Discordant CD38 measurement of CD8+ T lymphocytes using fluorescein conjugates in comparison with phycoerythrin conjugates

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Summary

Background: We have previously shown that monitoring of CD38 expression can be used as a marker for antiretroviral drug efficacy in HIV infected patients. However, the detection of CD38 expression may be affected by the sensitivity of the fluorochrome conjugated reagent.

Objective: In this study, we determined the level of CD38 expression using PE and FITC conjugated anti-CD38 monoclonal antibodies in different groups of HIV infected patients.

Methods: The frequency and mean fluorescence intensity of CD38 expression using PE and FITC conjugated anti-CD38 monoclonal antibodies were detected by flow cytometry either alone or in combination with HLA-DR. A correlation between CD38 expression and CD4 count, the percentage of CD4 or viral load in antiretroviral drug naïve HIV infected patients was performed. The results were compared with those for antiretroviral treated HIV infected patients who responsed to therapy and patients with virological failure.

Results: We found that while both reagents had the ability to detect a high frequency of CD38 expressing cells in untreated patients, only PE conjugated reagent provided correlation with markers for disease progression. More importantly, FITC conjugated reagent cannot monitor the increase in CD38 expression in patients who showed virological failure.

Conclusions: The results from this study suggest that a cautious selection of fluorochrome conjugated reagents and a method for utilizing the data are extremely critical in the use of CD38 expression as a monitoring tool for ART efficacy. (*Asian Pac J Allergy Immunol 2011;29:181-9*)

Key words: CD38, fluorescein, HIV, monoclonal antibody, phycoerythrin

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Abbreviations:

CD	=	Cluster of differentiation
HIV	=	Human immunodeficiency virus
FITC	=	Fluorescein isothiocyanate
PE	=	Phycoerythrin
HLA-DR	=	Human leukocyte antigen DR
ART	=	Anti-retroviral therapy
FACS	=	Fluorescence activated cell sorter
BDB	=	Becton Dickinson biosciences
PBS	=	Phosphate buffer saline
AIDS	=	Acquired immune deficiency syndrome
HAART	=	Highly active anti-retroviral therapy
EDTA	=	Ethylene diamine tetraacetic acid
CBC	=	Complete blood count
mAbs	=	Monoclonal antibodies
PerCP	=	Peridinin chlorophyll protein
APC	=	Allophycocyanin
MFI	=	Mean fluorescence intensity
PCR	=	Polymerase chain reaction
B-CLL	=	B-cell chronic lymphocytic leukemia
ABC	=	Antibody binding capacity
APC-Cy7	=	Allophycocyanin-cyanin 7
IL-4	=	Interleukin-4
IL-6	=	Interleukin-6

Introduction

HIV infection is one of the major health problems in Thailand with an estimated 1 million Thais infected by HIV and more than 450,000 who have died from HIV/AIDS.[1] Long-term treatment with antiretroviral therapy in these HIV infected patients was required in order to prevent disease progression. With this enormous increase in the number of antiretroviral drug treated patient, a specialized antiretroviral drug monitoring laboratory is essential. The availability at affordable cost of laboratory monitoring of antiretroviral therapy is important for therapeutic management. Among many laboratory tests, CD4 cell counts are the most important parameter for monitoring antiretroviral drug treatment outcomes. We have evaluated a simple protocol using generic monoclonal antibodies for CD4 cell counting which can reduce the cost from 10-20 US$ to less than 2 US$.[2] However, treatment failure as determined by CD4 cell counts will be detected relatively late, since antiretroviral drug treatment is initiated in patients with late stage disease. Thus, more sensitive parameters are needed to monitor the effects of therapy. Viral load is the most commonly used, but its relatively high cost makes monitoring of every patient impractical.

