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Analysis of Foxp3, CD25, and CD127 Expressed on Regulatory T Cells in Thai Subjects

Masatha Thongpan[1], Chagriya Kitiyakara[2], Yada Louischaroen[2], Prasit Keesukphan[3] and Sansanee C. Chaiyaroj[1]

SUMMARY Foxp3$^+$ natural regulatory T cells (nTreg) play a distinct role in maintaining self tolerance at the periphery. CD25^{hi} and CD127^{lo} were proposed for the identification and purification of nTreg but they have not been confirmed in non-Caucasian populations. This study examined the sensitivity and purity of Foxp3 nTreg identified by CD25 and CD127 in the peripheral blood of Thai subjects (13 males, 15 females with age range of 20-42 years old). The proportions of nTreg/CD4$^+$ as identified by the different markers were as follows: Foxp3$^+$, 18.3 ± 6.4%; CD25^{hi}, 6.4 ± 3.2%; and CD127^{lo}, 54.3 ± 14.2%. Sensitivity tests showed the following results: CD25^{hi}, 23.1%; CD127^{lo}, 40.6%; CD25^{hi}CD127^{lo}, 7.4%. Purity tests concluded as follows: CD25^{hi}, 63.6%; CD25^{int}, 24.9%; CD25^{lo}, 8.7%, CD127^{lo}, 26.5%; CD127^{hi}, 14.9%, and CD25^{hi}CD127^{lo}, 52.0%. In conclusion, the proportions of nTreg in Thai subjects are similar to Caucasian populations. CD25^{hi} is superior to CD127^{lo} for separating Foxp3$^+$ nTreg. Combining CD25^{hi} and CD127^{lo} does not improve the nTreg purity.
Abbreviations: CD25^{hi}, CD25 highly expressed cells; CD25^{int}, CD25 intermediately expressed cells; CD25^{lo}, CD25 low expressed cells; CD127^{hi}, CD127 high expressed cells; CD127^{lo}, CD127 low expressed cells.

Regulatory T cells (Treg) have important roles in maintaining self tolerance at the periphery to prevent autoimmunity. There are many types of T cells with regulatory functions, including CD8$^+$ suppressor T cells, double negative suppressor T cells, natural killer T cells, and γδ T cells. Recently, CD4$^+$ natural Treg (nTreg) cells have been described.[1] These cells can inhibit the immune response by suppressing T cell proliferation and reduce IL-2 and IFN-γ secretion of normal T cells by cell contact-dependent mechanisms.[2] These nTreg cells express several molecules such as cytotoxic T lymphocyte-associated antigen 4 (CTLA-4)[3] and interleukin-2 receptor α chain (CD25).[1] However, cell surface markers that allow a complete separation and isolation of nTreg remain elusive.

Earlier studies have found that isolated CD4$^+$CD25$^+$ cells are capable of mediating T cell suppression *in vitro*[1] and may be defective in autoimmune diseases.[4] However, CD25 is not only expressed by Treg but also on activated T cells. Therefore CD25 is not a specific marker for these Treg.[5] More recently, Hori *et al*. have shown that the transcription factor Foxp3 (Forkhead/winged-helix protein 3) is predominantly expressed in Treg cells isolated from the thymus as well as the periphery but

From the [1]Department of Microbiology, Faculty of Science, Mahidol University, Bangkok, Thailand, [2]Department of Medicine, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok, [3]Department of Family Medicine, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok, Thailand.
Correspondence: Sansanee C. Chaiyaroj
E-mail: scscy@mahidol.ac.th

not in other T cells[5] and subsequent studies have shown that Foxp3 is a specific marker for this subset of T cells. Foxp3 is an intracellular transcription factor directly involved in the commitment and maintenance of the nTreg lineage. $Foxp3^+$ nTreg are crucial to prevent autoreactive T cells in the periphery.[2] Unfortunately, since Foxp3 is an intracellular molecule and detection by flow cytometry requires cell permeabilization, Foxp3 cannot be used for nTreg functional studies.

