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CD4 and CD8 T cells with high intracellular glutathione levels are selectively lost as the HIV infection progresses

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Abstract

Maintenance of intracellular glutathione (GSH) levels has been implicated in blocking cytokine-stimulated HIV replication *in vitro*, in both acute and latent infection models. We demonstrate here that subsets of human peripheral blood mononuclear cells differ substantially in mean GSH levels, as measured on a cell-by-cell basis with the fluorescence-activated cell sorter (FACS): B cells have the lowest GSH levels; T cells are intermediate; and monocytes and macrophages have the highest levels. Furthermore, GSH levels subdivide the CD4 and CD8 T cell subsets into two classes each: high- and low-GSH cells, which cannot be distinguished by cell size or by currently known surface markers. Significantly, the high-GSH T cells are selectively depleted early during the HIV infection, and are effectively missing in all ARC and AIDS patients.

Introduction

Glutathione (GSH) is a tripeptide found in all animal cells and most plants and bacteria at relatively high concentrations (~1 – 10 mmol). It is an extremely important molecule from several standpoints: it is a cofactor for several enzymes; it reduces protein disulfides and thereby regulates sulfhydryl-dependent enzymes; it is essential for the synthesis of DNA precursors; and, perhaps most importantly, it protects cells against oxidizing agents, free radicals, and reactive metabolic intermediates ('oxidative stress') that damage DNA and other cellular components (1).

Lymphocyte function is particularly dependent on GSH levels. Soluble thiols have long been known to contribute to lymphocyte growth and activation (2), e.g. murine B and T cell lines grown *in vitro* often require the presence of 2-mercaptoethanol in the culture medium. Furthermore, several studies demonstrate that T cell function is correlated with intracellular GSH levels. For example, lymphocytes depleted of soluble GSH by 2-cyclohexene-1-one are strongly inhibited in their response to lectins (as mitogens) (3); L-2-oxothiazolidine-4-carboxylate, which increases GSH levels, acts synergistically with concanavalin A to stimulate T cells (4); and, as Droge *et al.* have demonstrated, increasing GSH levels in mice augments the *in vivo* activation of cytolytic T cells (5).

In studies presented here, we use multiparameter FACS (fluorescence-activated cell sorter) analyses to measure intracellular GSH levels (6) in human peripheral blood mononuclear cell (PBMC) subsets that are defined by concomitant staining with fluorescently conjugated monoclonal antibodies directed against cell surface antigens. Intracellular GSH levels in these studies are revealed by incubating cells with the fluorogenic reactant monochlorobimane (MCB). MCB is coupled to GSH by glutathione-S-transferase and thereby becomes fluorescent. The contribution of fluorescence from reaction with other cytoplasmic thiols is minimal; the resulting intracellular bimane fluorescence correlates linearly with cellular GSH levels (6,7).

Using these methods, we have shown that PBMCs from HIV-infected individuals demonstrate a decrease in intracellular GSH levels that is most pronounced in the CD4 and CD8 T cell subsets. [Analysis of 123 HIV+ individuals in various stages of disease, including GSH levels and subset analysis for all PBMC subsets, is beyond the scope of this paper and will be published elsewhere (F. J. T. Staal, M. Roederer, L. A. Herzenberg *et al.*, submitted for publication).] Decreased thiol levels, especially GSH, have previously been demonstrated in plasma, unfractionated leukocytes, and lung epithelial lining fluid from HIV-positive individuals (8,9), even when asymptomatic. However, these studies were unable to determine whether the observed intracellular decreases were evenly distributed across all cells, whether they were due to specific decreases in particular subsets, or whether they were due to the selective loss of a high-GSH-containing subpopulation. Data presented here underscore the importance of resolving these possibilities.

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Results and discussion

Examination of GSH levels in human leukocytes from normal individuals reveals a 10-fold variation in the amount of intracellular GSH in total PBMC taken as a whole (Figs 1–3). Much of this heterogeneity, however, is due to differences in GSH levels between T cells, B cells, and monocytes. As Fig. 1 shows, the variation within each of these subsets tends to be considerably less than the whole. The pattern of intracellular GSH levels shown in Fig. 1 (i.e. B < low-GSH T < monocyte/macrophage < high-GSH T) is representative of all samples of freshly isolated PBMC from normal subjects.

This pattern observed with human PBMC is different from that observed with murine PBMC. Murine T cells have less GSH than B cells. In addition, murine T cells show considerably less heterogeneity in GSH levels within the CD4 and CD8 subsets (M. Roederer *et al.*, in preparation).

