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Peroxisome Proliferator-Activated Receptor γ-Regulated Cathepsin D Is Required for Lipid Antigen Presentation by Dendritic Cells

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It is well established that dendritic cells (DCs) take up, process, and present lipid Ags in complex with CD1d molecules to invariant NKT cells. The lipid-activated transcription factor, peroxisome proliferator-activated receptor γ (PPARγ), has previously been shown to regulate CD1d expression in human monocyte-derived DCs, providing a link between lipid metabolism and lipid Ag presentation. We report that PPARγ regulates the expression of a lysosomal protease, cathepsin D (CatD), in human monocyte-derived DCs. Inhibition of CatD specifically expanded the invariant NKT cells and furthermore resulted in decreased maturation of saposins, a group of lipid transfer proteins required for lysosomal lipid Ag processing and loading. These results reveal a novel mechanism of lipid Ag presentation and identify CatD as a key component of this machinery and firmly place PPARγ as the transcriptional regulator linking lipid metabolism and lipid Ag processing.

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Abbreviations used in this article: CatD, cathepsin D; DC, dendritic cell; FABPA, fatty acid binding protein 4; GC, α-galactosylceramide; GC, galactosyl(1-2)galactosyl ceramide; IDC, immature dendritic cell; iNKT, invariant NKT; PPARγ, peroxisome proliferator-activated receptor γ; RAα, retinoic acid receptor α; RSG, rosiglitazone; RT-qPCR, real-time quantitative RT-PCR PCR; siRNA, small interfering RNA; TGM2, transglutaminase 2.

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transfer protein that is normally activated by limited proteolysis in the lysosomes and has been shown to play a role in loading of lipids to CD1d molecules. In summary, our results define a novel pathway/axis in which lipid activation of PPARγ enhances the iNKT cell function by upregulating CatD, which, in turn, generates the mature form of saposins, lipid transfer molecules, which facilitate lipid loading to CD1d molecules.

Materials and Methods

Ligands

Cells were treated with the following ligands as described in the Results: AM580 (BIOMOL), rosiglitazone (RSG) and GW9662 (Alexis Biochemicals), as well as AGN193109 (gift from R.A.S. Chandraratna, Allergan, Irvine, CA), 4-diethylaminobenzaldehyde (Fluka), bafilomycin (Calbiochem), as well as AGN193109 (gift from R.A.S. Chandraratna, Allergan, Irvine, CA), 4-diethylaminobenzaldehyde (Fluka), bafilomycin (Calbiochem), pepstatin A (Sigma-Aldrich), pepstatin A (Sigma-Aldrich), pepstatin A (Sigma-Aldrich), pepstatin A (Sigma-Aldrich), pepstatin A (Sigma-Aldrich), pepstatin A (Sigma-Aldrich).

Cell culture and ligand treatment

Monocytes (98% CD14+) were isolated from Buffy coats by Ficoll gradient centrifugation followed by magnetic bead separation using anti-CD14-conjugated microbeads (VarioMACS; Miltenyi Biotec). Buffoy coats were derived from healthy human donors according to the regulations of the institutional ethical review committee of the University of Debrecen. Differentiation of DCs was performed as previously described (22). Briefly, monocytes were plated in six-well tissue culture plates at 1.5 × 10⁶ cells/ml in RPMI 1640 (Sigma-Aldrich) media supplemented with 10% FBS (Invitrogen), penicillin/streptomycin, L-glutamine, and 500 U/ml IL-4 (PeproTech). Cells were cultured for 5–6 days, and the cytokine treatment was repeated on day 3. Ligands or vehicle control (50% DMSO/ethanol) was added to the cell culture at day 0, unless otherwise stated. AGN193109 and GW9662 administration was repeated on day 1, and the cytokine treatment was repeated on day 3.

