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Article

CROSSTALK BETWEEN PPARγ LIGANDS AND INFLAMMATORY-RELATED PATHWAYS IN NATURAL T-REGULATORY CELLS FROM TYPE 1 DIABETES MOUSE MODEL

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Abstract: Immunomodulation as means of immunotherapy has been studied in major research and clinical laboratories for many years. T-Regulatory (Treg) cell therapy is one of the modulator used in immunotherapy approaches. Similarly, nuclear receptor peroxisome proliferator activated receptor gamma (PPARγ) has extensively been shown to play a role as an immuno-modulator during inflammation. Given their mutual roles in downregulating the immune response, current study examined the influence of PPARγ ligands i.e thiazolidinedione (TZD) class of drugs on Foxp3 expression and possible crosstalk between PPARγ and nTreg cells of NOD and NOR mice. Results showed that TZD drug, ciglitazone and natural ligand of PPARγ 15d-prostaglandin downregulated Foxp3 expression in activated nTreg cells from both NOD and NOR mice. Interestingly, addition of the PPARγ inhibitor, GW9662 further downregulated Foxp3 expression in these cells from both mice. We also found that PPARγ ligands negatively regulate Foxp3 expression in activated nTreg cells via PPARγ-independant mechanism(s). These results demonstrate that both natural and synthetic PPARγ ligands capable of suppressing Foxp3 expression in activated nTreg cells of NOD and NOR mice. This may suggest that the effect of PPARγ ligands in modulating Foxp3 expression in activated nTreg cells is different from their reported effects on effector T cells. Given the capability to suppress foxp3 gene, it is possible to be tested as immunomodulators in cancer-related studies.

Keywords: T-regulatory cells, immune regulation, Foxp3, PPARγ, autoimmune diabetes, NOD mouse, Thiazolidinediones, ciglitazone.

1. Introduction

Over the past two decades since the identification of naturally-occurring CD4+CD25+Foxp3+regulatory T cells (nTreg), there have been intense research in delineating the immunobiology of nTreg cells in physiological and pathological conditions [1]. The master regulator in nTreg cells is the transcription factor, Forkhead box P3 (Foxp3), which plays an important role in the development and function of these cells [2, 3]. Foxp3 is expressed in the thymus by nTreg cells [4, 5] and is transiently expressed by peripheral CD4+CD25- conventional T cells (iTreg) [5]. In pathological conditions such as in autoimmune disorders, the recognition of self-tissues by auto-reactive T cells leads to the destruction of host tissues or organs. The immunosuppressive role of

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nTreg cells prevents such destruction from occurring by establishing peripheral self-tolerance toward these auto-reactive T cells [5]. This will thus hinder the development of debilitating autoimmune diseases from occurring. Hence mutation of Foxp3 gene results in the loss of immunoregulatory function of nTreg cells, predisposing the hosts towards autoimmune responses [3, 4].

It is well established that PPARγ activation is capable of inducing immunodownregulatory responses [6-9]. The ability of PPARγ in inducing anti-inflammatory responses in immune cells has been put forth. The activation of PPARγ by their ligands has been shown to downregulate the clonal expansion of activated T effector (Teff) cells [10]. PPARγ ligands such as the thiazolidinediones (TZDs) class of drugs alleviated adverse autoimmune conditions in allergic reactions and inflammatory bowel disease (IBD) [11, 12]. In addition, PPARγ ligands have been shown to ameliorate multiple sclerosis [13, 14]. The anti-inflammatory effect of 15d-prostaglandin-J2 (15d-PGJ2), a potent PPARγ ligand, significantly inhibited the activation of enchephalitogenic T cells and protected the development of encephalomyelitis in experimental autoimmune encephalomyelitis (EAE) [14]. The immunoregulatory function of PPARγ ligands may however act via PPARγ -dependent or -independent mechanisms [15]. The PPARγ ligand, pioglitazone, reduces allergic rhinitis in mice via PPARγ -dependent mechanism [16], while 15d-PGJ2 induces apoptosis in both T lymphocytes and Jurkat T cells via the activation of mitochondrial apoptotic pathway [17]. The correlation between PPARγ polymorphism with the increased risk of asthma in humans indicates that the role of PPARγ in anti-inflammatory reaction occurs at the gene expression level [18]. However, the possible mechanism of immunomodulation by PPARγ in nTreg cells is poorly understood. Therefore, this study was conducted to examine the possible crosstalk between immunoregulatory properties of nTreg cells and PPARγ in the murine autoimmune model (NOD mice). We also included the genetic control of the autoimmune mouse model, non-obese diabetic resistant (NOR) mice.

2. Experimental Section

Mice

Eight-week old female non-obese diabetic (NOD) and non-obese resistant (NOR) mice were purchased from The Jackson Laboratory (Bar Harbour, Maine, USA). The mice were maintained in the animal facilities under specific pathogen-free conditions in accordance with the guidelines and regulations of the Universiti Sains Malaysia and used at 12-week of age. All experimental protocols were approved by the USM Animal Ethics Committee (approval number: USM/Animal Ethics Approval/2009/ (43 [132]).

Antibodies and reagents

Mouse nTreg cells were isolated from spleen tissues of NOD and NOR mice by magnetic separation. Briefly, CD4+ cells were purified by negative and positive isolations using MACS CD4+CD25+ Treg isolation kit (Miltenyi Biotec, Cologne, Germany). Isolated CD4+CD25+ Treg cells were then stained with PE-anti mouse CD4, FITC-anti mouse CD25 and APC-anti Foxp3 mAbs to determine cell purity using the FACS Canto flow cytometer (BD Biosciences, New Jersey, USA). Labelled cells comprised ≥ 90% with > 80% Foxp3+CD4+CD25+ cells obtained. The isolated CD4+CD25+Foxp3+ cells were used as nTreg cells and were cultured in RPMI 1640 supplemented with 10% FBS (Thermo Fischer Scientific, Waltham, USA), 10 mM HEPES, 500 µl antibiotic stock solution containing 100 U/ml, 100 µg/ml streptomycin and 10 µM β–mercaptoethanol (Gibco BRL, Grand Island, New York). IL-2 was purchased from BD Biosciences (New Jersey, USA), ciglitazone and 15d-PGJ2 were purchased from Cayman Chemicals (Ann Arbor, Michigan, USA) and GW9662 was purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

Flow cytometry analysis

The expression of CD4 and CD25 surface markers and intracellular Foxp3 was evaluated using PE-conjugated anti-CD4, FITC-conjugated anti-CD25 and APC-conjugated anti-Foxp3. The phosphorylated ZAP-70 and STAT-5 proteins were evaluated by using FITC- conjugated anti-phosphorylated ZAP-70 and FITC-conjugated anti-phosphorylated STAT-5 antibodies and was assessed on FACS Canto Flow cytometer (BD Biosciences, New Jersey, USA). Mouse PE-and APC-conjugated IgG1 and FITC- conjugated IgG2a were used as isotype controls.

Total RNA isolation, cDNA synthesis and real-time PCR for the detection of PPARγ and Foxp3

Total RNA was extracted using the RNAeasy Mini kit (Qiagen, Germany) and subjected to cDNA synthesis using cDNA first strand synthesis (Qiagen, Germany). To quantify the concentration of unknown transcripts, a standard curve for PPARγ was generated. Briefly, a serial dilution of the target PPARγ DNA plasmid concentration, corresponding to 101 to 107 copy numbers was prepared and amplified to generate standard curves. PCR was performed using primers for PPARγ (forward: 5'-GCG GCT GAG AAA TCA CGT TC-3', Reverse: 5'-TTA AAA ATG TCC TGA ATA TCA GTG GTT C-3', Probe: 5'-GCT TCT TTC AAA TCT TGT CTG TCA CAC AGT-3'). Foxp3 gene with accession number AF277992.1 was selected from the NCBI database and quantified using TaqMan® Gene Expression assay for Foxp3 gene (assay ID Mm00475162_m1). The copy numbers for unknown samples were determined by extrapolating the data from these standard curves. The PPARγ expression level was reported as the number of mRNA transcripts per µg of total RNA (transcript/µg). Data were analysed using the ABI prism software (Applied Biosystem).

PPARγ-PPRE binding activity

PPARγ activation was measured by its binding to the response element, PPRE. This was measured by using ligand binding assay of PPARγ transcription factors (Cayman Chemical, Ann Arbor, Michigan, USA). The nuclear proteins of treated and untreated cells were extracted using the Nuclear Extraction kit (Cayman Chemical, Ann Arbor, Michigan, USA). A 96 well-plate, pre-coated with immobilized PPRE was used to detect the binding of activated PPARγ in the nuclear extract from samples. Using rabbit polyclonal primary antibody against PPARγ and goat anti-rabbit secondary antibody-conjugated with HRP, the plate was prepared for detection of colorimetric signal using ELISA plate reader at 450nm.

Signaling pathways modulation by PCR Array

This experiment was conducted using real-time PCR and the data obtained were analysed via PCR Array Data Analysis Software available online at http://www.pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php. This software extrapolate the data based on Ct values to report the fold differences between treated groups and untreated group using the 2^ (-ΔΔCt) formula. Total RNA was extracted from nTreg cells of NOD and NOR mice treated or untreated with PPARγ ligands. RNA extraction was performed using RNAeasy Mini kit (Qiagen, Germany) and subjected to cDNA synthesis using cDNA first strand synthesis (Qiagen, Germany). The cDNA was then used to perform Qiagen Mouse Signal Transduction PathwayFinder RT2 Profiler PCR ARRAY (catalog no. PAMM-014), (Qiagen, Germany). Two Arrays were performed for each mouse strain. Changes in cycle threshold (ΔCt) for each gene were obtained by subtracting the mean threshold cycle (Ct) of the housekeeping genes (Gusb, HPRT, Hsp90ab1, Gapdh and β-Actin) from the threshold cycle value of the gene. The fold regulation was calculated relative to the untreated group when genes that showed more than 5-fold difference were determined as have been upregulated while those less than 0.2 fold considered as downregulated.

Statistical analyses

Data from experimental analyses were presented as the mean of triplicates with standard error mean (mean ± SEM). The data were statistically analysed using the Minitab® 16.1.0 software (MiniTab Inc,

USA). The comparison between control and treated groups was tested for significance using one-way analysis of variance (ANOVA) test. Post-Hoc comparison test was performed to compare significant levels between treated groups. P value of less than 0.05 ($P < 0.05$) is considered significant.

