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A Standardized Flow Cytometry Procedure for the Monitoring of Regulatory T Cells in Clinical Trials

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Background: Quantification of regulatory T cells (Tregs) is crucial in immunomonitoring in clinical trials as this cell population has been shown to be involved in a wide range of diseases, including cancers, autoimmune diseases, infections, and allergies. Human Tregs are defined as $CD4^+$ $CD25^+$ $CD127^{low}$ $FoxP3^+$ cells, and the standardization of their staining by flow cytometry is a challenge, especially in multicenter clinical trials, notably because of the intracellular location of FoxP3.

Method: A flow cytometry staining procedure was settled and standardized to measure human Tregs in peripheral whole blood using precoated dried antibodies in ready-to-use tubes. It was compared with reference methods and implemented and validated to be suitable with different cytometer platforms.

Results: The standardized protocol developed with dried antibodies and reduced volumes of whole blood allows an optimal identification of Tregs. Compared with classical staining procedure, it reduces the number of steps required, in a very fast and simple technique. The accuracy of the method was confirmed by a multicenter comparison with different cytometer brands.

Conclusions: Our results highlight the reliability of this high-standard protocol that could become a reference method for the monitoring of Tregs in clinical trials. **© 2018 International Clinical Cytometry Society**

Key terms: flow cytometry; regulatory T cells; standardization

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Regulatory T cells (Tregs) are a key subset of T lymphocytes able to modulate the immune response by maintaining tolerance to self-antigens and preventing an excessive response of effector T cells (1,2). Tregs have been shown to play a critical role in multiple diseases, including autoimmune diseases (3) cancers (4), infections, cardiovascular diseases (5) and allergies (6). They are therefore a key parameter in various diseases and in the follow-up of patients in therapeutic trials (7). Human Tregs are usually defined as $CD3^{+}CD4^{+}CD25^{high}$ $CD127^{low}Foxp3^{+}$ cells (8). Treg assessment by flow cytometry is particularly tricky because the intranuclear localization of Foxp3 imposes numerous technical steps that are time-consuming and often yield poorly reproducible results. It is difficult to standardize these technics in laboratory routine for clinical trials or clinical research with large numbers of patients.

Recently developed reagents allow the labeling of limited amounts of whole blood and reduce processing times (9,10), but other pitfalls and sources of variation need to be addressed, that is, stability of antibodies over time with tandem degradation, antibody batch variations, risk of pipetting errors during preparation of antibody mixtures, and between-center differences in brand of cytometer.

Drying or lyophilization are the methods of choice for long-term storage and stabilization of fluorescent antibodies, which are otherwise unstable in liquid. Duraclone technology developed by Beckman Coulter® produces custom-designed panels of antibodies that are dried in individual tubes for direct labeling. These dried reagents are stable at room temperature and have a longer shelf-life than liquid antibodies, even for tandem dyes. This technology dramatically reduces pipetting errors, stability issues and batch-related bias, as it guarantees the same volume of antibodies in each test. Altogether, these features optimize assay reproducibility.

In the context of a European multicenter trial of type 1 diabetes patients treated with low-dose IL-2 (DIABIL-2 trial, *NCT02411253*), we aimed to measure Treg levels in 138 patients at 9 follow-up points over two years. Because samples had to be processed rapidly and as it was difficult and expensive to organize sample centralization in one laboratory, we developed a standardized method for Treg quantification using Duraclone tubes and implemented the method in the 18 laboratories across Europe involved in the trial.

MATERIAL AND METHODS

Blood samples from healthy donors were obtained from the Etablissement Français du Sang according to ethical guidelines and were collected in EDTA or heparin lithium tubes.

Antibody Labeling of Fresh Blood

Liquid reagents.

Antibody clones CD3-FITC (clone UCHT1, 1 µL), CD25-PE (clone B1.49.9, 10 µL), CD127-PCy7 (clone R34.34, 5 µL), Foxp3-AF647 (clone 259 D, 10 µL), CD8-KRO (clone 13B8.2, 5 µL), CD4-PB (clone B9.11, 5 µL), and Perfix-NC kits were from Beckman Coulter.

Dried format reagents.

