

Changes in antigen densities on leukocyte subsets correlate with progression of HIV disease

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Abstract

In a cross-sectional study of 154 HIV-infected and 33 uninfected healthy adults, we show that characteristic changes in the levels of expression of leukocyte surface antigens occur in the HIV-infected individuals. These changes, which collectively occur on virtually every leukocyte subset, are specific: a particular antigen may increase or decrease on one subset of PBMC but remain constant on another. Furthermore, within any particular subset, the levels of one or more antigens may change, while the levels of other surface antigens on the same cells remain constant. Some of these antigen density changes have been noted before, e.g. increased CD20 on B cells, and increased CD38 and HLA-DR on CD8 T cells. However, the multiparameter flow cytometry methodology used here reveals changes in a substantially larger number of surface markers, some of which are restricted to fine subsets of PBMC, such as naive or memory T cell subsets. For many of these antigens, the change in expression correlates with absolute CD4 counts; however, some antigens tend to differ most in individuals with CD4 counts $>500/\mu\text{l}$; others differ only in those with counts $<100/\mu\text{l}$. The changes in antigen densities we observe on B and T cells are consistent with the observation of a persistent quasi-activated state of these cells in HIV-infected individuals. Similarly, the altered expression of the signal-transducing molecules CD7 and CD16 that we demonstrate for NK cells may correlate with the functional defects previously demonstrated in NK cells. Thus, measurements of antigen densities such as those demonstrated here may provide surrogate markers for the altered functional capacities of PBMC subsets in HIV-infected individuals, and may thereby provide a much simpler assay for immunocompetence than *in vitro* functional assays.

Introduction

Currently, CD4 T cell number is the only immunologically based measurement that has been an acceptable surrogate marker for HIV disease. Indeed, on a population level, this number correlates with disease progression, e.g. at <200 CD4 T cells/ μl , HIV⁺ individuals have a significantly increased incidence of *Pneumocystis carinii* pneumonia. The fact that HIV infects only CD4-bearing cells has lent credence to this measurement as being relevant to the progression of disease; it appears that the steady-state level of CD4 T cells is governed in part by the long-term burden of virus always present in infected people (1,2).

CD4 T cells are not the only hematopoietic subset affected by HIV disease. Virtually all leukocyte subsets are affected in some way, even in otherwise healthy HIV-infected people. In fact, early in AIDS research it was established that B cells

show evidence of polyclonal activation (3). CD8 T cells, while not infectable with HIV, show dramatic alterations: an early increase in the total number of CD8 T cells (4) due to expansion of memory cells (5,6); a continuous loss of naive CD8 T cells (5,6); and, later in the progress of the disease, a substantial increase in the presence of CD8 cells bearing some activation markers (CD38 and HLA-DR) (4,7,8). NK cells, while unchanged in representation, exhibit a functional defect (9). Finally, there is evidence for altered functionality of antigen presenting cells, such as monocytes and dendritic cells (10-12).

Most of the observations of functional defects are based on rather complex *in vitro* assays of functional capacity. Therefore, it would be of significant value to have surrogate markers of functionality in cell subsets that could be easily

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measured. It is possible that correlates of cell functionality may be found by measuring the level of expression of proteins on the surface of that cell. Indeed, it is possible to detect changes in individual PBMC subsets by quantitative flow cytometric (FACS) measurements. For instance, we have shown that B cells from HIV-infected individuals often show an increase in CD20 expression on a per-cell basis (13). This increase was most profound in late-stage patients, although easily detectable at any stage. Since the variation of median CD20 expression on B cells amongst healthy adults is very small, it is relatively easy to identify a majority of HIV-infected patients based on CD20 expression only. Similarly, Scott *et al.* (14) demonstrated a specific decrease in the expression of complement receptor 2 (CR2, CD21) on B cells in HIV-infected individuals. The change in expression of CD20 and CD21 may reflect the polyclonal activation of B cells in HIV-infected individuals (3). By far the most antigen density observations have been made on monocytes (15–23), with sparse reports on neutrophils (20,24) and T cells (18,25,26).

The observation that there are specific changes in the expression of some proteins on the surface of cells in HIV-infected individuals led us to undertake quantitative phenotyping of over a dozen different common leukocyte antigens expressed on a variety of PBMC subsets. We established staining conditions which allowed for reproducible and quantitative measurement of these antigens on a per-cell basis, and appropriate statistical analyses of the data to provide quantitative expression information. This study was undertaken in concert with the analysis of T cell subset and PBMC subset frequencies in HIV-infected adults; thus, most of the antigens studied are those used for typical phenotyping studies. However, the data presented here demonstrate that many of these antigens show specific changes in expression on PBMC subsets, and that, on the basis of this cross-sectional study, these changes are often correlated with the progression of HIV disease as measured by CD4 counts.

Methods

Human samples

We recruited 154 HIV-infected adults from the San Francisco area. Since this study was part of a clinical trial in which an entry criterion was <500 CD4 T cells/ μ l, our cohort is weighted

towards infected individuals with fewer CD4 T cells than the HIV-infected population taken as a whole. Also excluded from participation were patients who had concurrent opportunistic infections, or were taking very large amounts of antioxidants, vitamins or minerals. The HIV infection status of each individual was confirmed by anti-p24 ELISA. All samples used in the analysis presented here were drawn from patients prior to inception of drug or placebo dosing. In addition, we recruited 33 HIV-uninfected adults, in good health, as control subjects. All clinical trial subjects signed informed consent forms.

From each HIV-infected patient, blood was drawn by venipuncture for FACS analysis, and additional tubes for complete blood count (CBC) and absolute CD4 and CD8 count (performed by an accredited commercial laboratory). From control subjects, only blood for FACS analysis was drawn. All samples were prepared within 8 h of the draw.

