combinations of these agents (1,2). However, most of these treatments are still not curative, hence newer treatments approaches are needed (3).

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear hormone receptor family (4,5). Three different isoforms have been identified: PPAR α , PPAR β/δ and PPAR γ . Upon ligand binding, they form a heterodimer with 9-*cis*-retinoic acid retinoid X receptors (RXRs). This complex binds to PPAR response elements (PPRE) located in the promoter regions of target genes (6,7). A key role for PPAR γ is in adipogenesis, where PPAR γ activation leads to the generation of adipocyte-specific genes (8). PPAR γ also participates in glucose homeostasis, cell cycle regulation, inflammation, atherosclerosis, apoptosis and carcinogenesis (9). PPAR γ can be activated by the naturally occurring prostaglandin D₂ metabolite 15-deoxy- $\Delta^{12,14}$ prostaglandin J₂ (15d-PGJ₂) (10,11), as well as by lysophosphatidic acid (12), and nitrolinoleic acid (13). PPAR γ is also activated by synthetic ligands including the thiazolidinedione class of clinically used anti-type 2 diabetic drugs (14).

PPAR γ and PPAR γ ligands have significant anti-inflammatory effects in immune cells (15). For example, macrophages treated with PPAR γ ligands are inhibited in terms of activation and production of inflammatory cytokines (16–18). In T lymphocytes, activation of PPAR γ inhibits proliferation and reduces their production of IFN γ and TNF α , as well as IL-2, by interfering with the transcription factor nuclear factor for activated T-cells (NFAT) (19). In addition, our laboratory has shown that mouse and human T cells express PPAR γ and treatment with PPAR γ ligands induces T cell apoptosis (20,21). PPAR γ also regulates B lymphocyte function. In PPAR γ -haploinsufficient mice, B lymphocytes exhibit increased proliferation and survival, enhanced antigen specific immune response and spontaneous NF- κ B activation (15,22). Furthermore, we and others have shown that PPAR γ is expressed in human normal and malignant B cells and exposure to certain PPAR γ ligands results in cell death (23–28).

The role of PPAR γ in the onset and development of cancer has been the recent focus of much attention. Cancer cells, including hematological cancers, display enhanced proliferation, in part, due to their failure to undergo terminal differentiation. The induction of differentiation and apoptosis by ligands of the nuclear hormone receptor family is a novel approach for cancer therapy; an example of this is the use of retinoic acid (RA) for the treatment of acute promyelocytic leukemia and other cancers (29–31). These studies have brought attention to the role of PPAR γ in the development and progression of cancer. The efficacy of PPAR γ ligands as potential anticancer drug therapies has been explored in several cancer models, including colon, breast, prostate, and lung (32). Recently, we and others have shown that activation of PPAR γ inhibits growth and induces apoptosis in hematological cancers, including multiple myeloma (26,33,34).

The tumor microenvironment contributes significantly to multiple myeloma resistance to chemotherapy (35) via the release of cytokines, chemokines and growth factors (e.g. IL-6) or by direct interaction between tumor cells and stromal cells (36,37). Some of the effects of PPAR γ ligands in the tumor microenvironment have been investigated in multiple myeloma (38,39). In these studies, PPAR γ ligands abrogated IL-6-dependent multiple myeloma cell growth by inhibiting the transactivation of IL-6/STAT3 (38). Moreover, PPAR γ ligands inhibited adhesion of multiple myeloma cells to BMSCs in a PPAR γ -dependent fashion through inhibition of the transcription factors NF- κ B and C/EBP β (39).

A major limitation of these studies is that only PPAR γ ligands were used to study the role of PPAR γ in cancer cell biology. Since PPAR γ ligands have been shown to have a variety of PPAR γ independent effects, the role of PPAR γ remains unclear (40,41). Indeed, research from our group and others exploring the viability of PPAR γ as a therapeutic target in B cell malignancies have used PPAR γ ligands in their experiment design. To unequivocally determine the central role of PPAR γ in the biology of multiple myeloma, we studied the functional significance of PPAR γ overexpression in human multiple myeloma cells using a lentiviral PPAR γ construct that we developed.

Materials and Methods

Reagents and Antibodies

Ciglitazone was purchased from Biomol (Plymouth Meeting, PA); 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). Antibodies against active caspase 3 (559565) and PARP (556362) were purchased from BD bioscience (San Diego, CA); a rabbit polyclonal anti-Mcl-1 (SC-819) antibody was purchased from Santa Cruz (Santa Cruz, CA) and the anti-XIAP antibody (MAB822) was from R&D systems (Minneapolis, MN). Total actin (CP-01) antibody was from Oncogene (Cambridge, MA). The GAPDH (CB1001) antibody and the caspase inhibitor VI (ZVAD-FMK) were purchased from Calbiochem (San Diego, CA).

Construction of Lentiviral vectors

The pcDNA-Flag-PPAR γ 1 wild type and the pcDNA-Flag-PPAR γ 1- L466A/E469A DN human PPAR γ 1 plasmid was a kind gift from VKK Chatterjee (University of Cambridge, U.K.). The cDNA encoding PPAR γ 1 protein tagged at its N-terminus with a FLAG epitope was amplified using Pfu DNA polymerase. The restriction enzymes *Nhe*I and *Not*I were introduced into the 5' and 3' PCR primers, respectively. The PCR product was then digested with *Nhe*I and *Not*I and subcloned into a *Nhe*I-*Not*I digested pCDH1-MCS1-EF1-copGFP vector (System Bioscience, Mountain View, CA). These vectors were named LV-PPAR γ and LV-PPAR γ -DN and the vector without PPAR γ cDNA was termed LV-empty. The insert sequences in all lentiviral vectors were verified by DNA sequencing.

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 $^{\circ}$ C using a Beckman SW 28 rotor. The concentrated virus stocks were titrated on RPMI 8226 cells based on GFP expression.

