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**Transitional B cells in quiescent SLE:  
an early checkpoint imprinted by IFN**

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1 **ABSTRACT**

2

3 Systemic lupus (SLE) is characterized by a break of B cell tolerance that plays a central role in  
4 disease pathophysiology. An early checkpoint defect occurs at the transitional stage leading to  
5 the survival of autoreactive B cells and consequently the production of pathogenic  
6 autoantibodies. The main purpose of our work was to determine whether transitional B cells, as  
7 the most immature naïve B cell subset upstream of pathogenic B cells, display specific features  
8 compared to healthy non SLE subjects. Through extensive analysis of transitional B cells from  
9 untreated or low treated, mostly Caucasian, SLE patients, we demonstrated that transitional (T1  
10 and T2) B cell frequencies were increased in SLE and positively correlated with disease activity.  
11 SLE transitional B cells displayed defects in two closely inter-related molecules (i.e. TLR9  
12 defective responses and CD19 downregulation). RNA sequencing of sorted transitional B cells  
13 from untreated patients revealed a predominant overexpression of interferon stimulated genes  
14 (ISGs) even out of flares. In addition, early transitional B cells from the bone marrow displayed  
15 the highest interferon score, reflecting a B cell interferon burden of central origin. Hence, the  
16 IFN signature in transitional B cells is not confined to African American SLE patients and exists  
17 in quiescent disease since the medullary stage. These results suggest that in SLE these 3 factors  
18 (i.e. IFN imprintment, CD19 downregulation and TLR9 responses impairment) could take part at  
19 the early transitional B cell stage in B cell tolerance by-pass, ultimately leading in periphery to  
20 the expansion of autoantibodies-secreting cells.

21

22 **KEY WORDS**

23 Systemic lupus erythematosus; Transitional B cells; Interferon; CD19; TLR9

## 24 1. INTRODUCTION

25

26 Systemic lupus erythematosus (SLE) is a severe autoimmune disease characterized by flares and  
27 mostly affecting young women. Patients produce pathogenic autoantibodies, specifically against  
28 double-stranded DNA (dsDNA) but also against ribonucleoproteins (SSA/Ro52, Sm) and  
29 phospholipids. The etiology of SLE is still not well understood but B cells and their  
30 autoantibodies play an essential role in disease pathophysiology [1,2]. There is currently a debate  
31 on SLE B cell fate and on the origin of ANA<sup>+</sup> IgG<sup>+</sup> cells [3]. Sequencing studies in young SLE  
32 patients and in the NZB/W lupus prone mice recorded greater percentages of ANA<sup>+</sup> cells in  
33 naïve or new emigrant/transitional B cells, and an early breakdown of B tolerance [4,5]. Other  
34 studies argue for a late loss of control, as demonstrated by the expansion of IgG<sup>+</sup> plasma cells in  
35 some patients with SLE and in the MRL/lpr model [5].

36 Strong data support the “early or naïve B cell model” in SLE. Autoreactive B cells including  
37 anti-nuclear and anti-ds DNA B cells are known to be controlled at the bone marrow (BM) level  
38 mainly by deletion or editing [6–8]. There is in patients affected with SLE an increased  
39 frequency of ANA<sup>+</sup> and anti-dsDNA<sup>+</sup> in naïve B cells compared to immature B cells suggesting  
40 defective selection at the transitional stage [4]. SLE mature naïve B cells display a specific  
41 decrease in the expression of CD19, which is necessary for efficient B cell signaling [9], and an  
42 altered *in vitro* toll like receptor 9 (TLR9) responses [10]. The latter observations are in  
43 agreement with disease worsening in TLR9 deficient lupus-prone murine models, and with  
44 TLR9 preventing the survival of autoreactive naïve B cells when co-engaged with B cell receptor  
45 (BCR) via nucleic acid-autoantibodies complexes [11].

46 Nucleic acid-containing immune complexes also activate myeloid and plasmacytoid dendritic  
47 cells (pDCs) leading to the secretion of type I and II interferons (IFN), key cytokines associated  
48 with SLE pathogenesis [12,13]. Mei Liu et al. showed that plasma from Chinese patients  
49 enhance transitional B cell survival in a type I IFN dependent manner [14] In addition, increased  
50 levels of IFN- $\beta$  were particularly detected in transitional B cells from African American SLE  
51 patients with nephritis and high levels of anti-ribonucleoproteins autoantibodies [15]. Hence,  
52 transitional B cells could be early key actors in SLE pathogenesis.

53 Considering these critical issues and the fact that transitional B cells are often increased in the  
54 blood of SLE patients independently of their ethnicity [10,16,17], we explored in depth  
55 transitional B cells from untreated or low treated, mostly Caucasian, SLE patients in quiescent or  
56 active phase of their disease. The main purpose of our work was to determine whether  
57 transitional B cells, as a potential checkpoint of tolerance and as the most immature naïve B cell  
58 subset upstream of pathogenic B cells, display specific features compared to healthy non SLE  
59 subjects. The question at hand is very important considering the needs in SLE for targeted or  
60 personalized therapy.

61 We confirm herein the increased frequency of transitional B cells in SLE, and more specifically  
62 of T1 and T2 subsets. This increase does not result from a proliferation or apoptosis defect but  
63 likely output from the BM. SLE transitional B cells display an abnormal phenotype with a CD19  
64 reduced expression and an impaired response to TLR9 stimuli. In addition, a strong IFN  
65 signature, of central origin, is present at this stage, even in quiescent patients. We suggest that  
66 these 3 factors (i.e. IFN imprintment, CD19 downregulation and TLR9 response impairment)  
67 could take part at the early transitional B cell stage in B cell tolerance by-pass.