In addition to viral load, many studies have investigated the possibility of using CD38 expression as a therapeutic monitoring marker.[3-10] CD38 was initially considered to be an activation antigen which usually increases in amount on the cell surface after cell activation. An increased expression of the CD38 molecule on peripheral blood CD8 + T cells from HIV-1 infected individuals was first reported by Giorgi et al in 1989.[11] Later, the level of CD38+ CD8+ T cells was reported as a strong predictor of HIV disease progression.[12-15] Many studies have shown a good correlation between CD38 expression on CD8+ T cells and the level of plasma viral load.[16-18] In addition, patients who received highly active antiretroviral therapy (HAART) showed a rapid decline of CD38 expression level in parallel with plasma viral load level[19], indicating a close relationship between CD38 expression and viral replication. We have shown that CD38 expression can be used as a therapeutic monitoring marker for the efficacy of antiretroviral therapy in HIV infected Thai patients.[20] However, we emphasized the need for caution in 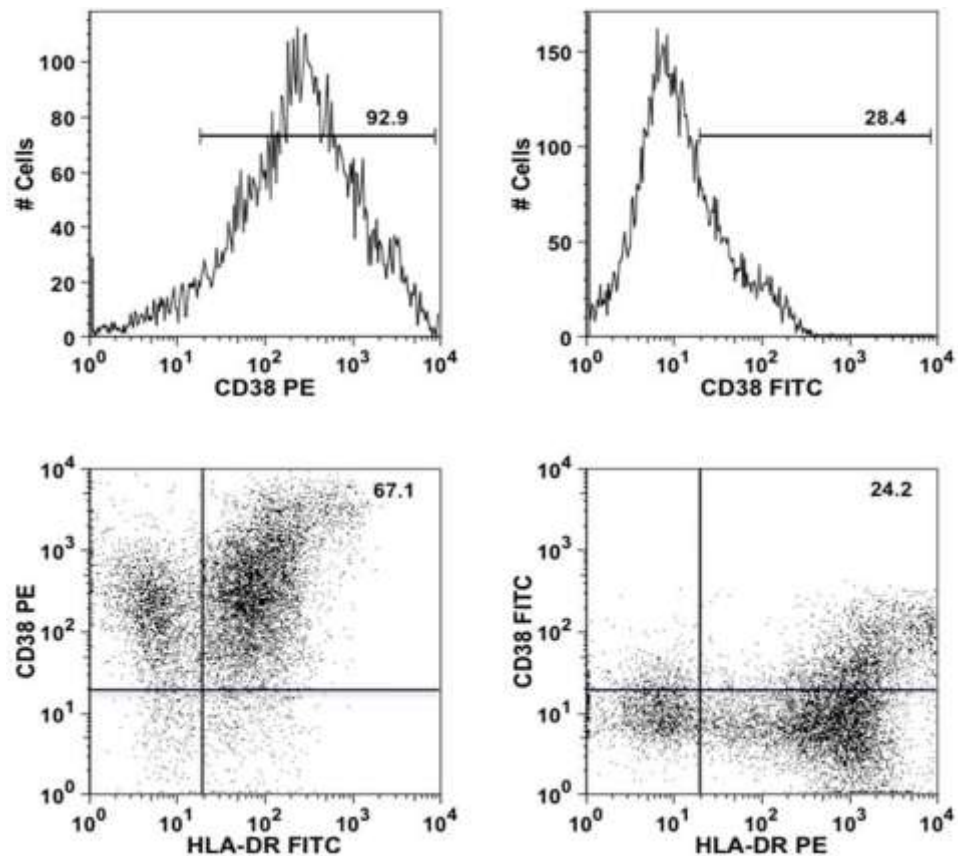the analysis of data on the expression of CD38, which should take into account the fluorochrome being utilized in the reported studies.

In this study, we investigated the level of CD38 expression utilized by fluorescein and phycoerythrin in HIV infected patients. The level of CD38 expression was compared between groups of patients and evaluated for the association with CD4 count and HIV plasma viral load.

Methods

Study population and sample collection

Peripheral blood samples were obtained from HIV infected patients who were recruited from the HIV Clinic, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. Appropriate informed consent was obtained prior to their enrollment in the study. The study was approved by the Institution Review Board of the Faculty of Medicine Siriraj Hospital. The patients were divided into 3 groups; the first group (n = 20) consisted of patients who did not receive any antiretroviral chemotherapy prior to or during the course of this study. The second group (n = 20) consisted of HIV infected patients who had been on antiretroviral therapy (ART) for at least 6 months and showed significant response to therapy and the third group (n = 5) consisted of HIV infected patients who had been on ART but had evidence of virological failure (viral load >50 copies/ml). Blood samples were collected in EDTA containing vacutainer tubes. Routine CBC based absolute lymphocyte counts and CD4+ T cell subset analysis by flow cytometry were carried out on aliquots of a blood sample from each volunteer. The values for CD4+ T cell subsets and viral load are shown in Table 1. Plasma was also collected by centrifugation of an aliquot of each blood sample and kept at –70 °C and used in batches for viral load determination.

