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Characterization of Circulating $CD4^+$ $CD8^+$ Lymphocytes in Healthy Individuals Prompted by Identification of a Blood Donor with a Markedly Elevated Level of $CD4^+$ $CD8^+$ Lymphocytes

HARRY E. PRINCE,* JONELLE GOLDING, AND JOANNE YORK

Cellular Immunology Laboratory, American Red Cross Blood and Tissue Services, Southern California Region, Los Angeles, California

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During flow cytometric analysis of lymphocytes from healthy donors, we identified a donor (donor A) with 22% $CD4^+$ $CD8^+$ cells (versus values of <4% for 65 other controls). To determine if $CD4^+$ $CD8^+$ cells from donor A and other controls were similar, we first defined the phenotypic profile of control $CD4^+$ $CD8^+$ cells. Enriched $CD4^+$ $CD8^+$ cell populations for 10 controls were prepared by a two-step positive selection scheme with anti-CD4-coated magnetic beads and anti-CD8-coated culture flasks; the selected population averaged 69% $CD4^+$ $CD8^+$ cells and 31% $CD4^+$ $CD8^-$ cells. For all 10 controls, two subsets of $CD4^+$ $CD8^+$ cells, $CD4^{dim}$ $CD8^{bright}$ and $CD4^{bright}$ $CD8^{dim}$, were observed. Phenotypic profiles of these two $CD4^+$ $CD8^+$ subsets were defined by pairing anti-CD8 with other monoclonal antibodies, and the profiles were compared with each other and with those of $CD4^+$ $CD8^-$, $CD4^-$ $CD8^{bright}$, and $CD4^-$ $CD8^{dim}$ cells. $CD4^{dim}$ $CD8^{bright}$ and $CD4^{bright}$ $CD8^{dim}$ cells differed in their proportions of $CD62\text{-}L^+$ cells and in their levels of CD11a and CD2 expression. Both $CD4^+$ $CD8^+$ subsets resembled $CD4^+$ $CD8^-$ cells in CD45RA, CD45RO, and CD25 expression; the comparable $CD4^-$ $CD8^+$ cells in CD62-L expression; and $CD4^-$ $CD8^{bright}$ cells in CD11b, CD16/56, and CD28 expression. CD38 expression in both $CD4^+$ $CD8^+$ subsets was decreased compared with those of other cell subsets. Whereas control $CD4^+$ $CD8^+$ cells averaged 33% $CD4^{dim}$ $CD8^{bright}$, $CD4^+$ $CD8^+$ cells from donor A were >90% $CD4^{dim}$ $CD8^{bright}$. Donor A $CD4^{dim}$ $CD8^{bright}$ cells exhibited proportional decreases in CD25 and CD62-L expression and increases in CD11b and CD54 expression compared with those of control $CD4^{dim}$ $CD8^{bright}$ cells. In response to immobilized anti-CD3, $CD4^+$ $CD8^+$ cells from donor A and a control synthesized DNA and showed an increase in the percentage of $CD25^+$ cells and increased intensity of CD2 expression. These findings show that two subsets of circulating, functionally competent $CD4^+$ $CD8^+$ cells in healthy controls can be identified and also describe a donor with a rare phenotypically aberrant $CD4^{dim}$ $CD8^{bright}$ population.

Although most cortical thymocytes coexpress CD4 and CD8 T-cell differentiation markers, they typically lose either CD4 or CD8 during the maturation process associated with the movement to peripheral circulation (16). The vast majority of circulating T cells are thus $CD4^+$ $CD8^-$ or $CD4^-$ $CD8^+$; however, a small percentage (usually <3%) of $CD4^+$ $CD8^+$ T cells is also found in the circulation (6). Little is known about the maturational steps giving rise to this minor $CD4^+$ $CD8^+$ T-cell population. They no longer express the thymocyte marker CD1a, indicating that they have undergone some degree of maturation (6). Studies by Blue et al. (5, 6) suggest that circulating $CD4^+$ $CD8^+$ cells may represent activated cells. This suggestion stems from in vitro studies showing that mitogen activation of $CD4^+$ $CD8^-$ or $CD4^-$ $CD8^+$ cells produced a subset of $CD4^+$ $CD8^+$ cells; prolonged culture of these $CD4^+$ $CD8^+$ cells resulted in reversion to the original phenotype, indicating that dual expression of CD4 and CD8 represented a transitional phenotype. However, the maturation/activation status and proliferative potential of the small population of circulating $CD4^+$ $CD8^+$ lymphocytes found in healthy individuals have not been assessed.

* Corresponding author. Present address: Microbiology Reference Laboratory, 10703 Progress Way, Cypress, CA 90630. Phone: (800) 445-0185. Fax: (714) 220-9213.

Our interest in $CD4^+$ $CD8^+$ lymphocytes arose from the identification of a healthy blood donor with a markedly increased level of circulating $CD4^+$ $CD8^+$ cells (>20% of lymphocytes). We sought to determine if the $CD4^+$ $CD8^+$ cells from this donor were phenotypically and functionally similar to the $CD4^+$ $CD8^+$ cells found in other donors with normal levels of these cells. Such a comparison required that we first design a method for obtaining a cell population enriched for $CD4^+$ $CD8^+$ lymphocytes and then establish the phenotypic profile and functional characteristics of $CD4^+$ $CD8^+$ lymphocytes from a group of donors with normal levels of these cells. In this report, we present data showing that (i) two subpopulations of $CD4^+$ $CD8^+$ cells, $CD4^{dim}$ $CD8^{bright}$ and $CD4^{bright}$ $CD8^{dim}$, were present in individuals with normal levels of $CD4^+$ $CD8^+$ cells, (ii) only the $CD4^{dim}$ $CD8^{bright}$ subpopulation was increased in the donor with an increased $CD4^+$ $CD8^+$ cell level, and (iii) the increased $CD4^{dim}$ $CD8^{bright}$ cell subset in this donor was phenotypically, but not functionally, aberrant from the comparable subset in donors with normal levels.

MATERIALS AND METHODS

Study subjects. The donor (donor A) with a markedly increased level of $CD4^+$ $CD8^+$ lymphocytes was a healthy 64-year-old white female blood donor recruited to serve as a control in ongoing retroviral studies. A routine phenotypic

analysis of lymphocyte subsets in a lysed whole blood sample revealed 22% $CD4^+$ $CD8^+$ lymphocytes for this donor, compared with values of <4% for 65 other similarly recruited controls. Additional blood specimens were obtained from donor A at 3, 6, 9, and 12 months after the initial visit.

The individuals who served as blood donors for the preparation and characterization of $CD4^+$ $CD8^+$ cells were healthy American Red Cross personnel (five males and five females) with <3% $CD4^+$ $CD8^+$ lymphocytes in lysed whole blood specimens.

