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Peroxisome Proliferator-Activated Receptor γ Is Required for CD4$^+$ T Cell-Mediated Lymphopenia-Associated Autoimmunity

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The nuclear hormone receptor peroxisome proliferator-activated receptor γ (PPARγ) was shown to play an immunoregulatory role in many immune-related cell types, and activation of PPARγ was reported to be an effective therapeutic approach in murine and human autoimmune disease. However, despite an association between lymphopenia and autoimmunity, there has been no study on the role of T cell PPARγ in lymphopenia-associated autoimmunity. In the present studies, we examined the role of PPARγ in CD4$^+$ T cells in two murine models of lymphopenia-associated autoimmunity. Surprisingly, we found that PPARγ expression in CD4$^+$ CD25$^-$ T cells (T effector cells [Teffs]) is actually required for development of autoimmunity under lymphopenic conditions. Mechanistically, the inability of PPARγ-deficient (T-PPAR) Teffs to mediate lymphopenic autoimmunity is associated with a significant decrease in accumulation of Teffs in the spleen, lymph nodes, and tissues after adoptive transfer. This abnormal accumulation of T-PPAR Teffs was associated with defects in both in vivo proliferation and survival. Additionally, T-PPAR Teffs demonstrated decreased cytokine production in inflammatory sites and decreased expression of the homing receptor α4β7. Finally, these abnormalities in T-PPAR Teff function were not elicited by lymphopenia alone but also required the additional activation involved in the mediation of autoimmunity. Thus, in contrast to its documented immunosuppressive role, we identified an unexpected function for PPARγ in Teffs: a role in Teff proliferation and survival in lymphopenia-associated autoimmunity. These findings highlight both the multifunctional role of PPARγ in T cells and the complexity of PPARγ as a potential therapeutic target in autoimmunity. ***The Journal of Immunology*****, 2011, 187: 000–000.**

Peroxisome proliferator-activated receptor γ (PPARγ) is a ligand-dependent nuclear hormone receptor that was originally described as a regulator of glucose homeostasis and fatty acid metabolism. Ligands for PPARγ, including the thiazolidinedione (TZD) class of drugs, are routinely used for the treatment of type 2 diabetes. In addition to these synthetic ligands, endogenous ligands, including fatty acid metabolites and unsaturated fatty acids, have been identified (1). We and other investigators showed that PPARγ is expressed in and mediates important immunoregulatory functions in conventional T cells, macrophages, and dendritic cells (2–6). In conventional T cells, ligation of PPARγ results in downregulation of IL-2, IFN-γ, and IL-4 production (2, 7, 8). Interestingly, a recent report also suggested that mice with a T cell-specific deletion in PPARγ show enhanced Th17 production in a murine model of autoimmunity (9). In addition, our laboratory and others described a critical role for PPARγ in natural regulatory T cell (Treg) suppressive function and in the differentiation and stability of inducible Tregs (10–12).

Consistent with the immunoregulatory effects of PPARγ, TZDs have been used to effectively treat a number of murine autoimmune and inflammatory models, including NOD mice, experimental autoimmune encephalomyelitis (EAE), colitis, asthma, and allergic disease (13–18). In humans, TZDs are effective in treating Crohn's disease and psoriasis, and initial studies in multiple sclerosis suggested a beneficial effect in secondary progressive disease (19–22). In addition to the effects of TZDs on the suppression of immune responses, it was demonstrated that PPARγ activation can influence immune cell survival. However, the role of PPARγ in T cell survival is controversial.

There is a strong associative link between lymphopenic proliferation and autoimmunity (23). Development of autoimmunity in multiple mouse models and some human diseases is associated with either a transient or sustained period of lymphopenic proliferation prior to development of autoimmune inflammation. Sjögren's disease, rheumatoid arthritis, celiac disease, and Crohn's disease have all been associated with decreased PBLs prior to development or exacerbation of disease (23). Paradoxically, the lymphodepletion of CD4$^+$ T cells by HIV has been associated with increased autoimmunity, including systemic lupus erythematosus, antiphospholipid syndrome, vasculitis, and Graves' disease (24). Recovery from lymphopenia following highly active retroviral therapy for HIV or T cell depletion with Campath-1h in multiple sclerosis patients has been associated with increased autoimmunity, particularly Graves' disease (24, 25). Although prior studies

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Abbreviations used in this article: cLP, colonic lamina propria; EAE, experimental autoimmune encephalomyelitis; GVHD, graft-versus-host disease; MHCII, MHC class II; MLN, mesenteric lymph node; PPARγ, peroxisome proliferator-activated receptor γ; SPL, spleen; Teff, T effector cell; T-PPAR, T cell-specific peroxisome proliferator-activated receptor γ-deficient; Treg, regulatory T cell; TZD, thiazolidinedione.

documented the immunoregulatory role of T cell PPARγ in non-lymphopenic autoimmune models, in the current study we set out to test the relevance of T cell PPARγ-mediated immunoregulation in lymphopenia-associated autoimmunity.

Surprisingly, we found an unexpected role for T cell PPARγ in lymphopenic autoimmune models. In contrast to its well-described function in downregulating autoimmunity and enhancing apoptosis, we found that, in the absence of PPARγ, $CD4^+$ T cells are unable to mediate lymphopenia-associated autoimmune disease.

Materials and Methods

Mice

RAG-$1^{-/-}$ and Bm12 mice were purchased from The Jackson Laboratories (Bar Harbor, ME). T cell-specific PPARγ-deficient (T-PPAR) mice were generated in our laboratory, as previously described (11). In brief, these mice were generated by crossing mice expressing Cre recombinase behind the CD4 promoter with a mouse expressing a floxed PPARγ gene locus to generate the T-PPAR mice. Because both parental strains were previously on a C57BL/6 background, this cross maintained the C57BL/6 lineage. T-$PPAR^{fl/fl}$ CRE^{neg} littermate control mice were used as controls in all experiments. T-PPAR mice show no observable clinical phenotype by 4 mo of age and have normal frequencies and numbers of all splenic populations (11). All experiments were performed using either male or female mice that were younger than 2 mo old. All animals were bred and cared for according to the University of Connecticut Center for Animal Care regulations.

Purification of $CD4^+CD25^-$ effector T cells for adoptive transfer

For all adoptive-transfer studies, $CD4^+CD25^-$ effector T cells (Teffs) were purified from T-PPAR or littermate spleens (SPLs) using a Miltenyi Treg purification kit (Miltenyi Biotec). Briefly, $CD4^+$ T cells were purified by magnetic bead negative selection of CD8, CD11b, B220, DX5, and Ter-119 cells, followed by a second negative selection column against CD25. The number of viable $CD4^+CD25^-$ T cells was determined by trypan blue exclusion prior to adoptive transfer.

Graft-versus-host disease

Eight to twelve-week-old female Bm12 mice were sublethally irradiated with 600 rad and allowed to rest for ≥5 h prior to injecting cells. $CD4^+CD25^-$ Teffs were purified by magnetic beads (Miltenyi Biotec) from female littermate and T-PPAR spleens. A total of 5×10^4 $CD4^+CD25^-$ T cells/mouse was injected i.v. Survival was monitored daily for 40 d.

