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Studies on the potential use of CD38 expression as a marker for the efficacy of anti-retroviral therapy in HIV-1-infected patients in Thailand

Nattawat Onlamoon[a,d], Sutchana Tabprasit[a], Surapol Suwanagool[b], Suda Louisirirotchanakul[c], Aftab A. Ansari[d], Kovit Pattanapanyasat[a,*]

[a]*Center of Excellence for Flow Cytometry, Division of Instruments for Research, Office for Research and Development, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand*
[b]*Department of Preventive and Social Medicine, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand*
[c]*Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand*
[d]*Department of Pathology and Laboratory Medicine, Room 2309 WMB, Emory University School of Medicine, 1639 Pierce Drive, Atlanta, GA 30322, USA*

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Abstract

The monitoring of the efficacy of anti-retroviral therapy (ART) is becoming an important issue in the developing world. The current use of CD4 counts, plasma viral loads, and monitoring of drug-resistant viruses are at present either uninformative or costly. Thus, more new cost-effective and practical techniques need to be established and implemented. Towards this goal, our lab has carried out studies on the potential use of CD38 frequency and density expression by flow analysis as a means to assess the efficacy of ART. Results of our studies using whole blood sample from normal healthy donors indicate that CD38 is expressed by a high frequency of not only CD4+ and CD8+ T cells but also most hematopoietic cell lineages analyzed. Detailed studies of CD38 expression along with other cell surface markers using whole blood sample from HIV-1-infected patients showed that the most discriminating change was the increased frequency and density of CD38 expression by CD3+CD8+ T cells. Of importance was our preliminary finding that a reversal of the increased frequency and density of CD38 expression by CD8+ T cells only appeared in the whole blood sample from patients who were responders to ART but not those who were drug failures. These initial data provide a platform and incentive for larger cohort studies including prospective pre- and post-ART for the institution of such monitoring techniques in resource limited settings.
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Keywords: CD38; HIV; Flow cytometry; Drug monitoring

Introduction

HIV infection continues to be a major public health problem in Thailand with approximately a million individuals that have been infected by the end of 2003 and so far with greater than 450,000 deaths associated with HIV/AIDS (Thai Working Group on HIV/AIDS Projection, 2003). It is also currently estimated that there are >600,000 Thai's living with HIV-1 infection who will require anti-retroviral therapy (ART) at some time point. As elsewhere in the world but particularly so in a developing country such as Thailand, the health care system is under increasing pressure to operate cost-effectively. ART available in the Western countries is too expensive for common Thai citizens which prompted the Government Pharmaceutical Organization of Thailand at the encouragement of the Royal Thai Government to produce generic anti-retroviral drugs. It has been estimated that only 13,000 HIV-1-infected Thai patients with a CD4 count <200 received the generic formulation of stavudine, lamivudine, and nevirapine (termed GPO-Vir) since 2003 and the plans are to make such ART available for an additional 50,000 HIV-1-infected Thai patients by the end of 2005 at a cost of US$1.00 per day. However, even such

* Corresponding author.
E-mail address: grkpy@mahidol.ac.th (K. Pattanapanyasat).

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costs are not trivial for not only Thailand but for other Asian and African countries and dictate a need to identify those patients that do not benefit from such therapy (drug failures) in efforts to maximize the benefit for others who could benefit from such therapy. Thus, a cost-effective and practical assay other than cost-prohibitive viral load and drug-resistant virus monitoring needs to be identified that could minimally be implemented at regional centers throughout Thailand to maximize the limited resources (Pattanapanyasat et al., 2003, 2005).

Flow cytometry is presently being utilized to monitor CD4 counts in all 60 provincial hospitals in Thailand accompanied by a centralized government-mandated quality control program. These thoughts prompted us to investigate the potential use of CD38 as a marker of cell activation which could be utilized in conjunction with CD4 counts for the monitoring of drug therapy in HIV-1-infected Thai patients. The expression of CD38 has been utilized by a number of laboratories as an activation marker (Anthony et al., 2003; Benito et al., 2002; Burgisser et al., 1999; Deeks et al., 2002; Evans et al., 1998; Hunt et al., 2003; Mildvan et al., 2004; Resino et al., 2004) and was first reported by Giorgi and Detels (1989) to be expressed significantly higher on peripheral blood CD8+ T cells from HIV-infected patients. Later, results of studies showed that the frequency of CD8+ CD38+ T cells was a good predictor of HIV-1 induced disease progression (Giorgi et al., 1993, 1999; Liu et al., 1996, 1997) which correlated well with viral load (Bouscarat et al., 1999; Liu et al., 1998; Orendi et al., 1998). The use of CD38 as a predictive marker was further supported by the finding that levels of this molecule were markedly decreased in patients following successful HAART and was closely related to viral load (Tilling et al., 2002). Of relevance to the studies performed herein was the finding that there appeared to be a transient increase in the levels of CD38 expressed in patients on HAART that correlated with levels of residual virus replication in these select HIV-infected patients (Benito et al., 2004) suggesting a direct link between replication of drug-resistant virus and either a failure to decrease CD38 expression and/or a rebound of CD38 expression. These studies prompted us to examine whether quantitation of CD38 expression can be utilized as a surrogate marker for the effectiveness of ART in the Thai population. The results of this preliminary study constitute the basis of this report.