3. Results

3.1.1 Expression of Foxp3 in nTreg cells of NOD and NOR mice

The influence of selected PPARγ ligands on Foxp3 in activated nTreg cells was tested by measuring the levels of Foxp3 mRNA expression in nTreg cells of NOD and NOR mice. These cells were treated with 15d-PGJ2 and ciglitazone in the presence or absence of the PPARγ inhibitor, GW9662. A group of untreated activated nTreg cells from both NOD and NOR mice was used as control for each of the mouse strain. The results obtained from the correlation analyses in NOD and NOR mice are shown in Figure 1. In both mouse strains, activated nTreg cells treated with ciglitazone expressed lower levels of Foxp3 mRNA compared to untreated group ($P < 0.01$). In both strains, the addition of GW9662, further reduced Foxp3 expression in these cells compared to ciglitazone-treated cells ($P < 0.05$). In NOD mice, treatment with 15d-PGJ2 significantly reduced the expression of Foxp3 mRNA in activated nTreg cells in comparison to untreated cells ($P < 0.01$). Similarly, Foxp3 expression in activated nTreg cells of NOR mice was undetectable after treatment with 15d-PGJ2 in comparison to untreated cells ($P < 0.01$).

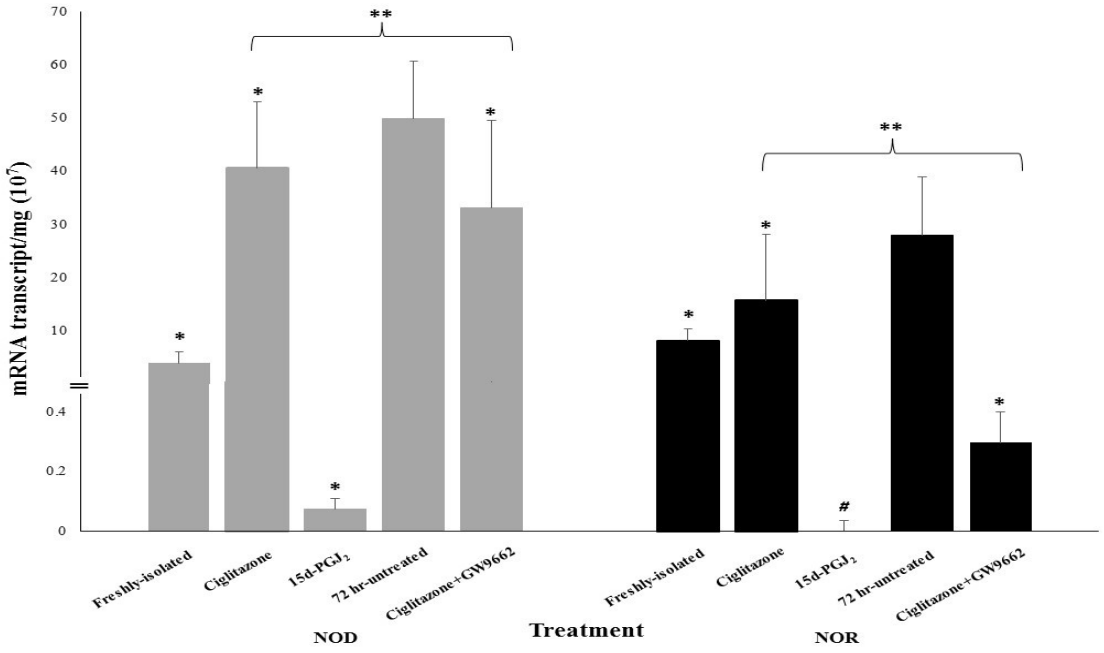

Figure 1 Foxp3 mRNA expression levels in nTreg cells from NOD and NOR mice following various treatment after 72 h culture. The grey bars represent Foxp3 gene transcripts from NOD mice while the black bars represent NOR mice. In NOD and NOR mice, the presence of ciglitazone significantly suppressed Foxp3 expression level in activated nTreg cells compared to untreated group. Moreover, the addition of GW9662 further downregulated Foxp3 level in these cells from both strains compared to untreated cells ($P < 0.01$) and ciglitazone-treated cells ($P < 0.05$). In NOD mice, Foxp3 expression in 15d-PGJ2-treated activated nTreg cells was suppressed compared to untreated cells ($P < 0.01$). Data are expressed as the amount of mRNA transcripts per ◎g of total RNA. This experiment was repeated twice and the graph was plotted based on the mean transcript values ±

SEM. Statistical analysis was performed using One-way ANOVA. Post-hoc comparison was performed to identify the significance between treated samples (n=4 mice/experiment).

*$P < 0.01$, sample groups vs untreated group.

**$P < 0.05$, ciglitazone-treated group vs ciglitazone + GW9662-treated group.

* $P < 0.01$, sample groups vs untreated group.

** $P < 0.05$, ciglitazone-treated group vs ciglitazone + GW9662-treated group.

the expression level was undetectable.

3.1.2 Binding activity between PPARγ and PPRE in nTreg cells of NOD and NOR mice

The specific sequence for PPARγ at the binding domain of DNA is known as Peroxisome-proliferator response elements (PPRE). Activation of PPARγ via its ligand results in its translocation into the nucleus and the binding of the activated receptor to PPRE, at the DNA binding domain after heterodimerization with retinoid X receptor (RXR) [19]. Thus, we investigated the binding activity of PPARγ and PPRE in activated nTreg cells from NOD and NOR mice following treatment with their ligands, with or without the PPARγ inhibitor. For this analysis, we postulated that following treatment with PPARγ ligands i.e. ciglitazone and 15d-PGJ2, activated PPARγ would bind to PPRE. Our result showed that there was negligible binding activity between PPARγ and PPRE in activated nTreg cells from both NOD and NOR mice after the respective treatments (Figure 2). This suggests that the activity of PPARγ ligand treatment of nTreg cells is independent of PPRE binding. On the other hand, adipose tissue nuclear lysate which was used as a positive control showed strong binding with the plate-bound PPRE.

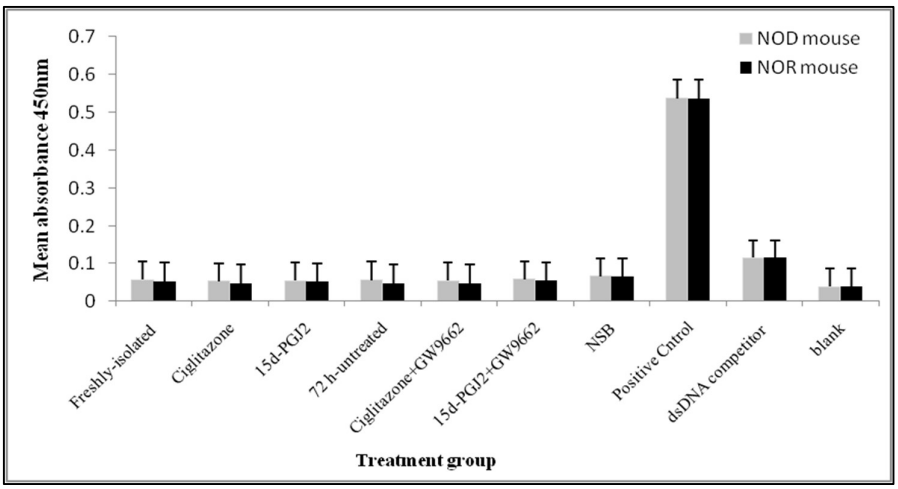

Figure 2 The binding activity between PPARγ **and PPRE in nTreg cells nuclear protein lysates from NOD and NOR mice.** The bar graphs show mean absorbance for each indicated treatment performed in duplicate. PPARγ double-stranded DNA (dsDNA) competitors were added onto dsDNA competitor wells while non-specific binding (NSB) wells were added with buffer without positive control or samples. Blank wells contained only buffer. This experiment was repeated twice and error bars represent mean ± SEM (n=3 mice/experiment).

3.1.3 Phosphorylation levels of ZAP-70 & STAT-5 transduction proteins

In newly activated T cells, phosphorylation of ZAP-70 initiates the activation of phosphorylation cascades in proximal intracellular signaling to propagate downstream responses. In order to examine the effect of PPARγ ligands on the proximal and distal signaling pathways in nTreg cells,

phosphorylation levels of ZAP-70 proteins were studied in activated nTreg cells of NOD and NOR mice. Following 72 h incubation with the PPARγ ligands, nTreg cells were stained with phosphorylated-ZAP-70 conjugated antibodies. Untreated nTreg cells were included as controls and isotype control was used to set the marker for fluorochrome staining. There was no significant difference in the levels of ZAP-70 phosphorylation in either treated or untreated cells of NOD or NOR mice (Figure 3 and 4). These findings may suggest that ZAP-70 phosphorylation is not involved in the activation of nTreg cells of NOD and NOR mice.

In T-lymphocytes, IL-2 binding to IL-2R will activate STAT-5 transcription factor, which in turn activate a set of genes that are involved in T cell development and inflammation [20]. On the other hand, in nTreg cells, the activation of STAT-5 by IL-2 signaling is important in Foxp3 expression and survival of nTreg cells [21]. Upon IL2-IL2R binding, activation of STAT-5 by phosphorylation is initiated to effect downstream signaling components such as JAK/STAT signaling [22]. Therefore, IL-2/STAT-5 signaling pathway is an important pathway in nTreg cells as it directly correlates with Foxp3 expression. Intervention of this pathway by PPARγ ligands may indicate the association between PPARγ and nTreg cells at the IL-2/JAK-STAT level. Following 72 h incubation with the PPARγ ligands, these cells were stained with phosphorylated-STAT-5 conjugated antibodies. Similarly, untreated nTreg cells were included as controls and isotype control was used to set the marker for fluorochrome staining. Activated nTreg cells of NOD and NOR mice demonstrated constitutive phosphorylation of STAT-5 with no significant influence from PPARγ ligand treatment. (Figure 5and Figure 6). Our current results may suggest that JAK/STAT signaling is required in activated nTreg cells for downstream events since we recorded high levels of phosphorylated STAT-5 in these cells. In addition, there was a negative association between STAT5 activation and PPARγ in nTreg cells of both NOD and NOR mice.

