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Technical Note

CD3 Bright Lymphocyte Population Reveal γδ T Cells

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Background: In routine CD3/CD4/CD8 T-cell analysis, a CD3 bright population of lymphocytes is frequently observed. The aim of the present study was to identify the immunological significance of such CD3 bright lymphocytes.

Methods: We analyzed samples from 31 healthy adult volunteers, 78 human immunodeficiency virus (HIV)-positive, and 78 renal transplanted patients.

Results: A clearly distinct CD3 bright (frequently $CD4^-/CD8^-$) T-cell fraction was observed in 84% of donors and was directly correlated with the fraction of γδ T cells ($r^2 = 0.64$). CD3 overexpression on γδ T cells was confirmed by a combination of monoclonal antibody staining (CD3-ECD, γδTCR-FITC, and αβTCR-PE-Cy5) or immunomagnetic purification of γδ T cells (i.e., MdFI 20 vs 8.86). The γδ T cells expressed CD8 polypeptide chains (α and β) in all possible combinations. The largest proportion, surprisingly, were cells expressing CD8ββ homodimers (43.8 ± 16.5%). CD8αα homodimers were expressed on 14.2% (± 12.3) of total γδ T cells, whereas CD8αβ heterodimers were expressed on 12.2% (± 7.5). We also observed a bimodal distribution of the intensity of CD3 fluorescence of γδ T cells in immunocompromised patients with a threshold at 105 cell/μl. CD3 bright γδ T cells were more frequently observed in HIV patients (29%) compared with renal transplant patients (11%) and healthy donors (3%; χ^2 test: $P = 0.0007$).

Conclusions: The simple observation of a CD3 bright T-cell subset on CD3/CD4/CD8 routine analysis suggests a high γδ T-cell fraction and, in our opinion, should be followed by a complementary analysis to determine precisely the number of γδ T cells and to identify their CD8α/β phenotype. When CD3 bright T cells/μl were more than 40%, high γδ T cells were detected in more than 87% of cases, with a specificity of 76%. Occasionally, the CD3 bright subset appeared to be strongly homogeneous, suggesting an oligoclonal proliferation that could possibly reveal a chronic localized stimulation or an early lymphoproliferative disorder. Because the γδ T cells have interesting immunological peculiarities, the clinical significance of their quantitative abnormality should be clarified in diseases such as HIV, organ transplantation, autoimmunity and lymphoma. © 2004 Wiley-Liss, Inc.

Key terms: γδ T cells; CD8αα; intraepithelial lymphocyte

Quantitative analysis of CD4/CD8 T-cell subsets is one of the most commonly requested analyses for immunological monitoring in conditions, such as acquired immunodeficiency disease syndrome (AIDS), post-bone marrow grafting, and during immunosuppressive treatment. Quality assurance (QA) standard protocols using four-color immunolabeling allow very reproducible identification of both CD4 and CD8 T lymphocytes in a single run (1). However, this analysis provides much more information that is rarely considered.

In CD3/CD4/CD8/CD45 routine analysis of peripheral T cells, we repeatedly observed a bimodal distribution of CD3 expression that suggested the presence of two distinct T-cell subsets.

Besides CD3, the antigen-specific T-cell receptor (TCR) is another pan-T-cell marker. There are two distinct types of TCR—γδTCR and αβTCR—with different ontogeny and functional properties. In the clinic, attention has been focused primarily on either CD4 or CD8 αβ T cells, which constitute the vast majority (>95%) of circulating T cells. The remaining 5% of cells are γδ T cells, dedicated to mucosal immunity (2–4) and develop only partially from thymus (5).

The aim of this report was to identify the immunological relevance of the $CD3^+$ bimodal distribution, firstly observed in immunocompromised patients, and confirmed in healthy donors.