Microarray analysis of RSG-treated DCs

The generation of the microarray data used for Fig. 1 is described in Szatmari et al. (23). Briefly, monocyte-derived DCs were cultured with or without the PPARγ agonist RSG and RNA was isolated at 6 h, 24 h, or day 5 of the culture. Hybridization of the RNA samples was carried out at the Microarray Core Facility of the European Molecular Biology Laboratory (Heidelberg, Germany). Analysis was carried out using GeneSpring GX7.3.1 (Agilent Technologies, Santa Clara, CA) software. Raw data (cell files) were analyzed by the GeneChip Robust multiarray analysis algorithm. All microarray data are available in the public Gene Expression Omnibus database under accession no. GSE8658: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE8658). For a full description of cell culture and differentiation, RNA isolation, hybridization, and data analysis, see Szatmari et al. (23) and the URL cited above.

Double immunofluorescence

Monocytes, DC, or RSG-treated DCs (6 × 10⁵ cells/group) were pelleted and fixed in 4% paraformaldehyde (pH 7.3) for 24 h at 4°C. Cell blocks were then embedded in paraffin followed by serial sectioning (4 μm thick). After deparaffinization and rehydration, sections from each group were mounted on the same glass slides and subjected to sequential double immunofluorescence staining for detection of PPARγ and CatD protein expressions, respectively. The following primary Abs were used: mAb to PPARγ (clone E8; Santa Cruz Biotechnology) at 1:75 dilution and poly–anti-rabbit IgG (clone C20; Santa Cruz Biotechnology) at 1:100. The following secondary Ab followed by biotinylated rabbit F(ab′)2 and streptavidin-Texas Red (Vector Laboratories). DAPI was used for nuclear counterstaining (Vector Laboratories). For negative controls, isotype-specific control IgG Abs (Dako) or a mixture of mAb to PPARγ and a specific blocking peptide were applied on separate slides in replacement of primary Abs. Normal human adipose tissue was included as positive control (not shown). Fluorescence microphotographs were captured using an Olympus BX51 microscope equipped with a tricolor excitation filter and an Olympus DP50 digital camera. For transferring and editing images for documentation, Viewfinder and Studio Lite software version 1.0.136 of Adobe Photoshop version 8.0 were used.

Western blot analysis

Fifty micrograms protein whole cell lysate was separated by 12.5% PAGE before being transferred onto polyvinylidene difluoride membrane (Bio-Rad Laboratories). Membranes were blocked using 5% nonfat dry milk in TBST at 4°C overnight before being probed with anti-CatD Ab (R20, sc-6487; Santa Cruz Biotechnology), and then membranes were stripped and reprobed with anti-GAPDH (ab8245-100; Abcam) according to the manufacturer’s recommendations. Saposin C cleavage product was detected by separating cell lysates on a Tris-Tricine 4–15% gradient gel (Bio-Rad) and the membrane was probed with anti-saposin (H-81, sc-32875; Santa Cruz Biotechnology) subsequent to transfer to polyvinylidene difluoride membrane.

Flow cytometry

Cell staining was performed using FITC- or PE-conjugated mAbs. Labeled Abs for flow cytometry included anti–CD1d-PE (BD Biosciences), anti-human iNKT cell (Vα24-Jαβ) Ab and isotype-matched controls (BD Biosciences) and anti-Vα24-FITC, anti–Vβ11-PE, and appropriate isotype-matched controls (Immunotech). Stained cells were assayed for fluorescence intensity using a FACSCalibur flow cytometer (BD Biosciences) and analyzed using CellQuest software.

iNKT cell expansion

DCs that were cultured in the presence of vehicle or RSG were treated with 100 ng/ml αGC or αGGC lipid Ag for 48 h to obtain lipid-loaded DCs. DCs (1 × 10⁵) were cocultured with monocyte-depleted autologous PBMCs for 5 d in 24-well tissue culture plates. For CatD inhibition experiments, DCs were treated with 1 or 10 μM pepstatin A (Sigma-Aldrich) at day 3 of DC differentiation. Prior to coculture, DCs were washed extensively and resuspended in fresh RPMI 1640 supplemented with 10% FBS and penicillin, streptomycin, and L-glutamine. Expansion of iNKT cells was measured by quantifying Vα24+Vβ11+ cells or Vα24+Vβ11+ cells by flow cytometry. Additionally, the invariant TCR α-chain was quantified by using real-time quantitative RT-PCR (RT-qPCR). In case of bafilomycin treatment, DCs were cultured in the presence of rosiglitazone, and bafilomycin (50 nM) was added on the fourth day of differentiation 15 min prior to the addition of lipid Ags.

