Evaluation of a simplified dual-platform flow cytometric method for measurement of lymphocyte subsets and T cell maturation phenotypes in Nouna, Burkina Faso

Running title: simplified dual-platform lymphocyte flow cytometry


aDepartment of Virology, and bDepartment of Tropical Medicine and Public Health, Institute of Hygiene, University of Heidelberg, Germany

cNouna Health Research Centre, Nouna, Burkina Faso
dBD Biosciences, Erembodegem, Belgium

Address for correspondence/proofs:

Thomas Böhler, MD
c/o Department of Virology
University of Heidelberg
Im Neuenheimer Feld 324
D-69120 Heidelberg, Germany
Tel. +49 –6221 565002
Fax +49 –6221 565003
thomas.boehler@med.uni-heidelberg.de
ABSTRACT

In the context of a larger clinical study in Nouna, Burkina Faso, we evaluated a simplified dual-platform flow cytometric method (DP FCM) that allows determination of major lymphocyte subsets in a single test tube. We compared phenotyping of lymphocytes with DP FCM and simultaneous measurements with standard single-platform flow cytometry (SP FCM) in 177 individuals. Analysis of comparative measurements revealed that DP FCM systematically underestimates the proportion of NK-cells, overestimates the percentage of CD3+ CD8+ lymphocytes, and yields proportions of B-cells and CD4+ T-cells comparable with SP FCM. Bland-Altman analysis showed a low bias between both methods and an acceptable precision for percent values of CD4+ T-cells (bias:precision –1±6%) and CD8+ T-cells (–3±6%). Absolute cell numbers of all lymphocyte subpopulations, however, were systematically biased towards lower values obtained by DP FCM. Reference values for the distribution of T-cell maturation phenotypes in 177 healthy adults using DP FCM were calculated. Mean (±standard deviation) CD4+-to-CD8+ T-cell ratio was 1.61±0.61, mean percentage of CD4+ T-cells was 42±7%, and of CD8+ T-cells 29±7%. Among CD4+ lymphocytes, 28±7% were classified as central memory (CD45RAlow CCR7+), 22±10% as naïve (CD45RAhigh CCR7+), 45±12% as effector memory (CD45RAlow CCR7neg), and 5±3% as terminally differentiated effector memory expressing CD45RA (CD45RAhigh CCR7neg). Among CD8bright lymphocytes, 3±2% had a central memory phenotype, 27±13% were naïve, 37±13% had an effector memory phenotype, and 34±12% were terminally differentiated effector memory cells expressing CD45RA.

Key words: lymphocytes, T-cells, central memory, effector memory, naïve, immunophenotyping, flow cytometry, reference values, sub-saharan Africa
INTRODUCTION

In the years 2004 and 2005 a population-based study was performed in Nouna, Burkina Faso in order to generate site- and gender-specific reference values for lymphocyte subsets in healthy adults in the context of an expanding program for prevention of mother-to-child transmission (PMTCT) of the human immunodeficiency virus type-1 (HIV-1). During that study single-platform (SP) flow cytometry (FCM) was used, a method, which is not available to most laboratories in developing countries due to its relatively high cost.

Since lymphocyte differentiation and counting by FCM is needed for immunological monitoring of antiretroviral treatment in resource-limited settings and immunological field studies on cohorts of young infants suffering from other diseases than infection with HIV-1 were planned in our research setting, we wanted to use a FCM test which allows determination of the complete lymphocyte differential. The test should be reliably performed with low volumes of venous and capillary blood and should be resistant against preanalytic errors. It should be as cheap as possible and should be run on a simple flow cytometer equipped with only one laser.

In the present study we evaluated such a simplified dual-platform (DP) FCM method for its clinical use in Nouna. The method allows determination of (a) the relative distribution of lymphocyte subsets in peripheral blood in a single test tube using a mixture of fluorochrome-conjugated monoclonal antibodies on a standard 3-color flow cytometer, and (b) the calculation of absolute values by using lymphocyte numbers obtained from a standard hematology analyzer. The results of simultaneous measurements using DP and SP FCM were compared.