Table 1. Values for CD4+ T cell subsets and viral load

GROUP	Absolute CD4 Count (Cells/μl)	%CD4	Viral load (copies/ml)
Untreated group (n=20)	381 ± 415	17.09 ± 11.78	74446 ± 78701
Successfully ART treated group (n=20)	337 ± 227	17.13 ± 8.84	< 50
Virological failure group (n=5)	212 ± 263	10.47 ± 10.43	142215 ± 179808

NOTE: Data were shown as mean ± SD.

Figure 1. Representative profile comparing CD38 expression using PE and FITC conjugated anti-CD38 monoclonal antibody in CD8+ T cells from untreated HIV infected subjects. Flow cytometry histograms and two-color dot plot are shown. The percentage of CD38 expressing cells are indicated by the bar of the histograms whereas the percentage of CD38 + HLA-DR+ co-expressing cells is listed in the upper right quadrant.

Monoclonal antibodies and reagents

The following anti-human monoclonal antibodies (mAbs) and their conjugated fluorochromes were commercially obtained from Becton Dickinson Biosciences (BDB: San Jose, CA) and utilized at the concentration recommended by the manufacturer: anti-human HLA-DR, clone L243, conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE); anti-human CD38, clone HB7, conjugated with FITC or PE; Anti-human CD8, clone SK1, conjugated with peridinin chlorophyll protein (PerCP); Anti-human CD4, clone SK3, conjugated with allophycocyanin (APC).

Immunofluorescent staining

The whole blood procedure was utilized for flow cytometric studies. Aliquots of 50 μl of EDTA blood were incubated with the following combination of mAbs: HLA-DR-FITC/ CD38-PE/ CD8-PerCP/ CD4-APC and CD38-FITC/ HLA-DR-PE/ CD8-PerCP/ CD4-APC. After the addition of the monoclonal antibody reagents, samples were gently mixed and incubated in the dark at 25°C for 15 minutes followed by the addition of 1 ml of FACS lysing solution (BDB) in order to lyse the red blood cells. The stained samples were gently mixed again and incubated in the dark at 25°C, for 10 minutes. Following centrifugation at 1,200 rpm for 5 minutes at room temperature, the supernatant was discarded and the cell pellet was re-suspended in 2 ml of wash buffer (PBS with 2% fetal bovine serum) followed by centrifugation at 1,200 rpm for 5 min at 25°C. Finally, the cell pellet was re-suspended in 300 μl of freshly prepared 1% paraformaldehyde in PBS pH 7.4 and subjected to flow analysis.

Flow cytometric analysis

Six-parameter analysis was performed on a FACSCaliber flow cytometer (BDB) using Cellquest software (BDB). Cells were gated using

Table 2. Correlation between CD38 expression and absolute CD4 count, percentage of CD4 and viral load in untreated HIV-1 infected patients

CD38 expression	P-value (type of correlation)		
	VS Abs CD4	*VS* % CD4	*VS* viral load
Percent of CD38 PE+ in CD8+ T cells	0.0007(-)	0.0034(-)	0.0133(+)
MFI of CD38 PE+ in CD8+ T cells	0.0112(-)	0.0107(-)	0.0137(+)
Percent of CD38 PE+ HLA-DR FITC+ in CD8+ T cells	0.0057(-)	0.0165(-)	NS
Percent of CD38 PE+ in CD4+ T cells	NS	NS	NS
MFI of CD38 PE+ in CD4+ T cells	NS	NS	NS
Percent of CD38 PE+ HLA-DR FITC+ in CD4+ T cells	<0.0001(-)	0.0007(-)	0.0054(+)
Percent of CD38 FITC+ in CD8+ T cells	NS	NS	NS
MFI of CD38 FITC+ in CD8+ T cells	NS	NS	NS
Percent of CD38 FITC+ HLA-DR PE+ in CD8+ T cells	0.0335(-)	NS	NS
Percent of CD38 FITC+ in CD4+ T cells	NS	NS	NS
MFI of CD38 FITC+ in CD4+ T cells	NS	NS	NS
Percent of CD38 FITC+ HLA-DR PE+ in CD4+ T cells	0.0104(-)	0.0040(-)	NS

NOTE: (+), direct correlation; (-), inverse correlation; NS, no significance.

the lymphogate technique for analysis of viable cells and at least 30,000 viable cells were analyzed per sample. CD8+ or CD4+ T cells were identified using a two-dimensional dot plot of CD8 versus CD4. Only the CD8+ bright population was used for analysis since this population was previously identified as cells enriched in those expressing the CD8 alpha/beta heterodimer. The frequency and density (mean fluorescence intensity, MFI) of CD38 expressing subsets were determined by a histogram plot whereas the percentage of the CD38+ HLA-DR+ cells was determined by a two-color dot plot (FITC vs PE). Isotype controls were used to define the positive and negative population.

