PPARγ LIGAND SUPPRESSES FOXP3 EXPRESSION IN T-REGULATORY CELLS DURING EXCESSIVE INFLAMMATION VIA MODULATING HISTONE ACETYLTRANSFERASE AND HDAC6/11 ACTIVITIES

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Abstract
Natural T-Regulatory (nTreg) cells represent approximately 8-10% of the total CD4+ T cell population and constantly expressing Foxp3 proteins. These cells are crucial for immune homeostasis, preventing over-inflammation and autoimmunity. Our previous study reported that PPARγ ligand, 15d-PGJ_2 negatively influences the expression of Foxp3 in nTreg cells, which reflexes the attenuation in immunosuppressive function of nTreg cells. This study aims to unveil the molecular mechanism of Foxp3 suppression by PPARγ in nTreg cells during autoimmune Type 1 Diabetes. Co-stimulatory proteins were measured using flow cytometry and methylation measurement of Foxp3 expression was measured based on histone modification activity. Nuclear proteins of isolated cells were extracted out to measure two HDAC and two HAT enzyme activities using ELISA. Purified nTreg cells were isolated using MoFlow Cell sorter and will be then cultured for 72 hrs to mimic the TCR activation and downstream signalling. The expression of Foxp3 in these cells were measured using flow cytometry analysis and were positively selected. Current data showed that histone acetylation activities were cross talked with PPARγ pathway in nTreg cells from diabetic, but in healthy mice. FoxP3 gene expression may be regulated via histone modification that in diabetic mice via PPARγ- independent pathways. Altogether, this study provides fundamental analysis on the putative role of PPARγ ligand 15d-PGJ_2 as HDAC6/11 inhibitors. Therefore, this may suggest that combination of 15d-PGJ_2 and GW9662 can be an alternative to HDAC6 inhibitor which is less toxic compared to pan-HDACi in treating inflammatory-related diseases. These ligands also potentially able to suppress the microenvironment of nTreg cells protecting tumour-bearing cells.

Keywords: Foxp3, PPAR ligands, HDAC inhibitors, Epigenetics, HDAC regulation, Autoimmune

Introduction

T-regulatory (Treg) cells naturally express surface markers CD4+CD25+ and intracellular transcription factor forkhead box P3 (FoxP3) (1). These cells are developed in the thymus, released to peripheral to maintained self-tolerance and immune homeostasis through their five putative suppressive mechanisms (1). High number of nTreg cells are correlated with tumorigenesis which indicate poor prognosis (2). In autoimmune diseases, low number of these cells lead to the development of over-reactive inflammation. Treg cells secrete IL-10 and TGF-β which are involved in immunosuppressive towards dendritic cells (DCs) (3), stimulate T lymphocytes such as T helper 1 (Th1), Th2 dan Th17 cell proliferation (4), mast cells, basophils and eosinophils (5). Genetic mutations on FoxP3 gene cause immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) in human and scurfy in mouse model which accelerate the development of autoimmune diabetes (6, 7, 8).

Constitutive expression of Foxp3 protein is important to maintain its suppressive effect by nTreg cells (9,10).

Meanwhile the matured nTreg cells exhibit hypomethylation or demethylated at CpG motif (11) on the Treg-specific demethylated region (TSDR) of the FoxP3 locus, indicating that FoxP3 expression is regulated by both genetic and epigenetic factors. Foxp3 protein suppressed two keyiImmune-related nuclear protein pathway NFAT and NF-κB, resulting in downregulation of other effector T cells cytokines including IL-2 (12, 13). FoxP3 requires direct deoxyribonucleic acid binding domain (DBD) to form oligomeric complexes with other proteins dynamically in regulating T cell activation. Thus, deficiency of Treg cells functions is associated with autoimmune diseases and inflammatory such as multiple sclerosis, SLE, T1D and rheumatoid arthritis. In contrast, upregulation of Treg activity can inhibit beneficial anti-tumor or anti-viral properties.

Similarly, the role of PPARγ as an immune suppressor has been widely studied. Our previous study showed that FoxP3 expression was found to be negatively regulated by PPARγ ligands in activated nTreg cells through PPARγ-independant mechanism (15).

PPARγ activation by its ligands lead to binding of PPAR/retinoid X receptor (RXR) heterodimer, causing subsequent conformational change of the ligand-binding domain (LBD) and lead to corepressor releasing bind on the coactivators, resulting in modulation of PPARγ activity (16, 17). PPARγ is an important nuclear receptor in diabetes as it acts as energy storage, fatty acid metabolism, adipogenesis differentiation and insulin sensitizer (18). The natural ligand and prostanoids to activate PPARγ translocation into nucleus is known as 15-Deoxy-Δ(12,14)-prostaglandin J2 (15d-PGJ_2) (19, 20). It was reported previously that the *in vivo* production of the 15d-PGJ_2 is insufficient to be a significant agonist (21). Thus, development of synthetic ligands to benefit from PPAR protein activation has been established in various diseases including diabetes mellitus. Due to its diverse potential as immune modulators, PPAR pathway has become the focal of interest in the field of immune modulations. Many researchers have shifted to uncover the role of epigenetic in relation to the mechanism of immune modulation.

In 2010, Grausenburger and co-workers demonstrated that HDACs capable to inhibit cytokine production from activated T cells in allergy model. Abnormalities in HDACs activity may lead to either proinflammatory cell activation or immune suppression (22). FoxP3 acetylation has been found to promote DNA binding and increased resistance to proteasomal degradation (23). The fact that HDAC6, HDAC9, HDAC11 and sirtuins 1 (sirt1) interact with FoxP3 in regulating its activity underline the possible interaction between these transcriptional proteins. Meanwhile, the inhibition of these HDAC members increased acetylation of FoxP3 expression (24). Acetylation of FoxP3 by HATs have been shown to help in maintaining its core histone i.e. ε-amino nitrogen specific lysine residues located in the amino terminal tails which is important to chromatin remodeling and gene activation (25, 26, 27).

Our findings reported on the influence of PPARγ activation in nTreg cells on co-stimulatory components, thus have underlined the crosstalk between histone proteins and FoxP3 protein regulatory roles during autoimmune diabetic condition. This is hoped to establish the understanding on the relation between these transcription factors. The potential synergistic effect between these molecules can be explore further for the establishment of molecular therapeutic target in treating immune-related diseases.

Material and Methods

Experimental animals

All experiments were performed in accordance with protocols approved by the USM Institutional Animal Care and Use Committee (USM IACUC) USM/Animal Ethics Approval/2015/(97) (704). Eight-week-old female NOD/ShiLtJ and seven- to eight-week-old female NOR/LtJ mice were purchased from Jackson Laboratory, Maine, USA while eight-week-old female Balb/c mice were purchased from Universiti Putra Malaysia. All animals were acclimatized at rodent quarantine room for 7 days before transferred to rodent experiment room. The room temperature was maintained at 21-23°C with humidity 51-57% and the light/dark cycle. All mice were kept in solid-bottom cages bedded with pre-autoclaved wood fiber. Three to four mice were placed per cage to minimize stress. Standard mouse diet pellet and reserve osmosis water were given ad libitum. Mice beddings were changed every three days and all mice were kept under room temperature 21-24°C with humidity 46-65%. All mice were labelled using ear notching system and tail colour code system, weighed, and measured for peripheral blood glucose level weekly. Scheduled sanitation where the mice were placed was performed to minimize contamination.

a) Measurement of NOD and NOR mice blood glucose level

Accu-Chek® Active strips with Accu-Chek Active glucometer system was used to measure peripheral glucose level from 1-2 µL capillary blood with the measuring interval 0.6-33.3 mmol/L. To reduce injury, mice were strained with a pre-clean modified 50ml Falcon tube as mice strainer (Figure 1). The tail of the mouse was disinfected with 70% alcohol swab and waited to dry before prick, and the first drop of the blood was clean to avoid alcohol contamination that may affect the reading. The subsequent blood drop was obtained for the testing and was run in duplicate.

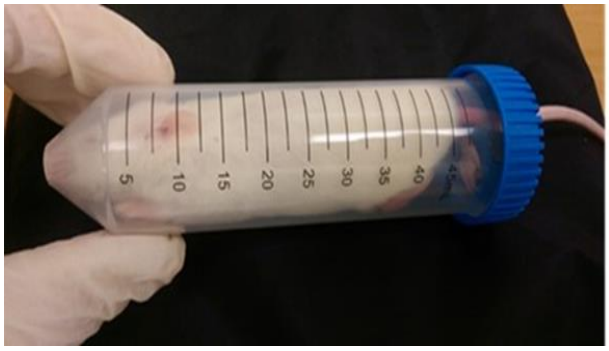
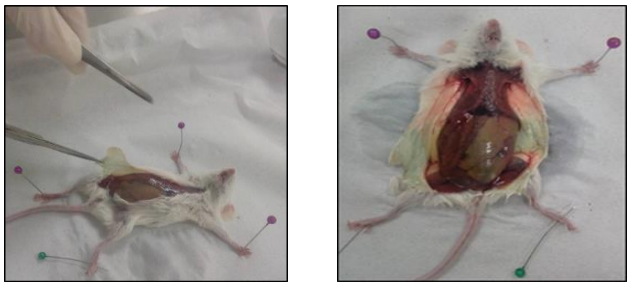


Figure 1: Pre-cleaned modified Falcon tube was used as mice strainer. This technique was created to minimize animal strangulation and injury during handling. One small hole was created at the bottom part of the tubes to allow air flow into the tube for breathing purposes.