Reagents

All fluorochrome-conjugated monoclonal reagents were obtained from PharMingen, San Diego, CA, with the exception of CD20 (clone 1F5, a kind gift of Ed Clark, University of Washington), and PE HLA-DR (Becton Dickinson, San Jose, CA). Ficoll-Paque was obtained from Pharmacia, Uppsala, Sweden. Biotin- and flavin-deficient RPMI 1640 (hereafter, deficient RPMI) was obtained from Irvine Scientific, Santa Ana, CA. Other chemicals were obtained from Sigma, St Louis, MO. The panel of three-color stains used in this study is shown in Table 1.

Preparation of mAbs

Quantitative analysis of antigen expression requires stringent staining conditions. These include the use of saturating concentrations of antibody wherever possible, strictly controlled incubation times, temperatures and volumes, and consistently calibrated and compensated flow cytometers. Changes in any of these parameters can influence accuracy to various extents and can significantly decrease precision.

All conjugated antibodies were titrated against PBMC from uninfected adults. Titrations were performed by serial 2-fold dilutions from a relatively high concentration of antibody (the concentration of many commercially available reagents, especially PE and Cy5-PE conjugates, is too low to achieve saturation). The concentration below which fluorescence began to decrease (plateau value) was chosen as the amount

Table 1. Immunofluorescence staining panel for antigen density study

Stain no.	FITC	PE	Cy5-PE	Purpose of stain (subsets identified)
1	CD14	CD16	CD45	total lymphocytes, monocytes
2	CD3	CD8	CD4	total CD4 or CD8 T cells
3	CD20	HLA-DR	CD5	B cell subsets
4	CD7	CD16	CD5	NK cells
5	CD3	CD38	CD4	activated CD4 or CD8 T cells
6	HLA-DR	CD8	CD4	activated CD4 or CD8 T cells
7	CD62L	CD45RA	CD4	naive and memory CD4 T cells
8	CD62L	CD45RA	CD8	naive and memory CD8 T cells
9	CD11a	CD45RA	CD4	naive and memory CD4 T cells
10	CD11a	CD45RA	CD8	naive and memory CD8 T cells

to use. For some antibodies, saturating concentrations required prohibitive amounts of antibody (>10 µg per test). However, the use of a constant incubation time allowed for quantitative measurements to be made even with these reagents. For some reagents (primarily the PE and Cy5-PE reagents), saturating concentrations of conjugated antibody resulted in cell staining that is off-scale on the flow cytometer; thus, for these reagents unconjugated antibody was added to reduce the intensity while maintaining the saturating characteristics. The inclusion of unconjugated antibody does not change the characteristic binding of the conjugated antibody other than by dilution; thus, neither the precision nor the accuracy is affected. An alternative method, the use of a neutral density filter to reduce fluorescence, is not possible since other reagents with the same fluor are much less bright and would then be off-scale at the low end of fluorescence. The preparation of monoclonal reagents for accurate and precise quantitative fluorescence measurements by FACS is described in detail elsewhere (27). Table 2 shows the amounts of every reagent used, and whether or not the stain was saturating. Staining intensity for all reagents was cell-number-independent (to at least 10 million cells per stain; all stains used ~1 million or less cells per test).

The final premixed three-color reagent combinations for each stain (a sufficient amount for this entire study) were titrated again prior to the initiation of the study; the saturation characteristics of the mixture were exactly as predicted by the titrations of the original unmixed reagents.

Because our instrument is calibrated daily to give the same

output values given the same input fluorescence intensity per cell, no normalization across samples or across experiments was necessary or performed. All values analyzed and presented here represent the raw channel values converted to linear fluorescence intensity by a simple, unvarying exponential function. Autofluorescence controls (unstained cells) for each sample were analyzed as well; autofluorescence is ~0.4 (in the fluorescence units presented in this manuscript) and did not vary across experiments. Samples from uninfected adults were analyzed concurrently with those from infected adults; usually, one or two uninfected adults and four to fifteen infected adults were analyzed in any particular experiment.

FACS analysis

PBMC were prepared from 6 ml of heparinized blood by Ficoll-Paque density centrifugation. Cells were washed once and stained with the appropriate amount of premixed reagents. The incubation was carefully timed to a total of 15 min (on ice). After staining, cells were washed three times in ice-cold deficient RPMI and then resuspended in ice-cold 0.5% paraformaldehyde in deficient RPMI. Flow cytometric analysis was performed on a dual laser (argon 360 nm, argon 488 nm) FACStarPlus (Becton Dickinson Immunocytometry Systems, San Jose, CA) interfaced to a VAX 6300 computer (Digital Computer, Maynard, MA). For each cell, data for forward scatter, side scatter and the fluorescences of fluorescein (515–545 nm bandpass filter), phycoerythrin (570–600 nm) and Cy5-PE (650–690 nm) were collected. For each stain, data from 30,000 (stains 1–6) or 50,000 (stains 7–10) cells were collected and analyzed by FACS-Desk software (28).

Statistical analyses

For analysis and display of statistical comparisons, we used JMP for the Apple Macintosh (SAS Institute, Cary, NC). Comparisons of distributions were performed by the nonparametric two-sample Wilcoxon rank test.

Results

Antigen density changes in PBMC from HIV-infected adults

The expression of CD20 is increased significantly, and that of CR2 is decreased significantly, when comparing B cells from infected adults with those from uninfected adults (13,14). Studies here extend these results by measuring the relative expression of a number of antigens on many leukocyte subsets for a cohort of 154 HIV-infected and 33 uninfected adults. In order to make measurements in experiments done over a relatively long time period (9 months) comparable, we premixed enough reagents for our panel of three-color stains to last through the entire study. In combination with our staining protocol, this ensured equivalent fluorescence measurements over this time period. The resulting fluorescence intensities on cells were independent of cell number added (data not shown).

