Improbable Flow Cytometric Measurements

Philippe Van de Perre¹*, Amandine Pisoni¹, Vincent Baillat², Nicolas Nagot¹, Edouard Tuaillon¹

¹Pathogenesis and Control of Chronic and Emerging Infections, University of Montpellier, INSERM, EFS, University of Antilles, CHU Montpellier, Montpellier, France
²Department of Infectious Diseases, CHU Montpellier, Montpellier, France

*Corresponding author: Philippe Van de Perre, Pathogenesis and Control of Chronic and Emerging Infections, University of Montpellier, INSERM, EFS, University of Antilles, CHU Montpellier, Montpellier, France

Abstract

Here we report an exceptional observation in clinical practice: the occurrence of strictly similar cytometry measurements involving five different parameters on two distinct blood samples collected seven months apart from the same HIV-infected patient.

Keywords: cytometry; Follow up HIV therapy; CD4 count

Introduction

Quantification of lymphocyte subpopulations by flow cytometry is routinely used to appreciate immune restoration induced by antiretroviral therapy (ART) in HIV-infected subjects and to estimate the capacity of the patient to mount an immune response [1]. Assessment of CD4 T cells by flow cytometry is automated, robust and has intra-run variation coefficient of approximately 1 to 3% [2]. However, CD4 T cell count is subject to significant intra-individual variability due to circadian changes in healthy individuals, and to factors such as treatments and comorbidities in persons living with HIV. Variation thresholds as high as 50% and 6.4% have been proposed for declines of CD4 T cells counts and percentage, respectively [2]. Here, we report and comment the improbable case of strictly identical flow cytometry measurement results involving several parameters on two distinct blood samples collected seven months apart from the same patient.

Materials and methods

The patient was a 60 years old HIV-infected man followed in the Department of Infectious Diseases of the University Teaching Hospital (CHU) in Montpellier, France for long-term ART (dolutegravir/abacavir/lamivudine). He was asymptomatic and considered as on fully suppressive therapy for several years. CD4 T cells were enumerated using four colours staining with a panel of antibodies specific for CD3, CD4, CD8, CD45, (CYTO-STAT1/tetra-CHROM™, Beckmann Coulter) on an automated flow cytometer (Navios, Beckman Coulter). The lymphocyte counts and lymphocyte subsets were enumerated at the last four 6-monthly visits, as displayed in the Table 1.
Table 1: Lymphocyte count and T cell subsets enumeration on four consecutive blood samples collected six months apart from the same patient.

| Date of sampling/analysis | Reference range | 12 May 2020 | 13 October 2020 | 11 May 2021 | 26 October 2021 |
|---------------------------|----------------|------------|----------------|------------|----------------|
| Lymphocytes count, cell/mm³ | 1500-4000 cells/mm³ | 1512 | 2178 | 2178 | 1628 |
| Lymphocytes TCD3+, %    | 60-80%          | 52         | 55           | 55         | 56           |
| Lymphocytes TCD3+, n/mm³ | 600-2100 cells/mm³ | 786        | 1198         | 1198       | 911          |
| Lymphocytes TCD4+, %    | 30-45%          | 28         | 31           | 31         | 31           |
| Lymphocytes TCD4+, n/mm³ | 690-1200 cells/mm³ | 423        | 675         | 675        | 504          |
| Lymphocytes TCD8+, %    | 20-35%          | 23         | 22           | 22         | 24           |
| Lymphocytes TCD8+, n/mm³ | 390-820 cells/mm³ | 348        | 479         | 479        | 391          |
| Ratio CD4+/CD8+         | 0.9-2.0         | 1.2        | 1.4          | 1.4        | 1.3          |
| Lymphocytes TCD8+CD38+/% | 32              | 44         | 49           | ND         |               |
| Lymphocyte CD8+CD38++, %| 1-7%            | 1.9        | 5.8          | 8.5        | ND           |

ND=Not done

Results

On October 13, 2020 and on May 11, 2021, not only were the lymphocyte counts strictly identical but lymphocytes subsets in percentages were also identical although strikingly different from the previous (12 May 2020) and the next (26 October 2021) six-month samples. A notable exception consisted of the activated CD8 T cells, which differed in the last two samples (although within the expected intra-laboratory variation coefficient) [3]. Based on the latter for total lymphocytes, CD3-, CD4- and CD8-T cell counts, the probability of having exactly the same results in May 2021 as in October 2020 for these 4 parameters, at the unit level, was 1.2 10-12.

