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Modulation of TH1 differentiation Peroxisome Proliferator-Activated Receptorα-Independent Manner

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ABSTRACT

The influence of PPAR α on numerous biological activities arises through its ability to carry out gene regulation. We examined the effects of PPARα on the expression of on key transcription factors involved in Th cell differentiation, T-bet, and determined whether PPARα mediates its effects. The T-cells from PPAR α null mice secreted higher IFN γ and lower IL-2 concentrations than WT T-cells. We demonstrate that PPAR α regulates the expression of these cytokines by CD4+ T cells, through its ability to negatively regulate TH1 differentiation via the transcription of T-bet both at mRNA and protein levels. T-cells from PPAR α null mice expressed higher p38 phosphorylation than WT T-cells. T-bet expression in CD4+ T cells was determined to be influenced by p38 mitogen-activated protein (MAP) kinase activation. The presence of WY14,643, PPAR α suppressed the phosphorylation of p38 MAP kinase in both the cell types. The pharmacological inhibitors of MAP kinases also downregulated T-bet in T-cells.

Abbreviations:
ERK, extracellular signal-regulated kinase; interleukin, IL; MAP, mitogen-activated protein; P38 MAPK, mitogen-activated protein kinase P38; PPARα, peroxisome proliferator-activated receptorα; T-bet, T-box expressed in T cells; WT, wild type.

Introduction
PPARs are ligand-activated transcriptional factors that regulate a large number of genes by transcriptional activation and repression (1). The three isotypes have been identified in lower vertebrates and mammals (2). PPARα, PPARβ (δ), and PPARγ exhibit different tissue distribution as well as different ligand specificities and functions (3). PPARα is highly expressed in the liver and brown adipose tissue and regulates lipid homeostasis. PPARα is activated by natural ligands, such as fatty acids, as well as the lipid-lowering fibrates, which are used clinically for the treatment of hypertriglyceridemia (4, 5). These agents have been shown to exert beneficial effects in autoimmune diseases and atherosclerosis (6-7).

PPARα controls positively the fatty acid transport and oxidation in the liver (2). Thus, PPARα plays an important role in the regulation of chronic diseases such as diabetes, obesity, and atherosclerosis.

In addition to adipocytes and liver, it has been shown that cells of monocyte/macrophage lineage express both PPARα and PPARγ, indicating a possible role of these receptors in immune function (8-9). Several investigators have reported that PPARα is expressed in B and T cells, and its expression wanes soon after lymphocyte activation (10, 11). Indeed, PPARα ligands have been shown to regulate inflammatory responses because they can inhibit production of IL-2, a T helper (Th) 1 cytokine, and T cell proliferation (6). PPARα ligands have also been shown to increase IL-4 expression, a Th2 cytokine (11). Most of these results argue for an immunosuppressive effect of PPARα that may promote Th2 immunity, necessary for a successful pregnancy (12).

PPAR positively regulate the expression of genes under their transcriptional control by binding to specific DNA sequences known as peroxisome proliferator response elements as a heterodimeric complex with the 9-cis-retinoic acid receptor. In the unliganded state PPARα is thought to be transcriptionally inert, due to its physical association with the nuclear co-repressors N-CoR and SMRT (13). Following ligand activation, the nuclear co-repressors dissociate from PPARα, thus enabling it to bind nuclear receptor co-activators such as SRC-1 and CBP/p300. These protein complexes restructure the chromatin template through histone acetylation, and allow the basal transcriptional machinery to access the promoter regions driving transcription of target genes under PPAR control (14, 15, 16, 17).

PPARα is highly expressed in the liver and brown adipose tissue and regulates lipid homeostasis. We have recently shown that the inflammation in the adipose tissues is also regulated by PPARα (18). PPARα is activated by natural ligands, like DHA, as well as, the lipid-lowering fibrates which are used clinically for the treatment of hypertriglyceridaemia (4, 5). These agents have been shown to exert beneficial effects in autoimmune diseases and atherosclerosis (11, 20). Several investigators have reported that PPARα is expressed in B and T-cells and its expression wanes soon after lymphocyte activation (10,11). Indeed, PPARα ligands have been shown to regulate inflammatory responses as they can inhibit production of IL-2, a T_H1 cytokine, and T-cell proliferation (6). PPARα ligands have also been shown to increase IL-4 expression, a T_H2 cytokine (12). Most of these results argue for an immunomodulatory effect of PPARα which may promote T_H2 immunity (11,18).