Mixed leukocyte reactions

DCs were harvested, extensively washed, and used as stimulator cells for CFSE-labeled allogeneic PBMCs (2 × 10⁵ cells/well). Briefly, PBMCs were harvested, washed, and resuspended (1 × 10⁶ cells/ml) in PBS supplemented with 10 μM CFSE (Molecular Probes) and incubated at 37°C for 15 min. Cells were then washed and incubated in PBS at 37°C for 30 min before PBMCs and immature or mature DCs (1 × 10⁴ cell/well) were cocultured in 96-well flat-bottom tissue culture plates. Cell proliferation was measured on day 5 by flow cytometry.

RT-qPCR

Total RNA was isolated using TRIzol reagent (Invitrogen). Reverse transcription was performed at 25°C for 10 min, 42°C for 2 h, and 72°C for 5 min from 4 μg RNA using SuperScript II reverse transcriptase (Invitrogen) and random primers (3 μg/ml; Invitrogen). Quantitative PCR was performed using real-time PCR (ABI Prism 7900; Applied Biosystems) or LC480 (Roche): 40 cycles at 95°C for 12 s and 60°C for 30 s using TaqMan assays. All PCR reactions were performed in triplicate and one reaction including no RT enzyme. The comparative threshold cycle method was used to quantitate the level of individual transcripts and normalized to cyclophilin A. Values are expressed as means ± SD. For detection of the iNKT cell-specific invariant TCR Vα–Jα junction, the following primers and probe were used: forward, 5′-AGCGATTCCAGGCCTTACATCT-3′; reverse, 5′-GTCAACTGAGTTCTCTCTCTTTAAAAG-3′; TaqMan probe, 5′-FAM-TGTGGTGAGGGCCAGAGGCTCCTAA-TAMRA-3′. Primers and probe was designed using Vector NTI software.

RNA interference

Small interfering RNA (siRNA) delivery was performed using electroporation of DCs as described (24). Briefly, DCs were harvested from six-well tissue culture plates and washed once with unsupplemented RPMI 1640 and once with PBS (all at room temperature). The cells were counted and resuspended in Opti-MEM without phenol red (Invitrogen Life Sciences) and 4 μg RNA using SuperScript II reverse transcriptase (Invitrogen) and random primers (3 μg/ml; Invitrogen). Quantitative PCR was performed using real-time PCR (ABI Prism 7900; Applied Biosystems) or LC480 (Roche): 40 cycles at 95°C for 12 s and 60°C for 30 s using TaqMan assays. All PCR reactions were performed in triplicate and one reaction including no RT enzyme. The comparative threshold cycle method was used to quantitate the level of individual transcripts and normalized to cyclophilin A. Values are expressed as means ± SD. For detection of the iNKT cell-specific invariant TCR Vα–Jα junction, the following primers and probe were used: forward, 5′-AGCGATTCCAGGCCTTACATCT-3′; reverse, 5′-GTCAACTGAGTTCTCTCTCTTTAAAAG-3′; TaqMan probe, 5′-FAM-TGTGGTGAGGGCCAGAGGCTCCTAA-TAMRA-3′. Primers and probe was designed using Vector NTI software.

RNA interference
medium supplemented with 10% FBS, penicillin, streptomycin, and l-glutamine in addition to GM-CSF and IL-4. Electroporation efficiency was assessed by fluorescent microscopy or flow cytometry 2–4 h post-electroporation using the siGLO Red transfection indicator (Dharmacon). Cells were monitored for cell viability using trypan blue exclusion and silencing efficiency on days 2–4 or the indicated days post-electroporation. Cell viability post-electroporation was routinely ≥90%.