## 68 **2. MATERIALS AND METHODS**

69

### 70 **2.1. Study approval**

71 The study was conducted in accordance with the principles of Helsinki declaration. Informed  
72 consents were obtained from all patients and healthy donors (HDs). The study was approved by  
73 the Clinical Research Ethic Committee of Strasbourg's University Hospital and the Human  
74 Subjects Institutional Review Board of the University of Rochester Medical Center (URMC).

75

### 76 **2.2. Patients and samples**

77 For blood samples, 23 patients (21 females and 2 males; 17 quiescent and 6 active patients) aged  
78 from 29 to 68 years with the diagnosis of systemic lupus erythematosus (SLE) were selected.  
79 The main characteristics of SLE patients are listed in Supplementary Table 1. All patients  
80 fulfilled the ACR SLE classification criteria [18]. Disease activity was measured by the modified  
81 Systemic Lupus Erythematosus Disease Activity Index (SLEDAI-2K) [19]. A SLEDAI-2K  
82 below 5 defines the quiescent patients associated with no activity in major organ systems, no  
83 vasculitis, no fever, no haemolytic anaemia, no new features of lupus activity or increase in  
84 treatment [20]. Only patients with no treatment (i.e. no immunosuppressive drugs, steroids and  
85 hydroxychloroquine within the last 6 months), or treated with low-dose steroids (<10 mg per  
86 day) with or without hydroxychloroquine were included. Patients treated with  
87 immunosuppressive drugs within the previous 6 months were systematically excluded. For  
88 transcriptomic analysis, only untreated patients were included, as hydroxychloroquine and  
89 corticosteroid therapy modify the lymphocyte transcriptomic profile [13]. Routine measurements  
90 were performed to determine ANAs (indirect immunofluorescence with Hep-2 cells, Zeus

91 Scientific, USA) and anti-dsDNA titers (screened by ELISA; Kallestad anti-DNA microplate  
92 EIA; BioRad, Hercules, CA, USA). ANAs were considered positive when  $> 1/160$ . SSA/Ro60,  
93 SSB/La, and Sm antibodies identification was performed using enzyme immunoassays  
94 (EuroImmun, Lubeck, Germany) according to manufacturer' specifications. Anticoagulated  
95 venous blood was drawn from SLE patients and from ethnicity, age and sex matched HDs and  
96 was subjected to density gradient centrifugation to get peripheral blood mononuclear cells  
97 (PBMC) and remove dead cells.

98 For bone marrow (BM) samples, 4 patients (4 females, all quiescent) aged from 24 to 55 years  
99 with the diagnosis of systemic lupus erythematosus (SLE) were selected. The main  
100 characteristics of SLE patients are listed in Supplementary Table 1. Research BM aspirates from  
101 SLE patients and HDs were obtained (i) at UPMC by aspiration from the iliac crest (n=3) and  
102 (ii) at Strasbourg University Hospital by aspiration from the sternum (n=1). Paired peripheral  
103 blood samples were obtained for 3 SLE patients and 3 HDs (2 at UPMC and 1 in Strasbourg).  
104 BM aspirate were subjected to density gradient centrifugation to get BM mononuclear cells and  
105 remove dead cells.

106

### 107 **2.3. Flow Cytometry**

108 Cell viability was assessed by incubation of cells with Fixable Viability Dye eFluor® 780  
109 (*eBioscience*, San Diego, CA, USA) following the manufacturer's protocol. Samples were  
110 incubated with antibodies for 15 minutes at 4°C. Intracellular staining was performed, for Ki-67  
111 using True Nuclear™ Transcription Factor Fix (BioLegend, San Diego, CA, USA) following the  
112 manufacturer's recommendations. The cells were washed twice in PBE (PBS, 0.5% Bovine  
113 Serum Albumin, 2mM EDTA) and analyzed on a Gallios flow cytometer (Beckman Coulter,



114 Villepinte, France). For the analysis of protein expression, level of the geometric mean  
115 fluorescence intensity (MFI) was used. Fluorescence Minus One (FMO) controls were  
116 performed. Data analysis was performed using Kaluza software (Beckman Coulter) with  
117 “logicle” compensation [21]. MFI ratio is determined as follow: [MFI of the considered  
118 marker]/[mean of the MFI of the considered marker in HD group]. The following monoclonal  
119 anti-human antibodies were used: CD10 (HI10a), CD19 (J3-119) and CD27 (B12701) from  
120 Beckman Coulter, Ki67 (c35/ki-67), CD3 (UCHT1), CD19 (HIB19), CD21 (B-ly4), CD24  
121 (ML5), CD27 (M-T271), CD38 (HIT2), CD69 (H1.2F3), CD86 (2331/FUN-1), IgD (HI10a) and  
122 IgM (G20-127) from BD Biosciences.

123

#### 124 **2.4. Apoptosis assay**

125 B cells were purified with magnetic separation with EasySep™ Human B Cell Isolation Kit  
126 (StemCell™ Technologies, Vancouver, Canada). The resultant post-sort purity was > 95%.  
127 Sorted B cells were plated at 100 000 cells/well in a 96 well-plate in RPMI 2% FBS for 8 hours,  
128 with DMSO 5%, positive control) or left untreated. Classical surface staining, to identify  
129 transitional B cells, and annexin V labeling (BD Bioscience), following the manufacturer’s  
130 recommendations, were performed. Cells were resuspended in a PBS solution containing DAPI  
131 and analyzed on a Gallios flow cytometer (Beckman Coulter).