Viral load measurement

HIV viral loads were determined by the automated Amplicor polymerase chain reaction (PCR) (CoBas Amplicor HIV-1 monitor test version 1.5; Roche Diagnostic). The sensitivity of detection by this assay was determined to be 400 HIV copies/ml of plasma utilizing the standard assay and 50 HIV copies/ml of plasma utilizing the ultrasensitive assay.

Statistical analysis

Data for each assay are presented as the mean ± S.D. of samples. The Mann-Whitney U test was used to determine the statistical significance of the differences observed between groups. The Pearson correlation coefficient test was used to analyze the association observed between different parameters. P values of < 0.05 were considered significant.

Results

Comparison of CD38 expression in antiretroviral drug naive HIV infected patients

Our previously published report had shown a significantly higher frequency of CD38 expression in both CD8+ and CD4+ T cells from healthy subjects when using PE conjugated anti-CD38 mAb as compared with using FITC conjugated mAb.[20] In order to determine the expression level for these two fluorochromes in HIV infected patients, we decided to compare the mean frequency and mean density of CD38 expression using PE and FITC conjugated anti-CD38 mAb in samples from 20 adult HIV infected patients who had no prior history of ART. Figure 1 demonstrates a representative flow cytometric profile which compares CD38 expression assessed by PE and FITC conjugated anti-CD38 mAb from untreated HIV infected patient. As shown in this figure, the frequency of CD38 expressing CD8+ T cells that were detected by PE conjugated anti-CD38 mAb was higher than the value from FITC conjugated anti-CD38 mAb. We found that a very high proportion of both CD8+ T cells (89.8 ± 5.0) and CD4+ T cells expressed CD38 (82.6 ± 10.8)
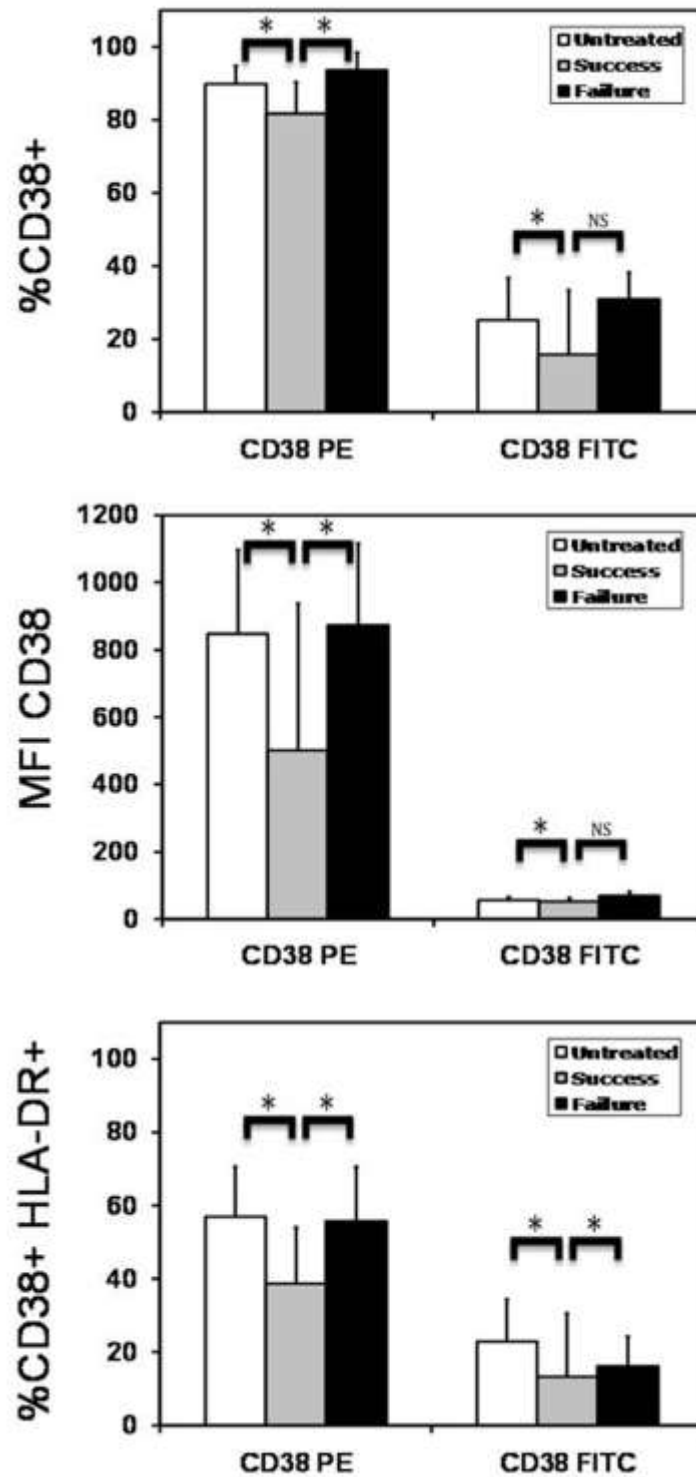
Figure 2. Comparison of CD38 expression using PE and FITC conjugated anti-CD38 monoclonal antibody in three different groups of subjects. Untreated HIV infected patients (open columns), successful antiretroviral treated HIV infected patients (gray columns), antiretroviral treated HIV infected patients who showed virological failure (black columns) are depicted as the average percentage of CD38 expression (a), the average mean fluorescence intensity of CD38 expression (b) and the average percentage of CD38+ HLA-DR+ co-expression (c) in CD8+ T cells using PE or FITC conjugated anti-CD38 monoclonal antibody. * indicates statistically significance whereas NS indicates no significance.