Cells and culture conditions

Human multiple myeloma RPMI 8226 cells (8226) were a kind gift from Dr. Robert G. Fenton (University of Maryland) (26). The human multiple myeloma U266 cells and the bone marrow stromal cell line (BMSC) HS-5 were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Both multiple myeloma cell lines are IL-6 independent. All cell lines 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 and human fetal lung fibroblasts (HFL-1) 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 microscopy

Human multiple myeloma cells were plated at a density of 1×10^6 cells/well in a 12 well plate and were infected with LV-empty or LV-PPAR γ or LV-PPAR γ -DN at MOIs of 0.5 to 1. Twenty four hours post-infection, the growth media was replaced. For morphologic analysis, RPMI 8226 and U266 cells were examined by phase-contrast microscopy using an inverted microscope (Olympus IX81) and images were captured for subsequent analysis using ImagePro 4.5.1 (Media Cybernetics, Silver Spring, MD). Lentiviral transduced cells in cultures were identified on the basis of the co-expressed GFP gene reporter.

PPAR γ gene reporter analysis

Human embryonic kidney 293FT cells were plated at a density of 1.5×10^5 cells/well in a 12-well plate. The following day, cells were co-transfected using lipofectamine LTX (Invitrogen). Briefly, 0.5 μ g of PPRE-luciferase reporter plasmid containing three copies of the ACO-PPRE (PPAR response element) from rat acyl CoA oxidase (a gift from Dr. B. Seed, Massachusetts General Hospital, Boston, MA) was co-transfected with 0.5 μ g of either the LV-empty vector, the LV-PPAR γ vector or the LV-PPAR γ -DN vector. Forty eight hours after transfection, luciferase activity was assayed using the Promega Luciferase Assay System (Madison, WI). Relative light units (RLU) were determined with a Lumicount Microplate Luminometer (Packard Instrument Co., Meriden, CT, USA).

BMSC and multiple myeloma co-culture conditions

BMSCs (HS-5) were plated in a 24-well plate at a density of 4×10^4 cells per well and allowed to settle overnight. 8226 cells were infected with either LV-empty or LV-PPAR γ (MOI=1) for 16 hr, after which viral supernatants were removed and new media was added to the cells. Some of the LV-infected multiple myeloma cells were added to the BMSCs and incubated at indicated time points. Seventy two hours after incubation, cells were visualized microscopically and cell supernatants were collected to assess IL-6 secretion.

For experiments where BMSCs were infected with the lentiviral constructs, BMSCs (HS-5) were plated in a 96-well plate at a density of 1.5×10^4 cells per well and incubated overnight. The next day, HS-5 were infected with either LV-empty or LV-PPAR γ (MOI=1) with polybrene (6 μ g/ml) for 24 hr. Viral supernatants were then removed and the cells were incubated in the presence or absence of uninfected multiple myeloma 8226 cells (4×10^5 cell/well). Some cells were treated with the PPAR γ ligand ciglitazone (5 μ M). Forty eight hours later, cell supernatants were collected to assess IL-6 secretion.

Annexin V and 7-AAD staining

Multiple Myeloma cells infected with LV-empty and LV-PPAR γ were collected 48 to 96 hours after infection. Cells were washed twice in PBS, and double-labeled with Annexin V-APC and 7-AAD according to the manufacturer's protocol (BD Pharmingen, San Diego, CA). Control populations consisted of unstained cells and cells stained with only Annexin V or 7-AAD. Cells were analyzed by flow cytometry using a Beckman Coulter flow cytometer. The fluorescence emission of 10,000 cells per treatment was collected in each channel.

Viability and proliferation assays

Cells were left untreated or were infected with LV-Empty or LV-PPAR γ for 72 h at a density of 4×10^4 cells per well of a 96-well flat bottom microtiter plate. MTT assay was performed to assess cell viability. Cell viability was also confirmed by trypan blue dye exclusion. MTT is taken up by cells via mitochondrial membrane potential and then reduced to a formazan residue by intracellular NAD(P)H-oxidoreductases in living cells with functional mitochondria (42). Ten microliters 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 infected for 48 hours and 1 μ Ci/well of 3 H-thymidine was added for the last 18 h of culture. The cells were harvested onto a 96-well filter plate and the 3 H-thymidine incorporation was detected as counts per minute (cpm) using a Topcount Luminometer (PerkinElmer, Boston, MA).

Propidium iodide analysis of DNA content

Multiple myeloma cells were infected with LV-empty and LV-PPAR γ for 96 hr, after which they were harvested, washed twice in PBS and fixed with 2% paraformaldehyde at room temperature for 30 min. Cells were then washed twice with PBS and post-fixed in ice-cold 70% EtOH for 10 – 14 hr. After fixation, the cells were washed in PBS and resuspended in 0.1% Triton X-100, 0.2 mg/ml RNase A (Sigma-Aldrich, St. Louis, MO), and 20 μ g/ml propidium iodide (Sigma-Aldrich) in PBS. The cells were incubated for 30 min at room temperature and immediately analyzed on a BD Biosciences FACS Calibur flow cytometer. The percentage of cells with sub-G₀/G₁ DNA content was determined using FlowJo software (Tree Star, Inc. Ashland, OR).

Cell cycle analysis

Cell cycle analysis of human multiple myeloma 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 infected for 24 hr and treated with ciglitazone (5 μ M) for an additional 48 hr. Cells were then pulsed for 30 minutes at a final BrdU concentration of 10 μ M and stained the APC-BrdU flow kit.

IL-6 ELISA

IL-6 production was assessed in cell culture supernatants by an enzyme-linked immunosorbent assay according to the manufacturer's instructions (BD Pharmingen, San Jose, CA).

