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1 **IL-6 SUPPORTS THE GENERATION OF HUMAN LONG-LIVED PLASMA CELLS**
2 **IN COMBINATION WITH EITHER APRIL OR STROMAL CELL SOLUBLE**
3 **FACTORS**

4

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ABSTRACT

The recent understanding of plasma cell (PC) biology has been obtained from murine models mainly. The current concept is that plasmablasts home to the BM and further differentiate into long-lived PCs (LLPCs). These LLPCs survive for months in contact with a complex niche comprising stromal cells (SCs) and hematopoietic cells both producing recruitment and survival factors. Using a multi-step culture system, we show here the possibility to differentiate human memory B cells into LLPCs surviving for at least 4 months *in vitro* and producing immunoglobulins continuously. A remarkable feature is that IL-6 is mandatory to generate LLPCs *in vitro* together with either APRIL or soluble factors produced by SCs, unrelated to APRIL/BAFF, SDF-1, or IGF-1. These LLPCs are out of the cell cycle, express highly PC transcription factors and surface markers.

This model shows a remarkable robustness of human LLPCs, which can survive and produce highly immunoglobulins for months *in vitro* without contact with niche cells, providing the presence of a minimal cocktail of growth factors and nutrients. This model should be useful to understand further normal PC biology and its deregulation in premalignant or malignant PC disorders.

Key words: Plasma cell; B cell; differentiation; microarray

53 **INTRODUCTION**

54 Mature memory plasma cells (PCs), termed long lived plasma cells (LLPCs), are
55 located in the bone marrow (BM) or mucosa and may survive for years, insuring long-
56 term immune memory.[1] LLPCs are rare cells (0.25 % of BM cells)[2,3] making their
57 study difficult in humans. Recent knowledge about their generation and maintenance
58 was obtained with murine models mainly. After selection of antigen (Ag) specific B
59 cells in the germinal center, involving mutations in immunoglobulin (Ig) variable
60 genes and isotype switching, centrocytes differentiate into memory B cells (MBCs) or
61 plasmablasts (PBs). These PBs migrate to medullary cords, exit into the lymph
62 through a sphingosine phosphate gradient and get to the peripheral blood.[4]
63 Circulating PBs have to find a specific niche in the BM or mucosa that will provide
64 them with the factors to survive and fully differentiate. The restricted number of
65 suitable PC niches is supposed to be the main limiting factor explaining LLPC rarity.[4]
66 In mice, Tokoyoda *et al*. have reported the PC niche to be a VCAM1$^+$ SDF-1$^+$ stromal
67 cell that could be shared by hematopoietic progenitors and pre-pro B cells.[5] Several
68 hematopoietic cell subsets have been described to be involve in PC retention,
69 maturation, and maintenance in the BM, including macrophages, eosinophils, and
70 megakaryocytes.[6] At least 3 growth factors and chemokines, produced by the
71 exquisite PC niche, are recognized to control PC survival: SDF-1, APRIL/BAFF, and
72 IL-6.[4] SDF-1, produced by SCs is essential to recruit mouse PBs into the BM.[5,7]
73 BAFF and APRIL are produced by hematopoietic cells and are critical to support PC
74 survival.[6] BAFF binds to 3 receptors, BAFF receptor, BCMA and TACI, whereas
75 APRIL binds to BCMA and TACI *in vivo*.[8] Although both APRIL and BAFF can
76 support murine LLPC survival,[9] APRIL appears to be more efficient than BAFF to
77 promote LLPC survival, at least in mice. First, APRIL binds to BCMA with a higher

affinity than BAFF,[8] and BCMA is highly expressed in PCs.[10] Secondly, APRIL and its receptor TACI bind to heparan sulfate chains, in particular to the proteoglycan syndecan-1,[11,12] which is a hallmark of mature PCs.[13] Thirdly, the long-term survival of transferred LLPCs is impaired in APRIL$^{-/-}$ mice, but unaffected in BAFF$^{-/-}$ ones.[14] IL-6, produced by SCs or dendritic cells, is essential for the generation of PBs, and its role in supporting LLPC survival is controversial in mice. The generation of Ag-specific PCs is dramatically decreased in IL-6$^{-/-}$ mice[15] and the ability of BM SCs to support the survival of purified BM LLPCs *in vitro* is inhibited by an anti-IL-6 monoclonal antibody (mAb) or lost using IL-6$^{-/-}$ mice SCs.[16,17] Similarly, the survival of LLPCs in the culture of intestine biopsies is impaired by an anti-IL-6 mAb or an APRIL/BAFF inhibitor.[18] But the persistence of LLPCs transferred into IL-6$^{-/-}$ mice is unaffected.[14]