In some recent studies the expression of CD127 or IL-7 receptor on the surface of T cells was shown to be inversely correlated with Foxp3 in human Treg.[6] $CD4^+$ T cells that expressed a low level of CD127 were found to express high levels of Foxp3 and these $CD4^+CD127^{lo}$ were highly suppressive *in vitro*.[6] Therefore $CD127^{lo}$ might be used as marker to identify nTreg. However, these studies were performed in a small number of subjects[2,3] and were not confirmed in non-Caucasian populations to date.

The objectives of this study were to determine the number of nTreg in adult Asian subjects using flow cytometry, to analyze the relationship between the expression of Foxp3, CD25, and CD127 and to evaluate the combination of $CD25^{hi}$ and $CD127^{lo}$ for the identification of $Foxp3^+$ nTreg.

MATERIALS AND METHODS

Subjects

Twenty-eight healthy volunteers were recruited to represent the normal population (13 males, 15 females with age range of 20-42 years old). The subjects were screened for underlying diseases by their medical history, physical examination, blood (5 ml) and urine tests. The laboratory parameters investigated included a complete blood count, liver function tests, hepatitis B surface antigen, serum anti-hepatitis C antibody, creatinine, blood sugar, anti-nuclear antibody, and a urinalysis. Subjects with active or recent infection (within two weeks) or with significant illness such as liver or kidney diseases or diabetes mellitus were excluded. All subjects gave their informed consent and the study was approved by the Ethics Committee of the Faculty of Medicine, Ramathibodi Hospital, Mahidol University.

Antibodies

Fluorescein isothiocyanate (FITC)-conjugated anti-FOXP3 MAb (clone PCH101) (eBioscience, San Diego, CA), PE-Cy7 conjugated anti-CD3 MAb (clone S4.1), PE conjugated anti-CD4 MAb (clone S3.5) and Allophycocyanin (APC) conjugated anti-CD25 MAb (clone CD25-3D10) (CALTAG™, Burlingame, CA), anti-CD127 MAb (BD Bioscience, San Jose, CA), irrelevant isotype-, fluorochrome-matched (CALTAG™) and secondary control antibodies (CALTAG™), were used in this study. The antibody concentrations used for cell staining were based on the data supplied by the manufacturers and initial optimization studies.

Surface and intracellular staining

Surface staining was performed after incubating the whole blood in red blood cell lysis buffer (155 mM NH_4Cl, 12 mM $KHCO_3$, 0.1 mM EDTA) for 15 minutes. The cells were incubated with fluorochrome-labeled antibodies to CD3, CD4, CD25, and CD127 (5 μg/ml, 10^6 cells/test). After incubation for 30 minutes at 4°C, they were washed twice with phosphate buffered saline (PBS) and fixed with 2% paraformaldehyde.

Intracellular staining was performed following the manufacturer's instructions (eBioscience). Briefly, cells were stained for the surface markers and washed twice with PBS. Then they were permeabilized with a Fixation/Permeabilization Buffer (eBioscience) at 4°C for 45 minutes in the dark. After washing twice with the permeabilization buffer, the cells were stained with anti-human Foxp3 MAb (15 μl/test) at 4°C for 30 minutes in the dark. Next, the cells were washed twice with PBS and fixed with 2% paraformaldehyde. The samples were then analyzed by flow cytometry.

Flow cytometric analysis

To assess the effects of the permeabilization on the cell surface markers, each sample was separated into 2 batches in order to compare between surface staining alone and surface staining followed by intracellular staining. The process was performed following the manufacturers' instructions with some modifications (eBioscience). In brief, background