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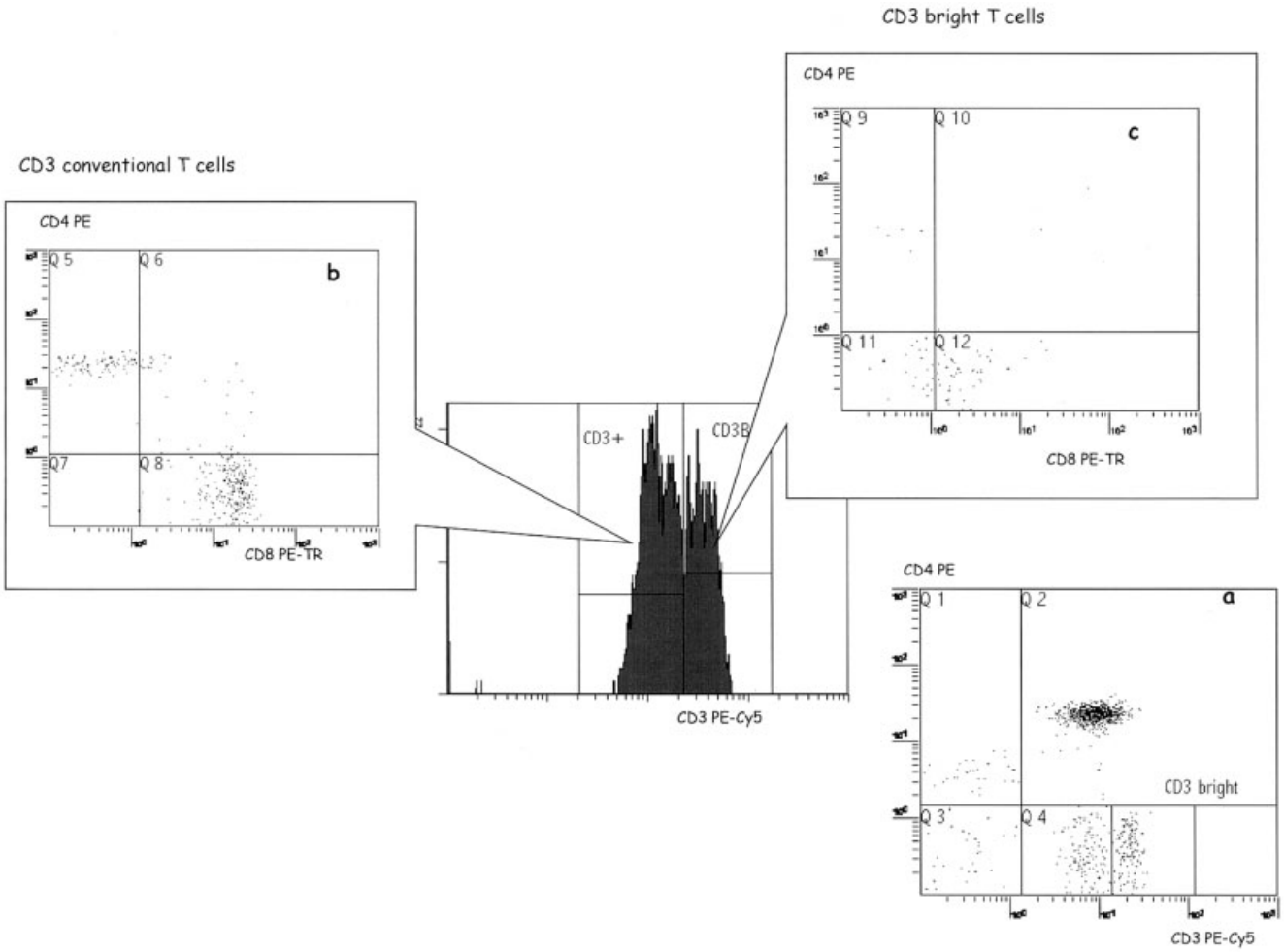

FIG. 1. Bimodal distribution of CD3 fluorescence intensity is frequently observed on CD3/CD4/CD8/CD45 routine lymphocyte analysis (one representative case). (**a**) CD3 bright T cells do not express CD4, but express low levels of CD8 (**c**). Conventional $CD3^+$ T cells express CD4 or CD8 (**b**).

PATIENTS AND METHODS

Lymphocyte analysis was performed on 187 samples from 31 healthy adult volunteers, 78 human immunodeficiency virus (HIV) patients, and 78 renal transplant patients. Lymphocytes were immunolabeled with a combination of CD3-PE-cyanine 5 (PE-Cy5, Clone UCHT1), CD4-PE-Texas Red (PE-TR, clone SFCI12T4D11), CD8α-phycoerythrin (PE, clone SFCI21thyD3), and CD45-fluorescein isothiocyanate (FITC, clone B3821F4A), (TetraChrome, Beckman-Coulter, Fullerton, CA). The TCR/CD8 chain expressions were analyzed using TCRα/β PE-Cy5 (clone BMA031), TCRγ/δ FITC (clone Immu 510), CD8α PE-TR (clone SFCI21thyD3), and CD8β PE (clone 2ST8.5H7) all purchased from Immunotech/Beckman-Coulter). TCR/CD3 expression was analyzed simultaneously in a few samples, using TCRα/β PE-Cy5 (clone BMA031), TCRγ/δ FITC (clone Immu 510), CD3 PE-TR (clone UCHT1), and CD8β PE (clone 2ST8.5H7) all purchased from Immunotech/Beckman-Coulter, USA).

The immunolabeling procedures were performed on whole blood according to the manufacturer's recommendations. Briefly, 100 μl of ethylenediaminetetraacetic acid (EDTA) anti-coagulated fresh peripheral blood was incubated with 10–20 μl of antibody, gently mixed, and incubated for 20 min at room temperature in the dark. Red blood cells were then lysed, and the remaining leukocytes were fixed with formaldehyde, using Immunoprep reagent on a T-Q prep machine (Beckman-Coulter). Samples were then stored in the dark, at room temperature, and analyzed within 1 h on a four-color EPICS XL flowcytometer (Beckman-Coulter). Immediately before analysis, CD3/CD4/CD8/CD45 labeled samples were mixed with an equal volume of standardized fluorescent beads (Flowcount, Beckman-Coulter) for absolute quantification. The analysis was performed on 5,000 $CD3^+$ T cells. Because the fluorescence intensity did not always have a normal distribution, we chose to consider the median (MdFI), instead of the mean (MFI), as calculated automatically by the machine software (System II, Beckman-Coulter).

The flowcytometer signal settings were checked every day using Flow Check® beads (Beckman-Coulter) and stabilized blood samples (ImmunoTrol®, Beckman-Coulter)