In humans, the factors that promote the differentiation of PBs into LLPCs are poorly identified *in vivo*, due to the rarity of bone marrow PCs (BMPCs) and the ethical difficulty to harvest the BM. The majority of the studies dealing with PC survival and growth were done with malignant PCs.[19,20] Using *in vitro* models of differentiation of B cells into PCs, we and others have shown that *in vitro* generated PCs are early PCs with a phenotype close to that of circulating PBs and PCs in healthy individuals.[10,21,22] These *in vitro* generated PCs expressed highly CD38 and CD31 and lack B cell antigens (CD20, CD22, CD24) except CD19. They express CD138 but at lower level than BMPCs, they express CD62L unlike BMPCs, and fail to express CD9, VCAM1, and CCR2.[10] Recent studies have proposed a role for osteoclasts[23] or SCs to support human PC survival *in vitro*.[24] In this last study, blood B cells could be differentiated into Ig-secreting LLPCs in contact with soluble factors produced by a mouse SC line. However, the molecular mechanisms of this supportive activity remain unknown.[24]

103 In the current study, we show that IL-6 is mandatory for the *in vitro* survival of LLPCs
104 in combination with either APRIL or BAFF or APRIL/BAFF-unrelated SC soluble
105 factors. These LLPCs are non-cell cycling, survive for months *in vitro*, while
106 producing Igs continuously.

107 **MATERIALS AND METHODS**

108 **Reagents**

109 Human recombinant IL-2, TACI-Fc, and APRIL were purchased from R&D Systems
110 (Minneapolis, MN), IFN-α (IntronA) from Merck Canada Inc. (Kirckland, Canada), IL-6
111 and IL-15 from AbCys SA (Paris, France), BAFF, SDF-1α, IGF-1, and IL-10, from
112 Peprotech (Rocky Hill, NJ, USA), the B-E8 anti-IL-6 mouse mAb from Diaclone
113 (Besançon, France), the SDF-1 inhibitor AMD3100 from Sigma (Sigma-Aldrich, St
114 Louis, MO), the IGF-1R inhibitor from Novartis Pharma (Basel, Switzerland), and
115 IKK16, a selective inhibitor targeting both IKK1 and IKK2, from R&D Systems.

116 **Cell samples**

117 Peripheral blood cells from healthy volunteers were purchased from the French
118 Blood Center (Toulouse, France) and $CD19^{+}CD27^{+}$ MBCs purified (≥ 95% purity) as
119 described.[10] When indicated, D10 early PCs ($CD20^{-}CD138^{+}$) were FACS-sorted
120 using FITC-conjugated anti-CD20 mAb and PE- or APC-conjugated anti-CD138
121 mAb. The purity of FACS-sorted cell populations was ≥ 95% as assayed by
122 cytometry. Resto-6 stromal cells were used as a source of SCs. These SCs were
123 obtained from a 15%/25% Percoll interface of dissociated human tonsil cells as
124 previously described.[25] Plastic-adherent cells were selected and expanded in RPMI
125 1640 culture medium and 10% fetal calf serum (FCS) yielding to the Resto-6 SCs
126 after 8 passages. Resto-6 SCs express usual mesenchymal stromal cell markers
127 (CD90, CD73, and CD105) and can acquire properties of fibroblastic reticular cells
128 (FRC) including expression of high levels of adhesion molecules and
129 gp38/podoplanin, production of a dense meshwork of transglutaminase, and
130 production of inflammatory and lymphoid chemokines upon stimulation by TNFα and
131 Lymphotoxin-α1β2.[25] Resto-6 SCs support efficiently the growth and survival of

132 normal B and T cells and of malignant lymphoma B cells, in particular after FRC-
133 commitment. They were used between passages 8 and 15.

