PPAR-δ senses and orchestrates clearance of apoptotic cells to promote tolerance

Lata Mukundan1,*, Justin I. Odegaard1,2,*, Christine R. Morel1,3,*, Jose E. Heredia1, Julia W. Mwangi1, Roberto R. Ricardo-Gonzalez1,4, Y.P. Sharon Goh1, Alex Red Eagle1,5, Shannon E. Dunn6, Jennifer U.H. Awakuni1, Khoa D. Nguyen4, Lawrence Steinman6, Sara A. Michie2, and Ajay Chawla1,4

1Division of Endocrinology, Metabolism and Gerontology, Department of Medicine, Stanford University School of Medicine, Stanford, California 94305-5103, USA
2Department of Pathology, Stanford University School of Medicine, Stanford, California 94305-5103, USA
3Graduate Program in Biomedical Sciences, Baylor Institute for Immunology Research, Baylor University, Dallas, TX 75204, USA
4Graduate Program in Immunology, Stanford University School of Medicine, Stanford, California 94305-5103, USA
5Department of Genetics, Stanford University School of Medicine, Stanford, California 94305-5103, USA
6Department of Neurology and Neurological Studies, Stanford University School of Medicine, Stanford, California 94305-5103, USA

Abstract

Macrophages rapidly engulf apoptotic cells to limit the release of noxious cellular contents and to restrict autoimmune responses against self antigens. Although factors participating in recognition and engulfment of apoptotic cells have been identified, the transcriptional basis for the sensing and silently disposing of apoptotic cells is unknown. Here we show that peroxisome proliferator activated receptor-δ (PPAR-δ) is induced when macrophages engulf apoptotic cells and functions as a transcriptional sensor of dying cells. Genetic deletion of PPAR-δ decreases expression of opsonins, such as C1qb, resulting in impairment of apoptotic cell clearance and reduction in anti-inflammatory cytokine production. This increases autoantibody production and predisposes global and macrophage-specific PPARδ−/− mice to autoimmune kidney disease, a phenotype resembling the human disease systemic lupus erythematosus. Thus, PPAR-δ plays a pivotal role in orchestrating the timely disposal of apoptotic cells by macrophages, ensuring that tolerance to self is maintained.
In vertebrates, resident and recruited macrophages are the professional phagocytes that rapidly clear apoptotic cells. This function protects neighboring cells from the noxious contents of dying cells, and prevents activation of the immune system by liberated cellular contents. Indeed, defects in apoptotic cell clearance make mice and humans susceptible to the autoimmune disease systemic lupus erythematosus. Despite its importance, how macrophages transcriptionally coordinate prompt apoptotic cell disposal remains poorly understood.

Two distinct groups of proteins facilitate the recognition and uptake of apoptotic cells by macrophages. First, factors secreted by macrophages, termed opsonins, serve as a bridge to link unique chemical moieties exposed on apoptotic cells to macrophage cell surface receptors. C1q, protein S, Gas6, thrombospondin-1, and milk fat globule-epidermal growth factor-8 (Mfge8) are examples of opsonins that enhance the recognition and phagocytosis of apoptotic cells by macrophages. Cell surface receptors that bind opsonins comprise the second category of proteins involved in the uptake of dying cells. Members of this diverse group include the macrophage scavenger receptors, integrin and complement receptors, tyrosine kinase Mer, calreticulin, and immunoglobulin and mucin domain containing TIM-4. In support of their pivotal role in apoptotic cell clearance, mice deficient in opsonins, such as C1q or Mfge8, or the engulfment receptor Mertk, exhibit heightened susceptibility to autoimmune disease on certain genetic backgrounds.

Engulfment of apoptotic cells brings large amounts of cellular lipids, including oxidized fatty acids and oxysterols, into the macrophage. We postulated that peroxisome proliferator activated receptors (PPARs), the genetic sensors of native and oxidized fatty acids, are ideally suited to sense this lipid influx. Because we and others have previously shown that PPARs regulate the macrophage program of alternative activation, it raised the question whether these lipid sensors also orchestrate tolerogenic responses in macrophages.