132

#### 133 **2.5. B-cell stimulation**

134 B cells from SLE patients without hydroxychloroquine treatment were purified with magnetic  
135 separation with EasySep™ Human B Cell Isolation Kit (StemCell™ Technologies). The  
136 resultant post-sort purity was >95%. Sorted B cells were plated at 100 000 cells/well in a 96

137 well-plate in RPMI 10% FBS and gentamycine 10µg/ml with 2µg/ml class B CpG (TLR9  
138 agonist, ODN 2006, InvivoGen, San Diego, CA, USA), 1µg/ml Gardiquimod (TLR7 agonists,  
139 InvivoGen) or 1µg/ml CD40 ligand (R&D System®, Mineapolis, MN, USA). Expression of  
140 surface activation markers was analyzed after 48 hours with Gallios flow cytometer (Beckman  
141 Coulter).

142

## 143 **2.6. Quantitative real-time RT-PCR analysis**

144 For blood samples, B cells were purified, from PBMC of HDs and of SLE patients, with  
145 magnetic separation with EasySep™ Human B Cell Isolation Kit (StemCell™ Technologies).  
146 Then, transitional B cells (CD19<sup>+</sup>CD27<sup>-</sup>IgM<sup>+</sup>IgD<sup>+</sup>CD24<sup>++</sup>CD38<sup>++</sup>) and mature naïve  
147 (CD19<sup>+</sup>CD27<sup>-</sup>IgM<sup>+</sup>IgD<sup>+</sup>CD24<sup>+</sup>CD38<sup>+</sup>) populations were sorted (FACS Aria II, BD Biosciences).  
148 For BM samples, pre/proB (CD19<sup>+</sup>CD24<sup>++</sup>CD38<sup>++</sup>IgM<sup>-</sup>IgD<sup>-</sup>), immature  
149 (CD19<sup>+</sup>CD24<sup>++</sup>CD38<sup>++</sup>IgM<sup>+</sup>IgD<sup>-</sup>) and early transitional (CD19<sup>+</sup>CD24<sup>++</sup>CD38<sup>++</sup>IgM<sup>+</sup>IgD<sup>+</sup>)  
150 populations were sorted. Cell viability was assessed with DAPI (Sigma-Aldrich) and the  
151 resultant post-sort purity was > 95%. RNA was isolated using RNeasy Microkit (Qiagen,  
152 Valencia, CA, USA) following the manufacturer's protocol, including a DNase digestion.  
153 Reverse transcription was performed on a T100™ Thermal cycler (BioRad) using High Capacity  
154 cDNA Reverse transcription Kit (Applied Biosystems, Foster City, CA) following the  
155 manufacturer's protocol. 5ng of cDNA were PCR-preamplified for 14 cycles using TaqMan®  
156 PreAmp Master Mix Kit (Applied Biosystems). Quantitative real-time PCR reactions were  
157 performed on a StepOnePlus realtime thermal cycler (Applied Biosystems) using the TaqMan®  
158 Gene Expression Master Mix (Applied Biosystems) following the manufacturer's protocol.  
159 Assays were plated in triplicate. Relative expression levels were calculated with the comparative  
160 Ct method using the mean of the Ct between *GAPDH* and *HPRT1* housekeeping gene for

161 normalization. As defined by Rice et al. [22], the median fold change of the 6 interferon-  
162 stimulated genes *IFI27*, *IFI44L*, *IFIT1*, *ISG15*, *RSAD2*, *SIGLEC1* were used to create an  
163 interferon score for each individual. The following list of TaqMan<sup>®</sup> probes (Applied Biosystems)  
164 was used: *GAPDH* (Hs99999905\_m1), *HERC5* (Hs00180943\_m1), *HERC6* (Hs00215555\_m1),  
165 *HPRT1* (Hs01003267\_m1), *IFI27* (Hs01086370\_m1), *IFI44L* (Hs00199115\_m1), *IFIT1*  
166 (Hs01675197\_m1), *ISG15* (Hs00192713\_m1), *RSAD2* (Hs01057264\_m1) and *SIGLEC1*  
167 (Hs00988063\_m1).

168

## 169 **2.7. Whole transcriptome sequencing (RNA-seq)**

170 B cells were purified, from PBMC of quiescent (n=3) and active (n=3) SLE patients and from  
171 PBMC of healthy donors (n=6), with magnetic separation with EasySep<sup>™</sup> Human B Cell  
172 Isolation Kit (StemCell<sup>™</sup> Technologies). Then, transitional B cells (CD3<sup>-</sup>CD19<sup>+</sup>CD27<sup>-</sup>  
173 IgM<sup>+</sup>IgD<sup>+</sup>CD24<sup>++</sup>CD38<sup>++</sup>) populations were sorted (FACSAria II, BD Biosciences). Cell  
174 viability was assessed with DAPI (Sigma-Aldrich) and the resultant post-sort purity was >95%.  
175 RNA was isolated using RNeasy Microkit (Qiagen) following the manufacturer's protocol,  
176 including a DNase digest. Sample quality was assessed using Agilent 2100 bioanalyzer (Agilent  
177 Technologies, Palo Alto, CA, USA). RNA-seq libraries preparation and sequencing were  
178 performed in the GenomEast platform (Strasbourg, France). Full length cDNA was generated  
179 from 1 ng of total RNA using Clontech SMART-Seq v4 Ultra Low Input RNA kit for  
180 Sequencing (Takara Bio Europe, Saint Germain en Laye, France) according to ma'ufacturer's  
181 instructions, with 12 cycles of PCR for cDNA amplification by Seq-Amp polymerase. 600 pg of  
182 pre-amplified cDNA were then used as input for Tn5 transposon tagmentation using the Nextera  
183 XT DNA Library Preparation Kit (Illumina, San Diego, CA) followed by 12 cycles of library