b) Diabetes development of NOD mice

Diabetes onset was monitored weekly from 11 to 41 weeks at rodent experiment room AMDI, USM. All animals were handled strictly according to the guidelines. Blood was obtained as described in section a). Diabetic mice and control strains were sacrificed to obtain their spleens according to procedure described earlier. All mice were sacrificed through cervical dislocation. The spleens were then harvested under aseptic technique (Figure 2) and collected in tubes containing ice-cold culture media. The carcasses were sent for proper disposal. All spleens were transported on ice to for further downstream analysis.



a) b)

Figure 2: Spleen harvest on sacrificed mouse. a) After cervical dislocation, the skin was opened, and 70% alcohol was sprayed before cut through the cavity part. (b) The cavity was opened, and spleen harvest was performed aseptically

Isolation and in vitro culture of CD4+ CD25+ FoxP3+ nTreg cells from splenocytes

Harvested spleens were washed twice with ice-cold PBS buffer before homogenized using 5ml syringe plunger. The cell suspension was transferred into a 15ml conical tube with 30 µM pre-separation filter (Miltenyi Biotech, German) on the top. The cells were centrifuged at 300 x g, 10 minutes and 4°C, and the supernatant was aspirated and the pellet was resuspended with 1ml of ice-cold isolation buffer for cell counting. Briefly, the cells were diluted in PBS buffer with 10 µl 0.4% (v/v) trypan blue and equal volume of diluted cells were transferred to haemacytometer chamber to differentiate the viable cells as indicated by trypan blue staining using inverted microscope.

Automated sorting of natural T regulatory cells from Balb/c mice

Cells were pelleted at 300 x g for 10 minutes and resuspended with 40 µl of staining buffer then labelled with CD4-FITC and CD25-PE multicolor antibodies at ratio 1:10. Unstained cells were used as control negative while control positive was prepared by adding antibody in 1:10 dilution as single staining of CD4-FITC and CD25-PE respectively. Labelled cells were mixed well and incubated for 10 minutes at 2-8°C. Cells were then washed with 1-2 ml of buffer, spun at 300 xg for 10 minutes then the supernatant was discarded. All labelled cells were resuspended with 500 µl buffer for automated sorting using MoFlow Cell sorter (Beckman Coulter).

Magnetic sorting of natural T regulatory cells from NOD and NOR mice

Magnetic isolation of natural T regulatory cells was performed using isolation kit for CD4+CD25+ Regulatory T cell (Miltenyi Biotec, Germany). The magnetic isolation was performed in a two-step procedure. Firstly, indirect labelling of non-CD4+ were magnetically labeled with a cocktail of biotin-conjugated antibodies and Anti-Biotin MicroBeads. These cells were subsequently depleted over a separation column containing magnetic beads. The flow-through fraction of unlabelled CD4+ T cells were then labeled with CD25 antibodies for positive selection of CD4+CD25+ regulatory T cells. Cells were then prepared for *in vitro* experiments.

Post sort analysis

The verification of cell sorting analysis is necessary upon cell isolation to maintain single cell population where up to $\leq 10^6$ cells were used for this purpose. The cells were stained for surface marker CD4-FITC and CD25-PE antibodies (Miltenyi Biotec, Germany) for 10 minutes, cold in the dark. Then followed by washing the with 1-2 ml of buffer and discarded the supernatant by centrifugation. These cells require fixed and permed method for the subsequent intracellular staining (as per manufacturer's protocol). The intracellular staining was performed by added 100µl of 1x Perm/Wash buffer mixed well with Foxp3 APC, incubated in the dark (40- 45 minutes) following rigorous mixing by vortex, then subjected to washing with perm/wash buffer to remove residues (Miltenyi Biotec, Germany). Next, cell pellet was resuspended in 500 µl of staining buffer to be analyzed by BD FACSCanto™ II Analyser flow cytometry.

Immunofluorescence staining of Foxp3 protein in

untreated and treated groups.

Following *in vitro* culture, treated and untreated nTreg cells were immobilized on a clean microscope slide to analyze Foxp3 protein expression following treatment using cytospin centrifugation at 800 rpm for 3 minutes. The cells were then fixed and permed and with freshly prepared 1x Fix/Perm buffer in the dark at 4°C for 45 minutes. All immobilized cells were washed by dipping into a coplin jar with prepared 1x Perm/Wash buffer for 1 minute and then proceed with blocking step by wetting the slide with 2% (w/v) BSA in 1x PBS at room temperature. Cells were then incubated overnight with Foxp3 primary antibody as per manufacturer's instruction. To prevent dehydration, all stained slides were wrapped in damp towel overnight before washing with 1x Perm/Wash buffer. Staining of Alexa Fluor 488 goat anti-rabbit diluted 1:200 in 0.1% (w/v) BSA in 1x PBS is used as secondary antibody and followed protocol as per manufacturer's instruction. 4', 6-diamidino-2-phenylindole (DAPI) solution was used to counter-stain following three times washing of the labelled slides. The slides were then sealed for viewing under immunofluorescence microscope. Foxp3 expression in treated nTreg cells were analysed using DP2-BSW digital camera software.

Acetylation and deacetylation activities binding assay preparation.

Acetylation and deacetylation were measured using colorimetric assays. Total 4 µg cell nuclear extract from respective treatment groups were loaded into the designated wells according to the manufacturer's protocol (EpiGENTEK). The final volume for each wells including blank, standard, and sample for HAT, HDAC6/HDAC11 binding were set at 100 µl per well. Measurement of acetylation/deacetylation activities were determined using the standard curve generated from serial dilution for each enzyme respectively. Slope at the linear plot was determined with coefficient of determination, $R2 > 0.95$. Levels and percentage of changes in activity for these enzymes were calculated using formula according to the manufacturer's protocol.

Results

Efficiency comparison between Beckman Coulter MoFlow automated cell sorter and MACS magnetic cell isolation for CD4+CD25+ cell single population

In the current study, we used both automated and manual cell isolation for CD4+CD25+ cell population. Figure 3 and Figure 4 showed that the efficiency of isolation was more than 90% for both sorting which indicate that both methods are reliable. Isolated CD4+CD25+ cell population were labelled with anti-FoxP3 to detect its expression levels on nTreg cells. It is interesting to note that we have found FoxP3 expression on Balb/c (Figure 3d) is slightly lower which is 44% compared to 63% in NOD whereas approximately 75% in NOR mice (Figure 4d). This may suggest that FoxP3 expression in these cells is also influence by genetic background.

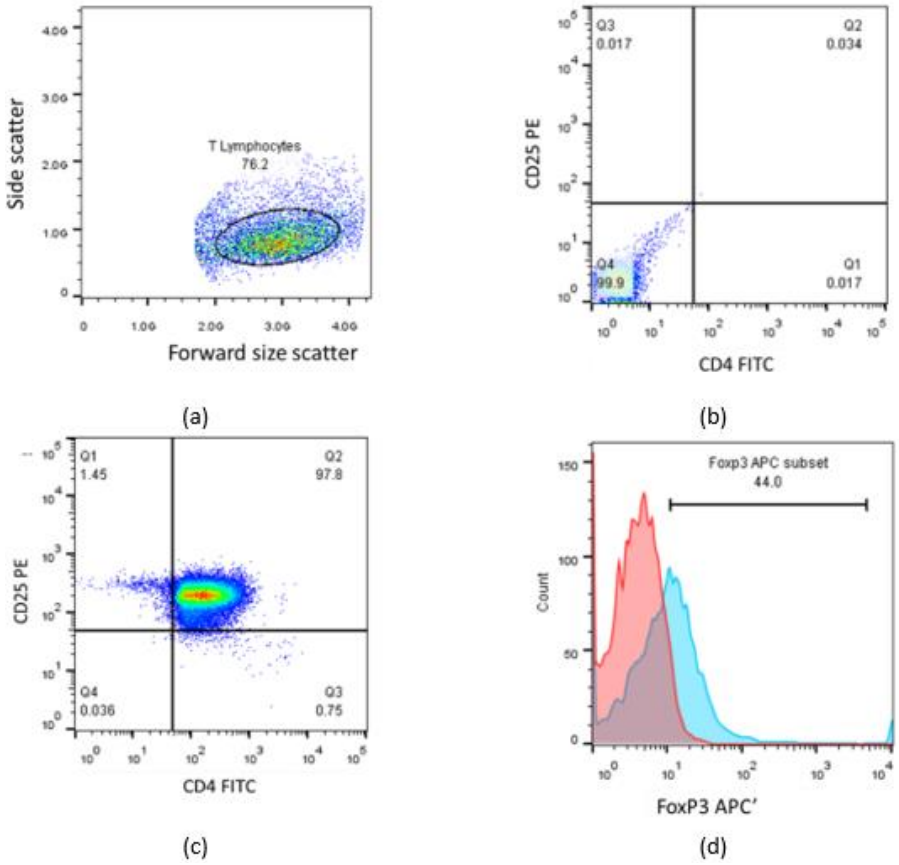


Figure 3: The efficiency of nTreg cell isolation from Balb/c mice using MoFlow sorter.
(a) Dot plot representing gated on T cells population from Balb/c mice splenocytes. (b) Unstained cells were used as negative control to get the gate for positive and negative population. (c) Bivariate dot plot representing sorted nTreg cells were stained with anti-CD4 FITC and anti-CD25 PE to indicate double positive population. (d) Histogram shows FoxP3 expression was 44% (blue) compared with unstained (pink)
Dot plot is a representative of three independant experiment (n=4 mice/experiment).