fluorescence was assessed using the appropriate isotype- and fluorochrome-matched control MAbs to determine the percentage of positive cells. $CD4^+$ T cells were gated on $CD3^+$ cells using the relevant labeled antibodies. The individual percentages of the $Foxp3^+$, $CD25^{hi}$, and $CD127^{lo}$ cells were based on the $CD4^+$ T cell population. The analysis was performed using FACScanto™ (CellQuest™ software; Becton Dickinson, San Jose, CA). The cells were divided into 6 groups by gating: $CD25^{hi}$, $CD25^{int}$, $CD25^{lo}$, $CD127^{lo}$, $CD127^{hi}$, and $CD25^{hi}CD127^{lo}$ cells under the $CD4^+$ population. The percentage of $Foxp3^+$ cells was determined for each selected group of markers (Fig. 1). A sensitivity test was performed to identify which cell surface markers can identify the largest number of $Foxp3^+$ cells. Sensitivity (%) was calculated: (number of cells positive for both Foxp3 and selected surface marker[s])/(number of $Foxp3^+$ cells) x 100. A purity test was performed to determine the ability of the cell surface markers to identify the $Foxp3^+$ cells with the lowest contamination by other cells. Purity (%) was calculated: (number of $Foxp3^+$ cells/number of cells positive for the selected marker[s]) x 100.

Statistical analyses

The normality of the distribution of the Treg levels in the subjects was determined using the Kolmogorov-Smirnov goodness-of-fit test. The percentage levels of the T-cell population subtypes and the mean fluorescence intensities of the stained cells were compared using the Student's *t* test for normally distributed populations and the Mann-Whitney ***U*** test for non-normally distributed populations. The parameters themselves were compared using the Pearson's correlation coefficient and Spearman's rank correlation test. Statistical analyses were performed using SPSS (SPSS, v11.5, SPSS Inc, Chicago, IL). A $p < 0.05$ was considered significant.

RESULTS

Flow cytometric analysis

Blood was collected from 28 subjects. After screening, three subjects were excluded from the study because one had diabetes mellitus, and two had a high titer of anti-nuclear antibody. The percentages of individual Treg markers in the CD4+ cells of the Thai subjects were as follows: $Foxp3^+/CD4^+$, 18.3 ± 6.4%; $CD25^{hi}/CD4^+$, 6.4 ± 3.2%; and $CD127^{lo}/CD4^+$, 54.3 ± 14.2% (Fig. 2).

Permeabilization of the cell membrane is required for intracellular staining of Foxp3. Therefore, we compared stained white blood cells (WBC) using permeabilization and non-permeabilization protocols

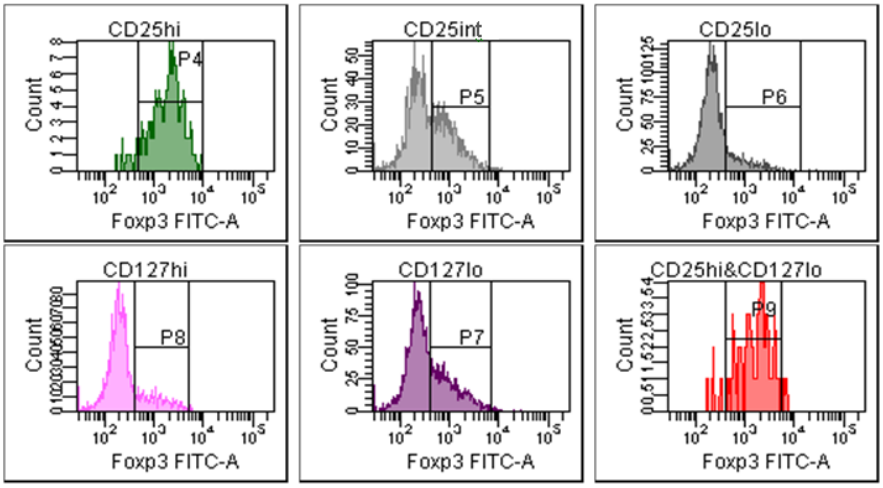

Fig. 1 Gating for %purity. Gating of $Foxp3^+$ cells in each population: $CD25^{hi}$, $CD25^{int}$, $CD25^{lo}$, $CD127^{lo}$, $CD127^{hi}$, $CD25^{hi}CD127^{lo}$ cells for the calculation of %purity.