184 amplification. Following purification with Agencourt AMPure XP beads (Beckman-Coulter), the  
185 size and concentration of libraries were assessed by capillary electrophoresis. Sequencing was  
186 performed on an Illumina HiSeq 4000 with 50 bp single-end reads. Image analysis and base  
187 calling were performed using RTA 2.7.3 and bcl2fastq 2.17.1.14. Adapters and low-quality  
188 sequences (Phred score < 20) were removed using cutadapt v1.10. After this preprocessing, reads  
189 shorter than 40 bases and reads mapping to rRNA sequences were discarded for further analysis.  
190 Reads were mapped onto the hg38 assembly of the human genome using STAR v2.5.3a [23].  
191 Gene expression was quantified using HTSeq v0.6.1p1 [24] and gene annotations from Ensembl  
192 release 91. Gene expression comparisons between quiescent SLE patients and HDs, active SLE  
193 patients and HDs and active versus quiescent SLE patients were performed using the method  
194 proposed by Love et al. [25] implemented in the DESeq2 Bioconductor library (v1.16.1).  
195 Adjustment for multiple testing was performed with the Benjamini and Hochberg method [26].  
196 Results were interpreted using Ingenuity Pathway Analysis 2.3 (Qiagen) and the second  
197 generation of the modular transcriptional framework developed by Chaussabel and Baldwin [27].  
198 Considering transcripts with a sum of normalized read count across all samples >150, a 2-fold  
199 change cut-off with an adjusted p-value<0.01 was set to identify differentially expressed genes.  
200 Variant calling was performed according to The GATK Best Practices workflow for variant  
201 calling on RNA-seq data [28]. Duplicate reads were marked using MarkDuplicates from Picard  
202 version 1.122. Further analyses were performed using GATK version 3.4-46: reads were split  
203 into exon segments and sequences overhanging intronic regions were hard-clipped using  
204 SplitNCigarReads, variant calling was then performed using HaplotypeCaller. Resulting variants  
205 were filtered according to GATK recommendations for RNA-seq data: clusters of at least 3 SNPs  
206 within a window of 35 bases were filtered and filtering based on Fisher Strand values (> 30) and

207 Quality by Depth (< 2) values were also performed. Variants were annotated using GATK,  
208 SnpSift [29] and SnpEff [30]. ClinVar database (<https://www.ncbi.nlm.nih.gov/clinvar/>) was  
209 used to assess if variants involved in monogenic forms of SLE, primary immunodeficiencies  
210 with SLE-like features or interferonopathies were found (clinical significance value:  
211 “pathogenic” or “risk factor”, 28 genes checked).

212

### 213 **2.8. Statistical analysis**

214 Data were analyzed using GraphPad Prism version 7 (GraphPad software Inc, San Diego, CA,  
215 USA). Statistical significances were calculated with two-tailed unpaired Mann-Whitney U-test or  
216 two-tailed Spearman rank correlation test. All values are mean  $\pm$ SEM. *P* values lower than 0.05  
217 were considered statistically significant, or lower than 0.01 for RNA-seq.

218 **3. RESULTS**

219

220 **3.1. Transitional B cell expansion in peripheral blood in SLE**

221 Transitional B cells represent a central developmental stage for B cells, reflecting ontogenesis in  
222 the BM before peripheral differentiation and selection into long-lived mature B cells. The  
223 classification of human transitional B cells into immature T1, intermediate T2 and T3 subsets is  
224 based on the expression of CD24, CD38, IgM, IgD markers in the CD19<sup>+</sup>CD27<sup>-</sup> lymphocyte  
225 gate, as described by Simons et al. and others (Fig. 1A) [17,31,32]. Immature T1 is the  
226 predominant subset in the BM while T2 is predominant in the peripheral blood [32]. We carried  
227 out detailed phenotypic analysis of these populations in the blood of 20 low treated or untreated  
228 SLE patients. All except one patient were Caucasian and their main clinical characteristics are  
229 presented in Supplementary Table 1. Most patients were female with at least 4 ACR criteria (see  
230 methods) but with different clinical and biological presentations of SLE. All patients were  
231 positive for anti-dsDNA antibodies. The frequency of transitional B cells (CD19<sup>+</sup>CD27<sup>-</sup>  
232 CD24<sup>++</sup>CD38<sup>++</sup>) was significantly higher in SLE patients than in healthy donors (HDs)  
233 (12.6%±1.2 versus 6.5%±0.3, p<0.0001) and correlated with the SLEDAI-2K score (r=0.66,  
234 p<0.01) (Fig. 1A, 1B). Transitional B cell increase in SLE patients was mostly the consequence  
235 of T1 and T2 subsets expansion (2.0±0.9% vs 0.9%±0.3 and 6.7%±3.9 vs 3.0%±0.9,  
236 respectively; p<0.0001) (Fig. 1B and Supplementary Fig. 1). Results were comparable  
237 considering either quiescent or active patients (Supplementary Fig. 2).

238

239 **3.2. Defect in transitional B cell responses to TLR9**

240 Based on our previous recent results [10], we hypothesized that transitional B cells present a  
241 lower expression of CD19, concomitantly with an impaired TLR9 response that could influence  
242 their selection. As shown in Fig. 1C, SLE transitional T1, T2 and T3 B cells displayed a decrease  
243 in CD19 expression. This downregulation was present in transitional B cells from quiescent and  
244 active SLE patients (Supplementary Fig. 3). In addition, we further tested TLR9 responses in  
245 rare non-treated SLE patients to circumvent hydroxychloroquine interference [10]. We  
246 confirmed that the induction of CD86 and CD69 activation markers on transitional B cells was  
247 defective after TLR9 activation when compared to HDs ( $31.9\% \pm 5.5$  vs  $57.3\% \pm 6.1$ ;  $p < 0.05$ ),  
248 while their responses after TLR7/TLR8 and CD40 stimulation were similar to HD counterparts  
249 (Fig. 1D).