**iNKT cell sorting and determination of cytokine production in DC–T cell cocultures**

Monocyte-derived DCs were cultured for 5 d as described in the experimental procedures in the absence or presence of 2.5 μM RSG. On day 5 of DC differentiation, DCs were pulsed with 100 ng/ml αGC or with vehicle. Autologous monocyte-depleted PBMCs were stained with FITC-conjugated TCR Vα24- and PE-conjugated TCR Vβ11-specific mAbs (IOTest; Beckman Coulter, Brea, CA). The double-positive iNKT population was separated by a FACSDiva cell sorter (BD Biosciences, Franklin Lakes, NJ).

Production of IL-10 and IL-13 cytokines by DCs alone or in the presence of separated iNKT cells (1:10 DC/iNKT cell ratio) were monitored by ELISA after 36 h coculture.

**iNKT apoptosis assay**

Lipid Ag-pulsed DCs were cocultured with autologous T cells. T cells were stained with PC5-labeled anti-human CD3 (BD Biosciences) and then labeled with propidium iodide and annexin V-FITC for the detection of apoptosis. CD3+ cells were analyzed for apoptosis by FACS.

**Statistical analyses**

A two-sample Student’s t test was used. The results were considered significant at the level of p < 0.05, as indicated by *p < 0.05, **p < 0.01, and ***p < 0.001.

**Results**

In this study we set out to uncover how PPARγ contributes to lipid Ag presentation events in monocyte-derived DCs in addition to regulating CD1d expression. We presumed that if PPARγ activation enhances lipid presentation by regulating a yet unidentified mechanism, then this must be reflected in the gene expression changes taking place upon PPARγ ligand activation. Therefore, we examined a microarray data set of differentiating human monocyte-derived DCs (23, 25). The microarray data set compared the gene expression profiles of differentiating DCs at early and late time points (6 h, 24 h, and 5 d) following treatments with or without RSG, a PPARγ agonist. Strikingly, this analysis revealed PPARγ-mediated regulation of genes involved in lipid Ag presentation such as lipid uptake and lysosomal delivery (apolipoprotein E) (26) and endogenous lipid Ag processing (β-hexosaminidase B) (11) (Fig. 1A). We assumed that a subset of the genes whose expression pattern followed that of known lipid presentation genes, but have previously not been linked to lipid presentation, might also participate in lipid Ag processing. Interestingly, cathepsins D, L, and S, but not E, the major aspartic protease involved in the MHC class II presentation pathway (27) (Fig. 1, Supplemental Fig. 1), were also upregulated upon treatment with the synthetic PPARγ agonist RSG. Cathepsins are lysosomal proteinases, of which both cathepsin L and S have been implicated in lipid Ag presentation in mice (28, 29). However, no role for CatD in lipid Ag presentation has been reported to date.

**FIGURE 1.** Activation of PPARγ in iDCs leads to upregulation of candidate lipid Ag-presenting genes. A, Heat map representation of microarray data of the indicated genes from monocyte-derived DCs in the presence or absence of RSG at the indicated time points are shown. B, Expression and regulation of cathepsins by PPARγ in iDCs confirmed by RT-qPCR. Normalized mRNA levels of the indicated genes from day 5 monocyte-derived DCs from three representative donors are depicted. CatD is the dominant cathepsin in DCs, as shown by RT-qPCR measurements. C, Western blot analysis of CatD protein levels from monocytes and day 5 monocyte-derived DCs treated with PPARγ ligands. D, Immunofluorescence detection of PPARγ (green) and CatD (red) using pelleted and fixed monocytes (Mo), iDCs, and iDCs treated with RSG (iDC RSG). Nuclei are visualized by DAPI (blue). Original magnification ×40. In the case of iDC RSG, a panel (labeled with white box) with further magnification (original magnification ×100) is also shown. GW, GW9662, RSG, PPARγ antagonist; veh, vehicle control (DMSO/EtOH).
Importantly, our data indicated a robust regulation of CatD by PPARγ in DCs (6- to 10-fold upregulation; Fig. 1B, upper panel), and, significantly, CatD was the dominant cathepsin in developing human DCs (Fig. 1B, lower panel). Taken together with previous reports, these data led us to investigate the possibility of PPARγ-regulated CatD as a novel component of the lipid Ag processing machinery to support iNKT cell expansion.