Our staining protocol ensured accurate and precise antigen density measurements primarily by the use of saturating antibody concentrations, as well as a rigorous staining protocol that carefully controlled incubation times, temperatures

Table 2. Amounts of antibody used in panel stains

Conjugate	µg/test ^a	µg unconj ^b	Saturating? ^c
FITC CD14	2.2	0	50%
FITC CD3	0.9	0	yes
FITC HLA-DR	1.1	0	yes
FITC CD7	2.2	0	yes
FITC CD62L	0.4	0	yes
FITC CD20	1.5	0	no
FITC CD11a	1.8	0	yes
PE CD16	0.8	0.4	yes
PE CD8	0.5	0.5	yes
PE CD38	0.5	0.5	50%
PE CD45RA	1.1	0.1	yes
PE HLA-DR	— ^d	0	50%
CyC CD45	0.26	1.0	yes
CyC CD4 ^e	0.17	0	no
CyC CD4 ^f	0.47	1.0	yes
CyC CD5	0.56	0.25	yes
CyC CD8	0.13	0.5	yes
CyC CD3	0.22	0	no

^aTotal amount of the conjugated antibody used per tube.

^bTotal amount of unconjugated antibody (identical clone as the conjugate) used per test.

^c'Yes': fluorescence achieved in a 15 min stain is >80% of the maximally obtainable fluorescence; 50%: fluorescence achieved is approximately half-maximal; 'no': fluorescence increased linearly with antibody concentration near the concentration actually used.

^dThe mass of antibody in this stain is unknown.

^eUsed for stains 5–7 and 9 (CD4 was not saturating).

^fUsed for stain 2 (saturating CD4; used for antigen density quantitation).

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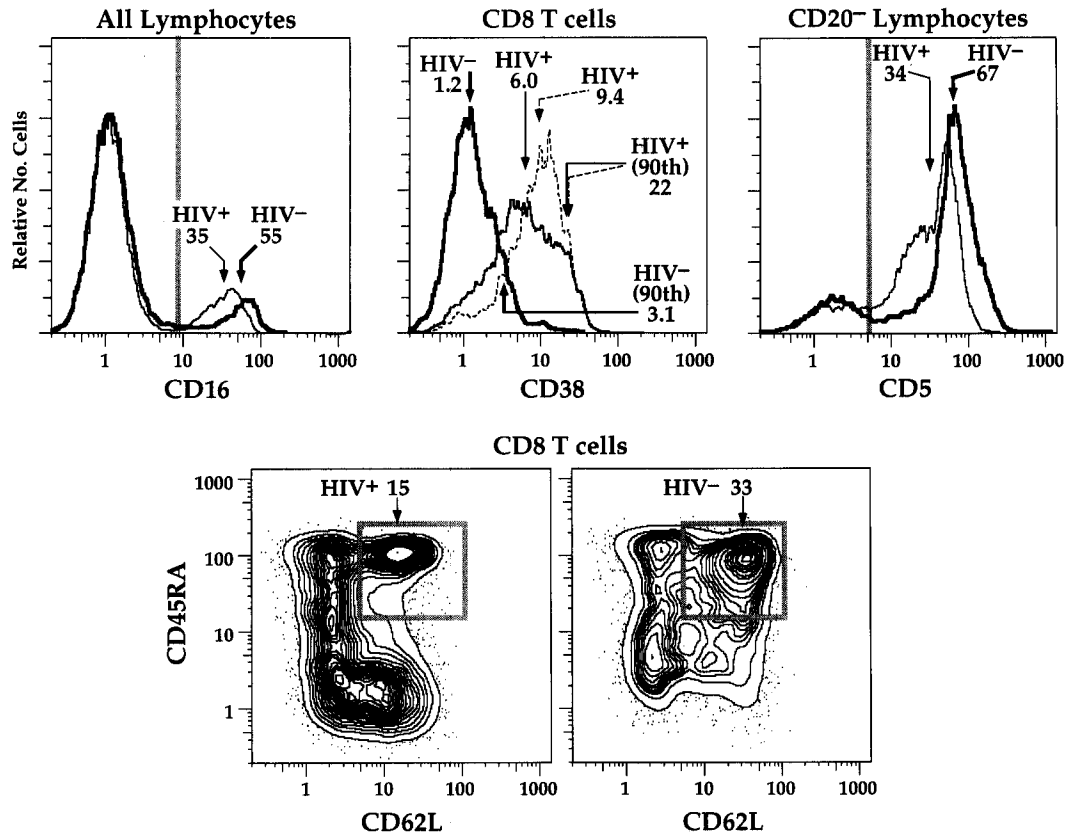


Fig. 1. Examples of antigen densities that are different in HIV-infected and uninfected adults. Four different examples are given of how the expression of antigens on a variety of leukocyte subsets is altered in HIV-infected adults. Grey lines indicate analysis gates used when computing statistics. Numbers are the median (50th percentile) fluorescences for subpopulations, except where noted. (Top left) Expression of CD16 on all lymphocytes (panel stain 2). The CD16⁺ cells are NK cells; there is less CD16 per cell on NK cells from the HIV-infected adult than from the uninfected adult. (Top middle) The expression of CD38 is shown for CD3⁺CD4⁻ lymphocytes (consisting primarily but not exclusively of CD8 T cells; panel stain 5). Two examples of HIV-infected adults are shown. Both median fluorescences and 90th percentile fluorescences are shown. (Top right) Expression of CD5 on CD20⁻ lymphocytes is shown (panel stain 3). The CD20⁻CD5⁺ cells are primarily NK cells; statistics were computed only on the CD20⁻CD5⁺ population. CD8 T cells express less CD5 than do CD4 T cells—thus, two populations of CD5-expressing cells are apparent in this HIV-infected adult, because of the significantly elevated proportion of CD8 to CD4 T cells. (Bottom) Comparison of the phenotypes of naive (CD62L⁺CD45RA⁺) and memory (all other combinations) CD8⁺ lymphocytes (panel stain 8). The median CD62L fluorescence for the naive cells (box) is shown. In this example, the proportion of naive CD8 T cells is decreased in the HIV-infected adult; in addition, the phenotype of the naive T cells is changed in that they express less than half of the CD62L per cell than the control.

and volumes. It is important to note that the use of subsaturating antibodies is still quantitative under these conditions. For these reagents, the antibody is in vast excess of antigen even at subsaturating concentrations; thus, cell number (in the ranges obtained from Ficoll separations) becomes irrelevant (27). In a previous study (13) we found a variation of well under 10% between healthy individuals in the expression of CD20, a reagent which was not used at saturating concentrations.