Using Beckman Coulter's custom design service and its dry coating technology, custom tubes containing the same amount of CD3-FITC, CD25-PE, CD127-PE-Cy7, Foxp3-AF647, CD8-KRO, and CD4-PB antibodies as used in liquid staining were produced.

Staining protocol.

Total blood (60 µL) sampled with anticoagulant was mixed with 6 µL of Perfix-NC R1 buffer, vortexed immediately for 2–3 s and incubated for 15 min at room temperature in the dark. Perfix-NC R2 buffer (360 µL) was added and, after homogenization, a 360 µL aliquot was transferred to a Duraclone tube or mixed with the liquid antibody mix. For the compensation set-up, single color staining was performed with liquid or the Duraclone technique. After vortexing for 10 s, tubes were incubated for 60 min at room temperature in the dark. PBS 1× (3 mL) was added to the tubes, incubated for 5 min at room temperature in the dark before centrifugation for 6 min at 250*g*. The supernatant was removed to leave the pellet dried and the cells were resuspended in 3 mL of 1× Perfix-NC R3 buffer prior to another 6-min centrifugation at 250*g*. The pellet was dried and resuspended in 300 µL of 1× R3 buffer. Tubes were protected from light and stored at 4°C until the acquisition on a cytometer within the next 24 h.

Staining of Peripheral Blood Mononuclear Cells

Antibody clones CD3-AA700, CD4-ECD, CD8-AA750, and CD25-PE were incubated for 15 min in 100 µL of 2% PBS-FBS containing one million cells and washed in PBS. Cells were then resuspended in 1 mL of 1× Fix/Perm buffer (eBioscience), prepared according to manufacturer's instructions, and incubated for 30 min at 4°C in the dark. Cells were washed with 2 mL of 1× permeabilization buffer (eBioscience) and incubated for 15 min at 4°C in the dark in 2% rat blocking serum (eBioscience). FoxP3-APC antibody was then added prior to

incubation for 30 min at 4°C in the dark. Cells were finally washed with permeabilization buffer and resuspended in 2% PBS-FBS. Tubes were protected from light and stored at 4°C until the acquisition on a cytometer within the next 24 h.

Acquisition.

Rainbow Calibration Particles, 8 peaks (3.0–3.4 μm) (Biolegend) were prepared by adding one drop of beads in 300 μL of deionized water.

Acquisitions were performed on a Navios cytometer (Beckman Coulter) maintained daily according to the manufacturer's recommendations with Flow Check Pro and Flow Set Pro fluorospheres, or on an LSRII (Becton Dickinson). Acquisitions were performed, respectively, using Navios software or DIVA, and all analyses were done with Kaluza 1.3 software (Beckman Coulter).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA). The normal distribution of each set of variables was analyzed using the D'Agostino & Pearson omnibus normality test. Paired data were compared using Student's *t* test. Correlation tests were performed using the Pearson test. A P value < 0.05 was considered significant.

RESULTS

Panel Design: Choice of Markers and Method

Most routine techniques for staining of Tregs use peripheral blood mononuclear cells (PBMCs) isolated by density gradient. Here, whole blood staining was preferred to PBMCs to limit handling and to reduce blood sample volume. Duraclone technology was used to reduce the number of technical steps and to minimize bias, as it ensures the same quantity of antibodies from the same batch and is very stable over time (certified for one year by the manufacturer). We used the Perfix-NC kit for intranuclear permeabilization and staining, so as to simplify and accelerate labeling. Compared with the reference method performed on PBMCs, the technique developed for our study reduced the technical steps from 10 to 4, likewise the time from 165 to 100 minutes, as shown in Figure 1.

The centers involved in the clinical trial were equipped with cytometers from different manufacturers, that is, Navios, Gallios, and CyanADP from Beckman Coulter and LSR II, LSR Fortessa and Canto II from Becton Dickinson, all of which have three lasers (405, 488, and 638 nm). A panel of fluorochrome-conjugated antibodies was first designed for use with these different cytometry platforms and to limit machine settings for labs unaccustomed to compensation.

