UNIVERSITI SAINS MALAYSIA
GERAN PENYELIDIKAN UNIVERSITI PENYELIDIKAN
LAPORAN AKHIR

IMMUNE REGULATION OF AUTOIMMUNE DIABETES: THE ROLE OF REGULATORY T(T-REG) CELLS & PPAR GAMMA

PENYELIDIK

PROFESOR DR. NORAZMI MOHD. NOR

PENYELIDIK BERSAMA

PROF. DR. NIK SORIANI YAAKOB

2015

UNIVERSITI SAINS MALAYSIA

RU GRANT
FINAL REPORT CHECKLIST

Please use this checklist to self-assess your report before submitting to RCM
Checklist should accompany the report.

NO.	ITEM	PLEASE CHEC		
		PI	JKPTJ	RCMO
1	Completed Final Report Form	√	✓	
2	Project Financial Account Statement (e-Statement)	√	✓	
3	Asset/Inventory Return Form *(Borang Penyerahan Aset/Inventori)*	√	✓	
4	A copy of the publications/proceedings listed in Section D(ii) (Research Output)	√	✓ 4th ICGMB hdak dikeluarkan	
5	Comprehensive Technical Report	√	✓	
6	Other supporting documents, if any	√	✓ surat kebenaran tajaan kementerian pelajaran	
7	Project Leader's Signature	√	✓	
8	Endorsement of PTJ's Evaluation Committee	√	✓	
9	Endorsement of Dean/ Director of PTJ's	√	✓	

Project Code :
(for RCMO use only)

UNIVERSITI SAINS MALAYSIA

RU GRANT FINAL REPORT FORM

Please email a softcopy of this report to rcmo@usm.my

A	**PROJECT DETAILS**
i	**Title of Research:** **Immune regulation of autoimmune diabetes : "The role of regulatory T-cells (Treg) and PPARγ"**
ii	
iii	**Name of Research Leader:** Prof. Dr. Norazmi Mohd Nor
iv	**Name of Co-Researcher:** 1. Prof. Dr. Nik Soriani Yaakob 2. 3.
v	**Duration of this research:** **a) Start Date : 15 July 2012** **b) Completion Date : 14 July 2014** **c) Duration : 3 years** **d) Revised Date (*if any*) : 14 July 2015**
B	**ABSTRACT OF RESEARCH**
	(An abstract of between 100 and 200 words must be prepared in Bahasa Malaysia and in English. This abstract will be included in the Report of the Research and Innovation Section at a later date as a means of presenting the project findings of the researcher/s to the University and the community at large) Fungsi pengawalaturan sel regulatori semulajadi (nTreg) adalah kritikal bagi tolerans imun periferal. Molekul transkripsi yang dinamakan Foxp3 telah dikenalpasti sebagai penting untuk fungsian sel nTreg. Satu lagi molekul anti-inflamatori, iaitu reseptor proliferator peroksisom teraktif γ (peroxisome-proliferator activated receptor, PPARγ) juga memainkan peranan sebagai pengawalatur imun. Kajian ini telah dijalankan untuk mengenalpasti mekanisme pengawalaturan imun sel nTreg dengan menggunakan ligan PPARγ ke atas sel nTreg mencit BALB/c, serta mencit diabetes jenis 1 (type 1 diabetes, T1D) atau diabetes autoimmun; iaitu mencit diabetes tak obes (non-obese diabetic, NOD) serta kontrolnya, mencit rentan diabetes tak obes (non-obese diabetic resistant, NOR). Tahap optimum jangkamasa, kepekatan IL-2, dan analisis fungsian telah dilaksanakan ke atas kultur *in vitro* sel nTreg mencit BALB/c. Tahap ekpresi isoform-isoform PPARγ serta aktiviti penggabungan PPARγ terhadap elemen tindak-balas proliferator proksisom (peroxisome-proliferator response element, PPRE), telah diteliti di dalam sel-sel nTreg yang dirawat dengan ligan PPARγ, ciglitazone, untuk menentukan sama ada kesan yang diperolehi adalah melalui laluan PPARγ atau tidak. Hasil kajian menunjukkan bahawa tiada aktiviti penggabungan PPARγ-PPRE diperolehi apabila sel nTreg BALB/c, NOD mahupun NOR dirawat dengan ciglitazone. Rawatan

dengan ligan PPARγ itu juga tidak menyebabkan pemfosforilan faktor-faktor transkripsi ZAP-70 dan STAT-5 dalam sel nTreg mencit-mencit kajian. Sel nTreg mengekspres PPARγ1 dan PPARγ2 pada tahap rendah. Rawatan dengan ciglitazone mengaruh ekspresi PPARγ1 melalui laluan bergantung-PPARγ (P <0.01), tetapi tidak PPARγ2. Ligan PPARγ merencat pengekspresian Foxp3 dalam sel nTreg daripada ketiga-tiga jenis mencit melalui laluan tak bergantung PPARγ berbanding sel yang tak dirawat (P <0.01). Didapati pelbagai gen sasaran yang berkaitan dengan laluan proinflamatori direncat dalam sel nTreg NOD apabila dirawat dengan ligan PPARγ berbeza daripada mencit NOR. Perencatan ekspresi Foxp3 oleh ligan PPARγ dalam sel nTreg mungkin boleh merencatkan fungsi imuno-modulatori sel nTreg dalam mencit, terutamanya mencit autoimun diabetes.

