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Brief Communication

Inter-Laboratory Assessment of Flow Cytometric Monocyte HLA-DR Expression in Clinical Samples

Julie Demaret,^{1,2} Alexandre Walencik,³ Marie-Christine Jacob,³ Jean-François Timsit,⁴
Fabienne Venet,^{1,2} Alain Lepape,^{2,5} and Guillaume Monneret^{1,2*}

¹Hospices Civils de Lyon, Hôpital Edouard Herriot, Laboratoire d'Immunologie, Lyon F-69003, France

²EAM 4174, HCL-Université Claude Bernard Lyon 1, Lyon, France

³Centre Hospitalo-Universitaire Albert Michallon, Laboratoire d'immunologie, Grenoble, France

⁴Université Grenoble 1, UFR Santé, Albert Michallon Hospital, Medical ICU, Grenoble, France

⁵Hospices Civils de Lyon, CH Lyon-Sud, Réanimation, Lyon, France

Background: Diminished expression of human leukocyte antigen DR on circulating monocytes (mHLA-DR) is a reliable indicator of immunosuppression in critically ill patients, predictive of both adverse outcome and septic complications. The objective of the present work was to test, in an inter-laboratory clinical study, a standardized protocol for mHLA-DR measurement by flow cytometry.

Methods: mHLA-DR was assessed in fresh whole blood according to a standardized staining protocol. Cells were analyzed on different flow cytometers (FC500, Navios, FACS Canto II) in different laboratories (Lyon and Grenoble). Results were expressed as numbers of antibodies bound per cell (AB/C).

Results: Correlations between results were excellent (Pearson and interclass correlation coefficients > 0.98). Coefficients of variations for intra-assay precision ranged from 1.9 to 3.2%. **Conclusion:** The present report highlights the robustness of this standardized flow cytometric protocol for mHLA-DR measurement in multicentric clinical studies. © 2012 International Clinical Cytometry Society

Key terms: HLA-DR; monocyte; sepsis; flow cytometry; standardization

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After severe stress/injury, the development of a state of immunosuppression is now a well-documented phenomenon in intensive care unit (ICU) patients. This might directly participate in detrimental outcome and in lowering patients' resistance to secondary infections (1,2). Therefore, it is now hypothesized that restoring immune functions in ICU patients could represent a major therapeutic avenue (3). Preliminary clinical trials in the field, testing either IFN-g or GM-CSF, have shown promising results (4–6). Nevertheless, a crucial aspect is our capacity to identify only the most immunosuppressed patients (i.e., who could benefit the most from immunostimulating therapies).

To date, a diminished expression of human leukocyte antigen-DR on circulating monocytes (mHLA-DR) measured by flow cytometry is consensually accepted as a

reliable indicator of immunosuppression in critically ill patients (2). In terms of functionality, monocytes with low HLA-DR expression are known to be unable to mount a pro-inflammatory response to any subsequent bacterial challenge or to properly present antigens to T

Julie Demaret and Alexandre Walencik contributed equally to the work.

*Correspondence to: Guillaume Monneret, Immunology Laboratory, Hopital E. Herriot, Hospices Civils de Lyon, 5 place d'Arsonval, 69437 Lyon Cedex 03, France.

E-mail: guillaume.monneret@chu-lyon.fr

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cells (2,7). In terms of clinical information, decreased mHLA-DR has been shown to be predictive of both adverse outcome and septic complications after trauma, surgery, pancreatitis, burn, and septic shock (8–11).

Recently, mHLA-DR has been used to stratify the administration of GM-CSF in a clinical trial including a small cohort of septic patients (6). This biomarker-guided GM-CSF therapy appeared safe and effective in restoring monocytic immunocompetence and shortening the duration of mechanical ventilation and hospital/ICU stay (6). Therefore, the next challenge would be to design a multicentric clinical trial to test this hypothesis in a large cohort of patients. However, a prerequisite is to ensure a standardized flow cytometric protocol for mHLA-DR measurement, so as to obtain reproducible results between centers. Regarding this aspect, pre-analytical requirements have been published in 2002 (12). Then, an international group of experts established a consensually accepted standardized protocol in 2005 (13). Using this standardized protocol, the objective of the present work was to assess the reproducibility of flow cytometric mHLA-DR measurements in an inter-laboratory clinical study.