In addition, we generated reference values of T-cell maturation phenotypes for healthy adults living in Nouna, Burkina Faso using the linear differentiation model of CD4+ and CD8+ T-cells which is based on the expression of the long isoform of the common leukocyte antigen CD45RA and the chemokine receptor CCR7. According to this model, CD45RA\text{high} CCR7+ naïve T-cells develop into CD45RA\text{low} CCR7+ central memory cells.
upon stimulation with their cognate antigen, and may then switch to the CD45RA<sup>low</sup> CCR7<sup>neg</sup> effector memory phenotype upon re-stimulation. Infection with HIV-1 was shown to influence the distribution of these T-cell maturation phenotypes by increasing the percentage of terminally differentiated, CD45RA<sup>high</sup> CCR7<sup>neg</sup> CD4<sup>+</sup> T-cells – a population which is very small in healthy individuals and has not been well characterized<sup>1,11</sup>. Data on frequency and absolute numbers of these T-cell subpopulations are not available for populations in Sub-Saharan Africa.

MATERIALS AND METHODS

The study took place in north-western Burkina Faso (West-Africa) in the research zone of the Nouna Health Research Center (Centre de la Recherche en Santé de Nouna, CRSN). From July 2004 until September 2005 the CRSN conducted a population-based clinical study<sup>9</sup> recruiting 364 individuals for the generation of immunohematological reference values in collaboration with the Centre Medical avec Antenne Chirurgicale (CMA) in Nouna, the Institute of Virology at the University of Heidelberg, Germany, and BD Biosciences Europe, Erembodegem, Belgium. The study was part of the ongoing longitudinal PMTCT trial in Nouna, which was approved by both the National Ethics Committee in Burkina Faso and the Institutional Ethics Committee of the University of Heidelberg, Germany. The inclusion of healthy adults for the purpose of generating laboratory reference values in the local population was additionally approved by the Institutional Ethics Committee at the Nouna Health Research Center.

In the study presented here we evaluated a simplified DP FCM method which allows simultaneous analysis of the major lymphocyte subpopulations in peripheral blood within a single test tube. Measurements using both SP FCM<sup>10</sup> and the simplified DP FCM method were performed in 177 study participants. In addition, reference ranges for lymphocyte subpopulations and T-cell maturation phenotypes were calculated using complete data sets.
from 177 healthy adults (68 males and 105 females). 18 percent of these individuals were below 20 years of age, 49 percent were between 20 and 29 years of age, 16 percent were between 30 and 39 years of age and 17 percent were at least 40 years of age.

Details of blood sample collection and serological testing are described elsewhere. All FCM measurements were performed on a 3-color instrument (BD FACScan™) generously provided by BD Biosciences. Standard SP FCM was done with BD MultiSET™ software, BD TruCount™ tubes, and BD TriTEST™ reagents as recommended by the manufacturer.

Hematology testing with an automated device (Sysmex KX21N, Sysmex Corporation, Kobe, Japan) was used to calculate absolute cell numbers of lymphocyte subpopulations with DP FCM.

Samples for the DP FCM test were prepared at climatised room temperature (range 20 – 30 °C) using a monoclonal antibody reagent mixture as recommended by the manufacturer (Tube 1: CD8-FITC and CD19-FITC, CD56-PE and CD3-PE, CD4-PerCP; Tube 2: CD45RA-FITC, CCR7-PE, CD4-PerCP; Tube 3: CD45RA-FITC, CCR7-PE, CD8-PerCP; Tube 4: IgG-Isotype control coupled to FITC and PE, CD4-PerCP). In preparatory experiments on blood samples from healthy laboratory personnel in Heidelberg, CD8-FITC, CD19-FITC, CD56-PE, CD3-PE and CD4-PerCP were used in combination with monoclonal antibodies coupled with allophycocyanine (CD14-APC, CD45-APC, or CD94-APC, respectively) in order to check for purity of electronic gates and to further characterise lymphocyte subpopulations on a four-color flow cytometer (FACScalibur™, BD). Stained cells were kept in the dark at 2 to 8 °C until acquisition on the flow cytometer; measurements were always performed within 12 hours of staining.