The immunodownregulatory function of nTreg cells is critical in mediating peripheral self-tolerance. The transcription factor, Foxp3, has been established to be crucial for the function of nTreg cells. Another anti-inflammatory molecule, peroxisome proliferator-activated receptor γ (PPARγ has also been previously shown to have immunoregulatory properties. The current study was conducted to determine the modulatory mechanism of nTreg cells following treatment with the PPARγ ligand, ciglitazone, in isolated nTreg cells of BALB/c, as well as in the Type 1 diabetes or autoimmune mouse model, Non Obese Diabetic (NOD) as well as in the control, Non Obese Resistant (NOR) mice. Optimization of culture duration, concentration of IL-2, and functional analyses were performed on cultured nTreg cells from BALB/c mice *in vitro*. The expression levels of PPARγ isoforms as well as PPARγ binding activity to its response elements, PPRE, was examined in treated and untreated nTreg cells to determine whether the oberved responses were PPARγ-dependant or –independant. Results showed that there were no binding of PPARγ to PPRE in the treated nTreg cells of BALB/c, NOD or NOR mice. PPARγ ligand treatment also did not cause phosphorylation of the transcription factors ZAP-70 and STAT-5 in these mice. nTreg cells was found to constitutively expressed low levels of PPARγ1 and PPARγ2. Upon treatment with ciglitazone PPARγ1 but not PPARγ2 expression was induced via PPARγ-dependant pathway ($P < 0.01$). PPARγ ligand downregulated the expression of Foxp3 in nTreg cells of BALB/c, NOD and NOR mice via a PPARγ-independant pathway compared to untreated group ($P < 0.01$). Various pro-inflammatory pathway-related target genes were downregulated in NOD nTreg cells following treatment with ciglitazone compared to those of NOR mice. The downregulation of Foxp3 expression by PPARγ ligand in nTreg cells may downregulate the immuno-regulatory function of these cells in mice, especially autoimmune diabetic mice.

C BUDGET & EXPENDITURE

i

ii **Equipment Purchased Under Vot 35000**

No.	Name of Equipment	Amount (RM)	Location	Status
		None	Purchased	

 # Please attach the Asset/Inventory Return Form (Borang Penyerahan Aset/Inventori) – Appendix 1

D RESEARCH ACHIEVEMENTS

i Project Objectives *(as stated/approved in the project proposal)*

No.	Project Objectives	Achievement
1	To quantify the gene expression levels of PPARγ and Foxp3 in T-reg cells of NOD and NOR mice using Real-Time PCR	Completed
2	To quantify the expression levels of relevant intracellular signaling pathways in T-reg cells of NOD and NOR mice by PCR Array analyses	Completed
3	To determine the correlation between the gene expression profiles in Objectives 1 and 2	Completed

ii Research Output

a) Publications in ISI Web of Science/Scopus

No.	Publication (authors,title,journal,year,volume,pages,etc.)	Status of Publication (published/accepted/ under review)
1	Nor Effa, S Z, Soriani, Y S, Norazmi M N. " Putative crosstalk between Foxp3 and PPARγ in natural T-regulatory (nTreg) cells in experimental autoimmune diabetes", intended for submission to Clinical & Experimental Immunology, (ISSN: 1365-2249, impact factor 3.03)	In preparation
2	Nor Effa, S Z, Soriani, Y S, Norazmi M N. "Fundamental crosstalk between Foxp3 and PPAR isoforms in natural T-regulatory (nTreg) cells in BALB/c mice", intended for submission to Journal of Immunology Research (ISSN: 2314-88612314-8861, impact factor 2.93)	In preparation

b) Publications in Other Journals

No.	Publication (authors,title,journal,year,volume,pages,etc.)	Status of Publication (published/accepted/ under review)
1	Nor Effa SZ, Yaacob NS, Norazmi MN (2015). Isolated natural T-regulatory (nTreg) cells from BALB/c mice circumvent TCR and co-stimulatory signaling activation to suppress T-effector cells in vitro. Int J Chem Environ Biol Sci (IJCEBS) 3(5) ISSN 2320–4087 (Online)	Published

c) Other Publications
(book,chapters in book,monograph,magazine,etc.)

No.	Publication (authors,title,journal,year,volume,pages,etc.)	Status of Publication (published/accepted/ under review)
1		

d) **Conference Proceeding**

No.	Conference (conference name,date,place)	Title of Abstract/Article	Level (International/National)
1	1st International Conference on Medical and Health Sciences, 22nd-24th May 2013, Kota Bharu	The role of PPARγ in inducing Foxp3 gene expression in natural T-regulatory (nTreg) cells of Type 1Diabetes mice	International
2	4th International Conference on Genetics and Molecular Biology, 25 August 2015, Grand Season Hotel, Kuala Lumpur	Isolated natural T-regulatory (nTreg) cells circumvent TCR and co-stimulatory signaling activation to suppress T-effector cells *in vitro*	International

 # Please attach a full copy of the publication/proceeding listed above

iii **Other Research Ouput/Impact From This Project**
(patent, products, awards, copyright, external grant, networking, etc.)

Since this is primarily a fundamental-type of research, the findings are still at the laboratory stage and the primary impact is through publication of the work

E HUMAN CAPITAL DEVELOPMENT

a) **Graduated Human Capital**

Student	Nationality (No.)		Name
	National	International	
PhD	1		1. Nor Effa Syazuli, (graduated 2015) 2.
MSc	(1)		1. Mohd Khairi (did not complete – left to start work) 2.
Undergraduate			1. 2.

b) **On-going Human Capital**

Student	Nationality (No.)		Name
	National	International	
PhD			1. 2.
MSc			1. 2.
Undergraduate			1. 2.

c) **Others Human Capital**

Student	Nationality (No.)		Name
	National	International	
Post Doctoral Fellow			1. 2.
Research Officer			1. 2.
Research Assistant	1		1. Nur Hidayati Mohd Balia 2.
Others (.....)			1. 2.