Conjugated antibodies directed against gating markers (CD3-FITC, CD4-PB, CD8-KRO, CD25-PE, and CD127-PC7) were selected with fluorochromes to limit compensation with the anti-FoxP3 conjugated fluorochrome (Alexa Fluor-647). As shown in Figure 2A–E, the resulting staining clearly individualized Tregs, defined as $CD3^+CD4^+CD25^+CD127^{-/low}FoxP3^+$ cells. Moreover, the staining index of FoxP3, calculated as (median of fluorescence of positive cells – median of fluorescence of negative cells)/(2 × standard deviation of negative cells), for CD4+ lymphocytes was significantly higher than with the reference staining on PBMCs (shown in Fig. 2F), illustrating that Treg identification was easier with this new method (Fig. 2G). Compensations were performed with Duraclone single staining tubes and VersaComp antibody capture beads. As expected, very few compensations were needed with this panel, as shown by a representative example of a compensation matrix (Fig. 2H).

Validation of Duraclone Staining

Duraclone staining was first compared with its equivalent liquid combination. There was excellent agreement between the dried and liquid reagents, as dried reagents showed 8.109% (±1.935%) of the T cells to be Tregs while the liquid reagents showed 8.173% (±1.945%), thus validating the reliability of the dried antibodies (Fig. 3A).

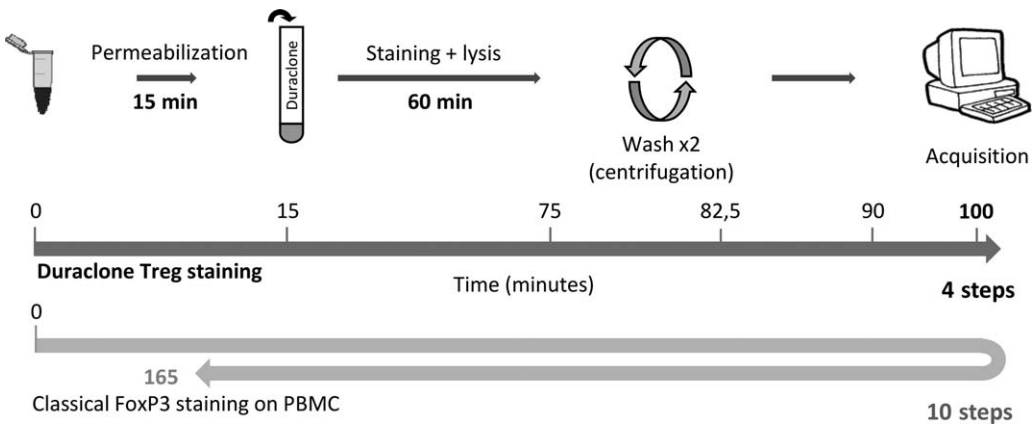


FIG. 1. **Protocol.** The time needed for each step of the staining is indicated on the dark gray arrow. For comparison, the total time needed for the classic technique previously used in the laboratory is indicated in light gray.