Colitis and RAG-$1^{-/-}$ cell transfers

$CD4^+CD25^-$ T cells were purified by magnetic beads (Miltenyi Biotec) from littermate or T-PPAR mice, and $0.75–1 \times 10^6$ cells were injected i.p. into RAG-$1^{-/-}$ mice. Weights were determined prior to injection and monitored weekly for 7–8 wk. At the end of 7–8 wk, the mice were sacrificed, and the colons were fixed, sectioned, stained with H&E, and scored in a blinded fashion by a pathologist. Histological evidence of colitis was scored as a sum of acute and chronic inflammation as follows: acute inflammation score: 1) neutrophil infiltration with rare or absent cryptitis; 2) cryptitis with rare or no crypt abscesses; 3) severe crypt abscesses; and 4) severe cryptitis with ulceration. Chronic inflammation score: 1) mild inflammatory expansion of the colonic lamina propria (cLP); 2) moderate to severe lamina propria infiltration with minimal transmural inflammation; 3) lifting of crypts and goblet cell depletion; and 4) lymphoid follicles and intramural inflammation. The extent of inflammation was determined as a percentage of the colon inflamed, and the total score was calculated by multiplying the extent by the sum of the acute and chronic inflammation scores. For cellular evaluation, SPL, mesenteric lymph nodes (MLNs), or cLP were harvested at day 7, 14, or 56 after adoptive transfer, as indicated. For $CD8^+$ T cell studies, 1×10^6 $CD8^+$ T cells, purified from littermate or T-PPAR SPLs by magnetic bead purification (Miltenyi Biotec), were injected i.p. into RAG-$1^{-/-}$ mice. SPLs and MLNs were harvested at day 7 or 14 after transfer. Naive T-PPAR mice had normal numbers and viability (by trypan blue exclusion) of splenic $CD8^+$ T cells (data not shown).

Ex vivo analysis of adoptively transferred cell populations

Single-cell suspensions were prepared from SPL and MLN cells, resuspended in RPMI 1640 media (containing 10% FBS, L-glutamine, HEPES, Pen/Strep, gentamicin, and 2-ME), counted by trypan blue, and stained with various fluorescently tagged Abs, as indicated. Single-cell suspensions of cLP cells were prepared by removing the colons below the cecum to the rectum and dissociating the tissue by sequential steps of dithioerythritol, EDTA, and dissociation buffer containing collagenase D (Roche), DNase (Sigma), and dispase (Worthington). Lymphocytes were then purified from total lamina propria cells by Percoll-gradient separation (44%/67%).

Ex vivo restimulation and intracellular cytokine analysis

The total number of viable SPL, MLN, and cLP cells was determined by trypan blue exclusion and restimulated in vitro with 10 μg/ml plate-bound anti-CD3 Ab (Clone 145-2C11; eBioscience), 2 μg/ml anti-CD28 Ab (BioLegend), and 5 μg/ml brefeldin A (Sigma Aldrich) for 4 h. Cells were then permeabilized with a BD Permeabilization kit (BD Biosciences), anti-FcR blocked (anti-CD16/CD32 Fc Block; BioLegend), and stained for CD4 (intracellular), IL-17, and IFN-γ. In some experiments, cells were also stained with an anti-MHC class II (MHCII) Ab for use in a dump gate.

FACS analyses for surface markers and markers of proliferation and apoptosis

Cells were blocked with anti-FcR prior to staining and stained with Abs to: CD4 (FITC, PE-Cy7, PE, e450 [eBioscience]; Alexa Fluor 700 [BioLegend]); MHCII (I-A/I-E PE-Cy7 [BioLegend]); IFN-γ allophycocyanin (BioLegend); CD69 PerCp-Cy5.5 (BD Biosciences); CD62L efluor780 (eBioscience); CD44 (allophycocyanin [Biosciences], Pacific blue [BioLegend]); CD25 a700 (eBioscience), Ki67 (clone B56; BD Biosciences), and CD127 PE (eBioscience). For Ki67 staining, cells were initially fixed with 3.7% formaldehyde, anti-FcR blocked, and surface stained. After surface staining, the cells were permeabilized with the Foxp3 permeabilization kit (eBioscience), anti-FcR blocked, and stained with Ki67 at room temperature in the dark for 1 h. For staining with Annexin V, cells were stained using an Annexin V detection kit (BD Biosciences). Briefly, cells were stained for CD4 and Annexin V in Annexin-binding buffer (BD Biosciences) for 15 min at room temperature, washed, and analyzed on either a FACSCalibur or an LSRII (BD Biosciences).

Recovery of $CD4^+$ T cells in irradiated mice

Female littermate or T-PPAR mice were sublethally irradiated with 600 rad. Reconstitution of the endogenous T cells in the SPL and MLNs was assessed at 7, 14, and 56 d after irradiation. Single-cell suspensions were prepared from the SPL and MLNs and stained for CD4 and Ki67 staining, as above.

Statistics

The p values were determined by the Student unpaired t test. All compilation graphs are shown as group means. Vertical bars represent SEM.

Results

PPARγ expression in $CD4^+CD25^-$ T cells is required for development of graft-versus-host disease

To determine the role of T cell PPARγ in lymphopenic models of immune-mediated inflammation, we used mice with a T cell-specific deletion of PPARγ (11). T cells from naive T-PPAR mice show no statistically significant difference in expression of activation markers compared with littermate T cells (data not shown).

We first studied T-PPAR T cells using the adoptive-transfer model of Bm12 graft-versus-host disease (GVHD) (26). In this model, splenic C57BL/6 (wild-type) $CD4^+CD25^-$ Teffs are injected i.v. into sublethally irradiated Bm12 mice. Bm12 mice express a mutant MHCII molecule recognized as foreign by the transferred wild-type Teffs, resulting in bone marrow failure and eventual death. Teffs were purified from T-PPAR mice or from littermate controls. As expected, Teffs from littermate controls mediated lethal GVHD when transferred into irradiated Bm12 mice, with the majority of mice dead by day 20 and all but 1 of 11 mice dead by day 31 (Fig. 1). Surprisingly, all mice injected with T-PPAR Teffs showed no signs of illness and remained healthy until the termination of the experiment at day 40 (Fig. 1). Thus, in contrast to the well-documented immunosuppressive function of PPARγ in T cells, these results indicated that expression of PPARγ in Teffs actually plays a role in the development of Bm12 GVHD.

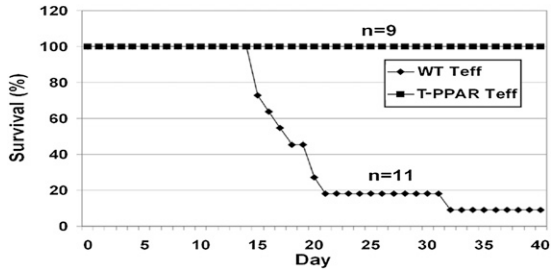

FIGURE 1. T-PPAR Teffs are unable to mediate GVHD. Splenic $CD4^+$ $CD25^-$ Teffs were purified from littermate or T-PPAR mice by magnetic bead purification. A total of 5×10^4 Teffs was injected i.v. into sublethally (600 rad) irradiated Bm12 mice. Survival was monitored daily for 40 d after adoptive transfer in four separate experiments.