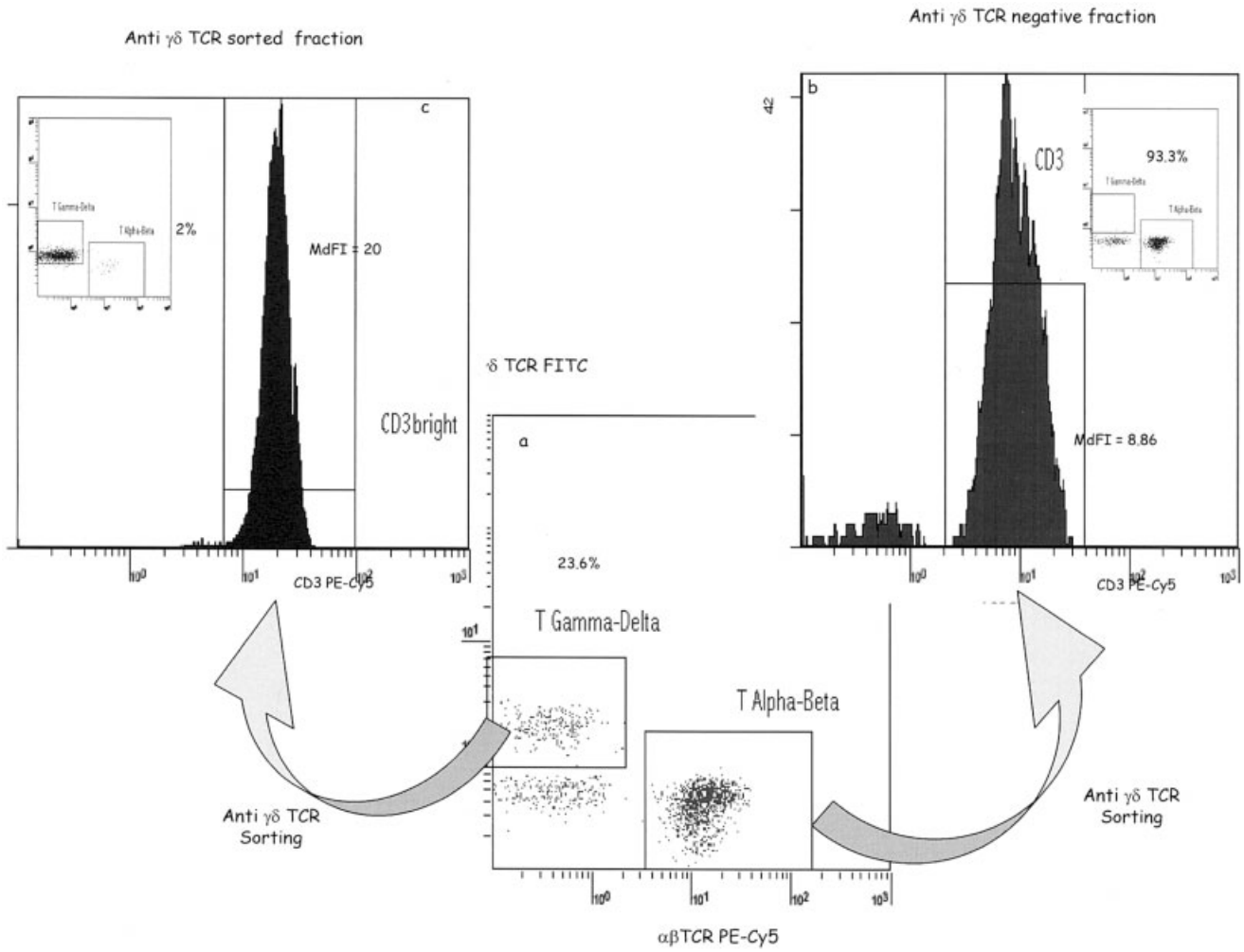

FIG. 2. γδT lymphocytes are more CD3 bright than αβ T cells. One patient had a very high level (23.6%) of γδ T lymphocytes. The γδT cells were purified with magnetic beads (Materials and Methods) after staining with γδ TCR-FITC and αβTCR-PE-CY5 (**a**). CD3 expression was higher in the γδ + sorted fraction (**c,** MdFI = 20) than in the γδ- remaining cells (**b:** MdFI = 8.86).

in order to check the day-to-day stability of the CD3, CD4, CD8 fluorescence signals. Signal amplification for each photomultiplicator (PMT) and compensation were automatically corrected once a week, after every maintenance and each time the daily test failed, using Flow Set® standardized beads and CytoComp reagent and cell kit® (Beckman-Coulter) in accordance the manufacturer's recommendations. The resulting settings were analyzed periodically by Levey-Jennings graphs on System II software (Beckman-Coulter). The external quality controls comprised an international survey Interlaboratory Quality Assurance Program (Beckman-Coulter) and United Kingdom National External Quality Assessment Schemes (NEQUAS; Clinical Pathology Accreditation Ltd., Nequas Sheffild, UK).

γδ T cells were purified from a γδ T-cell-rich (23.6%) sample, using magnetic beads. The peripheral blood mononuclear cells (PBMC) were separated on density gradient from EDTA anti-coagulated blood, washed in sterile, calcium free, phosphate buffered saline (Eurobio, Les Ullis, France) and immunolabeled with anti γδTCR monoclonal antibody (clone 11F2) conjugated with a hapten. The labeled cells were then sorted using anti-hapten antibody dually conjugated with the magnetic colloid and FITC (Miltenyi Biotech, GmbH, Bergisch Gladbach, Germany). Both sorted and negative fractions were analyzed using the same procedures previously described excluding the lysis step.

Statistical analysis was performed using the χ^2 test to compare frequencies in different groups, paired or unpaired Student's *t*-tests to compare means between or within groups and linear regression curve with coefficient of determination (r^2) to evaluate correlation of quantitative values. The results were expressed as mean ± 1SD. The receiver operating characteristic (ROC) was calculated at the website of the Russell H. Morgan Department of Radiological Science (Johns Hopkins University, Baltimore, MD) with a Java program by John Eng.