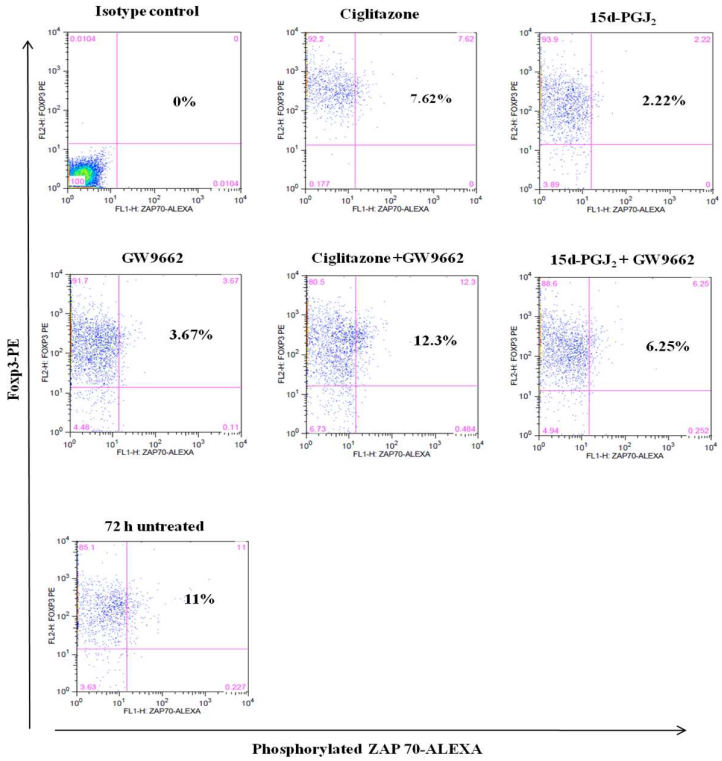

Figure 3 The expression of phosphorylated ZAP-70 in nTreg cells of NOD mice following various treatment after 72 h culture. Dot plot shows the levels of phosphorylated ZAP-70 in activated nTreg cells as indicated by Foxp3 expression. ZAP-70 phosphorylation was measured in nTreg cells treated

with ciglitazone, 15d-PGJ$_2$, GW9662, combination of ciglitazone + GW9662, and combination of 15d-PGJ$_2$ + GW9662. 72 h-untreated cells was assigned as control group. Data shown are representative of two independant experiments. (n=2 mice/experiment).

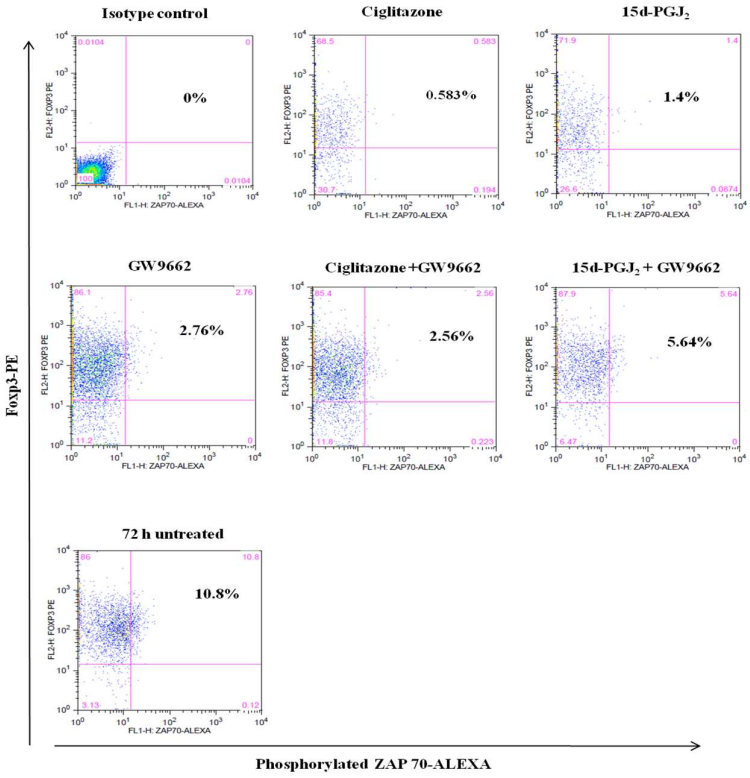

Figure 4 The expression of phosphorylated ZAP-70 in nTreg cells of NOR mice following various treatment after 72 h culture. Dot plot shows the levels of phosphorylated ZAP-70 in activated nTreg cells as indicated by Foxp3 expression. ZAP-70 phosphorylation was measured in nTreg cells treated with ciglitazone, 15d-PGJ$_2$, GW9662, combination of ciglitazone + GW9662, and combination of 15d-PGJ$_2$ + GW9662. 72 h-untreated cells was assigned as control group. Data shown are representative of two independant experiments. (n=2 mice/experiment).

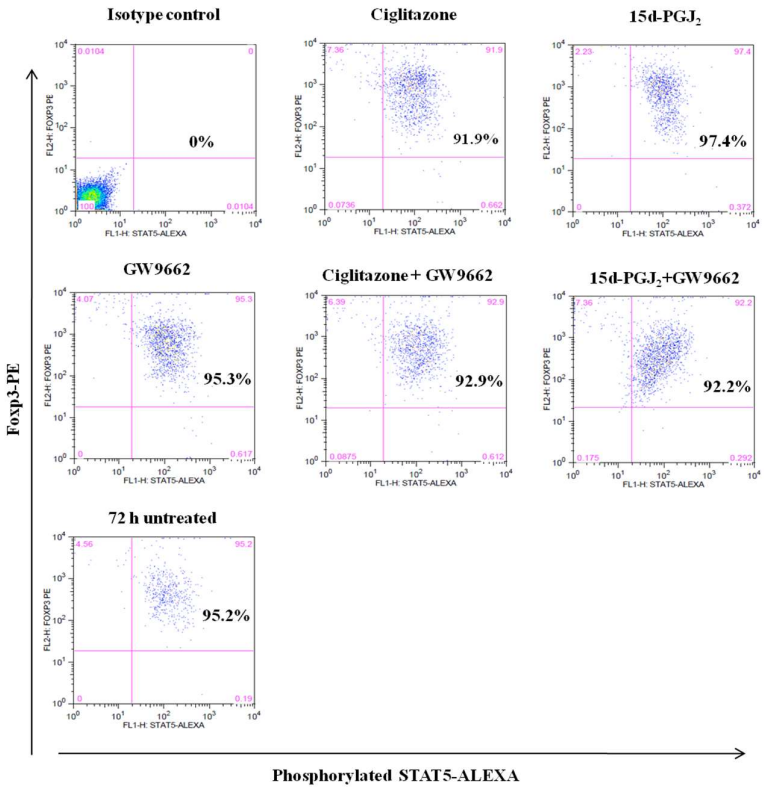

Figure 5 The expression of phosphorylated STAT-5 in activated nTreg cells of NOD mice following various treatment after 72 h culture. Dot plot shows the levels of phosphorylated STAT-5 in nTreg cells. STAT-5 phosphorylation level was measured in activated nTreg cells treated with ciglitazone, 15d-PGJ2, GW9662, combination of ciglitazone + GW9662, and combination of 15d-PGJ2 + GW9662. 72 h-untreated group was assigned as control group. Data shown are representative of two independant experiments (n=2 mice/experiment).

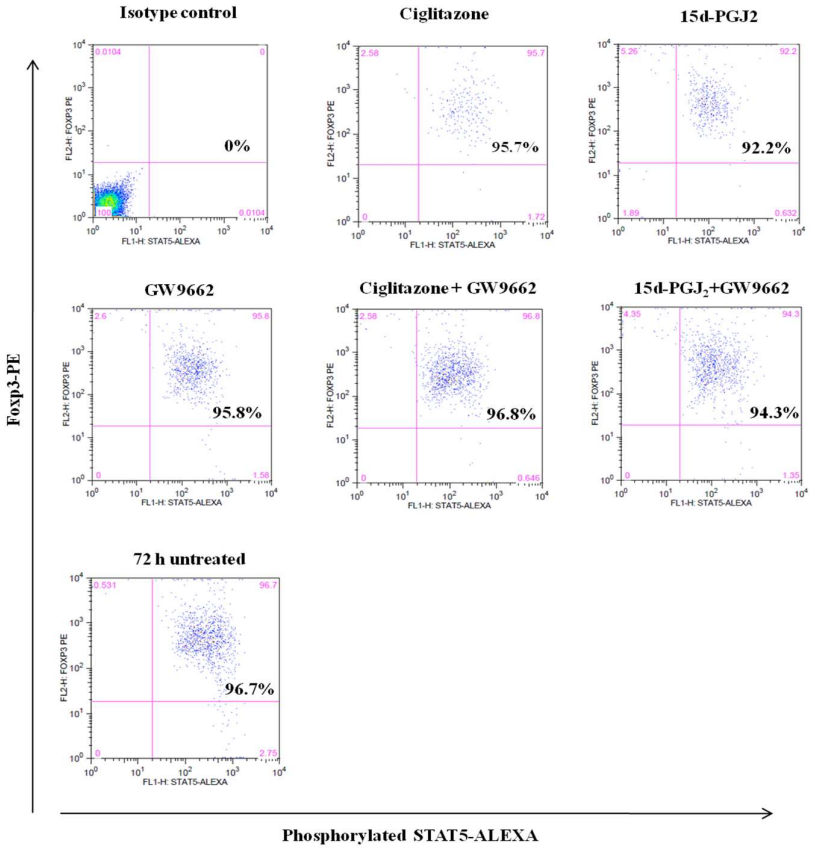

Figure 6 The expression of phosphorylated STAT-5 in activated nTreg cells of NOR mice following treatment after 72 h culture. Dot plot shows the levels of phosphorylated STAT-5 in nTreg cells. STAT-5 phosphorylation level was measured in activated nTreg cells treated with ciglitazone, 15d-PGJ2, GW9662, combination of ciglitazone + GW9662, and combination of 15d-PGJ2 + GW9662. 72 h-untreated group was assigned as control group. Data shown are representative of two independant experiments (n=2 mice/experiment).

3.1.4 Differential gene expression involved in signal transduction pathways in nTreg cells of NOD and NOR mice

In order to assess the possible crosstalk between PPARγ and signaling pathways in nTreg cells, we examined the modulation of selected gene expression in these cells following treatment with PPARγ ligands. By using the Signal Transduction PathwayFinder™ array, we analysed 84 important genes responsive to 18 different signal transduction pathways. Out of the 18 signaling pathways, 4 signaling pathways that are relevant in immune cells were chosen. The fold change cut-off value for gene expression was determined at > five-fold change in order to arbitrarily assign meaningful biological differences between expressed genes. This result is hoped to provide some information on biologically relevant genes in nTreg cells and their association with PPARγ. Table 1-4 depict the differentially regulated pathway-related target genes in NOD and NOR mice following treatment with PPARγ ligands. In NOD mice, most of inflammatory-related pathways were downregulated in PPARγ ligand-treated nTreg cells. In NOR mice, a few genes including cdkn1a, ccl20, il2ra, lta, nfkbia, nos2 and tank genes were upregulated in PPARγ ligand-treated nTreg cells and these genes are known to be involved in pro- inflammatory related pathways. Furthermore, cd5 gene that is responsible for the expression of cd5 antigen on cell surface was upregulated in ciglitazone-treated nTreg cells from NOR mice.

Table 1. Differentially expressed target genes of pathways related to immune cells in ciglitazone-treated nTreg cells from NOD mice.