Results

PPAR-δ orchestrates timely disposal of apoptotic cells

To investigate the role of PPARs in apoptotic cell clearance, we profiled the expression of all three mouse PPARs (α, δ and γ) in primary bone marrow-derived macrophages (BMDMs) after apoptotic cell feeding (Supplementary Fig. 1a online). PPAR-δ mRNA, but not PPAR-γ mRNA, was induced ~200% after apoptotic cell feeding (Fig. 1a), whereas PPAR-α mRNA was undetectable in macrophages. Immunoblotting confirmed that ingestion of apoptotic cells led to ~190% increase in PPAR-δ protein (Fig. 1b). To exclude the contribution of apoptotic cell-derived PPAR-δ to the signals detected in macrophages, we analyzed the expression of PPAR-δ protein in thymocytes. Intracellular staining failed to detect significant levels of PPAR-δ protein in thymocytes (Fig. 1c). In contrast, expression of PPAR-δ protein was specifically increased in macrophages fed apoptotic thymocytes (Fig. 1c). Moreover, we failed to detect expression of lymphocyte specific proteins, such as the Src kinase Lck or Cd3, in macrophages fed apoptotic thymocytes (Supplementary Fig. 1b, d online), whereas Lck and Cd3 proteins were readily detectable in thymocytes (Supplementary Fig. 1b, c online). Lastly, phagocytosis of latex beads, necrotic cells or opsonized sheep red blood cells (sRBCs), which are taken up via distinct pathways, did
not induce a significant increase in PPAR-δ mRNA (Supplementary Fig. 1e online) or protein (Supplementary Fig. 1f, g online). Altogether, these data demonstrate that phagocytosis of apoptotic cells induces expression of PPAR-δ in macrophages, suggesting its potential involvement in coordinating the macrophage’s transcriptional response to apoptotic cells.

To test this postulate, BMDMs from wild-type and PPARδ−/− mice were fed apoptotic thymocytes and phagocytosis was quantified. Since doublet discrimination can distinguish between engulfed and bound cells, engulfment of fluorescence-labeled apoptotic cells was monitored by flow cytometry18. Strikingly, compared to wild-type macrophages, phagocytosis of apoptotic cells was reduced by ∼75% and ∼60% at 30 and 60 minutes, respectively, in PPARδ−/− macrophages (Fig. 1d). Similar results were obtained when phagocytosis was quantified by microscopy (Supplementary Fig. 2a online). Furthermore, on an individual cell basis, PPARδ−/− macrophages were less efficient at taking up apoptotic thymocytes, as evidenced by the lower percentage of cells containing >2 thymocytes (Supplementary Fig. 2b and c online). This observed phagocytic defect was specific for apoptotic cells, because phagocytosis of opsonized apoptotic thymocytes, opsonized sRBCs or necrotic thymocytes was unaffected in PPARδ−/− macrophages (Supplementary Fig. 2d–f online). This impairment in phagocytosis was also independent of PPAR-δ’s known role in energy metabolism (Supplementary Fig. 2g online)19,20.

Two independent approaches were undertaken to investigate apoptotic cell clearance in vivo. First, wild-type and PPARδ−/− mice were injected with fluorescence-labeled apoptotic thymocytes, and splenic clearance was monitored9. Although similar numbers of apoptotic thymocytes were found in spleens 20 minutes after injection (Fig. 1e), the relative clearance of apoptotic cells was delayed in PPARδ−/− mice. At 4 hours, ~2-fold more fluorescent cells were present in the spleens of PPARδ−/− mice (1.38% vs. 2.7%) (Fig. 1e). Similarly, resident peritoneal macrophages of PPARδ−/− mice displayed reduced capacity for clearance of fluorescence-labeled apoptotic cells. Six hours after intraperitoneal injection of apoptotic thymocytes, the number of free thymocytes recovered from the peritoneum of PPARδ−/− mice was ~5-fold higher than in wild-type mice (0.43% versus 2.02%) (Fig. 1f). These findings thus demonstrate that PPAR-δ promotes timely disposal of apoptotic cells in various macrophage populations.

**PPAR-δ regulates opsonin gene expression in macrophages**

To identify the molecular targets by which PPAR-δ regulates apoptotic cell clearance, expression of opsonins and their cognate receptors was monitored by microarrays. Clustering analyses revealed that a number of genes encoding opsonins, including C1qa, C1qb, C1qc, Gas6, Mfge8, and Thbs1, were downregulated in PPARδ−/− macrophages (Fig. 2a). In contrast, the expression of macrophage receptors that recognize these “eat-me” signals was largely unchanged, with the notable exception of MerTK (Fig. 2a). Quantitative PCR (qRT-PCR) analysis confirmed that expression of C1qa, C1qb, Mfge8, MerTK and Thbs1 was reduced by 60-70% in PPARδ−/− BMDMs (Fig. 2b). In agreement, immunoblot analysis revealed ~300% decrease in C1qb protein in PPARδ−/− macrophages (Fig. 2c). To determine whether activation of PPAR-δ can enhance opsonin gene expression in
macrophages, wild-type BMDMs were treated with PPAR-δ agonist GW074221 and opsonin gene expression was monitored by qRT-PCR. Indeed, PPAR-δ activation induced C1qa, C1qb, Mfge8 and Thbs1 by ~200%-300% (Fig. 2d), in a PPAR-δ-dependent manner (Supplementary Fig. 3b online). Finally, since mutations in C1q are responsible for the monogenic form of systemic lupus erythematosus in humans22, we investigated whether C1qb is a direct transcriptional target of PPAR-δ. In silico analysis revealed a consensus PPAR-δ binding site in the C1qb gene located at ~1.6 kb. Deletion analysis of the mouse promoter affirmed that PPAR-δ/RXR heterodimers directly regulate C1qb gene expression (Fig. 2e, Supplementary Fig. 3b online).

