Distribution of CD38 Molecules on CD3+ and CD8+ T- lymphocyte in Adulthood HIV-1-uninfected Thais

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The expression of CD38 on CD8+ T-lymphocyte is a significant predictive value in disease progression of HIV infected individuals and in monitoring a response to therapy. CD38 molecules expressing on CD3+ and CD8+ T-cells were measured quantitatively by flow cytometry in 30 healthy Thai adults. In each experiment, the known amount of fluorochrome in CD38 antibodies bound per cell of QuantiBRITE PE beads was plotted, and set a regression line. With this line, the amount of CD38 molecules bound to CD3 and CD8 target cells was estimated. The aim of this study was to determine the reference value of CD38 molecules on CD8+ T-lymphocyte, which is the baseline in comparison to the CD38 molecule expressing on CD8+ T-lymphocyte in HIV-infected individuals. The present results showed that the amount of CD38 expressions on CD8+ T-lymphocyte in HIV negative Thai adults was about 2 times higher than those from Caucasian's lymphocyte. The reference range of CD38 molecules in the present study would best be used as baseline in prognosis and drug monitoring of HIV-1 infection in Thailand.

CD38 antigen expression on CD8^{+} T-lymphocyte is a marker of lymphocyte activation and is predictive of rapid HIV-1 disease progression[1]. CD38 is a broadly distributed marker molecule that not only manifests on mature T or B-cell but also appears on bone marrow precursors, thymocytes, plasma cells, NK cells and in vitro-activated T and B cells. The molecular weight of CD38 was 45 to 46 kDa, and does not display homology with any other known cell surface molecule but appears to transduce activation signals[2]. These molecules may function in a novel cellular signaling pathway, which may play a role in cellular maturation and activation[3]. CD38 molecules represent a continuum of expression whose precise function is largely unknown. In HIV-1 infection, CD38 has so far been studied mainly from a prognostic perspective. Its expression is high on peripheral blood lymphocytes in primary infection, decreases during transition to asymptomatic phase, and then increases during progression to AIDS [4]. High expression on peripheral blood CD8^{+} T-cell is a negative prognosis factor and is decreased by successful HAART (highly active antiretroviral therapy) [5]. Measurements of these molecules on CD8^{+} T lymphocyte by using relative fluorescence intensity (RFI), reflect the biological variation

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more appropriate than percent positive cell measurements[6].

Several methods have been reported for quantitation of antigen expression such as quantum simply cellular (QSC) or quantitative indirect immuno-fluorescence (QIF) used on flow cytometer or using radioisotope. The development of standard beads with a known amounts of fluorchrome has provided an alternative means of quantitating surface antigen expression. Calibrated bead standard with a known number of PE molecules per bead provides fluorescence intensity measurements in terms of mean of quantifying PE-stained lymphocyte with a flow cytometer. The bead standards are run under the same instrument settings as the patient samples. A regression line is generated that is used to estimate the antibodies bound per cell (ABC) of monoclonal antibody of interest[1]. Calibrated bead standards are used to determine either the antibody-binding capacity or the molecules of equivalent soluble fluorochrome of stained cell by flow cytometer. Using cursor set on a cell stained with an isotype control monoclonal antibody, the number of CD38 antibodies bound per $CD8^+$ T lymphocyte is the product of the median CD38 RFI and the RFI multiplier[1].

A standard quantitative methodology for defining levels of marker expression is of importance when cell population express heterogeneous levels of antigen characteristics. It has also been shown that CD38 expression on $CD8^+$ T lymphocyte is a strong prognostic marker for development of acquired immunodeficiency syndrome (AIDS) and a significant predictor of disease progression in HIV-infected individual[5,7-9]. Although the biological reasons for association between elevated expression of CD38 and rapid progression of AIDS remain unknown, measurements of CD38 expression provide an additional tool for staging human immunodeficiency virus-1 disease, and might be important in monitoring a response to therapy[10]. However, most of the reference ranges of this marker have been described in Caucasians. Although there are differences in both genetic and environmental factors, no published data in reference ranges of CD38 in Thai people has been reported. Therefore, the purpose of the present study was to determine the amount of CD38 measurement as a reference value for Thais and Southeast Asian persons. The reference value of CD38 in the present study might be useful in the clinical management of HIV-1 infection in Thailand.