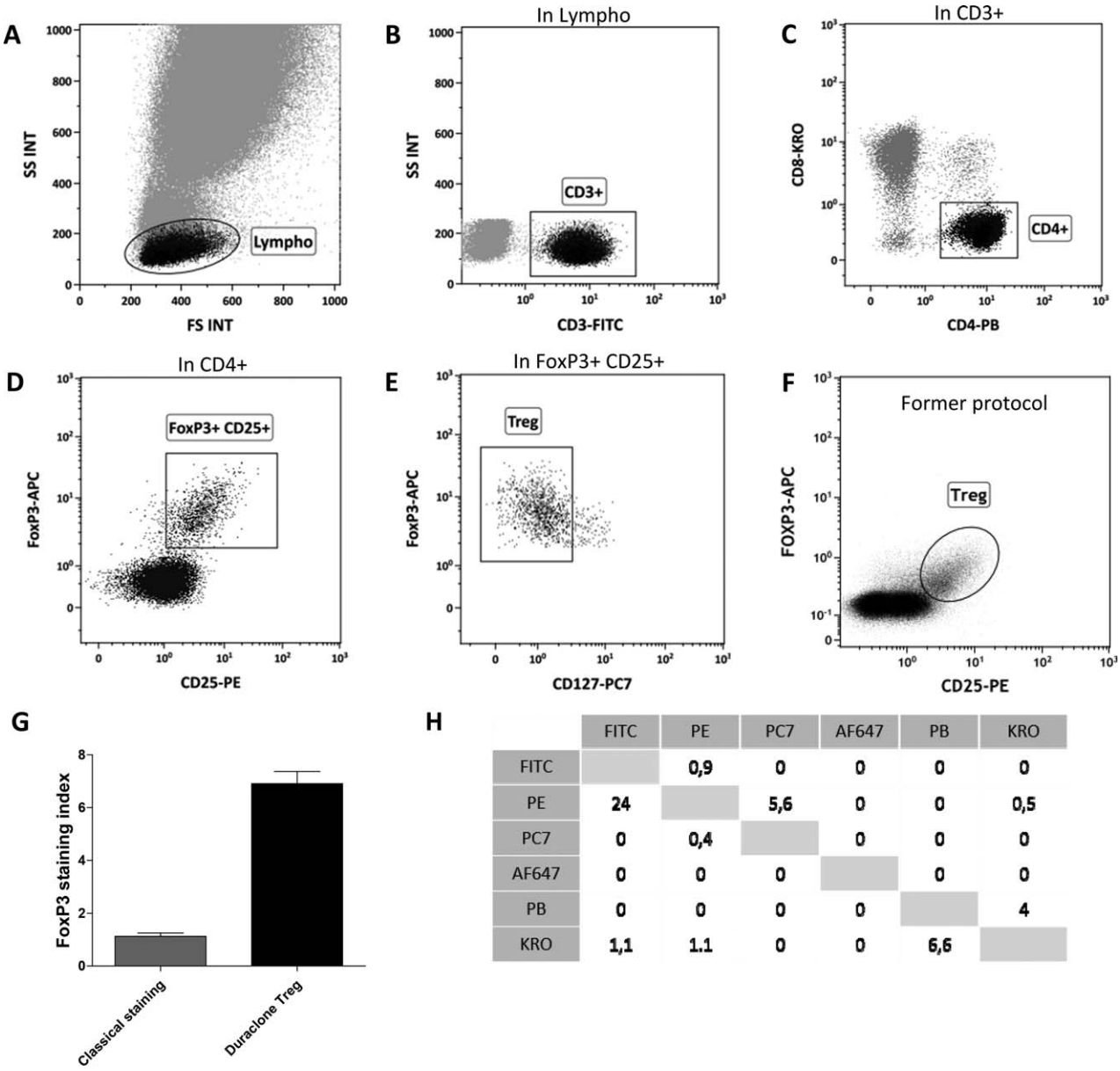


FIG. 2. Duraclone staining of Tregs. **A–E**: Example of the staining and gating strategy. After removal of the debris and doublets, lymphocytes are gated on the FS/SS graph (A), then a CD3+ population is defined from the "Lympho" gate (B), and a CD4+ population from the CD3+ population (C). Tregs are defined as Foxp3+ CD25+ CD127low cells in CD4+ T cells (D and E). **(F)** Representative former staining of Tregs on PBMCs. **(G)** Comparison of FoxP3 staining index between the classic techniques performed on PBMCs and the Duraclone staining of fresh blood. Statistical analyses were performed with the Mann-Whitney test, $***P < 0.0001$ **(H)** Example of a compensation matrix.

To assess whether the anticoagulant used for blood collection affects staining quality, we compared data obtained from the same donors with blood sampled in EDTA or lithium heparin tubes. No change in the quality of the labeling was observed and there was a strong correlation between the two conditions, although the percentage of Tregs among $CD4^+$ T cells was systematically slightly higher in the lithium heparin than in EDTA ($9.118 \pm 2.405\%$ vs. $8.028 \pm 2.378\%$; Fig. 3B).

To validate the method of Treg quantification developed with Duraclone tubes in different brands of machine, cytometers were calibrated using 8-peak fluorescent Rainbow Calibration Particles, to obtain superposition of fluorescent peaks at the same level. As shown in Figure 3C, results recorded with a Navios cytometer or an LSRII cytometer were similar and showed that the results were reproducible with these two cytometers ($7.549 \pm 1.902\%$ for Navios, $7.532 \pm 1.897\%$ for LSRII).

