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The authors regret that Nina Nowak’s name was incorrectly listed as Natalja Nowak in the original version of their paper. The final html and pdf versions of the article have been corrected.
BRIEF DEFINITIVE REPORT

CD4+ T helper (Th) cells differentiate into discrete subsets, which can be discriminated on the basis of their cytokine expression profiles. Besides the “classical” CD4+ T cell subsets (i.e., Th1, Th2, and regulatory T cells), a new subset characterized by secretion of IL-17 was identified (Harrington et al., 2005; Park et al., 2005). Th17 cells provide protection in certain infections, but more importantly, have been linked to development of autoimmunity, a function previously assigned to Th1 cells.

The nuclear receptor PPARγ selectively inhibits Th17 differentiation in a T cell–intrinsic fashion and suppresses CNS autoimmunity

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T helper cells secreting interleukin (IL)-17 (Th17 cells) play a crucial role in autoimmune diseases like multiple sclerosis (MS). Th17 differentiation, which is induced by a combination of transforming growth factor (TGF)-β/IL-6 or IL-21, requires expression of the transcription factor retinoic acid receptor–related orphan receptor γt (RORγt). We identify the nuclear receptor peroxisome proliferator–activated receptor γ (PPARγ) as a key negative regulator of human and mouse Th17 differentiation. PPARγ activation in CD4+ T cells selectively suppressed Th17 differentiation, but not differentiation into Th1, Th2, or regulatory T cells. Control of Th17 differentiation by PPARγ involved inhibition of TGF–β/IL-6–induced expression of RORγt in T cells. Pharmacologic activation of PPARγ prevented removal of the silencing mediator for retinoid and thyroid hormone receptors corepressor from the RORγt promoter in T cells, thus interfering with RORγt transcription. Both T cell–specific PPARγ knockout and endogenous ligand activation revealed the physiological role of PPARγ for continuous T cell–intrinsic control of Th17 differentiation and development of autoimmunity. Importantly, human CD4+ T cells from healthy controls and MS patients were strongly susceptible to PPARγ–mediated suppression of Th17 differentiation. In summary, we report a PPARγ–mediated T cell–intrinsic molecular mechanism that selectively controls Th17 differentiation in mice and in humans and that is amenable to pharmacologic modulation. We therefore propose that PPARγ represents a promising molecular target for specific immunointervention in Th17–mediated autoimmune diseases such as MS.

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We first investigated the influence of PPARγ on Th17 differentiation by investigating other classical markers of Th17 cells. In addition to IL-17A, we found that PPARγ activation by PIO suppressed expression of TNF and IL-22 (Fig. 1 e), as well as IL-17F, IL-21, and IL-23R, in T cells (Fig. 1 f). Likewise, expression of the chemokine receptor CCR6 and its ligand CCL20 were also strongly controlled by PPARγ activation (Fig. 1 g). This demonstrates that PPARγ, indeed, influenced differentiation of Th17 cells rather than merely suppressing IL-17A production.

Selectivity of PPARγ for Th17 differentiation
To further characterize the specificity of PPARγ on the differentiation of Th17 cells, we evaluated the effect of PIO on cytokine-induced CD4+ T cell differentiation into Th1, Th2, or regulatory T cells. Importantly, PIO did not modulate TGF-β-mediated induction of Foxp3+ regulatory T cells, IL-4-mediated induction of Th2 cells, or IL-12-mediated induction of Th1 cells (Fig. 2 a). This is in contrast to the effect of RA, which is a natural ligand of the nuclear RA receptor (Chambon, 1994) that has been shown to reciprocally regulate Th17 and regulatory T cell differentiation (Mucida et al., 2007). In direct comparison, RA and...
PIO both efficiently suppressed Th17 differentiation, whereas RA but not PIO induced TGF-β-mediated expression of Foxp3 (Fig. 2a). Accordingly, CD4-PPARγKO T cells did not show altered TGF-β-mediated Foxp3-induction (unpublished data). A further distinction between RA and PIO was observed on Th1 differentiation, as RA slightly but significantly impeded IL-12-mediated induction of IFN-γ expression in T cells (Fig. 2a), as has been previously reported (Iwata et al., 2003). Collectively, these data indicate that distinct molecular mechanisms were involved in PPARγ-mediated, as compared with RA-mediated, control of T cell differentiation.

