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1 **Towards standardization of immune functional assays**

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25
26 **Keywords:** Immune Functional Assay; Host Response; Stimulation; Independent Validation;
27 Standardization

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HV, healthy volunteers; IFA, immune functional assays; IFN- γ , interferon gamma; IGRA, interferon gamma release assays; LPS, lipopolysaccharide; LTI, latent tuberculosis infection; MI, milieu intérieur; PBMC, peripheral blood mononuclear cell; SEB, staphylococcal enterotoxin B;

34 Abstract

35

36 Recent advances in the immunotherapy field require evaluation of the immune function to
37 adapt therapeutic decisions. Immune functional assays (IFA) are able to reveal the immune
38 status and would be useful to further adapt/improve patient's care. However, standardized
39 methods are needed to implement IFA in clinical settings. We carried out an independent
40 validation of a published method used to characterize the underlying host response to
41 infectious conditions using an IFA. We evaluate the reproducibility and robustness of this IFA
42 and associated readout using an independent healthy volunteers (HV) cohort. Expression of a
43 44 genes-signatures and IFN γ protein secretion and gene-expression was assessed after
44 stimulation. We observed a strong host-response correlation between the two cohorts. We also
45 highlight that standardized methods for immune function evaluation exist and could be
46 implemented in larger-scale studies. This IFA could be a relevant tool to reveal
47 innate/adaptive immune dysfunction in immune-related disorders patients.

48 Words count : 148

49 1. Introduction

50

51 The increase in patients suffering from immune-related disorders led to the
52 improvement in precise knowledge concerning the components and function of the immune
53 system. Whether of physiological or pathophysiological origin, *i.e.* immunosenescence [1] or
54 autoimmune diseases [2, 3], the identification of immune alterations requires close monitoring
55 of the immune status. Their ever-increasing number and severity make these disorders a major
56 public health problem. Several therapies targeting the immune system, such as anti-
57 inflammatory agents or immune stimulatory therapy, are currently used to re-balance the
58 immune system in immunocompromised patients. Nevertheless, clinicians lack the tools to
59 decipher immune status, and more precisely, immune function, in order to adapt therapeutic
60 decisions and personalize patient care.

61 In some clinical situations, the use of immune functional assays (IFAs) could help improve
62 management and reveal valuable information compared to the use of circulating biomarkers
63 such as cell counting or measurement of soluble and cell surface biomarkers. Based on the
64 evaluation of the immune response after an *in vitro* stimulation with antigen, IFAs allow
65 assessing the immune function as well as anticipate the ability to respond to therapy or fight a
66 pathogen. In the last few years, T-cell mediated immunity (blood-based) assays, such as
67 interferon gamma release assays (IGRA), have been developed and have revolutionized the
68 detection of latent tuberculosis infection (LTI). This type of test enables clinicians to be more
69 accurate and effective in the management of patients suffering from specific disorders.
70 Research, as well as clinical studies, use and adapt these IFAs to try to respond to specific
71 questions, such as the capacity of specific patients to respond to vaccination [4 – 6]. Despite
72 encouraging results, the main challenge complicating the implementation of IFAs in clinical

73 settings is the need for standardization in order to perform these methods in any laboratory, in
74 a reliable and reproducible way [7].

75 Such tests should minimize the number of steps during sample preparation and ideally use the
76 biological matrix directly sampled from the patient to limit technical variability and eliminate
77 the pre-analytical error. As already described for cytokine release assay [8,9], use of direct
78 measurement in whole blood sampling would be the relevant solution to minimize the risk of
79 contamination and avoid causing non-specific cell activation or cell death [10]. Indeed, the
80 use of whole blood rather than peripheral blood mononuclear cell (PBMC) isolation preserves
81 all interactions between circulating immune cells and reflects the internal environment of the
82 subject [11]. Nevertheless, in addition to this pressing need for standardization, the second
83 difficulty encountered is the inter-individual variability exhibited by the immune system.