Surprisingly, the human CD4 and CD8 T cell subsets are each divided into two classes, one of which contains cells with more than twice as much intracellular GSH as cells in the other (Figs 1 and 2). Figure 2 shows several examples of these classes for normal individuals. Both the CD4 and CD8 T cells show considerably more heterogeneity in intracellular GSH than the other leukocyte subsets. In 7 of 15 individuals, CD8 T cells clearly resolve into two separate classes; in the other individuals, both classes are detectable, but the overlap precludes clear resolution.

CD8 is expressed at slightly lower levels (up to 20% less CD8)

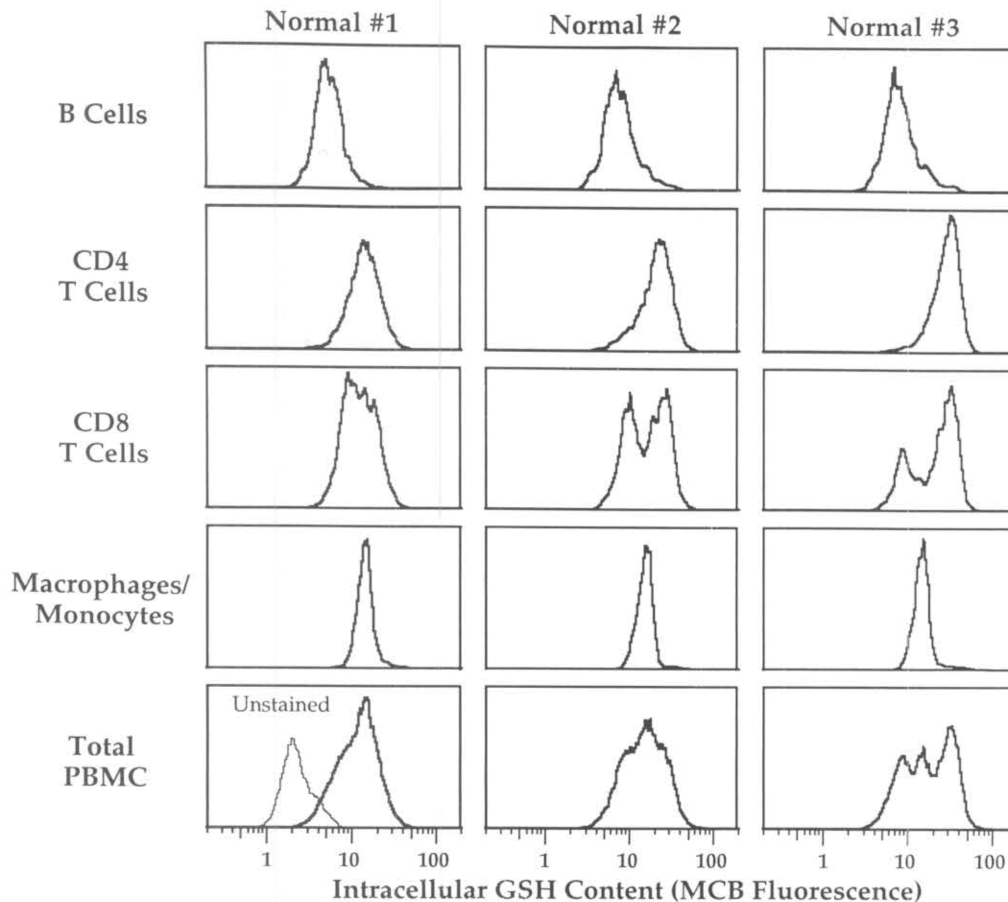

Fig. 1. Human leukocyte subsets have distinct intracellular GSH levels. One-dimensional histograms of MCB fluorescence (intracellular GSH level) are shown for surface-antigen-defined leukocyte subsets, for three individuals. B cells have the lowest intracellular GSH; T cells have a very heterogeneous level extending from just above B cells to well above the macrophage/monocyte level (see Fig. 2; the same three individuals are shown). Methods: fresh heparinized blood was fractionated on Ficoll-Paque to obtain PBMC. These were stained in 1 ml of staining media (SM: 10 mM HEPES, 0.1% NaN_3, in deficient RPMI) with MCB. MCB (10 mM in ethanol) was added to a final concentration of 40 μM for 10 min at room temperature. At the end of the incubation, 10 ml of ice-cold SM was added to quench the reaction; the sample was underlayered with serum and the cells recovered by centrifugation. Cells were then divided into individual samples for antibody staining. Fluorescein- and phycoerythrin- (PE) conjugated antibodies (kind gift of Becton Dickinson, San Jose, CA) were added at the appropriate dilutions and incubations were carried out for 15 min on ice in SM. Cells were washed twice, and then analyzed on a FACStarPlus (Becton Dickinson) equipped with two argon ion lasers. The primary laser was tuned to 488 nm; the secondary to 361 nm. Five parameters were collected for each cell: forward- and side-scattered light, and the fluorescent emission of fluorescein (515–545 nm), PE (562–588 nm), and MCB (excitation at 361 nm, emission at 515–565 nm). Hardware compensation was used to correct for spillover between fluorescein and PE. Software gating was used to obtain the histograms for each leukocyte subset, which were defined as follows: B cells, $CD20^+$ small cells; CD4 T cells, $CD4^+CD8^-$ small cells; CD8 T cells, $CD4^-CD8^+$ small cells (NK cells expressing low levels of CD8 were excluded); monocytes and macrophages, $CD14^+$ large cells; total PBMC, not gated by fluorescence or cell size. Cell types were also sorted and analyzed for absolute GSH levels by HPLC. Consistent with previous evidence (6,7), these determinations verified that the MCB fluorescence reflected intracellular GSH levels (data not shown).