F COMPREHENSIVE TECHNICAL REPORT

Applicants are required to prepare a comprehensive technical report explaining the project. The following format should be used (this report must be attached separately):

- Introduction
- Objectives
- Methods
- Results
- Discussion
- Conclusion and Suggestion
- Acknowledgements
- References

Please see attached document

G PROBLEMS/CONSTRAINTS/CHALLENGES IF ANY

(Please provide issues arising from the project and how they were resolved)

The increase cost of animals. Each mouse (NOD or NOR) averaged to about RM350 each excluding maintenance. Hence the number of animals that could be used was limited. Furthermore, the transportation of the animals took a long time (from Jackson Labs in US) and batches of animals (9-week old) need to be bred before transported.

H RECOMMENDATION

(Please provide recommendations that can be used to improve the delivery of information, grant management, guidelines and policy, etc.)

I	COMMENTS, IF ANY/ENDORSEMENT BY PTJ'S RESEARCH COMMITTEE
	Good output with one article and two conference proceedings have been published. In addition, one PhD student is trained and graduated in year 2015.

UNIVERSITI SAINS MALAYSIA
JABATAN BENDAHARI
KUMPULAN WANG UNIVERSITI PENYELIDIKAN (RU)
PENYATA PERBELANJAAN SEHINGGA 31 JULAI 2015

	RM		
Jumlah Geran :	234,380.00	Ketua Projek :	PROFESOR NORAZMI MOHD. NOR
Peruntukan JULAI 2012 : (Tahun 1)	126,940.00	Tajuk Projek:	IMMUNE REGULATIION OF AUTOIMMUNE DIABETES: THE ROLE OF REGULATORY T (T-REG) CELLS & PPAR GAMMA
Peruntukan JULAI 2013 : (Tahun 2)	107,440.00	Tempoh :	3 Tahun (15/07/2012-14/07/2015)
		No. Akaun :	1001/PPSK/813063

Kwgan	Akaun	PTJ	Projek	Peruntukan Projek	Perbelanjaan Terkumpul sehingga Tahun lalu	Peruntukan Semasa	Tanggungan Semasa	Bayaran Tahun Semasa	Belanja Tahun Semasa	Baki Projek
1001	11000	PPSK	813063	71,880.00	11,435.47	60,444.53	-	7,043.30	7,043.30	53,401.23
1001	14000	PPSK	813063	-	-	-	-	-	-	-
1001	15000	PPSK	813063	-	-	-	-	-	-	-
1001	21000	PPSK	813063	10,000.00	2,266.00	7,734.00	1,044.00	3,567.64	4,611.64	3,122.36
1001	22000	PPSK	813063	-	150.00	(150.00)	-	-	-	(150.00)
1001	23000	PPSK	813063	-	6,066.93	(6,066.93)	-	6.49	6.49	(6,073.42)
1001	24000	PPSK	813063	-	-	-	-	-	-	-
1001	25000	PPSK	813063	-	-	-	-	-	-	-
1001	26000	PPSK	813063	-	15,160.00	(15,160.00)	-	-	-	(15,160.00)
1001	27000	PPSK	813063	147,000.00	152,738.80	(5,738.80)	-	8,924.00	8,924.00	(14,662.80)
1001	28000	PPSK	813063	2,500.00	95.00	2,405.00	-	-	-	2,405.00
1001	29000	PPSK	813063	3,000.00	21,512.52	(18,512.52)	-	3,814.56	3,814.56	(22,327.08)
1001	32000	PPSK	813063	-	-	-	-	-	-	-
1001	35000	PPSK	813063	-	-	-	-	-	-	-
				234,380.00	209,424.72	24,955.28	1,044.00	23,355.99	24,399.99	555.29

International Journal of Chemical, Environmental & Biological Sciences (IJCEBS) Volume 3, Issue 5 (2015) ISSN 2320–4087 (Online)

Isolated Natural T-Regulatory (nTreg) cells from BALB/c Mice circumvent TCR and Co-stimulatory Signaling Activation to Suppress T-effector cells *in vitro*

Nor Effa, S Z[1,4], Yaacob, N S[2], Norazmi, M N[1, 3]

Abstract---Natural $CD4^+$ $CD25^+$ $Foxp3^{hi}$ T-regulatory cells nTreg) cells are required to induce tolerance against self-antigens uring inflammation. The expression of Foxp3 protein by these cells erald the ability to suppress $CD4^+$ T effector (Teff) cell activation, ence act as the master regulator for nTreg cell function. In the resence of exogenous IL-2 cytokine, nTreg cells are viable *in vitro* pon isolation, however the optimal time-point is uncertain. The urrent study identified the optimal *in vitro* culture conditions for Treg cells isolated from BALB/c mice. The suppressive function of olated cells towards Teff cells was also measured to determine the bility to retain their suppressive function after *in vitro* culture. The sults indicated that single population of nTreg cells from plenocytes using magnetic isolation method is > 90% purity. The ptimal time-point was set at 72-hr, similar to other Teff cells. In ddition, in the absence of TCR stimulation, nTreg cells capable of uppressing Teff cell activation, indicating the requirement of D3/CD28 ligation on nTreg cells surface is dispensable for their uppressive effect.

Keywords---Foxp3, Immune regulation, T-regulatory cells, nmunosuppression.

I. Introduction

OVER the past twenty years since the identification of naturally-occurring $CD4^+CD25^+Foxp3^+$ Regulatory T cells (nTreg), there have been intense researches in elineating the immunobiology of nTreg cells in hysiological and pathological conditions (1). The master egulator in nTreg cells is the transcription factor Forkhead ox P3 (Foxp3) which plays an important role in the evelopment and function of nTreg cells (2, 3). Foxp3 is xpressed in the thymus by nTreg cells (4, 5) and is ansiently expressed by $CD4^+CD25^-$ conventional T cells Treg) peripherally (5).