84 In this context, the "*Milieu Intérieur*" team (MI) used a whole blood, semi-closed stimulation
85 system built into syringe-based medical devices, with the ambition of defining the boundaries
86 of a healthy immune response [12]. Using a cohort of 25 healthy volunteers (HV) (aged
87 between 30–39 years) stratified by gender (13 women, 12 men), they challenged whole blood
88 with complex stimuli (mimicking fungi, bacteria, and virus infection) and established a 44-
89 gene expression signature, capturing the diversity of complex immune responses. In this
90 study, an independent validation of the protocol developed by Urrutia *et al.* was performed
91 step by step in order to evaluate the reproducibility of the technical process before its potential
92 implementation for the management of patients with immune-related disorders in clinical
93 settings.

94

95 2. Material and methods

96 2.1 Blood stimulation

97

98

99 According to EFS standardized procedures for blood donation and to provisions of the
100 articles R.1243–49 and following ones of the French public health code, a written non-
101 opposition to the use of donated blood for research purposes was obtained from HV. The
102 blood donors' personal data were anonymized before transfer to our research laboratory. We
103 obtained the favourable notice of the local ethical committee (Comité de Protection des
104 Personnes Sud-Est II, Bâtiment Pinel, 59 Boulevard Pinel, 69,500 Bron) and the acceptance of
105 the French ministry of research (Ministère de l'Enseignement supérieur, de la Recherche et de
106 l'Innovation, DC-2008-64) for handling and conservation of these samples.

107 Whole blood from healthy volunteers (n=20), stratified by gender (10 women and 10 men),
108 and with a median age of 52 years (range: 25-65 years), was distributed (1 mL) into pre-
109 warmed TruCulture tubes (Myriad Rbm, Austin, TX, USA) containing either medium alone
110 (Null), staphylococcal enterotoxin B (SEB; 400 ng/mL), or lipopolysaccharide (LPS; 100
111 ng/mL (E.coli, O55:B5)). These were then inserted into a dry block incubator and maintained
112 at 37°C. After 24 hours of stimulation, the pellet was separated from the supernatant using a
113 valve and kept in TRI Reagent® LS (Sigma-Aldrich, Saint-Louis, MO, USA). This protocol
114 was adapted from Urrutia *et al* [12].

115

116 2.2 Cytokine and transcriptomic assays

117

118 Interferon gamma (IFN- γ) release was quantified from Truculture's supernatant using
119 an immunoassay in the VIDAS® platform (bioMérieux, Marcy l'Etoile, France).

120 As previously described, RNA extraction was performed using a modified protocol of the
121 NucleoSpin 96 RNA tissue kit (Macherey-Nagel GmbH&Co. KG, Düren, Germany) and using
122 a vacuum system [12]. Nanostring technology was used for mRNA detection of a 44 gene

123 panel (details in supplementary table S1), an hybridization-based multiplex assay
124 characterised by its amplification-free step; 300 ng of RNA were hybridized to the probes at
125 67°C for 18 hours using a thermocycler (Biometra, Tprofessional TRIO, Analytik Jena AG,
126 Jena, Germany). After removal of excessive probes, samples were loaded into the nCounter
127 Prep Station (NanoString Technologies, Seattle, WA, USA) for purification and
128 immobilization onto the internal surface of a sample cartridge for 2-3 hours. The sample
129 cartridge was then transferred and imaged on the nCounter Digital Analyzer (NanoString
130 Technologies) where colour codes were counted and tabulated for the 44 genes.

131

132 *2.3 Genes expression analysis*

133

134 As previously described, each sample was analyzed in a separate multiplexed reaction
135 that each included eight negative probes and six serial concentrations of positive control
136 probes. Negative control analysis was performed to determine the background for each
137 sample. Data was imported into nSolver[®] analysis software (version 4.0) for quality checking
138 and normalization of data. Briefly, to normalize differences in RNA input, geometric means
139 were calculated over four housekeeping genes (*RPL19*, *TBP*, *POLR2A*, and *HPRT1*)
140 according to the *Milieu Intérieur* study (details in supplementary material and method S1)
141 [12].