PPARγ expression in Teffs is required for the development of colitis

An inherent difficulty in using the Bm12 GVHD model is that the fate of the Teffs after transfer cannot be easily tracked. To be able to track the cells and determine the effect of PPARγ deletion on T cell activation, cytokine production, and proliferation in lymphopenic autoimmunity, we used an adoptive-transfer model of colitis with a defined inflammatory site and draining lymph nodes (27). In this model, Teffs are injected into RAG-$1^{-/-}$ mice, and these mice begin developing progressive colitis within 2 wk, characterized by weight loss, diarrhea, and inflammation developing along the entire length of the colon. To determine the requirement for PPARγ expression in Teffs for the mediation of this disease, Teffs from littermate or T-PPAR mice were adoptively transferred into RAG-$1^{-/-}$ mice. Mice receiving littermate Teffs began to lose weight by week 3 posttransfer. In contrast, mice receiving T-PPAR Teffs continued to gain weight throughout the experiment (Fig. 2*A*). Histological examination at 7 wk posttransfer revealed that littermate Teffs caused severe colitis involving crypt abscesses, neutrophilic infiltration of crypts, goblet cell depletion, crypt elongation, and lamina propria expansion. In contrast, mice receiving T-PPAR Teffs showed no histological signs of colitis. This difference in the mediation of colitis can be seen in typical

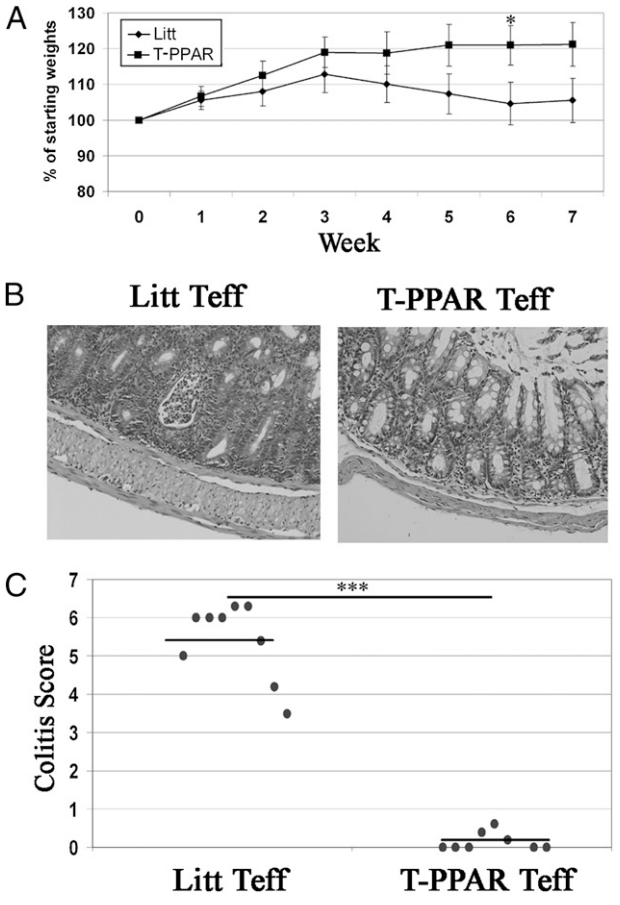

FIGURE 2. T-PPAR Teffs are unable to mediate colitis. Splenic $CD4^+$ $CD25^-$ Teffs were purified from littermate or T-PPAR mice by magnetic bead purification. A total of 0.75–1 $\times$ 10^6 Teffs/mouse were injected i.p. into RAG-$1^{-/-}$ mice. *A*, Mice were weighed once per week. Mean of weekly weights are shown as percentage of mean starting weights (littermate, $n = 14$; T-PPAR, $n = 13$). Error bars represent SEM. *B*, Typical examples of colon histology at 7 wk (original magnification $\times$10). *C*, Compilation and means of histological colitis scores (littermates, $n = 8$; T-PPAR, $n = 8$). The horizontal lines represent the means of the colitis scores. (The top horizontal line shows the two groups that were compared and the statistical significance in the difference of their means.) $^*p < 0.05$, $^{***}p < 0.001$.

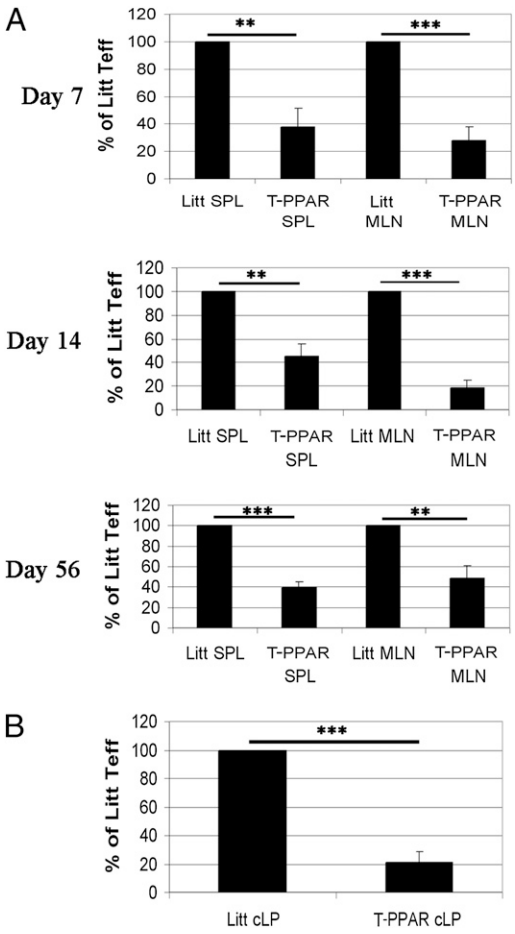

FIGURE 3. T-PPAR $CD4^+$ T cells are defective in accumulating in the SPL, MLN, and cLP. Splenic $CD4^+CD25^-$ Teffs were purified from littermate or T-PPAR mice by magnetic bead purification. A total of 1×10^6 Teffs/mouse was injected i.p. into RAG-$1^{-/-}$ mice. On days 7, 14, and 56 postinjection, SPL and MLN were harvested, counted, and stained for $CD4^+MHCII^-$ cells. *A*, The total number of $CD4^+MHCII^-$ T cells recovered in the SPL and MLN at days 7, 14, and 56 was normalized, with littermate Teffs set at 100% in each individual experiment. Day 7, $n = 7$; day 14, $n = 9$; and day 56, $n = 6$. *B*, The total number of $CD4^+MHCII^-$ T cells recovered in the cLP at day 14 was normalized, with littermate Teffs set at 100% in each individual experiment ($n = 5$). Results of $CD4^+$ T cell recovery in *A* and *B* are depicted as mean percentage of littermate Teffs. Error bars represent SEM. $^{**}p < 0.01$, $^{***}p < 0.001$.

histological examples (Fig. 2*B*) and in the histological scores (Fig. 2*C*). These findings confirmed the results seen with GVHD and indicated that expression of PPARγ in Teffs also plays a role in the development of this adoptive-transfer model of colitis.