In pathological conditions such as autoimmune disorders, ie recognition of self-tissues by auto-reactive T cells leads to ie destruction of host tissues or organs. The nmunosuppressive role nTreg cells prevent such destruction from occurring by establishing peripheral self-tolerance toward auto-reactive T cells. This will thus hinder the development of debilitating autoimmune diseases from occurring. Mutation of Foxp3 gene in mouse models results in the loss of immunoregulatory function of nTreg cells, predisposing the hosts towards autoimmune responses (5).

It is fairly well-established that IL-2Rα-chain receptor, also known as CD25 receptor mediates nTreg cell suppressive activity. Binding of IL-2 to IL2-Rα chain, together with IL2Rβ and γ-chain subunits, will initiate the heterotrimer formation which subsequently initiate signal transduction (6). IL-2 is expendable in thymic maturation of nTreg cells but paradoxically critical in maintaining nTreg cell viability in the periphery (5, 7, 8).

Adoptive transfer of nTreg cells to $IL2B^{-/-}$ mice significantly diminished the tendency to develop lethal immune response (6). The inability of nTreg cells to produce IL-2 is due to failure of chromatin remodelling at the IL-2 promoter region. The binding of IL-2 to IL-2R initiates the activation of Signal Transducers and Activators of Transcription 5 (STAT5) in activated conventional T cells as well as activated nTreg cells. However, unlike conventional T cells, STAT5 activation in nTreg cells enhances the capacity of these cells to survive but not production of cytokine (9, 10, 11).

Therefore, this study was conducted to examine the preliminary *in vitro* culture condition and functional analysis of isolated nTreg cells from BALB/c mice following *in vitro* culture. The findings is hope to cater for the fundamental knowledge before more comprehensive analyses, such as elucidation of suppressive mechanisms adopted by nTreg cells during *in vitro* and *in vivo*.

II. Materials And Methods

Mice

Female BALB/c mice were purchased from the Animal Research and Service Centre (ARASC), Universiti Sains Malaysia. The mice were maintained in the animal facilities under specific pathogen-free conditions in accordance with the guidelines and regulations of ARASC, University Sains Malaysia and used at 12-week. All experimental protocols were approved by the institutional animal ethics committee. The animal ethic approval was obtained prior animal purchase. (Animal ethics approval number: USM/Animal Ethics Approval/2009/ (43 (132).

Nor Effa, S.Z. School of Health Sciences, Universiti Sains Malaysia, 5150, Kubang Kerian, Malaysia, & Regenerative Medicine Cluster, dvance Medical And Dental Institute, Universiti Sains Malaysia, Kepala atas, 13200, Malaysia. (Email: effa@usm.my)

Yaacob, N.S. School of Medical Sciences, Universiti Sains Malaysia, elantan, 16150, Kubang Kerian, Malaysia. (Email: niksoriani@usm.my)

Norazmi, M.N. Institute for Research in Molecular Medicine NFORMM), Universiti Sains Malaysia, Kelantan, 16150, Kubang Kerian, alaysia. (Email: norazmimn@usm.my)

page 379 - 382

International Journal of Chemical, Environmental & Biological Sciences (IJCEBS) Volume 3, Issue 5 (2015) ISSN 2320–4087 (Online)

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Norazmi, M.N. Institute for Research in Molecular Medicine FORMM), Universiti Sains Malaysia, Kelantan, 16150, Kubang Kerian, aysia. (Email: norazmimn@usm.my)

Abs and reagents

Mouse nTreg cells were isolated from the spleen tissues of BALB/c mice by magnetic separation. Briefly, $CD4^+$ cells were purified by negative and positive isolations using MACS $CD4^+CD25^+$ Treg isolation kit (Miltenyi Biotec). 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 by using FACS Canto flow cytometry (BD Biosciences). Cells labelled were ≥ 90% purity with > 80% $Foxp3^+$ was obtained. The isolated $CD4^+CD25^+Foxp3^+$ cells were used as nTreg cells and $CD4^+CD25^-$ cells were used as Teff cells. The cells were cultured in RPMI 1640 supplemented with 10% FBS (Hyclone), 10 mM HEPES, 10 mM, 500 µl antibiotic stock solution containing 100 U/ml, 100 µg/ml streptomycin and 10 µM β–mercaptoethanol. IL-2 was purchased from BD Biosciences, ciglitazone and 15d-$Prostaglandin_2$ were purchased from Cayman Chemicals. GW9662 was purchased from Sigma-Aldrich.

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. Mouse PE-and APC-conjugated IgG1 and FITC-conjugated IgG2a were used as isotype controls for fluorescein-conjugated antibodies used. Carboxyfluorescein succinimidyl ester (CFSE) was used to track cell division.

Statistical analysis

Data from experimental analyses were presented as the mean of triplicates with standard error mean (mean ± SEM). The data were statistically analysed using Minitab® 16.1.0 software. 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. The *P* value of less than 0.05 ($P < 0.05$) is considered significant.

III. Results

Efficiency of natural $CD4^+CD25^+Foxp3^{hi}$ T regulatory (nTreg) were successfully isolated from BALB/c mice

Single cell population of nTreg cells are necessary for downstream experiments to be performed. Splenocytes from BALB/c mice were processed and nTreg cell population would be separated out by cell isolation procedure as described in materials and methods. The process of nTreg cell isolation was performed by negative selection using magnetic labelling of non-$CD4^+$ cells with Biotin-Antibody Cocktail and anti-Biotin Microbeads. In parallel, $CD25^+$ cells were stained with CD25-PE. This was followed by depletion of non-$CD4^+$ cells from the mixture after these cells were retained in the magnetic field of a MACS column placed in a MACS separator. The flow-through contained unlabelled pre-enriched $CD4^+$ T cells.