142

143 *2.4 Statistical analysis*

144 Data obtained from our independent healthy volunteers cohort were compared to public
145 *Urrutia et al.* dataset (Gene Expression Omnibus (GEO) DataSets (GSE85176)). Non-
146 parametric Deming regression and Spearman's correlation were calculated for all parameter

147 comparisons using R software V3.6.1. A p-value <0.05 was considered as statistically
148 significant.

149

150 3. Results

151 3.1 Transcriptomic response

152

153 Immune response after stimulation with SEB (Fig. 1A) or LPS (Fig. 1C) showed a
154 strong correlation between the two independent healthy cohorts for the 44-gene expression
155 signature, after followed the same protocol than Urrutia et al. [12]. This was confirmed by
156 Deming regression on medians (Spearman's correlation coefficient $\rho=0.99$ for SEB and
157 0.90 for LPS; p-values <0.001; Fig. 1B and 1D) and on inter-deciles (Spearman's correlation
158 coefficient $\rho=0.96$ for SEB and 0.94 for LPS; p-values <0.001; details in supplementary
159 figure S1).

160

161 3.2 Interferon gamma response

162

163 In a second time, following SEB stimulation of HV, a comparison of *ifn- γ* gene
164 expression (in the corresponding TruCulture) and IFN- γ release in the supernatant (Fig. 2)
165 was performed. The latter is described as a marker of T-cell mediated immunity and used as a
166 diagnostic tool in research and clinical settings (IGRA) [13,14]. A strong correlation was
167 observed between protein and gene expressions for the 20 HV from Lyon (Fig. 2A) and the 25
168 HV from MI (Fig. 2B) (Spearman's correlation coefficient $\rho \geq 0.64$ for both cohorts and p-
169 value <0.0005).

170

171 4. Discussion and conclusion

172

173 In this study, we carried out an independent validation of a published method used to
174 characterize the underlying host response to infectious conditions using an IFA. We evaluate
175 the reproducibility and robustness of this IFA and associated readout using an independent
176 HV cohort.

177 The strong host-response correlation between the two cohorts highlights that standardized
178 methods for immune function evaluation exist and could be implemented in larger-scale
179 studies.

180 Also, based on the strong correlation observed, IFN- γ mRNA measurement might be a
181 reliable and robust readout to assess T-cell functionality. Besides, the 24-hour stimulation of
182 whole blood in standardized TruCulture tubes, which limits technical manipulation bias,
183 renders this method a versatile tool for clinical use [11].

184 In conclusion, this standardized IFA could be a relevant tool to reveal innate and adaptive
185 immune dysfunction in patients suffering from immune-related disorders.

186

187

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192

193 Conflict of Interest Disclosure

194 William Mouton, Chloé Albert Vega, Guy Oriol, Alexandre Pachot, Julien Textoris, François
195 Mallet and Karen Brengel-Pesce, are employed at bioMérieux, an *in-vitro* diagnostic
196 company,