when PE conjugated anti-CD38 mAb was utilized as compared with data obtained utilizing FITC conjugated anti-CD38 mAb which gave values of 25.2 ± 11.6 for CD8+ T cells and 25.2 ± 9.3 for CD4+ T cells. When the densities of CD38 expression were examined, the MFI of CD38 expression using the PE-conjugated mAb was 847.4 ± 324.1 for CD8+ T cells and 1159.1 ± 632.4 for CD4+ T cells as compared with only 57.6 ± 7.2 for CD8+ T cells and 60.3 ± 11.4 for CD4+ T cells when using the FITC conjugated mAb. Furthermore, the frequency of CD8 + T cells that co-expressed CD38 and HLA-DR using PE conjugated anti-CD38 and FITC conjugated anti-HLA-DR mAb was 57.0 ± 13.6, as compared with values of 23.0 ± 11.6 when using FITC conjugated anti-CD38 and PE conjugated anti-HLA-DR mAb. The percentages of CD4+ T cells that co-expressed CD38 and HLA-DR using the same combination of PE and FITC conjugated antibodies were 38.5 ± 27.0 and 11.9 ± 9.9, respectively.

Correlation between CD38 expression and CD4 count, percent CD4 and viral load

To determine whether different data obtained by different fluorochromes might affect the correlation between CD38 expression and a marker of disease progression, a correlation between CD38 expression and CD4 count, percent CD4 and viral load in antiretroviral drug naïve HIV infected patients was performed and this data is shown in Table 2. When the data was obtained using PE conjugated anti-CD38 reagent, there was an inverse correlation between frequency and MFI of CD38 expression on CD8+ T cells and the CD4 count or the percentage of CD4, whereas a direct correlation with plasma HIV viral load was observed. When data on CD38 and HLA-DR co-expression was examined in samples from HIV infected patients, an inverse correlation between the frequency of CD38+ HLA-DR+ on CD8+ T cells and CD4 count or the percentage of CD4+ T cells was observed whereas no correlation with HIV viral load was found. In contrast, such correlations were not apparent when observed between these parameters and CD38 expression on CD4+ T cells or when using the FITC conjugated anti-CD38 reagent, suggesting that correlations with disease progression are only valid with the analysis of CD38 expression on CD8+ T cells and with the use of the PE conjugated anti-CD38 reagent.