Although demonstrated to be both transactivation and transrepression competent within lymphocytes, the role(s) of PPARα in lymphocyte biology remains largely unknown. To gain further insight into the physiological function of PPARα within lymphocytes, we investigated physiological responses by T cells isolated from $PPAR\alpha^{null}$ mice as well as responses elicited by T cell lines that overexpress PPARα. In this study, we present experiments that describe a role for PPARα in T cell activation. We show that unliganded PPARα has the ability to negatively regulate the transcription of T-bet, an inducible transcription factor in lymphocytes that is important in the initiation and termination of activation-induced cytokine gene transcription (19). By controlling the initiation of T-bet transcription, PPARα was able to indirectly influence the level of activation- induced IFN-γ pro-

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duced by CD4+T cells. Furthermore, we report that the control of PPARα over T-bet expression occurs via a DNA-binding independent mechanism, mediated through the ability of PPARα to repress the phosphorylation of p38 mitogen-activated protein (MAP) kinase following T cell activation.

Materials and Methods

Animals

The study was performed on wild type (WT) mice (Charles River, Les Oncins, France) and homozygous PPARα^{null} (PPARα-knockout) mice of C57BL/6J genetic background (20). (The Jackson Laboratory, Bar Harbour, ME, USA). Mice were housed individually in wood chip-bedded plastic cages at constant temperature (25°C) and humidity (60±5%) with a 12-h light-dark cycle. The derivation and phenotypic characteristics of these animals have previously been reported (20). PPARα^{null} mice fail to express a functional PPARα protein in all tissues, including CD4^{+} T cells (20).

The experimental protocol has been approved by Benin's ethic commission in experimental research with animals according to the international conventions.

Dynabead cell enrichment

For the preparation of CD4+ T-cells, freshly isolated splenic lymphoid cells were suspended at a concentration of $2x10^7$ cells/ml in RPMI-1640 medium, containing 5% foetal bovine serum (v/v). The erythrocytes present in the cell suspension were lysed by brief treatment with sterile aqueous 0.83% (w/v) ammonium chloride. The cell suspension was incubated with 2 µg/ml each of biotinylated anti-CD45R/B220, anti-CD11b, and anti-CD8 antibodies (BD PharMingen, San Diego, CA) for 20 min on ice. Following washing with phosphate buffered saline (PBS), the cells were resuspended with M-280 magnetic Dynabeads coated with streptavidin (Dynal, New York, NY), and incubated at a bead/cell ratio of 1:1 for 20 min with agitation at 4°C. The residual cells were collected, washed, and separated for use in culture or for mRNA analysis. The level of purity of the cell preparations was assessed, in FACS, by staining cells with FITC-anti-mouse CD4, FITC-anti-mouse CD8, and FITC-anti-mouse B220 antibodies. The level of cell purity was routinely >90%.

ELISA

Freshly isolated CD4+ T-cells were activated in multiwell plates, pre-coated with immobilized anti-CD3 (2 µg/ml) and anti-CD28 (1 µg/ml) antibodies, at 37°C in an atmosphere of 5% CO_2 in air. Cell culture supernatants were collected for quantitative evaluation of immunoactive IL-2, IFN-γ and IL-4 by ELISA, as described elsewhere (21). Rat anti-murine cytokine mAbs and murine rIL-2 and rIFN-γ cytokine standards were purchased from BD PharMingen (San Diego, CA).

RT-PCR quantification assay:

Total mouse liver's RNA was isolated by extraction using Trizol reagent (Invitrogen Life Technologies, Groningen, the Nederland) according to the manufacturer's instructions. The integrity of RNA was electrophoretically checked by ethidium bromide staining and by the OD absorption ratio OD_{260nm}/OD_{280nm}.A microgram of RNA was reversibly transcribed with Superscript II RNAze H-reverse transcriptase using oligo (dT) according to the manufacturer's instructions (Invitrogen Life Technology, France).

Real time -PCR was performed on an iCycle, real time of detection system (Bio-rad, Hercules, CA, USA),and amplification was done by using SYBR Green I detection(SYBR Green Jumpstart, Taq Ready Mix for quantitative PCR, Sigma-Aldrich, St Louis; MO USA). Oligonucleotide primers (***Table 1)***, used for mRNA analysis, were based on the sequences of mice gene in the Gene Bank database.