Finally, to validate the technic in our multicenter clinical trial, samples from the same 4 donors were sent to 5 different laboratories involved in the trial. As shown in Figure 4, the values from each donor were very similar

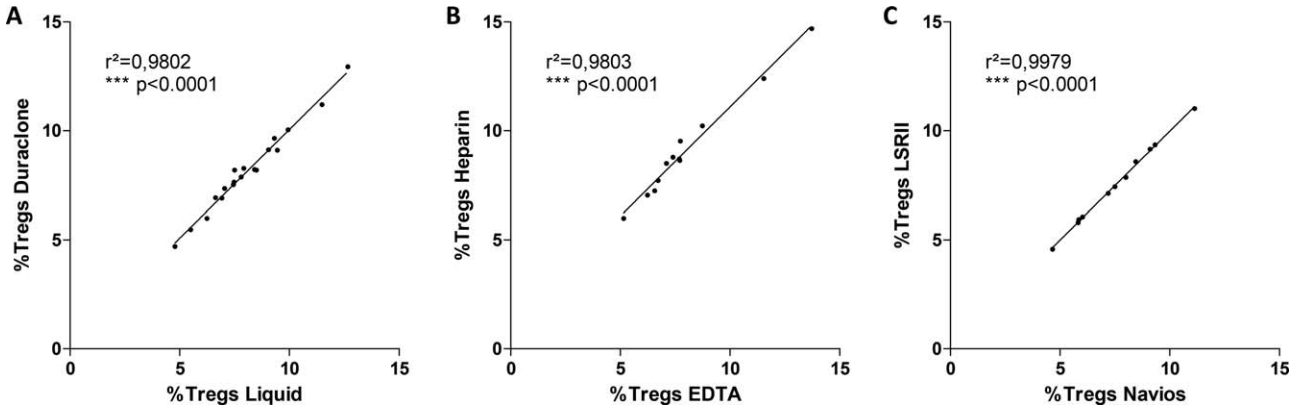


FIG. 3. Validation of the Duraclone Treg staining. (**A**) Correlation analysis between staining of Tregs from healthy donors with liquid antibodies and with Duraclone tubes ($n = 19$). **(B)** Correlation analysis of staining performed with EDTA or lithium heparinate as anticoagulant ($n = 12$). **(C)** Correlation analysis of staining acquired on a Navios or an LSRII cytometer ($n = 11$). The statistical analysis were performed using Pearson's correlation test, *** $P < 0.0001$.

across the centers, with <10% variation for all values (mean difference of $4.04 \pm 3.30\%$) in comparison to our reference value.

DISCUSSION

In the context of a multicenter clinical trial, we developed a standardized method for flow cytometry monitoring of Tregs using Duraclone technology that requires very few technical steps, minimal compensations and much less time than classic intracellular staining. This study validates the procedure and demonstrates the excellent reproducibility of the results.

Duraclone technology has already been shown to provide high reproducibility and efficient standardization in large-scale projects such as the ONE study (11) and the PRECISESADS study (12). Our study gives further evidence of its advantages, because Treg Duraclone staining yielded very consistent results compared with those obtained with liquid antibodies, and the separation of the

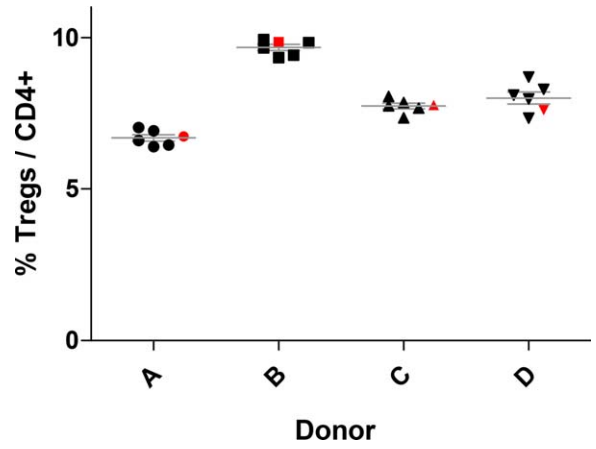


FIG. 4. Intercenter reproducibility of the Duraclone Treg staining. Blood samples from the same four healthy donors (A–D) were stained with Treg Duraclone tubes in five different centers involved in the DIABIL-2 clinical trial. Treg percentages among CD4+ T cells from each center are shown in black. Results from our laboratory are in red. [Color figure can be viewed at wileyonlinelibrary.com]