For subtyping of lymphocytes with the single-tube method, at least 10,000 lymphocytes were acquired using a forward scatter (FSC) / side scatter (SSC) gate (Fig. 1 A). For the determination of T-cell maturation phenotypes on the FACScan™ flow cytometer, at least 2,500 lymphocytes expressing CD4+ (Fig. 2 A) or lymphocytes with a high expression of CD8 (Fig. 2 C) were acquired using a SSC / fluorescence channel (FL)-3 gating strategy. List mode data were analysed using the CellQuest Pro™ software. In the single-tube lymphocyte
differential (tube 1 = T1), the T-, B- and natural killer (NK)-lymphocyte subpopulations were
identified due to differences in fluorescence intensities of defined surface antigens and their
typical combinations as shown in Fig. 1 A to D.

T-cell maturation phenotypes were defined by differential expression of the long isoform
of the common leukocyte antigen CD45RA and the C-C-chemokine receptor CCR7 as
originally described by Sallusto et al.\textsuperscript{13}. CD4\textsuperscript{+} lymphocytes were identified as low SSC cells
with a high FL-3 staining intensity (Fig. 2 A) and analysed in a FL-1 vs. FL-2 dot plot (Fig. 2
B) for expression of CD45RA and CCR7. In the CD8\textsuperscript{+} T-cell subpopulation, maturation
phenotypes were only determined in cells expressing high levels of CD8 (CD8\textsuperscript{bright}
lymphocytes) in order to exclude CD8\textsuperscript{+} lymphocytes with intermediate and low CD8
expression. Such cells comprise both NK-cells and T-cells with NK-like functions; they
express only the long isoform of the CD45 antigen (CD45RA) and appear not to participate in
the dynamic maturation pathway seen in CD8\textsuperscript{bright} lymphocytes which are nearly 100% T-
cells\textsuperscript{18}.

Markers for the discrimination of T-lymphocyte maturation phenotypes were set using
mouse IgG isotype control antibodies to define the boundary between CCR7\textsuperscript{+} and CCR7\textsuperscript{neg}
cells. The boundary on the FL-1 axis was fixed at 100 arbitrary fluorescence units in order to
clearly distinguish cells with high expression of CD45RA (CD45RA\textsuperscript{high}) from cells with low
CD45RA staining intensity. Thus, the upper right quadrant in Fig. 2 B contains naïve CD4\textsuperscript{+}
subpopulation (CD45RA\textsuperscript{low} CCR7\textsuperscript{+}), the upper left quadrant the central memory
subpopulation (CD45RA\textsuperscript{low} CCR7\textsuperscript{+}), the lower left quadrant effector memory cells
(CD45RA\textsuperscript{low} CCR7\textsuperscript{neg}), and the lower right quadrant terminally differentiated effector
memory cells expressing CD45RA (CD45RA\textsuperscript{high} CCR7\textsuperscript{neg}). CD8\textsuperscript{bright} lymphocytes were
identified as low SSC cells with a fluorescence intensity above 100 arbitrary units in the FL-3
channel (Fig. 2 C) and analysed as described before (Fig. 2 D).

The calculation of reference ranges (5\textsuperscript{th} and 95\textsuperscript{th} percentiles, arithmetic mean and
standard deviations of all parameters) was performed using SAS for Windows. The
comparison of relative and absolute lymphocyte counts obtained by SP and DP FCM was
done as described by Bland and Altman.

RESULTS

Strengths and weaknesses of the simplified DP FCM method. First, we studied
potential limitations of the simplified single-tube DP FCM method in blood samples from
healthy laboratory personnel using a four-color flow cytometer (FACScalibur™) in
Heidelberg. Fig. 1 shows the typical result of such an experiment. An electronic gate (G1 =
R1) set on the cell population with low forward scatter and low side scatter characteristics
was used to identify peripheral blood lymphocytes (Fig. 1 A). The expression of different
surface antigens, simultaneously stained with different fluorescence-labeled monoclonal
antibodies and analysed in a FL-1 vs. FL-2 dot plot (Fig. 1 B) allowed the separation of
CD19+ B-cells as events with high FL-1 (FITC) signal intensity from CD3+ T-cells exhibiting
high FL-2 (PE) signal intensity (Fig. 1 B and C, regions R5 and R3, respectively). NK-cells
were identified as CD56+ cells with lower FL-2 intensity than CD3+ T-cells (region R4 in Fig.
1 B and C).