The pre-enriched $CD4^+$ cells were subsequently used for positive selection, by magnetically labelled CD25-PE positive cells with Anti-PE microbeads, and separating them out. Subsequently the eluted cells were confirmed comprise high $CD4^+CD25^+$ cell population. The purity of $CD4^+CD25^+$ ce population obtained was > 90% as determined by FAC analysis (Figure 1). The percentage of $Foxp3^+$ cells were 80% from the total $CD4^+CD25^+$ population.

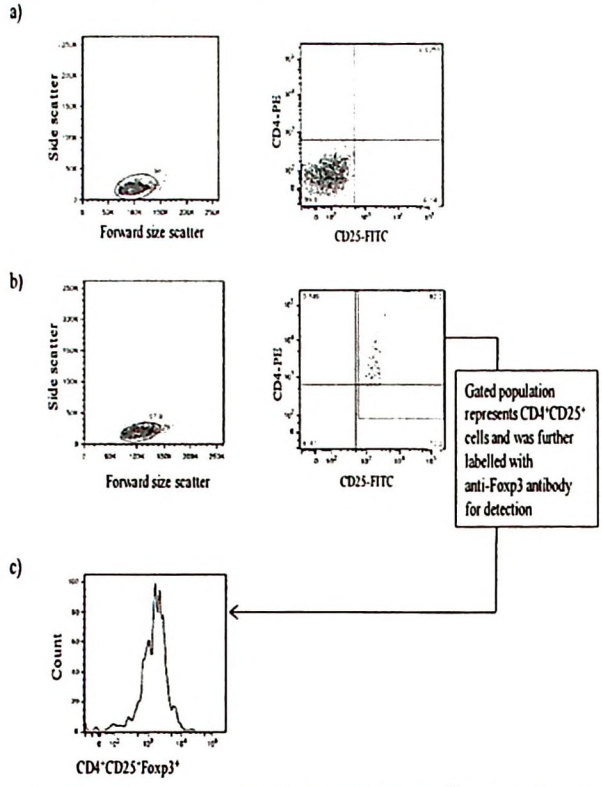
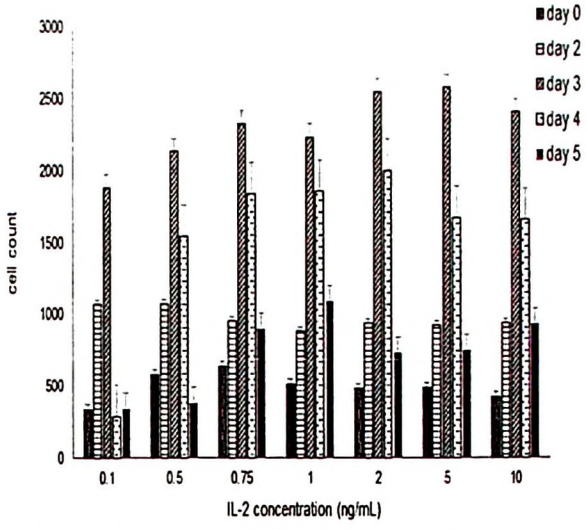


Fig. 1 Efficiency of natural $CD4^+CD25^+Foxp3^{hi}$ Treg (nTreg) cell isolation from splenocytes.

a) Dot plot represents $CD4^+$ T-lymphocytes stained wit IgG1-PE and IgG2a-FITC isotype control. b) Dot plot show $CD4^+$ T-lymphocytes stained with PE-conjugated rat an mouse CD4 and FITC-conjugated rat anti-mouse CD25. c Histogram shows the expression of $Foxp3^{hi}$ cells (black) gate on $CD4^+$ $CD25^+$ T-lymphocytes compared with the isotyp control (grey). Data are representative of one out of thre experiments.

Optimization of in vitro culture condition of nTreg cells

Natural Treg (nTreg) cells at 1 x 10^5 cells in 100 complete RPMI media were cultured on day zero, two, th four and five. This was performed to determine the optir time-point and IL-2 concentration for these cells to prolifera *vitro* at various concentrations of IL-2, ranging from 0.1 ng to 10 ng/mL. Natural Treg (nTreg) cells were stained with 5 CFSE dye prior to *in vitro* culture followed by stimulation anti-CD3/CD28 antibodies. After each culture day, proliferation of nTreg cells was measured by flow cytometry day three, nTreg cells recorded the highest cell numbe comparison to other time-points regardless of the concentra of IL-2 used (Figure 2). This indicates that the optimal nTreg proliferation capacity is on day three. Data was analysed u the Proliferation Platform FlowJo software (TreeStar).



ig. 2 The proliferation of stimulated nTreg cells in the presence of IL-2 cytokine. The optimal proliferation time for nTreg cells was recorded on day three of *in vitro* culture.

At various concentrations of IL-2, the rate for optimal Treg cell proliferation was recorded on day three before it minished on subsequent days of culture. In addition, g/mL IL-2 showed to be the optimal concentration for these lls to expand *in vitro*. Data represent as mean of three dividual experiments. The mean values are plotted to nstruct the graph. Error bars represents ± SEM. (n = 5/ each periment).

Suppressive function analysis of isolated nTreg cells

The functional analysis of isolated splenic nTreg cells was termined by observing their ability to suppress $CD4^+$ T-fector (Teff) cells *in vitro* (Figure 3). In the figure, data owed that when stimulated, Tresponder cells co-cultured th Treg cells resulted in significantly reduced proliferative pacity, compared with the Tresponder:Tcontrol group ($P <$ 01). When unstimulated, Tresponder cells co-cultured with eg cells were suppressed and had reduced cell division, mpared to the stimulated Tresponder:Tcontrol group ($P <$ 01). Stimulated Tresponder:Tcontrol co-cultured cells were corded to have higher levels of divided cells in comparison th other groups. Stimulated Tresponder:Treg co-cultured lls were observed to have more potent suppressive effect in mparison to unstimulated Tresponder:Treg co-cultured ls. The freshly-isolated Tresponder:Tcontrol unstimulated oup (0 h) was the initial population of cells before culture. e Tresponder:Tcontrol unstimulated was set as a growth arker for Tresponder:Tcontrol stimulated group. These sults indicate the ability of nTreg cells to suppress activated 4^+ T cells, but only partially suppressed inactive $CD4^+$ T ls. Furthermore, the involvement of secondary signals was spensable for nTreg cells to suppress Teff cells, as partial ppressive effect was observed in unstimulated esponder:Treg co-culture cells.