T-PPAR Teffs show decreased in vivo accumulation and cytokine production

The colitis model allowed us to assess the accumulation and cytokine production of T-PPAR and littermate Teffs in SPL and MLNs at 7, 14, and 56 d posttransfer. In addition, the accumulation and cytokine production of the transferred Teffs were determined in the cLP on day 14 (at the onset of colitis). Although there was significant variation from experiment to experiment with regard to the total number of $CD4^+$ T cells recovered from the SPL, MLN, and cLP, comparison of the total number of littermate versus T-PPAR $CD4^+$ T cells in each experiment showed a consistent and dramatic decrease in the accumulation of T-PPAR $CD4^+$ T cells in these tissues (e.g., cLP at day 14: littermate Teffs: 0.39×10^6 [SEM, $\pm$ 0.194] and T-PPAR Teffs: 0.07×10^6 [SEM, $\pm$ 0.045]; MLN at day 7: littermate Teffs: 0.15×10^6 [SEM, $\pm$ 0.053] and T-PPAR Teffs: 0.023×10^6 [SEM, $\pm$ 0.01]). Normalizing the results from each individual experiment, such that the littermate $CD4^+$ T cell recovery was set at 100%, we found that the absolute number of T-PPAR $CD4^+$ T cells was decreased 60–80% in the SPL, MLN, and cLP compared with littermate controls. The greatest decrease was noted in the MLN at days 7 and 14 (70 and 80% reduction in total $CD4^+$ T cell numbers; Fig. 3*A*) and in the cLP at day 14 (80% reduction in total $CD4^+$ T cell numbers; Fig. 3*B*).

To determine cytokine production by the $CD4^+$ T cells, SPL and MLN cells were harvested at day 14 after transfer, restimulated, and stained for intracellular IFN-γ and IL-17. The frequency of $CD4^+$ T cells that were IFN-γ^+, IL-17^+, or IFN-γ^+IL-17^+ in the SPL and MLN was similar between littermate and T-PPAR T cells (Fig. 4*A*). The results were similar for SPL and MLN $CD4^+$ T cells at days 7 and 56 after transfer (data not shown). Thus, the significantly fewer T-PPAR $CD4^+$ T cells that are present in the SPL and MLN demonstrate normal frequencies and patterns of cytokine production. However, in contrast to the SPL and MLN, there was a 40–50% decrease in the frequency of IFN-γ^+ T-PPAR $CD4^+$ T cells in the cLP (Fig. 4*A*). Furthermore, despite a decrease in IFN-γ production by T-PPAR cLP $CD4^+$ T cells, these cells showed only a slight increase in the frequency of IL-17 production (Fig. 4*A*). Thus, in contrast to a previous report using a nonlymphopenic autoimmune model (9), we did not find an increase in Th17 T cells at the site of inflammation with PPARγ-deleted $CD4^+$ T cells in our lymphopenic autoimmune model. Overall, when absolute numbers of littermate versus T-PPAR $CD4^+$ T cells are compared, IFN-γ^+, IL-17^+, or IFN-γ^+IL-17^+ T-PPAR T cells were decreased 60–90% in the SPL, MLN and the cLP at day 14 (Fig. 4*B–D*). These results suggested that T-PPAR Teffs are defective in accumulating in both secondary lymphoid organs and tissues and that there is a specific defect in their ability to produce cytokines in inflammatory sites.

T-PPAR $CD4^+$ T cell expression of IL-7Rα and markers of activation

IL-7 is critical in driving and maintaining lymphopenic proliferation. Therefore, we assessed the expression of IL-7Rα in SPL and MLN $CD4^+$ T cells at days 7, 14, and 56 after adoptive transfer into RAG-$1^{-/-}$ mice. We found a decrease in the T-PPAR

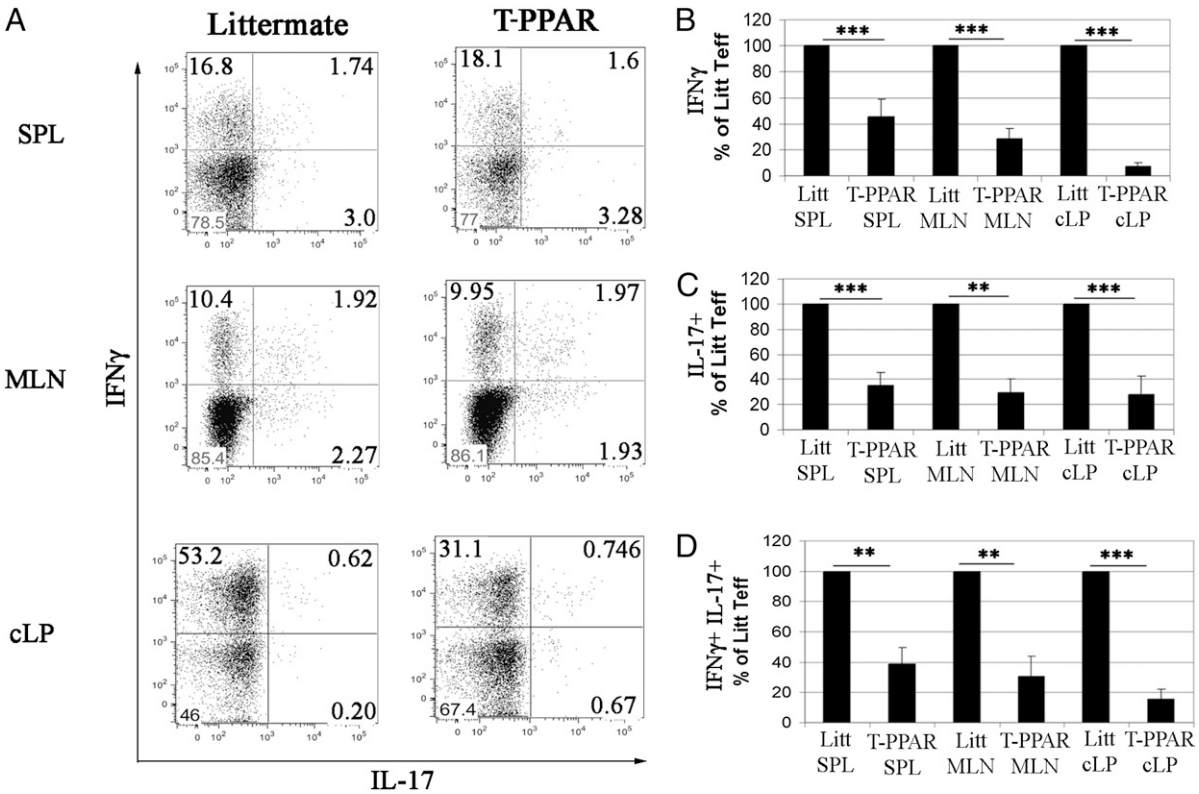

FIGURE 4. T-PPAR $CD4^+$ T cell cytokine production. Splenic $CD4^+CD25^-$ Teffs were purified from littermate or T-PPAR mice by magnetic bead purification. A total of 1×10^6 Teffs/mouse was injected i.p. into RAG-$1^{-/-}$ mice. On day 14, the SPL, MLN, and cLP were harvested; restimulated with plate-bound anti-CD3 Ab, anti-CD28 Ab, and brefeldin A for 4 h; and stained for CD4, MHCII, IL-17, and IFN-γ. *A*, Representative FACS plots, gated on $CD4^+MHCII^-$ cells. *B–D*, Total number of cytokine-producing cells, gated on $CD4^+MHCII^-$ T cells, normalized with littermate Teffs set at 100% in each individual experiment. *B*, IFN-γ^+. *C*, IL-17^+. *D*, IFN-γ^+IL-17^+. IFN-γ: SPL ($n = 7$), MLN ($n = 6$), cLP ($n = 4$). IL-17^+: SPL, MLN, and cLP ($n = 4$). IFN-γ^+ IL-17^+: SPL, MLN, and cLP ($n = 4$). Error bars represent SEM. $^{**}p < 0.01$, $^{***}p < 0.001$.