Material and Method
Subjects

A total of 30 healthy adults enrolled for check ups at Princess Mahachakri Sirindhorn Hospital in Nakhon Nayok province. Medical examination had been performed before the time of blood collection. Random selected specimens from a set of 300 subjects, all subjects meeting study criteria, including all parameters of CBC are in normal ranges. Serological study of Hepatitis B virus (HBV) profile had been performed. Acute, chronic HBV or HIV-1 infection had been ruled out. The ages and sex within two groups, male and female, have been matched. The age range in the present study was 18-60 years but 14 subjects (nearly 50%) were 20 years old (min-max = 18-60). The present study was approved by the institutioní's ethics committee and informed consent was obtained from each subject. Blood samples were collected into EDTA vacutainer tubes and transported to the laboratory within 12 hours. Complete blood count, absolute leukocyte number and percentage of lymphocyte were obtained using a Coulter Counter T540 (Coulter Electronics, Hialeah, FL).

Monoclonal Antibodies

All monoclonal antibodies in the present study were purchased from Becton Dickinson Immunocytometry systems (BDIS; SanJose, CA). Three-color immunofluorescence antibodies were conjugated to Fluorescein isothiocyanate (FITC), Phycoerythrin (PE) or Peridinin chlorophyll protein (PerCP). In the present study, PerCP conjugated CD3 in combination with FITC conjugated CD8 and PE-conjugated CD38 were used to measure the amount of CD38 molecules. In identifying and separating T- lymphocyte subpopulations, three color combination of monoclonals to CD3 (FITC)/CD4 (PE)/ CD8 (Per CP) were used. To establish and verify positive cell measurement, cursor sets on cells stained with an isotype control ($IgG_1FITC/IgG_{2a}PE$) for marker position were used.

Sample Preparation

EDTA bloods were stained, lysed and fixed within 18 hours according to manufacturerí's protocol. Briefly, 100 microliters of blood sample was incubated with fluorochrome-conjugated monoclonal antibodies for 15 minutes at room temperature in the dark. Then FACS lysing was added and further incubated for 10 minutes at room temperature in the dark. After the incubation period, the specimen

was centrifuged (at 300g 5 minutes) and supernate was discharged. The pellets were washed with phosphate buffer saline (PBS) (pH7.2), fixed with 0.5 ml of 1% paraformaldehyde and analyzed on a FACS Calibur with Cellquest software (BDIS).

Quantitative flow Cytometer Analysis of CD 38:

QuantiBRITE system beads were reconstituted in phosphate buffer saline with azide. These beads were run on the flow cytometer by using the same instrument setting as routine calibrates with Calibrite 3. QuantiBRITE beads which contained four levels of fluorescent intensity were performed and FL_2 axes in terms of PE molecule were assayed to provide geomean of four bead populations in histogram analysis (Fig. 1). QuantiBRITE 1:1 PE: mAb reagents ensure that PE molecule per cell translate into antibody molecule per cell. The geomeans of CD38 expression were keyed in QuantiQuest Software and it will automatically be plotted as

QuantiBRITE PE beads versus relative fluorescence. The linear regression obtained using the Quantibrite PE bead is used to convert the FL2 linear fluorescence staining of cell population into the number of PE molecules bound per cell (ABC). By this program, CD38 molecules were calculated in terms of antibodies bound per cell (ABC) (Table 1). In analysis of CD38 data, using quadrants defined by a non-antigen specific binding, yielded results those were either positive or negative for cell surface expression of the relevant antigen. In each experiment, these beads were run as first tube and sample tubes that stained with CD8-FITC/CD38-PE/CD3-PerCP were run in parallel. Dot plots for representative subjects from normal group qualitatively illustrate the expression of CD38 on CD8^{+} T-lymphocyte (Fig. 2). The measurements of these molecules were obtained on the CD3^{+} and CD8^{+} T lymphocyte of HIV-1 seronegative in terms of relative fluorescence intensity and then converted to the number of CD38-ABC.