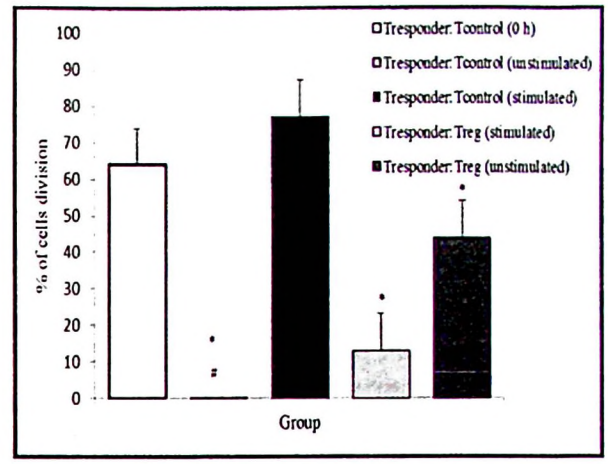


Fig. 3 Suppressive function analysis of isolated nTreg cells. The suppressive function of isolated nTreg cells towards Teff cells was maintained after 72 h *in vitro* culture.

CFSE-labelled T-effector cells (Tresponder) were added together with unlabelled nTreg (Treg) or Teff (Tcontrol) in a co-culture setting at ratio 10:1, in the presence (stimulated) or absence (unstimulated) of CD3/CD28 antibodies for 72 h. The freshly-isolated unstimulated Tresponder:Tcontrol (0 h) was used as the reference CFSE intensity at the start of culture. The CFSE intensity of these cells was measured to mark the initial CFSE intensity before culture. Stimulated Tresponder:Tcontrol group was assigned as the experimental control group. In addition, unstimulated Tresponder:Tcontrol group was used to compare the kinetic of cell growth in the experimental control group. The suppression activity of nTreg cells towards Teff cells were measured by the capacity of Teff cells to proliferate. This experiment was repeated three times and the mean values were used to construct the graph. Error bars represent ± SEM. (n=3 mice/experiment). *P value < 0.01, versus Tresponder:Tcontrol stimulated group. # Low CFSE intensity due to cell death.

IV. Discussion

Our preliminary experiments demonstrated that isolated nTreg cells from BALB/c splenocytes optimally expand *in vitro* at 72 hr time-point, at 5ng/mL IL-2 (Figure 2). Furthermore, they retained the suppressive function towards Teff following *in vitro* culture (Figure 3). This was demonstrated by the modulation of labelled-Teff cell proliferation during co-culture with unlabelled nTreg cells. When stimulated, Treg cells suppressed Teff cell division by more than 60% compared to stimulated Teff alone. These results suggest that nTreg cells effectively induce anergy in activated Teff cells, demonstrating their suppressive capacity as previously reported (12). Interestingly, unstimulated nTreg cells were capable of suppressing unstimulated Teff cells by 30% implying that nTreg cells are able to suppress Teff cells even without antigenic stimulation during *in vitro* expansion. This may indicate that the suppressive effect of nTreg cells may not require TCR and costimulatory activation.

It has been suggested that nTreg cells do not need to be activated in order to suppress Teff cells *in vitro* (13). Theoretically, the absence of polyclonal stimulation during *in*

vitro co-culture prevents activation of both nTreg cells and Teff cells. However our current data suggest the partial suppressive effect is produced by nTreg cells on Teff cells in the absence of polyclonal stimulation. This may be partly due to the presence of exogenous IL-2 that indirectly drives partial activation of Teff cells. Furthermore, previous reports showed that the absence of TCR cognates and costimulatory signals did not hinder the suppressive capacity of nTreg cells (13, 14, 15). This is presumed to be due to the phenotypic features of nTreg cells, such as high expression of CD25 and CTLA-4 (16, 17). This allows nTreg cells to suppress autoreactive Teff cells without TCR activation via a response known as bystander suppression (14, 18). In addition, the kinetics of suppression by nTreg cells is driven by the activation state of the Teff cells, not the time for nTreg cells to acquire suppressive function (19). It was shown that nTreg cells start to suppress Teff cell proliferation in as little as 2 h following *in vitro* culture, suggesting that nTreg cells are capable of suppressing activated Teff cells in a small kinetic window (19). This rapid kinetic of suppression between nTreg cells and Teff cells *in vitro* illustrates that nTreg cells do not require TCR activation to mediate suppression. Apart from that, nTreg cells may also not require co-stimulatory activation in order to become suppressive. A study showed that the CD28-deficient nTreg cells were capable of hindering the activity of Teff cells, suggesting that costimulatory activation is not required by nTreg cells to suppress Teff cell function (20). Moreover, cotransfer of $CD4^+CD25^+$ cells from $CD28^-/^-$ mice capable of protecting SCID mice recipients from experimental colitis (21).

Taken together, the previous studies and current data suggest that TCR and co-stimulatory activation in nTreg cells is dispensable for nTreg cell suppressive effect. Previous studies have demonstrated that the differentially-activated pathways involved in nTreg cells and Teff cells may explain the differences in stimulatory requirements of these cells (22). This data is important in order to provide the fundamental direction on experimental design in studies involving adoptive transfer of nTreg cell *in vitro* and *in vivo*.