Table 1 *Primer and sequences used in mRNA quantification by real-time PCR*

GENES	PRIMER'S SEQUENCES	
	Forward	Reverse
β-actin	5'- GGCACCACACC TTCTACAATGAGC -3'	5'- CGACCAGAGGCAT ACAGGGACAG -3'
T-bet	5'- AGTATGTCGTG GAGTCTA -3'	5'- CATACTTGGCAGG TTTCT -3'
IFN-γ	5'- CTT CCT CAT G GC TGT TTC TGG-3'	5'- CGA CTC CTT TTC CGC TTC CTG -3'

The reverse transcriptase reaction was diluted and an aliquot was subjected to amplification by PCR with the gene-specific primers listed.

The amplification was carried out in a total volume of 25µl containing 12.5µl SYBR Green Taq Ready Mix, 0.3µM of each primer and diluted cDNA. Cycling condition consisted to an initial denaturation step of 95°C for 3 minutes a hot start followed by 40 cycles of 95°C for 30 sec or at 60°C for 30sec with a simple fluorescence detection point at the end of the relevant annealing or extension segment.

At the end of the PCR, the temperature was increased from 60 to 90°C for 15sec and at 58±2°C for 60sec, and the fluorescence was measured every 15sec to draw the melting curve. The standard curves were generated for each protein or β-actin using serial dilution of positive control template in order to establish PCR efficiencies. All determinations were performed at least in duplicates using two dilutions of each assay to achieve reproducibility. Results were evaluated by iCycler iQ software including standard curves, amplification efficiency(E) and threshold cycle(Ct). Relative quantization of mRNA expression was determine using the ΔΔCt in which ΔΔCt= ΔCt (gene of interest)−ΔCt(β-actin). ΔCt= Ct (interest group) –Ct (control group). Relative quantity (RQ) was calculated as follow: $RQ= (1+E)^{(-\Delta\Delta Ct)}$.

Preparation of nuclear extracts and immunoblot analysis

Nuclear extracts were prepared from T-cells following treatment for various times with immobilized anti-CD3 and anti-CD28, as described elsewhere (22). Briefly, cells were washed twice with ice-cold PBS containing 1 mM PMSF, resuspended in 250 µl buffer A (10 mM HEPES, pH 7.8, 0.1 mM EDTA, 10 mM NaCl, 3 mM $MgCl_2$, 300 mM sucrose, 10 µg/ml aprotinin, 100 µM leupeptin, 1 mM DTT, and 1 mM PMSF), and incubated on ice for 10 min. Then, 25 µl of 1% Nonidet P-40 was added and mixed carefully. Cells were collected by centrifugation at 800 x g for 1 min at 4°C and washed with 200 µl buffer A. Nuclei were then resuspended in 50 µl buffer B (20 mM HEPES, pH 7.8, 3 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 25% glycerol, 10 µg/ml aprotinin, 100 µM leupeptin, 1 mM DTT, and 1 mM PMSF) and incubated for 15 min on ice. Nuclear debris was removed by centrifugation at 16,000 x g for 1 min. In some experiments, cells were pretreated with the extracellular signal-regulated p38 MAP kinase inhibitor SB202190 (Alexis Biochemicals, San Diego, CA) before activation. Whole cell extracts used in the analysis of the MAP kinases were generated, as described elsewhere (26, 23).

The supernatant was then removed, and protein content was determined by Bradford Assay. Equal amounts of nuclear protein were subjected to 10% SDS-PAGE and polyvinylidene difluoride membrane (Millipore, Bedford, MA) (23). After blocking with 5% nonfat milk TBS, blots were incubated with either anti-T-bet or anti- IFN-γ antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). The MAP kinases in the cytosolic fractions were detected by the antibodies raised against phosphorylated forms of p38, (Ozyme/Cell Signaling, Beverly, MA). Membranes were incubated with these antibodies for 1hr and then washed, and incubated with goat anti-rabbit HRP conjugate (1/2000 dilution in TBS-Tween) for 45 min at room temperature. After washing, bands were visualized using a chemiluminescence kit, according to the manufacturer's instructions (Santa Cruz Biotechnology, Santa Cruz, CA).

Statistical analysis
Results are shown as means±SEM. The significance of the differences between mean values was determined by two-way ANOVA (STATISTICA, Version 4.1, Stat soft, Paris, France), followed by the least significant difference (LSD) test. Differences were considered significant at $P < 0.05$.