Preparation of an enriched $CD4^+$ $CD8^+$ lymphocyte population. Peripheral blood mononuclear cells were obtained from 75 ml of heparinized venous blood by centrifugation over sodium metrizoate-Ficoll solution (Accuprep; Accurate Chemical and Scientific Corp., Westbury, N.Y.) and resuspended in phosphate-buffered saline containing 1% human AB serum (PBS-HAB) at a concentration of 3×10^6 lymphocytes per ml. A small aliquot of cell suspension (approximately 1×10^5 lymphocytes) was used to determine the total percentages of CD4 cells and CD8 cells by routine flow cytometric procedures. Anti-CD4-coated magnetic beads (Dynal, Lake Success, N.Y.) were then added to peripheral blood mononuclear cells at a bead/CD4 cell ratio of 4:1; this mixture was incubated for 1 h at room temperature (RT). Beads (with attached CD4 cells) were then drawn to the side of the tube with a magnet; the residual cell suspension was saved for later use as a population enriched for $CD4^-$ $CD8^+$ cells. Beads were washed twice by resuspension in PBS-HAB followed by application of the magnet. Beads were then resuspended in 0.1 ml of detach-a-bead reagent (Dynal) and incubated for 1 h at RT. Two milliliters of PBS-HAB was added to the bead suspension, and beads were drawn to the side of the tube with a magnet. Released CD4 cells were washed three times with PBS-HAB and resuspended in PBS-HAB at a concentration of 5×10^6 cells per ml. This cell suspension was added to anti-CD8-coated culture flasks (Applied Immune Sciences, Santa Clara, Calif.) at 4 ml of suspension per flask; usually two flasks per donor were required. Flasks were incubated at RT for 1 h; unattached cells were then resuspended by gently rocking the flasks and saved for later use as a $CD4^+$ $CD8^-$ cell population. Following two washes with 4 ml of PBS-HAB, each flask received 4 ml of PBS-HAB and then attached cells ($CD4^+$ $CD8^+$ enriched) were dislodged with a disposable cell scraper (Becton Dickinson Labware, Lincoln Park, N.J.). All three of the resulting cell populations (CD8 enriched, $CD4^+$ $CD8^-$, and $CD4^+$ $CD8^+$) were >90% viable as assessed by trypan blue exclusion.

Phenotypic analysis. Aliquots of 0.05 ml of whole blood were treated with the following monoclonal antibody pairs (all monoclonal antibodies were from Becton Dickinson Immunocytometry Systems, San Jose, Calif.): phycoerythrin (PE)-conjugated mouse immunoglobulin G1 (IgG1) and fluorescein isothiocyanate (FITC)-conjugated mouse IgG1, PE–anti-CD8 and FITC-IgG1, PE-IgG1 and FITC–anti-CD4, and PE–anti-CD8 and FITC–anti-CD4. Following lysis of erythrocytes with 0.15 M ammonium chloride, centrifugation, and resuspension in PBS-HAB, the fluorescence of cells within a lymphocyte gate was assessed with a FACSort flow cytometer (Becton Dickinson Immunocytometry Systems). The first pair was used to optimize fluorescence detectors, the second and third pairs were used to adjust fluorescent compensation, and the fourth pair was used to determine the percentages of $CD4^-$ $CD8^+$, $CD4^+$ $CD8^+$, and $CD4^+$ $CD8^-$ lymphocytes. The absolute number of circulating lymphocytes in lysed whole blood samples was determined by flow cytometry as previously described (14).

Suspensions of enriched $CD4^+$ $CD8^+$ cells, $CD4^+$ $CD8^-$ cells, and $CD4^-$ $CD8^+$ cells were phenotypically characterized by dual-color flow cytometry; the monoclonal antibody pairs utilized consisted of PE- or FITC-conjugated anti-CD8 paired with an additional monoclonal antibody (bearing the reciprocal fluorescent conjugate) recognizing CD4, HLA-DR, CD62-L (Leu-8), T-cell receptor α,β (TCR-α,β), TCR-$\gamma\delta$, CD11a, CD2, CD45RA, CD45RO, CD38, CD11b, CD16/56, CD25, CD54, or CD28. Two additional antibody pairs, PE–anti-CD8 and FITC–anti-CD3 as well as PE–anti-CD3 and FITC–anti-CD8, were included for $CD4^-$ $CD8^+$ cells. The indicated second markers paired with anti-CD8 were selected to yield information on the lineage, maturation/activation status, and function properties of the cells analyzed. CD3, TCR-α,β, and TCR-γ,δ expression is indicative of T-cell lineage (18). CD16/56 expression is characteristic of natural killer cells, including a CD8 cell subset with natural killer activity (9). CD45RA expression is characteristic of naive T cells, whereas CD45RO expression is characteristic of memory T cells (1). CD25 and HLA-DR are activation markers found in association with CD45RO expression (memory cell status) (10, 15, 21); CD38 expression, in contrast, is decreased in association with CD45RO expression by peripheral T cells (15). Expression patterns of the adhesion markers CD62-L (Leu-8 and L-selectin), CD2, CD11a, and CD54 are modulated (CD62-L expression decreased while the expression of all the others increased) by activation, and these same patterns are associated with memory cell status (1, 11). CD11b is a marker of suppressor CD8 cells (8), and CD28 is a costimulatory molecule found on CD4 and CD8 cells with the capacity to respond to mitogenic anti-CD3 monoclonal antibody (3, 4).

After optimizing fluorescence detectors for cells in a lymphocyte gate and adjusting fluorescent compensation, 10,000 ungated events were then collected (if possible) for each tube. The fluorescence (PE-stained cells on the x axis and FITC-stained cells on the y axis) of cells within a lymphocyte gate was analyzed with LYSYS II software. Quadrants were set by using cells treated with PE-IgG2a and FITC-IgG2a for all three cell populations analyzed. In addition, modified quadrants set as previously described (13) were used for further analysis of the $CD4^-$ $CD8^+$ cell population. Briefly, for $CD4^-$ $CD8^+$ cells treated with PE–anti-CD8 and FITC–anti-CD3, the horizontal quadrant line was raised from its regular position to a position just above the $CD8^{dim}$ $CD3^-$ cell subset, separating $CD8^{dim}$ cells from $CD8^{bright}$ cells (>95% $CD3^+$). Similarly, for $CD4^-$ $CD8^+$ cells stained with PE–anti-CD3 and FITC–anti-CD8, the vertical quadrant line was moved to a position just to the right of $CD3^-$ $CD8^{dim}$ cells. Cell fluorescence was then reanalyzed for all monoclonal antibody pairs containing anti-CD8, and the proportion of $CD8^{dim}$ or $CD8^{bright}$ cells expressing the indicated second marker was calculated by the subtraction method previously described (13). An identical modified quadrant approach, based on PE– or FITC–anti-CD8 staining intensity, was also used to distinguish between $CD4^{bright}$ $CD8^{dim}$ and $CD4^{dim}$ $CD8^{bright}$ cells. The difference in anti-CD4 staining intensity for these two subsets was less marked than the difference in anti-CD8 staining intensity but was nevertheless clear when anti-CD4 was paired with anti-CD8.