We next investigated whether PPARγ also affected expression of the key transcription factors determining CD4+ T cell differentiation. PPARγ-activation selectively suppressed TGF-β/IL-6-mediated expression of RORγt, the transcription factor...
required for Th17 induction, whereas the expression of the transcription factors determining Th1, Th2, and regulatory T cell differentiation, i.e., T-bet, GATA-3, and FoxP3, was not influenced by PIO (Fig. 2 b), again confirming that PPAR-γ acted specifically on the differentiation of Th17 cells. Other transcriptional regulators have been reported to influence Th17 differentiation. Foxp3 has been shown to directly antagonize RORγt activity, and thus prevent Th17 differentiation (Zhou et al., 2008). Furthermore, several groups have reported that the aryl hydrocarbon receptor elicits either regulatory T cell or Th17 responses when activated by distinct ligands; however, the underlying mechanisms do not seem to involve RORγt regulation (Quintana et al., 2008; Veldhoen et al., 2008). Additionally, the nuclear orphan receptor NR2F6 seems to regulate Th17-dependent autoimmunity, but with no apparent involvement of RORγt (Hermann-Kleiter et al., 2008). It

Figure 2. Selectivity of PPAR-γ for Th17 differentiation. (a) CD4+ T cells were subjected to Th1, Th2, Th17, and regulatory T cell differentiation protocols, as described in the Materials and methods section, and the influence of RA and PIO on the induction of lineage markers was determined by flow cytometry and analyzed as described in Materials and methods. (b) CD4+ T cell differentiation was induced as described in Materials and methods, and the influence of PIO on expression of the lineage-determining transcription factors T-bet, GATA-3, RORγt, and Foxp3 was determined by quantitative real-time PCR and normalized to β-actin levels after 48 h. Data in a and b are representative of at least three independent experiments.
can therefore be concluded that several receptors are involved in the T cell–intrinsic control of Th17 responses, but that the molecular pathways involved in these processes are distinct. Even among the family of PPARs, the regulatory effect on Th17 differentiation is not a general feature, as lack of PPARα in T cells did not result in altered IL-17 expression levels (Dunn et al., 2007).

**PPARγ inhibits Th17 differentiation by controlling RORγt induction**

We next evaluated whether PPARγ influenced RORγt expression in T cells. In PPARγKO T cells, we observed enhanced cytokine-induced RORγt induction compared with PPARγWT T cells (Fig. 3a). The suppressive effect of PPARγ activation by PIO on the one hand and the increased expression of RORγt in PPARγKO T cells on the other hand illustrate the dynamic range of PPARγ-mediated control of Th17 differentiation. We substantiated the influence of PPARγ activation on RORγt expression using reporter mice, which express GFP under control of the RORc(γt) promoter (Lochner et al., 2008). In such T cells, we observed that PIO strongly reduced TGF-β/IL-6–mediated GFP expression (Fig. 3b). Importantly, both the frequency of GFPpos T cells and the mean fluorescence intensity of GFP-expressing T cells were reduced by PIO (Fig. 3b and c). These results indicated that most CD4+ T cells failed to express RORγt under the influence of PPARγ activation, thus giving rise to less Th17 cells. Furthermore, the decreased mean fluorescence intensity of GFP in PIO-treated RORc(γt) reporter T cells (Fig. 3c) revealed that upon PPARγ activation there was less GFP, i.e., RORγt, on a per cell basis, suggesting that PPARγ reduced RORγt transcription on a single-cell level.