Western blots

One million human multiple myeloma cells were mock infected or infected with LV-empty or LV-PPAR γ . Seventy two hours post-infection, whole cell lysates were prepared 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 an 8–16 % Precise™ protein gel (Pierce, Rockford, IL) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA). The membranes were analyzed for immunoreactivity with indicated primary antibody, washed and then incubated with an appropriate horseradish peroxidase-conjugated secondary antibody. The membranes were then 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 PPAR γ 1 overexpression in multiple myeloma cell lines

We have previously demonstrated that both normal and malignant human B cells, including multiple myeloma cells, modestly express PPAR γ and that exposure to certain PPAR γ agonists induces cell death (25–28), suggesting that PPAR γ maybe a therapeutic target for multiple myeloma. To begin to determine both the physiological role of PPAR γ in multiple myeloma and the functional consequences of its overexpression, we constructed a lentivirus for PPAR γ gene delivery. Although human B lineage cells are generally difficult to transduce, our pilot experiments revealed that a lentivirus would provide an efficacious gene delivery system. We constructed a lentiviral vector that contains a Flag-tagged PPAR γ 1 cDNA under the control of the CMV promoter. The vector also expresses copepod green fluorescent protein (CopGFP) under the control of the elongation factor 1 (EF1)- α promoter. As additional controls, the backbone lentiviral vector, which expresses CopGFP, but not the PPAR γ 1 gene (LV-empty), and the lentiviral vector expressing a dominant negative form of PPAR γ 1 (LV-PPAR γ -DN) were used (Figure 1A). This dominant negative contains two amino acid substitutions that inhibit the release of co-repressors and reduce co-activator recruitment to the PPAR promoter (43).

To evaluate the function of PPAR γ in human multiple myeloma cells, we used the well characterized multiple myeloma cell RPMI 8226 (44). First, we transduced 1×10^6 RPMI 8226 cells with LV-empty or LV-PPAR γ or LV-PPAR γ -DN at an MOI of 1. Forty eight hours post-infection, GFP expression was assessed by flow cytometry. More than 80% of the cells were transduced (Figure 1B). We next measured the presence of Flag-tagged PPAR γ protein using an anti-FLAG antibody and found that infection with the LV-PPAR γ and LV-PPAR γ -DN, but not the LV-empty resulted in FLAG expression, indicating that transduction with LV-PPAR γ and LV-PPAR γ -DN resulted in increased levels of exogenous PPAR γ -WT or PPAR γ -DN protein, respectively (Figure 1C). To test whether this overexpressed PPAR γ protein was functional and could then bind to its promoter sequence, we used a luciferase reporter assay generated by co-transfecting human 293FT cells with a luciferase reporter construct containing 3 PPRE elements (PPRE-LUC) and either LV-empty, LV-PPAR γ or LV-PPAR γ -DN. Forty eight hours post-transfection cell lysates were collected. Cells transfected with LV-PPAR γ vector exhibited a dramatic increase in promoter activity, as assessed by an increase in luciferase, compared to that of LV-empty and LV-PPAR γ -DN (Figure 1D), indicating that the PPAR γ that is overexpressed is indeed functional.

Lentiviral PPAR γ overexpression reduces cell proliferation and induces cell cycle arrest

We next determined the effect of PPAR γ overexpression on cell proliferation, by assessing cell number and ^3H -thymidine incorporation. In Figure 2A, the first bar indicates the cell number before infection (0 hr). For multiple myeloma cells that were uninfected or infected with LV-empty, the cell number almost doubled by 48hr post-infection. In contrast, the cells infected with LV-PPAR γ showed a substantial reduction in cell number compared to that of LV-empty infected cells. To assess whether this was due, at least in part, to a decrease in proliferation, ^3H -thymidine incorporation was measured. Figure 2B shows that proliferation was not significantly influenced by LV-empty transduction ($p=0.06$). However, RPMI 8226 proliferation was reduced by approximately 50% in LV-PPAR γ expressing cells compared to that of RPMI 8226 transduced with LV-empty ($p=0.02$). Given that PPAR γ overexpression results in a decrease in proliferation, as assessed by ^3H -thymidine incorporation, we next analyzed the effects of PPAR γ overexpression on cell cycle progression using BrdU and 7-AAD staining (Figure 2C). Cells positive for BrdU represent cells that have entered the S phase, whereas 7-AAD reveals cell subsets in the G₁ and G₂/M phases. Multiple myeloma cells infected with LV-PPAR γ displayed a four-fold decrease of cells in S phase compared to that of LV-empty infected cells (7% in LV-PPAR γ infected cells vs. 28% in LV-empty infected cells); and a two fold increase in cells in G₂/M phase (60% in LV-PPAR γ infected cells vs. 30% in LV-empty infected cells) by 48 hours. Taken together, PPAR γ overexpression results in a decrease in RPMI 8226 cell proliferation, as a consequence of a G₂/M cell cycle arrest.

PPAR γ overexpression reduces multiple myeloma cell viability by eliciting apoptosis

We next determined whether the decrease in proliferation of RPMI 8226, as a result of PPAR γ overexpression, was due to the induction of apoptosis. Cells undergoing apoptosis show characteristic morphological features such as shrinkage and membrane blebbing (45). To determine whether overexpression of PPAR γ induced apoptosis, RPMI 8226 cells were infected with LV-PPAR γ , LV-empty or LV-PPAR γ -DN. Cellular morphology was then assessed by light and fluorescence microscopy. Seventy two hours after infection, cells infected with either LV-empty or LV-PPAR γ DN showed their characteristic round morphology, while PPAR γ overexpressing cells (infected with LV-PPAR γ) showed morphological changes characteristic of apoptosis, such as plasma membrane blebbing (Figure 3A, *arrow*) and cytoplasmic convolution (Figure 3A, *arrow head*). To test whether PPAR γ overexpression has the same effect in another multiple myeloma cell line, we infected U266 multiple myeloma cells at an MOI of 1. These cells showed similar changes in cellular morphology as RPMI 8226 multiple myeloma cells (Figure 3B). To further determine whether the increased expression of PPAR γ influenced multiple myeloma cell viability, RPMI 8226 cells were infected with LV-empty or LV-PPAR γ and cell viability was assessed by MTT assay. Seventy two hours post-infection, viability was reduced by 50% in cells overexpressing PPAR γ compared to that of uninfected and LV-empty control infected cells (Figure 3C). Taken together, these results indicate that PPAR γ overexpression has pro-apoptotic effects resulting in a deleterious effect on multiple myeloma cell survival. Interestingly, PPAR γ overexpression did not induce cell death in human fibroblasts nor in embryonic kidney cells (*data not shown*), suggesting that this effect is cell type specific.