Histogram Statistics

File: Quantibrite 5.001
Sample ID: Q5-29OCT04
Tube:
Acquisition Date: 29-Oct-04
Gated Events: 9278
X Parameter: FL2-H (Log)

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: G1
Total Events: 10000

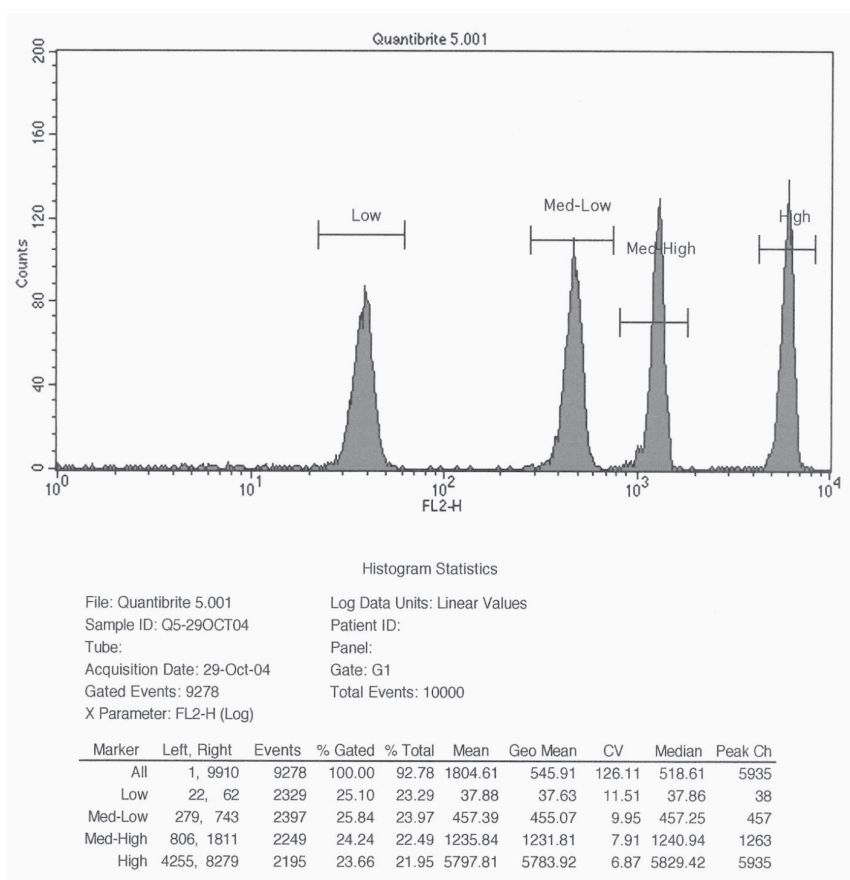| Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	9278	100.00	92.78	1804.61	545.91	126.11	518.61	5935
Low	22, 62	2329	25.10	23.29	37.88	37.63	11.51	37.86	38
Med-Low	279, 743	2397	25.84	23.97	457.39	455.07	9.95	457.25	457
Med-High	806, 1811	2249	24.24	22.49	1235.84	1231.81	7.91	1240.94	1263
High	4255, 8279	2195	23.66	21.95	5797.81	5783.92	6.87	5829.42	5935

Fig. 1 Histogram plot of QuantiBRITE PE beads on a FACScalibur flow cytometer set up with CaliBRITE calibration beads for lyse/no wash procedure

Statistical Analysis

Data were analyzed using SPSS (v.11) software. Descriptive statistics (range, mean ± SD) used to describe demographic and laboratory

Table 1. The number of PE molecules per bead from package insert and geometric mean of bead peaks in linear fluorescence was entered to plot a linear regression line

PE molecules per bead from package insert	Geometric means of bead peaks (linear fluorescence)
1400	37.9
14000	457.3
36600	1240.9
182000	5829.4

characteristics. The distribution of CD38 markers was compared between $CD3^+$ and $CD8^+$ T-lymphocyte using the nonparametric Wilcoxonís signed rank test. P values < 0.05 were consi-dered significant.