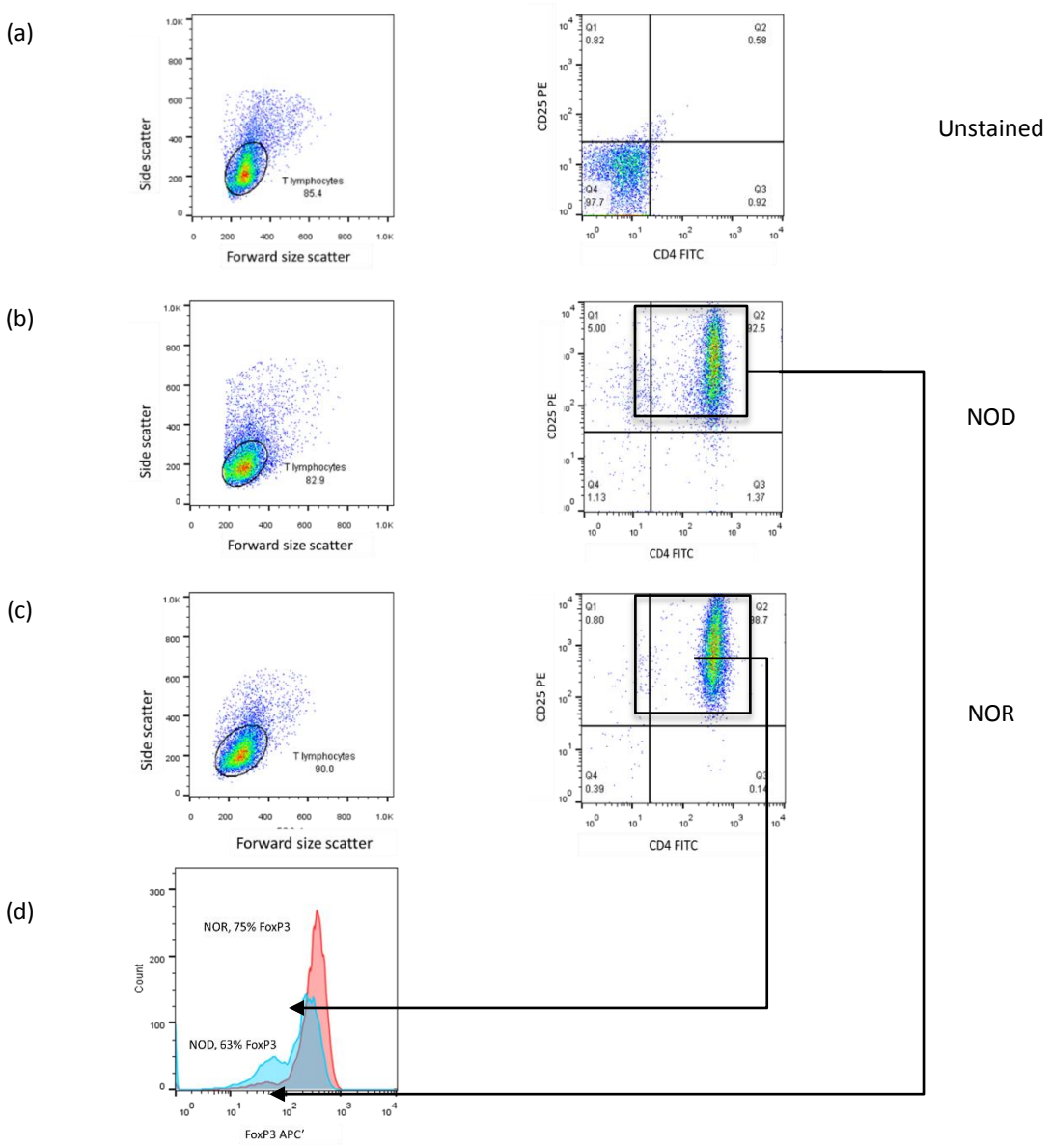


Figure 0: Efficiency of nTreg cell isolation from NOD and NOR mice using magnetic selection sorting. Dot plot representing gating strategy on T cells population performed on unstained population. Unstained population was used as control negative. (b) Bivariate dot plot representing isolated nTreg cells from NOD and (c) NOR were stained with anti-CD4 FITC and anti-CD25 PE to indicate double positive population. (d) CD4+CD25+ population was stained with anti-FoxP3 APC to further analysed FoxP3 expression.
Dot plot is a representative of three independant experiment (n=4 mice/experiment).

PPARγ ligands and its inhibitor did not affect nTreg cell stimulation and proliferation in vitro

Following 72hrs in vitro culture, isolated nTreg cells from Balb/c and NOD mice with respective treatment groups were recorded to remain viable as observed under the microscope as shown in Figure 5 and Figure 6. As shown in the figures, cells appeared to be uniform in size and clumping, which indicated in vitro cells underwent moderate stimulation and proliferation activities. Based on our findings, we recorded that nTreg cell stimulation and differentiation from both strains were not significantly affected by ciglitazone and 15d-PGJ_2 following in vitro culture, similarly observed in control untreated cells. Addition of GW9662 also did not reverse the effect, as shown in Figure 6(c).

The noticeable low number of cells in Figure 5 (a) and (b) was due to lack of nTreg cells harvested from Balb/c mice as more cells were used for optimization of intracellular staining. Due to limited budget and humane animal handling, we used low number of animals for the experiment which directly affect with number of cells yielded in the study. Overall, cells cultured under the conditioned culture media with respective treatment did not show any cytotoxic effect, since the vehicle control group showed normal cell

proliferation and activation.

Even though these ligands did not affect the cell proliferation and addition of their inhibitor did not reverse any of these during in vitro, we postulated that the molecular events in these cells were affected given the role of PPARγ as immune modulators is well-established.

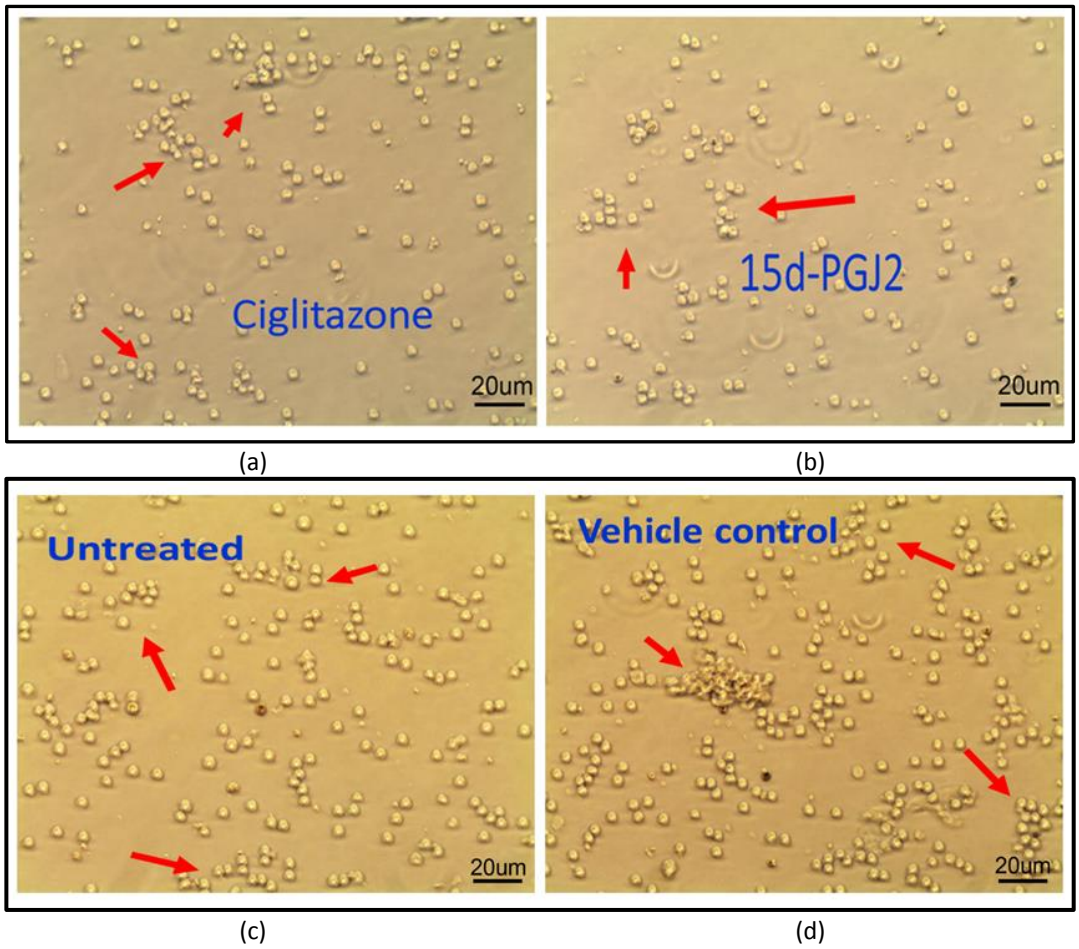
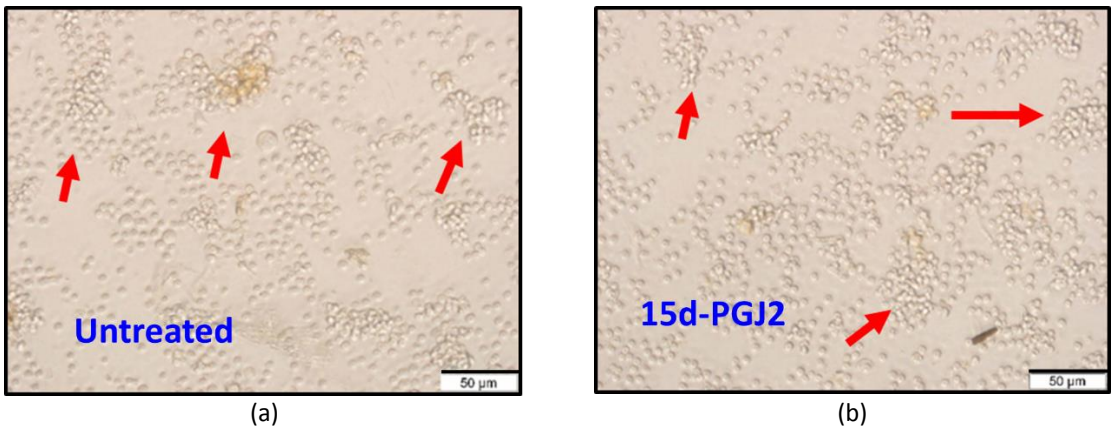
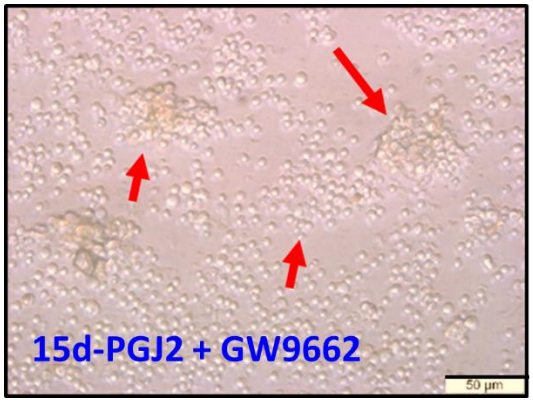