in the high-GSH subset in roughly half of the individuals studied. When present, this difference makes it easier to distinguish the two classes (Fig. 2, middle panel). Aside from this difference, we have not as yet been able to find any flow cytometric marker that distinguishes the high- and low-GSH T cells; i.e. the differences in GSH levels in these T cells are not simply due to differences in cell volume since forward and orthogonal light scatter measurements do not reveal any size or morphological differences. Similarly, using three-color analysis, we find that surface markers that distinguish functionally distinct T cells (e.g. activated versus resting) do not distinguish the low- and high-GSH T cells. These include: CD45RA, a marker that distinguishes between memory and virgin T cells; IL-2 receptors (CD25) and transferrin receptors, which distinguish activated from resting T cells; and CD7, HLA-DQ, and HLA-DR [note that *in vitro*, T cell activation leads to a decrease in GSH (10)]. CD3 (T cell receptor), CD4, CD5, CD26, CD27, and CD28 surface antigens also do not distinguish the GSH-defined classes.

In some individuals, there is a clear distinction based on intracellular GSH for the high- and low-GSH T cell classes; however, in many individuals there is a continuous distribution of intracellular GSH content, with no clearly visible subpopulations (Fig. 2). This is to be expected when the mean content for the two classes is close, and the frequency of cells in one of the classes is substantially higher than in the other (i.e. a shoulder appears in the distribution of GSH, indicating the presence of two adjacent, overlapping populations). The lack of resolution of these peaks increases the error in quantitating the relative frequencies of the classes. Nevertheless we have found that, although the frequencies of the GSH-defined T cell classes vary considerably among normal individuals (Table 1), the relative frequencies of the classes in the same individual tend to remain constant for at least several months.

Measurement of GSH levels in the T cell classes shows that the amount of intracellular GSH is highly conserved between individuals. In fact, this is true for all subsets of leukocytes in PBMC, i.e. the absolute intracellular GSH level in any given leukocyte subset (and class) varies $<15\%$ among 15 normal individuals (Fig. 3). Furthermore, preliminary evidence indicates that there is a strong correlation between the GSH levels among different lymphocyte subsets within a given individual (e.g. the highest correlation, $r = 0.88$, was for GSH levels in total CD4 versus total CD8). Thus, GSH levels appear to be precisely (and perhaps coordinately) regulated in subsets of PBMC.

GSH levels in human immunodeficiency virus (HIV)-infected individuals, in contrast, do not appear to be precisely regulated, at least in terms of the representation of the high-GSH