No	Symbol	Description	GenBank Accession no	Unigene	Fold change[1]
MAP KINASE					
1	egr1	Early growth response 1	NM007913	Mm.181959	< 0.2
2	fos	FBJ osteosarcoma oncogene	NM010234	Mm.246513	< 0.2
3	jun	Jun oncogene	NM010591	Mm.275071	< 0.2
4	nab2	Ngfi-A binding protein 2	NM008668	Mm.336898	< 0.2
TGF-β					
5	cdkn1a	Cyclin dependent inhibitor 1A	NM007669	Mm.195663	< 0.2
6	cdkn1b	Cyclin dependent inhibitor 1B	NM009875	Mm.2958	< 0.2
7	cdkn2a	Cyclin dependent inhibitor 2A	NM0099877	Mm.4733	< 0.2
8	cdkn2b	Cyclin dependent inhibitor 2B, p15, inhibits CDK4	NM007670	Mm.423094	< 0.2
NF-κβ					
9	Ccl20	Chemokine (C-C) motif ligand 20	NM016960	Mm.116739	< 0.2
10	Cxcl1	Chemokine (C-X-C motif) ligand 1	NM008179	Mm.21013	< 0.2
11	Icam1	Intracellular adhesion molecule 1	NM010493	Mm.435508	< 0.2
12	Ikbkb	Inhibitor of kappa B kinase beta	Nm010546	Mm.277886	< 0.2
13	Il1a	Interleukin 1 alpha	NM010554	Mm.15534	< 0.2
14	Il2	Interleukin 2	Nm008366	Mm.14190	< 0.2
15	Il2ra	Interleukin 2 receptor alpha chain	NM008367	Mm.915	0.3686
16	Lta	Lymphotoxin A	NM010735	Mm.87787	< 0.2
17	Nfkbia	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	NM010907	Mm.170515	1.0718
18	Nos2	Nitric oxide synthase 2, inducible	NM010927	Mm.2893	< 0.2
19	Tank	TRAF-family-associated NF-k B activator	NM011529	Mm.244393	3.5540
20	Tnf	Tumor necrosis factor	NM013693	Mm.1293	0.3585
21	Vcam1	Vascular endothelial molecule 1	NM011693	Mm.76649	< 0.2
NFAT					
22	Cd5	CD5 antigen	NM007650	Mm.779	< 0.2
23	Fasl	Fas (TNF-receptor superfamily member 6) ligand	NM010177	Mm.3355	< 0.2
24	Il2	Interleukin 2	Nm008366	Mm.14190	< 0.2
PKC					
25	Csf2	Colony stimulating factor 2	NM009969	Mm.4922	< 0.2
26	myc	Myelocytomatosis oncogen	NM010849	Mm.2444	> 10.0
27	odc1	Ornithine decarboxylase, structural 1	NM013614	Mm.34102	> 5.0

[1] Fold change differences as compared to untreated control group. Fold change < 0.2 fold indicates downregulation and > 5 fold indicates upregulation. Genes with * are not differentially regulated as compared to control group.

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Table 2. Differentially expressed target genes of pathways related to immune cells in prostaglandin J_2-treated nTreg cells from NOD mice.

No	Symbol	Description	GenBank Accession no	Unigene	Fold change[1]
MAP KINASE					
1	egr1	Early growth response 1	NM007913	Mm.181959	< 0.2
2	fos	FBJ osteosarcoma oncogene	NM010234	Mm.246513	< 0.2
3	jun	Jun oncogene	NM010591	Mm.275071	< 0.2
4	nab2	Ngfi-A binding protein 2	NM008668	Mm.336898	< 0.2
TGF-β					
5	cdkn1a	Cyclin dependent inhibitor 1A	NM007669	Mm.195663	< 0.2
6	cdkn1b	Cyclin dependent inhibitor 1B	NM009875	Mm.2958	0.60
7	cdkn2a	Cyclin dependent inhibitor 2A	NM0099877	Mm.4733	< 0.2
8	cdkn2b	Cyclin dependent inhibitor 2B, p15, inhibits CDK4	NM007670	Mm.423094	0.37
NF-κβ					
9	Ccl20	Chemokine (C-C) motif ligand 20	NM016960	Mm.116739	< 0.2
10	Cxcl1	Chemokine (C-X-C motif) ligand 1	NM008179	Mm.21013	< 0.2
11	Icam1	Intracellular adhesion molecule 1	NM010493	Mm.435508	< 0.2
12	Ikbkb	Inhibitor of kappa B kinase beta	Nm010546	Mm.277886	< 0.2
13	Il1a	Interleukin 1 alpha	NM010554	Mm.15534	< 0.2
14	Il2	Interleukin 2	Nm008366	Mm.14190	< 0.2
15	Il2ra	Interleukin 2 receptor alpha chain	NM008367	Mm.915	< 0.2
16	Lta	Lymphotoxin A	NM010735	Mm.87787	0.5620
17	Nfkbia	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	NM010907	Mm.170515	< 0.2
18	Nos2	Nitric oxide synthase 2, inducible	NM010927	Mm.2893	< 0.2
19	Tank	TRAF-family-associated NF-k B activator	NM011529	Mm.244393	< 0.2
20	Tnf	Tumor necrosis factor	NM013693	Mm.1293	1.2968
21	Vcam1	Vascular endothelial molecule 1	NM011693	Mm.76649	< 0.2
NFAT					
22	Cd5	CD5 antigen	NM007650	Mm.779	< 0.2
23	Fasl	Fas (TNF-receptor superfamily member 6) ligand	NM010177	Mm.3355	< 0.2
24	Il2	Interleukin 2	Nm008366	Mm.14190	< 0.2
PKC					
25	Csf2	Colony stimulating factor 2	NM009969	Mm.4922	*
26	myc	Myelocytomatosis oncogen	NM010849	Mm.2444	*
27	odc1	Ornithine decarboxylase, structural 1	NM013614	Mm.34102	> 5.0

[1] Fold change differences as compared to untreated control group. Fold change < 0.2 fold indicates downregulation and > 5 fold indicates upregulation. Genes with * are not differentially regulated as compared to control group.

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Table 3. Differentially expressed target genes of pathways related to immune cells in ciglitazone-treated nTreg cells from NOR mice.

No	Symbol	Description	GenBank Accession no	Unigene	Fold change[1]
MAP KINASE					
1	egr1	Early growth response 1	NM007913	Mm.181959	3.650
2	fos	FBJ osteosarcoma oncogene	NM010234	Mm.246513	< 0.2
3	jun	Jun oncogene	NM010591	Mm.275071	< 0.2
4	nab2	Ngfi-A binding protein 2	NM008668	Mm.336898	< 0.2
TGF-β					
5	cdkn1a	Cyclin dependent inhibitor 1A	NM007669	Mm.195663	> 5.0
6	cdkn1b	Cyclin dependent inhibitor 1B	NM009875	Mm.2958	1.204
7	cdkn2a	Cyclin dependent inhibitor 2A	NM0099877	Mm.4733	< 0.2
8	cdkn2b	Cyclin dependent inhibitor 2B, p15, inhibits CDK4	NM007670	Mm.423094	< 0.2
NF-κβ					
9	Ccl20	Chemokine (C-C) motif ligand 20	NM016960	Mm.116739	> 5.0
10	Cxcl1	Chemokine (C-X-C motif) ligand 1	NM008179	Mm.21013	< 0.2
11	Icam1	Intracellular adhesion molecule 1	NM010493	Mm.435508	*
12	Ikbkb	Inhibitor of kappa B kinase beta	Nm010546	Mm.277886	*
13	Il1a	Interleukin 1 alpha	NM010554	Mm.15534	*
14	Il2	Interleukin 2	Nm008366	Mm.14190	*
15	Il2ra	Interleukin 2 receptor alpha chain	NM008367	Mm.915	> 5.0
16	Lta	Lymphotoxin A	NM010735	Mm.87787	> 5.0
17	Nfkbia	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	NM010907	Mm.170515	> 5.0
18	Nos2	Nitric oxide synthase 2, inducible	NM010927	Mm.2893	> 5.0
19	Tank	TRAF-family-associated NF-k B activator	NM011529	Mm.244393	> 5.0
20	Tnf	Tumor necrosis factor	NM013693	Mm.1293	*
21	Vcam1	Vascular endothelial molecule 1	NM011693	Mm.76649	*
NFAT					
22	Cd5	CD5 antigen	NM007650	Mm.779	> 5.0
23	Fasl	Fas (TNF-receptor superfamily member 6) ligand	NM010177	Mm.3355	*
24	Il2	Interleukin 2	Nm008366	Mm.14190	*
PKC					
25	Csf2	Colony stimulating factor 2	NM009969	Mm.4922	*
26	myc	Myelocytomatosis oncogen	NM010849	Mm.2444	> 5.0
27	odc1	Ornithine decarboxylase, structural 1	NM013614	Mm.34102	> 5.0

[1] Fold change differences as compared to untreated control group. Fold change < 0.2 fold indicates downregulation and > 5 fold indicates upregulation. Genes with * are not differentially regulated as compared to control group.

Table 4. Differentially expressed target genes of pathways related to immune cells in prostaglandin J_2-treated nTreg cells from NOR mice.

No	Symbol	Description	GenBank Accession no	Unigene	Fold change[1]
MAP KINASE					
1	egr1	Early growth response 1	NM007913	Mm.181959	1.501
2	fos	FBJ osteosarcoma oncogene	NM010234	Mm.246513	*
3	jun	Jun oncogene	NM010591	Mm.275071	2.020
4	nab2	Ngfi-A binding protein 2	NM008668	Mm.336898	< 0.2
TGF-β					
5	cdkn1a	Cyclin dependent inhibitor 1A	NM007669	Mm.195663	*
6	cdkn1b	Cyclin dependent inhibitor 1B	NM009875	Mm.2958	*
7	cdkn2a	Cyclin dependent inhibitor 2A	NM0099877	Mm.4733	*
8	cdkn2b	Cyclin dependent inhibitor 2B, p15, inhibits CDK4	NM007670	Mm.423094	*
NF-κβ					
9	Ccl20	Chemokine (C-C) motif ligand 20	NM016960	Mm.116739	0.4983
10	Cxcl1	Chemokine (C-X-C motif) ligand 1	NM008179	Mm.21013	0.1632
11	Icam1	Intracellular adhesion molecule 1	NM010493	Mm.435508	0.6713
12	Ikbkb	Inhibitor of kappa B kinase beta	Nm010546	Mm.277886	0.1632
13	Il1a	Interleukin 1 alpha	NM010554	Mm.15534	1.0317
14	Il2	Interleukin 2	Nm008366	Mm.14190	0.4846
15	Il2ra	Interleukin 2 receptor alpha chain	NM008367	Mm.915	0.695
16	Lta	Lymphotoxin A	NM010735	Mm.87787	0.3856
17	Nfkbia	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	NM010907	Mm.170515	2.6117
18	Nos2	Nitric oxide synthase 2, inducible	NM010927	Mm.2893	1.0755
19	Tank	TRAF-family-associated NF-kB activator	NM011529	Mm.244393	2.5758
20	Tnf	Tumor necrosis factor	NM013693	Mm.1293	1.0317
21	Vcam1	Vascular endothelial molecule 1	NM011693	Mm.76649	0.4132
NFAT					
22	Cd5	CD5 antigen	NM007650	Mm.779	2.2894
23	Fasl	Fas (TNF-receptor superfamily member 6) ligand	NM010177	Mm.3355	< 0.2
24	Il2	Interleukin 2	Nm008366	Mm.14190	< 0.2
PKC					
25	Csf2	Colony stimulating factor 2	NM009969	Mm.4922	*
26	myc	Myelocytomatosis oncogen	NM010849	Mm.2444	*
27	odc1	Ornithine decarboxylase, structural 1	NM013614	Mm.34102	> 5.0

[1] Fold change differences as compared to untreated control group. Fold change < 0.2 fold indicates downregulation and > 5 fold indicates upregulation. Genes with * are not differentially regulated as compared to control group.