CD38 expression in antiretroviral drug treated HIV infected patients

Since no obvious correlation between CD38 expression on CD4+ T cells and markers of disease progression was observed as shown above, only the results from CD8+ T cells are reported herein. In an effort to determine whether there is any difference in using different fluorochromes to detect CD38 expression following ART, we measured CD38 expression in 20 HIV infected patients who showed significant response to ART with stable control of plasma viremia (viral load < 50 copies/ml) after receiving ART and 5 HIV-infected patients who showed virological failure (viral load >50 copies/ml). As shown in Figure 2, a slight but statistically significant decrease in the frequency of CD38 expressing CD8+ T cells was observed in drug treated patients when compared to untreated HIV infected patients. Thus, the frequency of CD8+ T cells expressing CD38 using the PE conjugated anti-CD38 mAb was 81.7 ± 8.8 in the drug treated patients as compared with 89.8 ± 5.0 from the untreated patients ($p = 0.0034$). Similar analyses using FITC conjugated anti-CD38 mAb gave values of 15.8 ± 17.6 as compared with 25.1 ± 11.6 from the untreated patients ($p = 0.0015$). In contrast, an increase in the frequency of CD38 expressing CD8+ T cells, that was observed in patients with failure of ART as compared to patients with successful ART, are only valid with the use of the PE conjugated anti-CD38 reagent. Thus, the frequency of CD8+ T cells expressing CD38 using PE and FITC conjugated antibodies gave values of 93.7 ± 4.8 ($p = 0.0276$) and 31.0 ± 7.3 (non-significant), respectively.

When the MFI of CD38 expression was examined, a marked decrease was observed in drug treated patients when compared to untreated HIV infected patients. Thus, the MFI of CD38 expressing CD8+ T cells using the PE-conjugated mAb was 502.2 ± 435.2 in the drug treated patients as compared with 847.4 ± 324.1 from the untreated patients ($p < 0.0001$). Similar analyses using FITC conjugated anti-CD38 mAb gave values of 51.7 ± 10.2 as compared with 57.6± 7.2 from the untreated patients ($p = 0.0051$). Similar to data obtained for the frequency of CD38 expressing CD8+ T cells, an increase in the MFI of CD38 expressing CD8+ T cells that was observed in patients with failure of ART, as compared to patients with successful ART, are only valid with the use of the PE conjugated anti-CD38 reagent. Thus, the MFI of CD8+ T cells

expressing CD38 using PE and FITC conjugated antibodies gave values of 871.8 ± 244.3 ($p = 0.0043$) and 68.4 ± 13.5 (non-significant), respectively.

Furthermore, a significant decrease in the frequency of CD8+ T cells co-expressing CD38 and HLA-DR was also observed in drug treated patients when compared to untreated HIV infected patients. Thus, the frequency of CD8 + T cells that co-expressed CD38 and HLA-DR using PE conjugated anti-CD38 and FITC conjugated anti-HLA DR mAb was 38.6 ± 15.4 in the drug treated patients as compared with 57.0 ± 13.6 from the untreated patients ($p = 0.0005$). Similar analyses using FITC conjugated anti-CD38 mAb gave values of 13.2 ± 17.4, as compared with 22.9± 11.6 from the untreated patients ($p = 0.0012$). Interestingly, both PE and FITC conjugated antibodies can determine a significant increase in the frequency of CD8+ T cells co-expressing CD38 and HLA-DR that was observed in patients with failure of ART as compared to patients with successful ART, although only a slight increase which was statistically significance was observed when using FITC conjugated anti-CD38 mAb. Thus, PE and FITC conjugated antibodies gave values of 55.9 ± 14.7 ($p = 0.0297$) and 16.1 ± 8.1 ($p = 0.0209$), respectively.

Discussion

The results reported herein show that the level of CD38 expression depends on the sensitivity of the fluorochrome being utilized. Thus, the results of our studies in HIV infected patients showed a significantly higher frequency of CD38 expression in both CD8+ and CD4+ T cells when using PE conjugated anti-CD38 mAb as compared with using FITC conjugated mAb. These results not only suggested a difference in frequency detected by each fluorochrome, but also emphasize the need for caution in the analysis of the correlation between the expression of CD38 and a marker of disease progression. Furthermore, monitoring of ART efficacy was also affected by the use of different fluorochrome conjugated reagent, as demonstrated by an inability of FITC conjugated anti-CD38 reagent to discriminate between patients with failure of ART and patients with successful ART.