To confirm that the PPARγ-mediated regulation of CatD was also reflected at the protein level, Western blot analysis and immunofluorescence staining was performed (Fig. 1C, D). Western blot analysis verified PPARγ-mediated upregulation of CatD. Furthermore, immunofluorescence staining showed a nuclear staining pattern for PPARγ (green) and cytoplasmic localization for CatD (red), consistent with previous reports finding most CatD protein in the lysosomal compartments (Fig. 1D) (30). Next, to corroborate the PPARγ dependence of CatD obtained by pharmacological means, we silenced PPARγ in developing monocyte-derived DCs by the delivery of PPARγ-specific siRNA oligonucleotides. PPARγ itself was silenced at an efficiency of ~60% (Fig. 2A). This has completely downregulated the expression of the bona fide PPARγ target gene, fatty acid binding protein 4 (FABP4/aP2). The upregulation of CatD upon RSG treatment was clearly downmodulated (to 56.8%) in the presence of PPARγ-specific siRNA oligonucleotide, whereas this was not the case for nonsilencing oligonucleotide or mock-treated samples (Fig. 2A, lower panel). Based on the above findings, we concluded that CatD is regulated by PPARγ.

Next, we moved to further characterize the regulation of CatD by PPARγ in developing DCs. We have previously reported that a subset of the PPARγ-mediated gene expression changes can be mediated through the induction of retinoic acid synthesis and subsequent activation of retinoic acid receptor α (RARα) (25). Therefore, to fully map the signaling events from PPARγ activation to CatD upregulation, we wanted to determine the contribution of retinoid signaling to the upregulation of CatD. To characterize the signaling pathways important in CatD induction, we chose to monitor the expression levels of marker genes, FABP4 and transglutaminase 2 (TGM2), that are under control of PPARγ or RARα, respectively, and whose expression levels can reliably report existing PPARγ or RARα activity in DCs. As expected, the addition of the PPARγ agonist RSG could induce the bona fide PPARγ target gene, FABP4 (Fig. 2B). Similarly, the well-characterized RAR target gene, TGM2, was primarily induced by the RAR ligand AM580 (Fig. 2C). Interestingly, we found that both the PPARγ ligand RSG and the RARα ligand AM580 could induce CatD (Fig. 2D). Importantly, the CatD induction seen upon PPARγ activation could only be slightly reversed by a treatment with a RARα antagonist (AGN193109) or by a treatment with an...
inhibitor of endogenous retinoic acid synthesis, 4-diethylaminobenzaldehyde. The separate activation of PPARγ or RARα pathways in DCs could induce CD1d cell surface protein expression (Supplemental Fig. 2) and led to an enhanced iNKT expansion in T cell coculture experiments. Interestingly, the simultaneous activation of both pathways did not result in a synergistic further enhancement of iNKT expansion. This result substantiates our model that predicted that PPARγ and RARα participate in the same molecular pathway that modulates iNKT expansion. The above data collectively suggest that CatD is regulated by multiple nuclear receptors in developing DCs and that it is under dual control by both PPARγ and RARα.