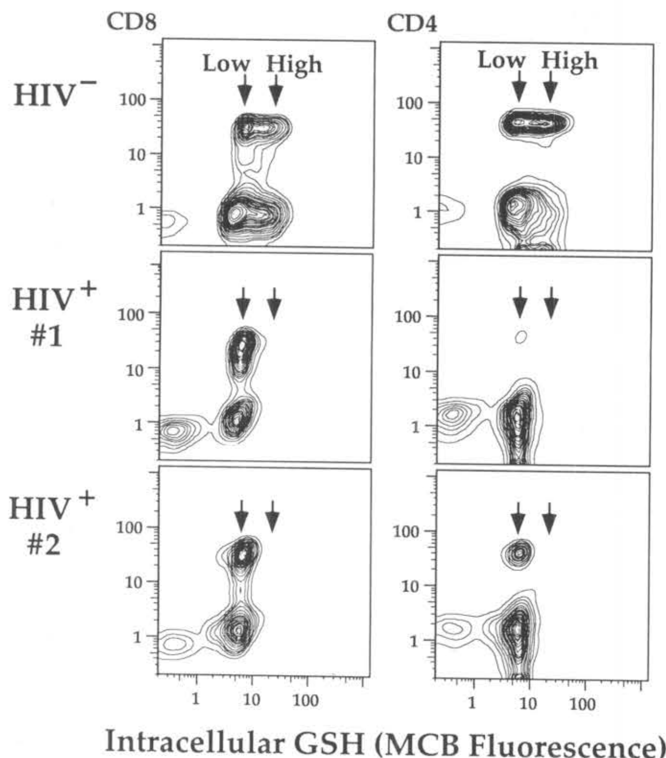

Fig. 2. Intracellular GSH levels subdivide T cells into classes. CD4- and CD8-bearing T cell subsets are resolved in two classes by MCB (low and high levels of GSH). In the two-parameter 'probability' contour plots shown, the number of contours in a given area is proportional to the frequency of cells within that area (5% per contour) (23). The three individuals shown range from a relatively low proportion of the high-GSH-containing subsets (top) to a relatively high proportion (bottom). There is an excellent correlation ($r = 0.85$, $n = 15$; $P < 0.001$) between the ratios of the frequencies of low- and high-GSH CD4 T cells and low- and high-GSH CD8 T cells (the frequency of high-GSH cells in each lineage was determined on the basis of a constant threshold value for GSH which demarcates the classes in those individuals in which the two classes are visually resolved). High- and low-GSH T cells were sorted by FACS and analyzed by HPLC for absolute GSH content to confirm previous findings (6,7) demonstrating that the differences in MCB fluorescence reflect differences in intracellular GSH content, and that GSH-S-transferase activity, required for coupling of MCB to GSH, is not a limiting factor in these experiments (data not shown).

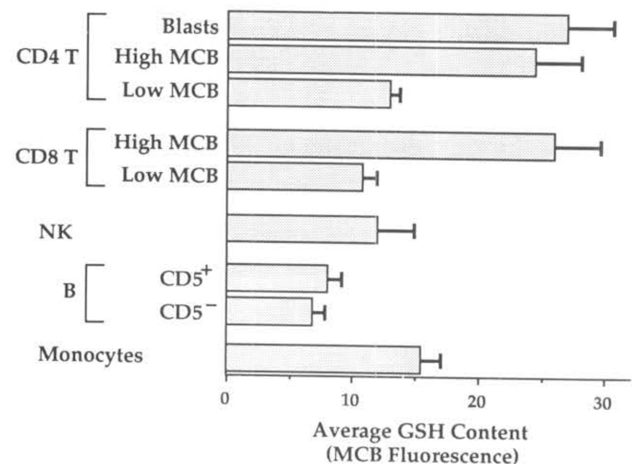

Fig. 3. GSH levels are consistent among individuals. The GSH levels were determined for 15 healthy volunteers using the MCB assay. All samples were stained simultaneously and treated identically. The populations were defined as follows: B cells, CD20+; NK cells, CD7+ CD16+; monocytes, CD14+. CD4 T cells and CD8 T cells were defined by the presence of the respective marker and the absence of the other; furthermore, large cells were excluded. CD4 blasts were defined as large cells that were CD3+CD4+CD5+. Low- and high-GSH T cells were defined on the basis of a constant threshold GSH value that split the T cells in those individuals with well-resolved populations. The median MCB fluorescence for each subset is displayed; error bars represent 1 SD for the 15 samples.

T cells. Individuals with AIDS (acquired immune deficiency syndrome) or ARC (AIDS-related complex) have sharply diminished frequencies of cells in the high-GSH T cell classes (Table 1 and Fig. 4). In fact, high-GSH T cells in both CD4 and CD8 subsets are the first to be lost when the HIV infection becomes manifest and are often gone well before AIDS patients lose the majority of their CD4 cells. The disappearance of these cells is reflected by decreases in the mean GSH levels for the overall CD4 or CD8 subsets (F. J. T. Staal *et al.*, in preparation).

Table 1. High-GSH-containing T cells are depleted during the course of AIDS

Subset	High-GSH cells in T cell subsets (%)		
	Normal	Asymptomatic and ARC	AIDS
CD4	74 ± 18 (48 – 96)	14 ± 10 (4.6 – 26)	11 ± 7.8 (0 – 24)
CD8	59 ± 15 (35 – 85)	7.0 ± 9.0 (1.3 – 26)	5.9 ± 11 (0.4 – 38)

The percentage of cells in each subset with high GSH was estimated from multiparameter FACS analysis (9). Entries represent the mean ± SD (number of individuals: normal, 15; asymptomatic and ARC, 7; AIDS, 11). The ranges are given in parentheses below each average. The average values for asymptomatic + ARC and AIDS are significantly different from normals ($P > 95\%$) but are not significantly different from each other (comparisons by the Scheffe *F*-test).