3.2. Figures, Tables and Schemes

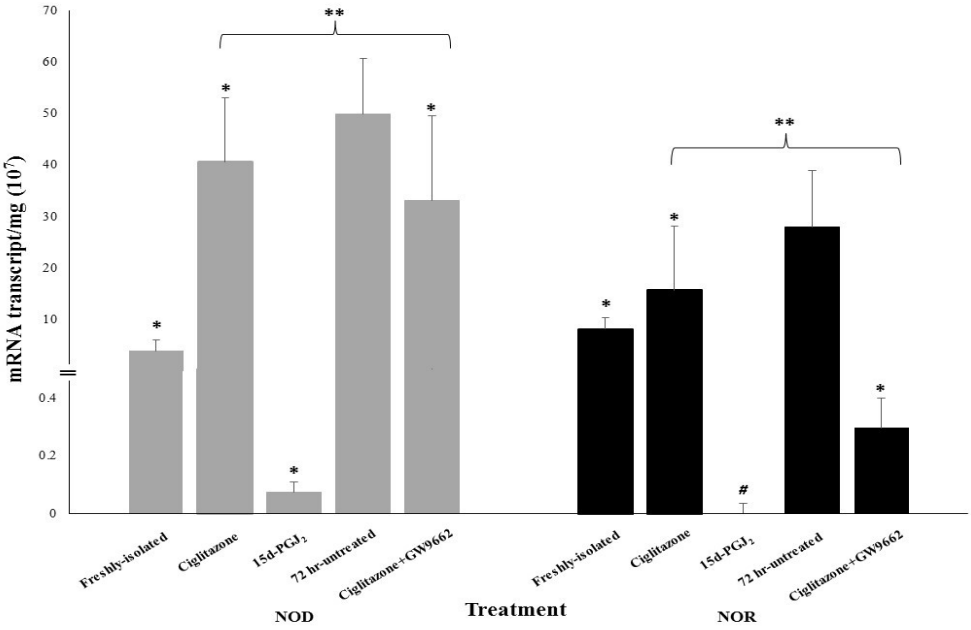

Figure 1 Foxp3 mRNA expression levels in nTreg cells from NOD and NOR mice following various treatment after 72 h culture. The grey bars represent Foxp3 gene transcripts from NOD mice while the black bars represent NOR mice. In NOD and NOR mice, the presence of ciglitazone significantly suppressed Foxp3 expression level in activated nTreg cells compared to untreated group. Moreover, the addition of GW9662 further downregulated Foxp3 level in these cells from both strains compared to untreated cells ($P < 0.01$) and ciglitazone-treated cells ($P < 0.05$). In NOD mice, Foxp3 expression in 15d-PGJ2-treated activated nTreg cells was suppressed compared to untreated cells ($P < 0.01$). Data are expressed as the amount of mRNA transcripts per ◎g of total RNA. This experiment was repeated twice and the graph was plotted based on the mean transcript values ± SEM. Statistical analysis was performed using One-way ANOVA. Post-hoc comparison was performed to identify the significance between treated samples (n=4 mice/experiment).

*P < 0.01, sample groups vs untreated group.
**P < 0.05, ciglitazone-treated group vs ciglitazone + GW9662-treated group.
* P < 0.01, sample groups vs untreated group.
** P < 0.05, ciglitazone-treated group vs ciglitazone + GW9662-treated group.
# the expression level was undetectable.

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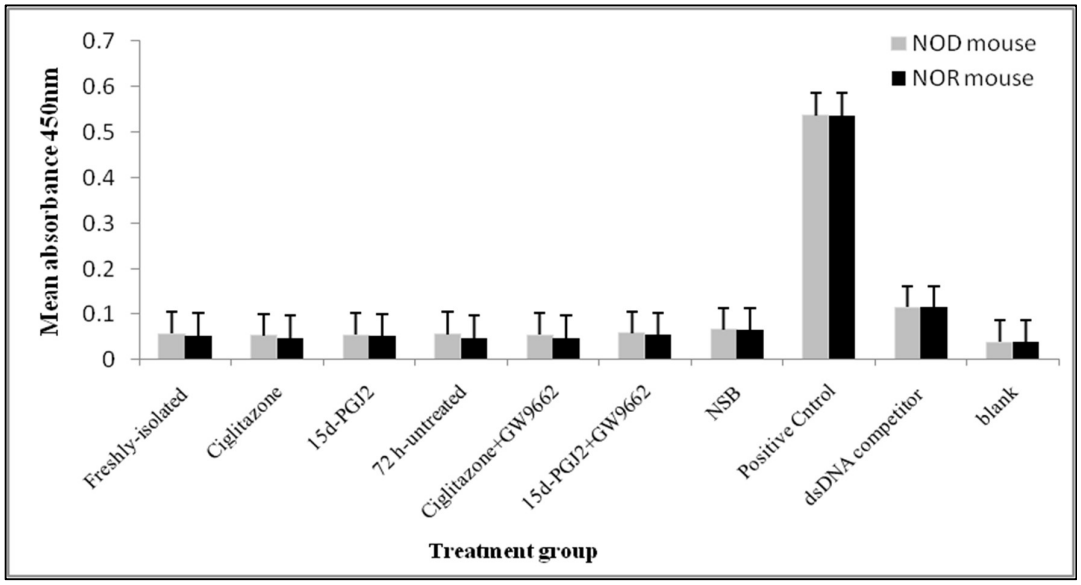

Figure 2 The binding activity between PPARγ **and PPRE in nTreg cells nuclear protein lysates from NOD and NOR mice.** The bar graphs show mean absorbance for each indicated treatment performed in duplicate. PPARγ double-stranded DNA (dsDNA) competitors were added onto dsDNA competitor wells while non-specific binding (NSB) wells were added with buffer without positive control or samples. Blank wells contained only buffer. This experiment was repeated twice and error bars represent mean ± SEM (n=3 mice/experiment).

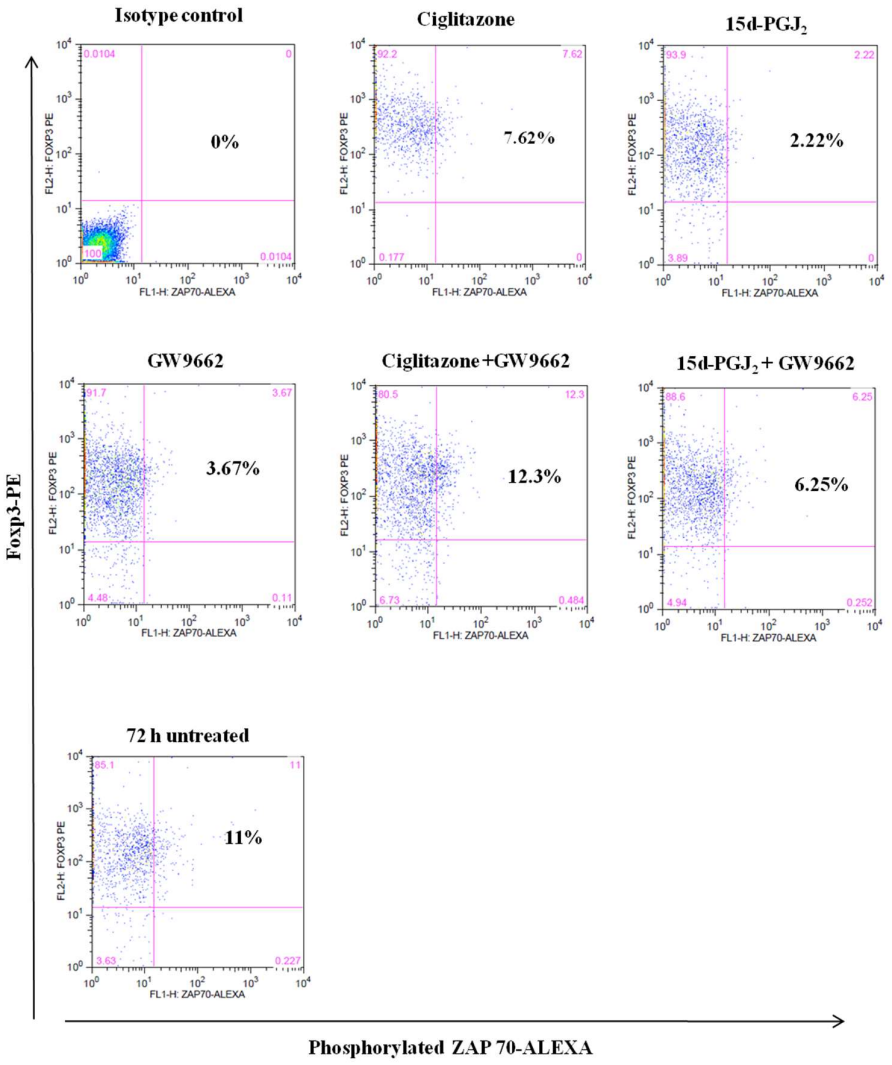

Figure 3 The expression of phosphorylated ZAP-70 in nTreg cells of NOD mice following various treatment after 72 h culture. Dot plot shows the levels of phosphorylated ZAP-70 in activated nTreg cells as indicated by Foxp3 expression. ZAP-70 phosphorylation was measured in nTreg cells treated with ciglitazone, 15d-PGJ_2, GW9662, combination of ciglitazone + GW9662, and combination of 15d-PGJ_2 + GW9662. 72 h-untreated cells was assigned as control group. Data shown are representative of two independant experiments. (n=2 mice/experiment).