Results

The percentages and absolute numbers of T-lympho-cyte subpopulation

Cell surface markers of T- lymphocyte were analyzed on 10,000 lymphocytes in gate. By three-color immunophenotypic data in HIV seronegative subjects, the mean percentage of $CD3^+$, $CD4^+$ and $CD8^+$ T-lymphocytes were 60.33, 34.39 and 24.06 respectively. For absolute number of T-lymphocyte subpopulations, the means of absolute number of $CD3^+$, $CD4^+$ and $CD8^+$ T-lymphocyte were 1289.69,
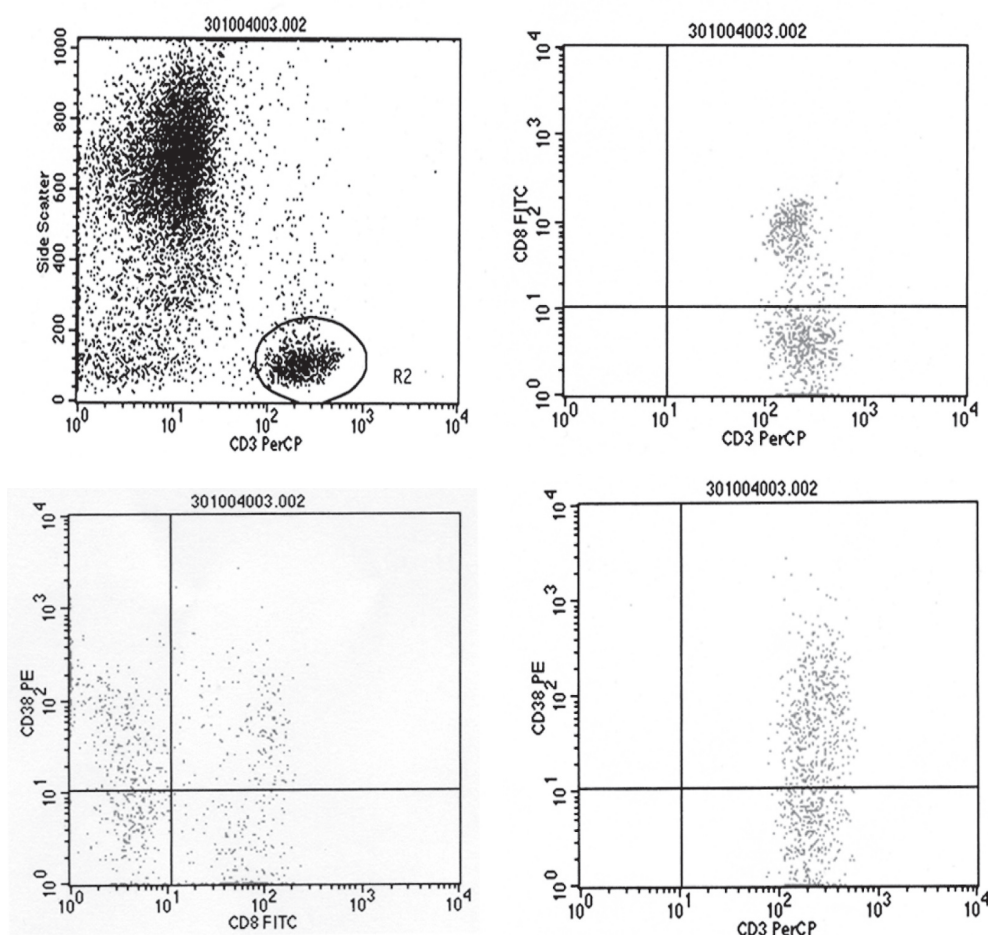
Fig. 2 Dot Plots of dual-color immunofluorescence staining for isotype control tube, a seronegative control. The cursor on the isotype control showed the position used to analyze CD38 antigen expression on $CD8^+$ T cell. Data from three-color CD8/CD38 /CD3 staining show CD 38 expression on $CD4^+$ and $CD8^+$ T-lymphocyte

745.63 and 507.59 cells/mm³ respectively. The percentages, absolute number of T-lymphocyte subpopulations and demographic data of subjects are summarized in Table 2.

The percentages and absolute numbers of CD3⁺ and CD8⁺ T-lymphocyte that express CD38 antigen

In the present study, the mean percentages of CD3⁺ and CD8⁺ T-lymphocyte that express CD38 were 22.17% (range from 9-44) and 7.49% (range from 3-19) respectively. The mean of absolute numbers of CD3⁺ and CD8⁺ T-lymphocyte that express CD38 were 486.79 cells/mm³ (range from 191.48-1147.52) and 159.74 cells/mm³ (range from 49.95-406.72) respectively as shown in Table 2.