250

### 251 **3.3. IFN signature in SLE PB transitional B cells**

252 These data prompted us to explore if SLE transitional B cells could harbor a specific  
253 transcriptomic signature, to explain their increased number, their phenotype, and more generally  
254 a breakdown of tolerance at this stage. We performed RNA-sequencing analysis on sorted  
255 transitional B cells ( $CD19^+CD27^-CD24^{++}CD38^{++}$ ) from 12 individuals including 3 SLE  
256 quiescent patients (mean SLEDAI-2K 3.3), 3 active patients (mean SLEDAI-2K 10) and 6 HDs  
257 (see Supplementary Table 1 for the main characteristics of these SLE patients). For  
258 transcriptomic analysis, only untreated patients (for the last six months) were included, as even  
259 hydroxychloroquine or corticosteroids can modify the lymphocyte transcriptomic profile [13].  
260 All SLE patients selected for this analysis displayed a high percentage of transitional B cells and  
261 lower CD19 levels on these cells than HDs. Genes with a minimum of 2-fold change and an  
262 adjusted  $p$ -value  $< 0.01$  were considered as differentially expressed genes (see Materials and

263 Methods section). Comparisons were performed between quiescent SLE patients versus HDs (19  
264 upregulated genes, 7 downregulated genes), active SLE patients versus HDs (33 upregulated  
265 genes, 13 downregulated genes), and in active versus quiescent SLE patients (4 upregulated  
266 genes, 1 downregulated gene) (Fig. 2 and Supplementary Tables 2-4). No difference was  
267 detected between SLE and HD transitional B cells considering the expression of genes involved  
268 in TLR7 or TLR9 signaling, of *CD19*, *CD21* and *PAX5* (which encodes a regulator of CD19), or  
269 of genes encoding molecules involved in BCR signaling cascade, namely *PI3K*, *BTK*, or *AKT*.  
270 These results were consistent with previous q-RT PCR analysis of sorted naive SLE B cells [10].  
271 This approach identified interferon stimulated genes (ISGs) as the most overexpressed genes in  
272 SLE patients compared to controls, i.e. 14 and 25 ISGs in sorted transitional B cells from  
273 quiescent and active SLE patients compared to HDs, respectively (Fig. 3A-B). Results were  
274 analyzed using Ingenuity Pathway Analysis, and the modular transcriptional repertoire  
275 developed by Chaussabel et al. [27], which describes 260 modules of genes sets in blood in  
276 various immunological conditions. The three IFN-related modules (M 1.2, M 3.4, and M 5.12)  
277 driven by type I IFN but also type II IFN (for M 3.4 and M 5.12), appeared up-regulated in SLE  
278 transitional B cells (Fig. 3C). IFN signature was already present in quiescent patients and higher  
279 in active patients. The analysis of RNA sequences did not highlight deleterious mutations in  
280 genes (SNPs and indels called as described in Materials and Methods section) related to  
281 monogenic interferonopathies or to IFN pathway (32 genes, including *TREX1*, *TMEM173*, *RIG-*  
282 *1*, *CECRI*, *RNASEH2A*, *RNASEH2B*, *RNASEH2C*) [22,33]. Genes coding for IFN were not over-  
283 expressed, neither genes coding for IFN receptors compared to controls. We did not detect  
284 significant transcriptomic signatures for other cytokines.



285 IFN signature in whole PBMCs from SLE patients has been previously associated to disease  
286 activity [12,13]. To confirm that IFN signature was reproducible in the transitional B cells of  
287 quiescent SLE patients (i.e. out of flares), we tested on sorted cells from additional quiescent  
288 SLE patients (n=6, mean SLEDAI-2K 3.3; see Supplementary Table 1 for the main  
289 characteristics of these SLE patients) the relative expression of eight ISGs, some of which  
290 (namely *IFI27*, *IFI44L*, *IFIT1*, *ISG15*, *RSAD2*, *SIGLEC1*) were previously used to determine an  
291 interferon score in inflammatory diseases [20]. *HERC5* and *HERC6* genes were added as they  
292 encode positive regulators of IFN pathways [34] and were found over-expressed in our RNA-Seq  
293 analysis. Transcripts coding for all these genes were very significantly amplified by RT-qPCR in  
294 sorted transitional B cells from quiescent SLE patients compared to HDs (Fig. 3D). Of note, the  
295 IFN-score correlated with the frequency of transitional B cells ( $r = 0.94$ ,  $p < 0.01$ ) (Fig. 3E).

296

### 297 **3.4. IFN signature in BM B cells**

298 The IFN signature in transitional B cells from SLE patients could be the consequence of higher  
299 levels of IFN in the peripheral blood or affecting the early transitional stage in the BM [35]. To  
300 assess this point, and despite the rarity of the samples, the IFN score of sorted BM B cells was  
301 compared to sorted peripheral B cells subsets in the same SLE patients (Fig. 4A). BM early  
302 transitional B cells displayed the highest IFN score, followed by a progressive decrease in the  
303 peripheral subsets, revealing a B cell IFN burden of central origin (Fig. 4B-C).