ACKNOWLEDGEMENTS

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The role of PPARγ ligands in inducing Foxp3 gene expression in natural T-regulatory (nTregs) Cells of Type 1 Diabetic (T1D) Mice

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Introduction: PPARγ is known to have anti-inflammatory properties in immune cells, while Foxp3 gene confers immunosuppressive effects on natural T-regulatory (nTreg) cells. Understanding the interaction between these transcription factors in nTreg cells could enhance our understanding of the immunopathogenesis of T1D.

Objective: To determine the influence of PPARγ ligands on the expression of Foxp3 gene in activated nTreg cells from T1D mice.

Methodology: Splenic nTreg cells from Non-obese Diabetic (NOD) and Non-obese Diabetic Resistant (NOR) mice were isolated and cultured with anti CD3/CD28 antibodies and IL-2, in the presence of the PPARγ agonists, ciglitazone or 15d-prostaglandin-J_2, with or without the presence of GW9662, a PPARγ antagonist. Cell proliferation was determined by CFSE-labelling and measured by flow cytometry. Foxp3 mRNA transcription was measured by Absolute Quantitative Real-Time PCR (qPCR).

Result: Ciglitazone induced Foxp3 mRNA expression in NOD, but not in NOR mice, whereas 15d-prostaglandin-J_2 did not alter Foxp3 expression in these mouse models. The addition of GW9662 reversed the effect of ciglitazone in NOD.

Conclusion: While the natural PPARγ ligand, 15d-prostaglandin-J_2, had no effect on Foxp3 expression, ciglitazone, a synthetic PPARγ ligand, upregulated the expression of Foxp3. Given the fact that PPARγ is involved in downregulation of the immune response, the findings in this study suggest the potential role of ciglitazone in dampening the pathogenesis of T1D. Furthermore, the potential protective effect of PPARγ ligands in other autoimmune conditions should be examined.

(229 words)

Modulation of FOXP3 Expression by PPARγ ligands in natural T-regulatory Cells

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Introduction: The characteristics and functions of natural $CD4^+CD25^+$ $FOXP3^+$ T-Regulatory (nTreg) cells is currently an area of great interest. These cells are important in maintaining homeostasis of the immune response.

Aim: We determined the influences of PPARγ ligands on the expression of FOXP3 gene in activated nTreg cells.

Methodology: Splenic nTreg cells from Balb/c mice were isolated and cultured with the presence of anti CD3/CD28 and IL-2 cytokine. Cell proliferation was determined by CFSE-labeling, measured by flow cytometry. FOXP3 mRNA transcription was measured by Quantitative Real-Time PCR (qPCR).

Result: FOXP3 mRNA transcription was recorded highest at 0 hour, and at its lowest when cells were treated with 15d-PGJ_2. GW9662-treated cells have pronounced mRNA expression compared to Ciglitazone, 15d-PGJ_2 and untreated group.

Conclusion: With the presence of the PPARγ ligands, FOXP3 mRNA expression was detected at minimal level. This finding may contribute to the future study in delineate PPARγ-dependant and independent mechanisms in nTreg cells during pathological autoimmune conditions.

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Technical report

IDENTIFICATION OF PUTATIVE CROSSTALK BETWEEN FOXP3 AND PPARγ IN NATURAL T-REGULATORY (nTreg) CELLS FROM HEALTHY AND AUTOIMMUNE DIABETIC NOD MICE

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Abbreviation:

CFSE Carboxyfluorescence Succinimidyl Ester

CNS Conserved non-coding sequence

DC Dendritic cell

Foxp3 FORKHEAD BOX P3

GALT Gut-associated lymphoid tissue

GPR40 G-Protein associated receptor 40

HIF-1 Hypoxia inducible factor-1

Abstract: The vital role play by natural Treg cells (nTregs) as immune-regulator during autoimmune conditions is of current insight in immune regulation. Similarly, the nuclear hormone receptor peroxisome proliferator-activated receptor γ (PPARγ) has been shown to play a role as an immuno-downmodulator during inflammation. However, the association between PPARγ and nTregs is still ambiguous. Given their mutual roles in downregulating the immune responses, the association between PPARγ ligands and nTregs was investigated. Current study examined the constitutive and induced expression of PPARγ isoforms in activated nTreg cells, the influence of PPARγ ligands on Foxp3 expression and the possible crosstalk between PPARγ and signaling pathways in nTreg cells of healthy and autoimmune diabetic mice. *In vitro*, activated nTreg cells constitutively downregulated PPARγ1, but upregulated PPARγ2 expression. Surprisingly, ciglitazone induced PPARγ1, but suppressed PPARγ2 expression in activated nTreg cells. In addition, we found that ligand-activated PPARγ negatively regulate Foxp3 expression in activated nTreg cells via PPARγ-independant mechanism. Overall, PPARγ ligands induced several pro-inflammatory-related pathways in activated nTreg cells of healthy mice, while downregulated most of pro-inflammatory-related pathways in autoimmune-diabetic mice. In conclusion, our data suggest that PPARγ isoforms are differentially involves in suppressing Foxp3 expression in activated nTreg cells during healthy and autoimmune conditions, as opposed to its activation in conventional $CD4^+$ T cells to becoming induced Treg (iTreg) cells.