(Fig. 3A). Without permeabilization, WBC could be separated into 3 populations, polymorphonuclear cells (PMN), lymphocytes, and monocytes using forward scatter (FSC) and side scatter (SSC) analysis whereas cells after permeabilization were shown to be shrunken and decreased in size (Fig. 3A). Since these cells could not be separated by FSC *vs.* SSC, thus, we used CD3 staining to select T cells from the whole population. $CD4^+$ T cells were then gated from the $CD3^+$ cells (Fig. 3B, upper panel). Subsequently, each Treg marker ($Foxp3^+$, $CD25^{hi}$, and $CD127^{lo}$) was gated from the $CD4^+$ cells (Fig. 3B, middle panel). Each $CD127^{hi}$ and $CD25^{int}$ population was gated in comparison to the IgG control (data not shown) and $CD25^{hi}$ cells were gated from the population with one logarithm higher of $CD25^{hi}$ than $CD25^{int}$ cells. This gating was used throughout this study. Lastly, the correlation of each pair of Treg markers such as Foxp3 vs. CD25, Foxp3 vs. CD127, and CD127 *vs.* CD25 was calculated (Fig. 3B lower panel).

The stained surface markers in permeabilized and non-permeabilized cells were compared by cell surface permeabilization (Fig. 4). The percentages obtained using non-permeabilized and permeabilized protocols of $CD3^+$ cells/WBC (40.7 ± 15.2 and 40.1 ± 14.0; $p = 0.59$), $CD4^+/CD3^+$ cells (54.7 ± 9.8 and 55.9 ± 10.6; $p = 0.17$) and $CD25^{hi}/CD4^+$ cells (6.6 ± 3.9 and 5.5 ± 3.9; $p = 0.27$) as determined by the two methods were not significantly different. However, the percentage of $CD127^{lo}/CD4^+$ cells decreased dramatically after treatment with permeabilization buffer (54.3 ± 14.2 and 31.6 ± 15.1; $p < 0.01$).

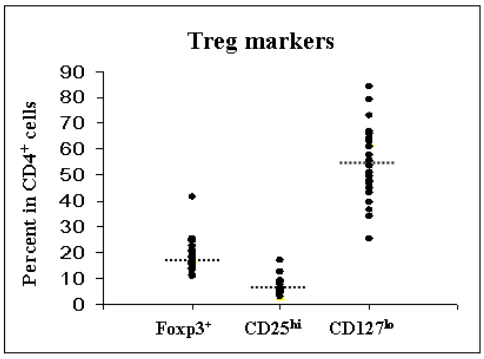

Fig. 2 Percentage of the Treg population in healthy subjects identified by different cell markers in $CD4^+$cells. The percentage of positively-stained cells was gated from the total $CD4^+$ cells. Percent permeabilized $Foxp3^+/CD4$ = 18.3 ± 6.4, percent nonpermeabilized $CD25^{hi}/CD4$ = 6.3 ± 3.2, percent nonpermeabilized $CD127^{lo}/CD4$ = 54.2 ± 14. Average line is shown as horizontal dotted line.

Correlation, sensitivity and purity of cells expressing CD25 and CD127 compared to $Foxp3^+$ cells.

Next, the relationship of the master Treg regulator, Foxp3, with the other suggested cell surface markers, *i.e.* CD25 and CD127, was determined. This analysis was performed to test whether surface markers can be used to identify Treg, using Foxp3 staining for a comparison. For each individual, a proportion of the cells positive for each surface Treg marker ($CD25^{hi}$ or $CD127^{lo}$) were plotted against the proportion of cells positive for Foxp3 (Fig. 5). For the whole group of subjects, there was no correlation between $\%Foxp3^+/CD4^+$ and $\%CD25^{hi}/CD4^+$ ($p = 0.24$) or $\%CD127^{lo}/CD4^+$ ($p = 0.42$). Although the correlation of $\%Foxp3^+/CD4^+$ with $\%CD25^{hi}$-$CD127^{lo}/CD4^+$ was significant ($p = 0.04$), the correlation coefficient was small ($R^2 = 0.175$).