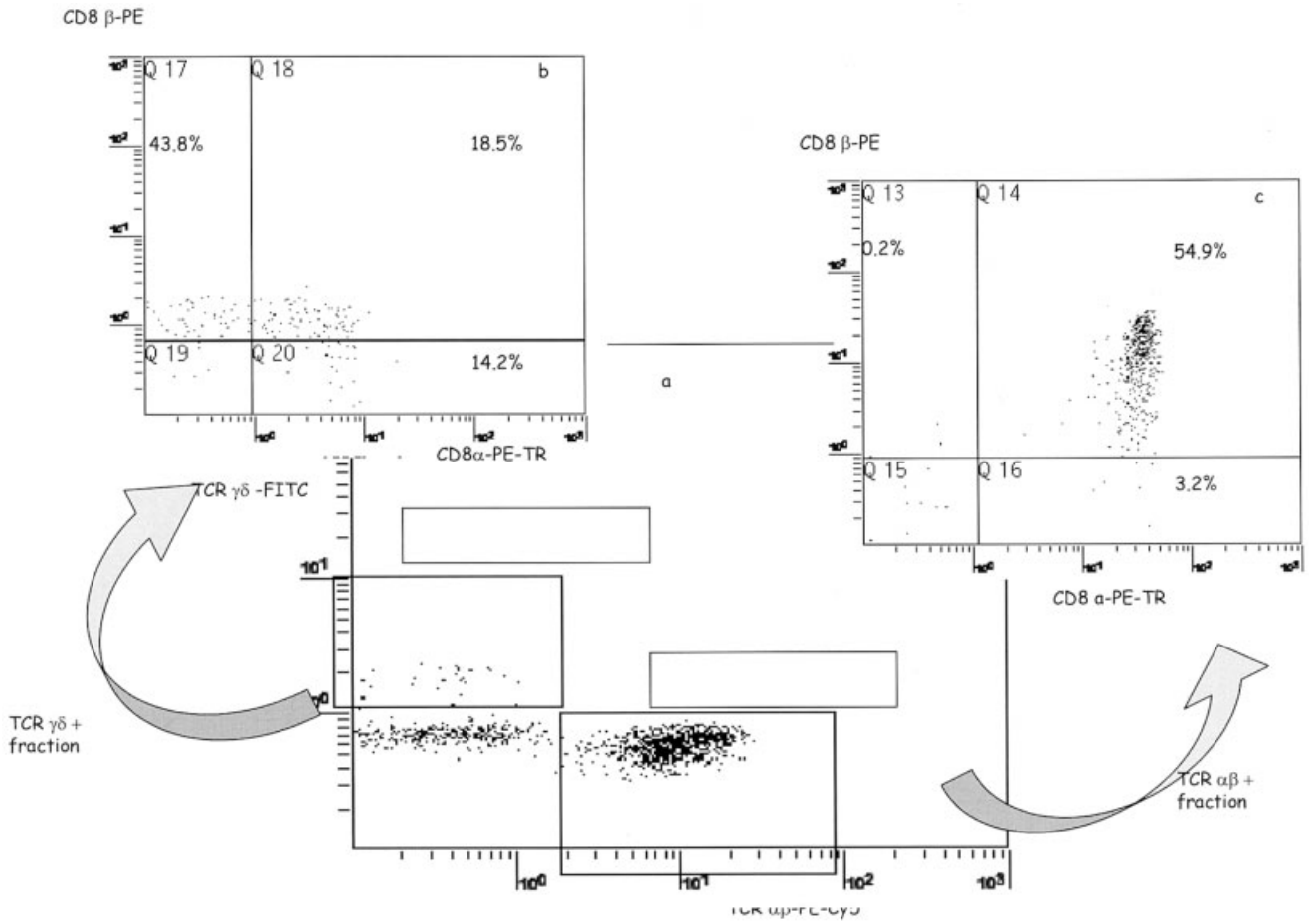

FIG. 3. γδ T cells express all possible combinations of the two CD8 polypeptide chains α and β. CD8 expression was analyzed on both αβ and γδ T-cell fractions using four-parameter labeling (CD8β-PE; CD8αPE-TR; γδTCR-FITC, αβ TCR-PE-Cy5) of whole blood with red blood cell lysing and no washing (Materials and Methods). $\gamma\delta TCR^+$ and $\alpha\beta TCR^+$ T cells were electronically selected (**a**) for analysis of CD8α/CD8β proportion (**b,c**) one representative sample is shown). γδ T cells expressed either CD8β chain alone (43.8 ± 16.5%), both α and β chains of CD8 (12.2α ± 7.5%), or CD8α only (14.2 ± 12.3%; **c**). CD8 fluorescence intensity was significantly lower on γδT cells compared with αβT cells, for CD8α chain (MdFI = 5.4 ± 3.2 compared with 32.4 ± 7.6, $P < 0.0001$) as well as CD8β chain (MdFI = 2.2 ± 1.0 compared with 10.3 ± 3.1, $P < 0.0001$; **b**). Less than 2% of cells had fluorescence intensity >1.0 with the isotype controls (not shown).

RESULTS

Evidence for a CD3 Bright T-Cell Subset in Healthy Donors

In the first set of experiments, we confirmed the presence of a CD3 bright fraction of T cells in 26 of 31 (84%) healthy donors (15 females, 16 males, aged 46.5 ± 13.3 years old). CD3 bright T cells constituted 3.15 ± 2.16% of the lymphocytes (Fig. 1a). The bimodal CD3 distribution could be confirmed with anti-CD3 monoclonal antibody obtained from another manufacturer (Dako; clone UCHT-1) and did not depend on the amount of antibody used (5, 10 -recommended-, 20 or μl per 100 μl of blood; data not shown).