$CD4^+$ T cell expression of IL-7Rα at day 14 in both the SPL and MLN compared with littermate $CD4^+$ T cells (Fig. 5*A*). Because of experiment-to-experiment variability in the percentage of IL-7Rα expression, this decrease did not reach statistical significance when all of the day 14 experiments were compiled. However, normalizing the results from each individual experiment, such that the percentage of littermate $CD4^+$ T cells expressing IL-7Rα expression was set at 100% within individual experiments, revealed a statistically significant decrease in T-PPAR $CD4^+$ T cell IL-7Rα expression in the MLN at day 14 (Fig. 5*B*). A decrease in T-PPAR $CD4^+$ T cell IL-7Rα expression was not seen in the MLN at days 7 or 56 after adoptive transfer, nor was there a statistically significant decrease in T-PPAR $CD4^+$ T cell IL-7Rα expression in the SPL or cLP at any time point (Fig. 5). These results suggested that a decreased expression of IL-7Rα is unlikely to be responsible for the abnormal accumulation of T-PPAR $CD4^+$ T cells in this colitis model. However, we cannot completely rule out this possibility, given the recent demonstration that even small decreases in IL-7Rα expression can diminish the proliferative response of $CD8^+$ T cells in vitro (28).

A defect in activation is another mechanism potentially underlying the abnormal accumulation of T-PPAR $CD4^+$ T cells. To address this question, we analyzed the expression of activation markers by T-PPAR and littermate $CD4^+$ T cells in the SPL and MLN at day 7 after adoptive transfer into RAG-$1^{-/-}$ mice (i.e., before the onset of colitis), as well as at days 14 and 56 (after the onset of colitis). We measured the expression of CD44, CD25, CD69, and CD62L and found no difference in these markers on T-PPAR versus littermate $CD4^+$ T cells at any time point (data not shown). These results suggested that there is no defect in the in vivo activation of T-PPAR Teffs.

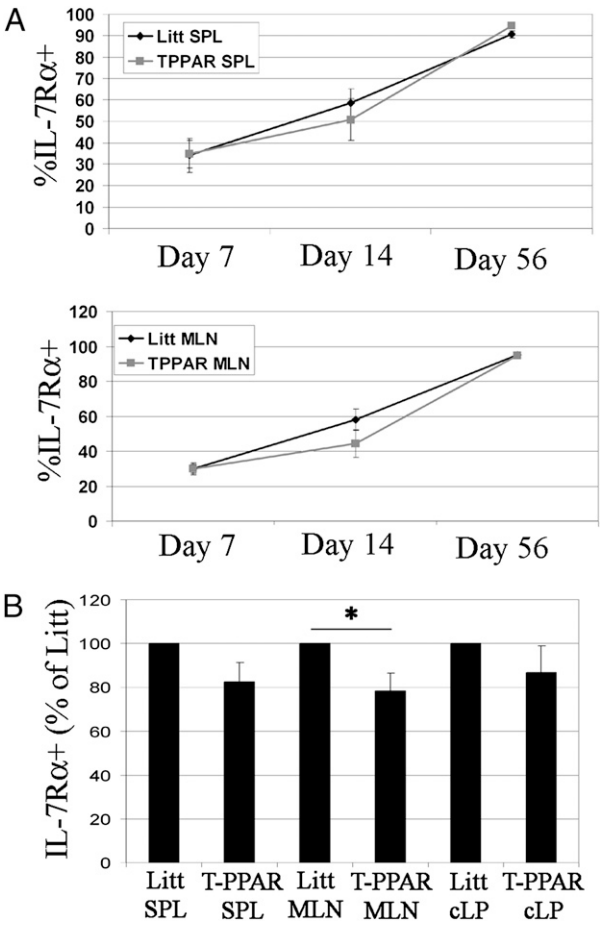

FIGURE 5. T-PPAR $CD4^+$ T cell expression of IL-7Rα. Splenic $CD4^+$ $CD25^-$ Teffs were purified from littermate or T-PPAR mice by magnetic bead purification. A total of 1×10^6 Teffs/mouse was injected i.p. into RAG-$1^{-/-}$ mice. On the days indicated, SPL and MLN were harvested and stained for CD4, MHCII, and IL-7Rα. *A*, Mean percentage of IL-7Rα expression, gated on $CD4^+MHCII^-$ cells, on day 7 ($n = 5$), day 14 ($n = 8$), and day 56 ($n = 6$) after adoptive transfer in the SPL and MLN. *B*, Mean percentage of IL-7Rα expression at day 14, gated on $CD4^+MHCII^-$ cells, in SPL ($n = 7$), MLN ($n = 8$), and cLP ($n = 4$), normalized with littermate Teffs set at 100% in each individual experiment. Error bars represent SEM. $*p < 0.05$.

T-PPAR $CD4^+$ T cells show decreased expression of the gut-homing molecule α4β7

The deletion of Teff PPARγ may control the development of colitis by altering the ability of Teffs to home to the gut. To address this possibility, $CD4^+$ T cells in the MLN and SPL were stained for the gut-homing molecule α4β7 at day 7 after adoptive transfer. We found that there was a statistically significant decrease in expression of α4β7 in T-PPAR versus littermate $CD4^+$ T cells in both the MLN (48% littermate, 32% T-PPAR) and SPL (20% littermate, 13% T-PPAR) at day 7 (Fig. 6*A*).

Although this decreased expression of α4β7 in T-PPAR $CD4^+$ T cells could theoretically play a role in the decreased development of colitis after adoptive transfer of T-PPAR Teffs, it is unlikely to explain the inability to mediate GVHD. The pathology and disease manifestations in the Bm12 model of GVHD are known to be related to bone marrow failure, with little, if any, involvement of colitis (29, 30). Furthermore, we confirmed that Bm12 mice undergoing GVHD do not demonstrate histological changes of colitis (data not shown). The fact that T-PPAR Teffs are incapable of mediating Bm12 GVHD, taken together with the concept that the decreased accumulation of T-PPAR $CD4^+$ T cells in the SPL and MLN after transfer into RAG-$1^{-/-}$ mice cannot be accounted for by a decrease in α4β7 expression, suggested that the defect in α4β7 expression is likely not the primary abnormality underlying the inability of T-PPAR Teffs to mediate lymphopenic autoimmune disease.