**Figure 3.** PPARγ inhibits Th17 differentiation by controlling RORγt induction. (a) Th17 differentiation from PPARγKO and wild-type T cells was induced as described in Materials and methods; RORγt expression was determined by quantitative real-time PCR and normalized to β-actin levels. (b and c) CD4+ T cells from Rorc(γt)-GFPGFP reporter mice were treated with PIO, and Th17 differentiation was induced. After 14 h, GFP expression was assessed by flow cytometry and analyzed for frequency of GFPpos cells (b) and for MFI of GFP-expressing cells (c). One out of three independent experiments is shown. (d) PPARγ was recombinantly expressed (Fig. S3a), and interaction of recombinant PPARγ with the murine RORγt promoter was determined by surface plasmon resonance analysis. Sensograms show the binding of indicated concentrations of PPARγ at either the RORγt promoter or the murine AP2 promoter containing a bona fide PPRE site as positive control; shown are a representative sensogram (left) and a quantitative analysis (right). The bar graph shows mean ± SEM from three independent experiments. (e) Signal-dependent clearance of SMRT from the RORγt promoter is prevented by PIO. ChIP experiments were performed for SMRT in mock-treated CD4+ T cells and in CD4+ T cells stimulated with TGF-β/IL6 in the presence or absence of PIO. ChIP assay was performed with αSMRT or IgG for control of specificity. Immunoprecipitated DNA was analyzed by quantitative PCR using primers specific for the RORγt promoter; as control, binding of SMRT to a nonrelated DNA control (exon 1 of the RORγ gene) was investigated and set as 1. Two independent experiments were performed, and mean results ± SEM are shown.
Figure 4. PPARγ in T cells controls CNS autoimmunity and restricts Th17 differentiation in vivo. (a) MOG-EAE was induced in CD4-PPARγKO mice and CD4-PPARγWT littermates (n = 8 per group, 3 experiments), and the clinical disease score was assessed daily. (b) In a separate experiment, mice...
The control of PPARγ over RORγt transcription led us to examine whether the RORγt promoter contained a bona fide PPARγ-binding site (PPRE), which might permit direct interaction of PPARγ with the RORγt promoter. Bioinformatic analysis did not reveal any known PPRE sequence within the mouse RORγt promoter (unpublished data). In addition, we excluded direct interaction of PPARγ with the RORγt promoter by examining the binding of recombinant PPARγ to the full-length RORγt promoter using surface plasmon resonance analysis. In contrast to strong and specific binding of PPARγ to the AP2 promoter, which contains a PPRE site (Frohnert et al., 1999), we did not observe significant binding to the RORγt promoter (Fig. 3 d).

The lack of a high-affinity PPARγ binding site in the RORγt promoter raised the possibility that PPARγ might negatively regulate RORγt transcription through a trans-repression mechanism that does not require direct DNA binding. One such mechanism involves the ability of ligand-activated PPARγ to inhibit signal-dependent clearance of NCoR or SMRT corepressor complexes from promoters of regulated genes (Pascual et al., 2005; Ghisletti et al., 2009). To investigate this possibility, we used chromatin immunoprecipitation (ChIP) to screen of genomic sequences surrounding the RORγt promoter (unpublished data) for corepressor binding. These studies revealed the binding of the corepressor SMRT, but not NCoR, at the RORγt promoter in unstimulated mouse CD4+ T cells (Fig. 3 e and not depicted). Importantly, stimulation of CD4+ T cells with TGF-β and IL-6 resulted in rapid and nearly complete loss of SMRT from the RORγt promoter (Fig. 3 e), indicating that SMRT clearance precedes RORγt activation. Interestingly, this cytokine-induced clearance of SMRT from the RORγt promoter was prevented by the PPARγ agonist PIO (Fig. 3 e). These data suggest that the retention of SMRT results in persistent repression of RORγt in the presence of activating cytokines, and are consistent with prior studies demonstrating that PPARγ suppresses activation of inflammatory response genes in macrophages by preventing NCoR/SMRT turnover (Ghisletti et al., 2009). Interference of SMRT clearance from the RORγt promoter thus provides a previously unrecognized mechanism by which ligand-activated PPARγ may control Th17 differentiation in T cells. However, these findings do not exclude other mechanisms, such as modulation of STAT3 or IRF4 signaling (Nurieva et al., 2007; Huber et al., 2008).