304

### 305 **3.5. Activation and proliferation status**

306 Finally, RNA-sequencing analysis of peripheral transitional B cells showed an over-expression  
307 of genes related to the regulation of cell-cycle and coding for pro- or anti-apoptotic proteins

308 (Supplementary Tables 2-4). *Per se* transitional B cell activation could not explain such gene  
309 regulation as the frequency of CD86<sup>+</sup> B cells and the intensity of HLA-DR expression in SLE  
310 patients were comparable to that of HDs (Supplementary Fig. 4). We tested the hypothesis that  
311 SLE transitional B cells proliferate more or die less than HDs transitional B cells. However, flow  
312 cytometric analysis did not highlight any difference in proliferation (Fig. 5A) or early apoptosis  
313 in freshly isolated transitional SLE B cells (Fig. 5B), arguing for an accelerate differentiation  
314 from immature B cells to transitional stage in SLE patients.

315 **4. DISCUSSION**

316

317 Organ damage in SLE is driven by immune complexes deposition and anti-nuclear antibodies  
318 [2]. Hence, lots of work to elucidate SLE pathogenesis focused on the behavior of autoreactive B  
319 cells to explain how pathogenic B cells producing high affinity auto-antibodies can arise.  
320 Autoreactive B cells are submitted to stringent selection in the BM, via editing and deletion [36–  
321 39], and during B cell differentiation their percentage dropped significantly in HDs between the  
322 transitional stage and the mature naive stage [6]. However, a percentage of ignorant or anergic  
323 autoreactive B cells remain and they will be ultimately control in the periphery [40].

324 Herein, we describe that transitional B cells in SLE patients displayed, as naive B cells [10], an  
325 impairment in TLR9 response, and low levels of CD19 concerning all 3 subsets. TLR9  
326 deficiency in lupus-prone mice leads to disease worsening, and Sindhava et al. showed in  
327 humans the crucial role of TLR9 in controlling the survival of autoreactive naive B cells when  
328 co-engaged with BCR [11]. CD19 downregulation could participate for diminished TLR9  
329 response in early SLE B cells [9,10]. It may also favor *per se* abnormalities in selection at the  
330 BM level, considering autoreactive B cell fate in CD19 deficient patients [41], in CD19 deficient  
331 transgenic cell lines [42] or in mice [43]. Thus, the defect in transitional B cells of two closely  
332 inter-related molecules (i.e. CD19 and TLR9) may favor the B-cell tolerance breakdown  
333 described in SLE at this stage.

334 We detected an IFN signature in sorted transitional B cells from both peripheral blood and BM.  
335 Domeier et al. recently reported in mice, that type I IFN maintains BCR signaling beyond the  
336 threshold required for effective tolerance, driving autoreactive B cell development into the  
337 germinal center (GC) pathway [44]. Since IFN influence appears in the BM at the early

338 transitional stage, it could affect the BCR signaling threshold also at the medullary B-cell stages  
339 and not only in the periphery.

340 IFN signature and abnormalities in CD19/TLR9 signaling could have different causes.  
341 CD19/TLR9 defects, are undoubtedly present in quiescent SLE patients. Munroe et al. were able  
342 to examine type I and type II interferon activities, autoantibodies and cytokines levels in samples  
343 collected before and after diagnosis of SLE and it appears that IFN activities precede the disease  
344 [45]. Studies in ANA+ healthy subjects or in other autoimmune conditions may answer about the  
345 causal/relationship of our findings in SLE and in autoantibody mediated diseases. For example,  
346 transitional B cells are expanded in Sjögren syndrome [17], although the expression of CD19  
347 remains normal [10]. Thus, investigations in other conditions and in prospective longitudinal  
348 cohorts could help to precise if the frequency of transitional B cells is a reflection of IFN burden  
349 without real disease specificity and whether or not it can predict the occurrence of some of them.

350 We confirmed that IFN imprintment in SLE is not confined to African American patients,  
351 especially to those with nephritis and anti-Ro antibodies [15] but also concerns Caucasian  
352 patients with anti-dsDNA. Transitional B cells from African American SLE patients displayed an  
353 autocrine production of IFN- $\beta$  [15]. However, we did not detect overexpression of genes coding  
354 for type I or type II IFN in our cohort, suggesting an extrinsic influence in Caucasian patients,  
355 i.e. IFN secretion by other cells. This is consistent with the low transcription of genes coding for  
356 type I IFN in another group of European American SLE patients compared to African American  
357 SLE in sorted transitional B cells [15]. In this view, Palanichamy et al. described in SLE a  
358 significant production of IFN- $\alpha$  and  $\beta$  by resident BM cells, associated with a reduction in the  
359 fraction of precursor B cells and a T1 and T2 B cell subsets expansion [35]. The correlation of  
360 the IFN score with the percentage of transitional B cells, together with the normal status of

361 peripheral transitional B cells in terms of activation, proliferation and survival, argue for the  
362 influence of IFN on early BM differentiation. We cannot rule out that for some reasons,  
363 transitional B cells in SLE are more sensitive to IFN although transcriptional levels of IFN  
364 receptors did not differ in our analysis.

365 In our previous work, we correlated the CD19 low expression on B cells to the presence of anti-  
366 dsDNA antibodies, suggesting that apoptotic bodies and immune complexes containing DNA  
367 participate to CD19 and TLR9 regulation in B cells in the BM. Immune complexes could also  
368 trigger IFN production via TLR7, or directly via DNA-sensors in myeloid cells [46], and favor  
369 the output of transitional B cells, interfering with the elimination of autoreactive B cells at this  
370 stage [47]. Interestingly, *TLR7* can escape X chromosome inactivation in immune cells and its  
371 expression is higher after puberty in female and correlates with sex hormones levels [48].  
372 However, we did not observe significant differences in responses of SLE transitional B cells to  
373 TLR7/TLR8 agonist compared to controls, neither differences in TLR7/TLR9 transcription in  
374 transitional B cells via RNA sequencing analysis. It would be however of great interest to focus  
375 on this point.