The amount of CD38 molecule expressing on CD3⁺ and CD8⁺ T lymphocyte

In the present study, both mean $\pm$ SD and Median (interquartile) were reported in order to compare with other studies. CD38 molecules expressing on CD8⁺ T-lymphocyte from HIV- negative Thais ranged from 1453-3453 molecules/cell (mean $\pm$ SD = 1953 $\pm$ 452.44). For CD3⁺ T-lymphocyte, expression of CD38 molecules were ranged from 1621-3362 molecules/cell (mean $\pm$ SD = 2280 $\pm$ 479.39). These figures indicated that expressions of CD38 on these cells were highly variable among HIV-negative adults. The difference in expression of these molecules on CD3⁺ and CD8⁺ T-lymphocytes

was significant with $p < 0.001$. For non-parametric analysis, medians (interquartile ranges) of CD38 expressing on CD8⁺ and CD3⁺ T-lymphocytes were 1807.0 (1650) and 2156 (1981), respectively as shown in Table 2.

Discussion

The present study showed that the percentages of CD3⁺, CD4⁺ and CD8⁺ T-lymphocytes are in agreement with previous studies from both Thais and Caucasian populations[11-13]. Despite showing similar percentages of lymphocyte sub-population, the absolute numbers of CD3⁺, CD4⁺, and CD8⁺ T-lymphocytes are slightly different. This source of variation might be due to the method in processing differential white blood cells by various automated cell counts.

Characterization of lymphocyte subpopulation in healthy Thais is becoming more important especially with the increasing burden of the HIV epidemic in Thailand where about 600,000 persons are estimated to live with this infection[14]. Knowledge of the immune status of healthy Thai adults could, thus, contribute to the clinical management of HIV-infected individuals.

In analyzing the immune activation marker, CD38 expression could provide an additional tool for staging of HIV-disease progression even after adjustment for the level of CD4⁺ T lymphocytes[15]. This activation marker can be used as a guide in the

Table 2. Demographic and laboratory characteristics of study population (30 healthy adults)

Charateristics	Mean $\pm$ SD	Range
Age	28$\pm$6	18-60 years
Gender ratio	male:female	1:1
Percentage of CD3⁺ T-cells	60.33$\pm$5.52$^\alpha$	49-73
Absolute number of CD3⁺ T-cells	1289.69$\pm$338.46$^\beta$	634.41-2138.40
Percentage of CD4⁺ T-cells	34.39$\pm$3.24	30-40.3
Absolute number of CD4⁺ T-cells	745.63$\pm$207.44	363.63-1166.4
Percentage of CD8⁺ T-cells	24.06$\pm$5.95	14-37
Absolute number of CD8⁺ T-cells	507.59$\pm$174.95	267.12-1135.44
Percentage of CD3⁺ T-cells that express CD 38 expression	22.17$\pm$7.55	9-44
Absolute number of CD3⁺ T-cells that expressed CD 38 expression	486.79$\pm$236.64	191.48-1147.52
CD38 molecule expressing on CD3⁺ T-cells	2280.40$\pm$479.39	1621-3362
Percentage of CD8⁺ T-cells that express CD 38 expression	7.49$\pm$3.97	3-19
Absolute number of CD8⁺ T-cells that expressed CD 38 expression	159.74$\pm$96.54	49.95-406.72
CD38 molecule expressing on CD8⁺ T-cells	1953.47$\pm$452.44	1453-3453

Expressed as cells/mm³
$^\alpha$ Expressed as the percentage of CD3⁺, CD4⁺, CD8⁺ T- lymphocyte; value are mean $\pm$ SD
$^\beta$ Expressed as the absolute number of CD3⁺, CD4⁺, CD8⁺ T- lymphocyte; value are mean $\pm$ SD

initiation of prophylactic treatment of opportunistic infections and in monitoring responses to antiviral therapy. In this sense, CD38 antigen is being intensively investigated and numerous data have been obtained from flow cytometry. By translating the mean fluorescent intensity (MFI) into antibody binding per cell (ABC), as described above, the figures were amplified and resulted in greater sensitivity for estimation of CD38 molecules. However, many effects have been shown to influence the results of quantitative CD38 on flow analysis. The range of CD38 expression may vary from laboratory to laboratory based on the techniques of measurements. The periodic variation of the quantitation standard bead that used to run with each assay and the requiring careful validation of instrument performance were included.