Result

High level of CD38 expression in healthy Thai individuals

In efforts to initiate studies on the potential use of CD38 as a marker for the monitoring of the efficacy of ART in HIV-1 infected patients, a baseline study was first performed to define CD38 expression on 20 normal adult Thai volunteers. Previously published reports had shown low levels of CD38 expression on CD4+ or CD8+ cells on normal healthy individuals with a marked increase in both the frequency of cells and the level (density) of expression of this molecule following in vitro cell activation. We thus decided to first of all compare FITC vs. PE-conjugated anti-CD38 monoclonal antibody (mAb) reagents to determine the frequency and density of CD38 expression by CD4+ and CD8+ T cells in these 20 healthy adult Thai volunteers. All the values reported below for either the frequency or the density of cell surface expression are reported as mean ± SD. Interestingly, we found that a very high frequency of both CD8+ T cells (73.5 ± 8.7) and CD4+ T cells expressed CD38 (74.0 ± 5.8) when PE conjugated anti-CD38 mAb was utilized as compared with data obtained utilizing FITC-conjugated anti-CD38 mAb which gave values of 7.2 ± 3.2 for CD8+ T cells and 18.2 ± 5.5 for CD4+ T cells (Figs. 1a and b). When the densities of CD38 expression were examined for the same data sets, the mean density of CD38 expression using the PE-conjugated mAb was 315.3 ± 106.0 for CD8+ T cells and 530.3 ± 104.9 for CD4+ T cells as compared with only 50.1 ± 7.4 for CD8+ T cells and 45.2 ± 6.3 for CD4+ T cells when using the FITC-conjugated antibody (Figs. 1c and d). These data emphasize caution in the analysis of data on the expression of CD38 which should take into account the fluorochrome being utilized for the reported studies. In addition, while all remaining studies were performed using both FITC- and PE-conjugated anti-CD38 mAb, only the results with the PE-conjugated reagent are reported herein for simplification of data analysis. In efforts to determine the relationship between the expression of CD38 and another independent marker of cell activation,

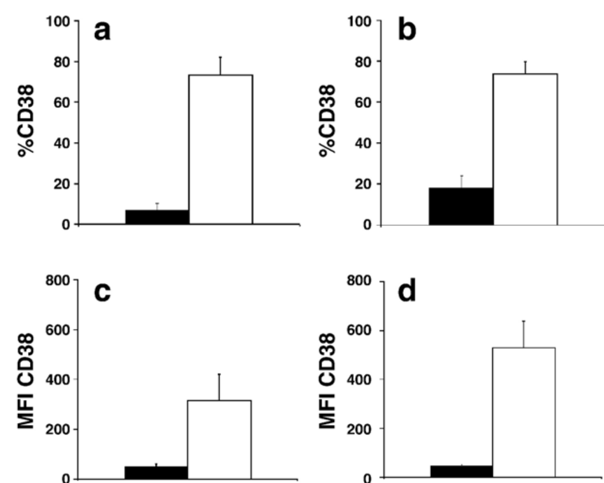

Fig. 1. Comparison of CD38 expression using FITC- and PE-conjugated anti-CD38 monoclonal antibody in healthy adult Thai individuals. CD8+ (a, c) and CD4+ (b, d) T cells were stained with FITC (black column)- or PE (open column)-conjugated anti-CD38 monoclonal antibody. CD38 expression is noted as either the mean frequency (%) of CD38 expressing cells (a, b) or as the mean density (MFI) of CD38 expression (c, d).

we chose to examine the correlation between HLA-DR and CD38 expression by CD4+ and CD8+ T cells. The frequency of CD8+ and CD4+ T cells that co-expressed CD38 and HLA-DR was 14.5 ± 7.0 and 4.4 ± 2.5, respectively. These results suggest that although the frequencies and densities of CD38 expression using the PE-conjugated anti-CD38 antibody on resting CD4+ and CD8+ T cells in the normal Thai population is high, most of these cells do not co-express HLA-DR on their cell surface. This was true even when a PE-conjugated anti-HLA-DR reagent was utilized.

These findings of high levels of CD38 expression by both CD8+ and CD4+ T lymphocytes in normal blood donors prompted us to examine the expression of CD38 on other cell lineages in the same normal donors using the same PE-conjugated anti-CD38 mAb. Studies were therefore carried out to examine the frequencies of CD38 expressing CD19+ B cells, CD56+ NK cells, the TCR gamma–delta+ T cells, and for purposes of control, the total lymphoid cell lineage. As shown in Fig. 2, a high frequency of CD38 expressing cells was observed in all lymphocyte subsets. The highest mean frequency of >90% was observed within the NK cell lineage. These data suggest that the previously published data on the expression of CD38 on lymphoid cell lineages need to be re-evaluated based on the fluorochrome one utilizes for such studies.