376 SLE differs from one patient to another, depending of genetic factors and environment. The idea  
377 that central tolerance and early escape of autoreactive B cells participate to the genesis of SLE  
378 has been harmed by elegant studies demonstrating the extra-follicular activation of autoreactive  
379 B cells and of plasmocytes in periphery [5]. In view of our results, early B cells are abnormal  
380 likely since the BM, considering frequency or signaling, in many SLE patients even out of flares.  
381 Thus, both early and late mechanisms could interfere with survival and activation of autoreactive  
382 B cells in SLE patients and further analysis should clarify at what point it is in a personalized  
383 way.

384 **AUTHOR CONTRIBUTIONS**

385 Y.D., V.G., E.M., J.A. and A.S.K. designed the research.

386 Y.D., V.G., A.G., C.K., B.A., L.J., B.J., N.D., V.P, T.M, J.A. and A.S.K. enrolled patients and  
387 performed the experiments.

388 Y.D., V.G., A.G., C.K., J.A., E.M., T.M., P.S.S. and A.S.K. analyzed the data.

389 Y.D., V.G., E.M, J.A and A.S.K. wrote the paper.

390

391 **CONFLICT-OF-INTEREST DISCLOSURE**

392 The authors declare no competing financial interests.

393

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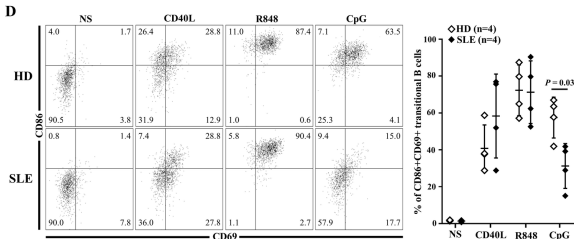
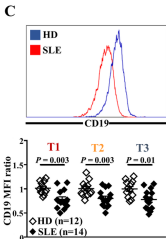
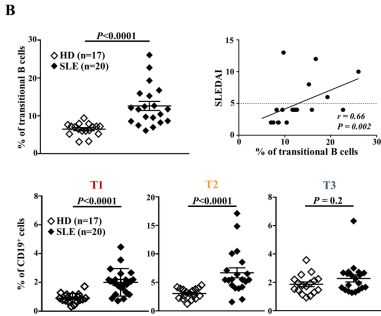
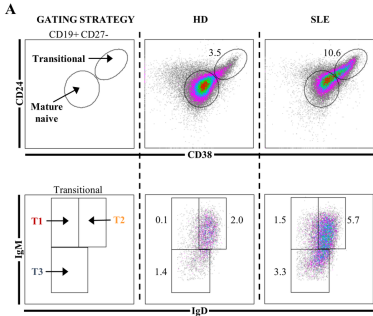
581 **Fig. 1.** (A) Representative dot plots of transitional B cells and their subsets in one HD and one  
582 SLE patient. (B) Frequency of transitional B cells (*top left panel*) and their subsets (*bottom*  
583 *panel*) (% of CD19<sup>+</sup>) in HDs compared to SLE patients. Correlation between transitional B cells  
584 (% of CD19<sup>+</sup>) and disease activity assessed by the SLEDAI-2K score (*top right panel*). (C) MFI  
585 of CD19 in transitional B cells subsets from HDs and SLE patients. (D) Frequency of  
586 CD86<sup>+</sup>CD69<sup>+</sup> transitional B cells from HDs or SLE patients after no stimulation (NS) or *in vitro*  
587 stimulation with CD40L (CD40 ligand), R848 (TLR7 agonist) or CpG (TLR9 agonist) for 2  
588 days. HDs: healthy donors; MFI: mean of fluorescence intensity; SLE: Systemic lupus  
589 erythematosus.

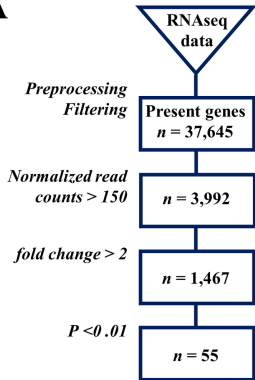
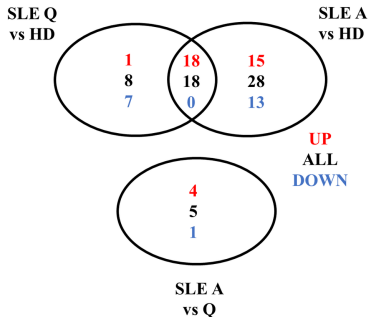
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591 **Fig. 2.** (A) Flow chart of RNA Sequencing analysis. (B) Venn Diagram representing genes  
592 significantly up (*in red*) or down-regulated (*in blue*) between quiescent SLE patients, active SLE  
593 patients and HDs. A: active; HDs: healthy donors; Q: quiescent; SLE: systemic lupus  
594 erythematosus.