Results
PPARα modulated the secretion of cytokines
Figure.1 shows that, in both the strains of mice, under the same activating conditions it is interesting to note that T-cells of PPARα^{null} mice secreted higher IFNγ, but CD4$^+$ T cells from PPARα^{null} mice produced lower amounts of IL-2 than T cells from the WT animals.

T-bet activated in the absence of anti-IFN-γ
Since PPARα modulated the secretion of cytokines into the extracellular environment, we measured the expression of T-bet mRNA in T-cells of WT and PPARα ^{null} mice. T-bet protein levels in the nuclear fractions were analyzed by Western blot at 24 h postactivation. Freshly isolated T-cells were activated in multiwell plates, pre-coated with immobilized anti-CD3

(2 μg/ml) and anti-CD28 (1 μg/ml) antibodies the absence or presence of 10 g/ml anti- IFN-γ., at 37°C in an atmosphere of 5% CO2 in air. WT T cells were severely compromised in their ability to up-regulate expression of T-bet mRNA when activated in the presence of anti- IFN-γ, but were able to up-regulate expression of T-bet when activated in the absence of anti- IFN-γ (Figure.2).

IFN- γ produced in PPARα ^{null} activated in the presence of anti-IFN-γ
Under the same activating conditions it is interesting to note that in the presence of anti- IFN-γ, they were induced to express both T-bet mRNA and protein. This correlated with retention in their ability to express mRNA for IFN-γ. PPARα regulates the modulation of TH1 differentiation by independent of IFN-γ signalling (Figure.3).

WY14, 643, an agonist of PPARα, exerts inhibition of T-bet expression independently of PPARα
In order to examine the role of PPARα gene in modulated T-cell activation, we employed WY14,643 an agonist of PPARα. The level of T-bet mRNA was quantitated at 6 h postactivation and protein contents were analyzed in nuclear fractions by western blot at 24h postactivation. In WT T-cells, WY14,643 highly downregulated the expression of T-bet both at protein and transcript levels (Fig. 4). As far as PPARα^{null} T-cells are concerned, we observed that WY14,643 inhibited T-bet expression both at protein and mRNA levels.

A chemical inhibitor of p38 MAP kinase downregulated the expression of T-bet mRNA and protein expression.
We also pretreated CD4$^+$ T cells with SB202190, a chemical inhibitor of p38 MAP kinase. The T cells were then activated with anti-CD3 plus anti-CD28 antibodies. As shown in Figure 5, SB202190 downregulated the expression of T-bet mRNA transcripts both in WT and PPARα^{null} T-cells.

WY14,643 inhibited the phosphorylation of the p38 MAPK in both WT and PPARα^{null} T-cells

The densitometric analysis of western blots revealed that PPARα^{null} T-cells exhibited higher phosphorylated p38 MAPK than WT T-cells. We observed that WY14,643 inhibited the phosphorylation of the three MAP kinases in both WT and PPARα^{null} T-cells (Fig. 6). We observed that WY14,643 inhibited the phosphorylation of p38 MAPK in both WT and PPARα^{null} T-cells.

Discussion
In both the strains of mice, under the same activating conditions it is interesting to note that T-cells of PPARα^{null} mice secreted higher IFN-γ, but CD4$^+$T cells from PPARα ^{null} mice produced lower amounts of IL-2 than T cells from the WT animals. Interestingly, T-bet was originally isolated based on its ability to bind to the IL-2 promoter and was later demonstrated to actually repress IL-2 expression by T cells in in vitro experiments.

PPARα has been extensively studied in tissues that utilize fatty acids as a primary energy source, such as heart, liver, muscle and kidney (3, 24). This receptor isoform has also been found to be expressed in several other tissues and cell types such as chondrocytes, keratinocytes and cells of the immune system (25, 26, 27, 28, 29).

Ligand activation of PPARα or PPARγ in macrophages can effectively inhibit activation-induced inflammatory cytokine production through the active repression of several crucial transcription factors (29).

The observed differences in cytokine production were due to kinetic differences in the transcription of the IFN-γ and IL-2 genes postactivation. Similar to what has been reported previously (30). From these results it is apparent that the presence of PPARα in CD4$^+$ T cells contributes to the regulation of IFN-γ and IL-2 expression in response to activation. Based on the reported ability of T-bet to repress IL-2 expression as well as to transactivate the IFN-γ gene (22, 31, 32). The initiation of T-bet transcription postactivation was kinetically accelerated in PPARα^{null} CD4$^+$ T cells. Thus, differences of T-bet expression might contribute to the differences observed in cytokine production in WT and in PPARα^{null} T cells.