Figure 1 shows four examples of antigen density in which expression levels are significantly different between infected and uninfected adults. In data presented here, we focus primarily on the phenotype of cells within a particular subset (i.e. the amount of expression of particular antigens), rather than their representation (i.e. the relative frequency of cells within a parent population or within all lymphocytes). It is

important to note that a change in the representation of a subset does not predicate a change in the phenotype of the cells within that subset, nor does a change in the representation necessarily result from a change in the phenotype.

Figure 2 correlates these same four antigen densities with disease progression, as estimated by the absolute number of CD4 T cells per μ l of whole blood. Table 3 summarizes these changes, as well as changes for other antigens (see below).

For example, these data show that the amount of CD16 per NK cell in infected adults is 60% that for the uninfected adults (Figs 1 and 2, and Table 3). Typically, NK cells are uniquely identified on the basis of this marker (granulocytes expressing CD16 are excluded by scatter gating; the few T cells that express CD16 are too dim to be included in this gate). For our cohort, the representation (frequency) of NK cells within PBMC as defined by CD16 is not significantly changed (data

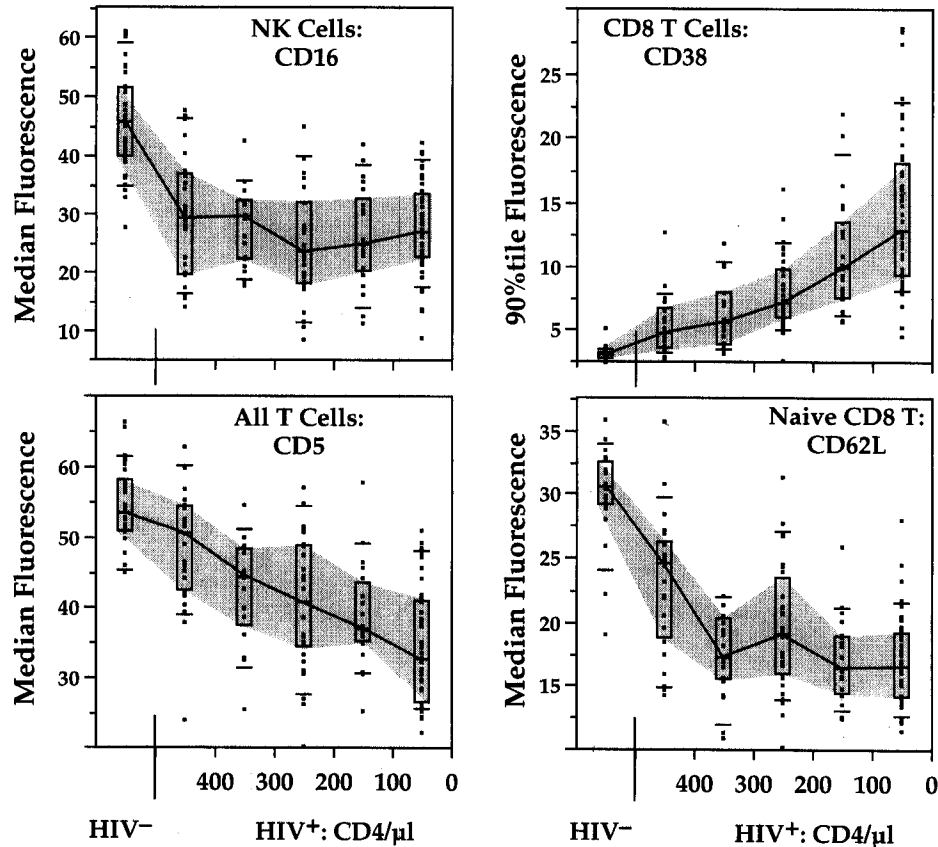


Fig. 2. Correlation of antigen density changes with disease stage. The four antigen density changes exemplified in Fig. 1 are shown for our entire cohort. Each point represents the median antigen density (or 90th percentile for CD38) for an individual, as determined by the median (or 90th percentile) of the relative fluorescence units of the population noted (cf. Fig. 1). Individuals were grouped according to infection status and, for infected adults, by the absolute CD4 count. Individuals with CD4 counts $>400/\mu\text{l}$ whole blood were grouped in the first HIV⁺ group; subsequent groups are based on $100/\mu\text{l}$ divisions. The lines connect the median values for each group of individuals; the boxes enclose the interquartile ranges for each group, and are connected by the shaded region; the small horizontal bars are the 10th and 90th percentiles for each group. Number of individuals: HIV⁻, 33; HIV⁺: CD4 count $<100/\mu\text{l}$, 50; $100\text{--}200/\mu\text{l}$, 24; $200\text{--}300/\mu\text{l}$, 32; $300\text{--}400/\mu\text{l}$, 22; $>400/\mu\text{l}$, 26.

not shown). However, the phenotype of the NK cells is quite different: the expression of both CD16 and CD7 (Table 3) is significantly decreased. In contrast, the very low expression of CD16 on monocytes is increased in HIV-infected adults (Table 3) and the high expression of CD16 on granulocytes is unchanged (data not shown). Thus, the change seen on NK cells is a specific change in the regulation of the expression of CD16 in that cell type only.