The ability to detect different frequencies of CD38 positive cells by FITC and PE conjugated mAbs could be explained by both the sensitivity of flow cytometer and CD38 expression level per cell. Since our previous study showed that CD38 was already expressed at relatively low levels per cell in normal individuals[20], the ability to detect increased frequencies of CD38 positive cells in HIV infected patients depends on the ability to differentiate a small increase of CD38 expression per cell. More importantly, since the sensitivity of flow cytometer depends on the ability to distinguish dimly positive cells from their background, this ability relies on the brightness of the fluorochorome. It is important to note that FITC has a very low relative brightness compare to PE which resulted in lower ability of FITC to distinguish weakly positive cells.

The inability of FITC conjugated mAb to detect specific molecules has been previously observed.[21] In this study, the investigators measured CD20 expression on B-cells from normal samples and from B-cell chronic lymphocytic leukemia (B-CLL) samples which show down-regulation of CD20. By using FITC and PE conjugates from the same mAb to determine the antibody binding capacity (ABC) value, the study showed that similar ABC values were obtained on normal B cells. In contrast, while ABC values can be determined from normal B cells using FITC conjugated mAb, CD20 FITC staining on B-CLL B cells was too weak for accurate quantification. Furthermore, a later study from the same investigators showed discrepancies in measuring CD4 expression on T cells using FITC and PE conjugates mAb.[22] Thus, CD4 expression can be consistently quantified in ABC units using CD4-PE conjugated mAb whereas quantification with CD4-FITC conjugated mAb is not as consistent. These two studies suggested that while FITC and PE conjugated mAb can detect surface molecules with high expression density, such as CD4 molecule, consistency of the results should be a concern when using FITC conjugates. In contrast, detection of molecule with low expression density could be problematic when using FITC conjugates. We suggest that the selection of fluorochrome conjugated reagents is a critical concerned when molecules with low expression density are being studied.

Since the selection of fluorochrome conjugated antibody plays an essential role in the detection of cell surface molecules that express at low density, knowledge of the brightness of a fluorochrome is important in the selection of the right one. Generally, the staining index was used to compare the brightness of the same clone of a monoclonal antibody that conjugated with the different fluorochromes. While FITC gives a low staining index, many fluorochromes have comparable staining index to FITC such as Alexa Fuour® 488

and PerCP. Some fluorochromes, such as APC-Cy7, Alexa Fluor® 700 and Pacific Blue, have staining indices lower than FITC. The selection of these fluorochromes might result in the inablity to detect low density molecules. More importantly, basic knowledge about the expression level of the molecule of interest is essential since many molecules, such as the receptor for cytokine and growth factor are normally present at low density. For example, the receptor for IL-4 and IL-6 were found at a few hundred copies per cell. The brightest fluorochrome should be reserved for cell surface molecules that express at low density.

Currently, the percentage of CD38 positive cells is determined by two methods, one which utilizes a cut-off based on using an isotype control[12] and another which has a cut-off based on an internal negative and positive control, using the expression of CD38 by granulocytes (negative) and monocytes (positive).[19] While the first method is widely used in flow cytometric analyses, the second method was introduced as being a more accurate and reliable method for the monitoring of cells from HIV-1 infected individuals. Thus, while granulocytes have an intermediate level of CD38 expression, monocytes have a relatively higher level of CD38 expression. Using such criteria, only CD8+ T cells with high CD38 expression (based on the density expressed by monocytes) are to be classified as positive. Based on this strategy, we found that all of the CD38 expressing CD8+ T cells in healthy individuals showed CD38 expression level comparable to granulocytes when using PE conjugated mAb and were therefore considered negative. In contrast, overlap between granulocytes and monocytes was observed when using FITC conjugated mAb, which makes determination of CD38 expression more difficult. We submit that while this is a valid approach, for purely scientific reasons, it is important to utilize isotype staining as a negative control, because use of the level expressed by granulocytes as a negative control gives a false impression of the expression of CD38 by hematopoietic cells.

In conclusion, the results obtained in the present study strongly suggest that a careful selection of fluorochrome conjugated reagent and a method for collecting the data are very critical in the use of CD38 expression as a monitoring tool for ART efficacy. However, with a proper control, the analysis of CD38 expression can be use for monitoring of patients receiving ART.

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