Activation of $CD4^+$ $CD8^+$ lymphocytes. $CD4^+$ $CD8^+$ cell preparations from donor A and one control donor were resuspended at 2.5×10^5 cells per ml in RPMI 1640 culture medium supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml), glutamine (2 mM), and 10% HAB; 0.2 ml of this cell suspension was added to each of 10 uncoated micro-

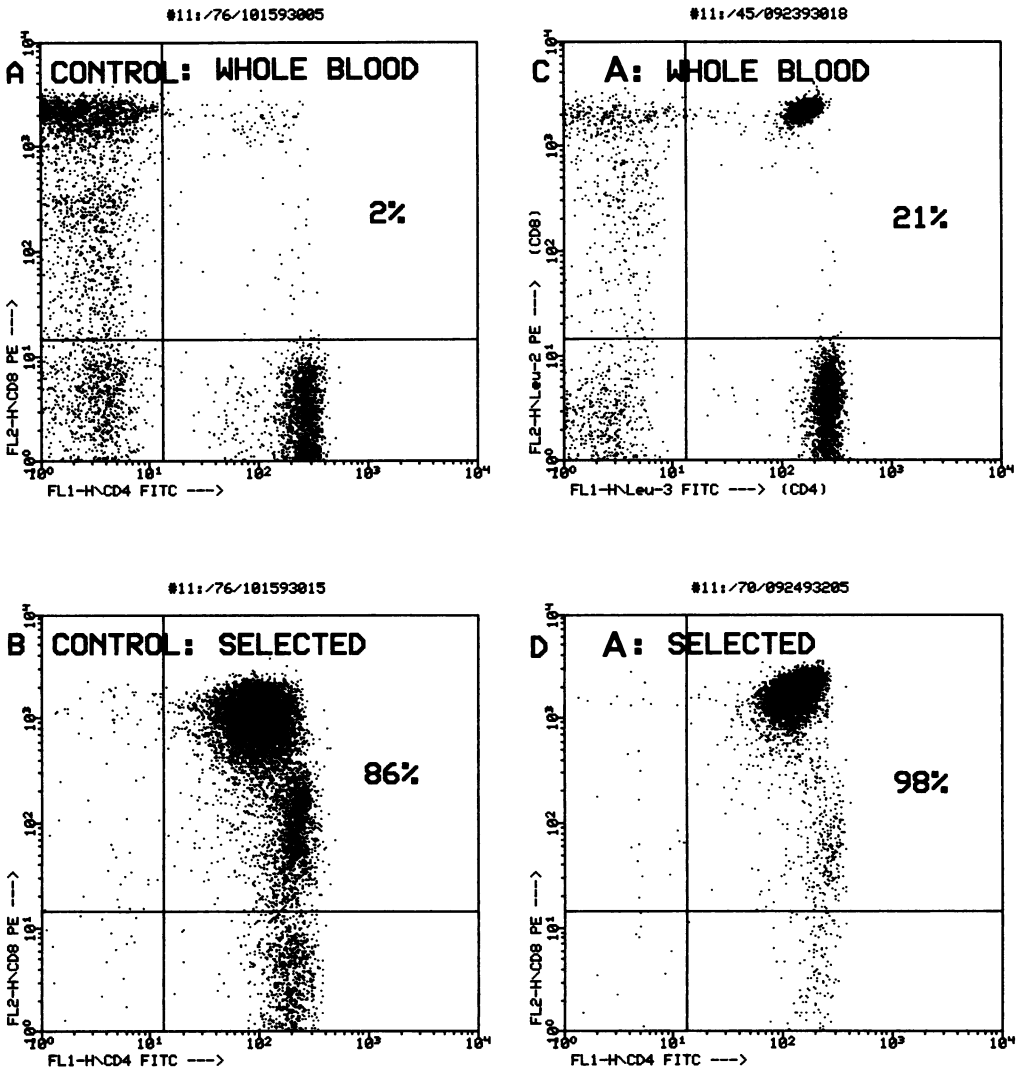

FIG. 1. Flow cytometric dot plots representing $CD4^+$ $CD8^+$ lymphocytes in lysed whole blood (A and C) and $CD4^+$ $CD8^+$-enriched (selected) (B and D) preparations from a representative control (A and B) and donor A (C and D). The percentage of lymphocytes with a $CD4^+$ $CD8^+$ phenotype is indicated in the upper right quadrant of each panel. Visual examination of $CD4^+$ $CD8^+$ cells in all panels, but especially panels B and D, clearly reveals a $CD4^{dim}$ $CD8^{bright}$ subset and a $CD4^{bright}$ $CD8^{dim}$ subset.

titer wells and 10 microtiter wells containing immobilized anti-CD3 monoclonal antibody. These wells were prepared by adding 0.05 ml of a solution of 62 ng of anti-CD3 (Becton Dickinson Immunocytometry Systems) per ml–0.05 M carbonate buffer (pH 9.0) per well, followed by incubation at RT for 90 min; wells were then washed three times with PBS-HAB. Microtiter plates were incubated in 5% CO_2 at 37°C; on day 3 of culture, three wells per culture type received 0.5 μCi of [^{3}H]thymidine, and after an additional 4 h of incubation, cells were harvested and counted for radioactivity. The contents of seven additional wells per culture type were pooled, washed, and stained with the following panel of monoclonal antibody pairs: PE-IgG1 and FITC-IgG1, PE–anti-CD8 and FITC-IgG1, PE-IgG1 and FITC–anti-CD25, PE–anti-CD8 and FITC–anti-CD4, PE–anti-CD8 and FITC–anti-CD25, and PE–anti-CD8 and FITC–anti-CD2. The fluorescence of cells within a scatter gate, including both resting and blastoid cells, was analyzed as described in the previous section with regular quadrant settings.

Statistics. Comparisons of the proportions of various CD4 CD8 cell subsets expressing a given second marker were conducted by the unpaired Student t test. P values were corrected for multiple comparisons by the Bonferoni method; thus, P values were multiplied by 70 (the number of comparisons made). Corrected P values of <0.05 were considered significant.

RESULTS

$CD4^+$ $CD8^+$ cells in donor A and controls. In Fig. 1 are representative flow cytometric dot plots demonstrating the relative percentages of $CD4^+$ $CD8^+$ cells in whole blood and the selected $CD4^+$ $CD8^+$ cell population for a control and donor A. A comparison of Fig. 1A and C clearly shows that the percentage of circulating $CD4^+$ $CD8^+$ cells in donor A was much higher than that in the control and that nearly all of these cells were $CD4^{dim}$ $CD8^{bright}$. Fig. 1B demonstrates that two

TABLE 1. Characteristics of $CD4^+$ $CD8^+$ cells in controls and donor A

Parameter	Value for group or individual					
	Control[a]	Donor A at:				
		Initial visit	3 mo	6 mo	9 mo	1 yr
Whole blood $CD4^+$ $CD8^+$ lymphocytes (%)	1 ± 0.7	22	ND[b]	16	21	20
Absolute no. of:						
$CD4^+$ $CD8^+$	25 ± 22	578	ND	343	448	428
$CD4^+$ $CD8^-$	723 ± 118	1,217	ND	932	1,005	1,081
$CD4^-$ $CD8^+$	551 ± 178	379	ND	410	297	325
$CD4^+$ $CD8^+$ cells within enriched population (%)	69 ± 20	99[c]	99	98	99	91
Enriched $CD4^+$ $CD8^+$ cells $CD4^{dim}$ $CD8^{bright}$ (%)	33 ± 26	90	96	87	96	91
Proportion (%) of $CD4^+$ $CD8^+$ cells expressing:						
HLA-DR	11 ± 7	4	1	2	1	2
CD62-L (Leu-8)	51 ± 14	5	6	8	7	8
TCR-α,β	97 ± 1	99	99	99	98	99
TCR-γ,δ	1 ± 0.6	<1	<1	<1	<1	<1
CD45RA	36 ± 17	35	50	27	28	26
CD45RO	91 ± 5	96	97	97	95	98
CD38	23 ± 8	4	2	4	3	5
CD16/56	10 ± 8	4	2	3	3	3
CD25	29 ± 11	3	1	5	3	4
CD11b	25 ± 19	72	83	76	74	68
CD54	4 ± 4	ND	94	65	71	35
CD28	94 ± 6	94	96	97	94	96