Region R8 in Fig. 1 B contains cells that were neither stained with antibodies against
CD3, nor CD8, CD56 or CD19. This cell population was excluded from analysis by electronic
gating (region R2 in Fig. 1 B). The dot plot used for final analysis is shown in Fig. 1 C. CD3+
T-cells were further separated into CD4+ and CD8+ subpopulations in a FL-3 (PerCP) vs.
FL-1 (FITC) dot plot (Fig. 1 D; CD4+ T-cells = region R6, CD8+ T-cells = region R7). As
shown in the histogram in Fig. 1 E, nearly all NK-cells identified as CD56+ lymphocytes were
positive for the NK-cell marker CD94; the electronic gate (G4 = R1 and R4) contained only
very few CD14+ monocytes. Contamination of the B-cell gate (G5 = R1 and R5) by CD14+
monocytes or CD94+ NK-cells was also minimal (Fig. 1 F). Thus, gate purity for detection of
NK-cells and B-cells was high. However, the use of CD56 as the only NK-cell marker lead to the loss of specific NK-cell subsets from analysis. As shown in Fig. 1 G, unstained cells in region R8 comprised both CD14+ monocytes and CD94+ NK-cells (electronic gate G8 = region R1 and region R8).

When adding unlysed erythrocytes to the measurement tubes, these erythrocytes appeared in the lower left part of region R8; they could be electronically excluded and did not disturb analysis of lymphocyte subpopulations (data not shown). The results of lymphocyte subset analysis were also not influenced by increasing numbers of unstained cells in the test tube (data not shown). Thus, the single-tube FCM method in combination with the electronic gating procedure described here was resistant against typical preanalytic confounders of clinical flow cytometry. Intraindividual coefficients of variation in the distribution of lymphocyte subsets were as follows: CD3+ T-cells 3%, CD4+ T-cells 4%, CD8+ T-cells 4%, CD19+ B-cells 7%, and CD56+ NK-cells 13% (average results of up to 10 measurements on consecutive days in five healthy individuals).

Methodological comparison of DP and SP FCM. We then applied the simplified DP FCM method in a field study on blood samples from 177 healthy adults in Burkina Faso and observed the following distribution of the lymphocyte differential (mean ± standard deviation):

- CD3+ T-cells represented the most abundant subpopulation (77±8%), followed by CD19+ B-cells (13±5%) and CD56+ NK-cells (10±6%). The mean CD4+-to-CD8+ T-cell ratio was 1.61±0.61, the mean frequency of CD4+ T-cells was 42±7%, that of CD8+ T-cells 29±7%.
- Absolute numbers of CD3+ T-cells were 1,799±537 µL⁻¹, of CD19+ B-cells 316±168 µL⁻¹, and of CD56+ NK-cells 238±186 µL⁻¹. Mean CD4+ T-cell counts were 980±293 µL⁻¹, mean CD8+ T-cell counts were 676±300 µL⁻¹.

In order to examine whether the DP FCM method and the standard SP FCM method agreed sufficiently to be regarded as interchangeable we analysed data on simultaneous measurements with both methods according to Bland and Altman. Both the simplified DP
FCM method and the standard SP FCM method gave nearly identical percentages of B-cells, while percentages of T-cells were higher by DP FCM compared to SP FCM, and percentages of NK-cells were lower (Fig. 3). Absolute numbers of these lymphocyte subpopulations (Fig. 4) were higher if measured by SP FCM compared to DP FCM. The bias for T-cells was about 10% of the average T-cell number, for B-cells about 20% of the average B-cell number, and for NK-cells about 50% of the average NK-cell number. Thus, the mean bias of absolute cell numbers of these lymphocyte subpopulations was clinically relevant.

*Fig. 5* illustrates the comparison between percent values and absolute numbers of CD4+ T-cells (Fig. 5 A and B) and CD8+ T-cells (Fig. 5 C and D) generated with the two FCM methods. Bland-Altman analysis indicates that both methods gave nearly identical percentages of CD4+ T-cells, while percentages of CD8+ T-cells were higher if measured by DP FCM. Absolute numbers of both T-cell subpopulations measured by SP FCM were higher than those obtained with DP FCM. Comparison of percent values showed a low bias with an acceptable precision both for CD4+ and CD8+ T-cells. The bias in terms of absolute cell numbers was approximately 15% of the average cell number in CD4+ T-cells and approximately 5% of the average cell number in CD8+ T-cells, thus being clinically relevant.