Identification of the CD3 Bright T Cells in Healthy Donors

Of these CD3 bright T cells, 55.8% were both CD4 and CD8 negative (Fig. 1c). Therefore, we suspected that they could be γδ T cells. In healthy donors, we observed that 2.4 ± 1.4% (46 ± 26 cells/μl, range: 2–105) of peripheral blood lymphocytes expressed the γδTCR. The size of the CD3 bright and γδ T-lymphocyte fractions were directly correlated ($0.73x + 0.370$; $R^2 = 0.644$, $P = 0.012$; not shown).

γδ T Cells Expressed High Levels of CD3

To confirm that high levels of CD3 are indeed expressed on γδ T cells, we used a CD3 PE-TR labeling combined with γδ T FITC and αβ T PE-Cy5, in five patients. We observed that electronically selected γδ T cells expressed a higher CD3 fluorescence intensity than the αβ T cells (e.g., MdFI = 15.2 vs 12.1). Similarly, sorted γδ T cells, which contained only 1.2% of αβ T cells and less than 1% of non-T cells, had a higher CD3 median fluorescence intensity (MdFI = 20) compared with the γδTCR-negative fraction (MdFI = 8.86; Fig. 2), which contained less than 0.25% of γδTCR (labeled by the fluorescent magnetic colloids or free FITC-antibody we added).

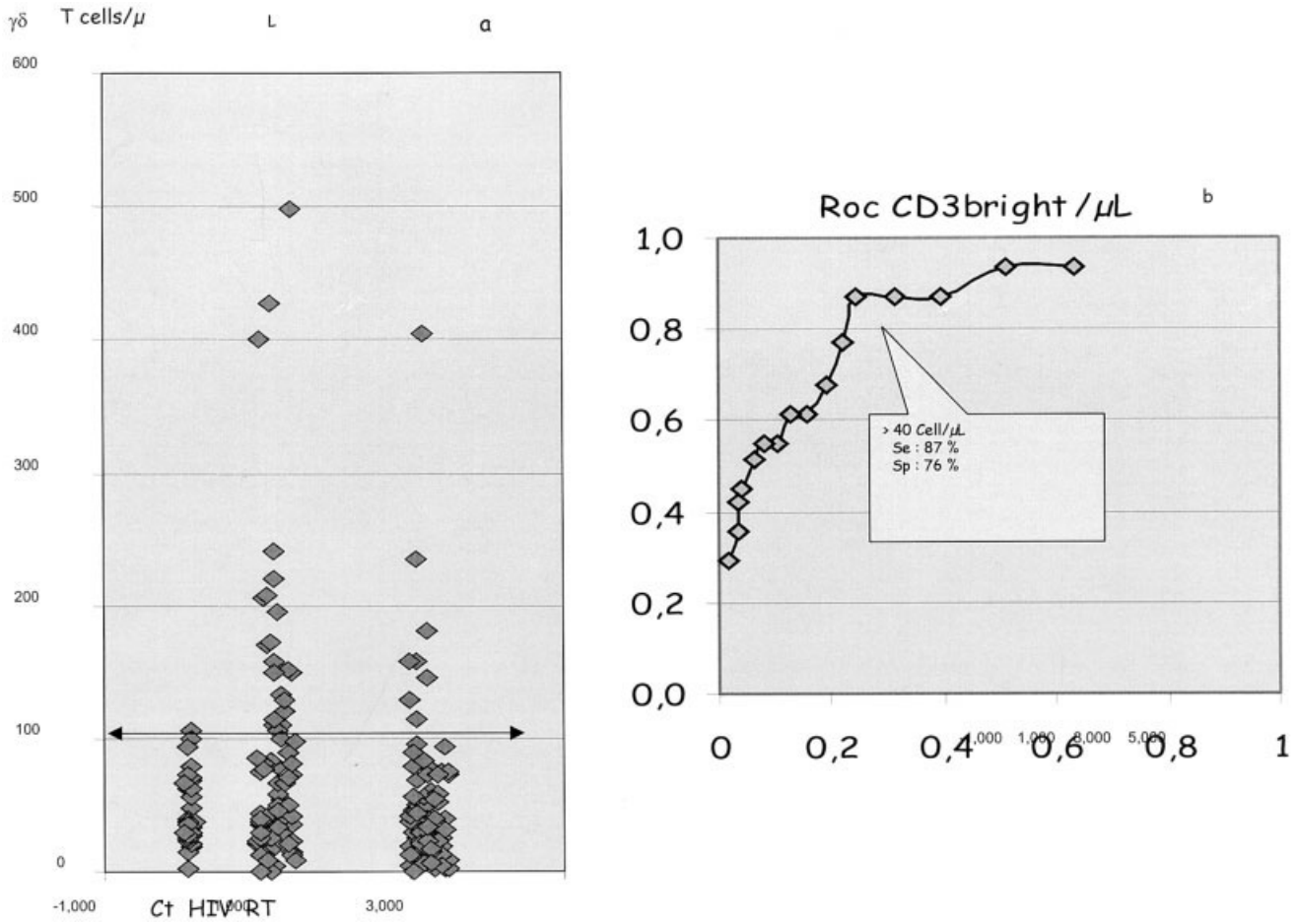

FIG. 4. Increased CD3-bright cells highlight the possibility of abnormal increase in γδ T cells. γδ T lymphocyte analysis is shown (**a**) in 31 healthy donors (ct), 78 HIV patients (HIV), and 78 renal transplant patients (RT). Immunosuppressed patients had elevated peripheral γδ T cells (>105 cell/µl) specially among HIV (29.3%) and RT (10.5%) patients compared with healthy donors (3%, χ^2 test: $P < 0.001$). The receiver operating characteristic (ROC) curve (**b**) shows that more than 87% of patients with high γδT cells could be identified when CD3-bright cells were higher than 40 cells/µl (specificity 76%).