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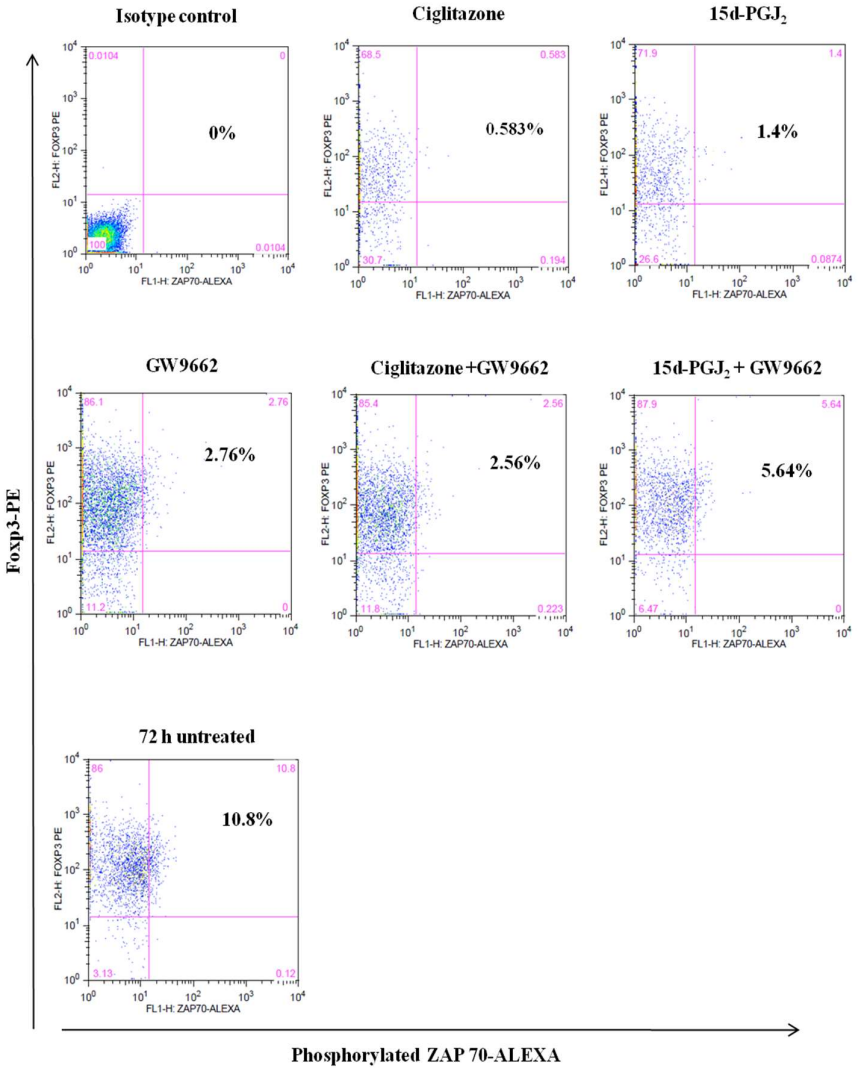

Figure 4 The expression of phosphorylated ZAP-70 in nTreg cells of NOR mice following various treatment after 72 h culture. Dot plot shows the levels of phosphorylated ZAP-70 in activated nTreg cells as indicated by Foxp3 expression. ZAP-70 phosphorylation was measured in nTreg cells treated with ciglitazone, 15d-PGJ2, GW9662, combination of ciglitazone + GW9662, and combination of 15d-PGJ2 + GW9662. 72 h-untreated cells was assigned as control group. Data shown are representative of two independant experiments. (n=2 mice/experiment).

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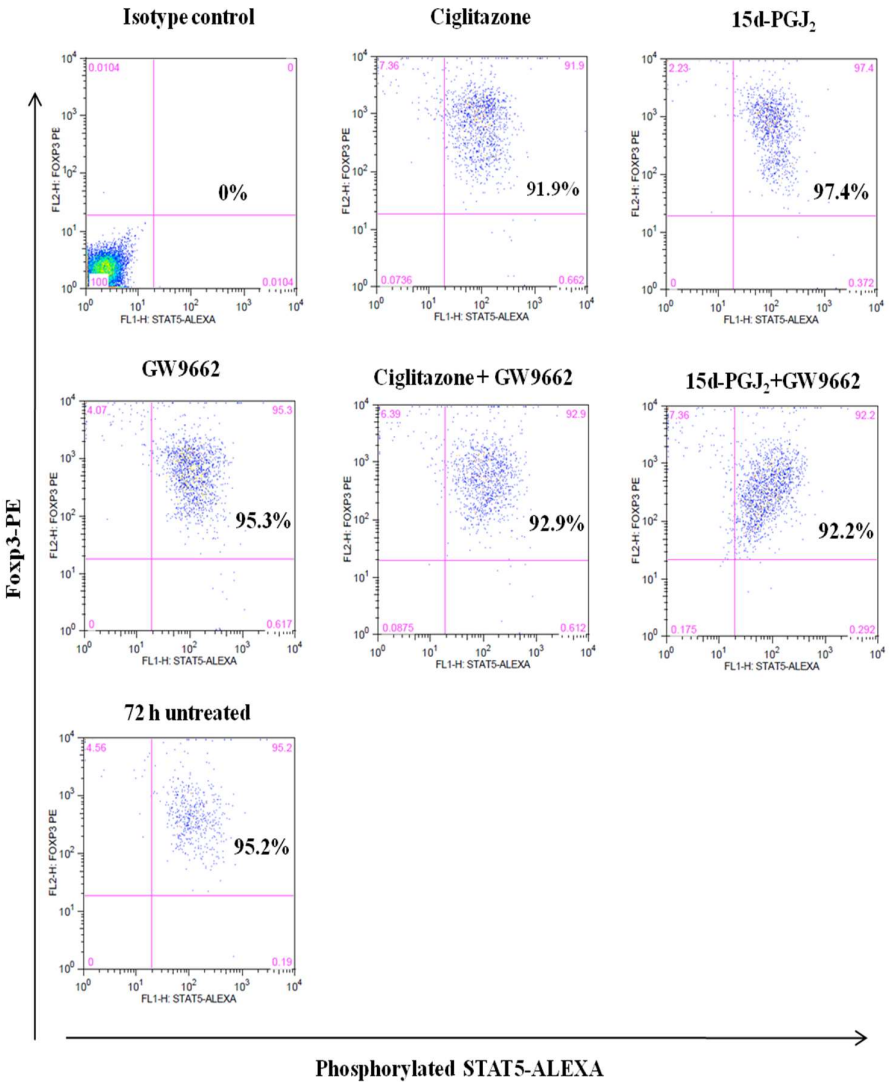

Figure 5 The expression of phosphorylated STAT-5 in activated nTreg cells of NOD mice following various treatment after 72 h culture. Dot plot shows the levels of phosphorylated STAT-5 in nTreg cells. STAT-5 phosphorylation level was measured in activated nTreg cells treated with ciglitazone, 15d-PGJ2, GW9662, combination of ciglitazone + GW9662, and combination of 15d-PGJ2 + GW9662. 72 h-untreated group was assigned as control group. Data shown are representative of two independant experiments (n=2 mice/experiment).

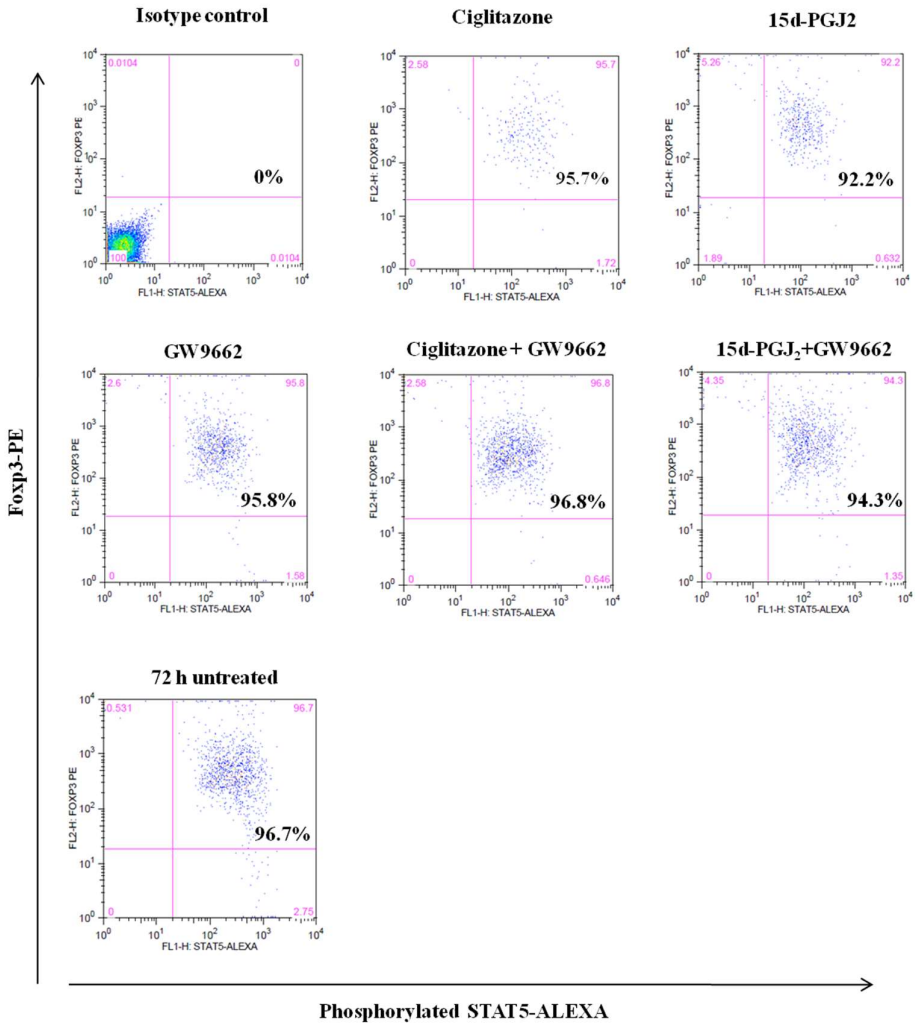

Figure 6 The expression of phosphorylated STAT-5 in activated nTreg cells of NOR mice following treatment after 72 h culture. Dot plot shows the levels of phosphorylated STAT-5 in nTreg cells. STAT-5 phosphorylation level was measured in activated nTreg cells treated with ciglitazone, 15d-PGJ$_2$, GW9662, combination of ciglitazone + GW9662, and combination of 15d-PGJ$_2$ + GW9662. 72 h-untreated group was assigned as control group. Data shown are representative of two independant experiments (n=2 mice/experiment).

Table 1. Differentially expressed target genes of inflammatory pathways in ciglitazone-treated nTreg cells from NOD mice.