**Reference values for T-cell maturation phenotypes.** Reference values of the relative distribution (percentages and absolute numbers) of T-lymphocyte maturation phenotypes obtained from 177 healthy adults living in Nouna, Burkina Faso, are shown in Table 1. The characteristic differences between CD4+ and CD8bright lymphocytes are also illustrated in Fig. 2.
DISCUSSION

We developed a DP FCM method that allows (a) phenotyping of major lymphocyte subsets in a single test tube (T1) by flow cytometry and (b) quantification of lymphocyte subsets by simultaneous determination of lymphocyte numbers with an automated hematology counter. Preparatory experiments and analysis of comparative measurements between simplified DP and standard SP FCM revealed that DP FCM systematically underestimated the percentage of NK-cells, probably because these cells are not completely identified by the exclusive use of CD56 as the sole NK-cell marker. It has been shown that human NK-cells follow a maturational pathway leading from CD16+ CD56neg precursor cells to mature CD16+ CD56+ and CD16neg CD56+ NK-cells. NK precursor cells are not detected in the T1 assay. Thus, the use of this assay does not permit quantification of specific subpopulations such as NK-cell precursors and is limited to screening for a skewed distribution of major subtypes of lymphocytes.

Additionally, the CD56bright NK-cell subset may appear in the T-cell region of the T1 analysis, thereby decreasing the percentage of NK-cells and concomitantly increasing the percentage of CD3+ and potentially also of CD8+ T-cells. These subpopulations were slightly overestimated by DP FCM compared to SP FCM, whereas similar proportions of B-cells and of CD4+ T-cells were detected with both methods. Despite these shortcomings, the T1 FCM method seems to be suitable for immunological field studies in developing countries. Miniaturization of the assay allows the measurement to be performed in samples of 25 µl of blood at a price of less than 3 US $. It can reliably be performed by skilled laboratory personnel after a short training even on very low volumes of capillary blood from newborn infants (von Au, unpublished results), and it is suitable for immunological screening during infectious diseases (or after vaccinations) even in infants suffering from malnutrition, which may influence the distribution of lymphocyte subpopulations other than CD4+ T-cells. Several cheap methods have been developed for...
CD4+ T-cell counting in developing countries, however, these assays usually do not yield the complete lymphocyte differential count. A FCM method similar to the T1 single-tube protocol (Lymphogram) was published by Bellido et al. No systematic comparison of the Lymphogram method and standard FCM was performed on a larger patient cohort or on samples from healthy controls. Giustolisi et al. compared Lymphogram measurements to standard multicolor FCM of lymphocyte samples from patients with suspected leukemia and stated that the results “showed no major discordance”. No calculation of absolute numbers of T-, B- and NK-cells was attempted.

In our study, we did not only compare percentages of lymphocyte subpopulations but also absolute cell numbers and found a significant systematic bias, showing that SP FCM constantly yielded higher absolute cell counts than DP FCM. This systematic bias had already been described by Nicholson et al., who found higher absolute cell counts (increased by 5 to 10%) with SP FCM using MultiSET/TruCount methodology compared to conventional DP FCM (SimulSET™ and automated hematology). In our cohort in Nouna, Burkina Faso, absolute CD4+ T-cell counts obtained with SP FCM were approximately 200 cells µL⁻¹ higher than absolute CD4+ T-cell counts obtained with DP FCM (calculated by multiplication of the MultiSET™ data on percent CD4+ T-cells and the lymphocyte number simultaneously measured with the Sysmex hematology analyser). Thus, the observed bias between DP FCM and SP FCM corresponds to values reported in the literature.

In the second part of our study we used a panel of monoclonal antibodies in addition to the single-tube DP FCM method in order to assess the relative distribution of naïve and memory subpopulations of peripheral blood T-cells. To our knowledge, this is the first description of T-cell maturation phenotypes in a larger cohort of West African healthy adults, which is based upon expression patterns of CD45RA and CCR7. Among CD4+ lymphocytes, such expression patterns have been previously described in 8 healthy controls in the USA. In that study, 28±2% (mean ± SEM) of CD4+ T-cells were T naïve (CD45RA+ CCR7+), 59±2% were T CM (CD45RAneg CCR7+), 11±1% were T EM (CD45RAneg CCR7neg), and 2±1% were…
TEMRA (CD45RA+ CCR7neg). In another study\textsuperscript{11} results on CD4+ T-cells from 11 normal US-American healthy adult volunteers were reported (median and range): 35 (20 to 50)% of cells were TEMRA, 49 (15 to 70)% were TEM, 12 (2 to 27)% were TEM, and 2 (0 to 22)% were TEMRA.

