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

Britt Nakken,*†1,2 Tamas Varga,*† Istvan Szatmari,* Lajos Szeles,* Adrienn Gyongyosi,* Petr A. Illarionov,‡ Balazs Dezso,§ Peter Gogolak,∥ Eva Rajnavolgyi,∥ and Laszlo Nagy*†

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 reduced the expansion of 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. The Journal of Immunology, 2011, 187: 240–247.

Abbreviations used in this article: CatD, cathepsin D; DC, dendritic cell; FABPA, fatty acid binding protein 4; αGC, α-galactosylceramide; αGCG, galactosyl(1,2)-galactosyl ceramide; iDC, immature dendritic cell; iNKT, invariant NKT; PPARγ, peroxisome proliferator-activated receptor γ; RA, 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 ligand activation of PPARγ enhances the iNKT induction capability of DCs 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-dimethylaminobenzaldehyde (Fluka), bafilomycin (Sigma-Aldrich), pepstatin A (Sigma-Aldrich), oG, and galactosyl(1-2) galactosyl ceramide (oGCC) (Kirin Brewery and from P.A. Illarionov, School of Biosciences, University of Birmingham, Birmingham, U.K.).

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 (VarisMACS; Miltenyi Biotec). Buffy 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, 800 U/ml GM-CSF (Leucomax), and 500 U/ml IL-4 (PeproTech). Cells were cultured for 5–6 d, 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 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 multiasarray analysis algorithm. All microarray data are available in the public Gene Expression Omnibus database (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 polyclonal goat anti-CatD (clone C20; Santa Cruz Biotechnology) at 1:100 dilution. In brief, PPARγ was detected by incubating sections 1 h at room temperature with primary Ab followed by HRP-labeled anti-mouse secondary IgG-F(ab’2) and FITC-conjugated tyramide (PerkinElmer Life Sciences) and, 500 U/ml IL-4 (PeproTech). Cells were cultured for 5–6 d, 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 3.

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-JαQβ) Ab and isotype-matched controls (BD Biosciences) and anti-Vα24–FITC, anti–Vβ11-PE, and appropriate isotype-matched controls (Immunotech). Stained cells were assessed for fluorescence intensity using a FACScan 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 aG or oG as a 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+Jα18+ 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 × 10⁵ 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 (AB1 Prism 7900; Applied Biosystems) or LC480 (Roche): 40 cycles at 95°C for 12 s and at 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–Jo junction, the following primers and probe were used: forward, 5′-AGCCATTACGGCTCTTACATCT-3′; reverse, 5′-GTCAACTGATTTCCCTTCTTCACCAGGTTGATAGCCTAA-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
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 siRNA targeting PPARγ using pelleted and fixed monocytes (Mo), iDCs, and iDCs treated with RSG (iDC RSG). Nuclei are visualized by DAPI (blue). Original magnification (Dharmacon, Lafayette, CO), or Qiagen siRNA targeting PPARγ (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.

Lipid Ag-pulsed DCs were cocultured with autologous T cells. T cells were stained with PC5-labeled anti-human CD3 (BD Biosciences) and then stained with PE-conjugated TCR Vα24+ and FITC-conjugated TCR Vβ11-specific mAbs (IOTest; Beckman Coulter, Brea, CA). The double-positive iNKT cell suspension was added, gently mixed, and incubated for 3 min before being pulsed in a Gene Pulser Xcell (Bio-Rad). Pulse conditions were square-wave pulse, 500 V, 0.5 ms. Immediately after electroporation, cells were transferred to RPMI 1640 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 postelectroporation. Cell viability postelectroporation 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 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 stained with PE-conjugated TCR Vα24+ and FITC-conjugated TCR Vβ11-specific mAbs (IOTest; Beckman Coulter, Brea, CA). The double-positive iNKT cell suspension was added, gently mixed, and incubated for 3 min before being pulsed in a Gene Pulser Xcell (Bio-Rad). Pulse conditions were square-wave pulse, 500 V, 0.5 ms. Immediately after electroporation, cells were transferred to RPMI 1640 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 postelectroporation. Cell viability postelectroporation was routinely ≥90%.

Statistical analyses

A two-sample Student 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), were 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](http://www.jimmunol.org/). 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, 1D). 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-diethylamino-benzaldehyde. The separate activation of PPARγ or RARs pathways in DCs could induce CD1d cell surface protein expression (Supplemental Fig. 2C) 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 RARs 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 RAR.