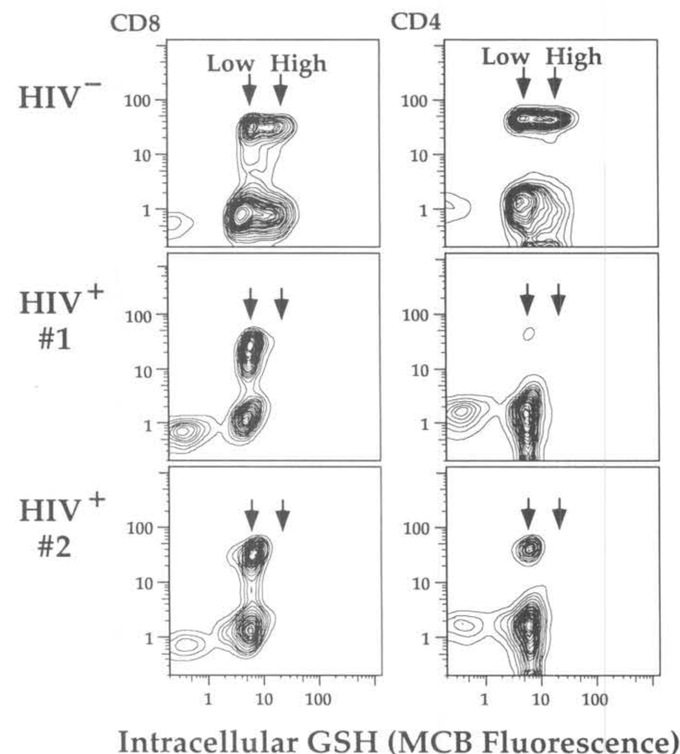

Fig. 4. HIV+ individuals selectively lose high-GSH T cells. PBMC from two persons infected with HIV (middle and bottom panels) and an uninfected individual (top panels) were stained for GSH, and CD4 (left) or CD8 (right). GSH levels were significantly lower in T cells from virtually all HIV+ individuals studied; the decreases are correlated with the progression of the disease. Clearly, the high-GSH-containing T cell classes are completely lacking in these individuals, as highlighted by the arrows. The two HIV+ individuals were classified as ARC and had no concurrent infections. The three individuals shown here had absolute CD4 T cell counts (cells/μl whole blood) of 806, 304, and 53 respectively, and CD8 T cell counts of 524, 974, and 393 respectively.

The overall decrease in GSH in HIV-infected individuals (8,9) may be due directly to the actions of TNF-α and other inflammatory cytokines which are present at substantially increased levels in these individuals (11 – 15). Since inflammatory cytokines are known to cause oxidative stress (16 – 18), the loss of the high-GSH T cells in these individuals could be due to a higher sensitivity of these cells to oxidative stress, which results in the selective loss (or sequestration out of the periphery) of these cells, or their conversion to cells that are low in GSH. Alternatively, the high-GSH T cells may be developmentally distinct from their low-GSH counterparts within the CD4 and CD8 lineages, and the development or survival of these cells may be limited by the presence of inflammatory cytokines (or other aspects of the HIV infection).

In any case, the overall decrease of GSH in PBMC from HIV-infected individuals, coupled with the selective loss of the high-GSH subsets, correlates with the progressive loss of T cell function in ARC and AIDS patients (19,20). In fact, in view of the sensitivity of T cell (and other lymphocyte) function to GSH levels (3 – 5), the loss of the high-GSH subsets may partially explain the immunodeficiency in these individuals: Fidelus *et al.* have shown that, *in vitro*, increasing intracellular GSH 50% potentiates T cell activation, whereas a 50% depletion can lead to virtually complete inhibition of T cell response to concanavalin A. Furthermore, supplementation of GSH *in vivo* can augment T cell function (5,10). Finally, because the high-GSH classes are selectively lost early in the HIV infection, and low GSH levels promote cytokine-dependent HIV replication (18,21,22), the loss of these cells and the decrease in overall GSH levels may play important role(s) in the evolution of the disease as well as providing a potentially useful prognostic indicator for disease progression.

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Abbreviations

AIDS	acquired immune deficiency syndrome
ARC	AIDS-related complex
FACS	fluorescence-activated cell sorter
GSH	glutathione
HIV	human immunodeficiency virus
MCB	monochlorobimane
PBMC	peripheral blood mononuclear cells
PE	phycoerythrin

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