Figures 1 and 2 also show the altered expression of CD62L on naive CD8 T cells. We have previously shown that naive CD8 T cells are lost during the progression of HIV disease, in terms of both frequency and absolute count (5,6). As we show here, in addition to the change in frequency, those naive CD8 T cells which remain show a changed phenotype (decreased CD62L). The decrease in the expression of CD62L is not sufficient to prevent resolution of this subset from memory cells that do not express CD62L (Fig. 1). Note that the expression of CD45RA on this subset of cells is unchanged between infected and uninfected adults (Table 3)—again demonstrating a specific change in the regulation of a molecule in these cells. The change in the expression of CD62L on

naive T cells (or on memory T cells) does not correlate with the representation of the naive T cells in blood, in terms of either percentage or absolute count (data not shown). Thus, for naive CD8 T cells, the change in representation and the change in phenotype occur independently.

Quantitation of T cell activation markers

Consistent with previous reports, we find increased expression of the 'activation markers' HLA-DR and CD38 on CD8 cells (4,7,8), as well as CD4 cells (Figs 1 and 2, and Table 3). While these are typically expressed on activated T cells, the T cells that express these markers in HIV-infected adults do not seem to express other classical activation markers, such as CD25, CD69 and CD71 (29–32; M. Roederer, unpublished observations).

The presence of cells bearing a surface antigen is typically expressed as the frequency of cells having more fluorescence than (for instance) cells stained with an isotype control. However, the proportion of CD38-expressing cells is very difficult to quantitate, because the distribution of expression is continuous with the non-expressing cells. Therefore, the

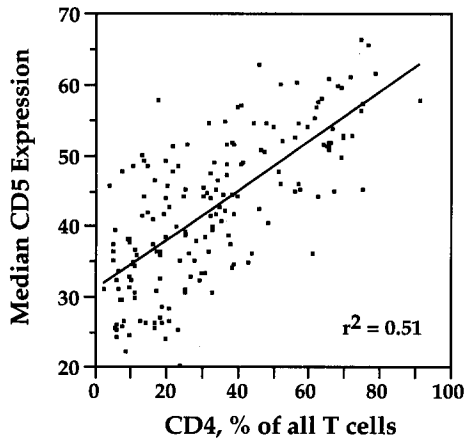


Fig. 3. Antigen density changes can signify changes in the representation of underlying subsets. CD4 T cells express approximately twice as much CD5 as CD8 T cells. Thus the median CD5 expression (expressed as relative fluorescence units) on all T cells will depend on the relative frequency of CD4 and CD8 T cells. The correlation of CD5 expression on all T cells with the fraction of T cells that are CD4 is shown. The correlation coefficient indicates that 50% of the variation in CD5 expression can be accounted for by the representation of the CD4 T cell subset within the entire T cell population.

fraction of CD38-positive cells will (artefactually) depend on the sensitivity of the instrument, as well as the brightness of the reagent and the quality of the background controls.

This frequency measurement is also unsatisfactory in that it does not reflect the amount of antigen being expressed by the positive cells. For this reason, the median fluorescence (or mean fluorescence, if data are collected with linear amplification) is more attractive. Even so, for some distributions the median may not be very informative, e.g. if only 40% of the cells express a marker, then the median is still within the negative population. For these antigens, we also quantitate the 90th percentile of antigen expression (Fig. 1), as this value is much more sensitive to low-frequency subpopulations. Indeed, for CD38 expression the 90th percentile shows greater statistical power than does the median (data not shown), primarily because its value increases considerably more than does the median. For all other antigens that we analyzed, the distribution of expression is much more uniform and the median fluorescence is a satisfactory statistic.

Subset representation changes may lead to apparent antigen density changes

A change in the expression of an antigen may reflect a change in the regulation of expression of the protein on a cell-by-cell basis. On the other hand, it might reflect a different frequency (representation) of unresolved underlying subsets that differentially express the antigen. As an example, the expression of CD5 on all T cells is significantly reduced in HIV-infected adults. However, the loss of CD4 T cells largely accounts for this decrease (Fig. 3), since CD4 T cells express more CD5 than CD8 T cells (33; data not shown). The average CD5 expression for all T cells will be the average expression of CD4 and CD8 T cells, weighted by their relative frequencies. Therefore, the expression of CD5 must in part correlate with

the fraction of T cells which are CD4 T cells. Our panel of stains did not allow us to determine if CD5 expression is altered on CD4 or CD8 T cells from infected adults.

Summary of antigen density changes

Table 3 details the statistics regarding the change in antigen densities associated with HIV infection. Every major subset of PBMC has a demonstrable change in the expression of one or more antigens. While these changes are statistically significant, not all are large in absolute magnitude. Two computed statistics (grouped under 'statistical strength') are shown so that the magnitude of the change can be appraised. The first gives the percentage of the infected cohort which are 'outliers', in that their value is greater than (or less than) 95% of the uninfected population (i.e. approximately the fraction of HIV-uninfected adults whose expression value is >2 SD from the normal distribution). CD62L expression on memory CD8 T cells has the highest index for this statistic: for 94% of HIV-infected adults, the expression of CD62L for these cells falls below the 5th percentile of CD62L expression in uninfected adults. The second statistic is the percentage change in the amount of surface expression between uninfected and infected adults. The largest changes, in relative expression levels, are the 'activation' markers (CD38 and HLA-DR), because their expression on uninfected adults is essentially nil.

The utility of the antigen density values as indicators of disease or status requires that their measurement be reproducible. Table 3 also lists the variability in determination of these antigen densities, quantitated by two independent means: the variability in measurements of a single healthy HIV⁻ adult over a 9 month period; and the variability within 28 HIV-infected adults measured three times each over a 3 week period. The variability is always less than the absolute change in the expression [with the exception of CD8 on one CD8 memory subset, for which the increase is quite small (17%) but still statistically significant ($P < 0.0004$)].

Table 3 also lists some antigen densities which do not differ between infected and uninfected adults. In fact, none of the other antigen densities that are measurable by our panel of stains differed with HIV infection status.