CD38 expression in anti-retroviral drug naive HIV-infected patients

In the light of the above findings, we next conducted a series of studies on how the results of the above data would impact on the use of CD38 expression as a marker of HIV disease and, in particular, its application for the monitoring of the efficacy of ART. We first compared the frequency and mean density of expression of CD38 in whole blood samples from 20 adult HIV-infected patients who had no prior history of ART with samples from 31 normal adult Thai volunteers. Fig. 3 demonstrates a representative flow cytometric profile which compares data on CD38 expression by CD8+ T cells from 2 healthy individuals and 2 untreated HIV-infected patients and Table 1 lists the mean values of the CD38 expressing CD4+ and CD8+ T cells from both HIV-infected patients and normal controls. As seen, the frequency of CD8+ T cells expressing CD38 was significantly higher (P = 0.0001) in the 2 HIV-infected patients (88.9 and 92.4 % with a mean of 89.8 ± 5.0 for the 20 patients) than the 2 normal controls (71.4 and 88.2% with a mean of 70.3 ± 11.3 for the 31 normal individuals). Similarly, the frequency of CD4+ T cells expressing CD38 was also somewhat higher giving a mean value of 79.2 ± 11.1 for samples from HIV-1-infected patients (see Table 1) as compared to 71.6 ± 7.8 for the normal controls ($P <$ 0.039). In these same analyses, the mean density of CD38 expression by CD4+ and CD8+ T cells was also determined and found to be significantly increased ($P <$ 0.0001) in each of these subsets from the HIV-1-infected patients as compared to controls (see Table 1).

Although high levels of CD38 expressing cells were found in healthy Thai individuals, most of these cells did not express HLA-DR, as shown above. We thus reasoned that it would be important to examine the relationship between HLA-DR expression on CD38 expressing CD4+ and CD8+ T cells from HIV-infected patients as compared with normal individuals. In contrast to normal control subjects, CD38+ HLA-DR+ co-expressing cells were a dominant phenotype in HIV-infected patients (see Fig. 3). The mean frequency of CD38+ HLA-DR+ co-expressing cells was 57.0 ± 13.6 for CD8+ T cells and 29.4 ± 23.4 for CD4+ T cells as compared with 14.5 ± 7.0 and 4.4 ± 2.5 for CD8+ and CD4+ T cells, respectively, from control subjects (each giving values of $P <$ 0.0001 when compared to controls).

CD38 and HLA-DR co-expression by CD8+ T cells correlates with CD4 count but not viral load

Since the objective of this study was to determine the potential of using CD38 expression as a marker for disease progression, a correlation between CD38 expression, CD4 count, and viral load in anti-retroviral drug naïve HIV-infected patients was performed and the data are shown in Fig. 4. As seen, whereas there is an inverse correlation between frequency and density of CD38 expression on CD8+ T cells and absolute count and/or percentage of CD4+ T cells, there was a direct correlation between frequency and density of CD38 expression and plasma HIV viral copies/ml (see Fig. 4). When data on CD38 and HLA-DR co-expression was examined in samples from HIV-1-infected patients, whereas there was an inverse correlation between the frequency of CD8+ T cells that co-expressed HLA-DR and absolute CD4 count (and/or the percentage of CD4+ T cells), there was no statistically significant correlation between the frequency of CD8+ T cells that co-expressed

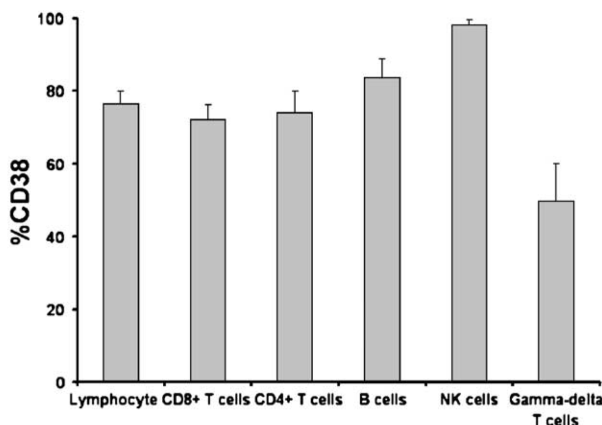

Fig. 2. Expression of CD38 by different lymphoid cell subsets depicted as mean frequency within each cell lineage. A combination of subset-specific monoclonal antibodies with PE-conjugated anti-CD38 monoclonal antibody was used.