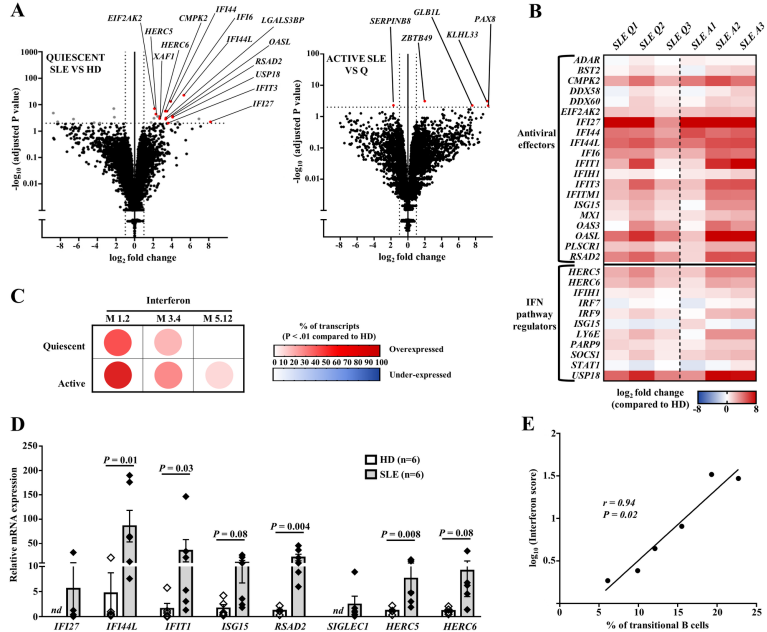
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596 **Fig. 3.** (A) Volcano plot representation of RNA-Seq data comparing transitional B cells from  
597 HDs (n=6) versus untreated quiescent SLE patients (n=3) (*left panel*) and transitional B cells  
598 from untreated quiescent versus active patients (n=3) (*right panel*). (B) Hierarchical  
599 representation of interferon stimulated genes (ISGs) expression in transitional B cells from each  
600 patient compared to HDs. (C) Modular analysis of RNA-Seq data representing the % of  
601 transcripts over/under-expressed (P<0.01) in untreated SLE patients (quiescent, n=3; active, n=3)  
602 compared to HDs (n=6). (D) qRT-PCR of ISGs in sorted transitional B cells of quiescent SLE  
603 patients compared to HDs. (E) Correlation between transitional B cells (% of CD19<sup>+</sup> cells) and  
604 the interferon score. HDs: healthy donors; ND: not detected; SLE: Systemic lupus  
605 erythematosus.

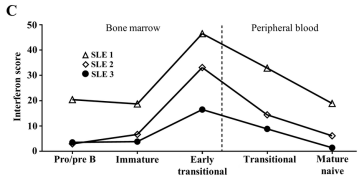
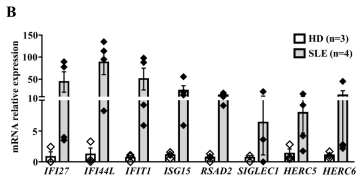
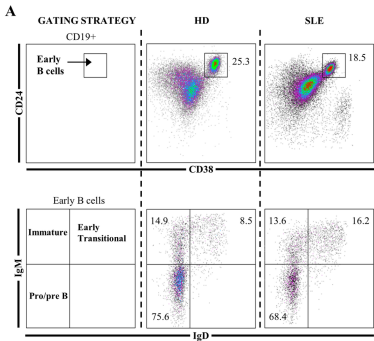
606  
607 **Fig. 4.** (A) Gating strategy for BM analysis of pre/proB (CD19<sup>+</sup>CD24<sup>++</sup>CD38<sup>++</sup>IgM<sup>-</sup>IgD<sup>-</sup>),  
608 immature (CD19<sup>+</sup>CD24<sup>++</sup>CD38<sup>++</sup>IgM<sup>+</sup>IgD<sup>-</sup>) and early transitional B cells  
609 (CD19<sup>+</sup>CD24<sup>++</sup>CD38<sup>++</sup>IgM<sup>+</sup>IgD<sup>+</sup>). (B) qRT-PCR of eight interferon stimulated genes in sorted  
610 early transitional B cells from BM of SLE patients compared to control. (C) Representation of  
611 the interferon score in BM and peripheral B cell subpopulations in three SLE patients. BM: Bone  
612 marrow; ND: not detected; SLE: Systemic lupus erythematosus.

613  
614 **Fig. 5.** (A) Representative dot plots of Ki-67<sup>+</sup> cells (*top panel*) and Ki-67 MFI (*bottom left*  
615 *panel*) in gated transitional B cells from one HD and one SLE patient; frequency of Ki-67<sup>+</sup>  
616 transitional B cells from HDs and SLE patients (*bottom right panel*). (B) Frequency of early  
617 apoptotic (DAPI<sup>-</sup> AnnexinV<sup>+</sup>) transitional B cells in HDs and SLE patients. FMO: Fluorescence  
618 Minus One; HDs: healthy donors; MFI: mean of fluorescence intensity; SLE: systemic lupus  
619 erythematosus.

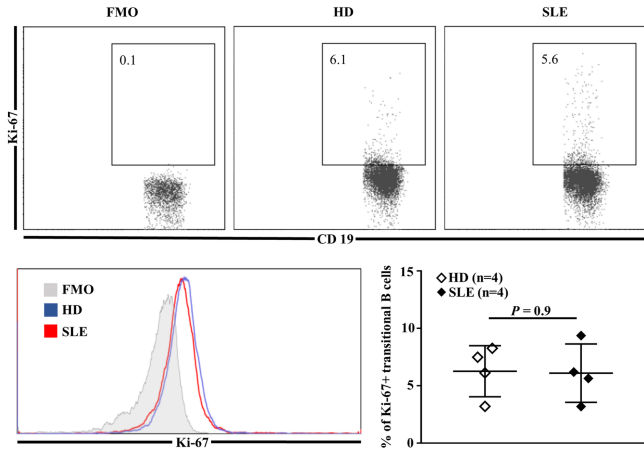


**A****B**







**A****B**