Lentiviral overexpression of PPAR γ induces apoptosis and activation of caspases in human multiple myeloma cells

A characteristic of the early stages of apoptosis is the exposure of phosphatidylserine from the inner leaflet of the membrane to the external surface (45). To further characterize the cytopathic effects caused by PPAR γ overexpression on apoptotic cell death, double staining was performed on LV-empty and LV-PPAR γ infected cells using anti-Annexin V-APC mAb and the DNA stain 7-AAD, followed by analysis using flow cytometry (Figure 4A). Cells infected

with LV-PPAR γ exhibited a dramatic increase in Annexin-V/7-AAD double positive population compared to that of LV-empty control (Figure 4A, *upper right quadrants of panels*). These results are even more evident at the 96 hr time point where the cells infected with LV-PPAR γ showed a further increase in AnnexinV/7AAD double positive population, supporting the fact that PPAR γ -overexpression induces apoptosis. We also observed an increase on 7-AAD single-positive cells at 96 hour time point, which may suggest secondary necrosis. Rates of apoptosis after LV-PPAR γ infection were also determined by DNA staining with propidium iodide and flow cytometric analysis. Apoptotic cells exhibit reduced DNA content that is detected by the appearance of a sub-G₀/G₁ cell population by flow cytometry. As shown in Figure 4B, LV-empty control cells display a background level between 7 to 12%. In contrast, the number of sub-G₀/G₁ events in RPMI 8226 cells infected with LV-PPAR γ increased over time from 23% at 72hr to 40% at 96 hr post-infection. These results further confirm the induction of apoptosis by PPAR γ overexpression.

Cells undergoing apoptosis also display an increase in nuclear chromatin condensation; these compact nuclei become brightly fluorescent when labeled with nuclear stains, such as DAPI (46). To further support PPAR γ -induced cell death, multiple myeloma cells left uninfected or infected with either LV-empty or LV-PPAR γ were stained with DAPI. Fluorescence microscopy revealed nuclear condensation and fragmentation 96 hours after LV-PPAR γ infection (Figure 4C, *Right panel, arrows*). In contrast, nuclei from non-infected and LV-empty infected cells predominantly appeared uniformly stained without condensation (Figure 4C, *left and middle panels*). Quantification of apoptotic nuclei in cultures exposed to LV-PPAR γ exhibited 37% of cells with apoptotic nuclei, whereas only 4 % of cells with apoptotic nuclei were detected in cells infected with LV-empty (Figure 4C). These results further support the findings that apoptotic changes of cell and nuclear morphology are induced by PPAR γ overexpression. Together, these changes reveal an induction of a non-proliferating, apoptotic phenotype in cells overexpressing PPAR γ .

Caspase-3 has a key role in apoptosis, being responsible for the proteolytic cleavage of many key proteins, including the nuclear enzyme poly (ADP-ribose) polymerase (PARP)(47). To examine the molecular basis for the PPAR γ -dependent induction of apoptosis, we measured caspase activity after LV-PPAR γ transduction. Human multiple myeloma RPMI 8226 cells were infected with either LV-empty or LV-PPAR γ and 72 hours later cell lysates were collected. The active form of caspase 3, as well as the cleavage of PARP was determined by western blot analysis. Cells that were infected with LV-PPAR γ showed an increased level of active caspase 3, compared to that of uninfected and LV-empty infected cells (Figure 4D). PARP cleavage was determined by the appearance of an 85-kDa fragment by western blot analysis. Cells that were left uninfected or that were infected with LV-empty control virus contain predominantly the full length (116 kDa) PARP, whereas the 85 kDa cleaved PARP appeared in the cells that were infected with LV-PPAR γ (Figure 4D). These results suggest that PPAR γ overexpression induces caspase activation.

Growth inhibition and induction of apoptosis by ciglitazone treatment

Thiazolidinediones, a class of anti-diabetic drugs, are high-affinity synthetic ligands of PPAR γ (14). To assess whether a model PPAR γ ligand further enhanced human multiple myeloma cell death in cells that overexpress PPAR γ , multiple myeloma cells were infected at a lower MOI of 0.5 with either LV-empty or LV-PPAR γ . At this MOI, ~50% of the cells were transduced as assessed by analysis of GFP by flow cytometry (data not shown). We hypothesized that RPMI 8226 cells would proliferate less with modest PPAR γ overexpression. We further speculate that treatment with ciglitazone would augment apoptosis in cells expressing higher PPAR γ protein levels. Twenty four hours after transduction, cells were either untreated or treated with 5 μ M ciglitazone for an additional 48 hours. We previously showed

that this concentration of ciglitazone does not induce apoptosis in multiple myeloma cells (26). Cells were then pulsed with BrdU for 30 minutes, fixed, permeabilized and stained intracellularly with anti-BrdU-APC antibody. Cells were then analyzed for both GFP expression and BrdU incorporation by flow cytometry. As Figure 5A shows, LV-PPAR γ infected cells showed somewhat reduced incorporation of BrdU, especially the higher expressers of PPAR γ (GFP-high, *upper right quadrant*). When cells were treated with 5 μ M ciglitazone, there was an enhanced inhibition in DNA synthesis in cells that overexpressed PPAR γ but not in the cells infected with LV-empty or uninfected cells. At the same time, the percentage of apoptotic cells (7-AAD positive) increased from 16% in LV-empty infected cells treated with ciglitazone to 27% in cells infected with LV-PPAR γ and treated with ciglitazone, while it remained almost the same in LV-empty infected cells (Figure 5B). These data support the hypothesis that increased levels of PPAR γ further sensitize the cells to PPAR γ ligand-induced growth arrest and cell death.