[a] Data are means ± 1 SD for 10 individuals.
[b] ND, not determined.
[c] For the initial visit, cyropreserved peripheral blood mononuclear cells were used to prepare enriched $CD4^+$ $CD8^+$ cells; for all other visits, fresh peripheral blood mononuclear cells were used.

distinct subsets of $CD4^+$ $CD8^+$ cells, a $CD4^{dim}$ $CD8^{bright}$ subset and a $CD4^{bright}$ $CD8^{dim}$ subset, were present in the control.

Data for the entire $CD4^+$ $CD8^+$ cell population in controls and donor A are shown in Table 1. Both the percentage and absolute number of $CD4^+$ $CD8^+$ cells in donor A were markedly increased on all occasions tested compared with the mean values for 10 other controls; however, the absolute levels of $CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$ cells in donor A were not remarkably different. On average, just over two-thirds (69%) of the cells in enriched $CD4^+$ $CD8^+$ cell populations from controls expressed the $CD4^+$ $CD8^+$ phenotype, with the remaining cells expressing a $CD4^+$ $CD8^-$ phenotype (Fig. 1B); in contrast, essentially all cells in the enriched $CD4^+$ $CD8^+$ population from donor A were of the expected phenotype. Over 90% of $CD4^+$ $CD8^+$ cells from donor A expressed the $CD4^{dim}$ $CD8^{bright}$ phenotype, compared with an average of only 33% for the control group. The increased absolute number of $CD4^+$ $CD8^+$ cells in donor A thus mainly represented an increase in the number of $CD4^{dim}$ $CD8^{bright}$ cells rather than $CD4^{bright}$ $CD8^{dim}$ cells.

Also shown in Table 1 are findings from phenotypic studies of the $CD4^+$ $CD8^+$ population as a whole. Virtually all $CD4^+$ $CD8^+$ cells from both the control group and donor A were T cells. However, $CD4^+$ $CD8^+$ cells from donor A were remarkably different from those of the control group at all time points tested in the proportion expressing CD62-L, CD38, and CD25 (all were decreased) as well as the proportion expressing CD11b and CD54 (both were increased).

Phenotypic comparison of $CD4^+$ $CD8^+$ subsets with other subsets defined by CD4 and CD8 expression in the control group. By capitalizing on the differences in the level of CD8 expression, we were able to phenotypically characterize the $CD4^{dim}$ $CD8^{bright}$ and $CD4^{bright}$ $CD8^{dim}$ cell subsets of controls. The results of this characterization and comparisons with other cell subsets defined by CD4 and CD8 expression are shown in Fig. 2 and Table 2. In Fig. 2, the cell subset designations are as follows: A, $CD4^+$ $CD8^-$; B, $CD4^{bright}$ $CD8^{dim}$; C, $CD4^{dim}$ $CD8^{bright}$; D, $CD4^-$ $CD8^{dim}$; E, $CD4^-$ $CD8^{bright}$. For each window, the middle horizontal line represents the mean for the group of 10 controls, the lower horizontal line represents the mean minus 2 standard deviations (SD), and the upper horizontal line represents the mean plus 2 SD. The dots in Fig. 2 represent the values for donor A and should be disregarded for now; these data are discussed below. Table 2 indicates significant (+) and nonsignificant (−) *P* values in pairwise comparisons of the two control group $CD4^+$ $CD8^+$ subsets with each other and with other control group subsets defined by CD4 and CD8 expression.

Combined examination of the findings in Fig. 2 and Table 2 revealed several notable points. First, the $CD4^{bright}$ $CD8^{dim}$ and $CD4^{dim}$ $CD8^{bright}$ subsets significantly differed only in the proportion expressing CD62-L; coexpression proportions of all other markers tested were similar. Second, in comparisons of the $CD4^+$ $CD8^+$ subsets with other subsets, five general patterns (indicated by number in Table 2) for the proportions of cells expressing second markers were apparent. In the first pattern, characterizing HLA-DR and CD54 expression, no notable differences in marker expression by either of the $CD4^+$ $CD8^+$ cell subsets compared with those of the other three subsets ($CD4^+$ $CD8^-$, $CD4^-$ $CD8^{bright}$, and $CD4^-$ $CD8^{dim}$) were observed. In the second pattern, characterizing CD25, CD45RA, and CD45RO expression, both $CD4^+$ $CD8^+$ subsets were most similar to $CD4^+$ $CD8^-$ cells. In the third pattern, characterizing CD11b, CD16/56, and CD28 expression, both $CD4^+$ $CD8^+$ subsets were most similar to $CD4^-$ $CD8^{bright}$ cells. In the fourth pattern, characterizing CD62-L expression, the $CD4^+$ $CD8^+$ subsets were similar to the comparable $CD4^-$ $CD8^+$ subset defined by CD8 staining intensity (i.e., $CD4^{dim}$ $CD8^{bright}$ cells were like $CD4^-$ $CD8^{bright}$ cells, and $CD4^{bright}$ $CD8^{dim}$ cells were like $CD4^-$ $CD8^{dim}$ cells). In the fifth