Several observations led us to think that the induction of CD1d expression is not the only mechanism by which PPARγ activation can enhance iNKT expansion. The finding that inhibiting PPARγ activity at day 3 of DC differentiation by siRNA-mediated knockdown did not lead to an alteration in the CD1d transcript level in DCs, whereas modulation of iNKT activation could still be detected under these conditions (Fig. 2E and data not shown), suggested that PPARγ activation in DCs enhanced iNKT expansion by regulating other proteins as well, including CatD. This finding is in line with our demonstration that CD1d is an indirect and late target of PPARγ activation (25). To investigate the possible role of CatD in lipid Ag presentation and iNKT cell stimulation, we used two different approaches: pharmacological inhibition of CatD proteolytic activity by a selective inhibitor, pepstatin A, and silencing of CatD gene expression by siRNA strategy. In line with our previous results (22), we found that RSG treatment of DCs did not give rise to a measurable iNKT expansion in the absence of a lipid Ag. However, when the precursor form of a ligand for the lipid-presenting CD1d molecule, namely αGGC, was also included in the DC culture, then RSG-treated immature DCs (iDCs) showed a substantial increase in their ability to trigger iNKT expansion (Fig. 3A). Importantly, we observed that treatment of RSG-treated iDCs with pepstatin A, an inhibitor of aspartyl proteases, such as CatD, inhibited αGGC-induced iNKT cell expansion in a dose-dependent manner (Fig. 3A, 3B). Additionally, we found evidence of a correlation between iNKT cell counts quantitated by flow cytometry using Vα24/Vβ11 double-staining and RT-qPCR detection of the invariant Vα-chain (Vα24-Jα18) (Fig. 3A, 3B), validating the utility of this RT-qPCR strategy in quantitation of iNKT cells.

The endosomal events involved in lipid Ag presentation to iNKT cells remain mostly undefined. We reasoned therefore that due to the fact that CatD is a lysosomal protease and that CD1d is dependent on lysosomal localization for efficient iNKT cell induction, CatD might be involved in a specific lysosomal event important for lipid Ag presentation in the context of CD1d. To explore this possibility further, we took advantage of two synthetic CD1d-presented model lipid Ags, αGGC, which can readily bind to surface CD1d (31), and its precursor, αGGC, which strictly requires the uptake and delivery to lysosomes where it is converted to the active antigenic form, αGC, by α-galactosidase A (32). We investigated how the blocking of endosomal acidification by the addition of bafilomycin in DCs could affect iNKT expansion. We found that blocking endosomal acidification in RSG-treated DCs inhibited iNKT expansion in the presence of the precursor lipid αGGC but not in the presence of the mature form of αGC (Fig. 3C), suggesting that PPARγ activation in DCs enhanced iNKT expansion by inducing an endosomal process that contributed to an effective lipid presentation. Interestingly, we observed that only the iNKT expansion induced by the precursor form, αGGC, was sensitive to pepstatin A treatment (a pharmacological inhibitor or CatD) (Fig. 3D), suggesting the involvement of CatD in the processing of lipid Ags. To exclude the possibility that pepstatin A nonspecifically inhibited lysosomal processes, we showed that this compound did not affect peptide Ag-mediated conventional T cell proliferation in MLR reactions (Supplemental Fig. 3).

In a second approach to confirm the role of CatD in lipid Ag processing or presentation, we used again an siRNA strategy to

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** Inhibition of CatD leads to decreased iNKT cell expansion in response to lipid Ag. A, iNKT cell expansion in autologous iDC-PBMC cocultures as measured by two-color flow cytometry (Vα24-FITC/Vβ11-PE) in the presence of the indicated doses of pepstatin A. B, Correlation of the flow cytometric and RT-qPCR measurements (iNKT TCR α-chain) of the observed iNKT expansion in the same experiments in the presence of αGGC. Applied pepstatin A concentrations, 0, 1, and 10 μM in A and B, and 0 and 10 μM in D. C, Effect of the inhibition of endosomal acidification by bafilomycin (0 or 50 nM) on iNKT expansion. D, Effect of pepstatin A on the iNKT expansion seen in the presence of the lipid Ag αGGC, which requires lysosomal processing, or in the presence of the processed form αGC, which binds to CD1d molecules without further processing. Filled bars show pepstatin A-treated samples. Pepstatin A concentrations (0 and 10 μM) are indicated. Veh, vehicle.
silence the expression of CatD in developing iDCs before using these as APCs in the autologous iNKT cell expansion assay as previously described. We observed (Fig. 4A) that a successful silencing of CatD expression did not alter CD1d mRNA expression level or cell surface protein expression, yet it led to a significant decrease in the iNKT expansion induced by RSG-treated DCs loaded with αGC (Fig. 4C). These results clearly confirm the specific role of CatD in lipid Ag presentation on iNKT cell induction and show that CatD-mediated enhancement of iNKT expression is not due to the regulation of CD1d levels.