Phenotypes of γδ T Cells in Healthy Donors

On CD3/CD4/CD8 labeling, the CD3 bright T cells never expressed CD4 but frequently (39.5 ± 20.7%) expressed the CD8α (Fig. 1c), although at a lower level than the conventional $CD8^+$ αβ T cells (Fig. 1b). The frequency of γδ T cells expressing CD8 was correlated neither to the age of the patients nor to the $CD4^+$ T-cell or $CD8^+$ T-cell absolute count (results not shown). On CD8α/CD8β/γδTCR labeling, a substantial fraction of the γδ T cells did express CD8α chain either alone (CD8αα: 14.2 ± 12.3%) or with the CD8β chain (CD8αβ: 12.2 ± 7.5%). Moreover, most (43.8 ± 16.5%) of γδ T cells expressed (low levels of) the CD8β chain alone (Fig. 3). The CD8 MFI on γδ T cells was much lower than on αβ T cells for both α chain (5.4 ± 3.2 compared with 32.4 ± 7.6, paired *t*-test: $P < 0.0001$) and β chain (2.2 ± 1.0 compared with 10.3 ± 3.1, paired *t*-test: $P < 0.0001$) while most (98%) of lymphocytes labeled with the isotype controls or the $CD4^+$ T cells had a CD8 fluorescence intensity below 1.0 (MdFI = 0.15 and 0.13 respectively; not shown).

Possible Clinical Significance of γδ T Cells in Diseases

To investigate whether the CD3 bright/γδ T-cell fraction has any pathological significance, we analyzed their distribution in 156 immunocompromised patients: 78 HIV patients (22F/56M: 40.3 ± 10.2 years) and 78 renal transplant patients (26 F/52M: 52.3 ± 14.1 years). All HIV patients were treated and had a wide range of $CD4^+$ T-cell levels (511 ± 288 cells/µl). The renal patients had renal transplant for more than 6 months (1–8 years follow-up), were treated with Cyclosporin A, low doses of steroids (10–30 mg/day) and azathioprim and had normal levels of $CD4^+$ T cells (790 ± 402 cells/µl).

The mean of the absolute number of γδ T cells was significantly increased in HIV patients (88 ± 53 cells/µl) compared with renal transplant patients (51 ± 32/µl; *t*-test: $P = 0.0056$) and healthy donors (51 ± 38/µl; *t*-test: $P = 0.037$). We did not find any correlation with age or sex of patients (not shown). In fact, the graphic analysis (Fig. 4) shows a bimodal distribution. All but one (3%) healthy donors had less than 105 γδ T cells/µl. A signifi-

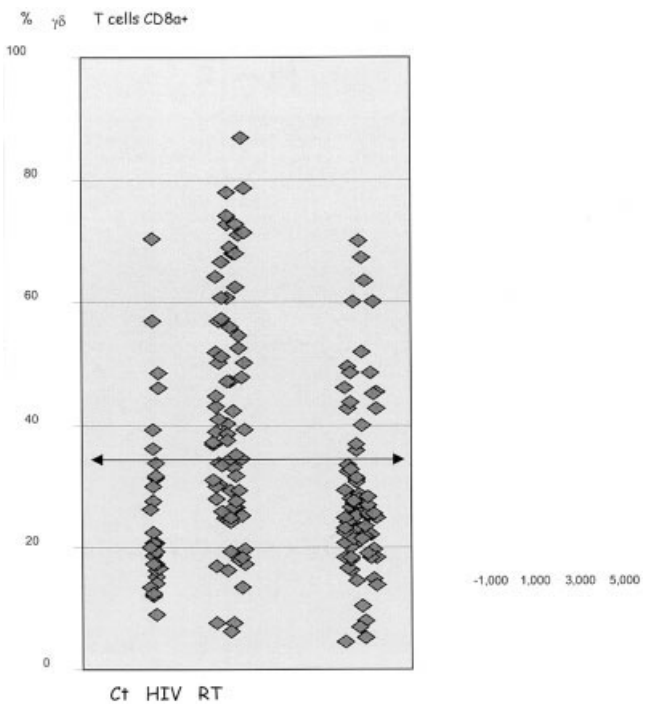

FIG. 5. γδT cells express more CD8α chain in HIV (41.7 ± 19.4%) compared with renal transplant (RT) patients (28.8% ± 14.1; $P < 0.0001$) and healthy donors (26.4 ± 14.6%; $P = 0.002$). 35% of HIV patients had more than 35% of γδT cells expressing CD8 compared with 8% of RT patients and 7% or donors (χ^2: $P = 0.0033$).

cantly high number of HIV patients (n = 22–29%, χ^2 test: $P = 0.0007$) had raised γδ T cells (105–498 cells/μl). The finding of increased γδ T cells was less frequent in renal transplant patients (n = 8–10.5%; from 115–405). The increased γδ T cells in HIV patients may be related to the rise in CD8 T cells in HIV patients (1167 ± 980 cells/μl) compared with renal transplant patients (457 ± 25.1 cells/μl, t-test: $P < 0.0001$) and healthy donors (518 ± 217 cells/μl, t-test: $P = 0.0004$). Indeed, the γδ T-cell counts were (poorly) correlated to the $CD8^+$ αβ T-cell counts ($r^2 < 0.497$), but not to the CD4 T cytopenia in HIV ($r^2 < 0.024$). This was similar in patients with renal transplants.