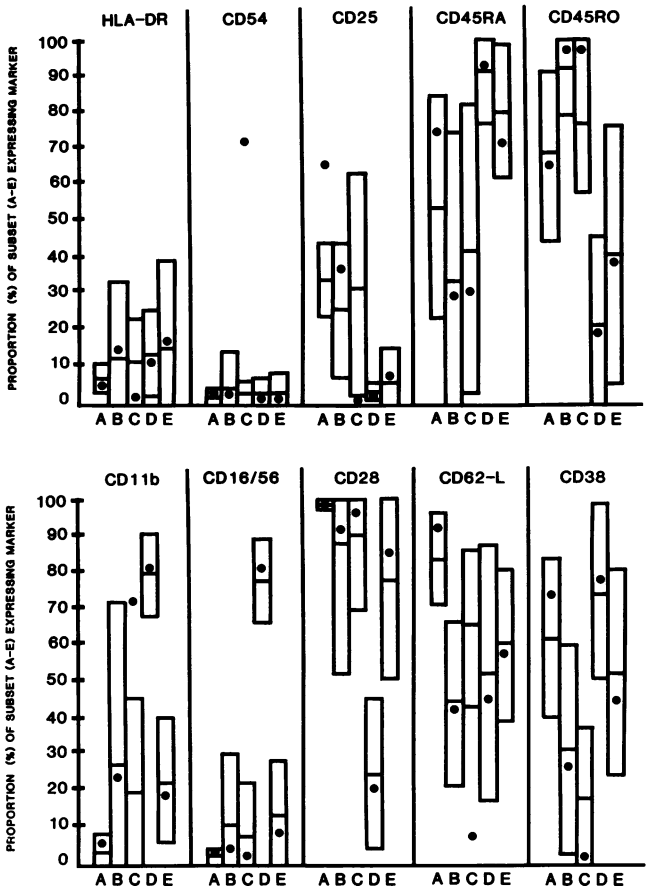

FIG. 2. Phenotypic characteristics of the five lymphocyte populations defined by CD4 and CD8 expression in a group of 10 controls and donor A. The middle horizontal line of each window represents the mean proportion of cell subset A to E expressing the indicated surface marker, the upper horizontal line of each window equals the mean plus 2 SD, and the lower horizontal line of each window represents the mean minus 2 SD. Each dot represents the value for that parameter in that subset from donor A. Subset designations are as follows: A, $CD4^+$ $CD8^-$; B, $CD4^{bright}$ $CD8^{dim}$; C, $CD4^{dim}$ $CD8^{bright}$; D, $CD4^-$ $CD8^{dim}$; E, $CD4^-$ $CD8^{bright}$. *P* values for pairwise comparisons of control group subsets are summarized in Table 3.

pattern, characterizing CD38 expression, both $CD4^+$ $CD8^+$ subsets were notably different from the other three cell subsets. Thus, both $CD4^+$ $CD8^+$ cell subsets resembled conventional CD4 cells in some aspects and conventional CD8 cells in other aspects but were distinctive in CD38 expression.

We also examined CD11a and CD2 expression by the five cell subsets defined by CD4 and CD8 expression in the control group. Figure 3 presents histograms of CD11a (A to E) and CD2 (F to J) expression from a representative control donor. CD11a expression by the $CD4^{bright}$ $CD8^{dim}$ subset was unimodal and similar to that observed with $CD4^+$ $CD8^-$ cells. In contrast, CD11a expression by the $CD4^{dim}$ $CD8^{bright}$ subset was bimodal and similar to that observed with $CD4^-$ $CD8^{bright}$ cells. CD11a expression by the $CD4^-$ $CD8^{dim}$ subset was brighter than those of the other four subsets. CD2 expression by the $CD4^{bright}$ $CD8^{dim}$ subset was essentially unimodal, corresponding to the brighter peak observed with $CD4^+$ $CD8^-$ cells. In contrast, CD2 expression by the $CD4^{dim}$ $CD8^{bright}$ subset was bimodal and resembled the distribution observed with $CD4^-$ $CD8^{bright}$ cells. The $CD4^-$ $CD8^{dim}$ subset contained some $CD2^-$ cells, and the $CD2^+$ cells were broadly distributed.

Aberrant phenotypic profile of $CD4^{dim}$ $CD8^{bright}$ cells from donor A. The dots in Fig. 2 are the values for cells from the 9-month blood specimen of donor A. With only one exception, all the values for $CD4^+$ $CD8^-$ cells, $CD4^{bright}$ $CD8^{dim}$ cells, $CD4^-$ $CD8^{dim}$ cells, and $CD4^-$ $CD8^{bright}$ cells were within the windows defining the ranges of the control group; the one exception, increased CD25 expression by $CD4^+$ $CD8^-$ cells, was also increased in the 1-year specimen (62%). $CD4^{dim}$ $CD8^{bright}$ cells from donor A, however, were different from control $CD4^{dim}$ $CD8^{bright}$ cells in their expression of four additional markers; the proportions expressing CD62-L and CD25 were notably decreased, whereas the proportions expressing CD11b and CD54 were notably increased. The levels of CD11a and CD2 expression by cell subsets from donor A were similar to those of control subsets (data not shown). Further, no expression of the thymocyte marker CD1a in $CD4^+$ $CD8^+$ cell subsets from the 9-month donor A specimen or three control specimens was found (data not shown).

Activation of $CD4^+$ $CD8^+$ cells. As shown in Fig. 4, $CD4^+$ $CD8^+$ cells from both a control and donor A (the same cell populations shown in Fig. 1B and D) incubated with immobilized anti-CD3 exhibited increased CD25 expression (Fig. 4B and D) compared with those of cells incubated in medium alone (Fig. 4A and C). This increase in CD25 expression was accompanied by increased DNA synthesis; the proliferative responses for the cells represented in Fig. 4A to D were 204,

TABLE 2. Comparison of phenotypic markers in CD4 CD8 cell subsets

Phenotypic marker	Pattern	Significance of *P* values[a]					
		$CD4^{bright}$ $CD8^{dim}$ vs $CD4^+$ $CD8^-$	$CD4^{dim}$ $CD8^{bright}$ vs $CD4^+$ $CD8^-$	$CD4^{bright}$ $CD8^{dim}$ vs $CD4^-$ $CD8^{bright}$	$CD4^{dim}$ $CD8^{bright}$ vs $CD4^-$ $CD8^{bright}$	$CD4^{bright}$ $CD8^{dim}$ vs $CD4^-$ $CD8^{dim}$	$CD4^{dim}$ $CD8^{bright}$ vs $CD4^-$ $CD8^{dim}$
HLA-DR	1	−	−	−	−	−	−
CD54	1	−	−	−	−	−	−
CD25	2	−	−	+	+	+	+
CD45RA	2	−	−	+	+	+	+
CD45RO	2	+	−	+	+	+	+
CD11b	3	−	−	−	−	+	+
CD16/56	3	−	−	−	−	+	+
CD28	3	−	−	−	−	+	+
CD26-L	4	+	+	−	−	−	−
CD38	5	+	+	+	−	+	+

[a] By using the unpaired Student *t* test and the Bonferoni method for corrections, corrected *P* values of <0.05 were considered significant (+). −, nonsignificant. The two subsets of $CD4^+$ $CD8^+$ cells, $CD4^{dim}$ $CD8^{bright}$ and $CD4^{bright}$ $CD8^{dim}$, significantly differed only in the proportion expressing CD62-L (data not shown).