When the theoretical model of CD4+ and CD8+ effector and memory T-cell generation was first described\textsuperscript{12,16}, authors made the cautionary comment that the markers used for discrimination of these T-cell subpopulations are rapidly and transiently modulated upon cell activation and the phenotypic characterisation only applies to resting cells, i.e. those that are not engaged in an antigen-driven response\textsuperscript{12}. Especially for the CD8+ T-cell compartment it has been shown that both the effector memory and the central memory subsets contain populations at intermediate stages of differentiation which may develop into both directions of the differentiation pathway\textsuperscript{15}. The method used in our study is more reliable in the CD4+ than in the CD8+ T-cell subset. We focused our study on CD8\textsuperscript{bright} T-cells because these cells represent the major CD8+ T-cell subpopulation (>95%). We explicitly excluded CD8\textsuperscript{dim} lymphocytes (representing mainly NK-cells and NK-T-cells) from analysis of maturation phenotypes by electronic gating. Both CD8\textsuperscript{dim} NK-cells and CD8\textsuperscript{dim} T-cells express high levels of CD45RA and their inclusion leads to falsely elevated proportions of the naïve subpopulation. Our gating strategy allows us to exclude these cells (which seem not to participate in the dynamic maturation process of the major CD4+ and CD8+ T-cell subpopulations) despite the use of a flow cytometer equipped with only a single laser.

Recently, Saule et al.\textsuperscript{14} obtained data on the relative distribution of naïve and memory subpopulations of CD4+ and CD8+ T-cells in 101 healthy blood donors in France and showed changes in the distribution patterns with advancing age of the donors. When comparing our data with those of Saule et al.\textsuperscript{14} for the age range of 20 to 40 years (65% of the Nouna donors were in that age range), the median percentage of naïve T-cells in West African donors were only half of those in European donors, while effector memory T-cells were nearly twice as high (CD4+ T\textsubscript{naïve}: 22% vs. 50%, TEM: 44% vs. 19%; CD8+ T\textsubscript{naïve}: 26%
In terms of absolute cell numbers, these differences were also impressive (CD4+ T naïve: 204 vs. 357 cells µL⁻¹, T EM: 405 vs. 136 cells µL⁻¹; CD8+ T naïve: 151 vs. 190 cells µL⁻¹, T EM: 215 vs. 72 cells µL⁻¹)⁴. CD4+ T-cells from European donors aged above 75 years were 34% T naïve (216 cells µL⁻¹) and 24% T EM (156 cells µL⁻¹), CD8+ T-cells were 12% T naïve (49 cells µL⁻¹) and 27% T EM (84 cells µL⁻¹)⁴.

In a study on healthy adults from Wonji, Ethiopia⁸, T-cell maturation phenotypes were defined by differential expression of CD45RA and CD27 and the following distribution patterns were observed (median and range): among CD4+ T-cells, 20 (5 to 47)% were naïve (CD45RA+ CD27+), 53 (40 to 66)% had a memory phenotype (CD45RAneg CD27+), 21 (9 to 45)% showed an effector memory phenotype (CD45RAneg CD27neg), and 1 (0 to 5)% were cytotoxic effector cells (CD45RA+ CD27neg). Among CD8+ T-cells, 29 (5 to 60)% were naïve (CD45RA+ CD27+), 21 (6 to 48)% had a memory phenotype (CD45RAneg CD27+), 9 (2 to 53)% showed an effector memory phenotype (CD45RAneg CD27neg), and 34 (8 to 78)% were cytotoxic effector cells (CD45RA+ CD27neg). Compared to healthy controls from Amsterdam, the Netherlands, HIV-negative Ethiopians had significantly reduced naïve and increased effector subsets in both CD4+ and CD8+ T-cell compartments and increased cytotoxic effector cells in the CD8+ T-cell compartment:

In summary, data from Burkina Faso (our study) and Ethiopia⁸ indicate substantial T-cell activation in African adults compared to people living in France¹⁴. The distribution pattern of T-cell maturation phenotypes may be a surrogate parameter for environmentally driven immuno-senescence. Even compared to Europeans with a chronological age above 75 years, young West Africans showed a skewed distribution of T-cell maturation phenotypes with higher numbers of effector memory and lower numbers of naïve CD4+ T-cells.