Figure 5: Microscopic observation at magnification 400x after 72 hours *in vitro* culture on treated and untreated nTreg cells isolated from Balb/c mice. The red arrows show the cell clumping in the treated and untreated groups indicating cell interaction and activation: 20 µM ciglitazone (a), 10 µM 15d-PGJ_2 (b), untreated (c) and 1% DMSO as vehicle control (d). Scale bar=20µM
Figure representative of three independant experiment (n=4 mice/experiment).





(c)

Figure 6: Morphological observation at magnification 200x on nTreg cells isolated from NOD mice under different treatment after 72 hours *in vitro* culture.
The red arrows show the cell clumping in all the groups indicating stimulated and differentiated cells: untreated (a) 10 µM 15d-PGJ_2 (b), 10 µM 15d-PGJ_2 and 10 µM GW9662 (c). Scale bar = 50 µM
Figure is a representative of three independant experiment (n=4 mice/experiment).

TIGIT and ICOS surface expressions were not altered by PPARγ signalling pathway in activated nTreg cells in vitro

Surface marker expressed by these cells determined their suppressive function, therefore we asked whether TIGIT and ICOS protein expressions were affected in thse cells following respective treatments. Our results showed that there were no significant differences measured on both surface protein from isolated nTreg cells treated with or without PPARγ ligands as compared to untreated groups (Figure 7). Both 15d-PGJ_2 and ciglitazone not significantly affect the expression of co-inhibitor TIGIT and co-stimulator ICOS receptors on isolated nTreg cells from Balb/c mice. Since this data showed no significant findings in Balb/c, the similar analysis was not performed on NOR and NOD mice due to limited cell numbers to be used for downstream experiments.

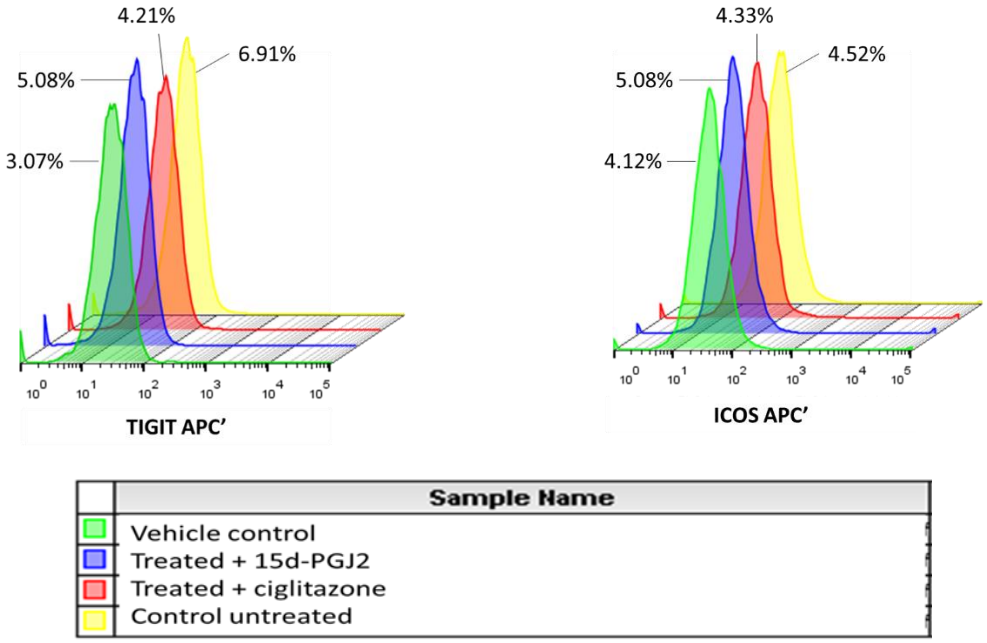


Figure 7: TIGIT and ICOS expressions did not influenced by PPARγ ligands after treated with ciglitazone and 15d-PGJ_2 when compared to untreated groups
Histogram representative of three independant experiment (n=4 mice/experiment).

15d-PGJ_2 did not altered FoxP3 expression while GW9662 reversed the effect in NOR, not NOD mice

To better characterize activated nTreg cells following treatment with PPARγ ligands, we examined the intracellular expression of FoxP3 in activated nTreg cells to correlate their crosstalk in autoimmune model.

Current results showed that following 72-hrs in vitro, activated nTreg cells from NOD expressed low levels of intracellular Foxp3 proteins and that treatment with 15d-PGJ_2 with or without its inhibitor did not change or induced its expression as compared to untreated group (Figure 8). Meanwhile, activated nTreg cells from NOR mice fairly expressed intracellular Foxp3 expression and treatment with 15d-PGJ_2 did not significantly alter its expression as compared to control group, shown in Figure 9.

Interestingly, it was observed that when these cells were treated with 15d-PGJ_2 in the present of its inhibitor, GW9662, the expression levels of intracellular FoxP3 were significantly reduced in number as compared to control group and 15d-PGJ_2- treated group (Figure 9). Thus, we concluded that 15d-PGJ_2 altered Foxp3 expression in activated nTreg cells in vitro but its inhibitor capable to reverse the effect via PPARγ independent pathway.

HAT, HDAC6 and HDA11 differentially regulated by 15d-PGJ_2 in activated nTreg cells in vitro

To further testify the crosstalk between PPARγ ligands and FoxP3, we analyzed the effect of PPARγ ligands on histone acetylation and deacetylation processes. These processes are mediated by HAT and HDAC enzymes in activated nTreg cells. Enzyme activities were determined by quantifying HAT, HDAC6 and HDAC11 deacetylation activities in activated nTreg cells following respective treatments in vitro.

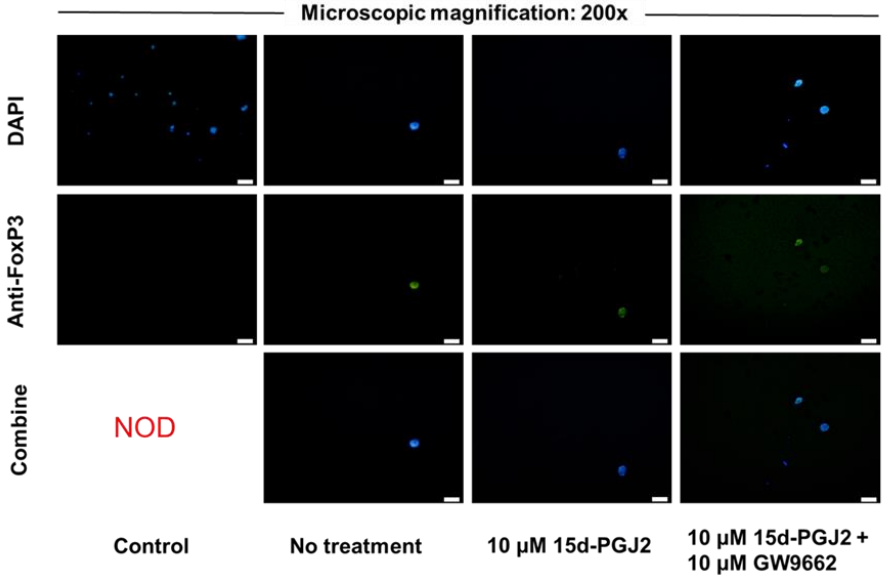


Figure 8: Expression of intracellular Foxp3 by activated nTreg cells isolated from NOD was analyzed using conventional fluorescence microscopy. Blue color at DAPI is nuclear staining. Green colour at Anti-FoxP3 indicated positive FoxP3 antigen. Scale bar = 50 µM
Figure is a representative of three independant experiment (n=4 mice/experiment).