Moreover, the γδ T cells expressed the CD8α chain more frequently in HIV patients (41.7 ± 19.4% of γδ T) compared with renal transplant patients (28.8 ± 14.1%; t-test: $P < 0.0001$) and healthy donors (26.4 ± 14.4%; t-test: $P = 0.002$; Fig. 5). In some instance, a clear extra CD3bright CD8+ dot plot was observed (Fig. 6a). This was correlated with a raise of γδ T cells (Fig. 6b). The extra T cell subset were γδT cell expressing heteroimeric (αβ) form of CD8 (Fig. 6c).

Predictive Value of Increased CD3 Bright γδ T Cells

The presence of a CD3 bright subset of T cells (>40 cells/μl) allowed the detection of more than 87% of patients with high γδ T cells (>105 cells/μl) without further testing (area under ROC curve was 0.851 with 77.6% accuracy; Fig. 4).

DISCUSSION

Knowledge of fundamental immunology has improved exponentially over the past few years [reviewed in refs. (6,7)]. In contrast, the information conveyed to clinicians after analysis in clinical immunology remains at a very basic level (i.e., numbers of $CD4^+$ and $CD8^+$ T cells). Improved technology and quality assurance of certified protocols provide us with high degree of confidence in the analysis of minor circulating cell subsets with unconventional phenotypes. Using the current clinical protocol for sample analysis, much more reliable information can be provided to clinicians with no extra cost. Until now, this information was not made available for clinical use. Even if the clinical significance of much of these new data has yet to be elucidated, our aim was to give clinicians the opportunity to get more detailed information, which could then be correlated with appropriate clinical circumstances.

The increase in CD3 bright T cells is an observation we make frequently. We propose that this information should be provided to the clinician with relevant interpretative comments pertaining to our current level of understanding of its immunological significance. In the present study, we have shown several lines of evidence suggesting that the CD3 bright fraction was γδ T cells. Thus, the commonly used CD3/CD4/CD8 analysis allows the indirect detection of circulating γδ T-cell variation that is rarely considered in clinical practice, despite the increasing availability of experimental evidence for their pathological involvement (5,6). By analyzing γδ and αβTCR T-cell phenotypes, our data suggest that γδ T cells can rise in a significant number of patients. A threshold of 105 cells/μl differentiates normal levels from a group of patients who show a large (up to 5 times) increase of peripheral γδ T cells. This threshold is in accordance with published work (7). Because the frequency of raised γδ T cells only concerned 1 of 5 patients and was rare in healthy donors, it would be unnecessarily expensive to routinely perform additional analysis for γδ T-cell counting. This is why we believe that a rise in CD3 bright T cells could be a good screening criterion for further analysis specifically focused on γδ T cells.

The threshold between normal and bright CD3 fluorescence intensities was not always easy to set because of the two peaks overlap. This could explain the relatively poor correlation we observed between CD3 bright and γδ T-cell fractions. The CD3 bright fraction was more or less easily distinguished by its relative size and the fluorochrome conjugated to the anti-CD3 antibody. In some instances, when the γδ T-cell subset was low, the CD3 bright fraction was almost indistinguishable from electronic noise. The PE-Cy fluorochrome appeared more discriminant for CD3 labeling than PE-Texas red. However, the bimodal distribution of CD3 was not dependent on the source of monoclonal antibodies, at least under saturating concentrations of the anti-CD3 antibodies used. Because CD3 bright T cells never expressed CD4, the dot plot of the $CD3/CD4^+$ subset could be a very convenient tool to