197 François Bartolo is an employee of Soladis, a services company specialized in data-related
198 projects management,

199

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259

260

261 Supplementary material and method S1

262 *Genes expression analysis*

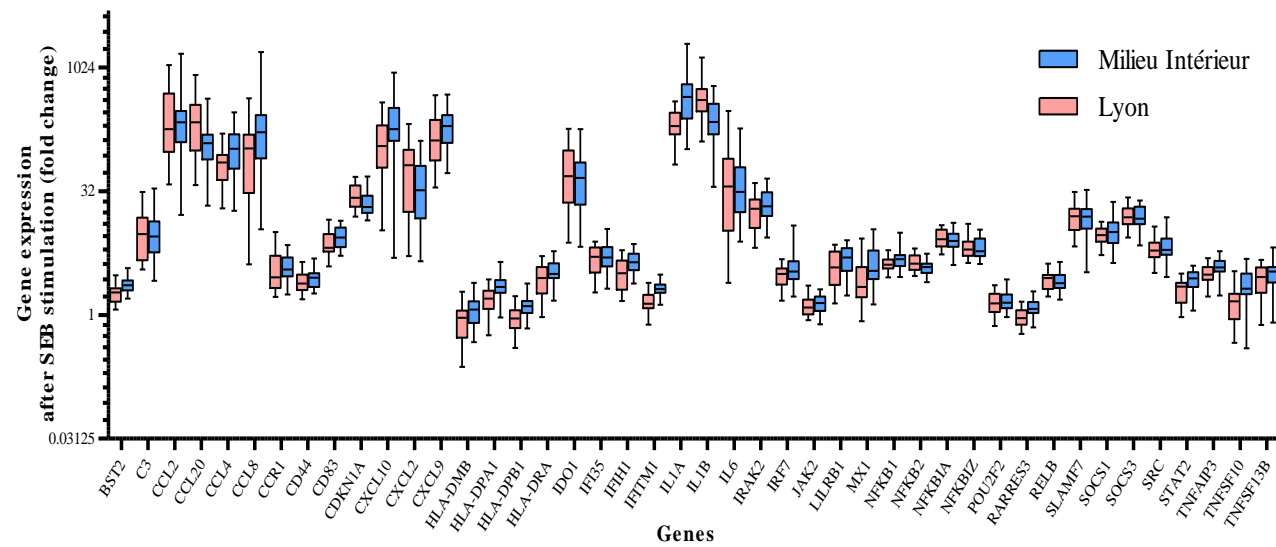
263 A first step of normalization using the internal positive controls allowed for correction of
264 potential sources of variation associated with the technical platform. To do so, the geometric
265 mean of the positive probe counts was calculated for each sample. A mean of geometric
266 means was then calculated for all subjects. A scaling factor for each subject was determined
267 as follows: geometric mean of the subject / mean of geometric means for all subject. For each
268 subject, each gene count was divided by the corresponding scaling factor. Next, the
269 background level was calculated as the mean +2 SD across the six negative probe counts. This
270 protocol was already described in *Urrutia et al.* study.

Figure legends

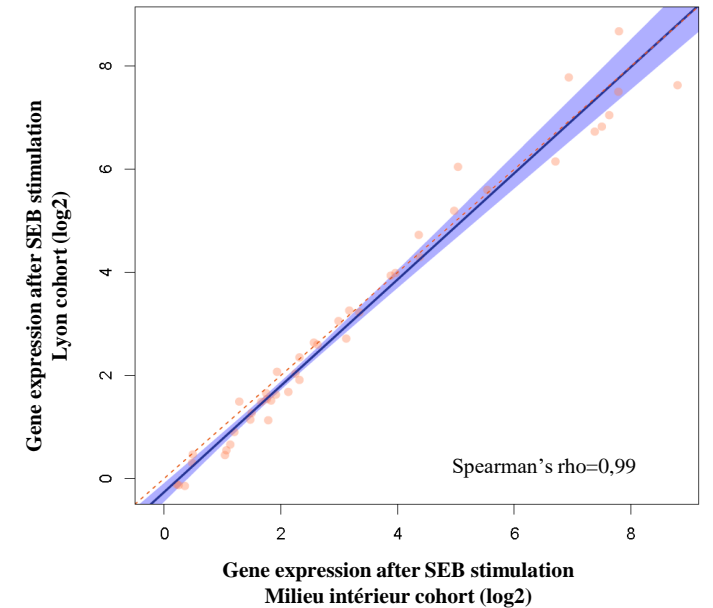
Figure 1. Gene expression after SEB- and LPS-whole blood stimulation.