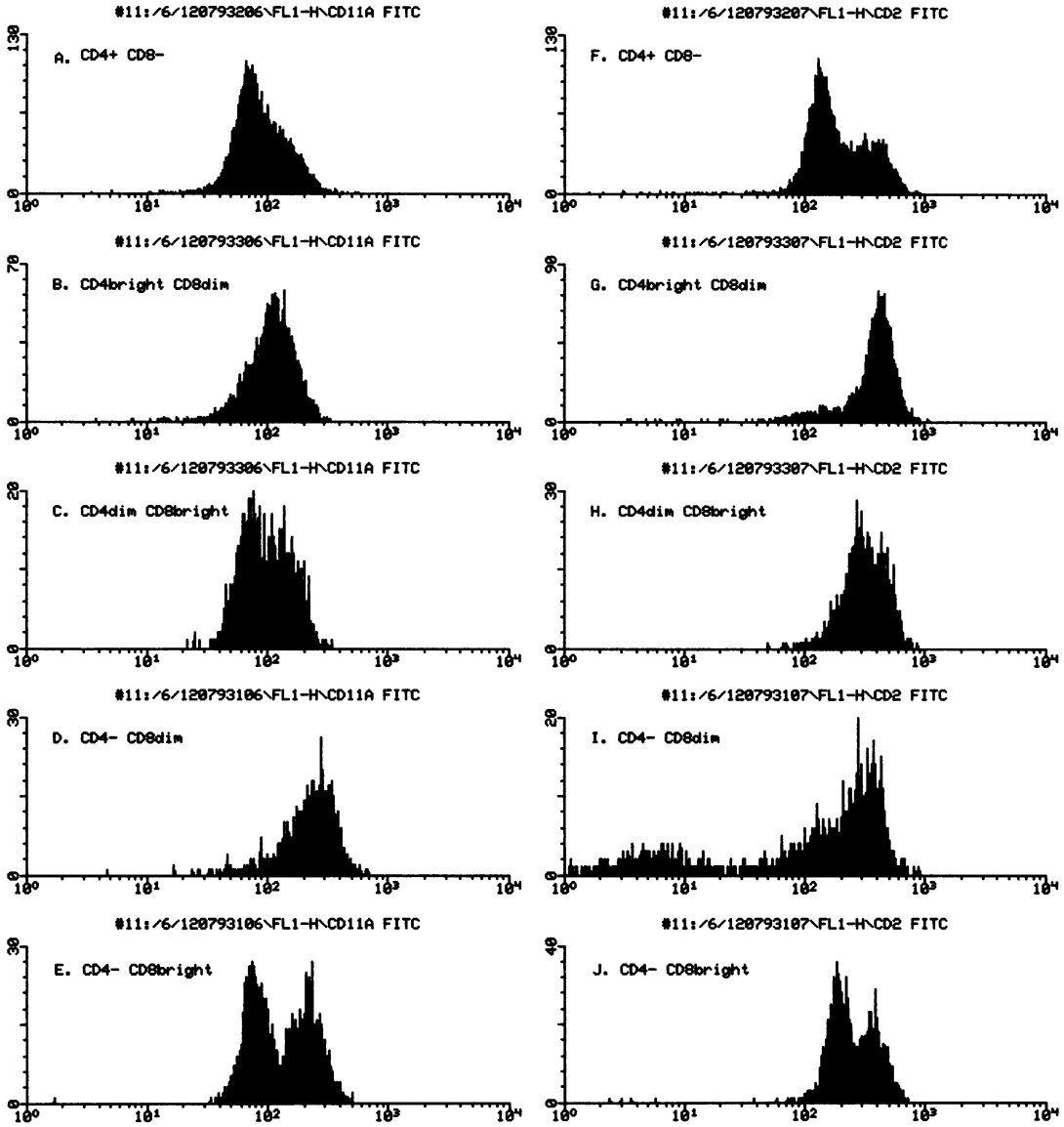

FIG. 3. Histograms demonstrating CD11a (A to E) and CD2 (F to J) expression by CD4 CD8 subsets in the control group. The *x* axis in each panel represents FITC-associated fluorescence on a logarithmic scale, and the *y* axis represents the number of cells per channel. For all cell subsets but $CD4^+$ $CD8^-$, the cells shown fell within a double gate in which the first gate defined lymphocytes on the basis of forward- and right-angle scatter and the second gate defined PE–anti-CD8-labeled cells with the indicated fluorescent intensity (bright or dim). Because the $CD4^+$ $CD8^-$ subset was 99% pure, cells shown in $CD4^+$ $CD8^-$ cell panels (A and F) represent all cells within a lymphocyte gate.

23,518, 116, and 18,228 cpm, respectively (means of triplicate cultures). Visual inspection of the dot plots for activated cell cultures (Fig. 4B and D) revealed that CD25 expression characterized both $CD8^{bright}$ and $CD8^{dim}$ subsets, corresponding to $CD4^{dim}$ $CD8^{bright}$ and $CD4^{bright}$ $CD8^{dim}$ subsets, respectively (data not shown demonstrated that all CD8 expression was associated with $CD4^+$ $CD8^+$ cells). In addition to enhanced CD25 expression, the intensity of CD2 expression by $CD4^+$ $CD8^+$ cells from a control and donor A was markedly increased after culture with immobilized anti-CD3 (Fig. 5). These data indicate that $CD4^+$ $CD8^+$ cells of both controls and donor A behaved as conventional T cells in response to activation by anti-CD3.

DISCUSSION

The findings presented here represent, to our knowledge, the first description of the phenotypic profile of $CD4^+$ $CD8^+$ T-cell subsets from healthy individuals with nonelevated levels of these cells. Two clearly distinguishable subsets of $CD4^+$ $CD8^+$ cells, differing in their relative levels of CD4 and, particularly, CD8 expression, were present. Compared with each other, the $CD4^{bright}$ $CD8^{dim}$ and $CD4^{dim}$ $CD8^{bright}$ subsets were quite similar in their expression of most of the additional markers measured; however, the $CD4^{bright}$ $CD8^{dim}$ subset contained a lower proportion of CD62-L^+ cells and mostly expressed higher densities of CD11a and CD2 compared with those of the $CD4^{dim}$ $CD8^{bright}$ subset. Published