Sensitivity and purity tests were calculated at the single cell level for each individual. Sensitivity tests for $CD25^{hi}$ and $CD127^{lo}$ markers were also calculated using Foxp3 as a gold standard (Fig. 6). The results showed that $CD127^{lo}$ had the highest sensitivity (mean %sensitivity = 40.6) compared to $CD25^{hi}$ (mean of %sensitivity = 23.1) and $CD25^{hi}CD127^{lo}$ (mean of %sensitivity = 7.4). The sensitivity of $CD127^{lo}$ was also significantly higher than the other two markers ($p < 0.01$). However, the mean of %sensitivity of $CD127^{lo}$ did not even reach half of the total cells which means that more than 50% $Foxp3^+$ cells were not $CD127^{lo}$ cells.

Then the purity tests were performed (Fig. 7). Using CD25 as a marker, the mean of %purity of Foxp3 turned out to be as follows: $CD25^{hi}$ (63.6%), $CD25^{int}$ (24.9%) and $CD25^{lo}$ (8.7%). The $CD25^{hi}$ population was found to contain the highest number of $Foxp3^+$ cells, significantly more than the $CD25^{int}$ and $CD25^{lo}$ populations ($p < 0.01$). Using CD127 as marker, the $CD127^{lo}$ population contained more

Foxp3^{+} cells than the CD127^{hi} (%purity = 26.5 and 14.9, respectively, $p < 0.01$). However, comparing CD25 and CD127, CD25^{hi} cells had a higher purity than CD127^{lo} cells ($p < 0.01$). Using CD25^{hi}CD127^{lo} as a combination marker, the %purity was also relatively high (52.0%) but not significantly different from CD25^{hi} alone. However, using the combined marker, a higher variation of the %purity (SD = 23.0) was observed compared to CD25^{hi} (SD = 12.2). Therefore, for Treg purification, CD25^{hi} expression