After verification of the validity of 11 May 2021 results, analyses were validated and notified to the practitioner and to the patient. ART was maintained unchanged. In the last two years, the automated method used for lymphocyte count [4] had not changed and the flow cytometer was used by means of exactly the same gating rules. The flow cytometer is calibrated on a monthly basis, Levey-Jennings graphs are generated and interpreted every month and an international quality assessment/quality control program (UK NEQAS) applied every other month. Cytometry analyses are routinely performed on fresh blood samples collected on EDTA tubes. The primary blood samples are discarded directly after measurements have been validated, which excludes redundant measurements on the same sample. In order to exclude artefactual observations, the screenshots of the two-flow cytometry analyses were compared (Figure 1). These graphs displayed discrete differences in cell populations’ distribution that are not distinguishable based on crude enumeration data (percentages and absolute counts). We concluded that the analyses done on 13 October 2020 and on 11 May 2021 had effectively been performed on distinct blood samples and that the similarity of results is not artefactual but attributable to chance only.
Figure 1: Phenotypic analysis of T cell subsets in blood samples collected and analysed seven-month apart. The figure consists of screenshots of the scatter plots of the following selected parameters produced by the flow cytometer.

A. CD3+CD4+
B. CD3+CD8+
C. CD8+CD38+

It shows discrete differences in cell distributions for CD3+CD4+ and CD3+CD8+ cells and a clear difference for CD8+CD38+ cells.

Discussion

Our laboratory is performing lymphocytes immune-phenotyping by flow cytometry on a routine basis on more than 60 samples a week since more than 25 years and it is the very first time that such event – two identical results involving five parameters on two separate samples collected several months apart – occurs. Based on repeatability and reproducibility assessments of the parameters’ measurements (Supplemental material) and the normal value ranges, the chance of obtaining two strictly identical measurements of several parameters on two separate blood samples from the same individual is deemed infinitesimal.

Conclusion

In the case of biologic phenomenon, very improbable observation can occur and should not be a priori disregarded.

References

1. Autran B, Carcelain G, Li TS, Blanc C, Mathez D, et al (1997) Positive effects of combined antiretroviral therapy on CD41 T cell homeostasis and function in advanced HIV disease. Science 277: 112-116.
### Supplemental appendix

#### REPEATABILITY

| Samples name       | Assays (N) | Mean    | Standard deviation | Variation coefficient (%) |
|--------------------|------------|---------|--------------------|---------------------------|
| Ly T CD3+ cells/µL | Sample 1   | 10      | 707.8              | 26                        | 3.7                        |
|                    | Sample 2   | 10      | 2774.5             | 128.5                     | 4.6                        |
| Ly T CD3+, %       | Sample 1   | 10      | 75.6               | 0.5                       | 0.7                        |
|                    | Sample 2   | 10      | 76.2               | 0.5                       | 0.6                        |
| Ly T CD3+ CD4+ cells/µL | Sample 1   | 10      | 93.8               | 9.3                       | 9.9                        |
|                    | Sample 2   | 10      | 1529.6             | 84.4                      | 5.5                        |
| Ly T CD3+ CD4+, %  | Sample 1   | 10      | 10                 | 0.7                       | 7.3                        |
|                    | Sample 2   | 10      | 42                 | 0.9                       | 2.1                        |
| Ly T CD3+ CD8+ cells/µL | Sample 1   | 10      | 577.6              | 19.2                      | 3.3                        |
|                    | Sample 2   | 10      | 1226.9             | 56.5                      | 4.6                        |
| Ly T CD3+ CD8+, %  | Sample 1   | 10      | 61.7               | 0.8                       | 1.3                        |
|                    | Sample 2   | 10      | 33.7               | 0.8                       | 2.5                        |

#### REPRODUCIBILITY

| Samples name       | Assays (N) | Mean    | Standard deviation | Variation coefficient (%) |
|--------------------|------------|---------|--------------------|---------------------------|
| Ly T CD3+ cells/µL | Immunotrol low | 30      | 425.63             | 31.65                     | 7.44                       |
|                    | Immunotrol  | 35      | 778.37             | 76.82                     | 9.87                       |
| Ly T CD3+, %       | Immunotrol low | 30      | 54.18              | 1.13                      | 2.09                       |
|                    | Immunotrol  | 35      | 71.7               | 0.72                      | 1.01                       |
| Ly T CD3+ CD4+ cells/µL | Immunotrol low | 30      | 130.83             | 11.26                     | 8.61                       |
|                    | Immunotrol  | 35      | 540.63             | 52.62                     | 9.73                       |
| Ly T CD3+ CD4+, %  | Immunotrol low | 30      | 16.65              | 0.64                      | 3.87                       |
|                    | Immunotrol  | 35      | 49.82              | 0.7                       | 1.4                        |
| Ly T CD3+ CD8+ cells/µL | Immunotrol low | 30      | 269.77             | 21.52                     | 7.98                       |
|                    | Immunotrol  | 35      | 218.11             | 23.52                     | 10.78                      |
| Ly T CD3+ CD8+, %  | Immunotrol low | 30      | 34.32              | 0.91                      | 2.65                       |
|                    | Immunotrol  | 35      | 20.07              | 0.46                      | 2.28                       |

Supplemental material: Repeatability and reproducibility assessments of the cytometry parameters measurements.