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# 1                    **Towards standardization of immune functional assays**

2 William Mouton<sup>1, 2, 3\*</sup> ; Chloé Albert Vega<sup>1, 2\*</sup> ; Mathilde Boccard<sup>1</sup> ; François Bartolo<sup>1, 4</sup> ; Guy  
3 Oriol<sup>1, 2</sup> ; Jonathan Lopez<sup>5, 9, 10</sup> ; Alexandre Pachot<sup>2</sup> ; Julien Textoris<sup>2, 6, 7</sup> ; François Mallet<sup>1, 2, 6</sup> ;  
4 Karen Brengel-Pesce<sup>1, 2</sup> ; Sophie Trouillet-Assant<sup>1, 3</sup>.

5 <sup>1</sup>Joint Research Unit Hospices Civils de Lyon-bioMérieux, Hospices Civils de Lyon, Lyon  
6 Sud Hospital, Pierre-Bénite, France.

7 <sup>2</sup>Medical Diagnostic Discovery Department (MD3), bioMérieux S.A., Marcy l'Etoile, France.

8 <sup>3</sup>Virologie et Pathologie Humaine - Virpath Team, Centre International de Recherche en  
9 Infectiologie (CIRI), INSERM U1111, CNRS UMR5308, ENS Lyon, Claude Bernard Lyon 1  
10 University, Lyon, France.

11 <sup>4</sup>Soladis, Lyon, France

12 <sup>5</sup>Hospices Civils de Lyon, Plateforme de Recherche de Transfert en Oncologie, Department of  
13 Biochemistry and Molecular biology, Lyon Sud Hospital, Pierre-Bénite, France.

14 <sup>6</sup>Joint Research Unit Hospices Civils de Lyon-bioMérieux, EA 7426 Pathophysiology of  
15 Injury-Induced Immunosuppression, PI3, Claude Bernard Lyon 1 University, Edouard Herriot  
16 Hospital, Lyon, France.

17 <sup>7</sup>Hospices Civils de Lyon, Department of Anesthesia and Critical Care Medicine, Edouard  
18 Herriot Hospital, Lyon, France.

19 <sup>8</sup>Hospices Civils de Lyon, Immunology Laboratory, Edouard Herriot Hospital, Lyon, France.

20 <sup>9</sup>Université de Lyon, Claude Bernard Lyon 1 University, Faculté de Médecine Lyon Est,  
21 Lyon, France.

22 <sup>10</sup>Centre de Recherche en Cancérologie de Lyon, INSERM U1052, CNRS UMR5286, Lyon,  
23 France.

24 \*Equally contributed to this work

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- $\gamma$ , 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 $\gamma$  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- $\gamma$ ) 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<sup>®</sup> 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- $\gamma$*  gene  
164 expression (in the corresponding TruCulture) and IFN- $\gamma$  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- $\gamma$  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

200 Corresponding author information

201 William Mouton  
202 Laboratoire Commun de Recherche Hospices Civils de Lyon-bioMérieux.  
203 Centre Hospitalier Lyon Sud, Bâtiment 3F, Pierre-Bénite, 69495, France.  
204 Tel : +33(0)4 72 67 87 81; Fax : +33(0)4 72 67 87 87  
205 Email: william.mouton1@biomerieux.com

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259

260

261 Supplementary material and method S1

262 *Genes expression analysis*

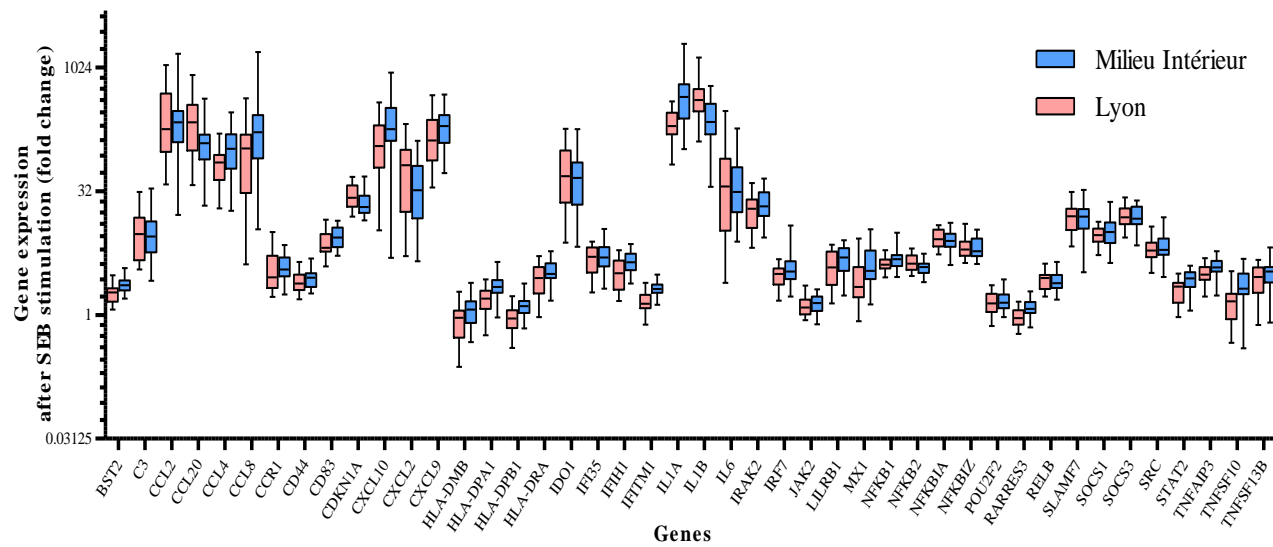
263 A first step of normalization using the internal positive controls allowed for correction of  
264 potential sources of variation associated with the technical platform. To do so, the geometric  
265 mean of the positive probe counts was calculated for each sample. A mean of geometric  
266 means 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