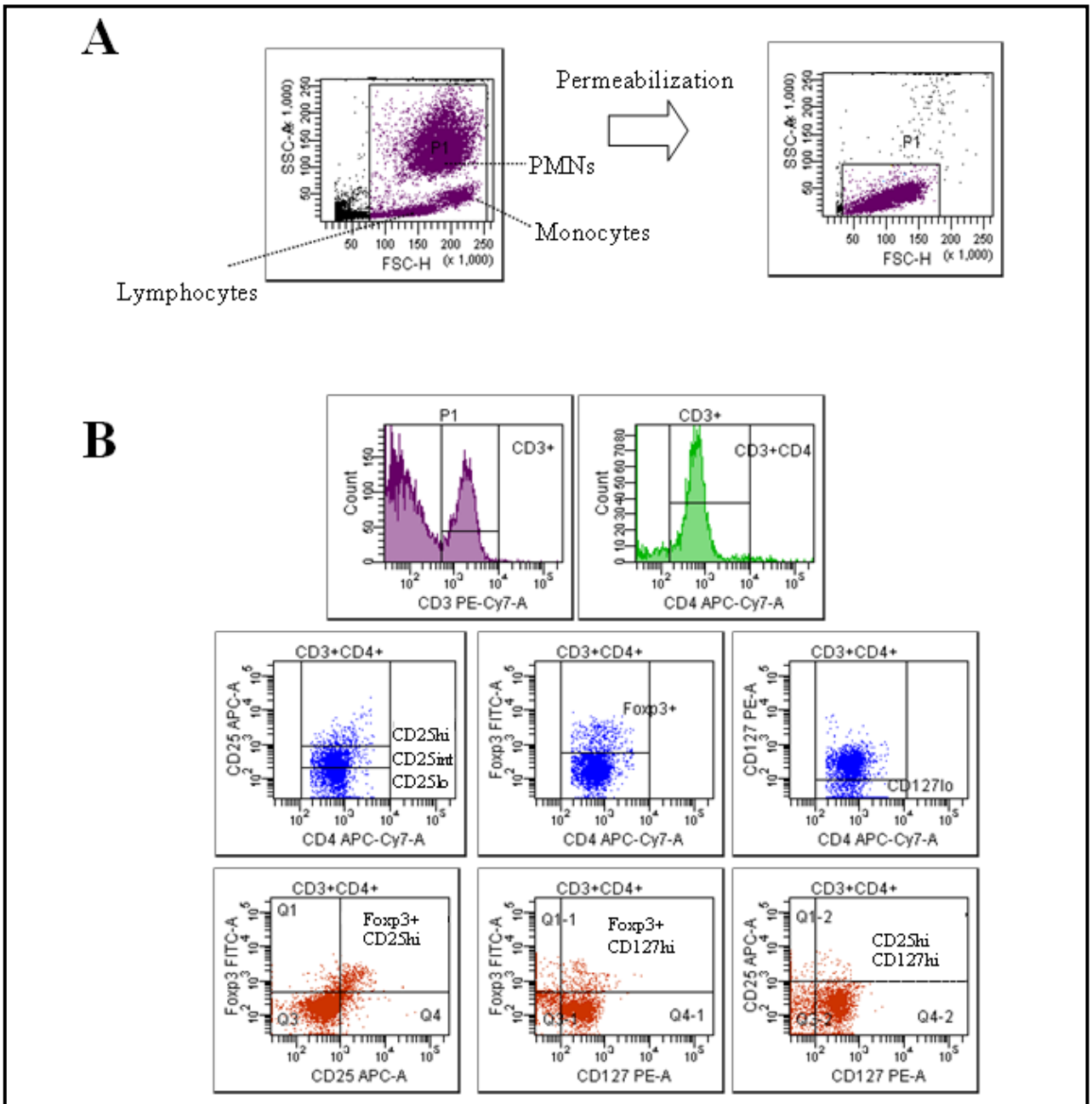

Fig. 3 Surface and intracellular staining of WBC. The blood sample was separated into two aliquots to perform surface staining alone and surface staining followed by intracellular staining. **A,** Cell population analyzed by FSC *vs.* SSC before and after permeabilization. **B**, Representative of gating for CD3^{+} and CD4^{+} T cells (upper panel), gating for Foxp3, CD25^{hi} and CD127^{lo} cells from CD4^{+} cells (middle panel) and the expression level of each pair of Treg markers (lower panel).

is the marker of choice.

DISCUSSION

In this study, we determined the percentage of nTreg in adult Thai subjects using three different markers namely CD25, CD127 and Foxp3. We found that $CD4^+CD25^{hi}$ was the most specific for $Foxp3^+CD4^+$ cells and that $CD127^{lo}$ either with CD4 or in combination with $CD25^+$ did not increase the purity for isolating $Foxp3^+CD4^+$ cells.

Previous studies in Caucasian populations have reported the number of $Foxp3^+$ cells as a percentage of different reference cell populations. For example $Foxp3^+$ cells have been shown to represent 1.2%[7] to 2.36%[8] of PBMC, 6.5%[9] of lymphocytes, and 2.9%[10] to 22%[11] of $CD4^+$ cells. In this experiment, we identified Treg using anti-Foxp3 MAb and flow cytometry to determine the percentage of $Foxp3^+$ cells in the CD4 population. Our mean percentage of $Foxp3^+$ in the CD4 population was 18.3% which is comparable to other studies in Indian (11.2%) and Taiwanese populations (18.0%).[12,13]

$CD4^+CD25^+$ was identified as a Treg marker both in mice and humans.[1] The percentage of $CD4^+CD25^+$ can range from 2%[14] to 13%.[15] However, each study used a different reference WBC population to calculate the percentage of $CD4^+CD25^+$ such as PBMC,[14] lymphocytes,[16] total WBC,[17] $CD3^+$,[18] and $CD4^+$ cells.[15] This is one reason why the percentage of Treg varied between the studies. Since $CD25^+$ cells include activated T cells, re-