Treg population was very satisfying, especially the Foxp3 staining, as shown by the staining index. A standardized Treg lyophilized panel of antibodies has already been proposed by the Human Immunophenotyping Consortium (13), and yielded a reproducible percentage of Tregs cells across different centers, although there was no FoxP3 included in the panel. We believe FoxP3 is necessary to specifically quantify Tregs, as it is usually considered as the "gold standard marker" of Tregs (14). In the past, the necessity of an intracellular labeling to stain FoxP3 could be an issue to design standardized staining, but our results using recently developed reagents highlight the fact that this is no longer an issue (10).

Moreover, dried antibodies tubes also make much easier the supplying of material to different centers involved in a trial, as (i) they can be stored at ambient temperature and not at 4°C like their liquid or lyophilized counterpart, and (ii) as one tube is used for one patient visit, it is very easy to provide the exact number of tubes needed for a patient follow-up.

Because this staining was developed in the context of a multicenter clinical trial, we chose fluorochromes suitable for all the cytometers of the trial centers, to simplify settings and minimize fluorochrome compensation. As a result, the staining can be acquired on any cytometer with blue, red, and violet lasers, as demonstrated here by the comparison between acquisitions performed on Navios and LSRII cytometers. Staining was highly reproducible, as there was <10% variation compared with our reference value in 5 trial centers with the same blood samples. The quality of the results was acceptable whatever the anticoagulant used, keeping in mind that the same anticoagulant should be used throughout a given study, as the Treg cell percentages differ slightly depending on the anticoagulant. Furthermore, this technique can be used with small volumes (<100 μL) of whole blood, which is a great advantage when repeated samples are taken or for pediatric patients, as in the DIABIL-2 trial.

Our Treg Duraclone assay has so far been successfully implemented in 15 centers, and no major issue has

arisen. Duraclone tubes, all needed reagents, and detailed standard operating procedures were provided by us. It is important to note that even if the Treg Duraclone assay allows to overcome multiple biases related to technical steps, other factors are critical in multicenter reproducibility, such as instrument setup and data analysis. To address these potential sources of variability, we provided each center with Rainbow Calibration Particles, all from same batch, together with target values to standardize instrument setup. To avoid biases related to data analysis, which can lead to high variability when manual gating is performed in each local center (13), data analysis was centralized and performed blindly. Moreover, even though the manufacturer guarantees the stability of the Duraclone tubes for 1 year, perfect staining was obtained >2 years after production.

Because in many clinical trials only frozen PBMCs are available, our method was tested on cryopreserved PBMCs, and we observed that the staining remains satisfactory (Supporting Information Figure S1). Treg percentages obtained from frozen PBMCs are slightly lower than those obtained from whole fresh blood, probably due to a loss of some Tregs during the freezing/thawing steps. This result raises the possibility to use the Treg Duraclone assay on frozen sample, keeping in mind that values obtained from different sample types cannot be compared, and that staining should be performed on whole blood when possible.

In this study, we used the Duraclone formulation, but other manufacturers currently propose comparable products, such as lyophilized antibodies, that would probably yield similar efficiency regarding the stability and reproducibility of the results. Indeed, lyophilized products, particularly Lyoplates® and Lyotubes® from BD Biosciences, have already been shown to be very stable overtime (15,16), to strongly correlate with liquid formulations (15,17) and to allow a very satisfactory standardization in cross-center studies (13,17). Together with our results, these studies tend to demonstrate that the new generation of stabilized reagents, either dried or lyophilized, will be critical in the future to reduce variability over time and in inter-institutional studies.

In conclusion, this study demonstrates that the staining procedure we designed to monitor Tregs in a multicenter clinical trial is very reproducible and reliable. It will be used in several other clinical trials starting this year and we believe it could become a reference technique for the monitoring of Tregs in clinical trials, especially in multicenter trials, because it is easy to use and robust.

AUTHORS CONTRIBUTION

MR, FP, and DK contributed to concept development and methodological design. FP and MB performed the experiments and analyzed data. GM, CB, PP, CL, JT, and ML were involved in the cross-center validation staining. FP, DK, and MR wrote the manuscript.

No potential conflict of interest relevant to this article was reported.

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