Mechanism whereby PPAR γ overexpression inhibits multiple myeloma cell proliferation and induces cell death

In the next set of experiments, we determined whether PPAR γ overexpression-induced caspase activation led to multiple myeloma cell apoptosis by using a broad-spectrum caspase inhibitor, ZVAD-FMK. Briefly, multiple myeloma RPMI 8226 cells were left uninfected or were infected with LV-empty or LV-PPAR γ . Six hours after infection, cells were treated every day with either DMSO or with 50 μ M ZVAD-FMK. ZVAD-FMK treatment alone results in no toxicity to the cells (data not shown). Cells were collected at different time points and apoptosis was analyzed by the DNA stain 7-AAD. This 7-AAD solution incorporates into cells that have their plasma membrane integrity disrupted. Multiple myeloma cells that were infected with LV-PPAR γ exhibited a time-dependent increase in the percentage of 7AAD+ cells, in comparison to that of uninfected and LV-empty infected cells (Figure 6A). The percentage of 7-AAD+ cells in ZVAD-FMK-treated cells was four-fold lower than that of the LV-PPAR γ infected cells without ZVAD-FMK-treatment. ZVAD-FMK treatment, however, did not completely inhibit the induction of apoptosis by PPAR γ overexpression (Figure 6A). We then evaluated whether treatment of PPAR γ overexpressing cells with ZVAD-FMK inhibited caspase activation. After five days of infection, cells were lysed and probed for the active form of caspase 3 and PARP cleavage. PARP cleavage was observed in the PPAR γ -overexpressing cells and treatment with ZVAD-FMK slightly reduced PARP cleavage. Moreover, cells that were transduced with LV-PPAR γ exhibited a dramatic increase in active caspase 3. However, when these cells were treated with ZVAD-FMK, this activation was completely abolished (Figure 6B). We next evaluated the levels of X-linked inhibitor of apoptosis protein (XIAP), which regulates cell death by inhibiting caspases (48). We found that XIAP levels were downregulated in the cells overexpressing PPAR γ , and treatment with the caspase inhibitor restored XIAP levels back to normal (Figure 6B). We also examined the levels of the anti-apoptotic protein Mcl-1 in PPAR γ -overexpressing cells. Multiple myeloma cells infected with LV-PPAR γ down-regulated Mcl-1 levels; however when cells were treated with the caspase inhibitor, the levels of Mcl-1 were restored (Figure 6B). Taken together, these results indicate that PPAR γ -overexpression induces cell death by activating caspases and by down-regulating anti-apoptotic proteins.

PPAR γ overexpression-induced multiple myeloma cell death can not be overcome by BMSCs

Tumor cell proliferation and survival are regulated by the local microenvironment. Stromal cells are a critical component of this tumor microenvironment. Some characteristics include multiple myeloma interaction with stromal cells, as well as growth factors secreted into the local environment. Importantly, adhesion of multiple myeloma cells to stromal cells confers drug resistance (37). Therefore, we tested whether co-incubation with bone marrow stromal cells would rescue multiple myeloma cells from undergoing apoptosis mediated by PPAR γ

overexpression. Multiple myeloma 8226 cells were infected with either LV-empty or LV-PPAR γ and cultured them with BMSCs. Similar morphological changes were observed as described earlier, where 8226 cells transduced with LV-PPAR γ showed plasma membrane blebbing (Figure 7A, *middle panel*) and cytoplasmic convolution (Figure 7A, *bottom panel*), while LV-empty transduced cells kept their characteristic round morphology (Figure 7A, *top panel*). Multiple myeloma cells were further analyzed for cell death by using 7-AAD staining. To distinguish between multiple myeloma cells and BMSCs, an anti-CD38 antibody was used, which is expressed by multiple myeloma cells but not by BMSCs. Again, multiple myeloma cells infected with LV-PPAR γ had a time-dependent increased in 7-AAD positive cells in comparison to LV-empty or uninfected cells (Figure 7B). When cells were co-cultured with BMSCs, there was a modest baseline increase in 7-AAD positive cells in uninfected cells and cells infected with LV-empty. However, when PPAR γ overexpressing multiple myeloma cells were co-incubated with BMSCs, there was a time-dependent increase in 7-AAD+ cells; this 7-AAD+ population was higher than the 7-AAD+ population observed in the absence of BMSCs (Figure 7B). These results indicate that co-incubation of multiple myeloma cells with BMSCs does not rescue multiple myeloma cells from undergoing apoptosis induced by PPAR γ overexpression.

PPAR γ overexpression in multiple myeloma or BMSCs inhibits basal and MM-induced IL-6 production by BMSCs and the inhibitory effect is enhanced by ciglitazone

We next analyzed whether PPAR γ overexpression had any effect on multiple myeloma-induced IL-6 production by BMSCs. Multiple myeloma cell adhesion to BMSCs is known to trigger IL-6 production from BMSCs (49). IL-6 is a cytokine that regulates growth and survival of multiple myeloma (49). We infected multiple myeloma cells with either LV-empty or LV-PPAR γ and then added them to BMSC-coated plates. Culture supernatants were collected 72 hr after co-incubation. We observed an increase in IL-6 production when BMSCs were co-incubated with either uninfected or LV-empty infected multiple myeloma cells. However, when BMSCs were co-incubated with LV-PPAR γ overexpressing multiple myeloma cells, there was a significant decrease in IL-6 production by BMSCs. Interestingly, the levels of IL-6 were two-fold lower than the levels produced in the absence of multiple myeloma cells (Figure 8A). These results suggest that PPAR γ overexpression in multiple myeloma cells inhibits IL-6 production by BMSCs.