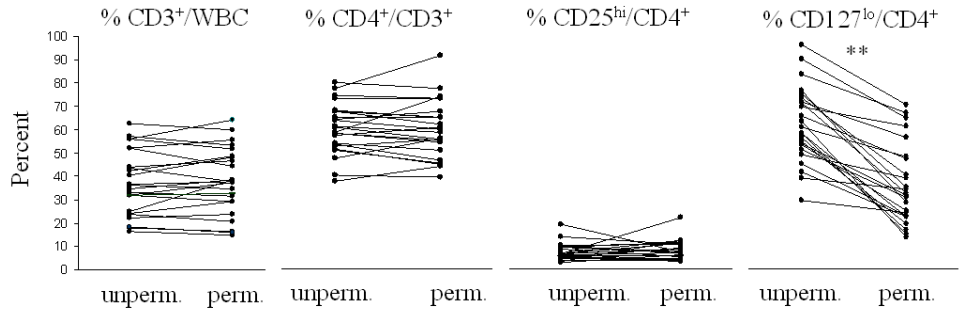

Fig. 4 Effects of cell permeabilization on cell surface staining. The expression of each marker in aliquots with and without permeabilization was compared. unperm., represents cells without permeabilization; perm., represents cells with permeabilization. ** $p < 0.01$.

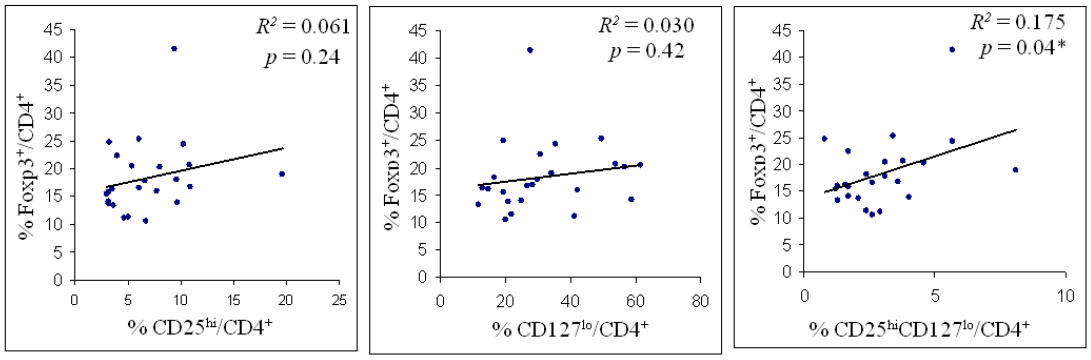

Fig. 5 The proportion of cells positive for different markers in each subject. The correlation between the numbers of cells positive for Foxp3 and CD25, CD127 was determined in $CD4^+$ cells for each individual. * $p < 0.05$.

searchers tried to exclude this population from Treg by selecting cells with a high expression of CD25.[19-21] Again, because of the use of different cell gating criteria for the calculations as mentioned above, there is a range of $CD4^+CD25^{hi}$ from 0.25%[19] to 6%[22] in the Caucasian population. In our study, the mean percentage of $CD4^+CD25^{hi}$ in the CD4 population was 6.4% which is similar to reports in Japanese populations (5%, 6.5%).[23,24]

Recently, Liu *et al.*[6] proposed $CD127^{lo}$ as a marker of Treg. However, few studies have examined the specificity of CD127 as a marker of nTreg. Moreover, CD127 is not only a Treg marker but it also represents the IL-7 receptor alpha chain which plays a role in T cell survival and phenotype memory.[25] Hence, it has not been used as a Treg marker by itself, but usually in combination with CD25,[26] Foxp3[27] or both.[28] Foxp3 is an intracellular marker and staining of Foxp3 requires cell permeabilization, which is not practical. Therefore other surface markers that can identify Treg were investigated and previous studies reported that CD127 could be used.[6,29] However, the investigators did not examine the effect of the permeabilization on the CD127 expression while co-staining with both Foxp3 intracellularly and CD127 on the cell surface. Our study suggests that caution might be necessary in using CD127 as a surrogate for Foxp3 expression since the expression of CD127 is decreased by the permeabilization process. Furthermore, in our study, the correlation between the numbers of $CD127^{lo}$ in permeabilized cells and the numbers of $Foxp3^+$ was not significant (data not shown).

Next, we investigated whether the surface markers CD25 and CD127 could be used to identify cells with Foxp3 expression, but the correlation between Foxp3 and each surface marker was poor. Sensitivity testing showed that the sensitivity of CD127 and CD25 to Foxp3 was never more than 50% of the total cell numbers. Studies using CD127 or CD25 as markers for Treg determination are likely to obtain different results compared to Foxp3. Therefore, all studies of Treg determination must state clearly which marker is used.