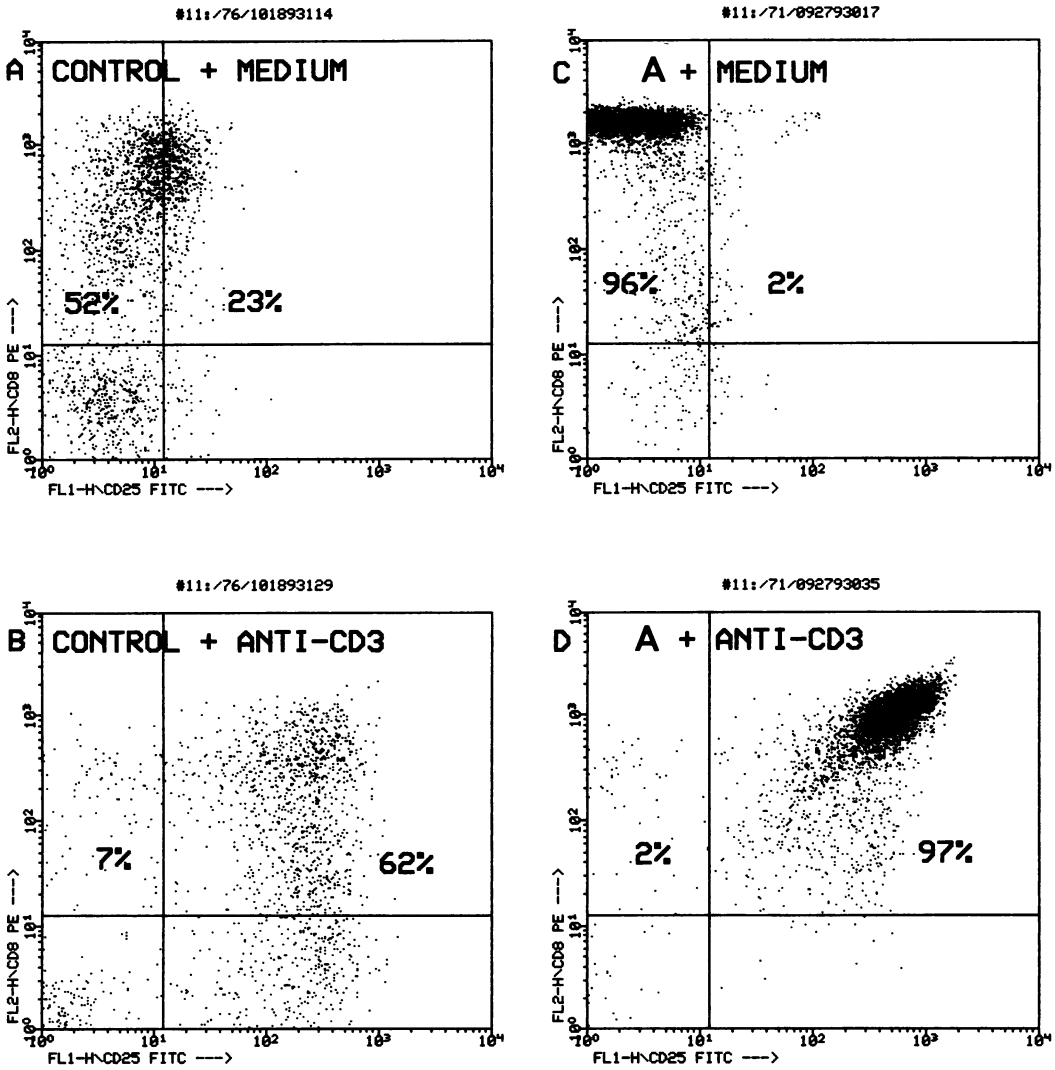

FIG. 4. Influence of activation on CD25 expression by $CD4^+$ $CD8^+$ cells. Enriched $CD4^+$ $CD8^+$ cell preparations from a control (A and B) and donor A (C and D) were incubated with medium (A and C) or immobilized anti-CD3 (B and D) for 3 days. Each value in an upper quadrant of each panel represents the percentage of cells within that quadrant; their sum for a given panel represents the total percentage of $CD4^+$ $CD8^+$ cells.

studies have shown that T cells lose CD62-L expression and acquire high-density CD11a and CD2 expression following in vitro activation (1, 11). These data thus suggest that on the basis of its phenotypic pattern of low CD62-L expression and high CD11a and CD2 expression, the $CD4^{bright}$ $CD8^{dim}$ cell subset may contain a higher proportion of memory cells than the $CD4^{dim}$ $CD8^{bright}$ subset does.

Compared with $CD4^+$ $CD8^-$ and $CD4^-$ $CD8^{bright}$ T-cell populations, both $CD4^+$ $CD8^+$ cell subsets more closely resembled $CD4^+$ $CD8^-$ cells in some aspects (CD25, CD45RA, and CD45RO expression) and $CD4^-$ $CD8^{bright}$ cells in other aspects (CD62-L, CD11a, CD28, and CD16/56 expression). These observations suggest that $CD4^+$ $CD8^+$ cell subsets may represent hybrid cells from a functional standpoint, capable of exhibiting effector functions normally associated with either conventional CD4 cells or conventional CD8 cells. Additional studies are thus needed to assess the proliferative response of $CD4^+$ $CD8^+$ cell subsets to recall antigens such as tetanus toxoid or influenza; a positive response would indicate major histocompatibility complex class II-restricted antigen recognition typical of conventional CD4 cells. Similarly, studies of the cytotoxic activities of $CD4^+$ $CD8^+$ cells in a system employing autologous Epstein-Barr virus-transformed B-cell lines as targets are required to assess the major histocompatibility complex class I-restricted cytotoxic activity typical of conventional CD8 T cells. Our experiments did show, however, that $CD4^+$ $CD8^+$ cells synthesized DNA and underwent upregulation of CD25 and CD2 expression (1, 20) following activation by mitogenic anti-CD3, indicating that they possess the potential for functional competence.

Our interest in $CD4^+$ $CD8^+$ cells arose from the identification of a healthy blood donor (donor A) with a markedly increased percentage and absolute number of circulating $CD4^+$ $CD8^+$ cells. Further analyses showed that the $CD4^+$ $CD8^+$ population in this donor consisted almost totally of $CD4^{dim}$ $CD8^{bright}$ cells. From a comparison with $CD4^{dim}$ $CD8^{bright}$ cells from individuals with normal levels of these cells, four notable phenotypic differences were identified; the

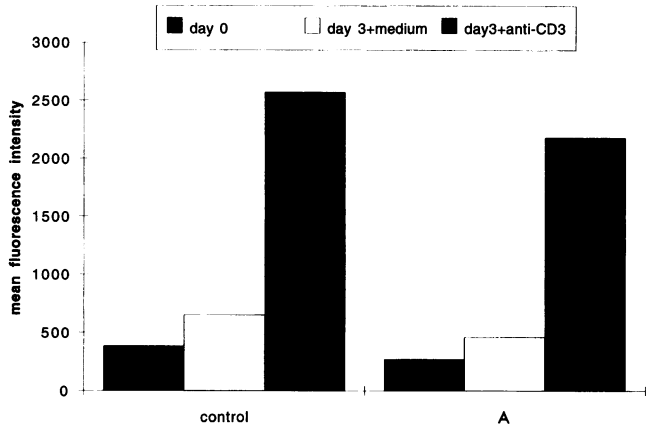

FIG. 5. Changes in CD2 fluorescence intensity following activation of $CD4^+$ $CD8^+$ cells by immobilized anti-CD3. Each value represents the mean fluorescence intensity (linear value calculated by LYSYS II software) of CD2 expression by $CD4^+$ $CD8^+$ cells from a control donor or donor A on the day of culture initiation (day 0) or after 3 days of culture either alone (+medium) or in the presence of immobilized anti-CD3 (+anti-CD3).

proportions of cells expressing CD62-L and CD25 were markedly decreased, whereas the proportions expressing CD11b and CD54 were increased. The maturational status of $CD4^{dim}$ $CD8^{bright}$ cells with this aberrant phenotypic profile remains unknown. Because decreased CD62-L expression and increased CD54 expression characterize mitogen-activated T cells, it is possible that these cells represent a previously activated (i.e., memory) cell subset (1, 11). Consistent with this hypothesis, other memory cell phenotypic patterns, such as a high percentage of $CD45RO^+$ cells, a relatively low percentage of $CD45RA^+$ cells, and a very low percentage of $CD38^+$ cells (11, 15), were found for donor A $CD4^{dim}$ $CD8^{bright}$ cells. However, a relatively high percentage of $CD25^+$ cells is usually associated with memory cell status (10, 15); the virtual absence of CD25 expression from donor A $CD4^{dim}$ $CD8^{bright}$ cells thus raises questions about the validity of the hypothesis of prior activation.

Likewise, the functional capacities of this aberrant $CD4^{dim}$ $CD8^{bright}$ cell subset from donor A remain unknown. Conventional $CD8^{bright}$ cells expressing CD11b have been shown to represent suppressor cells (8), but it is unclear if this association also applies to $CD4^+$ $CD8^{bright}$ cells. The CD54 molecule serves as a ligand for the heterodimeric adhesion structure composed of CD11a and CD18 (7); increased CD54 expression by the $CD4^{dim}$ $CD8^{bright}$ cells of donor A may thus be indicative of potent adhesive properties under the appropriate conditions.