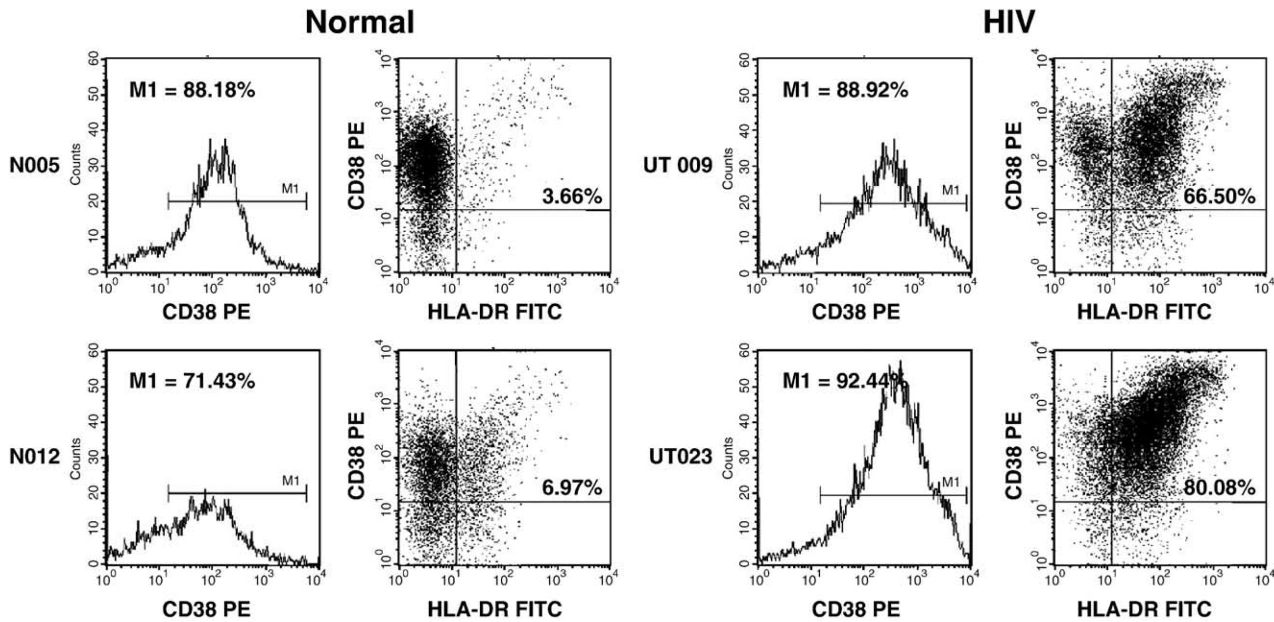

Fig. 3. Representative profile comparing CD38 expression in CD8+ T cells from 2 healthy individuals (Normal) and 2 untreated HIV-infected subjects (HIV). Flow cytometry histograms and two-color dot plot for the 4 samples are shown. The frequency of CD38 expressing cells are indicated by the M1 bar of the histograms whereas the frequency of CD38 + HLA-DR+ co-expressing cells are listed in the upper right quadrant.

CD38 and HLA-DR ($P < 0.1388$) with plasma HIV viral copies/ml (see Fig. 4).

Decreased CD38 expression in anti-retroviral drug-treated HIV-infected patients

In efforts to determine whether CD38 expression can be used as a therapeutic marker following ART, we measured CD38 expression in 23 HIV-infected patients who showed significant response to ART with stable control of plasma viremia (viral load <50 copies/ml) after receiving ART. As shown in Fig. 5a, a slight but statistically significant decrease in the frequency of CD38 expressing CD8+ T cells was observed in treated drug responder patients when compared to untreated HIV-infected patients. Thus, the frequency of T cells expressing CD38 was 79.2 ± 13.0 for CD8+ T cells and 70.8 ± 8.4 for CD4+ T cells in the drug-treated responder patients as compared with 89.8 ± 5.0 for CD8+ T cells ($P = 0.0011$) and 79.2 ± 11.1 for CD4+ T cells ($P < 0.017$) from the untreated patients.

In contrast to the data comparing the frequency of CD8+ and CD4+ T cells that expressed CD38 between treated and untreated HIV-infected patients where statistically significant differences were noted, there was even a greater difference when we compared the mean density of CD38 expression selectively on CD8+ T cells. Thus, as seen in Fig. 5b, the mean density of CD38 expression on CD8+ T cells was 475.3 ± 411.8 for samples from drug-treated responder patients as compared with values of 847.4 ± 324.1 for CD8+ T cells from untreated HIV-1-infected patients ($P < 0.0001$). The density values of CD38 expression by CD4+ T cells from drug-treated as compared to untreated controls were not statistically significant. It is important to note, however, that the values for CD38 expression in the drug-treated responder HIV-infected patients were still higher than seen in normal non-HIV-infected subjects and thus do not return back to normal values at least at the stages that the samples were tested from this cohort of HIV-infected patients.

The next obvious analyses we performed were to determine whether values for the co-expression of HLA-DR with CD38 would provide an even more optimal discriminatory function between patients receiving ART and those without drug therapy. As seen in Fig. 5c, the frequency of CD8+ T cells co-expressing CD38 and HLA-DR was significantly ($P < 0.0001$) decreased in the ART receiving drug responder patients (37.0 ± 15.9) as compared to the non-drug-treated HIV-infected patients (57.0 ± 13.6). The values for CD4+ T cells that co-expressed HLA-DR and CD38 in the drug-treated responder patients (18.0 ± 13.5) were not significantly different from the untreated patients

Table 1
Comparison of the relative frequencies (%) and mean density (MFI) of CD38 expression by CD4+ and CD8+ T cells from HIV-1-infected drug naïve and normal controls

Source of cells (*n*)	Phenotype	CD38 expression	
		%	MFI
Normal (31)	CD4	71.6 ± 7.8	518.9 ± 106.9
HIV-1 (20)	CD4	79.2 ± 11.1#	885.3 ± 2 91.5*
Normal (31)	CD8	70.3 ± 11.3	312.4 ± 94.9
HIV-1 (20)	CD8	89.8 ± 5.0*	847.4 ± 324.1*

Note. *$P < 0.0001$ when compared with normal, #$P < 0.05$ when compared with normal.