MATERIALS AND METHODS

Cell Preparation and Flow Cytometric Analysis

Samples of peripheral blood were collected in EDTA anticoagulant tubes. The protocol (thereafter called “standardized protocol”) has been extensively described in 2005 (13). Briefly, whole blood (50 μ L) was stained with 20 μ L of QuantiBrite HLA-DR/Monocyte mixture (QuantiBrite anti-HLA-DR PE (clone L243)/Anti-monocytes (CD14) PerCP-Cy5.5 (clone M Φ P9), Becton Dickinson San Jose, CA, USA) at room temperature for 30 min in a dark chamber. Alternatively, cells were stained with FITC-labeled anti-CD14 (BeckmanCoulter, Miami, FL) and PE-labeled anti-HLA-DR (clone L243, Becton Dickinson-Pharmingen, San Jose, CA) at room temperature for 30 min in a dark chamber according to a previously used protocol (thereafter called “alternate protocol”) (11). Samples were then lysed using the FACS Lysing solution (Becton Dickinson) for 15 min. After a washing step, cells were analyzed on three different flow cytometers: FC500, Navios (BeckmanCoulter) and FACSCanto-II (Becton Dickinson). A lyse-no wash procedure was also evaluated for some samples. Monocytes were first gated out from other cells on the basis of CD14 expression and mHLA-DR expression was then measured on their surface (mono-parametric histogram) as median of fluorescence intensity related to the entire monocyte population (as recommended by manufacturer). These results were then transformed in AB/C (number of antibodies fixed per cell) thanks to calibrated PE-beads (BD QuantiBrite™ - PE Beads, Becton Dickinson) that were run on each flow cytometer.

Results obtained with FC500 and Navios flow cytometers, both located in the same laboratory in Lyon (Immunology Laboratory, Hôp. E. Herriot), were first compared. In this case, cell stainings (i.e., standardized

and alternate protocols) were performed within 2 h after blood collection. Every sample was obtained from our routine workout (17 septic shock patients, 12 HIV-infected patients, and 13 healthy donors). In a second experiment, comparison was made between results obtained in Lyon (Navios) and Grenoble (FACSCanto-II) according to the standardized protocol. Samples were obtained in Lyon ICU ($n = 5$), Grenoble ICU ($n = 6$) and from healthy donors ($n = 3$). As there is a 90 min-drive between the cities of Lyon and Grenoble, samples were collected on the same day as follows. First, Lyon samples were obtained at 8:30am immediately placed on ice and driven to Grenoble. Meanwhile, in Grenoble, samples were collected at 9:30am and stored on ice. At 10:00am in Grenoble, every sample (i.e., from Lyon and Grenoble) were split in two aliquots. The first batch of aliquot was driven to Lyon on ice, the second stayed on ice in Grenoble. Finally, at 12:00am, staining procedure was simultaneously started in both centers after phone call to ensure a perfect synchronization.

Statistical Analysis

Correlations were evaluated using the Pearson's correlation coefficient and intra-class correlation coefficient tests. The statistical analyses were performed using SPSS software (version 16.0, SPSS, Chicago, IL).

RESULTS

FC500 (Alternate Protocol)/Navios (Standardized Protocol) Comparison

In a previous study (11), we set up a homemade protocol based on the same principle as the standardized procedure for mHLA-DR measurement (i.e., monocyte identification through CD14 expression / PE-HLA-DR - clone L243, Becton for HLA-DR expression measurement and QuantiBrite beads for calculation of AB/C) but without using the ready-to-use QuantiBrite mixture. As a first step, we compared this protocol run on a FC500 flow cytometer to the standardized protocol run on a Navios analyzer. For this purpose, we included 42 samples. Results are shown in Figure 1A. An excellent correlation was obtained between protocols ($y = x + 582$, $r: 0.98$, $p < 0.001$) without any outlier.

Navios (Lyon) / FACSCanto-II (Grenoble) Comparison

In a second step, we compared results of clinical samples stained according to the standardized protocol but analyzed simultaneously in two different laboratories on flow cytometers provided by different manufacturers (Navios - BeckmanCoulter and FACSCanto-II - BectonDickinson). Again, we observed an excellent correlation ($y = 1.05x + 258$, $r: 0.99$, $p < 0.001$ and intra-class correlation coefficient: 0.996 , $p < 0.001$). Indeed, results were thus found to be identical between centers (Fig. 1B).