The permeabilization process for Foxp3 staining leads to cell lysis. As such nTreg cannot be isolated for functional studies using Foxp3. Therefore, a surface marker that can identify Foxp3 with a high purity would be preferred for Treg isolation. The percent purity of a single marker or combined markers was then examined in order to investigate the usefulness or each surface Treg marker for Treg purification. The results showed that $CD127^{lo}$ had a higher purity of $Foxp3^+$ cells than $CD127^{hi}$, similar to the study done by Valencia and colleagues.[30]

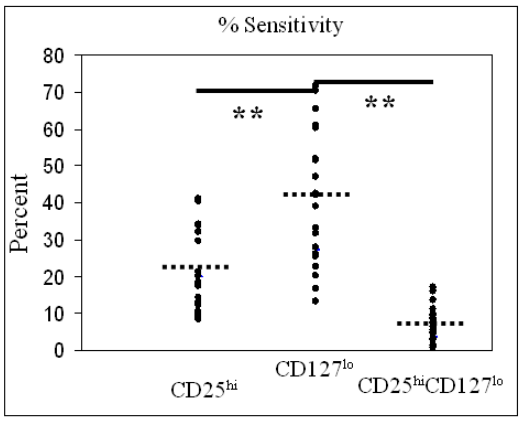

Fig. 6 Percent sensitivity of $CD25^{hi}$, $CD127^{lo}$, and $CD25^{hi}CD127^{lo}$ cells compared with $Foxp3^+$ cells. For each surface marker the sensitivity to Foxp3 was calculated by: percent sensitivity = (%positive marker with $Foxp3^+$ / % all $Foxp3^+$) x 100, $^{**}p < 0.01$. Average line is shown as horizontal dotted line.

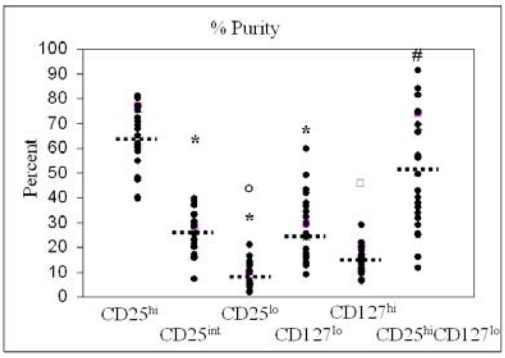

Fig. 7 Percent purity of the Treg population identified by CD25 and CD127 compared with those by Foxp3. Percent purity = (% $Foxp3^+$ cells / %positive marker cells) x 100. Average line is shown as horizontal dotted line. $^{\#}p > 0.01$ *vs.* $CD25^{hi}$, $^{*}p < 0.01$ *vs.* $CD25^{hi}$; $^{\circ}p < 0.01$ *vs.* $CD25^{int}$; $^{\square}p < 0.01$ *vs.* $CD127^{lo}$.

CD25 ^{hi} had a higher purity than CD25^{int} and CD25^{lo} (Fig. 7). Overall, CD25^{hi} gave the highest purity. Bonelli *et al*.[4] also found that the percentage of Foxp3^{+} cells is highest in the CD25^{hi} population. The percentage of Foxp3^{+} cells in the CD25^{hi} population can range from 78%[4] to 84%.[16] The combination of CD25^{hi} and CD127^{lo} also gave a high sensitivity but had a wide range of the standard deviation. Thus, CD25^{hi} with CD127^{lo} is not preferred to CD25^{hi} alone in isolating Foxp3^{+} cells.

In conclusion, we found that the numbers of Treg in normal Thai subjects using three markers, *i.e.* Foxp3, CD25, and CD127 are similar to other non-Caucasian and Caucasian populations. CD25 and CD127 markers could not be used as representatives of Foxp3. Cell permeabilization decreases cell surface staining and therefore CD127 staining. CD127 is not a useful marker to isolate Foxp3 cells because it has a poor specificity. CD25^{hi} appears to be the marker that would lead to the highest population of Treg for Treg separation.

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