We next tested whether pharmacologic activation of PPAR-δ enhances phagocytosis of apoptotic cells. Notably, treatment of wild-type mice with GW0742 enhanced the ability of CD11b+ splenic macrophages to phagocytose labeled apoptotic thymocytes by ~200% (Fig. 3a). To extend these findings to human cells, primary human monocyte-derived macrophages were treated with PPAR-δ activator GW0742. Both opsonin gene expression (Fig. 3b) and apoptotic cell uptake (Fig. 3c) was enhanced in human macrophages stimulated with GW0742, indicating conservation of this pathway between mice and humans.

Kupffer cells are the primary source of opsonins that circulate in the serum23-25, prompting us to investigate whether opsonin expression was reduced in livers of PPARδ−/− mice. Indeed, significant decreases in expression of C1qa, C1qb, Thbs1, and Mfge8 (~40-60%) were seen in livers of PPARδ−/− mice (Supplementary Fig. 3c online). Conversely, treatment of wild-type mice with GW0742 increased hepatic opsonin gene expression by ~50-90% (Supplementary Fig. 3d online). Congruent with these observations, circulating levels of C1qb (Fig. 3d), and to a lesser extent, Mfge8 (Supplementary Fig. 3e online) were lower in PPARδ−/− mice. To determine whether the reduction in opsonins contributes to the phagocytic defect of PPARδ−/− macrophages, we performed a series of experiments using sera from wild-type and PPARδ−/− mice. The observed phagocytic defect in PPARδ−/− macrophages was largely rescued by sera from wild-type mice (Fig. 3e), whereas incubation of wild-type macrophages with PPARδ−/− sera reduced their phagocytic capacity by ~35% (Fig. 3e). Similarly, addition of purified human C1q protein to PPARδ−/− macrophages restored their phagocytic activity to ~90% of wild-type macrophages (Fig. 3f). We thus conclude that PPAR-δ orchestrates the prompt disposal of dying cells in mouse and human macrophages by inducing expression of opsonins.

**PPAR-δ is a transcriptional sensor of apoptotic cells**

Apoptotic cells contain large amounts of native and oxidized fatty acids, leading us to ask whether they might activate PPAR-δ to enhance their own clearance. To explore this possibility, transient transfection assays were performed using PPAR reporter gene. Treatment of transfected cells with apoptotic thymocytes increased luciferase reporter gene activity only in the presence of PPAR-δ (Supplementary Fig. 4a online). Moreover, reporter gene assays with GAL4-PPAR-δ chimeric constructs confirmed that the ligand binding domain of PPAR-δ was sufficient to transduce the transcriptional effects of apoptotic thymocytes (Supplementary Fig. 4b online). Congruent with these data, apoptotic cell...
feeding induced expression of opsonins (C1qb, Mfge8 and Thbs1), Mertk, Tgfb1, and the PPAR-δ target gene (Plin2) in thioglycollate-elicited wild type macrophages (Fig. 4a), whereas the induction of all of these genes was diminished or absent in PPARd−/− macrophages (Fig. 4a). Moreover, challenge of wild-type but not PPARd−/− macrophages with apoptotic cells led to ∼650% increase in C1q proteins (a, b and c) (Fig. 4b), leading us to postulate that apoptotic cells might enhance their own clearance via activation of PPAR-δ.

To test this idea, a double feeding experiment was performed with BMDMs. Notably, priming of wild-type macrophages by apoptotic cells increased their phagocytic capacity by ∼60%, whereas there was no significant difference in the phagocytic capacity of macrophages lacking PPAR-δ (Fig. 4c). Finally, this transcriptional activation of PPAR-δ by apoptotic cells was specific, because necrotic cells and opsonized sRBCs failed to activate PPAR-δ (Supplementary Fig. 4c, d online).