Correlation of antigen density changes with HIV disease

Finally, we determined whether the changes in antigen density that we observed were related to the CD4 counts, as an estimate of HIV disease progression. Figure 4 correlates the antigen density measurements as a function of the absolute CD4 count. Assuming that these cross-sectional data are representative of longitudinal progression, we can break the antigen density changes into three classes based on when they change. The first class includes antigens which change early in disease. For instance, the expression of CD16 on NK cells was low on virtually all HIV-infected adults, independent of their CD4 count. Therefore, the change in the expression of this marker must have occurred prior to the CD4 counts dropping as low as $500/\mu\text{l}$; some markers may even have changed at the point of infection or seroconversion. The second class of antigens is exemplified by CD38 expression on CD8 T cells, which progressively increases during all stages of disease. The third class includes those that only

Table 3. Antigen density changes associated with HIV infection

Measurement ^a		Antigen density (AD) ^b		Statistical strength		Variability		Time of change ^g	
Cell type	Antigen	HIV ⁻ mean (SD)	HIV ⁺ mean (SD)	>2σ ^c (%)	Chg ^d (%)	HIV ⁻ CV ^e	HIV ⁺ CV ^f	(CD4/μl)	
Subset								500 ... 300 ... 0	
<i>Antigen densities which are significantly changed in infected adults (P < 0.001)^h</i>									
Monocytes	CD16	2.7 (1.0)	4.7 (2.2)	42	74	17	23		
	CD45	160 (35)	230 (35)	42	41	15	9.1		
Lymphocytes (all)	CD45	105 (9.8)	110 (12)	25	8	5.0	7.1		
	CD20	88 (13)	130 (37)	63	42	11	13		
B	CD7	38 (12)	22 (9.4)	30	-43	6.7	13		
	CD16	45 (8.8)	27 (8.6)	69	-40	5.1	11		
NK	CD38	3.2 (1.0)	6.9 (4.1)	54	120	30	15		
	CD7	23 (5.9)	13 (5.5)	73	-45	10	8.1		
T cells (all)	CD5	55 (5.9)	40 (9.4)	70	-27	6.5	10		
	CD38 ^{ij}	4.2 (0.8)	10 (8.1)	67	190	15	17		
CD4 T	HLA-DR ^j	4.5 (3.0)	48 (53)	84	960	16	25		
	CD62L	28 (4.3)	21 (4.6)	65	-25	4.8	10		
Naive ^k	CD62L	23 (5.2)	14 (4.9)	79	-37	7.6	9.5		
	CD8 T	CD8	52 (5.8)	44 (6.4)	39	-16	8.4		8.0
CD8 T	CD38	1.2 (0.1)	2.8 (2.0)	86	130	9.5	16		
	CD38 ^j	3.1 (0.5)	9.7 (5.5)	92	210	12	16		
Naive ^k	HLA-DR ^j	5.5 (3.0)	17 (9.3)	73	210	12	14		
	CD62L	30 (3.6)	19 (4.5)	83	-38	6.7	12		
M2 ^k	CD8	76 (6.6)	70 (11)	35	-9	4.9	9.4		
	CD62L	22 (6.9)	12 (2.0)	94	-45	11	5.6		
M3b ^k	CD8	57 (13)	67 (10)	20	17	4.9	7.9		
	M1+M2 ^k	CD11a	37 (5.9)	43 (5.7)	16	17	8.2	5.9	
<i>Antigen densities which are unchanged in infected adults</i>									
Monocytes	CD14	58 (12)	60 (14)			16	13		
	HLA-DR	10 (4.1)	8.9 (4.0)			19	27		
B	HLA-DR	24 (7.7)	23 (7.2)			17	19		
	CD4 T	CD3	61 (7.0)	60 (11)		8.8	12		
T	CD4	43 (2.6)	42 (6.0)			4.4	8.6		
	M1 ^k	CD4	61 (11)	58 (14)		16	19		
CD8 T	CD3	49 (5.3)	49 (7.1)			9.9	8.1		
	Naive ^k	CD45RA	81 (9.6)	79 (13)		8.4	9.4		
M1 ^k	CD8	75 (8.1)	73 (7.6)			8.4	7.5		
	M2 ^k	CD8	78 (7.9)	78 (8.3)		5.2	7.1		
M3a ^k	CD8	78 (8.0)	75 (8.4)			5.7	7.9		

^aThe antigen and on which cell it is quantitated. The antigen densities (AD) are expressed as relative fluorescence units. Their absolute value depends on a number of factors, including the specific fluorophore used, the number of fluorophores/protein and the detection efficiency. Thus, absolute values for different antigen measurements are not comparable.

^bAD is computed as the median (50th percentile) fluorescence for intensity of a given antigen on a given subset of cells, except as noted below (i). Shown are the mean (SD) of the median fluorescences for the infected and uninfected populations. For all measurements, at least 30 uninfected and 120 infected individuals were averaged.

^cThe percentage of HIV-infected individuals whose AD falls outside the values for 95% (~2 SD) of the uninfected cohort, i.e. the percentage of 'outliers' from the distribution for HIV⁻ adults.

^dThe percentage change in AD in absolute terms (0% is no change). A negative value indicates that the expression of the given marker is lower in infected individuals.

^eThe coefficient of variation (mean divided by SD × 100%) in the AD for a single uninfected individual measured eight times over a period of 9 months.

^fThe coefficient of variation in the AD for HIV-infected individuals measured three times each (over a period of 3 weeks), averaged over 28 individuals.

^gQualitative, graphical representation of the stage (defined by CD4 count) at which the AD displays the greatest amount of change: unshaded, no change; lightly shaded, slight change; darkly shaded, largest change in fluorescence intensity.

^hHIV⁺ versus HIV⁻, two-sample Wilcoxon rank test.

ⁱCD4 cells expressing so much CD38 that its fluorescence is off-scale (usually <1–5% of CD4 cells) were excluded from analysis. This subpopulation is distinct from the majority of CD38-bearing cells.

^jFor these antigens, the 90th percentile (rather than median) was computed.