We next investigated the effects of PPAR γ overexpression in BMSCs. We infected BMSC HS-5 cells with LV-empty or LV-PPAR γ . BMSCs were cultured alone or were cultured with multiple myeloma cells, in the presence or absence of ciglitazone (5 μ M). There was a slight decrease in the basal production of IL-6 from BMSCs that were infected with LV-PPAR γ (Figure 8B). In the presence of ciglitazone, basal IL-6 production was further reduced from PPAR γ -overexpressing BMSCs. When these BMSCs were co-incubated with multiple myeloma, LV-empty-infected BMSCs upregulated IL-6 production. However, when PPAR γ -overexpressing BMSCs were co-incubated with multiple myeloma cells, BMSCs failed to produce IL-6 (Figure 8B). Taken together, these results suggest that increasing the levels of PPAR γ in either multiple myeloma or BMSCs decreases both basal and MM-induced IL-6 production.

DISCUSSION

Multiple myeloma is a prevalent hematological cancer with poor prognosis, with an average survival of only 3–5 years. PPAR γ and PPAR γ ligands, represent new targets in the treatment of B cell malignancies. In addition to the well-known adipogenic role of PPAR γ , we propose that PPAR γ is a tumor suppressor. There is emerging evidence for a direct role of PPAR γ functional mutations in the initiation of several common human cancers, including lung, breast,

prostate, pancreas, and ovary (50,51). Moreover, the expression levels and/or transactivation of PPAR γ are impaired in certain cancers. For example, in human lung cancer, decreased expression of PPAR γ correlates with poor prognosis (52). In addition, well-differentiated adenocarcinomas have higher PPAR γ expression compared to poorly differentiated samples, implying that PPAR γ regulates tumor progression (53). The PPAR γ gene is found in the human chromosome 3, band 3p25 (54). Chromosomal abnormalities, such as 3p deletions have been identified in several hematological cancers (55). Here, we report that PPAR γ overexpression in multiple myeloma cells reduces proliferation and induces apoptosis. Importantly, treatment with ciglitazone, a selective PPAR γ agonist, augments these effects. These observations suggest that loss of PPAR γ expression and/or transactivation potential via post-translational modifications or inactivating mutations may augment the risk of developing multiple myeloma. Therefore, determining whether the expression levels and/or activation status of PPAR γ changes during progression from normal plasma cells to monoclonal gammopathy of undetermined significance (MGUS) and finally to multiple myeloma may be an important prognostic indicator.

The role, however, of PPAR γ in the development of hematological malignancies has not been extensively studied. We have previously demonstrated that both normal and malignant human B cells, including multiple myeloma cells, express moderate levels of PPAR γ , and that exposure to certain PPAR γ agonists induces apoptosis (25–28). However, the biological relevance of PPAR γ in multiple myeloma has, to date, not been explored.

PPAR γ overexpression has been used by other groups to study its effects on inflammatory diseases such as colitis, where PPAR γ levels were reduced as a consequence of inflammation (56). Moreover, when PPAR γ was overexpressed in thyroid carcinoma cells, where it induced cell cycle arrest and cell death (57). In concordance with these studies, we demonstrated that increased PPAR γ expression leads to inhibition of proliferation and induction of apoptosis in multiple myeloma cells, even in the absence of an exogenous PPAR γ ligand. Since transduction with LV-PPAR γ alone achieved therapeutic effects, we suggest that increasing the levels of PPAR γ in these cells may allow for interactions with endogenous PPAR γ ligands that activate the anti-proliferative actions associated with PPAR γ . Moreover, in the presence of the PPAR γ ligand ciglitazone, cells overexpressing PPAR γ had an even greater reduction in proliferation, further supporting the concept that PPAR γ plays an important role in regulating multiple myeloma cell growth, as also reported in other cell types (32,58–60).

A reduction in ³H-thymidine incorporation and BrdU incorporation (Figure 2) suggest that PPAR-overexpressing cells do not progress through S-phase. Moreover, this reduction in S phase transit occurs before the cells display apoptotic changes, suggesting the inhibition of cell proliferation precedes apoptosis. Rapid cell shrinkage and increased granularity are the most evident morphological changes associated with apoptosis, which also accompany nuclear condensation and membrane blebbing (45). In addition, cells at early stages of apoptosis expose phosphatidylserine from the inner side of the membrane to the membrane surface (45). Herein, we observed dramatic changes in multiple myeloma cellular morphology, as well as an increased number of Annexin V/7-AAD double positive subset after infection with LV-PPAR γ . Coincident with these events, we observed an increase in cells displaying sub-G₀/G₁ DNA content, as well as an induction of nuclear condensation in the cells that overexpressed PPAR γ , but not in the LV-empty control infected cells. These results are indicative that apoptotic induction is PPAR γ dependent, as infection with LV-empty control virus or the dominant negative form of PPAR γ did not cause any changes in cellular or nuclear morphology.

Cells undergoing apoptosis experience mitochondrial damage that leads to activation of caspases (47). Caspases are cystine proteases responsible for cleaving cellular substrates during apoptosis, including DNA cleavage, which is characteristic of apoptosis (47). The PPAR γ -

dependent morphological changes correlated with a loss of mitochondrial membrane integrity and cleavage of pro-caspase 3 and PARP, confirming activation of the intrinsic mitochondrial cell death pathway. Furthermore, co-incubation with a general caspase inhibitor inhibited PPAR γ overexpression-induced cell death and inhibited caspase 3 activation, as well as reduced PARP cleavage.

The inhibitors of apoptosis proteins (IAPs) are a family of caspase inhibitors that specifically inhibit caspases 3, 7, and 9 to prevent apoptosis (48). One of the members of this family is X-linked inhibitor of apoptosis protein (XIAP). Increased expression of the IAP family proteins, including XIAP, correlate with poor prognosis and chemotherapy-induced drug resistance in multiple myeloma (61). Myeloid cell leukemia-1 (Mcl-1) is an anti-apoptotic protein belonging to the Bcl-2 family (62). Mcl-1 protects multiple myeloma cells against spontaneous and chemotherapy-induced apoptosis (62). Moreover, downregulation of Mcl-1 induces multiple myeloma cell death (62). Herein, the levels of both anti-apoptotic protein XIAP and Mcl-1 were downregulated in the cells overexpressing PPAR γ , but not in the cells treated with caspase inhibitor or the LV-empty infected cells. Although the exact mechanism of PPAR γ -overexpression-induced cell death remains uncertain, these results indicate that PPAR γ -overexpression appears to target several pathways in multiple myeloma, including the activation of caspase-dependent and independent pathways and the down-regulation of inhibitors of apoptosis.