Results presented elsewhere have established the reference ranges of CD 38 antigen in French[16] and Spanish populations[17]. There is a remarkable difference in CD38 expression on CD8+ T-lymphocyte between two adult groups. The present results proved consistent with a previous report from a Spanish population[17]. The mean levels of CD38 molecules on CD8+ T-lymphocyte in Thais and Spanish are very similar, 1953 and 1978 respectively. However, earlier reported by Biocytex, CD38 molecules on CD8+ T-lymphocyte ranged from 300-1800 (mean = 800). CD38 expression on CD8+ T-lymphocyte in the present study was approximately two fold higher than those from a French study. The amount of CD38 molecules expression on CD8+ T-lymphocyte from French, Spanish and Thai are shown in Table 3.

In order to explain the discrepancy, regarding CD38 expression, one of the possible factors is the age range of the presented subjects. Despite the wide age range (18-60 years), nearly 50% of them were young adults (20 years old). Tsegaye et al reported that T-cell expressed the CD38 antigen eventually declines with age[18]. For the other factors, immune alterations in the Thai population might be environ-mentally driven. The environmental factors seemed to be mediated by food, pollution and tropical diseases. However, all subjects looked healthy and medical examination had been performed before the time of blood collection. Hepatitis B virus and HIV-1 infection had been rule out. Nevertheless, the involvement of a genetic factor is not excluded.

To the authorsí knowledge this is the first publication in describing the expression of CD38 on CD8+ T-lymphocyte in Thais. Compared to plasma HIV RNA levels, this prognostic marker for staging, progression or antiretroviral management of HIV is cheaper and easier to perform[7].

In conclusion, the presented data suggested that CD38 expression on CD8+ T-lymphocyte is highly variable among different genetic populations. In Thai adults, the number of CD38 molecules was expressed in higher concentrations than those in French and Spanish studies. The level of expression might be affected by both genetics and the environment. In this regard, the authors emphasize the necessity for close matching of the control group with the same population group for reference values in routine diagnosis or research.

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Table 3. Comparison CD38 molecules expressing on CD8+ T-lymphocyte in French, Spanish and Thais

	Mean (min-max)	Median (interquartile range)
French (n = 50)	800 (300-1800)	-
Spanish (n = 30)	-	1978 (1358)
Thais (n = 30) (Our study)	1953 (1448-3453)	1807 (1650)

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ปริมาณ CD38 บนผิวของ ที-ลิมโฟซัยท์ ชนิด CD8+ และ CD3+ ในคนไทยวัยผู้ใหญ่ที่ไม่ติดเชื้อ HIV

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ปริมาณ CD38 ที่อยู่บนผิวของ ที-ลิมโฟซัยท์ ชนิด CD8+ มีความสำคัญทั้งต่อการพยากรณ์ และการติดตามผลการรักษาโรคในคนที่ติดเชื้อ Human Immunodeficiency Virus (HIV) การวัดจำนวนของ CD38 บนผิวเซลล์โดยใช้ flow cytometry ได้ทำการตรวจวัดในคนไทยทั้งหมด 30 คน ในการทดสอบแต่ละครั้ง มีการสร้างสมการการถดถอย โดยใช้ QuantiBRITE PE beads ที่ทราบจำนวนโมเลกุลของ CD38 หลาย ๆ ระดับ จากสมการดังกล่าวทำให้สามารถคำนวณหาจำนวน CD38 บนผิวเซลล์ได้ วัตถุประสงค์ของการศึกษานี้ คือ การหาค่าอ้างอิงของจำนวน CD38 บนผิวเซลล์ ชนิด ที-ลิมโฟซัยท์ ในคนทั่วไปในภาวะที่ไม่ติดเชื้อ HIV เพื่อใช้เปรียบเทียบกับภาวะที่มีการติดเชื้อ การศึกษานี้พบว่าปริมาณของ CD38 บนผิวของที-ลิมโฟซัยท์ ในคนไทยมีมากกว่าชนชาติอื่นที่เคยมีรายงานไว้ประมาณ 2 เท่า ดังนั้น ค่าจำนวนโมเลกุลของ CD38 ที่ได้จากการศึกษานี้ จึงมีความเหมาะสมในการใช้เป็นค่าอ้างอิง สำหรับการพยากรณ์และติดตามการรักษาโรคติดเชื้อ HIV ในคนไทย