Whole blood from 25 healthy volunteers enrolled in the Milieu Intérieur cohort (blue) and from 20 healthy volunteers included in the Lyon study cohort (red) was stimulated in TruCulture tubes during 24h. After mRNA extraction, gene expression was quantified using Nanostring nCounter[®] technology and normalized using NSolver[®] software using four housekeeping genes (*HPRT1*, *POLR2A*, *RPL19* and *TBP*). Fold changes are used to describe gene expression variation between the stimulated condition (SEB (A) or LPS (C)) and the control condition (NULL) for the 44-gene signature studied. The Milieu Intérieur data was obtained from Gene Expression Omnibus (GEO) DataSets (GSE85176) [12]. Comparison of both cohorts was performed using a Deming regression analysis on gene expression medians following SEB (B) or LPS (D) stimulation. A Spearman's correlation coefficient was calculated for each condition and indicated on each graphical representation. The orange dots represent individual gene expression, the blue beam represents the confidence interval, the blue line the Deming regression and the red dotted line the theoretical 100% identity.

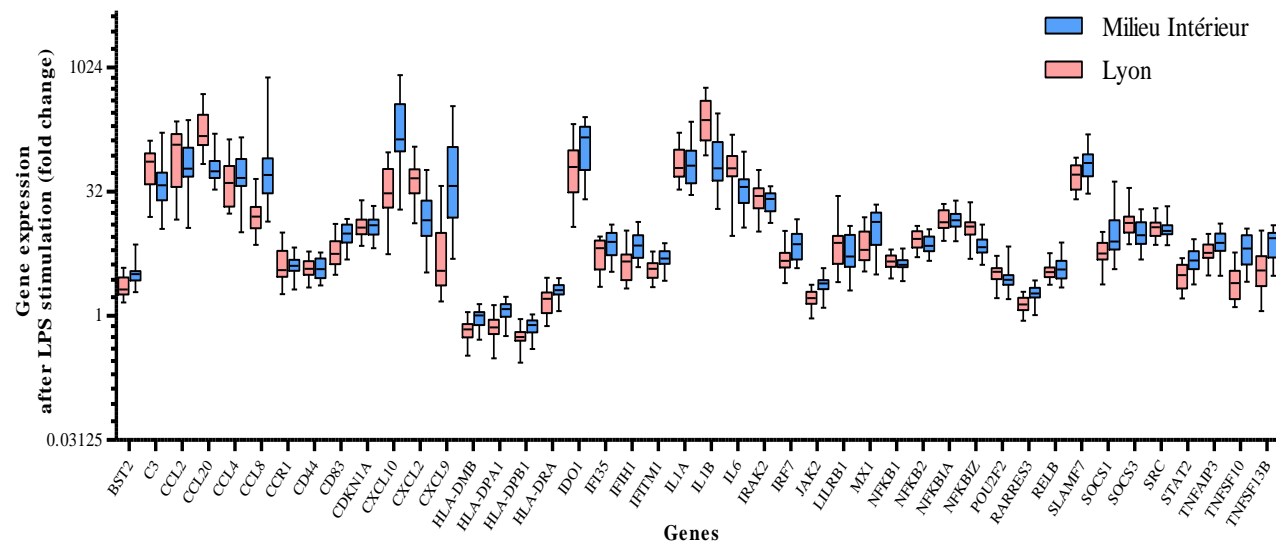
A



B