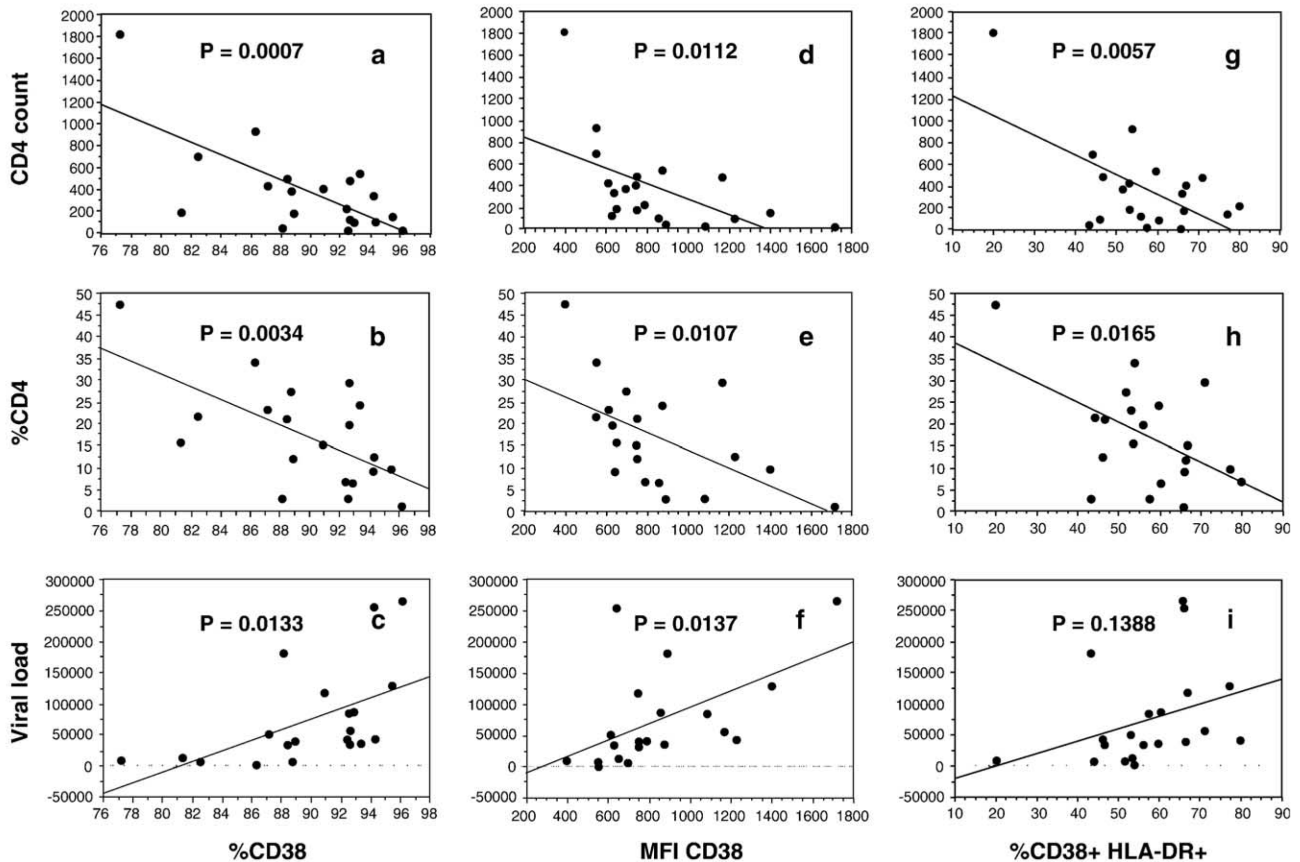

Fig. 4. Linear regression analysis of the correlation between CD38 expression in CD8+ T cell and absolute CD4 count, percent CD4, and viral loads in the group of untreated HIV-infected patients. Correlation between the frequency (%) of CD38 expression (a–c), mean density (MFI) of CD38 expression (d–f), and the frequency (%) of CD38+ HLA-DR+ co-expression (g–i) is shown.

(29.4 ± 23.4). These data appear to suggest that the analysis of HLA-DR and CD38 co-expression on CD8+ T cells as a monitoring device further increases the sensitivity of the therapeutic drug monitoring. Unlike data obtained on the untreated HIV-infected patients, no correlation was noted between absolute CD4 count, the percentage of CD4+ T cells and CD38 expression in the anti-retroviral drug-treated patients (data not shown).

Comparative analyses of the data from the anti-retroviral drug-treated vs. untreated patients indicate that whereas a decrease in the density of CD38 expression and frequency of CD38/HLA-DR co-expression in anti-retroviral drug-treated HIV-infected patients can be used as a therapeutic marker, unfortunately no specific cutoff points could be established for distinguishing untreated patients from treated patients. Clearly, either a larger cohort needs to be studied in order to establish such values or data have to be analyzed on a per patient basis prior to and following ART.

Increased CD38 expression in anti-retroviral drug-treated patients who showed virological failure

It is also recognized that inclusion of HIV-1-infected patients that fail ART in such analyses of CD38 expression by CD8+ T cells would be very useful for the implementation of CD38 expression as a therapeutic drug monitoring tool. Thus, we measured CD38 expression in 5 HIV-infected patients who showed virological failure (viral load > 50 copies/ml) after receiving ART and compared to patients with successful ART. As shown in Fig. 5a, a slight but statistically significant increase in the frequency of CD38 expressing CD8+ T cells was observed in patients who were classified as ART failures. Thus, the frequency of T cells expressing CD38 was 93.7 ± 4.8 for CD8+ T cells ($P = 0.0053$) and 81.0 ± 5.7 for CD4+ T cells ($P = 0.0128$). A statistically greater significant increase was observed when we compared the mean density of CD38 expression. As seen in Fig. 5b, the mean density of CD38 expression was 871.8 ± 244.3 for CD8+ T cells ($P = 0.0030$) and 1041.6 ± 384.5 for CD4+ T cells (non-significant) in samples from patients who were classified as ART failures. The frequency of CD8+ and CD4+ T cells co-expressing CD38 and HLA-DR was also significantly increased in ART treated patients who showed virological failure with values of 55.9 ± 14.7 for the CD8+ T cells ($P = 0.0286$) and 32.7 ± 15.9 for CD4+ T cells (non-significant) as shown in Fig. 5c. Unlike data obtained on the ART-treated responder HIV-infected patients, no significant difference of CD38 expression by CD4+ and CD8+ T cells was noted between the patients who