Previous work has established that phagocytosis of apoptotic cells suppresses autoimmune responses by releasing immunosuppressive cytokines (IL-10 and TGF-β) and inhibiting production of pro-inflammatory cytokines (IL-12 and TNF-α)3,26,27. Since PPAR-δ functions as a sensor of apoptotic cells, we asked whether it might mediate this molecular switch in cytokine secretion. For these experiments, thioglycollate-elicited wild-type and PPARd−/− macrophages were stimulated with lipopolysaccharide (LPS) in the presence or absence of apoptotic cells, and cytokine secretion was monitored by ELISA. As expected, apoptotic cell feeding increased secretion of the anti-inflammatory cytokine IL-10 (Fig. 4d), while suppressing the release of pro-inflammatory cytokines IL-12 and TNF-α in wild-type macrophages (Fig. 4e, f). Importantly, this switch in cytokine secretion pattern was absent in PPARd−/− macrophages (Fig. 4d–f), suggesting that immunosuppressive effects of apoptotic cells require an intact PPAR-δ signaling pathway in macrophages.

**PPARd−/− mice develop autoimmune disease**

Defective clearance of apoptotic cells triggers immune response to self antigens, manifesting as lupus-like autoimmunity in mice8,9,11,28. Since we observed decreased expression of opsonins and impaired clearance of apoptotic cells in PPARd−/− mice, we evaluated the onset of autoimmune disease in these animals. Notably, sera of PPARd−/− female mice (n=9) had ∼200%-600% higher levels of autoantibodies, including those directed against nuclear antigens (antinuclear antibody, ANA; double-stranded DNA, dsDNA; single-stranded DNA ssDNA), and cardiolipin, than age-matched wild-type controls (n=7; Fig. 5a-d). Furthermore, these changes in autoantibody titers were comparable to those found in wild type Balb/c mice treated with pristane, an established model for inducing lupus-like autoimmunity29,30. A hallmark of systemic lupus erythematosus is the deposition of autoantibodies as immune complexes in the kidney, leading to inflammation and destruction of the glomeruli31. Indeed, immunofluorescence staining revealed that glomeruli of all PPARd−/− female mice (n=7) had a ribbon-like pattern of IgG deposition, whereas glomeruli of wild-type female mice were largely spared (Fig. 5e). Accordingly, increased urinary protein excretion, a sign of kidney dysfunction, was present in PPARd−/− female mice (Fig. 5f). Moreover, perivascular inflammation, a common histologic finding in autoimmune kidney disease32,33, was increased by ∼600% in the kidneys of PPARd−/− female mice

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Similar results were obtained in an independent cohort of wild-type and \( \text{PPARd}^{-/-} \) female mice (\( n=8/\text{genotype} \); Supplementary Fig. 6 online).

To determine whether the lupus-like autoimmunity in \( \text{PPARd}^{-/-} \) female mice results from changes in lymphocyte subsets, we analyzed the spleens of 13-week old female mice before they had any evidence of autoimmune disease. Importantly, total numbers of B-cells, and CD4\(^+\) and CD8\(^+\) T-cells were not different between wild-type and \( \text{PPARd}^{-/-} \) female mice (Supplementary Figs. 7a, 8a and 9b online). The numbers of regulatory, memory, naïve, and activated T cells, and activated B cells were also similar amongst the genotypes (Supplementary Fig. 7a, 8a, 8b and 9a online). Furthermore, spleen weights and splenic lymphocyte composition was similar in 14 month wild-type and \( \text{PPARd}^{-/-} \) female mice (Supplementary Figure 7b, c online), suggesting that the autoimmunity in these animals is not due to aberrant proliferation or activation of lymphocytes.

To further explore this idea, we investigated whether macrophage-specific \( \text{PPARd}^{-/-} \) (Mac-\( \text{PPARd}^{-/-} \)) mice exhibited similar defects in clearance of apoptotic cells. Genetic deletion of PPAR-\( \delta \) in macrophages was sufficient to reduce opsonin gene expression by 35-75\% (Fig. 6a), and impair clearance of CMFDA-labeled apoptotic thymocytes by \( \sim 350\% \) in the spleen (Fig. 6b). Remarkably, this delay in clearance of apoptotic cells resulted in higher levels of ssDNA and cardiolipin autoantibodies in 3-6 month old female Mac-\( \text{PPARd}^{-/-} \) mice (Fig. 6c–e; \( n=8/\text{genotype} \)). Since Mac-\( \text{PPARd}^{-/-} \) mice were generated on the C57Bl/6J background, a strain known to be very resistant to spontaneous lupus-like autoimmune disease, we injected apoptotic thymocytes in a separate cohorts of mice to potentiate autoimmunity. Injection of syngeneic apoptotic cells increased titers dsDNA and ssDNA antibodies by \( \sim 200\% \) (Fig. 6f, g) in Mac-\( \text{PPARd}^{-/-} \) mice, resulting in increased perivascular inflammation and immune complex deposition in the kidney (Fig. 6h, i; \( n=6-7/\text{genotype} \)). In aggregate, these data demonstrate that deletion of PPAR-\( \delta \) in macrophages is sufficient to delay clearance of apoptotic cells, thereby providing the antigenic stimulus for breakdown of tolerance.