WT T cells were severely compromised in their ability to up-regulate expression of T-bet mRNA when activated in the presence of anti- IFN-γ, but were able to up-regulate expression of both these genes when activated in the absence of anti- IFN-γ. It has recently been reported that IFN-γ exposure rapidly up-regulates the expression of T-bet following activation of CD4$^+$ T cells (33,34). Similar to what has been reported previously (35), WT T cells were severely compromised in their ability to up-regulate expression of T-bet or IFN-γ mRNA when activated in the presence of anti- IFN-γ, but were able to up-regulate expression of both these genes when activated in the absence of anti-IFN-γ when PPARα^{null} CD4$^+$ were activated in the presence of anti- IFN-γ, they were induced to express both T-bet mRNA and protein. This correlated with a retention in their ability to express mRNA for IFN-γ The accelerated IFN-γ production in the PPARα ^{null} T cells upon restimulation most likely arises through a retained ability to express T-bet, as WT T cells that failed to express T-bet under the same conditions also failed to produce IFN-γ upon restimulation. PPARα regulates the expression of T-bet by antagonizing a signaling pathway that is independent of IFN-γ signaling. It is well recognized that activated PPARs can suppress the expression of many distinct genes using a variety of molecular mechanisms (36).

In an attempt to define the mechanism through which PPARα regulates T-bet expression, we analyzed the influences that ligand-activated PPARα would have on T-bet expression by activated T cells. CD4$^+$ T cells isolated from WT mice were treated with increasing doses of the highly specific PPARα ligand WY14, 643. These observations suggest that WY14,643 is modulating T-cell proliferation in a PPARα -independent fashion, at least in PPARα ^{null} T-cells.

The T-cells from PPARα^{null} mice exhibited less proliferation and secretion of IL-2 as compared to those from WT mice. It is possible that less IL-2 secretion may account for less T-cell proliferation in PPARα^{null} mice as T-cell blastogenesis is an IL-2-dependent phenomenon.

The differentiation of naive T-cells into T_H1 and T_H2 subsets is tightly regulated through the activities of specific signaling pathways and transcription factors (35). The T-box transcription factor, T-bet, represents a key regulator of T_H1 cell development through its ability to transactivate the IFN-γ gene while concomitantly repressing IL-4 gene expression (37). We have observed that the absence of PPARα gene resulted in the upregulation of T-bet mRNA transcripts. We also observed that IFN-γ was highly

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secreted by PPARα^{null} T-cells. Our observations corroborate several reports which have shown that PPARα ^{null} T-cells abundantly express T-bet mRNA and protein (38, 39) Hence, it has been suggested that IFN-γ signalling in PPARα ^{null} T-cells may rapidly induce the expression of T-bet in these cells (40). These observations also indicate the high pro-inflammatory status of PPARα ^{null} animals, contributed by IFN-γ these results suggest that the ability of PPARα to suppress T-bet expression is independent of PPARα activation and that ligand activation of PPARα abrogates its normally suppressive effects.

In this study, we have experimentally demonstrated in CD4$^+$T cells that unliganded PPARα negatively regulates the activation induced expression of T-bet. CD4$^+$T cells lacking PPARα undergo an early termination of induced IL-2 gene expression and protein production, and a concomitant overexpression of IFN-γ.

In WT T-cells, WY14,643 exerted effects on the downregulation of T-bet PPARα exerts its regulatory influences over T-bet by suppressing the activation-induced phosphorylation of p38 MAP kinase, a signaling molecule whose activity is associated with the expression of IFN-γ (41 - 42). We demonstrate in this study that p38 MAP kinase activation also contributes to inducing the expression of T-bet following TCR-mediated activation of CD4$^+$ T cells. The data we present, linking regulation of p38 MAP kinase activation to transcription of T-bet, are consistent with previous reports demonstrating that activity of the p38 MAP kinase is associated with Th1 T cell differentiation and IFN-γ production. IFN-γ signaling in CD4$^+$T cells rapidly induces the expression of T-bet (39). It has also been determined that T-bet expression becomes compromised without sufficient IFN-γ signaling.