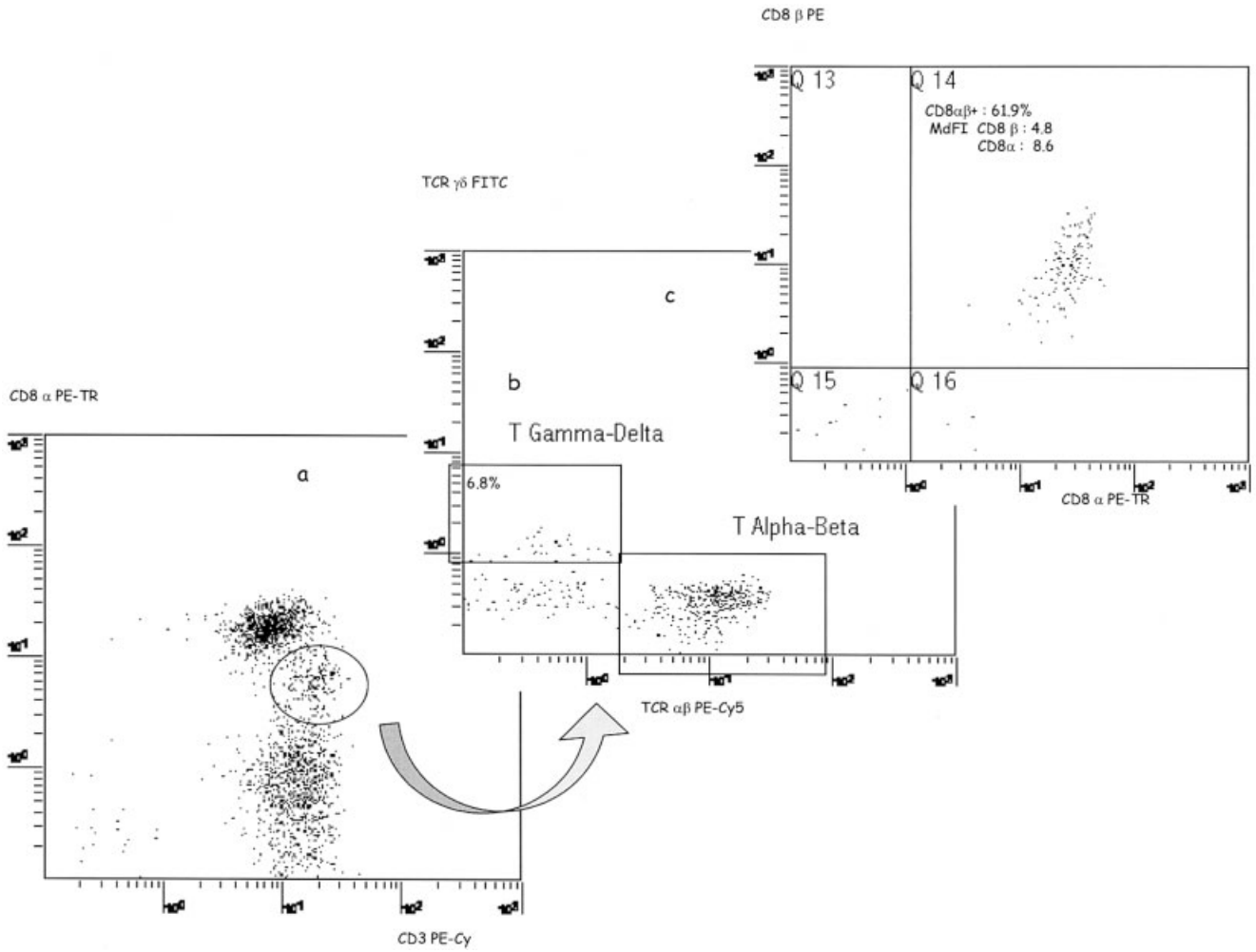

FIG. 6. Extra CD3 bright/CD8 dim T cells (**a**) are enriched for γδT cells expressing CD8αβ. A representative sample from one donor is shown. γδ T cells were more frequent (**b:** 6.8% of lymphocytes) and expressed unusually higher levels (lower than αβ T cells) of both CD8β (MdFI : 4.8) and CD8α (MdFI : 8.6) chains (**c:** 61.9%).

determine the confident interval of conventional CD3 distribution and the threshold for defining CD3 bright T cells.

γδ T cells have been extensively studied relatively recently. They have very restricted diversity (6), poor MHC restriction, and recognize antigens that are widely distributed and are not usually recognized (nonpeptidic phosphoantigens) by αβ T cells [reviewed in (6–7,9)]. The γδ T cells can enhance the immune response by producing high amounts of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), or chemokines (7,10,11). Moreover, γδ T cells have effector (cytotoxic) activity on their own (12). From an evolutionary standpoint, γδ T cells have a unique place between the highly specific αβ T cells and the innate immune system to fulfill the immune defense of the body against environmental pathogens. Experimental data have shown that γδ T cells were essential in immunity against organisms such as *Mycobacterium*, *Borellia*, *Francisella tularensis*, *Salmonella* (7,11,13,14), or virus (15,16), and the HIV virus (17–19). Moreover, γδ T cells have anti-tumor activity (20–24) and an increase in circulating γδ T cells has been described in patients with lymphoma (25). The γδ T cells are also involved in epithelium repair and homeostasis (26–28). In contrast, γδ T cells can be involved in immunopathologies such as diabetes (29) or autoimmune disorders (30–32) and asthma (25,33).

The γδ T cells have very rapid (starting after 4–6 days), high (×200 times), and prolonged (>7 months) proliferative capacity to antigen challenge (7). Moreover, because of their limited diversity, antigen-specific γδ T cells can reach as much as 48 –98% of circulating γδ T cells during an immune response (7). Thus, the detection of an increased circulating γδ T-cell subset may suggest a recent or ongoing chronic stimulation, particularly at mucosal or cutaneous sites. That information should alert clinicians for a possible slowly progressing infectious disease. It should be more particularly relevant in immunocompro-

mised patients and even more in HIV patients because their γδ T cells should be reduced due to virus-induced lymphopenia (17). Knowing that the distribution of the clonotype is strongly related to the site of residence (i.e., δ1 isotype from gut residents and δ2 from peripheral pool: 6), a more precise clonotyping of the peripheral γδ T-cell population may help to locate the origin of the proliferative stimulus.

In our study, we observed that a portion of γδ T cells expressed CD8αβ as previously described in rats (34). The CD8 appeared to be functional and not restricted to oligoclonal γδ T cells. Compared with αβ T-cell ontogeny, this observation may suggest that γδ T cells expressing CD8αβ could develop differently from other γδ T cells, and may even be of thymic origin (4).

We have also confirmed that γδ T cells could express homodimeric CD8αα. similar to a small fraction of the αβ T cells. Interestingly, both γδ T cells and CD8αα αβ T cells have shared tropism for mucosal barriers (2–4,35). Further characterization of the clonotype should determine if the CD8αα$^+$ γδ T cells are preferentially derived from the gut intraepithelial T-cell pool. Thus, an increase of peripheral CD8αα$^+$ γδ T cells could have clinical significance revealing an intestinal inflammatory or lymphoproliferative process.

Finally, we repeatedly observed that γδ T cells frequently expressed the CD8β chain alone? This has never been described before and needs to be confirmed by other investigators. However, the level of expression remains very low and may not be physiologically relevant.

During antigen recognition, both CD8αβ and CD8αα receptors strengthen the αβTCR binding to the MHC-peptide complex (36,37). It is generally accepted that γδ T-cell interact with nonclassical MHC molecules 6,7,9,14,38) and there is some evidence that CD8 molecules are involved in the interaction with these nonclassical MHC molecules (e.g., CD1, MICA, MICB) (6,9,14,38). It is relevant in this context that NK-T cells that mainly interact with classical MHC molecules express CD8 (14,39,40).

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