Systemic lupus erythematosus is a polygenic disease involving hits in multiple pathways, including B-cell signaling and activation, apoptotic cell clearance and toll like receptor signaling. Since global or macrophage-specific deficiency of PPAR-\( \delta \) primarily affects pathways of apoptotic cell clearance, we next investigated whether additional hits could potentiate autoimmunity in these animals. For these experiments, cohorts of wild-type (\( n=6-8 \)) and \( \text{PPARd}^{-/-} \) (\( n=8-10 \)) female mice were given a single injection of pristane, which induces lupus-like autoimmune disease by activating B-cells. As expected, wild-type female mice treated with pristane had higher levels of antibodies directed against nuclear and membranous antigens, including anti-cardiolipin, ANA, anti-dsDNA, and anti-ssDNA, than their saline-injected littermates (Supplementary Fig.10a–d online). Strikingly, the concentrations of these antibodies in sera of pristane-treated \( \text{PPARd}^{-/-} \) female mice were \( \sim 200-400\% \) higher than pristane-treated wild-type controls (Supplementary Fig. 10a-d online), and the penetrance of pristane-induced autoimmunity was increased from 28\% to 77\% in pristane-treated \( \text{PPARd}^{-/-} \) female mice (Supplementary Fig. 10e online). In agreement, massive deposits of complement-containing immune complexes were only seen in the glomeruli of pristane-injected \( \text{PPARd}^{-/-} \) female mice (Supplementary Fig. 10f online).
online). Furthermore, histological analysis revealed that while glomeruli of wild-type female mice had mild to moderate mesangial hypercellularity (precursor lesions), severe endocapillary proliferative glomerulonephritis with necrosis and crescent formation (end-stage lesions) was the dominant pathology present in the glomeruli of PPARδ−/− female mice (Supplementary Fig. 10g, h online). Altogether, these data demonstrate that genetic deficiency of PPAR-δ delays clearance of apoptotic cells and increases autoantibody production, leading to immune complex mediated glomerulonephritis, features reminiscent of the human disease systemic lupus erythematosus.

**Discussion**

During the past decade, numerous molecules responsible for recognition and uptake of apoptotic cells have been identified. Careful dissection of these pathways has revealed that prompt clearance of apoptotic cells by macrophages is essential for prevention of autoimmune disease. Remarkably, even during times of massive apoptosis apoptotic cells are rapidly cleared by macrophages40,41, implying that phagocytic programs are dynamic, allowing macrophages to adapt to their changing microenvironment. However, the transcription factors that coordinate expression of phagocytic genes remain unknown. Results presented here demonstrate that the nuclear receptor PPAR-δ functions as a sensor of dying cells to orchestrate the phagocytic response when macrophages are confronted with apoptotic cells.

After engulfment, apoptotic bodies are rapidly broken down into their molecular constituents. This results in a dramatic increase in the cellular pools of oxidized fatty acids and sterols, the normal components of the cellular membranes of apoptotic cells. Since nuclear receptors PPARs and liver X receptors (LXRs) are sensors of modified fatty acids and sterols, respectively12,42, we and others have postulated that they might coordinate the transcriptional response of macrophages during apoptotic cell clearance6,43. Indeed, evidence presented here provides strong support for a pivotal role of nuclear receptor signaling in the phagocytic response. Specifically, we demonstrate that apoptotic cell feeding potently induces and activates PPAR-δ, which then enhances the expression of opsonins, molecules that bridge apoptotic bodies to cell surface receptors on phagocytes. In addition, by sensing apoptotic cells, PPAR-δ functions as a molecular switch that discriminates between the pro-inflammatory and immunosuppressive actions of macrophages. Consequently, global or macrophage-specific deletion of PPAR-δ delays clearance of apoptotic cells, leading to increased production of autoantibodies and progressive lupus-like autoimmune disease.

