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

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***Abstract*---**Natural $CD4^+$ $CD25^+$ $Foxp3^{hi}$ T-regulatory cells (nTreg) cells are required to induce tolerance against self-antigens during inflammation. The expression of Foxp3 protein by these cells herald the ability to suppress $CD4^+$ T effector (Teff) cell activation, hence act as the master regulator for nTreg cell function. In the presence of exogenous IL-2 cytokine, nTreg cells are viable *in vitro* upon isolation, however the optimal time-point is uncertain. The current study identified the optimal *in vitro* culture conditions for nTreg cells isolated from BALB/c mice. The suppressive function of isolated cells towards Teff cells was also measured to determine the ability to retain their suppressive function after *in vitro* culture. The results indicated that single population of nTreg cells from splenocytes using magnetic isolation method is > 90% purity. The optimal time-point was set at 72-hr, similar to other Teff cells. In addition, in the absence of TCR stimulation, nTreg cells capable of suppressing Teff cell activation, indicating the requirement of CD3/CD28 ligation on nTreg cells surface is dispensable for their suppressive effect.

***Keywords*---**Foxp3, Immune regulation, T-regulatory cells, immunosuppression.

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 delineating the immunobiology of nTreg cells in physiological and pathological conditions (1). The master regulator in nTreg cells is the transcription factor Forkhead box P3 (Foxp3) which plays an important role in the development and function of nTreg cells (2, 3). Foxp3 is expressed in the thymus by nTreg cells (4, 5) and is transiently expressed by $CD4^+CD25^-$ conventional T cells (iTreg) peripherally (5).

In pathological conditions such as autoimmune disorders, the recognition of self-tissues by auto-reactive T cells leads to the destruction of host tissues or organs. The immunosuppressive 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).

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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 $CD4^+CD25^+$ cell population obtained was > 90% as determined by FACS analysis (Figure 1). The percentage of $Foxp3^+$ cells were > 80% from the total $CD4^+CD25^+$ population.

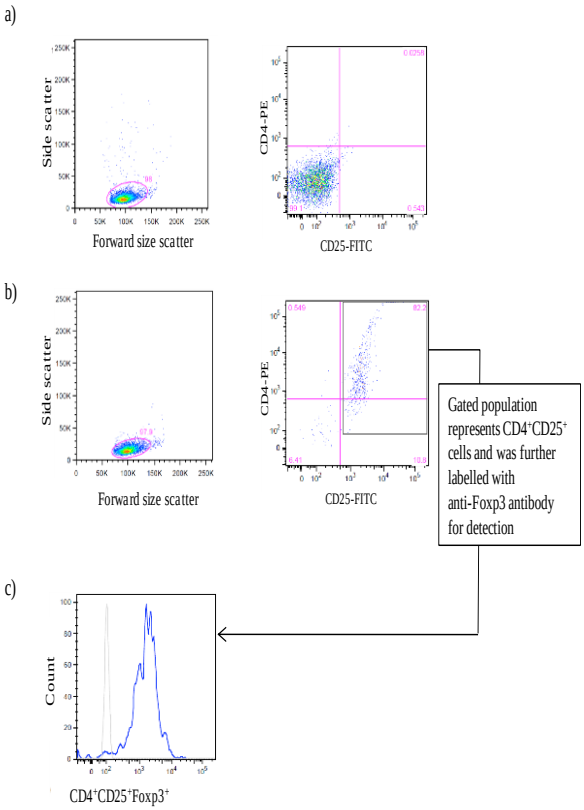

Fig. 1 Efficiency of natural $CD4^+CD25^+Foxp3^{hi}$ Treg (nTreg) cell isolation from splenocytes.

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

Optimization of in vitro culture condition of nTreg cells

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

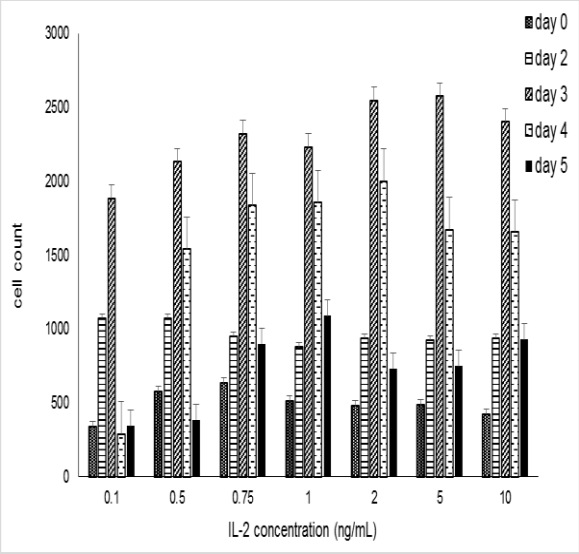

Fig. 2 The proliferation of stimulated nTreg cells in the presence of IL-2 cytokine. The optimal proliferation time for nTreg cells was recorded on day three of *in vitro* culture.

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

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 3). 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 dispensable for nTreg cells to suppress Teff cells, as partial suppressive effect was observed in unstimulated Tresponder:Treg co-culture cells.

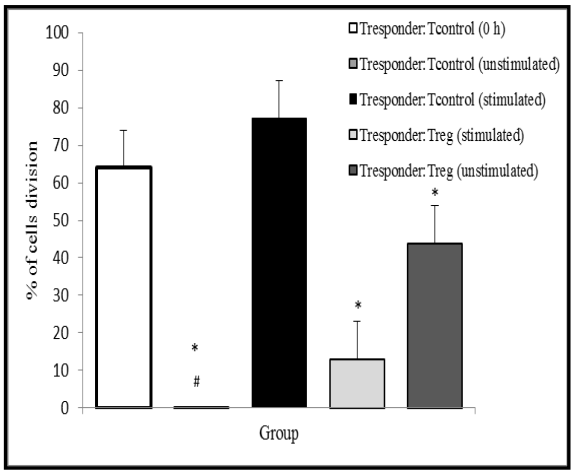

Fig. 3 Suppressive function analysis of isolated nTreg cells. The suppressive function of isolated nTreg cells towards Teff cells was maintained after 72 h *in vitro* culture.

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*.

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