Bone marrow microenvironment is a very important factor for multiple myeloma drug resistance and chemoresistance (37). BMSCs produce interleukin-6 (IL-6), a cytokine that protects multiple myeloma cells from drug-mediated apoptosis (36,63). Inhibition of IL-6 signaling sensitizes multiple myeloma cells to bortezomib-induced cell death (64). The involvement of PPAR γ ligands in influencing IL-6 production has been demonstrated by Farrar and colleagues, where PPAR γ ligands inhibited both multiple myeloma cell adhesion to BMSCs and multiple myeloma-induced IL-6 production by BMSCs (38,39). Here, we demonstrate that PPAR γ overexpression reduces IL-6 production by BMSCs, even in the absence of exogenous ligand (Figure 8). Thus, PPAR γ not only regulates multiple myeloma cells, but can impact the ability of BMSCs to produce factors such as IL-6 that promotes tumor progression. Moreover, PPAR γ overexpression-induced multiple myeloma cell death was not able to be rescued by BMSCs, indicating that PPAR γ overexpression might be able to overcome bone marrow microenvironment-mediated resistance.

Collectively, these results indicate that therapeutic attempts to induce PPAR γ expression in multiple myeloma cells may demonstrate synergistic activity with the use of PPAR γ ligands for therapy. Although appropriate targeting and delivery mechanisms would be required before PPAR γ gene therapy could become a reality, the observation made here does suggest that pharmacologic means of upregulating PPAR γ in cancer cells and multiple myeloma cells in particular, may be a potential new therapeutic target.

Statement of clinical relevance

Multiple myeloma is a disseminated neoplasm of differentiated plasma cells that is essentially incurable. Therefore, there is great interest in developing new therapeutic approaches for treating this deadly cancer. A transcription factor called PPAR γ and its ligands may represent a new target in treating hematological malignancies. Certain PPAR γ ligands inhibit proliferation and survival and are proposed to directly affect both the formation and progression of human tumors. Herein, we report that PPAR γ overexpression in multiple myeloma cells reduces their proliferation and induces apoptosis. Moreover, treatment with ciglitazone, a selective PPAR γ agonist, augments these beneficial effects. These new findings suggest that loss of PPAR γ expression and/or transactivation

potential may augment the risk of developing multiple myeloma. Therefore, PPAR γ expression levels and or activation status may be an important prognostic marker for multiple myeloma. Importantly, our investigation indicates that therapeutic efforts to up-regulate PPAR γ expression in multiple myeloma, in combination with administration of PPAR γ ligands, represents a potential new therapeutic strategy.

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Figure 1. Design of a lentiviral vector for PPAR γ overexpression in human multiple myeloma cells

A) Schematic diagram of the LV-empty, LV-PPAR γ -WT and LV-PPAR γ -DN vectors. The flag-PPAR γ 1-WT and flag-PPAR γ 1-DN 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)** Human multiple myeloma 8226 cells were transduced with LV-empty, LV-PPAR γ or LV-PPAR γ -DN (MOI=1). At 48hr post-transduction, cells were analyzed for their GFP expression using flow cytometry. LV-empty, LV-PPAR γ and LV-PPAR γ -DN infected cells showed 89% and 83% and 86% GFP positive cells, respectively. **C)** Exogenous PPAR γ 1 expression was evaluated by western blotting using an anti-FLAG antibody. Forty eight hours post-infection, the FLAG-PPAR γ protein was detected in the cells infected with LV-PPAR γ and LV-PPAR γ -DN, but not in the LV-empty infected cells. Total actin levels were assayed to normalize protein loading **D)** HEK 293FT cells were seeded in a 12-well plate and transiently co-transfected using

Lipofectamine LTX (invitrogen) with either LV-empty, LV-PPAR γ or LV-PPAR γ -DN and PPRE-Luciferase construct. At 48 hr after transfection, cells were lysed and a luciferase assay was performed. Cells transfected with LV-PPAR γ vector had a significantly increased luciferase activity compared to LV-empty and LV-PPAR γ -DN transfected cells ($p < 0.001$).

Figure 2. Lentiviral PPAR γ overexpression reduces cell proliferation and induces cell cycle arrest Human multiple myeloma 8226 cells were transduced with LV-empty or with LV-PPAR γ (MOI=1). **A)** At 48hr post-infection, the cells were harvested and counted in a hemocytometer. The 0 hr bar depicts the number of cells at time zero, the time of infection. **B)** Cells were infected for 24 hr and then pulsed for an additional 24 hr with 1 μ Ci of methyl ³H-thymidine per well. The cells were harvested and ³H-thymidine incorporation into DNA was measured. The bars indicate the standard deviation of the mean, p < 0.05. **C)** Cell cycle analysis. At 48hr post-infection LV-empty and LV-PPAR γ -transduced cells were incubated for 30 minutes with BrdU and labeled with anti-BrdU and 7-AAD. Cells that overexpressed PPAR γ showed a

reduction in the cells entering the S-phase of the cycle. This profile is representative of three separate experiments.

Figure 3. PPAR γ overexpression in two different multiple myeloma cell lines induces morphologic changes characteristic of apoptosis and reduces cell viability

A) Human multiple myeloma 8226 cells were transduced with LV-empty, LV-PPAR γ and LV-PPAR γ -DN (MOI=1). Light microscopy (*left panels*) and fluorescence microscopy for GFP expression (*right panels*) were performed after 72hr of culture. LV-empty and LV-PPAR γ -DN transduced cells display typical round morphology, whereas LV-PPAR γ infected cells show morphological changes of apoptosis including membrane blebbing (*arrow*) and cytoplasmic convolution (*arrow head*). Similar results were obtained in each of six experiments. **B)** Human multiple myeloma U266 cells were infected with LV-empty and LV-PPAR γ for 72 hr. Cells that were infected with LV-PPAR γ showed the morphological changes characteristic of

apoptosis, while the LV-empty transduced cells did not display any morphologic changes. Photomicrographs of representative microscope fields were taken with a 20X objective. **C)** Seventy two hours after infection, cell viability was assessed by MTT assay, as described in Materials and Methods. The bars indicate the standard deviation of the mean ($p < 0.05$).