The requirement for PPAR-δ in regulation of opsonin gene expression, such as C1qb, provides a direct link between this transcription factor, phagocytosis of apoptotic cells, and development of autoimmune disease in mice and humans. Notably, loss of PPAR-δ signaling in the macrophage reduces expression of key opsonins, including C1qa, C1qb, Mfge8, and Thbs1. Since genetic deletion of C1qb and Mfge8 delays clearance of apoptotic debris by macrophages11,28, the concomitant reduction of these opsonins provides a molecular explanation for the lower uptake of apoptotic cells by macrophages lacking PPAR-δ. Thus, we propose that the persistence of apoptotic debris and increased...
macrophage inflammatory responses to them in global or macrophage-specific $PPAR\delta^{−/−}$ mice provides the antigenic stimulus that potentiates autoimmunity in these animals. Importantly, increased titers of autoantibodies and progressive renal pathology collectively suggest that PPAR-$\delta$ deficient mice are a good model system for studying human systemic lupus erythematosus.

**Methods**

**Mice**

$PPAR\delta^{−/−}$ mice, backcrossed onto the 129/SvJ strain for eight generations, were utilized for these studies. 129/SvJ mice were used as wild-type controls. Spontaneous development of autoimmunity and its potentiation by systemically administered apoptotic cells was carried out using littermate control ($PPAR-\delta^{\text{flox/flox}}$) and macrophage-specific $PPAR\delta^{−/−}$ (Mac-$PPAR\delta^{−/−}$; $PPAR-\delta^{\text{flox/flox}}$; LysM$^{\text{Cre}}$) female mice on the C57Bl/6J background. These animals were backcrossed onto C57Bl/6J strain for greater than 10 generations, ensuring that their genetic makeup is primarily (99.9%) derived from the C57Bl/6J strain. BMDMs were prepared from wild-type and $PPAR\delta^{−/−}$ mice as previously described. Apoptotic targets were prepared by culturing thymocytes from 4-8 week old wild-type mice in serum free RPMI media for 16-18 hours or by treatment with dexamethasone (10 μM) for 3 hours. Prior to being fed to macrophages, apoptotic thymocytes were labeled by incubation with 2 μM 5-chloromethyl-fluorescein-diacetate (CMFDA, Molecular Probes) for 30 minutes.

**In vitro apoptotic cell uptake and clearance**

For uptake assays, apoptotic thymocytes were added to wild-type and $PPAR\delta^{−/−}$ BMDMs at a 5:1 thymocyte:macrophage ratio for 15, 30 or 60 min. Apoptotic cell uptake and clearance was determined by flow cytometry, which employed doublet discrimination to distinguish internalized from externally bound apoptotic cells. Phagocytic index was calculated by manually counting > 200 cells per sample and applying the following formula: \[
\frac{\text{[\# of macrophages containing 1 apoptotic cell] + \[\# of macrophages containing 2 apoptotic cells] \times 2 + \[\# of macrophages containing 3 apoptotic cells] \times 3 + \[\# of macrophages containing > 3 apoptotic cells] \times 4]}{\text{total \# of macrophages counted}}
\]

For studies with opsonized cells, apoptotic CMFDA-labeled thymocytes were opsonized with CD3 antibody (0.5 μg/1×10$^6$ cells; Pharmingen) for 30min at 37°C. Sheep RBCs (Colorado Scientific) were resuspended in PBS (1×10$^6$μl$^{-1}$), and opsonized with rabbit sRBC antibody (1:200) for 1h at RT. Unbound antibody was removed by PBS washes. Necrotic targets were generated by incubation at 56°C for 10min. Cellular necrosis was confirmed by Trypan-blue staining. For double feeding experiments, day 7 BMDMs, plated in 6-well plates, were treated with vehicle or apoptotic thymocytes (1:1) for 24h. One day later, macrophages were rechallenged with CMFDA-labeled apoptotic thymocytes and phagocytosis was quantified as described above. Rescue experiments were performed with BMDMs of both genotypes plated in DMEM (1 gl$^{-1}$) supplemented with m-csf (10 ngml$^{-1}$) and 10% sera of wild-type or $PPAR\delta^{−/−}$ mice. Phagocytosis of labeled apoptotic cells was quantified 1d later. For rescue experiments with recombinant C1Q, BMDMs were plated in DMEM (1gl$^{-1}$) supplemented with 1% FBS and m-csf (10 ngml$^{-1}$). Purified human C1Q (4 μl of 1.1 mgml$^{-1}$; Sigma) was
added to media (400 μl) 1h prior to feeding of CMFDA-labeled apoptotic thymocytes (1:2 ratio). Phagocytosis was quantified as described above.