The increased level and aberrant phenotype of $CD4^{dim}$ $CD8^{bright}$ cells from donor A have remained stable over 1 year. During this time, she has remained healthy and active. In experiments assessing TCR gene rearrangements conducted by Richard Gatti of the UCLA Medical Center, no evidence of a monoclonal T-cell expansion in donor A was found (8a).

Three other studies (12, 17, 19) have described a total of 24 apparently healthy individuals with increased levels of circulating $CD4^+$ $CD8^+$ lymphocytes. In 22 of 24 individuals, $CD4^+$ $CD8^+$ cells exhibited a $CD8^{dim}$ phenotype and, therefore, differed from the $CD4^+$ $CD8^+$ cells found in donor A. Further, in one of the two donors with increased levels of $CD4^+$ $CD8^{bright}$ cells, CD4 expression by these cells was very dim, also differing from donor A $CD4^+$ $CD8^+$ cells. The one donor with increased levels of $CD4^{dim}$ $CD8^{bright}$ cells similar in CD4 and CD8 expression to donor A $CD4^{dim}$ $CD8^{bright}$ cells was also a female in her 60s (19). However, her $CD4^{dim}$ $CD8^{bright}$ cells were notably different in phenotype from those of donor A in that they were 90% $CD56^+$, 95% $CD45RA^+$, and only 46% $CD45RO^+$ (versus 3, 28, and 95%, respectively, for those of donor A). The $CD4^{dim}$ $CD8^{bright}$ cell subset increase in donor A thus appears to represent a previously undescribed aberrant cell population. We plan to continue follow-up analysis of donor A in order to assess the long-term stability of the aberrant $CD4^+$ $CD8^+$ cell phenotype and to identify any changes in health status requiring clinical intervention.

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REFERENCES

1. **Akbar, A. N., M. Salmon, and G. Janossy.** 1991. The synergy between naive and memory T cells during activation. Immunol. Today **12:**184–188.
2. **Akbar, A. N., L. Terry, A. Timms, P. C. L. Beverley, and G. Janossy.** 1988. Loss of CD45R and gain of UCHL1 reactivity is a feature of primed T cells. J. Immunol. **140:**2171–2178.
3. **Azuma, M., J. H. Phillips, and L. L. Lanier.** 1993. $CD28^-$ lymphocytes. Antigenic and functional properties. J. Immunol. **150:**1147–1159.
4. **Baroja, M. L., K. Lorre, F. Van Vaeck, and J. L. Ceuppens.** 1989. The anti-T cell monoclonal antibody 9.3 (anti-CD28) provides a helper signal and bypasses the need for accessory cells in T cell activation with immobilized anti-CD3 and mitogens. Cell. Immunol. **120:**205–217.
5. **Blue, M.-L., J. F. Daley, H. Levine, K. A. Craig, and S. F. Schlossman.** 1986. Biosynthesis and surface expression of T8 by peripheral blood $T4^+$ cells in vitro. J. Immunol. **137:**1202– 1207.
6. **Blue, M.-L., J. F. Daley, H. Levine, and S. F. Schlossman.** 1985. Coexpression of T4 and T8 on peripheral blood T cells demonstrated by two-color fluorescence flow cytometry. J. Immunol. **134:**2281–2286.
7. **Dustin, M. L., and T. A. Springer.** 1988. Lymphocyte function-associated antigen-1 (LFA-1) interaction with intercellular adhesion molecule-1 (ICAM-1) is one of at least three mechanisms for lymphocyte adhesion to cultured endothelial cells. J. Cell Biol. **107:**321–331.
8. **Gane, P., O. Fain, I. Mansour, H. Roquin, and P. Rouger.** 1992. Expression of CD11b (Leu 15) antigen on $CD3^+$, $CD4^+$, $CD8^+$, $CD16^+$ peripheral lymphocytes. Estimation of $CD3^+8^+11b^+$ and $CD3^+4^-8^-11b^+$ T-cell subsets using a single laser flow cytometer. Scand. J. Immunol. **36:**395–404.

8a. **Gatti, R.** Unpublished data.

9. **Hogan, P. G., and A. Basten.** 1988. What are killer cells and what do they do? Blood Rev. **2:**50–58.
10. **Jackson, A. L., H. Matsumoto, M. Janszen, V. Maino, A. Blidy, and S. Shye.** 1990. Restricted expression of p55 interleukin 2 receptor (CD25) on normal T cells. Clin. Immunol. Immunopathol. **54:**126–133.
11. **Kanof, M. E., and S. P. James.** 1988. Leu-8 antigen expression is diminished during cell activation but does not correlate with effector function of activated T lymphocytes. J. Immunol. **140:** 3701–3706.
12. **Murakami, H., M. Hayakawa, T. Matsushima, M. Sawamura, J. Tamura, and K. Kubota.** 1992. Increased CD4/CD8 coexpressed cells in a healthy subject. J. Leukocyte Biol. **52:**133.
13. **Prince, H. E., S. Bermudez, and S. Plaeger-Marshall.** 1993. Preparation of $CD8^{bright}$ and $CD8^{dim}$ lymphocyte populations

using two positive selection methods in tandem. J. Immunol. Methods **165:**139–148.

14. **Prince, H. E., and W. J. Lesar.** 1989. Simultaneous determination of absolute total lymphocyte and CD4+ lymphocyte levels in peripheral blood by flow cytometry. Am. J. Clin. Pathol. **92:**206–209.
15. **Prince, H. E., J. York, and E. R. Jensen.** 1992. Phenotypic comparison of the three populations of human lymphocytes defined by CD45RO and CD45RA expression. Cell. Immunol. **145:**254–262.
16. **Reinherz, E. L., P. C. Kung, G. Goldstein, R. H. Levey, and S. F. Schlossman.** 1980. Discrete stages of human intrathymic differentiation: analysis of normal thymocytes and leukemic lymphoblasts of T-cell lineage. Proc. Natl. Acad. Sci. USA **77:**1588–1592.
17. **Richards, S. J., M. Sivakumaran, L. A. Parapia, I. Balfour, D. R. Norfolk, J. Kaeda, and C. S. Scott.** 1992. A distinct large granular lymphocyte (LGL)/NK-associated (NKa) abnormality characterized by membrane CD4 and CD8 coexpression. Br. J. Haematol. **82:**494–501.
18. **Rubin, B., G. Geisler, S. Caspar, and J. Arnaud.** 1992. The indispensable CD2-CD3 molecules: a key to T-cell differentiation and functional activation. Scand. J. Immunol. **36:**1–6.
19. **Sala, P., E. Tonutti, C. Feruglio, F. Florian, and A. Colombatti.** 1993. Persistent expansions of $CD4^+$ $CD8^+$ peripheral blood T cells. Blood **82:**1546–1552.
20. **Waldmann, T. A.** 1986. The structure, function, and expression of interleukin-2 receptors on normal and malignant lymphocytes. Science **232:**727–732.
21. **Zola, H., L. Flego, P. J. Macardle, P. J. Donohoe, J. Ranford, and D. Roberton.** 1992. The CD45RO (p180, UCHL1) marker: complexity of expression in peripheral blood. Cell. Immunol. **145:**175–186.