T-PPAR $CD4^+$ T cells demonstrate decreased proliferation and increased cell death in vivo

To determine whether there is a difference in proliferation between T-PPAR and littermate Teffs after adoptive transfer into RAG-$1^{-/-}$ mice, we assessed the expression of the cell cycle marker Ki67 in SPL and MLN $CD4^+$ T cells 7 d after transfer. We found that the mean percentage of $Ki67^+CD4^+$ T cells was significantly lower for T-PPAR cells than for littermate cells in both the MLN (30 and 52%, respectively) and SPL (15 and 30%, respectively) (Fig. 6*B*). These results suggested that T-PPAR Teffs have a significant defect in their ability to proliferate in vivo (as measured by the expression of Ki67) in this model of autoimmunity.

To determine whether there is a difference in cell survival between T-PPAR and littermate Teffs after adoptive transfer into RAG-$1^{-/-}$ mice, we stained SPL and MLN for CD4 and Annexin V 7 d after transfer. We found a significant increase in the frequency of MLN T-PPAR $CD4^+$ T cells that were Annexin V^+ and, thus, apoptotic compared with littermate $CD4^+$ T cells (84 and 58%, respectively) (Fig. 6*C*). We found no significant difference in the frequency of T-PPAR versus littermate Annexin V^+CD4^+ T cells in the SPL (data not shown). Taken together, these results suggested that T cell PPARγ is involved in regulating both the proliferation and survival of $CD4^+$ T cells in lymphopenic models of autoimmunity.

Both antigenic stimulation and lymphopenia are required for defective function of T-PPAR $CD4^+$ T cells

The colitis and GVHD models require both lymphopenia and antigenic stimulation for disease development. In the GVHD

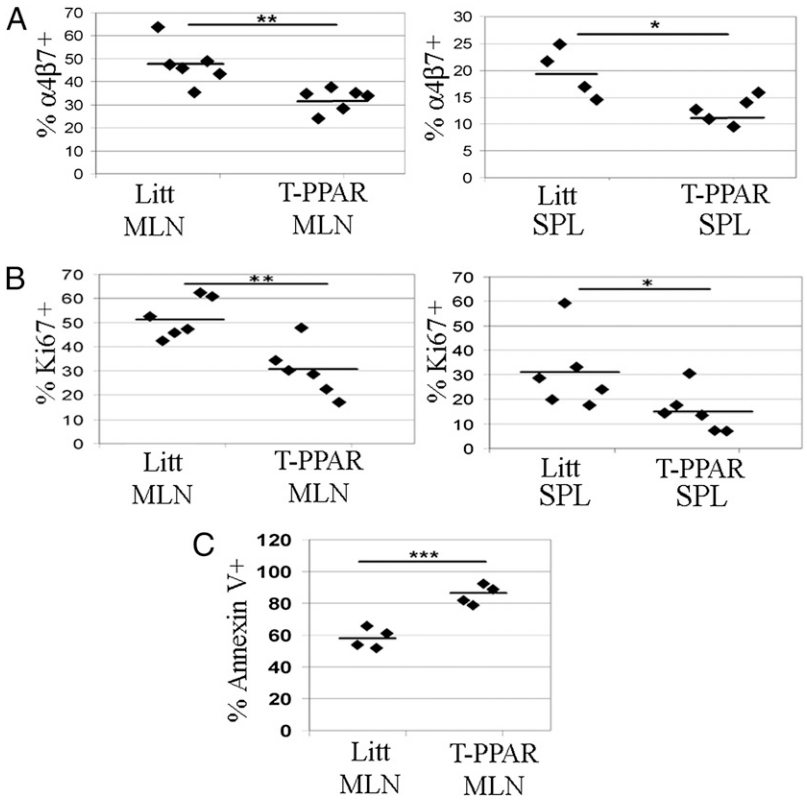

FIGURE 6. T-PPAR $CD4^+$ T cells show decreased expression of α4β7, decreased proliferation, and increased apoptosis. Splenic $CD4^+CD25^-$ Teffs were purified from littermate or T-PPAR mice by magnetic bead purification. A total of 1×10^6 Teffs/mouse were injected i.p. into RAG-$1^{-/-}$ mice. On day 7, SPL and MLN were harvested and stained for CD4, as well as stained for MHCII, as indicated. *A*, Expression of α4β7 on the $CD4^+MHCII^-$ population in the SPL and MLN. *B*, Expression of Ki67 on the $CD4^+MHCII^-$ population in the SPL and MLN. *C*, Expression of Annexin V on the $CD4^+$ population in the MLN. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

model, in addition to the lymphopenia resulting from the sublethal irradiation, antigenic stimulation results from the alloantigen response. In the colitis model, in addition to the lymphopenia inherent in the RAG-$1^{-/-}$ mice, the disease requires the presence of colonic bacteria and is postulated to involve antigenic stimulation by these bacteria (31). Given the combination of lymphopenia and antigenic stimulus involved in these autoimmune models, we next asked whether the defect in T-PPAR Teffs would be elicited by a lymphopenic environment alone. To address this question, littermate and T-PPAR mice were sublethally irradiated (600 rad), and the recovery and activation of the endogenous $CD4^+$ T cell populations were evaluated at days 7, 14, and 56 after irradiation.

In contrast to the substantial reduction in T-PPAR $CD4^+$ T cells recovered after transfer into RAG-$1^{-/-}$ mice, there was no statistically significant difference in the number of $CD4^+$ T cells recovered in the SPL or MLN from irradiated littermate versus T-PPAR mice at 7, 14, and 56 d postirradiation (Fig. 7). In addition, there were no differences observed in the proliferation (via Ki67 staining) of T-PPAR Teffs compared with littermate controls at these time points (data not shown). These results suggested that lymphopenia alone is not sufficient to elicit the defects in proliferation and survival that we found in T-PPAR Teffs and that antigenic stimulation, along with lymphopenia, is required for the elicitation of these abnormalities.

$CD8^+$ T cells from T-PPAR mice accumulate normally in RAG-$1^{-/-}$ mice

The transgenic CD4-Cre construct used in the generation of the T-PPAR mice (11) is first expressed in T cells at the double-positive stage of thymic development and was documented to be expressed in both mature CD4 and CD8 single-positive T cells (32). Thus, $CD8^+$ T cells, as well as $CD4^+$ T cells, in T-PPAR mice are deficient in PPARγ expression. To determine whether T-PPAR $CD8^+$ T cells would also have defects in proliferation and survival after adoptive transfer into RAG-$1^{-/-}$ mice, $CD8^+$ T cells were purified from the SPL of littermate and T-PPAR mice and transferred into RAG-$1^{-/-}$ mice. At 7 and 14 d after adoptive transfer, the total number of $CD8^+$ T cells in the SPL and MLN of the recipient RAG-$1^{-/-}$ mice was determined. Unlike T-PPAR $CD4^+$ T cells, $CD8^+$ T cells from T-PPAR mice showed no significant difference from littermate $CD8^+$ T cells in accumulation in the SPL or MLN