PPARγ in T cells controls CNS autoimmunity and restricts Th17 differentiation in vivo

To analyze whether PPARγ is involved in T cell–intrinsic control of CNS autoimmunity, we induced EAE in CD4-PPARγKO mice and wild-type littermates. CD4-PPARγKO mice showed a significantly earlier onset and aggravated disease course during the initial T cell–dependent phase of disease until d15 (Fig. 4 a). However, this difference was not observed in the effector phase, when disease activity is mainly determined by a local inflammatory response within the CNS governed by microglial cells (Heppner et al., 2005). Disease activity in CD4-PPARγKO mice directly correlated with the total numbers of infiltrating CD4+ T cells in the CNS (Fig. 4 b). Both at the beginning of clinical disease activity (day 8), and at the peak of disease in CD4-PPARγKO mice (day 13), we found significantly increased total CD4+ T cell numbers in the CNS. Later (day 18) disease score and T cell influx were not different from wild-type littermates. As expected, PIO-treated wild-type mice exhibited decreased T cell numbers within the CNS at all time points investigated (Fig. 4 b). Importantly, at the peak of disease in CD4-PPARγKO mice, the frequency of MOG35-55 peptide-specific, IL-17–producing CD4+ T cells in the CNS was increased by threefold, which, together with the increase in T cell influx, enhanced the numbers of IL-17–producing autoreactive T cells within the target organ by nearly fivefold (Fig. 4, b and c). In contrast, there was no alteration in antigen-specific IFN-γ–producing CD4+ T cells in these mice (Fig. 4 c).

The clinical symptoms and antigen-specific Th17 responses in CD4-PPARγKO mice both revealed that the kinetics of CNS autoimmunity in vivo were modulated by PPARγ in a T cell–intrinsic fashion. There was pronounced acceleration of IL-17–producing T cells in the CNS compared with the spleen in CD4-PPARγKO mice (Fig. 4 c), which may be caused by guided entry of Th17 cells into the CNS. A recent study demonstrated that CCR6-expressing Th17 cell function as “pioneer” cells, enabling immune cell entry into the CNS at the beginning of CNS autoimmunity (Reboldi et al., 2009). In this regard, the control of expression of both CCR6 and its ligand CCL20 by PPARγ activation (Fig. 1 g) may explain the decreased influx of T cells and the reduced disease activity in the CNS of PIO-treated wild-type mice. The protective effect of PIO on disease activity was greatly diminished in CD4-PPARγKO mice (Fig. S4 b), thus excluding off-target effects that had been reported previously (Chawla et al., 2001) and further demonstrating that...
PPAR-γ expression in T cells was required for full protective effect of PIO on CNS autoimmunity. The observation that PPAR-γ activation in vivo did not entirely protect from EAE development, despite its profound effect on Th17 differentiation, lends support for a key but not exclusive role of Th17 cells in CNS inflammation, as previously reported (Yang...
The persistent Th1 responses, which were not altered by PPARγ activation, may explain persistent disease activity, despite diminished Th17 responses.

As we also observed a significant increase in antigen-specific Th17 cell numbers in the spleen in CD4-PPARγKO mice at the peak of disease (Fig. 4 c), we next asked whether PPARγ influenced Th17 differentiation in vivo at early time points. To this end, we adoptively transferred CD90.2+ CD4+ T cells from OT-II mice, followed by immunization with OVA in CFA. Importantly, PIO treatment of these mice strongly interfered with the expression of activation markers (Fig. 4 d) and IL–17 production (Fig. 4 e) by the adoptively transferred T cells 4 d after immunization; this persisted for longer than 4 d (day 7; not depicted), demonstrating that PPARγ controls antigen-specific Th17 differentiation in vivo.

Collectively, the entire range of PPARγ-sensitive control of Th17 differentiation in vivo and CNS autoimmunity is reflected by the combination of pharmacological PPARγ activation on the one hand and by the absence of PPARγ activity in CD4-PPARγKO mice on the other hand.