In search of a mechanistic explanation for the observed CatD dependence of lipid Ag processing and presentation, we explored relevant proteins requiring processing by CatD. Intriguingly, it has been reported that a group of lipid transfer proteins, saposins, require proteolytic processing by CatD to obtain their functional properties (33, 34). Four saposins (A, B, C, D) are generated by proteolysis from the common precursor protein prosaposin. Recently, mature saposins were identified to facilitate lipid Ag presentation by mediating the proteolytic maturation of prosaposin into mature saposins, we investigated the possibility that CatD could play an essential role in lipid Ag presentation by mediating the proteolytic maturation of prosaposin to the individual saposins in developing monocyte-derived DCs. By separating DC protein samples on a high resolution Tris-Tricine peptide gel and probing with anti-saposin C Ab (also recognizing full-length prosaposin and intermediate cleavage products), we detected increased levels of mature saposin C peptide in PPARγ-stimulated iDCs as compared with control (Fig. 4D). Importantly, when pepstatin A was included during the iDC differentiation process, significantly diminished mature saposin C was detected (Fig. 4E). Taken together, these data clearly link the PPARγ-regulated increase of CatD protein levels with enhanced maturation of prosaposin to saposin and provide an attractive mechanistic explanation to the observed CatD dependence in lipid Ag presentation processes.

Importantly, the iNKT cells expanded in the presence of RSG-treated DCs possess INF-γ production capacity (25), which suggests that PPARγ activation in DCs can lead to the expansion of a distinct subset of functional iNKT cells. To further determine the regulatory role of PPARγ activity on iNKT expansion and activation, we pulsed RSG-treated DCs with αGC and investigated how RSG treatment modulated iNKT activation (Supplemental Fig. 4). We showed that RSG treatment of DCs modulated IL-10 and IL-13 production capacity of iNKT cells, which further underlined the importance of PPARγ in iNKT cell biology. Also note that this cell process is accompanied with a minimal amount of apoptosis (Supplemental Fig. 5).

**Discussion**

Lipid Ag processing and presentation are increasingly recognized as essential for a wide range of immunological functions. Our understanding of the processes including lysosomal Ag processing and presentation is increasing thanks to structural as well as mechanistic studies (28, 38–40). However, the regulation of the process, especially the role of transcriptional events underpinning the expression of the machinery responsible for lipid Ag uptake, processing, and presentation, is very poorly understood.

In the case of the analogous process, peptide Ag presentation by MHC, it was shown that both Ag processing and MHC expression are under a tight transcriptional regulation (41–44). Therefore, it is reasonable to assume that multiple aspects of the lipid Ag processing and presentation pathway are also transcriptionally regulated. We have already demonstrated that PPARγ transcriptionally regulates the expression of CD1d, the MHC class I-like molecule required for lipid Ag presentation via an indirect mechanism (25). Considering that in other processes regulated by PPARγ

**FIGURE 4.** Silencing of CatD confirms its role in iNKT cell expansion and CatD-mediated maturation of the lipid transfer protein prosaposin. A, siRNA-mediated silencing of CatD. mRNA expression of the RSG-regulated gene, CD1d, is not significantly reduced by silencing of CatD. B, Cell surface protein expression of CD1d is not reduced by silencing of CatD. C, Silencing of CatD expression in iDC diminishes iNKT cell expansion. CatD was silenced in iDC by the delivery of siRNA duplexes on day 3 of differentiation before being used as APCs in an autologous iNKT expansion assay. C, Enhanced maturation of prosaposin in vehicle-treated or PPARγ-instructed iDC determined by Western blot detection of the cleavage product saposin C (SapC). D, Inhibition of the maturation of prosaposin by pepstatin A (pepA; 10 μM) in iDCs. NS, non-silencing siRNA CatD; SIRNA, siRNA targeting CatD.
[including fat cell differentiation (45, 46) or macrophage lipo-protein uptake, processing and release], the receptor has multiple targets (47, 48), we hypothesized that, by analogy, lipid Ag processing and presentation might be similarly regulated at multiple steps.