No	Symbol	Description	GenBank Accession no	Unigene	Fold change[1]
MAP KINASE					
1	egr1	Early growth response 1	NM007913	Mm.181959	< 0.2
2	fos	FBJ osteosarcoma oncogene	NM010234	Mm.246513	< 0.2
3	jun	Jun oncogene	NM010591	Mm.275071	< 0.2
4	nab2	Ngfi-A binding protein 2	NM008668	Mm.336898	< 0.2
TGF-β					
5	cdkn1a	Cyclin dependent inhibitor 1A	NM007669	Mm.195663	< 0.2
6	cdkn1b	Cyclin dependent inhibitor 1B	NM009875	Mm.2958	< 0.2
7	cdkn2a	Cyclin dependent inhibitor 2A	NM0099877	Mm.4733	< 0.2
8	cdkn2b	Cyclin dependent inhibitor 2B, p15, inhibits CDK4	NM007670	Mm.423094	< 0.2
NF-κβ					
9	Ccl20	Chemokine (C-C) motif ligand 20	NM016960	Mm.116739	< 0.2
10	Cxcl1	Chemokine (C-X-C motif) ligand 1	NM008179	Mm.21013	< 0.2
11	Icam1	Intracellular adhesion molecule 1	NM010493	Mm.435508	< 0.2
12	Ikbkb	Inhibitor of kappa B kinase beta	Nm010546	Mm.277886	< 0.2
13	Il1a	Interleukin 1 alpha	NM010554	Mm.15534	< 0.2
14	Il2	Interleukin 2	Nm008366	Mm.14190	< 0.2
15	Il2ra	Interleukin 2 receptor alpha chain	NM008367	Mm.915	0.3686
16	Lta	Lymphotoxin A	NM010735	Mm.87787	< 0.2
17	Nfkbia	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	NM010907	Mm.170515	1.0718
18	Nos2	Nitric oxide synthase 2, inducible	NM010927	Mm.2893	< 0.2
19	Tank	TRAF-family-associated NF-k B activator	NM011529	Mm.244393	3.5540
20	Tnf	Tumor necrosis factor	NM013693	Mm.1293	0.3585
21	Vcam1	Vascular endothelial molecule 1	NM011693	Mm.76649	< 0.2
NFAT					
22	Cd5	CD5 antigen	NM007650	Mm.779	< 0.2
23	Fasl	Fas (TNF-receptor superfamily member 6) ligand	NM010177	Mm.3355	< 0.2
24	Il2	Interleukin 2	Nm008366	Mm.14190	< 0.2
PKC					
25	Csf2	Colony stimulating factor 2	NM009969	Mm.4922	< 0.2
26	myc	Myelocytomatosis oncogen	NM010849	Mm.2444	> 10.0
27	odc1	Ornithine decarboxylase, structural 1	NM013614	Mm.34102	> 5.0

[1] Fold change differences as compared to untreated control group. Fold change < 0.2 fold indicates downregulation and > 5 fold indicates upregulation. Genes with * are not differentially regulated as compared to control group.

Table 2. Differentially expressed target genes of inflammatory pathways in prostaglandin J_2-treated nTreg cells from NOD mice.

No	Symbol	Description	GenBank Accession no	Unigene	Fold change[1]
MAP KINASE					
1	egr1	Early growth response 1	NM007913	Mm.181959	< 0.2
2	fos	FBJ osteosarcoma oncogene	NM010234	Mm.246513	< 0.2
3	jun	Jun oncogene	NM010591	Mm.275071	< 0.2
4	nab2	Ngfi-A binding protein 2	NM008668	Mm.336898	< 0.2
TGF-β					
5	cdkn1a	Cyclin dependent inhibitor 1A	NM007669	Mm.195663	< 0.2
6	cdkn1b	Cyclin dependent inhibitor 1B	NM009875	Mm.2958	0.60
7	cdkn2a	Cyclin dependent inhibitor 2A	NM0099877	Mm.4733	< 0.2
8	cdkn2b	Cyclin dependent inhibitor 2B, p15, inhibits CDK4	NM007670	Mm.423094	0.37
NF-κβ					
9	Ccl20	Chemokine (C-C) motif ligand 20	NM016960	Mm.116739	< 0.2
10	Cxcl1	Chemokine (C-X-C motif) ligand 1	NM008179	Mm.21013	< 0.2
11	Icam1	Intracellular adhesion molecule 1	NM010493	Mm.435508	< 0.2
12	Ikbkb	Inhibitor of kappa B kinase beta	Nm010546	Mm.277886	< 0.2
13	Il1a	Interleukin 1 alpha	NM010554	Mm.15534	< 0.2
14	Il2	Interleukin 2	Nm008366	Mm.14190	< 0.2
15	Il2ra	Interleukin 2 receptor alpha chain	NM008367	Mm.915	< 0.2
16	Lta	Lymphotoxin A	NM010735	Mm.87787	0.5620
17	Nfkbia	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	NM010907	Mm.170515	< 0.2
18	Nos2	Nitric oxide synthase 2, inducible	NM010927	Mm.2893	< 0.2
19	Tank	TRAF-family-associated NF-k B activator	NM011529	Mm.244393	< 0.2
20	Tnf	Tumor necrosis factor	NM013693	Mm.1293	1.2968
21	Vcam1	Vascular endothelial molecule 1	NM011693	Mm.76649	< 0.2
NFAT					
22	Cd5	CD5 antigen	NM007650	Mm.779	< 0.2
23	Fasl	Fas (TNF-receptor superfamily member 6) ligand	NM010177	Mm.3355	< 0.2
24	Il2	Interleukin 2	Nm008366	Mm.14190	< 0.2
PKC					
25	Csf2	Colony stimulating factor 2	NM009969	Mm.4922	*
26	myc	Myelocytomatosis oncogen	NM010849	Mm.2444	*
27	odc1	Ornithine decarboxylase, structural 1	NM013614	Mm.34102	> 5.0

[1] Fold change differences as compared to untreated control group. Fold change < 0.2 fold indicates downregulation and > 5 fold indicates upregulation. Genes with * are not differentially regulated as compared to control group.

Table 3. Differentially expressed target genes of inflammatory pathways in ciglitazone-treated nTreg cells from NOR mice.

No	Symbol	Description	GenBank Accession no	Unigene	Fold change[1]
MAP KINASE					
1	egr1	Early growth response 1	NM007913	Mm.181959	3.650
2	fos	FBJ osteosarcoma oncogene	NM010234	Mm.246513	< 0.2
3	jun	Jun oncogene	NM010591	Mm.275071	< 0.2
4	nab2	Ngfi-A binding protein 2	NM008668	Mm.336898	< 0.2
TGF-β					
5	cdkn1a	Cyclin dependent inhibitor 1A	NM007669	Mm.195663	> 5.0
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NF-κβ					
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11	Icam1	Intracellular adhesion molecule 1	NM010493	Mm.435508	*
12	Ikbkb	Inhibitor of kappa B kinase beta	Nm010546	Mm.277886	*
13	Il1a	Interleukin 1 alpha	NM010554	Mm.15534	*
14	Il2	Interleukin 2	Nm008366	Mm.14190	*
15	Il2ra	Interleukin 2 receptor alpha chain	NM008367	Mm.915	> 20.0
16	Lta	Lymphotoxin A	NM010735	Mm.87787	> 5.0
17	Nfkbia	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	NM010907	Mm.170515	> 15.0
18	Nos2	Nitric oxide synthase 2, inducible	NM010927	Mm.2893	> 5.0
19	Tank	TRAF-family-associated NF-k B activator	NM011529	Mm.244393	> 5.0
20	Tnf	Tumor necrosis factor	NM013693	Mm.1293	*
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NFAT					
22	Cd5	CD5 antigen	NM007650	Mm.779	> 5.0
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PKC					
25	Csf2	Colony stimulating factor 2	NM009969	Mm.4922	*
26	myc	Myelocytomatosis oncogen	NM010849	Mm.2444	> 10.0
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Table 4. Differentially expressed target genes of inflammatory pathways in prostaglandin J_2-treated nTreg cells from NOR mice.

No	Symbol	Description	GenBank Accession no	Unigene	Fold change[1]
MAP KINASE					
1	egr1	Early growth response 1	NM007913	Mm.181959	1.501
2	fos	FBJ osteosarcoma oncogene	NM010234	Mm.246513	*
3	jun	Jun oncogene	NM010591	Mm.275071	2.020
4	nab2	Ngfi-A binding protein 2	NM008668	Mm.336898	< 0.2
TGF-β					
5	cdkn1a	Cyclin dependent inhibitor 1A	NM007669	Mm.195663	*
6	cdkn1b	Cyclin dependent inhibitor 1B	NM009875	Mm.2958	*
7	cdkn2a	Cyclin dependent inhibitor 2A	NM0099877	Mm.4733	*
8	cdkn2b	Cyclin dependent inhibitor 2B, p15, inhibits CDK4	NM007670	Mm.423094	*
NF-κβ					
9	Ccl20	Chemokine (C-C) motif ligand 20	NM016960	Mm.116739	0.4983
10	Cxcl1	Chemokine (C-X-C motif) ligand 1	NM008179	Mm.21013	0.1632
11	Icam1	Intracellular adhesion molecule 1	NM010493	Mm.435508	0.6713
12	Ikbkb	Inhibitor of kappa B kinase beta	Nm010546	Mm.277886	0.1632
13	Il1a	Interleukin 1 alpha	NM010554	Mm.15534	1.0317
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24	Il2	Interleukin 2	Nm008366	Mm.14190	< 0.2
PKC					
25	Csf2	Colony stimulating factor 2	NM009969	Mm.4922	*
26	myc	Myelocytomatosis oncogen	NM010849	Mm.2444	*
27	odc1	Ornithine decarboxylase, structural	NM013614	Mm.34102	> 5.0

[1] Fold change differences as compared to untreated control group. Fold change < 0.2 fold indicates downregulation and > 5 fold indicates upregulation. Genes with * are not differentially regulated as compared to control group.

4. Discussion

Since both PPARγ and Foxp3 are involved in immune suppression, we investigated whether there is cross-talk between these transcription factors in nTreg cells. To provide insight into the functional aspect of these cells, we utilized the autoimmune diabetic, NOD mouse model, to further dissect the interactions between these transcription factors. PPARγ ligands have been shown to induce anti-inflammatory responses in autoimmune models [23, 24, 25]. However, thus far whether PPARγ modulate the function of nTreg cells during autoimmune conditions has not been described.