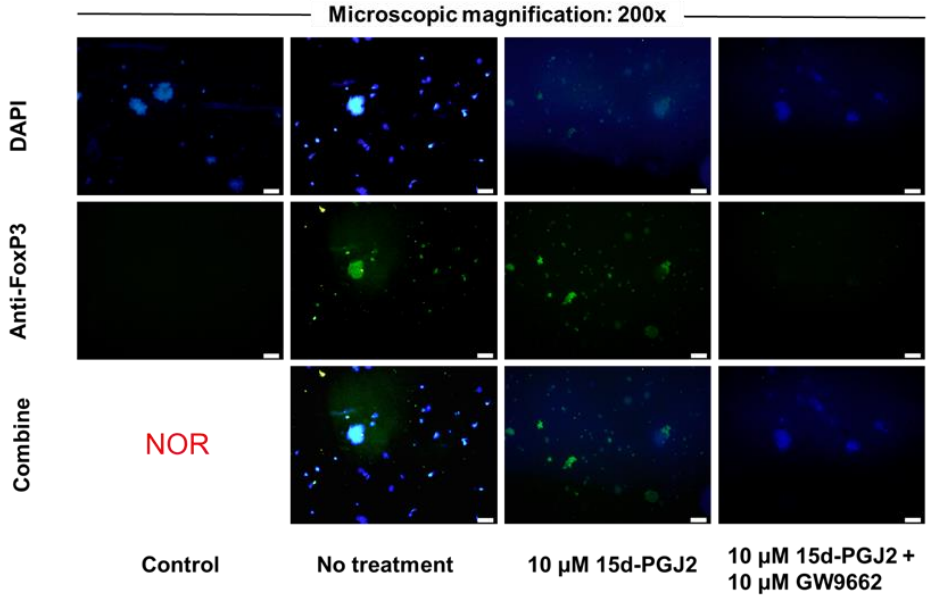


Figure 9: Natural Treg cells isolated from NOR was further analysed to measure the intracellular FoxP3 expression. Blue color at DAPI is nuclear staining. Green color at Anti-FoxP3 indicated positive FoxP3 antigen. Scale bar = 50 µM

In Figure 10, current data reported that the measured HAT activity in activated nTreg cells from NOR mice was not significantly changed in all treated groups when compared with untreated control group in vitro. Interestingly, in activated nTreg cells isolated from NOD mice, treatment with 15d-PGJ_2 slightly reduced $_{mean}$ HAT activity levels and addition of its inhibitor further suppressed HAT activity in these cells, as compared to control group.

Meanwhile, as shown in Figure 11, it was recorded that the measured mean HDAC6 activity levels in activated nTreg cells from NOR and NOD mice were high in control untreated groups following 72-hr in vitro culture. Following treatments, these cells from both NOR and NOD mice reduced their mean HDAC6 activity levels and addition of GW9662 further suppressed its activity in these cells. One-way ANOVA analysis in both NOR and NOD showed the differences of mean value obtained between groups were statistically significant as compared to control groups ($p<0.05$). Thus, we reported for the first time that PPARγ ligand significantly downregulated enzyme activity of HDAC6 in activated nTreg cells and its inhibitor further downregulated HDAC6 activity levels in activated nTreg cells in vitro. We measured the significant levels between 15d-PGJ_2-treated group with 15d-PGJ_2 in addition of GW9662 group using post-Hoc dunnet T3 test. It was shown that there was a significant difference in HDAC6 activity levels between activated nTreg cells treated with 15d-PGJ_2-treated group as compared to cells treated with 15d-PGJ_2 in combination with its inhibitor GW9662 ($p<0.05$) in NOR but not in NOD mice.

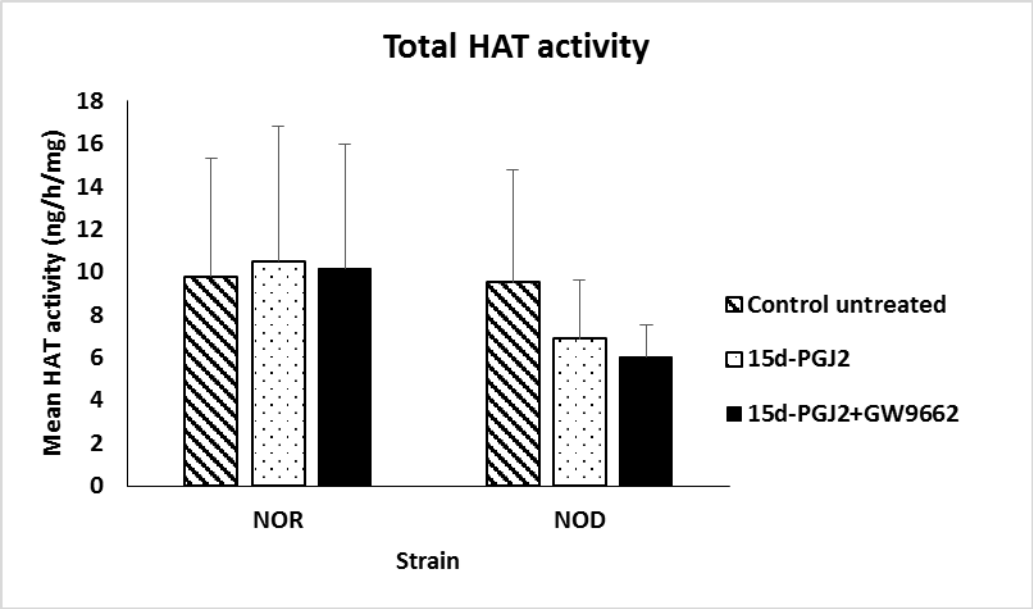


Figure 10 : Histone acetylation of HAT activity was measured in activated nTreg cells isolated from NOD and NOR mice. Bar chart shows total HAT activity (ng/h/mg) in treated nTreg cells with natural PPARγ ligand i.e. 15d-PGJ_2 additional with and without its inhibitor i.e. GW9662 from NOD and NOR mice compared to untreated group after 72 hours. In NOR, total HAT activity was not significant altered following each treatment. In addition, result indicates that there is no significant downregulation in NOD

Error bar indicates standard deviation. 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 Dunnett T3 was performed to identify the significance between treated samples (n =2)

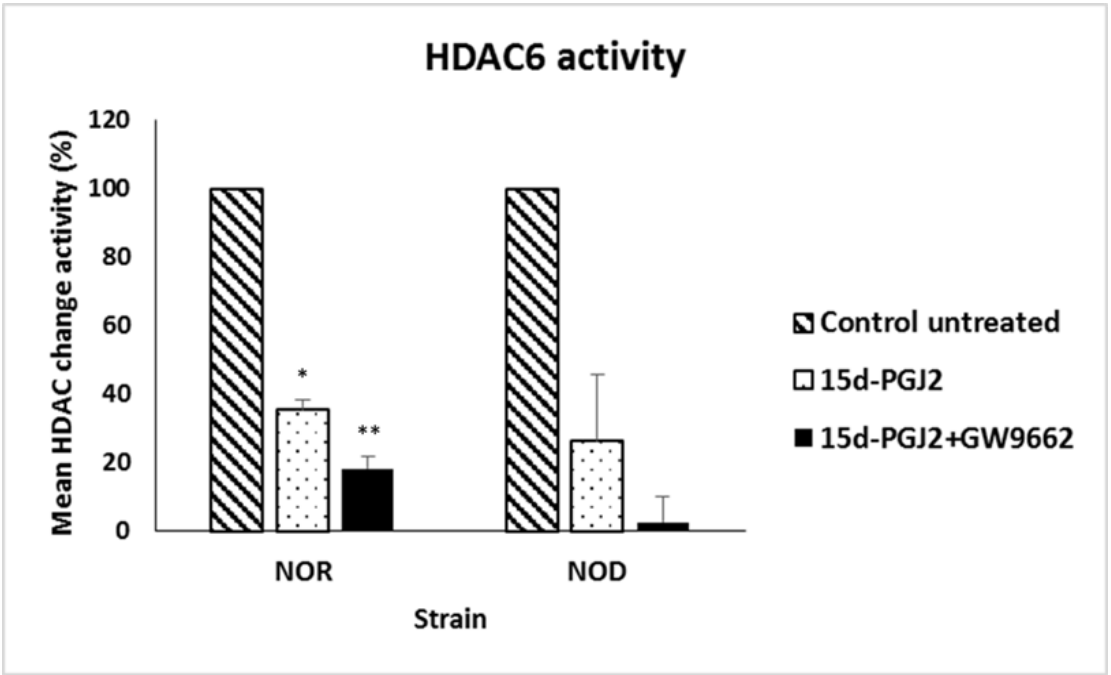


Figure 11: Histone deacetylation of HDAC6 activity was measured in activated nTreg cells isolated from NOD and NOR mice. Bar chart shows mean HDAC6 change activity in treated nTreg cells with natural PPARγ ligand i.e. 15d-PGJ_2 with and without its inhibitor i.e. GW9662 from NOD and NOR compared to control untreated group after 72 hours. In both mice, one-way ANOVA shows HDAC6 change activity significantly downregulated as compared to untreated groups. To analyse significant within treated groups, post-hoc analysis was done. It was shown that post hoc analysis within treated groups was significant in NOR but not in NOD

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 Dunnett T3 was performed to identify the significance with

the groups. Error bars indicate standard deviation (n = 2).
* $p < 0.05$, 15d-PGJ_2-treated group vs. untreated group.
** $p < 0.05$, 15d-PGJ_2+GW9662-treated group vs. untreated group.