In this study, we analyzed what other aspects of lipid presentation are regulated by PPARγ in monocyte-derived DCs. We used transcriptomics to provide us with the broadest possible scope and identified CatD, a lysosomal proteinase, whose expression in DCs is the highest among all cathepsins and closely matched that of genes with an already established function in lipid presentation, making it a suitable candidate for further analyses. By utilizing a combination of pharmacological activators and inhibitors of the relevant pathways, we determined that CatD is upregulated in response to both PPARγ and RARx stimulation. The retinoid-mediated regulation of CatD has been previously reported (49); however, our results suggest that CatD is dually and likely indirectly regulated by PPARγ and RARs in monocyte-derived DCs. This dual regulation is similar to that of an enzyme implicated in cholesterol metabolism and release, Cyp27β1 (50). Importantly, CD1d is also regulated by retinoid signaling, linking these two signaling pathways further (25).

We used two complementary techniques, FACS staining and RT-qPCR measurements (Fig. 3), to demonstrate that inhibition of CatD reduced the PPARγ-mediated enhanced induction of iNKT cell Ag presentation. Pepstatin A, an inhibitor of CatD, could block in a dose-dependent manner the presentation of the CD1d ligand precursor αG0C to iNKT cells. However, it did not affect the presentation of the processed αG0C lipid. This clearly indicated that CatD participated in a lysosomal processing step required for the processing and loading of the lipid precursor. Importantly, the inhibition of CatD could not block MHC class II-mediated Ag presentation (Supplemental Fig. 4), and therefore we could exclude the possibility that the increased iNKT induction seen upon PPARγ activation and CatD upregulation is due to a general and nonspecific increase in lysosomal processing events but is specific for the CD1d pathway. Interestingly, two other member of the same protein family, cathepsins L and S, were reported to be involved in the regulation of iNKT selection and activation. Cathepsin L-deficient mice showed impaired CD1d-mediated Ag presentation, although the cell surface expression of CD1d appeared to be normal (51). Cathepsin S-deficient mice, in contrast, showed impairment in the trafficking of CD1d molecules (29). In both cases the deficiency in cathepsin proteins appeared to block endosomal events required for potent CD1d-mediated Ag presentation. It is therefore feasible to propose that CatD regulates iNKT cell Ag presentation by modulating endosomal events required for loading of lipid Ags to CD1d molecules for an efficient lipid Ag presentation. As far as the relevant substrate is concerned, we identified prosaposin, a molecule already linked to lipid Ag transfer (35, 52). These data point to the existence of a PPARγ–CatD–prosaposin axis/pathway as a required mechanism in monocyte-derived DCs for efficient lipid presentation and for the enhancement of CD1d-mediated iNKT expansion. It is also noteworthy that the intracellular signal mediator ceramide has been shown to directly bind and regulate CatD activity (53). This raises the possibility of the existence of a ceramide–CatD–CD1d regulatory axis and directly ties in cellular glycolipid metabolism.

In conclusion, these data represent two conceptual advances. PPARγ is further established as an important regulator of lipid Ag processing and presentation and places CatD in the lysosomal process required for lipid Ag processing. As far as the ramifications of these data are concerned, identification of such a protease that is specifically involved in the lipid Ag presentation machinery could hold promise for modulation of protease activity for future drug targeting in disease conditions mediated by lipid-activated iNKT cells. In contrast, our data also reveal that PPARγ, a transcription factor whose activity can also be easily manipulated through pharmacological means, has a more coordinating role by regulating the expression of several components of the lipid Ag processing and presenting machinery. This suggests that lipid Ag presentation is a function of the cells extra- and intracellular lipid milieu and only gets activated if the necessary signaling has been engaged. It is intriguing to speculate about the nature of endogenous lipids activating PPARγ, which might set into motion this process. Oxidized fatty acids, components of modified low-density lipoprotein, apoptotic cells, or certain bacterial lipids might be involved. Further studies are needed to explore these possibilities and also to determine the utility of this pathway as a therapeutic target in inflammatory diseases.

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Disclosures

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References


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