C



D

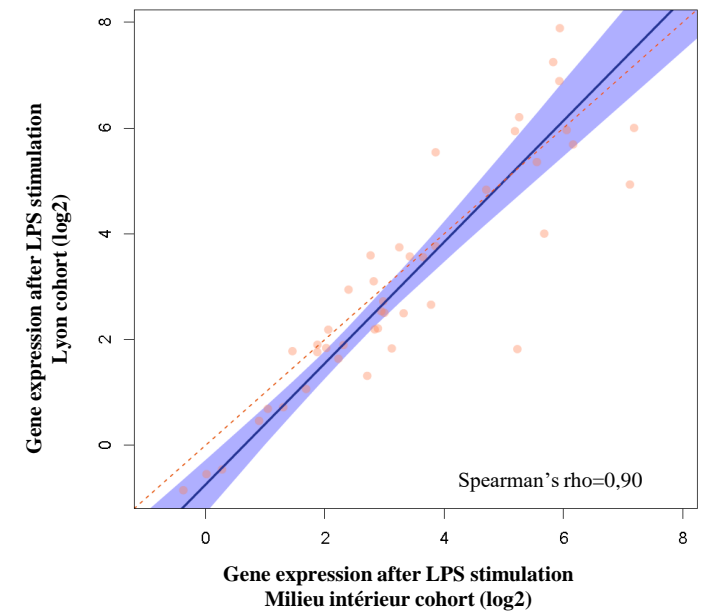
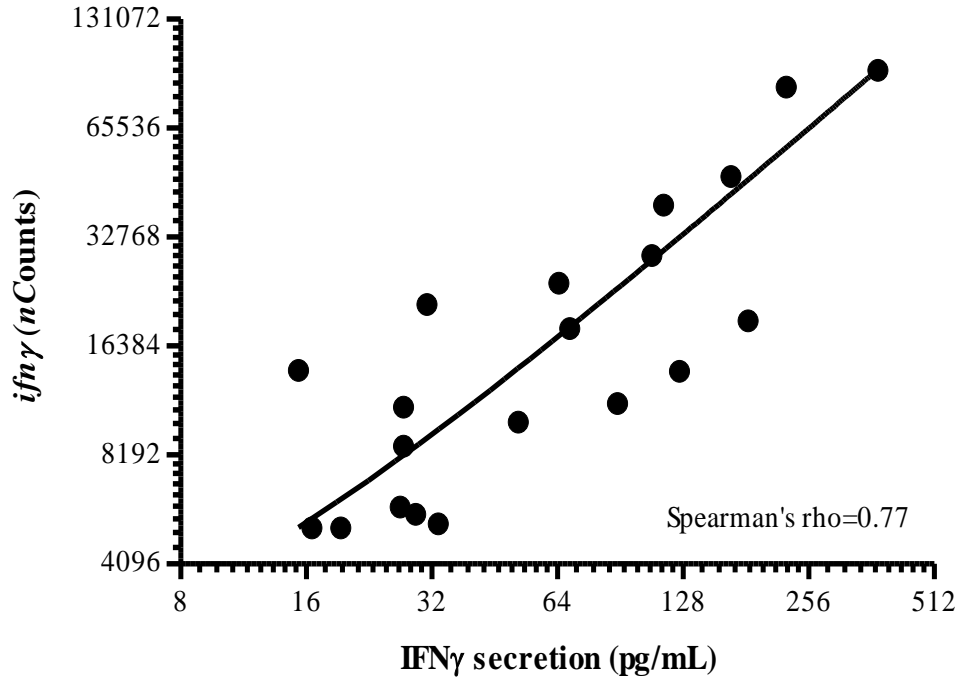


Figure legends

Figure 2. Interferon- γ induction after whole-blood SEB-stimulation.

Whole blood from 20 healthy volunteers included in the Lyon study cohort (A) and from 25 healthy volunteers enrolled in the Milieu Intérieur (MI) cohort (B) was stimulated with SEB in TruCulture tubes for 24h. Comparison between IFN γ mRNA and protein expression was assessed separately for each healthy cohort due to the use of a different method for cytokine secretion measurement. For protein quantification, IFN γ release was quantified in TruCulture supernatant using Luminex xMAP[®] for the MI cohort and using VIDAS[®] platform (bioMérieux) for the Lyon cohort. Count number for mRNA *ifn- γ* -gene expression, were normalized by the geometric mean of *HPRT1*, *POLR2A*, *RPL19* and *TBP* housekeeping genes. Gene data from the MI study was obtained from Gene Expression Omnibus (GEO) DataSets (GSE85176) [12] and protein data extracted from *Duffy et.al* supplementary information [15]. Spearman's correlation coefficient (ρ) was calculated for each condition and indicated on each graphical representation.

A



B