Similarly, our results on mean HDAC11 showed reduced in activity levels in these cells from both NOR and NOD mice following treatment with 15d-PGJ_2 and presence of its inhibitor further suppressed deacetylation activity in these cells in vitro by suppressed HDA11 activity levels (Figure 12). The significant level was unable to calculate due to insufficient experimental triplicate due to limited cell numbers.

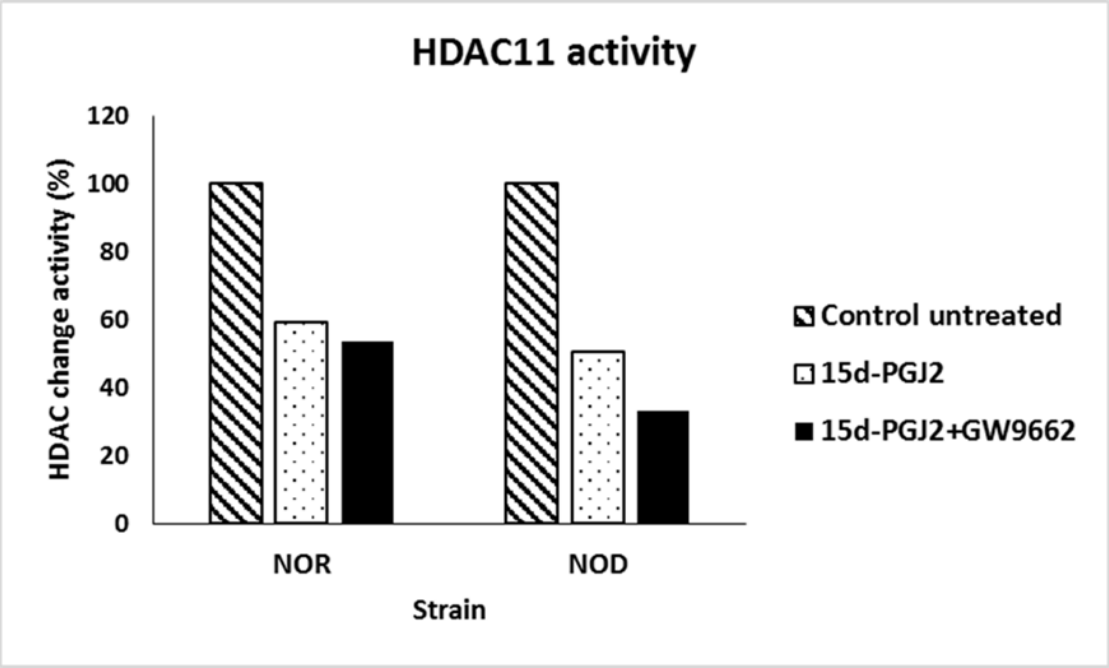


Figure 12: Histone deacetylation of HDAC11 activity was measured in activated nTreg cells isolated from NOD and NOR. Bar chart shows HDAC11 change activity in treated nTreg cells with natural PPARγ ligand i.e. 15d-PGJ_2 with and without its inhibitor i.e. GW9662 from NOD and NOR compared to untreated group after 72 hours. In both mice, the presence of 15d-PGJ_2 suppressed HDAC11 activity in activated nTreg cells compared to untreated group. The addition of GW9662 has further downregulated HDAC11 level in NOR and NOD mice compared to untreated group

Discussion

Current study explored on the possible crosstalk between Foxp3 expression nTreg cells with PPARγ in autoimmune models. Interest on PPARγ pathway has been well-established in the last 30 years ago as evidence suggested its role as immune modulators and potential of its naturally occurring ligands to be redefined for pharmaceutical purposes due to its safety and efficacy. The ability of 15d-PGJ_2 as a natural endogenous ligand to mediate activation of PPARγ through dependant and independant pathways open up wide opportunities of its pharmaceutical usage. The inhibition is mediated through various inflammatory-associated pathways including NF-κB signaling pathway (28, 29, 30, 31). This cyclopentenone types of prostaglandin J (PGJs) metabolite has various biological function including anti-inflammatory, anti-neoplastic, anti-viral and growth-regulatory activities in different cell types (28, 32).

Our study established the relationship between PPARγ pathway and Foxp3 expressions in autoimmune diabetes mellitus. Purpose of the study was to underline the significant influence of PPARγ ligands on the control of Foxp3 protein expression as the master regulator for nTreg cell suppressive activity. When Foxp3 expression is highly expressed, nTreg cell will effectively suppress excessive inflammatory reactions during autoimmune conditions. We tried to testify whether PPARγ ligands able to induce Foxp3 expression in these conditions, thus increase its effector function as immune suppressor during chronic excessive inflammation. Although current findings did not support our postulation, our current data have underlined the significant role on how PPARγ may affect Foxp3 expression in activated nTreg cells in vitro and the potential use of these ligands to manipulate the suppressive activity of nTreg cells during inflammatory-related diseases. In the current study, we demonstrated the crosstalk between these important proteins PPAR and Foxp3 using non-obese diabetes mouse models to highlight the potential relationship of the two.

In regard to separation efficiency, our findings reported that both isolation methods are comparable in terms of obtaining single cell population. However, variation between subspecies have been reported, mostly due to genetic background. The commonly used mouse strain in biomedical field, Balb/c is inbred experimental laboratory mouse strain whereas NOD is a polygenic strain which is commonly used to understand human T1D pathogenesis (33). While its long well-established healthy control strain for NOD is known as non-obese resistant mice (NOR) were bred from recombinant congenic strain-specific with endogenous retroviral profile, derived from outcross-backcross segregant from NOD reconstructed genome of C57BL/KsJ strain (34).

Our data showed that NOD recorded a slightly lower Foxp3 expression compared to its healthy control NOR. This result may suggest FoxP3 has lost its stability during adverse inflammatory condition, such as in NOD which may also contribute to the incidence of diabetes in these mice. Meanwhile, our measurement on co-inhibitory TIGIT and co-stimulatory molecules ICOS on nTreg cell surface revealed that PPARγ ligands did not affect suppressive function of nTreg cells via these molecules. Previous studies have reported that PPARγ is a potent negative regulator in inflammatory responses (29, 35,36, 37). Besides, it was reported that PPARγ activation downregulate the costimulatory molecule expression on DC (38) and mitogen activation in T cell (35, 39) leading to the impairment in cytokine production. Our findings may highlight another significant data on the potential suppressive mechanism of nTreg cells is not dependant on co-stimulatory and con-inhibitory surface protein ICOS and TIGIT since it was shown that these nTreg cells is highly plastic and capable to changes their phenotypic expression under different conditions. Most importantly, these changes are not mediated or cross-talked with PPARγ pathways, postulated to be via PPARγ-independent pathway.

Interestingly, ciglitazone significantly increased the capacity of TGF-β to mediate Teff cell conversion to iTreg via PPARγ–independant pathway. On the other hand, when ciglitazone co-administered in nTreg cells *in vitro*, the ligand tends to prolong the protection and survival of autoimmune models, thus suggesting that PPARγ-dependant pathway in Teff cells is dependant on the presence of nTreg cells (40). This may be an indication that the activation of nTreg cells regulates TIGIT and ICOS expression through PPARγ-independant pathway. Previous studies showed that activated nTreg cells upregulate TIGIT and ICOS expressions where TIGIT is found to co-express with FoxP3 when compared to naïve T cells (41, 41). To prevent adverse inflammatory immune response, TIGIT serves as inhibitory checkpoint in activated T lymphocytes particularly in Th1 and Th17 subsets (43) while activated ICOS+ nTreg cells inhibit DC maturation via IL-10 mediated response. Again, our data on ICOS/TIGIT expressions on nTreg cells by PPARγ ligands may highlight the characteristic of nTreg cell plasticity *in vitro*.

Our finding was supported by compilations of findings from Lei et al (2010) and Nor Effa et al (2018) where 15d-PGJ_2 has no significant difference on Foxp3 expression in nTreg cells including its suppressive activity (15, 44). Hughes et. al. (2014) revealed that an alternate binding site for PPARγ through structure-function studies, which suggested the competitive binding between synthetic ligands and endogenous ligands towards the canonical ligand-binding pocket (LBP) in PPARγ are non-overlapping (45). PPARγ ligands together GW9662 will synergistically block LBP, allowing for higher affinity of the alternate site to be bound by PPARγ ligands. The so called off-target effects of this ligand known as PPARγ-independant functional effects (45).

Previous study on autoimmune T1D showed that 15d-PGJ_2 and its inhibitor GW9662 capable to downregulate FoxP3 mRNA expression in activated nTreg cells (15). Similarly, current study reported that this ligand had no significant effect in inducing Foxp3 proteins following in vitro treatments. Although FoxP3 can be expressed in other immune cells, its immunosuppressive control display only by Treg cells, in which its expression in nTreg cells is TGF-β independant while TGF-β-dependant in iTreg cells (46). FoxP3 locus exhibit hypomethylation or demethylated at CpG motif (47), suggesting the epigenetic regulation of its expression. Its role as transcriptional repressor requires direct binding on DBD to regulate T cell activation to form oligomeric complexes with coactivators and corepressors such as HDACs/HATs dynamically (14).

Thus, we measured the enzymes activities of HAT, HDAC6 and HDAC11 to test whether the possible effect of Foxp3 suppression by 15d-PGJ_2 is via HAT/HDAC regulation. HDAC6 has been found to regulate FoxP3 acetylation as compared to sirt1 and HDAC9 in Treg cells (23). During inflammations, HSP60, HSP70 and HSP90 were upregulated and recognized by Treg cells to prevent host from self-injury through immunomodulatory effect (48). HDAC6 inhibition will cause HSP90 to loss its chaperone activity, leading to hyperacetylation-induced heat shock response, enabling cells to survive under inflammatory condition (23, 49, 50).