**In vivo apoptotic cell uptake and clearance**

For splenic uptake and clearance assays, 60×10⁶ CMFDA-labeled apoptotic thymocytes were injected intravenously into 8-12 week-old wild-type, *PPARδ−/−*, control or Mac-*PPARδ−/−* mice. Mice were sacrificed at the indicated time periods, and single-cell splenocyte suspensions were analyzed by flow cytometry⁹,¹⁰. For splenic macrophage uptake, CD11b⁺ splenocytes were positively selected using CD11b⁺ magnetic beads (Miltenyi) prior to flow cytometric analysis. Peritoneal uptake and clearance assays were performed similarly. Briefly, labeled apoptotic thymocytes were injected into the peritoneal cavity of naïve mice; 6h later, the population retrieved by peritoneal lavage was analyzed by flow cytometry.

**In vivo models of autoimmunity**

Autoimmunity was induced in female wild-type and *PPARδ−/−* mice with a single intraperitoneal injection of 1 ml pristane (Sigma) at 6 months of age and animals were sacrificed at 11 months of age⁵. Spontaneous autoimmunity was assessed in unmanipulated female mice at 3-6 months (*Mac-PPARδ−/−*) or 13-15 months (*PPARδ−/−*) of age. Provocative challenge with exogenously administered apoptotic cells was performed in control and *Mac-PPARδ−/−* female mice (n=6-7/genotype), as described by Mevorach³⁶. Briefly, 10×10⁶ apoptotic thymocytes were injected via tail vein into 12-15 week old female mice weekly for 4 weeks. Serum was collected prior to initiation of injections and 2d after the last injection, and analyzed for autoantibody production.