**PPARγ selectively controls Th17 differentiation in T cells from healthy controls (HCs) and MS patients**

The protective effects of PPARγ on both clinical manifestation and Th17-responses during EAE prompted us to investigate whether T cells from HCs and MS patients were susceptible to treatment with PPARγ agonists. Again, we focused on the effect of PPARγ activation on T cells by using direct stimulation with TGF-β/IL–21 in the absence of antigen-presenting cells. Pharmacologic PPARγ activation reduced the frequency of IL-17A–producing CD45RA+ CD4+ T cells both in HC and MS patients (Fig. 5 a). Although in our experiments there was no apparent difference in Th17 differentiation between HC and MS patients in vitro, it is important to note that PIO-treatment was equally effective in potent suppression of IL–17A release from T cells (Fig. 5 b). Moreover, no influence of PIO was observed during IFN-γ production (Fig. 5 b). Pharmacologic PPARγ activation prevented Th17 differentiation, as demonstrated by diminished expression of the Th17 markers IL–17F, IL–21, IL–22, and IL–23R upon PIO treatment (Fig. 5 c). Importantly, the specific effect of PPARγ activation on Th17 induction in human CD4+ T cells was further illustrated by selective regulation of RORγt expression, whereas T-bet and GATA-3 expression were not altered by PIO (Fig. 5 d).

In summary, we identify PPARγ as a defined molecular target to selectively modulate Th17 differentiation in a T cell–intrinsic fashion, which opens up new possibilities for specific immunomodulation in Th17-mediated autoimmune diseases such as MS.

**MATERIALS AND METHODS**

**Mice.** CD4-specific PPARγ knockout mice with the genotype PPARγ1/1 CD4-Cre+/- (i.e., CD4-PPARγKO mice) were generated by crossing PPARγ1/1 mice (He et al., 2003) with CD4-Cre+/- transgenic mice expressing Cre recombinase under control of the CD4 enhancer/promoter/silencer (Lee et al., 2001). Expression of Cre recombinase in CD4-expressing T cells leads to recombination at two loxP sites flanking exons two and three of the PPARγ gene, thus resulting in a T cell–specific PPARγ knockout (Fig. S1). We did not observe any alteration in immune cell frequencies in these mice (Fig. S1). CD90.2+ CD4–TCR transgenic OT II mice specific for the peptide ova223–238, BAC-transgenic Rorc(γt)-GFP1 mice, and C57BL/6 mice (Charles River Laboratories) were maintained under specific pathogen–free conditions. All animal experiments were performed according to the guidelines of the animal ethics committee and were approved by the government authorities of Nordrhein-Westfalen, Germany.

**Cell culture and adoptive cell transfer.** PBMCs were obtained from the peripheral blood of healthy volunteers or from patients with clinically defined relapsing-remitting MS according to the McDonald criteria, approved by the local Ethics Committee. CD4+CD45RA+CD45RO– CD25+ T cells were isolated by immunomagnetic separation using an AutoMACS (Miltenyi Biotech) and stimulated with plate-bound 1.5 μg/ml αCD3 antibody (OKT3), 1 μg/ml αCD28 antibody (28.2), 2.5 ng/ml TGF-β (R&D Systems) and 12.5 ng/ml IL–21 (Cell Systems) for 7 d in serum-free X-VIVO 15 medium (BioWhittaker; Yang et al., 2008). 10 μM PIO (Enzo Biochem, Inc.) was added when indicated. Mouse splenic CD4+ T cells were isolated by immunomagnetic separation using CD4-MACS beads (Miltenyi Biotech) and stimulated with plate-bound 4 μg/ml αCD3 antibody (145–2C11) and 4 μg/ml αCD28 antibody (3751) together with 5 ng/ml TGF-β and 20 ng/ml IL–6 (PeproTech) for Th17 differentiation; with IL–12 (10 ng/ml) for Th1 differentiation; with IL–4 (10 ng/ml) for Th2 differentiation or with TGF-β alone (5 ng/ml) for Regulatory T cell differentiation. In one experiment, MACS-isolated splenic DCs from B6 mice were cocultured with T cells in the presence of antigen (10 μg/ml ova223–238). The endogenous PPARγ agonist 13s-HODE (Cayman Chemicals) was used at a concentration of 10 μM. All-trans RA (Sigma-Aldrich) was used at a 1 μM concentration. TCR transgenic CD4+ T cells from OTII mice bearing the congenic marker CD90.1+ were isolated and 105 cells were adoptively transferred by bolus i.v. injection in 200 μl PBS into wild-type CD90.2+ congenic mice.