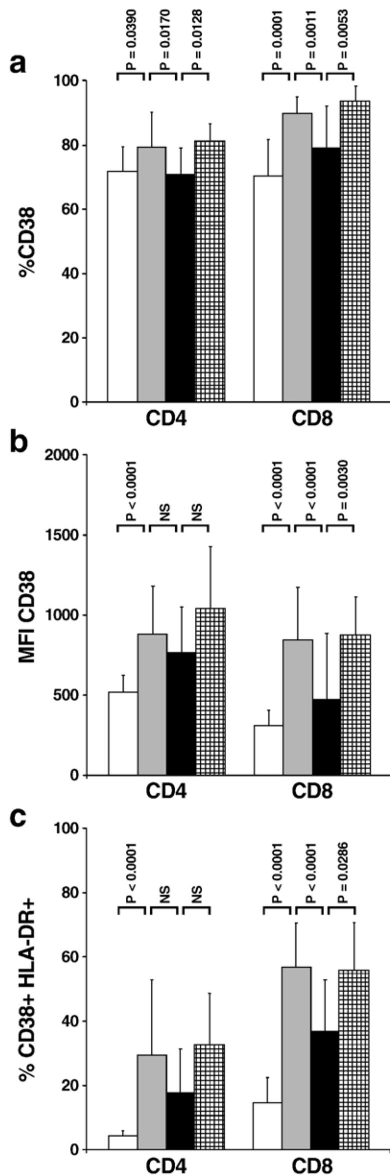

Fig. 5. Comparison of CD38 expression in four different groups of subjects. Healthy individuals (open columns), untreated HIV-infected patients (gray columns), successful anti-retroviral-treated HIV-infected patients (black columns), and anti-retroviral-treated HIV-infected patients who showed virological failure (line columns) are depicted as the mean frequency (%) of CD38 expression (a), the mean density (MFI) of CD38 expression (b), and the mean frequency (%) of CD38+ HLA-DR+ co-expression (c) in CD8+ and CD4+ T cells using PE-conjugated anti-CD38 monoclonal antibody.

were classified as ART failures and untreated patients as shown in Fig. 5.

Discussion

Chronic immune activation has been reasoned to be a significant contributor to disease progression in HIV-1-infected patients which has prompted the use of the expression of cell surface activation markers such as CD38 to monitor disease progression. Results from several such studies have documented a correlation between plasma viral load and the increased expression of CD38 as a strong predictor of disease progression (Bouscarat et al., 1999; Giorgi et al., 1993, 1999; Liu et al., 1996, 1997, 1998; Orendi et al., 1998). The level of expression of CD38 molecules has been shown to be relatively high in immature and activated T cells, relatively low in mature and resting naïve hematopoietic cells, and almost undetectable on resting memory T cells (Mehta et al., 1996).

In contrast to these previous findings of low expression on resting cells, the results reported herein show that the level of CD38 expression depends on the sensitivity of the fluorochrome being utilized. Thus, unlike the previous reported studies (Anthony et al., 2003; Benito et al., 2002; Tilling et al., 2002), results of our studies in healthy subjects 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. It is important to note that the detection of such differences in the frequencies using a brighter fluorochrome only applies to molecules that are expressed at relatively lower levels per cell (<1000 molecules/cell). Thus, as noted in the data presented herein, the increased frequencies were only noted when PE-conjugated anti-CD38 mAb was utilized in conjunction with FITC-conjugated anti-HLA-DR mAb. Use of PE-conjugated anti-HLA-DR mAb in conjunction with FITC-conjugated anti-CD38 mAb did not show any increase in the frequency of HLA-DR expressing cells. This is because the relative expression of HLA-DR per cell (>1000 molecules/cell which is readily detectable using most fluorochrome-conjugated antibodies including FITC) is significantly higher than CD38. Thus, use of FITC-conjugated anti-HLA-DR is sufficient to detect all cells expressing HLA-DR which is not true for CD38. However, use of PE-conjugated anti-HLA-DR mAb did increase the density values for this molecule but not the frequency of cells expressing HLA-DR. It is also important to note that the high frequencies of CD38 expressing cells were not restricted to just CD4 and CD8 cell lineages but were also noted in NK cells, B cells, and gamma–delta T cells. These data suggest that results of the studies of cell surface markers particularly those expressed at relatively lower levels need to be carefully evaluated in the context of the fluorochrome being utilized and the sensitivity of the flow cytometer/analyzer being utilized to acquire the data. A need for standardization is thus readily apparent. This issue becomes important not only for research-based studies but also for clinical studies which rely on such data for clinical decision making.