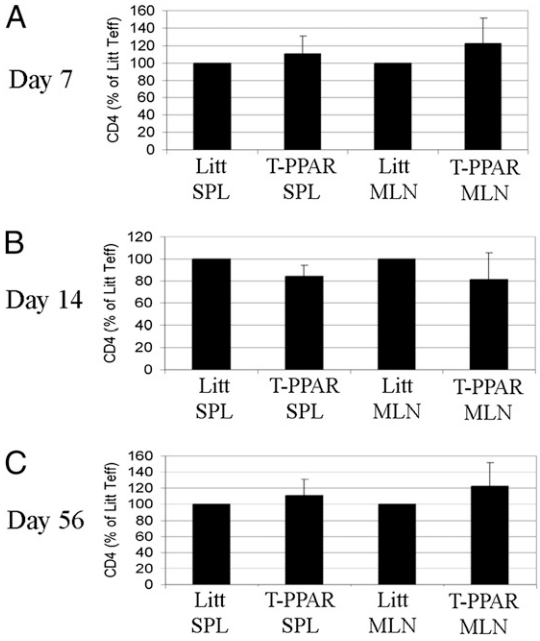

FIGURE 7. Irradiated T-PPAR mice show no defect in $CD4^+$ T cell accumulation. Littermate and T-PPAR mice were sublethally irradiated (600 rad), and SPL and MLN were harvested at (*A*) day 7 ($n = 6$), (*B*) day 14 ($n = 5$), and (*C*) day 56 ($n = 5$) after irradiation. Mean total T-PPAR $CD4^+MHCII^-$ cells are shown normalized to littermate Teffs. Error bars represent SEM.

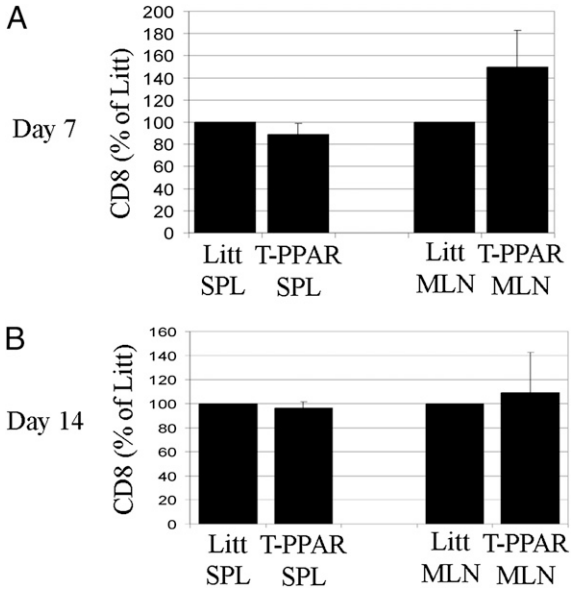

FIGURE 8. CD8$^+$ T cells from T-PPAR mice do not show a defect in accumulation. Splenic CD8$^+$ T cells were purified from littermate or T-PPAR mice by magnetic bead purification. A total of 1×10^6 CD8$^+$ T cells/mouse was injected i.p. into RAG-1$^{-/-}$ mice. MLN and SPL were harvested on days 7 and 14 after transfer and stained for CD8 and MHCII. *A*, Day 7 posttransfer (SPL, $n = 6$; MLN, $n = 6$). *B*, Day 14 posttransfer (SPL, $n = 3$; MLN, $n = 4$). Results shown are means of total CD8$^+$ T cells normalized to total littermate CD8$^+$ T cells. Error bars represent SEM.

(Fig. 8). Although not statistically significant, there was actually an increase in the total number of T-PPAR CD8$^+$ T cells in the MLN at day 7; however, this was not observed in the MLN at day 14, nor in the SPL at any time point. In addition, no differences were observed in the expression of activation markers by T-PPAR versus littermate CD8$^+$ T cells in the SPL or MLN (data not shown).

Finally, we also examined α4β7 expression and the percentage of Ki67$^+$ and Annexin V$^+$ cells among the SPL and MLN CD8$^+$ T cells at day 7 after adoptive transfer. We found that T-PPAR CD8$^+$ T cells did not differ from littermate CD8$^+$ T cells with regard to α4β7 expression or the percentage of Ki67$^+$ and Annexin V$^+$ cells (Fig. 9). Overall, these results suggested that PPARγ deletion in CD4$^+$ T cells, but not in CD8$^+$ T cells, results in the abnormalities in proliferation and survival that we found in lymphopenic models of autoimmunity.

Discussion

We and other investigators previously demonstrated that PPARγ mediates regulatory functions in conventional T cells, natural Tregs, macrophages, and dendritic cells and enhances the generation and stability of inducible Tregs (2–6, 10–12, 33). Numerous studies suggested that there is an important link between lymphopenic proliferation and autoimmunity (23). Despite the potential relevance of lymphopenia in the development of autoimmunity and the potential importance of PPARγ as an immunoregulator and therapeutic target in autoimmune diseases, there have been no prior studies of the role of PPARγ in regulating autoimmunity in lymphopenic conditions. We investigated this issue in the present studies and surprisingly found that, rather than having a down-regulatory role, T cell PPARγ is actually required for the mediation of autoimmunity.

Our current results demonstrated that T-PPAR Teffs are unable to cause disease in two murine models of lymphopenic autoimmunity: GVHD and colitis. Using the adoptive-transfer model of colitis, we found that T-PPAR Teffs demonstrated a number of abnormalities, the most glaring of which is a markedly decreased