**Statistical Analyses**

Continuous data are presented as mean ± SEM, and P values were calculated using two-tailed Student’s *t*-test for two samples of unequal variance. Ordinal data are presented as proportion, and P values were calculated using the non-parametric Mann-Whitney *U*-test. Statistical significance is indicated by a single asterisk (*P* < 0.05) or two asterisks (*P* < 0.01). The microarray data has been deposited in NCBI’s Gene Expression Omnibus (GEO) and are accessible through GSE17890.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
PPAR-δ orchestrates timely disposal of apoptotic cells. (a-c) Apoptotic cell feeding induces PPAR-δ expression in macrophages. Apoptotic thymocytes were fed to wild-type BMDMs (5:1), and expression of PPAR-δ mRNA (a) and protein (b) was quantified 6 and 24 hours later, respectively. (c) Intracellular staining confirmed absence of PPAR-δ expression in thymocytes, and its induction in macrophages after apoptotic cell feeding. Isotype control: gray histogram; PPAR-δ: unshaded histograms. (d) PPARd−/− macrophages are impaired in phagocytosis of apoptotic cells. Kinetics of phagocytosis of apoptotic cells in wild-type and PPARd−/− macrophages. Experiments were repeated five to six independent times, and a representative experiment is shown above. (e, f) Clearance of apoptotic thymocytes in vivo. Labelled apoptotic thymocytes were injected intravenously (e) or intraperitoneally (f), and clearance was monitored 4-6 hours later. Delayed clearance of apoptotic thymocytes by splenic (e) and peritoneal macrophages (f) in PPARd−/− mice (n=4-5). Recovery of fluorescently-labelled thymocytes is quantified in panels (e) and (f). Data is presented as mean ± s.e.m. *P < 0.05, **P < 0.01. ACs: apoptotic cells; AU: arbitrary units; WT: wild-type.
Figure 2.
PPAR-δ regulates expression of opsonins in macrophages. (a) Clustering analysis of microarray data from WT and PPARd−/− macrophages. Note the dramatic reduction in expression of opsonins in PPARd−/− macrophages. (b) Relative expression of opsonins and receptors in WT and PPARd−/− macrophages, as assessed by qRT-PCR. (c) Reduced expression of C1qb protein in PPARd−/− macrophages. (d) PPAR-δ agonist (GW0742 100nM) induces opsonin gene expression in wild type macrophages (n=3-4). (e) Activation of C1qb promoter by PPAR-δ. C1qb promoter fragments containing or lacking the PPAR response element (C1qb-Luc or mut-C1qb-Luc, respectively) were transfected into CV-1 cells and luciferase activity was assayed 18 hours later. Data is presented as mean ± s.e.m. *P < 0.05, **P < 0.01. Cycle time for the highest expressing gene is indicated inside its corresponding bar.
Figure 3.
PPAR-δ regulates phagocytosis of apoptotic cells via secretion of opsonins. (a) Treatment with PPAR-δ ligand enhances phagocytosis of apoptotic cells by splenic macrophages in vivo. Percentage of splenic macrophages (CD11b+) containing CMFDA-fluorescence was quantified 1 hour after intravenous injection of CMFDA-labelled apoptotic thymocytes (n=4-5). (b, c) PPAR-δ regulates pathways of apoptotic cell uptake in human macrophages. Treatment with GW0742 (100 nM) enhances opsonin gene expression (b) and phagocytosis of apoptotic cells (c) in single donor human monocyte-derived macrophages. (d) Decreased
levels of C1q in serum of \textit{PPARd}^{−/−} mice. Circulating C1qb was detected by immunoprecipitating C1q from serum followed by immunoblotting for C1qb. \textbf{(e)} Restoration of phagocytic capacity of \textit{PPARd}^{−/−} macrophages by serum from wild-type mice. BMDMs were incubated with wild-type or \textit{PPARd}^{−/−} macrophages for 24 hours prior to assaying their phagocytic capacity for apoptotic cells. \textbf{(f)} Enhancement of apoptotic cell uptake by purified human C1q in \textit{PPARd}^{−/−} macrophages. Data is presented as mean ± s.e.m. \textit{*} \( P < 0.05 \), \textit{**} \( P < 0.01 \). Cycle time for the highest expressing gene is indicated inside its corresponding bar.
Figure 4.
PPAR-δ is a transcriptional sensor of apoptotic cells in macrophages. (a-c) Apoptotic cells enhance their own clearance by activating PPAR-δ in macrophages. Apoptotic cell (AC) feeding induces expression of PPAR-δ target genes in a PPAR-δ-dependent manner (a). Apoptotic cells enhance expression of C1qb protein in wild-type but not PPARδ−/− macrophages (b). (c) Apoptotic cell feeding enhances phagocytic capacity of macrophages in a PPAR-δ-dependent manner. (d-f) PPAR-δ is required for the switch from inflammatory to immunosuppressive cytokine secretion when macrophages phagocyte apoptotic cells.
Apoptotic cells enhance secretion of IL-10 from LPS-stimulated WT, but not PPARd−/− macrophages (d). Apoptotic cells fail to suppress release of pro-inflammatory cytokines, such as IL-12p40 (e) and TNF-α (f), in PPARd−/− macrophages (compare LPS and LPS/AC samples). Data is presented as mean ± s.e.m. *P < 0.05, **P < 0.01.
Figure 5.
Mice lacking PPAR-δ spontaneously develop autoimmune disease. (a-d) Production of autoantibodies. Serum levels of ANA, and antibodies to dsDNA, ssDNA and cardiolipin were measured by ELISAs in 13-15 month old wild-type and PPARδ−/− female mice (n=7-9). For ssDNA antibody, ANA and dsDNA antibody, serum from pristane-treated Balb/cJ mice is presented as a positive control for induction of autoimmunity. (e) Increased deposition of immune complexes in glomeruli of PPARδ−/− mice. Paraffin-embedded sections were stained with FITC-conjugated antibody to mouse IgG. (f) Increased protein
excretion in \textit{PPARd\textsuperscript{-/-}} mice. Ratio of urinary protein to urinary creatinine was determined in 14 month WT and \textit{PPARd\textsuperscript{-/-}} female mice (\textit{n}=5). Data is presented as mean ± s.e.m. *\textit{P} < 0.05, **\textit{P} < 0.01 (nonparametric Mann-Whitney U-test). Bar=50 microns.
Figure 6.
Impaired clearance of apoptotic cells and increased autoimmunity in Mac-PPARd^{−/−} mice. (a) Reduced expression of opsonins in BMDMs from Mac-PPARd^{−/−} mice. (b) Decreased clearance of labelled apoptotic thymocytes in spleens of Mac-PPARd^{−/−} mice. 60×10^6 CMFDA-labeled apoptotic thymocytes were injected intravenously in control and Mac-PPARd^{−/−} mice (n=3), and presence of CMFDA-labelled cells was quantified 4 hours later in spleens. (c-e) Increased autoantibody production in young Mac-PPARd^{−/−} female mice. Serum levels of ssDNA antibodies (c, d) and cardiolipin antibodies (e) were quantified by
ELISAs in 3-6 month old control and Mac-PPARδ−/− mice (n=8/genotype). (f-i) Macrophage-specific PPAR-δ is required for tolerogenic responses to intravenously injected apoptotic cells. Control and Mac-PPARδ−/− mice were injected with 10^7 syngenic apoptotic thymocytes for 4 weeks, and changes in titers of ssDNA (f) and dsDNA (g) autoantibodies were quantified. Histologic and immunofluorescence analysis of kidneys performed 9 months after apoptotic cell injections (12 month old mice) revealed increased perivascular inflammation (h) and immune complex deposition in the glomeruli (i). Data is presented as mean ± s.e.m. *P < 0.05, **P < 0.01.