**EAE.** EAE was induced by s.c. injecting 50 μg MOG35–55 peptide (BIOTREND) emulsified in CFA (Difco) with 8 mg/ml heat-inactivated Mycobacterium tuberculosis and two i.p. injections of 200ng Bordetella pertussis toxin (List Biologicals) on days 0 and 2. Clinical assessment of EAE was performed daily using a scale ranging from 0 to 6: 0, clinically normal; 1, reduced tone of tail; 2, ataxia and/or slight hind-limb paresis; 3, severe hind-limb paresis; 4, hind limb plegia; 5, tetraparesis; 6, moribund/dead animals. Cell analysis from spleens and CNS was performed as indicated.

**Real-time RT-PCR.** Cells were washed with ice-cold PBS, and RNA extraction was performed using the RNeasy mini kit (QIAGEN) according to the manufacturer's protocol. Reverse transcription of RNA was performed with SuperScript III (Invitrogen). cDNA was analyzed using FAM-labeled TaqMan probes obtained from Applied Biosystems and used according to the manufacturer’s recommendations. mRNA expression levels of RORγt, T-bet, GATA-3, and Foxp3, as well as the Th17 markers IL–17A, IL17F, IL–21, IL–22, and IL–23R, were assessed using gene-specific primers. Gene expression was assessed in triplicates and normalized to β-actin. Amplification of cDNA was performed on an ABI Prism 7900 HT cycler (Applied Biosystems).

**Cytokine detection.** Mouse IL–17A and Foxp3 protein expression were examined by intracellular staining according to the manufacturer’s protocol. MOG-specific IL–17 and IFN-γ production was analyzed by specific ELISPOT assays according to the manufacturer’s procedures (R&D Systems), and spot numbers were counted with an automated ELISPOT reader (BIOREADER-2000). Human IL–17A and IFN-γ protein levels from cell culture supernatants were determined by ELISA (R&D Systems).
ChIP experiments. ChIP assays were performed as previously described (Pacual et al., 2005). Th17 differentiation was induced for the indicated time points before cross-linking for 10 min with 1% formaldehyde. Anti- 
SMRT (ABR) or control rabbit IgG (Santa Cruz Biotechnology, Inc.) were used for immunoprecipitation. A 150-bp region of the RORγt promoter was amplified spanning the most proximal transcription start site. Quantitative 
PCR was used with SYBR-GreenER (Invitrogen) and analyzed on a 7200 real time PCR system (ABI).

Surface plasmon resonance analysis. 6xHis-PPARγ was recombinantly expressed in the bacterial strain Escherichia coli BL21, eluted, and desalted using a PD-10 column and 10% glycerol in PBS. The promoter sequences of mouse RORγt and mouse AP2 were amplified by PCR using the oligonucleotides (5'-GGTCTCCACAGGACACCTGGAAG-3' and 5'-AGGACAGCACAACAGTGGCAGTG-3') for RORγt; and 5'-TCTAGAAG-GAAAGACACAGGG-3' and 5'-AGGGCGAAATGACATTCCACCTTAC-3' for AP2). For each reaction, one primer was biotinylated at the 5' end. As negative control, 2 kb of the mouse mannose receptor was amplified for 2 min using the "inject" mode. Measurements were done in the presence of 0.001 mg/ml heparin to reduce unspecific binding.

Online supplemental material. Fig. S1 shows the Th17 differentiation of highly purified naive CD4+ T cells. Fig. S2 shows the generation of T cell–specific PPARγ knockout mice and phenotypic characterization of immune cells. Fig. S3 shows the purification of recombinantly expressed full-length murine PPARγ; surface plasmon resonance analysis of PPARγ-binding to PPRE-oligonucleotides; and effect of PPARγ on SMRT-binding to the RORγt promoter in the absence of Th17 induc- ing conditions. Fig. S4 shows the effect of the CD4-Cre transgene on EAE disease course and the absence of a protective PPARγ effect in CD4+ PPARγ−/− mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20082771/D1C1.

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