Our analysis showed that PPARγ ligands downregulated Foxp3 expression in nTreg cells of both the autoimmune NOD as well as its control NOR mouse models. To further characterize whether this effect occurs via the PPARγ activation pathway, we treated the nTreg cells with its inhibitor, GW9662 and analysed the binding of PPARγ to its response element. Our current data demonstrated that both ciglitazone and 15d-PGJ2 promoted the downregulation of Foxp3 expression independently of PPAR. The addition of GW9662 did not abrogate the effect of the PPARγ ligand, but instead further suppressed Foxp3 expression in nTreg cells of both NOD and NOR mice. The synergistic effect of PPARγ ligands and GW9662 have in fact been demonstrated in growth inhibition of breast cancer cells [26] and suppression of IL-2 production in T-helper cells [27]. In fact, treatment with GW9662 alone was able to reduce IL-2 production in T-helper cells and Jurkat T-cell lines, suggesting that GW9662 has an intrinsic inhibitory property [27, 28]. The reasons for these observations is however still unclear. This finding is in agreement with a previous report by Lei et al [29] who showed that in human peripheral blood mononuclear cells, PPARγ agonists do not alter Foxp3 expression in nTreg cells and iTreg cells via PPRE. However, whether this effect is primarily due to the direct interaction between PPARγ and Foxp3 or secondary to interaction with other protein molecules require further investigation.

Our findings are contrary to previous reports that showed ciglitazone is not involved in modulating Foxp3 protein expression in nTreg cells from C57BL/6 mice [30] and human peripheral blood mononuclear cells [31]. In fact, the suppressive capacity of nTreg cells is not affected by PPARγ ligands, an indication that PPARγ agonists neither modify the Foxp3 expression nor the suppressive function of nTreg cells in healthy animals [31]. The first report on the effect of PPARγ ligands onTreg cells was demonstrated in a mice model of myasthenia gravis [31]. The study showed that co-transfer of wild type nTreg cells with ciglitazone positively promote survival of these mice compared to PPARγ -deleted nTreg cells. This suggests that PPARγ-expressing nTreg cell is important in mediating ciglitazone effect in vivo [31]. However, whether this effect is mediated by the level of Foxp3 in these cells was not studied. We speculate that the interaction between PPARγ and nTreg cells in in vivo setting may involve multiple players that results in the enhancement of nTreg cell function, but not the level of Foxp3 expression in nTreg cells.

PPARγ ligands negatively affect proinflammatory cytokine production by inhibiting the transcription factors AP-1, NF-κβ, NFAT and STATs in CD4+T cells [32, 33, 34]. On the other hand, Foxp3 expression requires binding of AP-1, NF-κβ and NFAT at the promoter and enhancer sites of nTreg cells [35, 36, 37, 38]. Thus the current findings suggest that PPARγ ligands may modulate Foxp3 expression in nTreg cells by interfering with the activation of these transcription factors which may in turn modulate the function of Foxp3 in nTreg cells. It would be interesting to determine whether administration of PPARγ ligands to NOD mice would worsen the autoimmune diabetic condition of the mice. Our study also demonstrated that, there was no significant PPARγ-PPRE interaction in PPARγ ligand-treated nTreg cells of both NOD and NOR mice, further indicating that the observed effect occurred independent of PPARγ activation.

Another possible explanation is that the suppressive effect of PPARγ ligands on Foxp3 expression in nTreg cells may be different from the transcriptional suppression/repression mechanism by PPARγ [39, 40]. As mentioned earlier, the negative influence of PPARγ ligands on the transcription factors

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AP-1, NF-kB and NFAT in CD4+ T cells has been proposed as the underlying mechanism for anti-inflammatory response by PPARγ. This downregulation by PPARγ ligands has been termed transrepression [39, 40]. This mechanism scavenges AP-1, NF-kB and NFAT from binding to the Foxp3 core promoter region [35, 36, 37, 38]. As a result, Foxp3 expression in nTreg cells is attenuated. Therefore, we postulate that PPARγ ligands may impose their immunodownregulatory effect on nTreg cells by ligand-dependant PPARγ transrepression.

Since our current data showed that PPARγ ligands negatively regulate Foxp3 expression in nTreg cells of NOD and NOR mice, we then examined whether this effect is due to PPARγ interference on the proximal or distal signaling components of nTreg cells. This was performed by measuring the phosphorylation levels of ZAP-70 and STAT-5. T-cell receptor complex activation results in the phosphorylation of tyrosine-based proteins such as ZAP-70. The activation of ZAP-70 results in activation of downstream signaling pathways [41]. STAT-5 signaling is required for nTreg cell function and survival [21]. Therefore, we analysed the level of phosphorylation of these mediators following treatment with PPARγ ligands.

Our current study showed that PPARγ ligands did not modify the phosphorylation levels of ZAP-70 or STAT-5 in nTreg cells from NOD and NOR mice. This finding suggests that PPARγ ligands do not suppress Foxp3 expression in nTreg cells of NOD and NOR mice by modulation ZAP-70 and STAT-5 signaling pathways. The current finding on PPARγ effect on STAT-5 is in line with a previous study that showed PPARγ do not hinder STAT-5 phosphorylation but rather affect the downstream signaling of the growth hormone- activated STAT-5 activation [42]. In addition, in this part of current study, we also noted that, PPARγ ligands did not altered the TCR proximal signaling activation i.e. ZAP-70 phosphorylation, in nTreg cells from both NOD and NOR mice. The levels of ZAP-70 phosphorylation in PPARγ treated and untreated nTreg cells were not significantly different. These findings are consistent with a previous report by [43] where they elucidated that the activation of ZAP-70 is extremely low in CD4+CD25+Foxp3+ cells.

Next, we examined the alterations in the relevant genes involved in signaling pathways in nTreg cells from NOD and NOR mice after treatment with PPARγ ligands using the PCR Array technology. We observed downregulation of pro- and anti-inflammatory pathway-related genes in PPARγ ligand-treated nTreg cells of NOD mice in response to PPARγ ligand treatment. Ciglitazone moderately induced myc and odc gene expression levels in nTreg cells of NOR and NOD mice. The stimulation of myc gene indicates a common response to a vast group of substances that elicit phosphoinositate and activate PKC signaling pathway [44] and myc protein is important to keep activated T cells functioning [59] whereas activation of odc1 gene promotes cell survival activities [45]. The activation of PKC pathway by ciglitazone is related on the survival of these cells in both mouse strains *in vitro* since PKC pathway activation in nTreg cells is canonical for cell proliferation and survival [46]. Furthermore, recent study showed that Foxp3 protein can regulate T cell metabolism by suppressing myc gene in T cell [58]. According to Angelin et al (2017), Foxp3 suppresses myc-dependant genes and thus regulate Treg cell metabolism to adapt in low-glucose/ high-lactate rich environments [58]. In the current findings, addition of ciglitazone inhibits Foxp3 expression in nTreg cells and induces myc expression in ciglitazone-treated nTreg cells in NOD and NOR mice. This may suggest that ciglitazone capable of reversing Foxp3 expression by upregulating myc expression *in vitro.*

12 target genes related to TGF-β, NF-κβ, and NFAT signaling pathways in PPARγ ligand-treated nTreg cells. TGF-β and p53-related gene, cdkn1a that encodes protein cyclin-dependant kinase inhibitor was slightly upregulated in nTreg cells following treatment with ciglitazone, but not 15d-PGJ_2. The upregulation may be due to cross-linkage with p53-dependant pathway since cdkn1a gene is under joint control of TGF-β and p53 signaling [47]. Also, overlapping cellular function by both components may indicate that they have potential convergence points in their signaling pathways [48].

Similarly, in NOR nTreg cells, ciglitazone induced NF-kB pathway-related genes i.e. il2ra and nf-kbia, but not 15d-PGJ2. Other genes, such as ccl20, lta and tank were slightly upregulated in ciglitazone-treated cells. These data may suggest that ciglitazone restricts NF-κB activation by upregulating nf-kbia expression, at the same time, inducing expression of CD25 molecules by upregulating il-2ra gene. The expression of il2-ra gene is also regulated by PKC signaling. Thus, upregulation of il-2ra gene induced by ciglitazone in nTreg cells is possibly via PKC dependant-NF-κβ pathway, since NF-κβ activation is preceded by PKC signaling. We also recorded that ciglitazone moderately upregulated NFAT-related target gene, cd5 in nTreg cells. This gene is responsible to encode for cd5 molecules on nTreg cell surfaces, responsible for suppressive function [49] thus this may correspond to the default function of nTreg cells as immunosuppressor of autoreactive immune cells.

In addition, we observed seven similar target genes that were downregulated in both NOD and NOR nTreg cells following treatment with PPARγ ligands. These target genes are nab2 gene of MAPK pathway and ei24 and igfbp3 genes of NFAT pathway. MAPK-related target gene, nab2 is associated with antigen recognition in CD4+ T lymphocytes [50]. Overall, the downregulation of pro- and anti-inflammatory related target genes by PPARγ ligands in NOD and NOR nTreg cells may suggest the correlation with Foxp3 expression in this cells. Therefore, this data help in narrowing down possible regulatory molecules responsible on PPARγ-Foxp3 crosstalk in activated nTreg cells.

5. Conclusions

Taken collectively, our current findings demonstrated the negative regulation of PPARγ ligands in inducing Foxp3 expression in nTreg cells of NOD and NOR mouse models. However, the underlying mechanism of Foxp3 suppression was differentially modulated in NOD and NOR nTreg cells. Our data from NOD and NOR mouse models suggest that PPARγ ligands attenuated Foxp3 mRNA expression in TCR-activated nTreg cells via PPARγ-independant pathways. Even though it is well-described that PPARγ ligands induce target gene expression via PPARγ-dependant manner, it is becoming increasingly clear that PPARγ ligands also mediate the non-genomic PPARγ-dependant and –independant manners [51, 52]. We speculate that in NOD nTreg cells, PPARγ ligands induced Foxp3 expression either via downregulation of multiple target genes in pro-inflammatory pathway related genes. These pro-inflammatory signaling pathways such as NFAT, NK-kB and PKC are essential for Foxp3 expression in nTreg cells, not for pro-inflammatory cytokine production [32-38]. An extensive analysis on Foxp3 expression in nTreg cells is also required in delineating the regulatory components involved in Foxp3 expression, particularly in committed nTreg cells. Although much remains to be learned about the basis of PPARγ and its ligands on Foxp3 expression in nTreg cells, the current study provided additional information to preliminary relationship between PPARγ ligands and Foxp3 expression thus put forward the redefinition of PPARγ ligands as immune modulators in tumor-associated conditions. In tumor microenvironment, population of Foxp3+Treg cells may indicate poor prognosis as they provide protection to tumor cells against tumour-infiltrating lymphocytes [53, 54, 55, 56, 57]. It is thus instrumental to look further into the potential function of TZD drugs on Foxp3+ Treg cells in tumor models as suggested by current study that it is potential to be used as immunomodulator in tumor immunotherapy better than autoimmune therapy.

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