Figure 4. Lentiviral overexpression of PPAR γ induces apoptosis in human multiple myeloma cells and activates caspases

A) Multiple myeloma RPMI 8226 cells were infected at an MOI of 1 and cultured for 48 hr and 96 hr. Cells were then labeled with Annexin-V-APC and 7-AAD and analyzed by flow cytometry. Data were analyzed using FlowJo Software. The numbers on each counter plot represent the percent of cells in the quadrant. Left: LV-empty infected cells for 48hr and 96 hr. Right panels: LV-PPAR γ infected cells for 48 hr and 96 hr. **B)** RPMI 8226 cells were harvested 72hr and 96hr post-infection and detection of sub-G $_0$ /G $_1$ cell population was performed by PI staining and analyzed by flow cytometry. These profiles are representative of three separate experiments. **C)** RPMI 8226 cells were left untreated (*Left*), infected with LV-

empty control (*Middle*) or infected with LV-PPAR γ (*Right*) for 96 hr. Cells were harvested and stained with DAPI. Arrows in right panel demonstrate apoptotic nuclei with condensed, fragmented chromatin. A quantification of cells with apoptotic nuclei revealed that PPAR γ overexpressing cells display a significant increase in the percentage of cells with apoptotic nuclei when compared to uninfected and LV-empty infected cells. **D)** After 72hr of infection, cells were harvested and total cell extracts were prepared. Twenty five micrograms of protein were loaded into an SDS-PAGE gel and transferred to a PVDF membrane. Membranes were then incubated with antibodies specific for FLAG, PARP and active-caspase-3. Total actin was used to normalize protein loading. This experiment was repeated three times with similar results.

Figure 5. Growth inhibition and induction of apoptosis by ciglitazone treatment

A) Human multiple myeloma 8226 cells were infected with either LV-empty or LV-PPAR γ at an MOI of 0.5 for 24 hr. Cells were left untreated (– Cig) or were treated with 5 μ M ciglitazone (+ Cig) for an additional 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 that of LV-empty and uninfected control. When cells were treated with ciglitazone, a significant reduction in BrdU incorporation was seen in the cells that express higher levels of PPAR γ , but not in the LV-empty or uninfected control cells. **B)** Apoptosis assay by flow cytometry using 7-AAD on multiple myeloma cells

infected with LV-empty or LV-PPAR γ with or without ciglitazone treatment. These profiles are representative of three separate experiments.

Figure 6. Mechanism whereby PPAR γ overexpression inhibits multiple myeloma cell proliferation and induces cell death

A) Multiple myeloma cells were mock infected or infected with LV-empty or with LV-PPAR γ at an MOI of 1. After 6 hours of incubation each treatment was divided into two wells, one was left untreated and the other was treated with 50 μ M concentration of ZVAD-FMK. ZVAD-FMK was added fresh every day for 5 days. Cells were collected at 48hr, 96hr and 120 hr post infection and analyzed for 7-AAD DNA binding by flow cytometry. The results are shown as a percentage of 7-AAD positive cells. Results are representative of three separate experiments. **B)** Five days after infections and treatments with ZVAD-FMK, cell lysates were collected and run in an SDS-PAGE gel and transfer to a PVDF membrane. Membranes were then incubated with antibodies specific for PARP, active-caspase-3, XIAP and Mcl-1. Total actin was used as a loading control.

Figure 7. Co-culture of multiple myeloma cells with BMSCs does not rescue PPAR γ -overexpression-induced cell death

A) Multiple myeloma 8226 cells were infected with LV-empty or LV-PPAR γ . Twenty four hours later, cells were washed and were culture alone or were added to HS-5 BMSC-coated plates for an additional 72 hr. Light microscopy (*left panels*) and fluorescence microscopy for GFP expression (*right panels*) were performed after 72hr of culture (*open arrows* indicate HS-5 cells). LV-empty infected cells displayed characteristic round morphology (*top panel*), while LV-PPAR γ infected cells showed apoptotic changes such as membrane blebbing (*middle panel, closed arrow*) and cytoplasmic convolution (*bottom panel, closed arrow*). Similar results were obtained in each of two experiments. **B)** Cells were collected using trypsin at 72hr and 96hr after co-incubation initiation. Cells were then analyzed for CD38 expression and for 7-AAD DNA binding by flow cytometry. The results are shown as a percentage of 7-AAD positive cells (gated on CD38⁺ population). Results are representative of two separate experiments.

A. PPAR γ overexpression in MM/ cultured with HS-5 **B. PPAR γ overexpression in BMSCs**

Figure 8. PPAR γ overexpression in BMSCs inhibits basal and MM-induced IL-6 production by BMSCs and the inhibitory effect is enhanced by addition of ciglitazone

A) Multiple myeloma 8226 cells were infected with LV-empty or LV-PPAR γ . Twenty four hours later, cells were washed and cultured alone or were added to HS-5 BMSC-dcoated plates for an additional 72 hr and cell supernatants were collected. IL-6 production was measured by ELISA. The experiment was performed in triplicate. IL-6 levels were undetectable in multiple myeloma 8226 cells cultured alone. (* $p < 0.05$; ** $p < 0.01$) **B)** HS-5 BMSCs were infected with either LV-empty or LV-PPAR γ for 24 hr. The next day, the media was changed and cells were cultured alone with or without ciglitazone (5 μ M) or in the presence of 8226 multiple myeloma cells with or without ciglitazone (5 μ M). Forty eight hours after co-incubation started, cell supernatants were collected and IL-6 production was analyzed by ELISA. (** $p \leq 0.01$; *** $p \leq 0.001$)