Flow cytometry has clearly provided a powerful tool in both clinical diagnostic and research applications. However, as stated above, an issue that is often ignored concerns the analysis and interpretation of the data acquired, especially as it relates to the sensitivity of the reagent and the flow cytometer being utilized. For clinical studies, there has always been a need for standardization

and thus most clinical evaluations require a standard and quality control of the reagents and the instruments. For research purists, there has also been a need to define expression in more concrete terms. Thus, is the molecule expressed on the cell surface or not? To make such a statement requires knowledge as to how many molecules of a given protein need to be expressed by a given cell for it to be detectable with the use of appropriate antibodies and the sensitivity of the instrument being utilized, the affinity of the antibody for the molecule, the stability of the interaction between the antibody and its ligand, the proximal distribution of the molecule on the cell surface, the ability of the molecule to undergo capping following ligation of the molecules with the appropriate antibody, and the state of the cell, i.e., is it resting or activated and the degree of activation. Thus, this becomes a complex issue. In this regard, it is important to note that there are a number of other flow cytometric-based procedures that have been utilized to increase the sensitivity for the detection of cell surface molecules. These procedures include the three-layer amplification technique (Mavrangelos et al., 2004; Zola et al., 1990), the use of magneto-fluorescent liposomes (Scheffold et al., 1995), enzymatic amplification staining (Earnshaw and Osbourn, 1999; Kaplan and Smith, 2000; Kaplan et al., 2001), and the use of new fluorophores, fluorescent microspheres, and Quantum dots (Bhalgat et al., 1998; Lidke et al., 2004; Panchuk-Voloshina et al., 1999). While these methods are clearly more sensitive for the detection of low-abundance cell surface molecules, they also have been shown to give variable results and high nonspecific staining (Mavrangelos et al., 2004). Some of these procedures are also relatively impractical for use in the clinical laboratory due to the complexities within the technique. For example, enzymatic amplification staining is not only time consuming but it also requires the use of many different reagents and involves many steps to be performed during the staining process.

Currently, there are a number of procedures available to monitor the outcome of ART. These include CD4 counts, viral load, drug-resistance testing and therapeutic drug monitoring (TDM). Successful ART refers to therapy that leads to a stable increase in CD4 counts compared with CD4 counts pre-ART or higher than what is considered to be protective of AIDS-defining illnesses (>200 cells/μl), a viral load that is undetectable (clearly based on the sensitivity of the method employed), the absence of drug resistance mutants assessed by phenotypic or genotypic resistance assays, and the maintenance of an effective drug concentration that provides maximal therapeutic effect with the least possible toxicity (Frenkel and Tobin, 2004; Grabar et al., 2000; Rakhmanina et al., 2004). Although these methods are very useful for the monitoring of patients receiving ART, each method has some disadvantage which is reflected by the accuracy of the measurements of the therapeutic outcome which in some cases make it impractical for clinical laboratory testing. Thus, treatment failure as determined by CD4 counts will be detected relatively late since no significant change in the level is normally observed during drug treatment (Deeks et al., 2002; Kaufmann et al., 1998; Piketty et al., 1998). While viral load can be used to monitor a success or failure of anti-retroviral treatment, the cost of viral load measurements remain expensive and inaccessible to most patients in the developing world (Stephenson, 2002). A genotypic resistance assay is currently available only in three teaching hospitals in Bangkok and TDM is only available at the HIV-NAT laboratory in Bangkok and each of these are difficult to implement and deemed impractical.

The above issues prompted a need for defining an affordable anti-retroviral therapeutic monitoring strategy and thus was the rationale for the studies reported herein. Thus, in contrast to previous studies that predominantly utilized the relative levels of CD38 expression as a prognostic marker for disease progression in HIV-1-infected patients, the studies reported herein were conducted in efforts to evaluate the potential usefulness of CD38 expression as a marker of anti-retroviral drug efficacy. Results from these initial studies basically show that HIV-1-infected patients who have received ART and have sustained plasma viral loads of <50 copies/ml do indeed demonstrate a somewhat lower frequency of CD8+ T cells which expressed CD38. However, a decrease in the mean density of CD38 expression in addition to the use of HLA-DR co-expression by CD8+ T cells provides a more reliable marker for therapeutic drug monitoring which is practical and more cost effective than the techniques outlined above. It should be noted that the studies reported herein were conducted as a cross-sectional study and it is highly likely that results of differences in the co-expression of CD38 and HLA-DR on CD8+ T cells from patients prior to and post-ART may provide an even more discriminatory tool.

The stable suppression of HIV replication is considered the main goal of ART. However, the inability to suppress viral replication or virological failure is a frequent finding (Clough et al., 1999; Deeks et al., 1999; Ledergerber et al., 1999) and has been associated with the presence of drug-resistant virus (Frenkel and Tobin, 2004). More importantly, virological failure has not always been associated with a fall in CD4 cell level which has led to the concept of "immunovirological discordance" (Moroni, 2003). There are 4 types of discordant responses in patients receiving ART, three of them based on virological failure associated with the presence of drug-resistance virus and a decrease (I), stable (II), or increase (III) in CD4 count which are described elsewhere (Belec et al., 2000; Kaufmann et al., 1998; Piketty et al., 1998; Sufka et al., 2003). Based on the data presented herein, we propose another explanation related to chronic immune activation as observed by CD38 expression. ART may suppress CD4+ T cell activation by the successful elimination of the high virulence wild-type virus which decreases CD4+ T cell turnover, thereby resulting in sustained or increased CD4