14. Saule, P., J. Trauet, V. Dutriez, V. Lekeux, J. P. Dessaint, and M. Labalette. 2006. Accumulation of memory T cells from childhood to old age: central and effector memory cells...


FIGURE LEGENDS

FIGURE 1. Quantification of peripheral blood lymphocyte subsets in a single FCM tube. Lymphocytes are identified as low forward scatter (FSC) and low side scatter (SSC) cells (region R1 in panel A). Details of the gating strategy are described in the text. Region R8 in panel B contains cells that are not stained with antibodies against CD3, CD8, CD56 or CD19. This cell population is excluded from analysis by defining the lymphocyte gate G1 = R1 and R2 (R2 = R3 or R4 or R5 but not R8 in panel B). The dot plot for final analysis is shown in panel C. Panels E and F show purity of gating of NK- and B-cells. The NK-cell gate (R1 and R4) contains mainly CD94+ NK-cells (panel E, bold line) and very few CD14+ monocytes (panel E, thin line). Neither CD94+ NK-cells nor CD14+ monocytes are found in the B-cell gate (R1 and R5) (panel F). Cells that are not stained with antibodies against CD3, CD8, CD56 or CD19 contain CD14+ monocytes (panel G, thin line) as well as CD56neg CD94+ NK-cells (panel G, bold line).
FIGURE 2. T lymphocyte maturation phenotypes. (panel A): CD4⁺ lymphocytes are identified as low side scatter (SSC) cells with a high staining intensity in fluorescence channel FL-3 (CD4 PerCP). (panel B): At least 2,500 events in the CD4⁺ lymphocyte gate were analysed for expression of CD45RA and CCR7: upper right, naïve (CD45RA<sup>high</sup> CCR7<sup>+</sup>), upper left, central memory (CM; CD45RA<sup>low</sup> CCR7<sup>+</sup>), lower left, effector memory (CD45RA<sup>low</sup> CCR7<sup>neg</sup>), and lower right, terminally differentiated effector memory expressing CD45RA (CD45RA<sup>high</sup> CCR7<sup>neg</sup>); a typical distribution pattern is displayed. (panel C): CD8<sup>bright</sup> lymphocytes were identified as low side scatter (SSC) cells with a fluorescence intensity above 100 arbitrary units in the FL-3 channel (CD8 PerCP). (panel D): At least 2,500 events in the CD8<sup>bright</sup> lymphocyte gate were analysed for expression of CD45RA and CCR7; a typical distribution pattern is displayed. (panel E): Markers for the discrimination of T lymphocyte maturation phenotypes were set using a highly purified mouse IgG isotype control to define the boundary between CCR7<sup>+</sup> (upper left and upper right quadrant in panel B, D and E) and CCR7<sup>neg</sup> (lower left and lower right quadrant). The boundary on the FL-1 axis was fixed at 100 arbitrary fluorescence units in order to identify cells with high CD45RA staining intensity (upper right and lower right quadrant). Quadrant statistics yield the percentages of the T-cell maturation phenotypes for CD4⁺ (panel B) and CD8<sup>bright</sup> lymphocytes (panel D).

FIGURE 3. Bland-Altman diagrams<sup>4</sup> of the relative distribution of T-, B- and NK-lymphocyte subpopulations determined by a single-platform lyse-no-wash FCM method (MultiSET™) and a simplified single-tube, dual-platform lyse-and-wash method (T1). The arithmetic mean of lymphocyte percentages (single-platform FCM plus dual-platform FCM / 2) is displayed on the x-axis, the difference of lymphocyte percentages (single-platform FCM minus dual-platform FCM) on the y-axis. Horizontal lines are indicating the mean difference (bias) ± 2 SD (precision) of simultaneous measurements with both methods in 177 adults.
FIGURE 4. Bland-Altman diagrams of absolute counts of T-, B- and NK-lymphocyte subpopulations determined by a single-platform lyse-no-wash FCM method (MultiSET™) and a simplified single-tube, dual-platform lyse-and-wash FCM method (T1). The arithmetic mean of absolute cell counts (single-platform FCM plus dual-platform FCM / 2) is displayed on the x-axis, the difference of absolute cell counts (single-platform FCM minus dual-platform FCM) on the y-axis. Horizontal lines are indicating the mean difference (bias) ± 2 SD (precision) of simultaneous measurements with both methods in 177 adults.