^kCD4 and CD8 T cell subsets are defined as follows: naive (CD45RA⁺CD62L⁺); memory M1 (CD45RA⁻CD62L⁻); memory M2 (CD45RA⁻CD62L⁺); memory M3a (CD45RA^{dim}CD62L⁻); and memory M3b (CD45RA^{br}CD62L⁻).

change significantly at late stages, e.g. CD38 expression on CD4 T cells. Although there is a small (and statistically significant) increase in all HIV-infected individuals, it is the individuals with <100 CD4 T cells/μl who show the greatest increase in CD38 expression on CD4 T cells.

Discussion

In this report we demonstrate that the phenotype of virtually every PBMC subset is altered in HIV-infected individuals. This altered phenotype is evidenced by a change in the expression

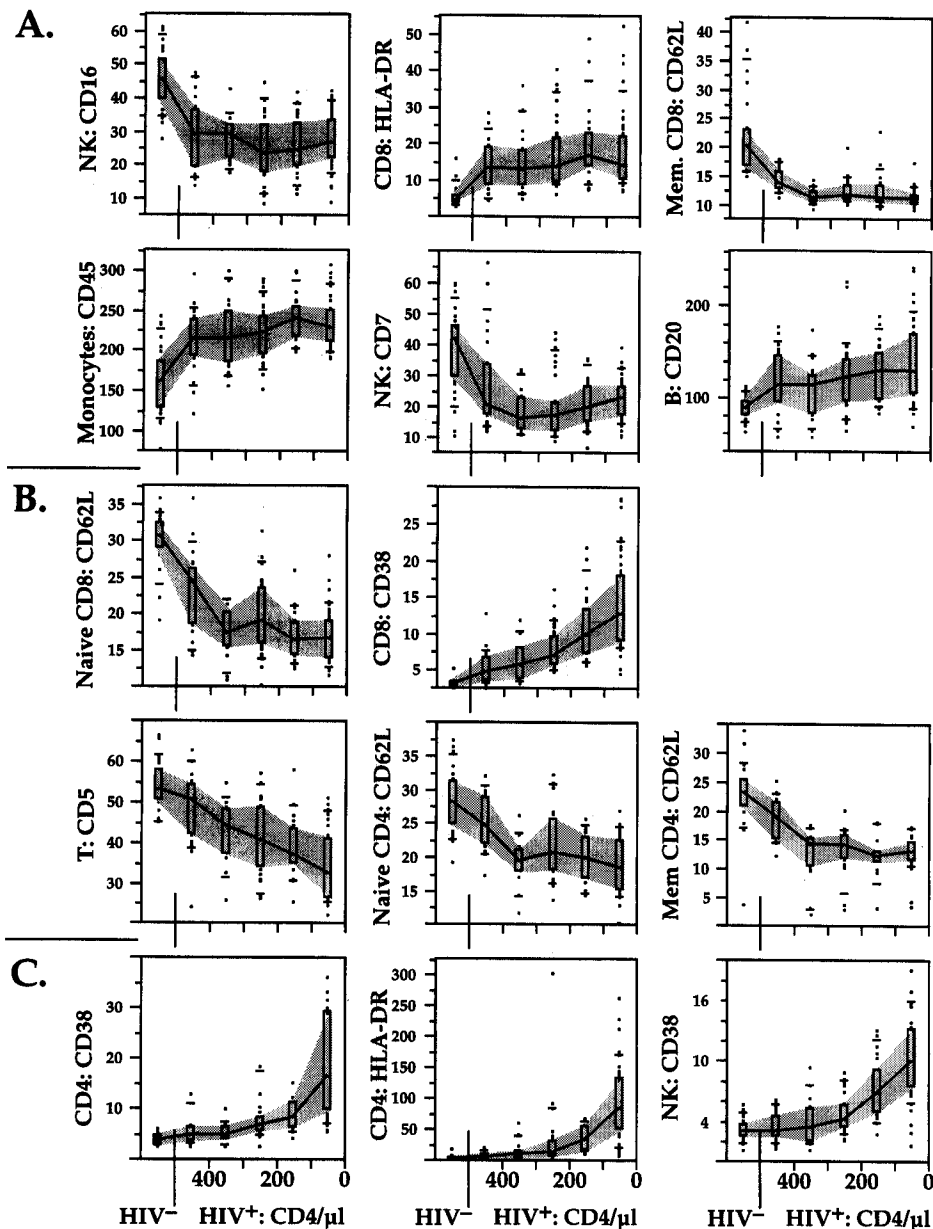


Fig. 4. Significant antigen density changes associated with HIV disease. Fourteen of the antigen density changes described in Table 3 are shown as a function of disease stage (as measured by CD4 count). The four examples from Fig. 1 are duplicated here for reference. For clarity, the individual points under the interquartile box plots are hidden. The plots are loosely grouped according to which stage most of the change in the antigen density occurred at. (A) Early, >400 CD4/ μ l; (B) throughout disease; (C) late, <200/ μ l. The vertical axes are labeled as 'cell type': 'antigen', which refers to the median fluorescence of the antibody against each particular antigen for each particular cell type. (Note: 'Mem' = memory; for CD62L expression on memory cells, a gate was defined for CD45RA⁻CD62L⁺ cells in either CD4 or CD8 lineages.)

of cell surface markers: some are increased whereas others are decreased. The changes in expression are not large in absolute terms: most are <50%. However, the expression of these markers is remarkably constant among healthy uninfected adults (Table 3), such that even small changes are statistically very significant and can be readily detected as unusual.

Our data are from the measurement of fluorescence intensities, and we did not attempt to convert these measurements

to absolute numbers of molecules per cell. This conversion is a difficult one, as evidenced by the fact that there are no uncontroversial methods for performing it. In any case, the conversion will always be a simple multiplication, i.e. the fluorescence is directly proportional to the number of surface antigens. Thus, the values expressed in Table 3 are true antigen densities even though the units are not molecules per cell. The crucial conclusions are derived from the comparisons of relative antigen density.