Our results showed that in untreated nTreg cells, these enzyme activities were highly detected following 72hrs in vitro conditions. However, when these cells were treated with 15d-PGJ_2 under the same in vitro conditions, their activity significantly reduced to the level that may indicate acetylation and deacetylation activities in activated nTreg cells mediated by the

ligand inhibit target transcription of these enzymes. In fact, GW9662 further augmented this effect. Current findings were in line with a previous report by Hironaka et al (2009) where they reported that HAT degradation activity is promoted by 15d-PGJ_2 to its inactive form and negate HAT conversion activity as well. Similar report also underlined the efficacy of 15d-PGJ_2 to convert active form of PCAF, p300 and CBP into inactive forms, indicating attenuation of gene expression such as p53, NF-κB and heat shock factor-dependant reporter (51). FoxP3 upregulation in Treg cells requires the orchestration of multiple proteins such as p53, NF-κB, HSP, p300/CBP and HAT coactivators to maintain their development and immunosuppressive function (52, 53, 54, 55, 56). Thus, since these protein co-activators are important for Foxp3 promoter binding activity, it affect the transcriptional process of Foxp3 in these cells following treatments. This may explain the current findings in our study. Altogether, the current data may suggest that naturally occurring PPARγ ligand has the potential to suppress Foxp3 expression in activated nTreg cells by modulating HAT and HDAC6/11 in these cells in healthy and autoimmune models, which means it altered histone deacetylation activities of Class II and Class IV HDAC.

Interestingly, HDAC6 has been found to control autophagosomes fusion to lysosomes without autophagy activation (57). It is capable to increase its efficient clearance of inflammasome component through autophagy pathway (57, 58, 59). Our results indicated that deacetylase activity by HDAC6 was downregulated in autoimmune NOD compared to healthy control when treated with PPARγ agonist and its inhibitor, thereby limiting inflammation. Meanwhile, it was reported that Foxp3 can co-associate with HDAC11 to promote its deacetylation in Treg cells, therefore deletion of HDAC11 gene may promote chromatin remodeling on FoxP3 locus, leading to augmentation suppressive function and DNA binding in these cells (60). Upon activation of T cells, HDAC11 expression shown to reduce in activity while significantly induces proliferation and pro-inflammatory cytokines production in these cells (61). In reverse, similar study showed that T effector cells lacking HDAC11 are susceptible towards Treg cell suppression activity (61).

Findings in the present study also demonstrated that prostaglandin metabolite significantly downregulates HDAC6/11 activities in diabetic model when compared to untreated group. It is interesting to note that the addition of GW9662 has further suppressed HDAC6-mediated deacetylation in healthy model when compared to untreated group at a significant level. It was reported that this antagonist can modulate gene expression through PPARγ-independant manners (15, 62). Histone deacetylation by 15d-PGJ_2 leads to transcriptional repression through PPARγ-independant TNF-α inhibition pathway (63). Thus, the activity of PPARγ ligand may also being regulated by PTMs, thus affecting protein expressions (64, 65).

Conclusion

In conclusion, we put forward the idea that crosstalk between PPARγ natural ligand, 15d-PGJ_2 has the potential repressive capacity on Foxp3 expression, mediated via histone modification. This putative role of 15d-PGJ_2 can be further explore for the development of HAT and HDAC inhibitors (HDACi) to be used for pharmaceutical drug repurposing approach. This is due to abundant of evidence that have highlighted the side effect of synthetic drug inhibitors for HAT and HDAC enzyme activities. Development of HDACi as a small-molecule inhibitor to regulate HDAC enzyme activities in inflammatory and cancer related areas has become major interests among pharmaceutical fields. Exploration on 15d-PGJ_2 as a potential HDACi can be further verified to be applied in tumor microenvironments due to the overwhelming hypermethylation status in many cancer cells.

References

1. Sakaguchi S. Naturally arising Foxp3-expressing CD25+ CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nature immunology*. 2005; *6*(4);345-352.
2. Adeegbe D.O. and Nishikawa H. Natural and induced T regulatory cells in cancer. *Frontiers in immunology*. 2013; *4*;190.
3. Onishi Y., Fehervari Z., Yamaguchi T. and Sakaguchi S. Foxp3+ natural regulatory T cells preferentially form aggregates on dendritic cells in vitro and actively inhibit their maturation. *Proceedings of the National Academy of Sciences*. 2008;*105*(29);10113-10118.
4. Corthay A. How do regulatory T cells work? *journal of immunology*. 2009;*70*(4);326-336.
5. Palomares O, Yaman G, Azkur AK, Akkoc T, Akdis, M and Akdis CA. Role of Treg in immune regulation of allergic diseases. *European journal of immunology*.2010;*40*(5);1232-1240.
6. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, *et al*. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nature genetics*. 2001;*27*(1);20.
7. Chen Z, Herman AE, Matos M, Mathis D and Benoist C. Where CD4+ CD25+ T reg cells impinge on autoimmune diabetes. *Journal of Experimental Medicine*. 2005;*202*(10);1387-1397.
8. Bacchetta R, Barzaghi F, and Roncarolo MG. From IPEX syndrome to FOXP3 mutation: a lesson on immune dysregulation. *Annals of the New York Academy of Sciences*. 2018;*1417*(1);5-22.

9. Horwitz DA, Zheng SG, Wang J, and Gray JD. Critical role of IL-2 and TGF-β in generation, function and stabilization of Foxp3+ CD4+ Treg. *European journal of immunology*. 2008; *38*(4);912-915.
10. Chen Z, Lin F, Gao Y, Li Z, Zhang J, Xing Y, *et al*. FOXP3 and RORγt: transcriptional regulation of Treg and Th17. *International immunopharmacology*. 2011;*11*(5);536-542.
11. Floess S, Freyer J, Siewert C, Baron U, Olek S, Polansky J, *et al*. Epigenetic control of the foxp3 locus in regulatory T cells. *PLoS biology*. 2007;*5*(2);38.
12. Bettelli E, Dastrange M, and Oukka M. Foxp3 interacts with nuclear factor of activated T cells and NF-κB to repress cytokine gene expression and effector functions of T helper cells. *Proceedings of the National Academy of Sciences*. 2005;*102*(14);5138-5143.
13. Lopes JE, Torgerson TR, Schubert LA, nover SD, Ocheltree EL, Ochs HD, *et al*. Analysis of FOXP3 reveals multiple domains required for its function as a transcriptional repressor. *The Journal of Immunology*. 2006;*177*(5);3133-3142.
14. Schubert LA, Jeffery E, Zhang Y, Ramsdell F and Ziegler S.F. Scurfin (FOXP3) acts as a repressor of transcription and regulates T cell activation. *Journal of Biological Chemistry*. 2001;*276*(40);37672-37679.
15. Nor Effa S, Yaacob N and Mohd Nor, N. Crosstalk between PPARγ Ligands and Inflammatory-Related Pathways in Natural T-Regulatory Cells from Type 1 Diabetes Mouse Model. *Biomolecules*. 2018;*8*(4);135.
16. Peters JM and Heuvel JPV. Proliferator-Activated Receptors (PPARs). *Cellular and molecular toxicology*. 2002;*14*;133.
17. Laudet V. and Gronemeyer H. *The nuclear receptor factsbook. 2nd Ed. Lyon, France.* Elsevier. 2002.
18. Stump M, Mukohda M, Hu C and Sigmund CD. PPARγ regulation in hypertension and metabolic syndrome. *Current hypertension reports*. 2015;*17*(12);89.
19. Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM and Evans RM. 15-deoxy-Δ12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPARγ. *Cell*, 1995;*83*(5);803-812.
20. Kliewer SA, Lenhard JM, Willson TM, Patel I, Morris DC and Lehmann JM. A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor γ and promotes adipocyte differentiation. *Cell*. 1995;*83*(5);813-819.
21. Erzin G and Çakır B. Assessment of serum IL-4, 15d-PGJ2, PPAR gamma levels in patients with bipolar disorder. *European Psychiatry*. 2017;*41*;S75.
22. Grausenburger R, Bilic I, Boucheron N, Zupkovitz G, El-Housseiny L, Tschismarov R, *et al*. Conditional deletion of histone deacetylase 1 in T cells leads to enhanced airway inflammation and increased Th2 cytokine production. *The Journal of Immunology*. 2010;*185*(6);3489-3497.
23. Beier UH, Akimova T, Liu Y, Wang L and Hancock WW. 2011. Histone/protein deacetylases control Foxp3 expression and the heat shock response of T-regulatory cells. *Current opinion in immunology*. 2011;*23*(5);670-678.
24. Ellmeier W and Seiser C. Histone deacetylase function in CD4+ T cells. *Nature Reviews Immunology*. 2018;1.
25. Allfrey VG, Faulkner R and Mirsky AE. Acetylation and methylation of histones and their possible role in the regulation of RNA synthesis. *Proceedings of the National Academy of Sciences*. 1964;*51*(5);786-794.
26. Vidali G, Gershey EL and Allfrey VG. Chemical studies of histone acetylation the distribution of ε-N-acetyllysine in calf thymus histones. *Journal of Biological Chemistry*. 1968;*243*(24);6361-6366.
27. Strahl BD and Allis CD. The language of covalent histone modifications. *Nature*. 2000;*403*(6765);41-45.
28. Straus DS, Pascual G, Li M, Welch JS Ricote M, Hsiang CH. Sengchanthalangsy LL, Ghosh G and Glass CK. 15-Deoxy-Δ12, 14-prostaglandin J2 inhibits multiple steps in the NF-κB signaling pathway. *Proceedings of the National Academy of Sciences*. 2000;*97*(9);4844-4849.
29. Chawla A, Barak Y, Nagy L, Liao D, Tontonoz P and Evans RM. PPAR-γ dependent and independent effects on macrophage-gene expression in lipid metabolism and inflammation. *Nature medicine*. 2001;*7*(1);48.
30. Hinz B. Brune K and Pahl A. 15-Deoxy-Δ12, 14-prostaglandin J2 inhibits the expression of proinflammatory genes in human blood monocytes via a PPAR-γ-independent mechanism. *Biochemical and biophysical research communications*. 2003;*302*(2);415-420.
31. Hovsepian E, Penas F and Goren NB. 15-deoxy-Δ12, 14 prostaglandin GJ2 but not rosiglitazone regulates metalloproteinase 9, NOS-2, and cyclooxygenase 2 expression and functions by peroxisome proliferator-activated receptor γ-dependent and-independent mechanisms in cardiac cells. *Shock*. 2010;*34*(1);60-67.
32. Straus DS and Glass CK. Cyclopentenone prostaglandins: new insights on biological activities and cellular targets. *Medicinal research reviews*. 2001;*21*(3);185-210.