Wash/No Wash Procedure and Method Precision

As the necessity for a washing step before data acquisition on flow cytometers was not definitely established

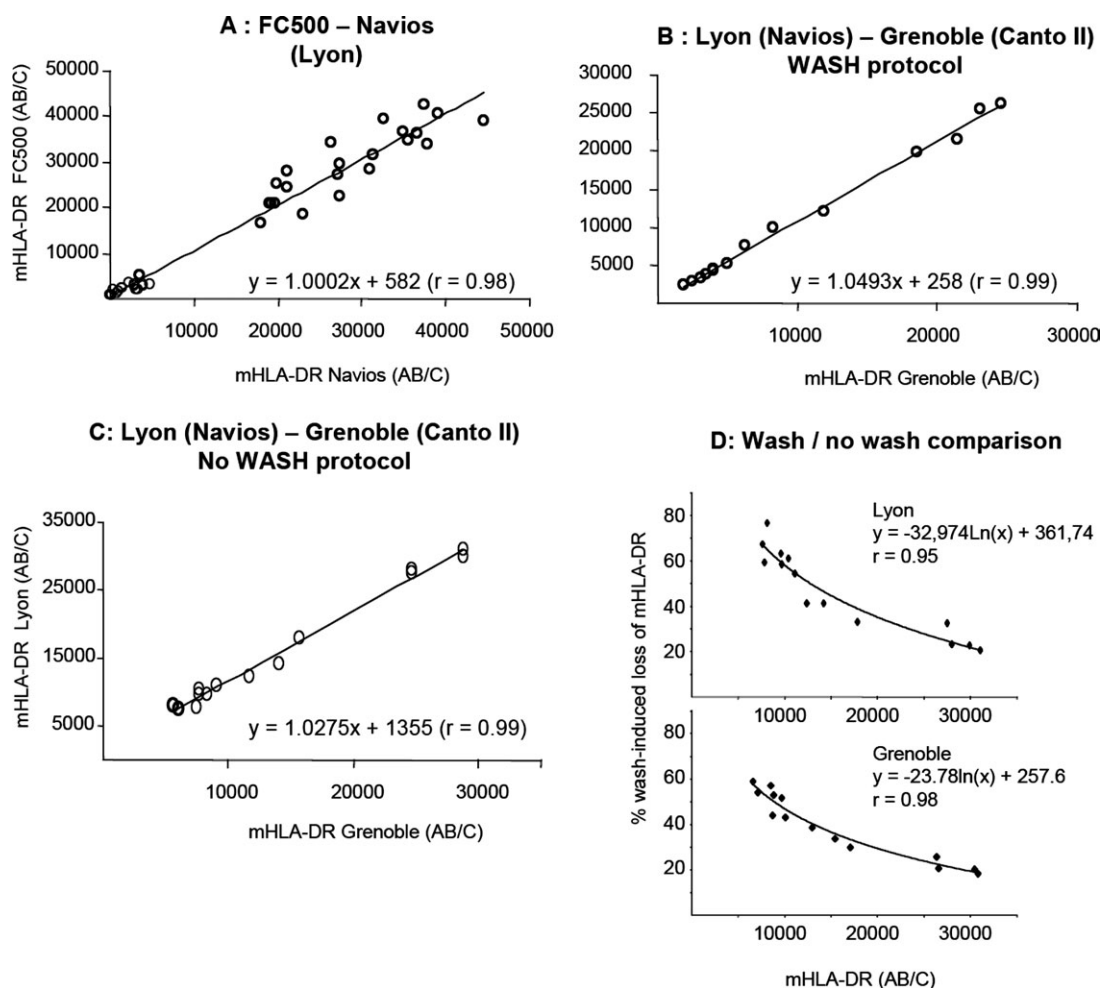


Fig. 1. Comparison of FC500 (alternate protocol) and Navios (standardized protocol) results in a same laboratory. 47 blood samples from patients (12 HIV-infected and 17 ICU patients) and 13 healthy donors were either treated according to homemade staining procedure (11) or the standardized protocol (13). Cells were washed after staining. Results were expressed as number of antibodies bound per cell (AB/C). **B**. Comparison of Navios – Lyon (standardized protocol) and FACS Canto II - Grenoble (standardized protocol) results. 14 blood samples from ICU patients ($n = 11$) and healthy donors ($n = 3$) were treated according to standardized procedure (13). Cells were washed after staining. Results were expressed as number of antibodies bound per cell (AB/C). **c**. No wash procedure results comparison (standardized protocol, Navios – Lyon and FACS Canto II – Grenoble). 14 blood samples from ICU patients ($n = 11$) and healthy donors ($n = 3$) were treated according to standardized protocol (13). Cells were not washed after staining. Results were expressed as number of antibodies bound per cell (AB/C). **d**. Wash-induced loss of mHLA-DR in Grenoble and Lyon (standardized protocol). The effect of a washing step was evaluated by calculating the percentage of mHLA-DR loss in comparison with the no wash procedure. 14 blood samples from ICU patients ($n = 11$) and healthy donors ($n = 3$) were treated according to standardized protocol (13). Cells were either washed or not after staining. Results were expressed as number of antibodies bound per cell (AB/C) and as logarithmic regression.