Comparison of antigen density measurements by different laboratories on different instruments and using different monoclonal reagents will require standardization of the measurement. While detailed discussion of this is beyond the scope of our report, such standardization will necessarily include reference reagents (for comparing different lots of monoclonal reagents) as well as reference fluorescence detection standards (e.g. combinations of fluorescent microspheres which cover the range of measured fluorescence intensities). The latter will standardize instrument configurations (including detection efficiency); the former will standardize staining configurations.

Antigen densities as potential surrogate markers?

Accompanying HIV disease is a severe dysfunctionality in most compartments of peripheral blood cells. It is very likely that alterations in the functionality of the immune system due to defects in individual subsets is a primary contributor to the progression of disease. Therefore, it is crucial to be able to determine the functional status of the immune system and its components in order to design appropriate therapeutic strategies. To date, these studies have been done by performing cumbersome functional assays on a specific subset of cells. Unfortunately, determination of the functional capacity of all the subsets is prohibitive, both in terms of time and reagent requirements, and in terms of the available sample size (blood volume) required to make these measurements. However, it is possible that surrogate markers of the functional capacity of PBMC subsets can be found. We propose that the frequency and phenotype (i.e. antigen densities) of cell populations may provide such surrogates.

Indeed, it is likely that changes in antigen densities similar to those we have observed for HIV may be found in a variety of immunopathogenic diseases. These types of quantitative measurements may be of general utility in the assessment of the functionality of an immune system.

Giorgi and colleagues have demonstrated that the expression of CD38 and HLA-DR on CD8 T cells is an important clinical correlate to HIV disease (8): patients who expressed primarily HLA-DR⁺ CD8 T cells had a much better prognosis than those with CD8 T cells expressing CD38. Quantitation of cells bearing these markers is made difficult by the continuity of the distribution with non-expressing cells. Defining positive versus negative cells is somewhat arbitrary, and depends heavily on controls (isotype staining) as well as the relative brightness of the conjugates and the sensitivity of the flow cytometer. Our approach was to estimate the relative expression by quantitating the 90th percentile of fluorescence for the subset (e.g. CD8 T cells). This value is very sensitive to the presence of low levels of activated cells, but is still robust in terms of insensitivity to outliers that skew mean values. It is likely that more advanced techniques for measuring antigen density distributions (e.g. multiparametric fits) will provide better statistics.

Potential functional correlates

We found that CD62L expression is significantly reduced on all T cell subsets that normally express this antigen (although sufficient CD62L remains to distinguish the expressing cells from the CD62L⁻ cells). CD62L is specifically down-regulated

immediately after stimulation of T cells through a specific proteolytic event (34). It is possible that the decreased expression reflects a low-level pan-T cell activation. Our panel did not allow us to determine if expression of this marker is reduced on other (non-T) PBMC subsets. These findings suggest that there may be significantly elevated levels of serum CD62L due to down-regulation by hydrolysis—akin to the increased levels of serum β_2 -microglobulin. Since CD62L is central to lymphocyte homing, altered expression of this molecule could lead to altered lymphocyte recirculation.

CD7 expression is also significantly reduced on all T cells and NK cells, and CD16 was significantly reduced on NK cells. Cross-linking of CD16 on NK cells induces calcium mobilization and other signaling events (reviewed in 35). CD7 can also transduce signals into either NK cells (36) or T cells (37–39). Thus, it is possible that the functional defect of NK cells may correlate with (or even be caused by) the reduced expression of CD7 and CD16 on these cells. It is interesting that the expression of CD16 on granulocytes, which is GPI-linked (in contrast to NK cell expression) and does not transduce signals (35), was unchanged (data not shown).

HIV infection of T cells has been shown to result in down-regulation of CD4 surface expression, through the specific interaction of virion proteins and CD4 (40). We could find no reduction in the expression of CD4 on T cells (Table 3), nor on monocytes (data not shown), in most HIV-infected adults. Since in most infected individuals the proportion of infected CD4⁺ cells is quite low, this is not surprising. However, there were six individuals from our cohort with unusually low CD4 expression (<32; cf. Table 3). Of these, five had CD4 counts below 15/ μ l and the other had 300/ μ l. It remains to be determined whether an usually low CD4 expression is correlated with a high proportion of HIV-infected cells.

Underlying causes of antigen density changes

A change in antigen density on a population of cells can arise from either of two independent mechanisms: a change in the representation of underlying subsets that express the antigen at different levels, or a change in the regulation of expression of the antigen on a cellular level. In the case of CD5 expression on T cells, change in the representation of underlying subsets (CD4 and CD8 T cells) accounts for a majority of the change (Figure 3). It is quite possible that altered expression of antigens on other subsets reflects a change in the representation of heretofore unknown subpopulations within those subsets.

Alternatively, the change in density may reflect a difference in the regulation of expression of such an antigen on a per-cell basis. This change may well reflect functional differences (if not even cause them). For instance, increased expression of CD45, a tyrosine phosphatase involved in many signal transduction pathways, on monocytes could have a significant impact on the responsiveness of these cells to stimuli; down-regulation of CD62L could impair homing of T cells bearing this integrin. Certainly, as discussed above, decreased expression in the signaling molecules CD7 and CD16 could significantly alter the functional responses of NK cells.

In any case, the quantitation of these antigens may provide useful insights into the observed functional defects of PBMC in HIV-infected adults. As our cross-sectional study suggests,

these measurements may also provide a surrogate for functional assays, allowing for much easier determination of the immunocompetence of an individual. Measurement of antigen density is a window into the functional state of cells that cannot be obtained by simply measuring their frequency. Finally, since some antigen density measurements correlate with disease progression, they may provide additional useful information for clinical diagnosis.

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Abbreviations

deficient RPMI biotin- and flavin-deficient RPMI 1640 medium

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