FIGURE 5. Bland-Altman diagrams of relative distribution and absolute cell counts of CD4+ and CD8+ T lymphocyte subpopulations determined by a single-platform lyse-no-wash FCM method (MultiSET™) and a simplified single-tube, dual-platform lyse-and-wash FCM method (T1). The arithmetic mean of lymphocyte percentages or absolute cell counts (single-platform FCM plus dual-platform FCM / 2) is displayed on the x-axis, the difference of lymphocyte percentages or absolute cell counts (single-platform FCM minus dual-platform FCM) on the y-axis. Horizontal lines are showing the mean difference (bias) ± 2 SD (precision) of simultaneous measurements with both methods in 177 adults.
TABLE 1. Reference values of relative (%) and absolute counts (µL⁻¹) of CD4⁺ and CD8bright lymphocyte subpopulations obtained from 177 healthy adults (109 female, 68 male) in Burkina Faso. Data are given as mean ± standard deviation, lower and upper limits of normal (i.e., 5th and 95th centiles) are shown in parenthesis.

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<th>central memory (CM)</th>
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<th>effector memory (EM)</th>
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<td></td>
<td>%</td>
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<td>CD4⁺ lymphocytes</td>
<td>28 ± 7 (18 - 40)</td>
<td>277 ± 104 (126 - 478)</td>
<td>22 ± 10 (5 - 41)</td>
<td>228 ± 146 (35 - 496)</td>
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<td>CD8bright lymphocytes</td>
<td>3 ± 2 (1 - 5)</td>
<td>16 ± 11 (4 - 34)</td>
<td>27 ± 13 (7 - 52)</td>
<td>173 ± 103 (36 - 363)</td>
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Abbreviation: EMRA = terminally differentiated effector memory cells expressing CD45RA
CD4+ lymphocytes (panel B)
CD45RA\textsubscript{low} CCR7\* = 41%
CD45RA\textsubscript{high} CCR7\* = 28%
CD45RA\textsubscript{low} CCR7\textsuperscript{neg} = 29%
CD45RA\textsubscript{high} CCR7\textsuperscript{neg} = 2%

CD8\textsuperscript{bright} lymphocytes (panel D)
CD45RA\textsubscript{low} CCR7\* = 4%
CD45RA\textsubscript{high} CCR7\* = 51%
CD45RA\textsubscript{low} CCR7\textsuperscript{neg} = 29%
CD45RA\textsubscript{high} CCR7\textsuperscript{neg} = 16%
Difference of NK cell numbers (µL\(^{-1}\))

Difference of B cell numbers (µL\(^{-1}\))

Difference of T cell numbers (µL\(^{-1}\))

Arithmetic mean of NK cell numbers (µL\(^{-1}\))

Arithmetic mean of B cell numbers (µL\(^{-1}\))

Arithmetic mean of T cell numbers (µL\(^{-1}\))

Bias 168 µL\(^{-1}\)

Precision ± 321 µL\(^{-1}\)

Bias 74 µL\(^{-1}\)

Precision ± 246 µL\(^{-1}\)

Bias 168 µL\(^{-1}\)

Precision ± 520 µL\(^{-1}\)
Arithmetic mean of CD4+ T cell counts (%)

Difference of CD4+ T cell counts (%)

Difference of CD4+ T cell numbers (µL\(^{-1}\))

Arithmetic mean of CD4+ T cell numbers (µL\(^{-1}\))

Arithmetic mean of CD8+ T cell counts (%)

Difference of CD8+ T cell counts (%)

Difference of CD8+ T cell numbers (µL\(^{-1}\))

Arithmetic mean of CD8+ T cell numbers (µL\(^{-1}\))

Bias –0.8%
Precision ± 6.5%

Bias –2.9%
Precision ± 5.8%

Bias 161 µL\(^{-1}\)
Precision ± 330 µL\(^{-1}\)

Bias 42 µL\(^{-1}\)
Precision ± 260 µL\(^{-1}\)