33. Awata T, Guberski DL and Like AA. Genetics of the BB rat: association of autoimmune disorders (diabetes, insulitis, and thyroiditis) with lymphopenia and major histocompatibility complex class II. *Endocrinology*. 1995;*136*(12);5731-5735.
34. Prochazka M, Serreze DV, Frankel WN and Leiter EH. NOR/Lt mice: MHC-matched diabetes-resistant control strain for NOD mice. *Diabetes*. 1992;*41*(1);98-106.
35. Clark RB, Bishop-Bailey D, Estrada-Hernandez T, Hla T, Puddington L and Padula SJ. The nuclear receptor PPARγ and immunoregulation: PPARγ mediates inhibition of helper T cell responses. *The Journal of Immunology*. 2000;*164*(3);1364-1371.
36. Schmidt MV, Brüne B and von Knethen A. The nuclear hormone receptor PPARγ as a therapeutic target in major diseases. *The Scientific World Journal*. 2010;*10*;2181-2197.
37. Hamaguchi M, and Sakaguchi S. Regulatory T cells expressing PPAR-γ control inflammation in obesity. *Cell metabolism*. 2012;*16*(1);4-6.
38. Nencioni A, Grünebach F, Zobywlaski A, Denzlinger C, Brugger W and Brossart P. Dendritic cell immunogenicity is regulated by peroxisome proliferator-activated receptor γ. *The Journal of Immunology*. 2002;*169*(3);1228-1235.
39. Cunard R, Ricote M, DiCampli D, Archer DC, Kahn DA, Glass CK, *et al*. Regulation of cytokine expression by ligands of peroxisome proliferator activated receptors. *The Journal of Immunology*. 2002;*168*(6);2795-2802.
40. Wohlfert EA, Nichols FC, Nevius E and Clark RB. Peroxisome proliferator-activated receptor γ (PPARγ) and immunoregulation: Enhancement of regulatory T cells through PPARγ-dependent and-independent mechanisms. *The Journal of Immunology*. 2007;*178*(7);4129-4135.
41. Yu X, Harden K, Gonzalez LC, Francesco M, Chiang E, Irving B, *et al*. The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. *Nature immunology*. 2009;*10*(1);48.
42. Tomoki I, Shino H, Yi-Hong W, Woong RP, Kazuhiko A, Laura BF *et al*. Two Functional Subsets of FOXP3+ Regulatory T Cells in Human Thymus and Periphery. *Immunity*. 2008;28(6)8;70-880.
43. Joller N, Lozano E, Burkett PR, Patel B, Xiao S, Zhu C, *et al.* Treg cells expressing the coinhibitory molecule TIGIT selectively inhibit proinflammatory Th1 and Th17 cell responses. *Immunity*. 2014;*40*(4);569-581.
44. Lei J, Hasegawa H, Matsumoto T and Yasukawa M. Peroxisome proliferator-activated receptor α and γ agonists together with TGF-β convert human CD4+ CD25− T cells into functional Foxp3+ regulatory T cells. *The Journal of Immunology*. 2010;*185*(12);7186-7198.
45. Hughes TS, Giri PK, De Vera IMS, Marciano DP, Kuruvilla DS, Shin Y, *et al*. An alternate binding site for PPARγ ligands. *Nature communications*. 2014;*5;*3571.
46. Fahlén L, Read S, Gorelik L, Hurst SD, Coffman RL, Flavell RA and Powrie F. T cells that cannot respond to TGF-β escape control by CD4+ CD25+ regulatory T cells. *Journal of Experimental Medicine*. 2005;*201*(5);737-746.
47. Floess S, Freyer J, Siewert C, Baron U, Olek S, Polansky J, *et al*. Epigenetic control of the foxp3 locus in regulatory T cells. *PLoS biology*. 2007;*5*(2);38.
48. Spierings J, and van Eden W. Heat shock proteins and their immunomodulatory role in inflammatory arthritis. *Rheumatology*. 2016;*56*(2);198-208.
49. Boyault C, Zhang Y, Fritah S, Caron C, Gilquin B, Kwon SH, *et al*. HDAC6 controls major cell response pathways to cytotoxic accumulation of protein aggregates. *Genes & development*. 2007;*21*(17);2172-2181.
50. Beier UH, Wang L, Han R, Akimova T, Liu Y and Hancock WW. Histone deacetylases 6 and 9 and sirtuin-1 control Foxp3+ regulatory T cell function through shared and isoform-specific mechanisms. *Sci. Signal.*2012;*5*(229);45.
51. Hironaka A, Morisugi T, Kawakami T, Miyagi I and Tanaka Y. 15-Deoxy-Δ12, 14-prostaglandin J2 impairs the functions of histone acetyltransferases through their insolubilization in cells. *Biochemical and biophysical research communications*. 2009;*390*(2);290-294.
52. Jung DJ, Jin DH, Hong SW, Kim JE, Shin JS, Kim D, *et al*. Foxp3 expression in p53-dependent DNA damage responses. *Journal of Biological Chemistry*. 2010;*285*(11);7995-8002.
53. Long M, Park SG, Strickland I., Hayden, M.S. and Ghosh, S. Nuclear factor-κB modulates regulatory T cell development by directly regulating expression of Foxp3 transcription factor. *Immunity*. 2009;*31*(6);921-931.
54. Brenu, E.W., Staines, DR, Tajouri L, Huth T, Ashton KJ and Marshall-Gradisnik SM. Heat shock proteins and regulatory T cells. *Autoimmune diseases*, *2013*.
55. Liu Y, Wang L, Han R, Beier UH, Akimova T, Bhatti T, *et al*. Two histone/protein acetyltransferases, CBP and p300, are indispensable for Foxp3+ T-regulatory cell development and function. *Molecular and cellular biology*. 2014;*34*(21);3993-4007.
56. Xiao Y, Li B, Zhou Z, Hancock WW, Zhang H and Greene MI. Histone acetyltransferase mediated regulation of FOXP3 acetylation and Treg function. *Current opinion in immunology*. 2010;*22*(5);583-591.

57. Lei J, Hasegawa H, Matsumoto T and Yasukawa M. Peroxisome proliferator-activated receptor α and γ agonists together with TGF-β convert human CD4+ CD25− T cells into functional Foxp3+ regulatory T cells. *The Journal of Immunology*, 2010;*185*(12);7186-7198.
58. Levine B, Mizushima N and Virgin HW. Autophagy in immunity and inflammation. *Nature*, 2011;*469*(7330);323-335.
59. Deretic V, Saitoh T and Akira S. Autophagy in infection, inflammation and immunity. *Nature Reviews Immunology*. 2013;*13*(10);722-737.
60. Huang J, Wang L, Dahiya S, Beier UH, Han R, Samanta A, *et al*. Histone/protein deacetylase 11 targeting promotes Foxp3+ Treg function. *Scientific reports*, 2017;*7*(1);8626.
61. Woods DM, Woan KV, Cheng F, Sodré AL, Wang D, Wu Y, *et al*. T cells lacking HDAC11 have increased effector functions and mediate enhanced alloreactivity in a murine model. *Blood*, 2017;*130*(2);146-155.
62. Seargent JM, Yates EA and Gill JH. GW9662, a potent antagonist of PPARγ, inhibits growth of breast tumour cells and promotes the anticancer effects of the PPARγ agonist rosiglitazone, independently of PPARγ activation. *British journal of pharmacology*. 2004;*143*(8);933-937.
63. Engdahl R, Monroy MA and Daly JM. 15-Deoxy-Δ12, 14-prostaglandin J2 (15d-PGJ2) mediates repression of TNF-α by decreasing levels of acetylated Histone H3 and H4 at its promoter. *Biochemical and biophysical research communications*. 2007;*359*(1);88-93.
64. Choi JH, Banks AS, Estall JL, Kajimura S, Boström P, Laznik D, *et al*. Anti-diabetic drugs inhibit obesity-linked phosphorylation of PPARγ by Cdk5. *Nature*. 2010;*466*(7305);451.
65. Tian L, Wang C, Hagen FK, Gormley M, Addya S, Soccio R, *et al*. Acetylation-defective mutants of Pparγ are associated with decreased lipid synthesis in breast cancer cells. *Oncotarget*. 2014;*5*(17);7303.