counts. This view is supported by the finding that drug treatment interruption in patients with drug-resistant viremia resulted in an increase in CD4+ T cell activation associated with decreased CD4 counts (Deeks et al., 2002). Accordingly, it is possible that patients with type I discordant response in fact were experiencing a failure in immune activation control instead of a role for direct pathogenicity from drug-resistant virus. The effect of immune activation on the failure to lead to an increase in CD4 counts (type IV discordant response) in the patients that control viremia but do not show an increase in CD4 counts (Kaufmann et al., 2002; Valdez et al., 2002) may be explained by two studies that showed high levels of T cell activation despite successful viral suppression (Anthony et al., 2003; Hunt et al., 2003). These data suggest that immune activation is central to the depletion of CD4+ T cells in HIV-infected patients and support the concept of using the analysis of CD38 expression for the monitoring of patients receiving ART. Studies aimed at analyzing data on CD38 expression from such groups of HIV-1-infected patients is the aim of our future studies.

Materials and methods

Study population and sample collection

Peripheral blood samples were obtained from otherwise normal healthy individuals and HIV-infected patients. Each study volunteer was explained the nature of the study in the Thai language and appropriate informed consent was obtained prior to their enrollment in the study. The study was approved by the Ethics Committee of the Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. Thirty-one local healthy adult Thai volunteers were recruited from laboratory staff of the hospital. Samples from 20 of these volunteers were used for baseline studies and all additional studies were conducted with an additional 11 volunteer samples. The HIV-1-infected patients were recruited from the HIV Clinic of the Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. The patients were divided into 3 groups, the first group (n = 20) consisted of patients who did not receive any ART prior to and during the course of this study, the second group (n = 23) consisted of HIV-infected patients who had been on 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 for the same time period but showed 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 determined on aliquots of a blood sample from each volunteer (Table 2). Plasma samples were also collected by centrifugation of an aliquot of each blood sample and kept at −70 °C and used in batches for viral load determination.

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 CD4, clone SK3, conjugated with allophycocyanin (APC), anti-human CD8, clone SK1, conjugated with peridinin chlorophyll protein (PerCP); anti-human CD3, clone SK7, conjugated with FITC, PE or PerCP; anti-human CD19, clone 4G7, conjugated with FITC; anti-human CD56, clone SNCAM16.2, conjugated with APC and anti-human γδ TCR, clone B1, conjugated with 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: Either CD3 or HLA DR-FITC/CD38-PE/CD8-PerCP/CD4-APC; and CD38-FITC/CD3 or HLA-DR-PE/CD8-PerCP/CD4-APC. After the addition of the monoclonal antibody reagents, samples were gently mixed by vortex, and incubated in the dark at 25 °C for 15 min followed by the addition of 1 ml of FACS lysing solution in order to lyse the red blood cells. The stained samples were gently mixed again by vortex, and incubated in the dark at 25 °C for 10 min. Following centrifugation at 1200 rpm for 5 min at room temperature, the supernatant was discarded and the cell pellet resuspended in 2 ml of wash buffer (PBS with 2% fetal bovine serum) followed by centrifugation at 1200 rpm for 5 min at 25 °C. Finally, the cell pellet was resuspended in 300 μl of freshly prepared 1% paraformaldehyde in PBS pH 7.4 and subjected to flow analysis. To determine the frequency of CD38 expressing cells within the lymphocyte

Table 2
Values of CD4+ T cell subsets and viral load

Group	Absolute CD4 count (cells/μl)	% CD4	Viral load (copies/ml)
Healthy individuals (n = 31)	781 ± 276	37.96 ± 8.65	N/A
Untreated group (n = 20)	381 ± 415	17.09 ± 11.78	76,658 ± 81,324
Successive ART-treated group (n = 23)	352 ± 216	16.99 ± 8.42	<50
Virological failure group (n = 5)	212 ± 263	10.47 ± 10.43	165,269 ± 198,908

subsets, the following combination of mAbs were used: CD19-FITC/CD38-PE/ CD3-PerCP/CD56-APC and CD19-FITC/CD38-PE/CD3-PerCP/γδ TCR-APC.

Flow cytometric analysis

Six-parameter analysis was performed on a FACSCaliber flow cytometer (BDB) using CellQuest software (BDB). Cells were gated using lymphogate for the analysis of only viable cells and at least 30,000 viable cells were analyzed per sample. The frequency of CD4+ or CD8+ T cells was identified by gating on either CD4 or CD8 vs. a side scatter dot plot. Only the CD8+ bright population was used for analysis since this population was previously identified as cells enriched for those expressing the CD8 alpha/beta heterodimer as compared with the low-density CD8 expressing cells that are predominantly alpha chain homodimer positive and likely represent a NK cell subset. The frequency (percentage) and density (mean fluorescent intensity, MFI) of CD38 expressing subsets of cells were determined utilzing a histogram plot whereas the percentage of the CD38+ HLA-DR+ cells were determined by two-color dot plot (FITC vs. PE) analysis. 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 mean ± SD of sample. The Mann–Whitney *U* test was used to determine the statistical significance of the difference observed between groups. The Pearson correlation coefficient test was used to analyze for the association observed between different parameters. P values <0.05 was considered significant.

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