1. Introduction

Over the past twenty years since the identification of naturally-occurring $CD4^+CD25^+Foxp3^+$ Regulatory T cells (nTreg), there have been intense researches in delineating the immunobiology of nTreg cells in physiological and pathological conditions

allergic reactions (18). Ciglitazone-contained nebulizer reduced the inflammatory reaction in asthmatic lung of asthmatic mouse model by modulating both immune and non-immune cells (19). PPARγ activation by ciglitazone also reduced mucus production by airway epithelial cells, TGF-β level and collagen deposition in the lower respiratory tract (19). Similarly, in mice sensitized with allergens, inflammatory reaction triggered following allergen exposure was reduced by PPARγ activation via inhibition of reactive oxygen species (ROS). This would directly limit the activation of NF-κB and hypoxia-inducible factor-1 (HIF-1) that are essential for inducing allergic reactions (20). 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 (21). However, the 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 genes in a murine autoimmune model. We tried to outline the influence of TZDs class of PPARγ ligands in modulating Foxp3 expression in nTreg cells of BALB/c, NOD and NOR mice.

2. Materials and methods

Mice

Female BALB/c mice were purchased from the Animal Research and Service Centre (ARASC), Universiti Sains Malaysia. Female Non-obese Diabetic (NOD) and Non-obese Resistant (NOR) mice were purchased from The Jackson Laboratory. The mice were maintained in the animal facilities under specific pathogen-free conditions in accordance with the guidelines and regulations of ARASC, University Sains Malaysia and used at 12-week. All experimental protocols were approved by the institutional animal ethics committee. The animal ethic

FITC- conjugated IgG2a were used as isotype controls for fluorescein-conjugated antibodies used. Carboxyfluorescein succinimidyl ester (CFSE) was used to track cell division.

Total RNA isolation, cDNA synthesis and real-time PCR for PPARγ isoforms and Foxp3

Total RNA was extracted using RNAeasy Mini kit (Qiagen) and subjected to cDNA synthesis using cDNA first strand synthesis (Qiagen). To quantify gene amplification of unknown samples, the standard curves for both PPARγ1 and PPARγ2 genes were used as standard reference. A serial dilution of known PPARγ1 and PPARγ2 DNA plasmid concentrations, from 10^1 to 10^7 copy numbers was prepared and then amplified to generate standard curves. PCR was performed using primers for PPARγ1 (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') PPARγ2, (forward: 5'-GGG TGA AAC TCT GGG AGA TTCTC-3', reverse: 5'-GTG GGC CAG AAT GGC ATC-3', probe: 5'-CAT CAG CGA AGG CAC CAT GCT CTG-3'). Foxp3 gene with accession number of AF277992.1 was selected from 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 analyzed using the ABI prism software (Applied Biosystem).

PPARγ-PPRE binding activity

PPARγ activity was measured by its binding to the response element, PPRE. This was measured by using ligand binding assay of PPARα, β, and γ transcription factors (Cayman Chemical). The nuclear proteins of treated and untreated cells were extracted using Nuclear

Data from experimental analyzes were presented as the mean of triplicates with standard error mean (mean ± SEM). The data were statistically analyzed using Minitab® 16.1.0 software. 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. The *P* value of less than 0.05 ($P < 0.05$) is considered significant.

3. Results

Suppressive function analysis of isolated nTreg cells

The functional analysis of isolated splenic nTreg cells was determined by observing their ability to suppress $CD4^+$ T-effector (Teff) cells *in vitro* (Figure 1). In the figure, data showed that when stimulated, Tresponder cells co-cultured with Treg cells resulted in significantly reduced proliferative capacity, compared with the Tresponder:Tcontrol group ($P < 0.01$). When unstimulated, Tresponder cells co-cultured with Treg cells were suppressed and had reduced cell division, compared to the stimulated Tresponder:Tcontrol group ($P < 0.01$). Stimulated Tresponder:Tcontrol co-cultured cells were recorded to have higher levels of divided cells in comparison with other groups. Stimulated Tresponder:Treg co-cultured cells were observed to have more potent suppressive effect in comparison to unstimulated Tresponder:Treg co-cultured cells. The freshly-isolated Tresponder:Tcontrol unstimulated group (0 h) was the initial population of cells before culture. The Tresponder:Tcontrol unstimulated was set as a growth marker for Tresponder:Tcontrol stimulated group. These results indicate the ability of nTreg cells to suppress activated $CD4^+$ T cells, but only partially suppressed inactive $CD4^+$ T cells. Furthermore, the involvement of secondary signals was

treated with ciglitazone significantly induced higher levels of PPARγ1 compared to the untreated group ($P < 0.01$). In cells receiving co-treatment of ciglitazone and GW9662, PPARγ1 expression was significantly higher than untreated group ($P < 0.01$), but was lowered compared with ciglitazone-treated cells ($P< 0.05$). Meanwhile, GW9662-treated cells showed higher level of PPARγ1 compared with untreated group ($P < 0.01$), but lower than ciglitazone-treated cells ($P < 0.05$). The mRNA level of PPARγ2 isoform in treated-activated nTreg cells were undetectable (results not shown). On the other hand, addition of the PPARγ ligands, ciglitazone and 15d-PGJ_2 significantly suppressed Foxp3 expression in these cells compared with untreated group ($P < 0.01$) (Figure 5). Addition of GW9662 did not reverse the effect produced by PPARγ ligands. Furthermore GW9662 had no effect in inducing Foxp3 expression in these cells after 72 h incubation. In summary, PPARγ ligands negatively regulate Foxp3 expression in activated nTreg cells from healthy BALB/c mice via PPARγ-independent pathways.

Induced expression levels of Foxp3 in nTreg cells of NOD and NOR mice

The effect of 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 PPARγ ligands in the presence or absence of the PPARγ inhibitor. A group of untreated nTreg cells from both NOD and NOR mice was used as control for each of the mouse strain respectively. The results obtained from the correlation analyzes in NOD and NOR mice are shown in Figure 6. In both mouse strains, stimulated nTreg cells treated with ciglitazone expressed lower levels of Foxp3 mRNA compared to untreated group ($P < 0.01$). In both strains, following the addition of GW9662, Post-hoc analysis revealed that Foxp3 expression was further reduced in these cells compared to ciglitazone-treated cells ($P <$