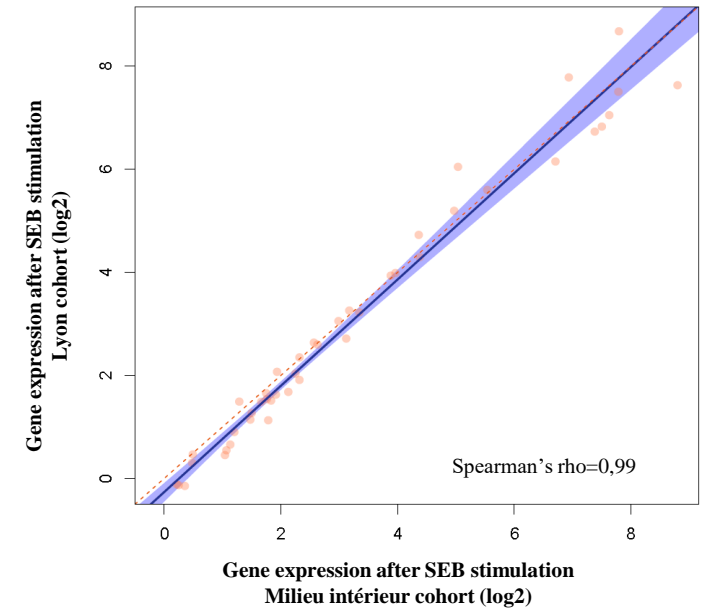
### **Figure 1. Gene expression after SEB- and LPS-whole blood stimulation.**

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<sup>®</sup> technology and normalized using NSolver<sup>®</sup> 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.