We observed a high expression of p38 MAPK in PPARα ^{null} T-cells. Jones et al. (34) have reported that PPARα ^{null} T-cells highly express p38 MAPK as unliganded PPARα repress p38 MAPK activation through a DNA-binding independent mechanism (43). These investigators proposed that high MAP kinase activity in PPARα ^{null} T cells could be accelerating indirectly T-bet expression. Several reports have well-shown that activity of the MAP kinase is associated with T_H1 T cell differentiation and IFN-γ production (44). It has been recently established that MAP kinases are activated in Th1 CD4$^+$ T cells through a signaling cascade that involves GADD45γ, a protein that can physically interact with PPARα (45). Alternatively, PPARα may suppress the activation of MAP kinase through an association with some secondary complex of proteins.

Nonetheless, it is clear that MAPK inhibition might modulate the expression of T-cell transcription factors as we further observed that SB202190, an inhibitor of P38 MAPK downregulated T-bet mRNA expression in T-cells. Interestingly, Owakai *et al.* (46) have demonstrated that both the MAPK (p38 and ERK1/2) might be involved in the T_H1 cell differentiation where p38 will act upstream of T-bet, and ERK1/2 will modulate the differentiation mechanism, and the inhibition of the activities of the two MAPK will completely block the T_H1 cell differentiation whereas the inhibition of p38 MAPK partially blocks this process. In PPARα^{null} T-cells, SB202190, also exerted the same action on the modulation of the expression of T-cell transcription factors. Bachmann *et al.* (47) have also shown that T-bet mRNA and protein expression was suppressed by the inhibition of p38 mitogen-activated protein kinase activity. The ability of PPARα to negatively regulate the activation-induced expression of T-bet in T-cells may influence the timing of the switch from T_H1 and T_H2 phenotypes.

Consequently, the enhanced p38 MAP kinase activity seen in stimulated PPARα^{null} T-cells CD4$^+$T cells could be accelerating T-bet expression indirectly, with the early activation of p38 MAP kinase resulting in increased IFN-γ expression and signaling. However, our present studies show that CD4$^+$ T cells lacking a functional PPARα retain their ability to express T-bet in the complete absence of IFN-γ signaling. This ability by activated PPARα^{null} T cells to express T-bet without IFN-γ signaling results in a rapid secretion of newly synthesized IFN-γ protein following restimulation. Wild-type CD4$^+$ T cells that are initially activated under conditions in which IFN-γ signaling is inhibited fail to express T-bet or IFN-γ following restimulation. These results indicate that the ability of PPARα to suppress the activation-induced expression of T-bet is mediated through an ability to antagonize aspects of TCR signaling, and is independent of regulation through IFN-γ signaling.

Conclusion: Our study show that the TH1 differentiation regulated in a PPARα-independent manner and, consequently, modulate the expression of certain pro inflammatory genes.

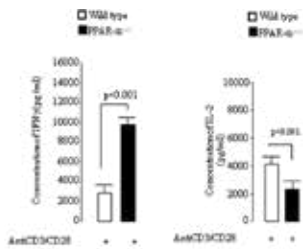

Figure 1 PPARα modulates secretion of IFN-γ, IL-2.

Freshly isolated T-cells were activated in multiwell plates, pre-coated with immobilized anti-CD3 (2 μg/ml) and anti-CD28 (1 μg/ml) antibodies, at 37°C in an atmosphere of 5% CO_2 in air. Following a 24-h activation period, the levels of IFN-γ, IL-2 in the culture supernatants were measured by ELISA. Values are means ± SEM, n = 15 per group of animals. Data were analyzed by two-way ANOVA.

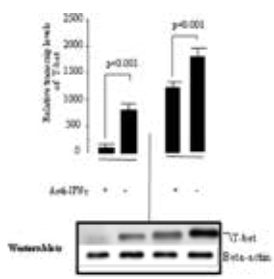

Figure 2 T-bet activated in the absence of anti-IFN-γ

Freshly isolated T-cells were activated in multiwell plates, pre-coated with immobilized anti-CD3 (2 μg/ml) and anti-CD28 (1 μg/ml) antibodies the absence or presence of 10 g/ml anti - IFN-γ at 37°C in an atmosphere of 5% CO2 in air. The expression of mRNA was quantitatively analyzed at 6 h postactivation by employing RT-PCR as described in materials and methods section. T-bet protein levels in the nuclear fractions were analyzed by Western blot at 24 h postactivation. Values are means ± SEM, n = 15 per group of animals. Data were analyzed by two-way ANOVA.