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.** 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 (pepA) 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 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 processing 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 demonstrate 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’s 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...
[including fat cell differentiation (45, 46) or macrophage lipoprotein 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, Cyp27 (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 enhancement of iNKT induction capacity of DCs. These experiments provided us with mechanistic links between CatD and lipid Ag presentation. Pepstatin A, an inhibitor of CatD, could block in a dose-dependent manner the presentation of the CD1d ligand precursor αGCC to iNKT cells. However, it did not affect the presentation of the processed αGCC 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 peptide 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 selection/activation 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 presentation. 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

The authors have no financial conflicts of interest.

References

Supplementary figure 2.

A

No NR ligand

B

RSG+AGN

RSG+AM

Vα24-Jα18 (iNKT) TCRα

Normalized mRNA

<table>
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<th>Condition</th>
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<td>0.004</td>
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<td>RSG+AM</td>
<td>0.004</td>
<td>0.008</td>
</tr>
</tbody>
</table>
Supplementary figure 3.

The figure shows a bar graph comparing the percentage of proliferating cells in iDC and mDC treated with different concentrations of Pepstatin A (0, 1, 10 μM) and different conditions (Control, PHA-M, IL-2, DC+T, T only). The bars are color-coded to represent Vehicle (white) and RSG (black).
Supplementary figure 4.

A

**IL-10 production**

B

**IL13 production**

- **A**
  - Bar chart showing IL-10 production in different conditions: veh, aGC, RSG, and DC only or DC+T cell co-culture.

- **B**
  - Bar chart showing IL13 production in different conditions: veh, aGC, RSG, and DC only or DC+T cell co-culture.

Legend:
- **No PPARγ ligand** (White)
- **RSG** (Black)
Supplementary online figure 5.

T cell apoptosis

% of total CD3+ cells

- PI labeling
- Annexin labeling

No DC  No lipid  aGC  aGGC  No lipid  aGC  aGGC

veh  DC-T cell co-culture  RSG
Supplementary figure 1: Expression levels of aspartic proteases in monocyte-derived DC. Since Pepstatin A is also capable of inhibiting other aspartic proteases than Cathepsin D, the expression level of additional aspartic proteases (Cathepsin E, Renin and Pepsin) were evaluated in mo-derived iDC with or without PPARγ-stimulation at 3 time points. The relative expression of aspartic proteases were assessed by using affymetrix microarray data. The line graph shows raw data of gene expression as per chip normalization. Microarray data was analyzed in GeneSpring 7.2 software (Agilent).

Supplementary figure 2: Effect of DC nuclear receptor activation on CD1d expression and iNKT expansion. (A) Lipid pulsed DCs were treated with the PPARγ agonist RSG, the RARα agonist AM580, the RARα antagonist AGN193109 or with the combination of above ligands. Cell surface expression of CD1d protein was measured by FACS. (B) Nuclear receptor ligand treatment of DCs modulates iNKT expansion. RT-qPCR measurements are shown.

Supplementary figure 3: Inhibition of cathepsin D by pepstatin A does not affect conventional MHCI/MHCII-mediated peptide presentation. iDC or mature DC (mDC) (1x10^4) differentiated in the presence of vehicle or 2.5μM RSG were harvested and co-cultured with allogeneic monocyte depleted CFSE-labeled PBMC (1x10^5) for 5 days. Cells were harvested and proliferation was assessed by CFSE-dilution measured by
flow cytometry. One representative experiment out of 4 is shown. The cathepsin D specific inhibitor, pepstatin A, was added to the differentiating DC cultures at day 3 at the indicated concentrations.

**Supplementary figure 4: Cytokine production by iNKT cells.** Cytokine production by sorted iNKT cells in the presence of PPARγ activated monocyte derived dendritic cells. For the exclusion of the cytokine production derived from DCs, samples in which only DCs were cultured are also shown. Production of IL-10 and IL-13 cytokines were monitored by ELISA after 36h co-culture. White bars: no PPARγ activation, black bars: PPARγ activation by RSG treatment of DCs.

**Supplementary figure 5: Measurement of T cell apoptosis in DC-T cell co-cultures.** DCs pulsed with lipid antigens were co-cultured with autologous T cells. T cells were stained with PC5 labeled anti-human CD3 and then labeled with propidium iodide (white bars) or annexin-FITC (black bars) for the detection of apoptosis. CD3+ cells were analyzed for apoptosis by FACS.