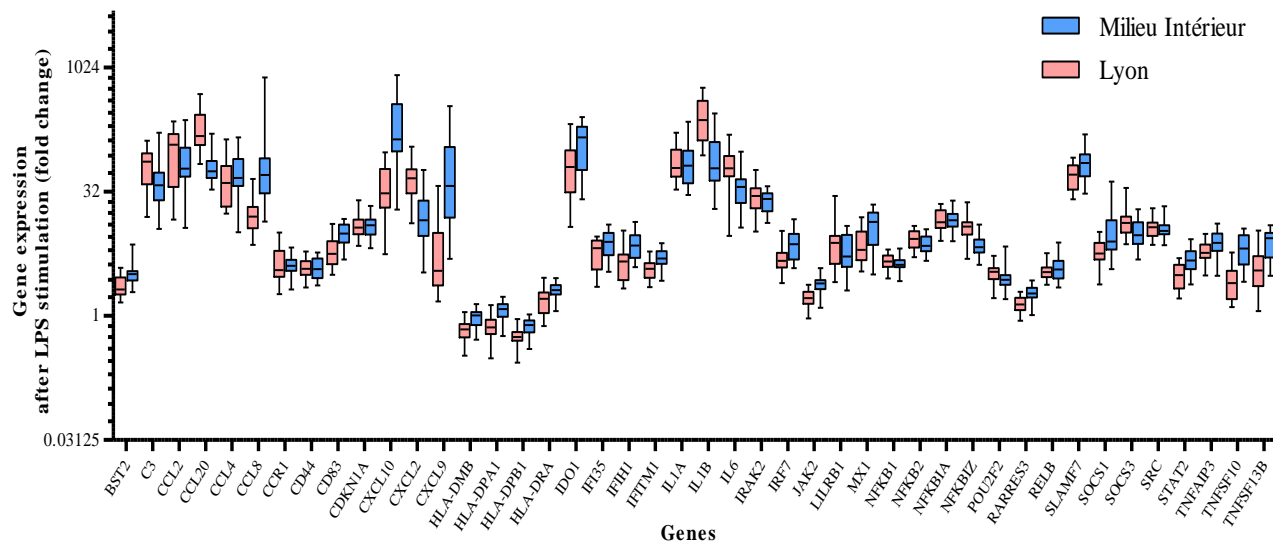
A



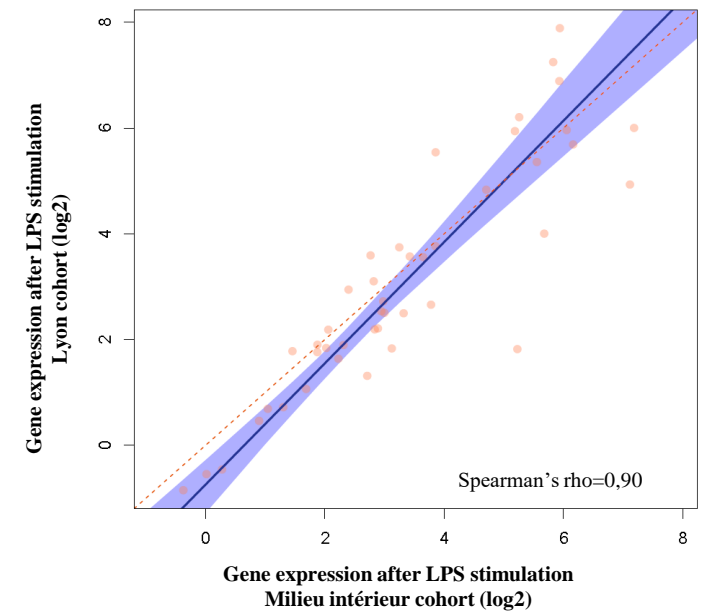
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C



D



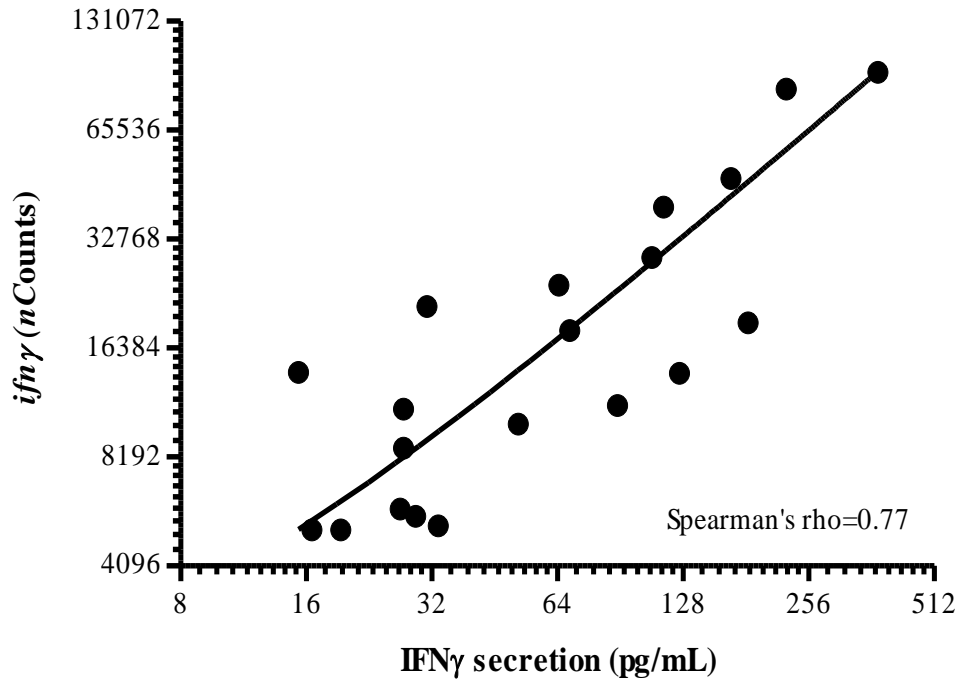
## Figure legends

### **Figure 2. Interferon- $\gamma$ 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 $\gamma$  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 $\gamma$  release was quantified in TruCulture supernatant using Luminex xMAP<sup>®</sup> for the MI cohort and using VIDAS<sup>®</sup> platform (bioMérieux) for the Lyon cohort. Count number for mRNA *ifn- $\gamma$* -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 ( $\rho$ ) was calculated for each condition and indicated on each graphical representation.



A



B