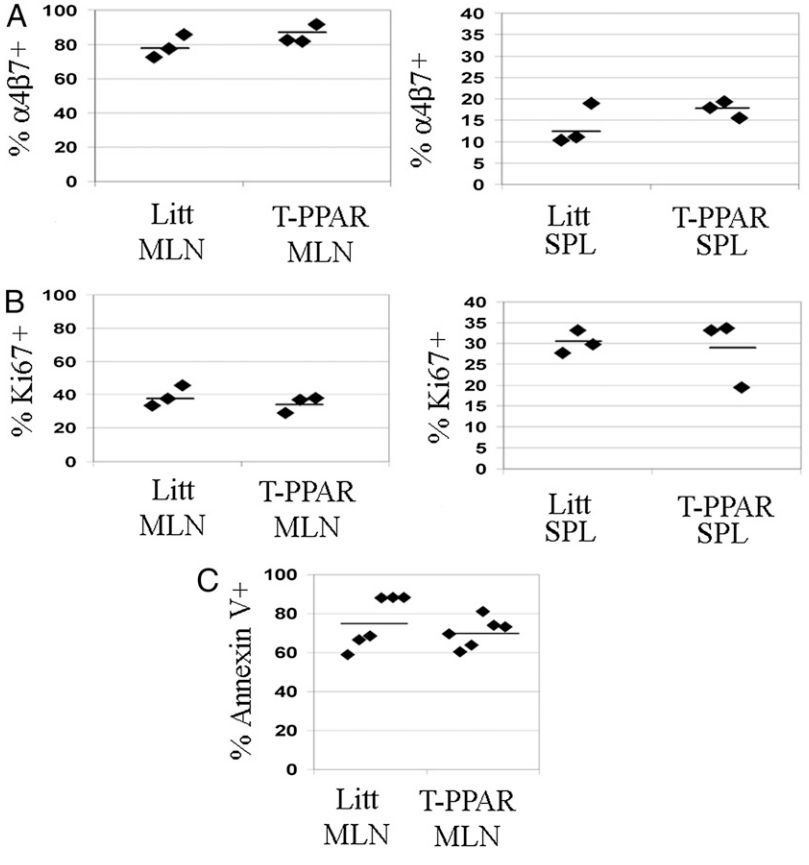

FIGURE 9. CD8$^+$ T cells from T-PPAR mice do not show abnormalities in expression of α4β7, proliferation, or apoptosis. Splenic CD8$^+$ T cells were purified from littermate or T-PPAR mice by magnetic bead purification. A total of 1×10^6 CD8$^+$ T cells/mouse was injected i.p. into RAG-1$^{-/-}$ mice. MLNs and SPL were harvested on day 7 and stained for CD8, MHCII, and (*A*) α4β7 or (*B*) Ki67. *C*, MLNs were harvested on day 7 and stained for CD8 and Annexin V. Results shown are gated on CD8$^+$MHCII$^-$ cells (*A*, *B*) or CD8$^+$ cells (*C*).

accumulation in SPL, MLN, and cLP. Mechanistically, this decreased accumulation of adoptively transferred T-PPAR Teffs seems to stem from both decreased proliferation (assessed by Ki67 staining) and increased apoptosis (assessed by Annexin V staining). We found no difference in littermate versus T-PPAR endogenous $CD4^+$ T cell accumulation after sublethal irradiation of littermate and T-PPAR mice themselves. It is important to note that the colitis and GVHD models involve both lymphopenia and antigenic stimulation in disease development. Thus, the results with the directly irradiated T-PPAR mice suggested that the defect in accumulation in T-PPAR $CD4^+$ T cells is not seen with lymphopenic stimulation alone but requires a combination of lymphopenia and an additional strong antigenic stimulus.

Studies of PPARγ's effects on survival of T cells in vitro have yielded conflicting results, with some studies showing a proapoptotic effect and others reporting a prosurvival effect (34). Harris and Phipps (4) demonstrated that activation of T cells in vitro in the context of TZDs led to increased T cell apoptosis, but this effect may have resulted from effects not mediated through PPARγ. However, consistent with the concept of increased T cell apoptosis with PPARγ activation, it was reported that PPARγ can play a role in the T cell apoptosis seen in sepsis (35, 36). In contrast to these proapoptotic effects of PPARγ activation in T cells, it was also demonstrated that activation of PPARγ resulted in increased in vitro survival of T cells and T lymphoma cells under conditions of cytokine withdrawal or serum starvation (34, 37, 38). Thus, our present findings may represent an in vivo equivalent to these in vitro cytokine-deprivation studies. It may be that the primary mechanism underlying the inability of PPARγ-deficient Teffs to mediate lymphopenic autoimmunity is an increased sensitivity to the limiting levels of growth factors available under these highly activating and proliferative conditions in vivo.

Our current findings suggested that the few T-PPAR $CD4^+$ T cells recovered in the SPL and MLN during the development of colitis showed a similar pattern of cytokine production as did the littermate $CD4^+$ T cells. However, the few T-PPAR $CD4^+$ T cells recovered in the cLP showed a decrease in IFN-γ production. It was recently reported that PPARγ plays a role in regulating Th17 differentiation and that PPARγ-deficient T cells demonstrate enhanced early disease severity in EAE associated with increased Th17 T cells infiltrating the CNS. These results led to the conclusion that T cell PPARγ, interacting with endogenous ligands, may normally play a role in downregulating T cell production of IL-17 (9). In the lymphopenic colitis model, we did not find a significant increase in IL-17 production from T-PPAR $CD4^+$ T cells in any of the tissues tested. The differences in IL-17 production between the EAE and colitis models provide further evidence of the multifunctional role of PPARγ in both driving and suppressing gene expression. Furthermore, this multifunctional role is likely dependent, at least in part, on the context in which the specific cells are functioning. As such, our results suggest a novel role for PPARγ, specifically in the context of lymphopenia-associated autoimmunity.

We identified decreased expression of $\alpha4\beta7$ by T-PPAR $CD4^+$ T cells in vivo. This decreased expression, seen in both the MLN and SPL at day 7, could theoretically play a role in the decreased development of colitis after adoptive transfer of T-PPAR Teffs. However, the fact that colitis does not play a significant role in the pathology of Bm12 GVHD (29, 30), taken together with the concept that the decreased accumulation of T-PPAR $CD4^+$ T cells in the SPL and MLN cannot be accounted for by a decrease in $\alpha4\beta7$ expression, indicated that the defect in $\alpha4\beta7$ expression likely is not the primary abnormality underlying the inability of T-PPAR Teffs to mediate lymphopenic autoimmune disease.

T-PPAR Teffs also demonstrated a decreased expression of IL-7Rα at day 14 in the MLN. This decrease in T-PPAR $CD4^+$ T cell IL-7Rα expression was not seen at day 7 or 56 in the MLN and was not significantly decreased at any point in the SPL. These results suggested that a decreased expression of IL-7Rα (and a resulting decrease in IL-7–mediated proliferation and survival) is unlikely to be responsible for the decreased accumulation of T-PPAR $CD4^+$ T cells in these lymphopenic autoimmune settings. However, we cannot completely rule out this possibility, given that even small decreases in IL-7Rα expression can diminish the in vitro proliferative response of $CD8^+$ T cells (28).

In contrast to T-PPAR $CD4^+$ T cells, we did not find an abnormality in accumulation, proliferation, or apoptosis in T-PPAR $CD8^+$ T cells. This suggested that these defects are specific for PPARγ-deficient $CD4^+$ T cells. The reason for this difference in $CD4^+$ versus $CD8^+$ PPARγ-deficient T cell accumulation is unclear. However, $CD8^+$ T cells adoptively transferred into RAG-$1^{-/-}$ mice do not mediate colitis and, therefore, may not receive the strong antigenic stimulation seen by the colitis-mediating $CD4^+$ T cells. Interestingly, these results may suggest that TZDs are particularly effective at suppressing inflammatory conditions that are primarily mediated by $CD8^+$ T cells, because they would maintain the immunoregulatory effects while not causing an increase in T cell survival. Future studies will explore this issue further.

Finally, the TZD class of PPARγ ligands was shown to be effective at treating a number of autoimmune conditions in both mice and humans, and there is significant interest in using these drugs clinically in autoimmune and other inflammatory conditions (19–22, 39). Although the immunoregulatory effects of PPARγ activation are well documented, our results now suggest a PPARγ paradox in autoimmunity; although ligation of PPARγ may ameliorate autoimmune inflammation under nonlymphopenic conditions, $CD4^+$ T cell PPARγ may also allow for increased survival of autoreactive T cells in lymphopenic conditions. Our results highlight the complexity of PPARγ function in T cells and may suggest a new caveat in the potential role of PPARγ activation as a therapeutic modality in autoimmunity.

Disclosures

The authors have no financial conflicts of interest.

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