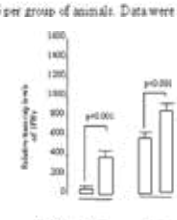

Figure 3 T-bet activated in the absence of anti-IFN-γ

Freshly isolated T-cells were activated in multiwell plates, pre-coated with immobilized anti-CD3 (2 μg/ml) and anti-CD28 (1 μg/ml) antibodies the absence or presence of 10 g/ml anti- IFN-γ at 37°C in an atmosphere of 5% CO2 in air. The expression of mRNA was quantitatively analyzed at 6 h postactivation by employing RT-PCR as described in materials and methods section. T-bet protein levels in the nuclear fractions were analyzed by Western blot at 24 h postactivation. Values are means ± SEM, n = 15 per group of animals. Data were analyzed by two-way ANOVA.

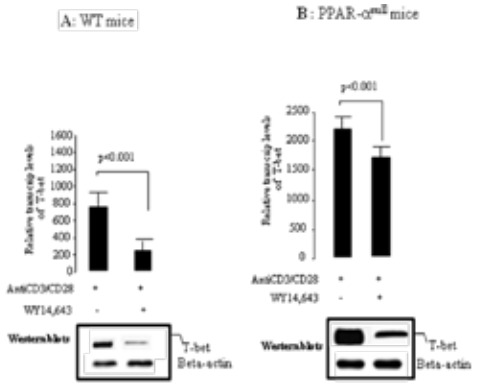

Figure 4 WY14, 643, an agonist of PPARα, exerts inhibition of T-bet expression independently of PPARα.

Freshly isolated T-cells were activated in multiwell plates, pre-coated with immobilized anti-CD3 (2 µg/ml) and anti-CD28 (1 µg/ml) antibodies at 37°C in an atmosphere of 5% CO2 in air. The expression of mRNA was quantitatively analyzed at 6 h postactivation by employing RT-PCR as described in materials and methods section. T-bet protein levels in the nuclear fractions were analyzed by Western blot at 24 h postactivation. The cells contained WY14,643 (25 µM). Values are means ± SEM, n = 15 per group of animals. Data were analyzed by two-way ANOVA.

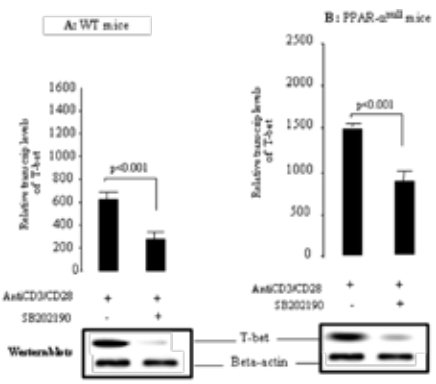

Figure 5 A chemical inhibitor of p38 MAP kinase downregulated the expression of T-bet mRNA and protein expression.

Freshly isolated T-cells were activated in multiwell plates, pre-coated with immobilized anti-CD3 (2 µg/ml) and anti-CD28 (1 µg/ml) antibodies at 37°C in an atmosphere of 5% CO2 in air. The expression of mRNA was quantitatively analyzed at 6 h postactivation by employing RT-PCR as described in materials and methods section. T-bet protein levels in the nuclear fractions were analyzed by Western blot at 24 h postactivation. The cells were incubated or not with the followings, SB202190 (10 µM), for 1h before activation with anti-CD3 and anti-CD28 antibodies. Values are means ± SEM, n = 15 per group of animals. Data were analyzed by two-way ANOVA.

Figure 6 WY14,643 inhibited the phosphorylation of the p38 MAPK in both WT and PPARα^null T-cells.

Freshly isolated T-cells were activated in multiwell plates, pre-coated with immobilized anti-CD3 (2 µg/ml) and anti-CD28 (1 µg/ml) antibodies at 37°C in an atmosphere of 5% CO2 in air. The T-cells from WT and PPARα^null mice were activated as mentioned in the legends of Figure 1. The cells contained WY14,643 (25 µM). The phosphorylated forms of MAPK were analyzed by Western blot at 24 h postactivation as described in the materials and methods section. Values are means ± SEM, n = 15 per group of animals. Data were analyzed by two-way ANOVA.

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