in the 2005 standardized protocol (13), we assessed again this aspect in both centers. As shown in Figure 1D, the wash procedure decreases mHLA-DR values. This loss was more pronounced for the lowest mHLA-DR values. Nevertheless, when comparing results obtained in both centers with the “no wash” protocol, we again observed an excellent correlation ($r: 0.99$, $p < 0.001$ and intra-class correlation coefficient: 0.994 , $p < 0.001$, Fig. 1C). In addition, in Lyon, the intra-assay precision was evaluated in the “wash” protocol by two ways: one sample stained once and analyzed five times or one sample stained five times and analyzed five times. Importantly, this showed that, by using the “wash” procedure, the intra-assay coefficients of variations (CVs) ranged from 1.9 to 3.2%.

DISCUSSION

The major result of the present report is to show the excellent correlation between mHLA-DR results obtained in two different laboratories when the standardized protocol is performed according to previously described pre-analytical recommendations (e.g., staining < 2 h after sampling or < 4 h at $+4^{\circ}\text{C}$ (12,13)). Measured on clinical samples, this observation was valid between laboratories, between different flow cytometers (Beckman-Coulter and Becton-Dickinson) and was even found when performed with slightly different staining procedures (alternate versus standardized protocols). To the best of our knowledge, such an inter-laboratory assessment on fresh clinical samples has never been performed since publication of the standardized protocol in

2005 (13). In the original paper, only two clinical samples were sent to participating laboratories and staining was started > 24 h after sampling (13). In this study, coefficients of variation (i.e., values from different laboratories) were found at 20% (mHLA-DR around 11,000 AB/C) and 15% (for mHLA-DR around 35,000 AB/C). Meanwhile, some multicentric studies have been published. However, whereas patients' inclusions were multicentric, mHLA-DR measurements were all performed in a single laboratory (6,14).

As initially reported (13), we observed that the final washing step before cell acquisition induces a decrease in mHLA-DR expression while both protocols remained well correlated. As, in some cases, this loss may not be reproducible due to non-specific antibody binding caused by inflammation-induced up-regulation of Fc γ receptors - which is obviously the case in ICU patients - the wash procedure may be preferred. Furthermore, this step clearly diminishes the non-specific background and thus ameliorates cell identification on dot plots. This may explain that we obtained an almost perfect regression line (slope equals 1, intercept at the origin of the y - and x -axes) when comparing results obtained using the wash protocol between centers (Fig. 1B). In line, regarding the wash procedure, intra-assay precision CVs were found below 5% which is excellent for such a manual technique. As far as we know, most groups that have published on mHLA-DR (and that we have contacted during completion of the manuscript) are currently performing this wash procedure, which is actually not significantly time consuming. Collectively, these data indicate that, although requiring one additional working step, the final wash step is preferable.

To date, the main indication of mHLA-DR measurement is to identify ICU patients at increased risk of nosocomial infections/death. One promising application in the future is to help these immunosuppressed patients in restoring their immune defenses. As an example, decreased mHLA-DR expression has recently been used to stratify the administration of GM-CSF in a small cohort of septic patients (6). This biomarker-guided GM-CSF therapy has been demonstrated to be safe and effective for restoring monocytic immunocompetence and shortening the time of mechanical ventilation and hospital/ICU stay (6). Patients were included into this prospective, randomized, double-blinded, placebo-controlled trial on the basis of a low mHLA-DR (i.e., below 8000 AB/C) on two consecutive days. The next step would be to conduct multicentric studies to validate these promising GM-CSF effects on larger cohorts of patients by using this standardized mHLA-DR procedure providing reproducible results between centers.

More generally, regarding the measurement of mHLA-DR after severe stress, a key challenge will be to delineate, for each subgroup of ICU patients (i.e., burn, trauma, sepsis, pancreatitis), the best thresholds defining the risk of secondary infection / death. Indeed, it is very likely that these thresholds will be different between septic shock, trauma or burn patients. Obviously, such per-

spective will rely on the use of flow cytometry, which is now ready for clinical applications when standardized protocols are properly designed and validated (15-17).

In conclusion, the present report and previous results (13) highlight the robustness of this standardized flow cytometry protocol for mHLA-DR measurement in multicentric studies.

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