134 **Cell cultures**

135 PCs were generated through a four-step culture. All cultures were performed in
136 Iscove's modified Dulbecco medium (IMDM, Invitrogen) and 10% FCS. In step 1,
137 purified peripheral blood MBCs (1.5 x 10^5/ml) were activated for 4 days by CpG
138 oligodeoxynucleotide and CD40 ligand (sCD40L) - 10 μg/ml of phosphorothioate
139 CpG oligodeoxynucleotide 2006 (Sigma), 50 ng/ml histidine tagged sCD40L, and
140 anti-poly-histidine mAb (5 μg/ml), (R&D Systems) - with IL-2 (20 U/ml), IL-10 (50
141 ng/ml) and IL-15 (10 ng/ml) in 6 well culture plates. In step 2, PBs were generated by
142 removing CpG oligonucleotides and sCD40L and changing the cytokine cocktail (IL-
143 2, 20 U/ml, IL-6, 50 ng/ml, IL-10, 50 ng/ml and IL-15, 10 ng/ml). In step 3, PBs were
144 differentiated into early PCs adding IL-6 (50 ng/ml), IL-15 (10 ng/ml) and IFN-α500
145 U/ml) for 3 days. In step 4, early PCs were differentiated into LLPCs using either
146 coculture with SCs, transwell culture, or a cytokine cocktail, and the cultures
147 maintained for months. Confluent monolayers of SCs were generated in 6-, 24-, or
148 48-well flat-bottom culture plates and PCs were then added onto the monolayers
149 together with various cytokines. These cocultures of PCs and SCs could be
150 maintained for months with the same SC monolayer, adding fresh culture medium
151 and growth factors once by week. Cocultures of PCs and SCs without cell contact
152 were done seeding SCs in the lower chamber of 6-well transwells and PCs in the
153 upper compartment, both compartments being separated by a 0.4 μm-polycarbonate
154 membrane (Corning, New-York, NY). SC-conditioned medium (SC-CM) was obtained
155 by culturing confluent monolayers of SCs for 5 days with culture medium. The culture
156 supernatant was 0.2 μM filtered and frozen and 50% of SC-CM was added to PC

157 cultures, and renewed every week. Finally, LLPCs were also obtained adding IL-6
158 (10 ng/ml) and either APRIL (200 ng/ml) or BAFF (200 ng/ml).

159 **Assay for cell viability and cell growth**

160 Cell concentration and viability were assessed using trypan blue dye exclusion test.
161 The number of metabolic active cells was also determined using intracellular ATP
162 quantitation with a Cell Titer Glo Luminescent Assay (Promega Corporation,
163 Madison, WI).

164 **Cell cycle analysis, immunophenotypic analysis, and cytology**

165 The cell cycle was assessed using DAPI staining (Sigma-Aldrich) and cells in the S
166 phase using incubation with bromodeoxyuridine (BrdU) for 1 hour and labelling with
167 an anti-BrdU antibody (APC BrdU flow kit, BD Biosciences) according to
168 manufacturer's instructions. Cells were stained with a combination of 4 to 7 mAbs
169 conjugated to different fluorochromes. The Cytofix/Cytoperm kit (BD Biosciences)
170 was used for intracellular staining of IgM, IgA, IgG or Ki67 antigen.[10] Flow cytometry
171 analysis was performed with a FACSAria cytometer using FACSDiva 6.1 (Becton
172 Dickinson, San Jose, CA) and with a Cyan ADP cytometer driven by the Summit
173 software (Beckman Coulter). Kaluza software (Beckman Coulter) was used for data
174 analysis. The fluorescence intensity of the cell populations was quantified using the
175 stain index (SI) formula: [mean fluorescence intensity (MFI) obtained from a given
176 mAb minus MFI obtained with a control mAb]/[2 times the standard deviation of the
177 MFI obtained with the same control mAb].[10] Cytospin smears of cell-sorted $CD20^-$
178 $CD138^+$ D30 PCs were stained with May-Grünwald-Giemsa.

179 **Analysis of Ig secretion**

180 **ELISA.** Flow cytometry sorted PCs were cultured at 10^6 cells/ml for 24 hours and
181 culture supernatants harvested. IgM, IgA, or IgG concentrations were assessed by

182 ELISA using human IgM, IgA, and IgG ELISA kits from Bethyl Laboratories
183 (Montgomery, TX), according to the manufacturer's recommendations.
184 **ELISPOT.** The number of IgM, IgA, or IgG secreting PCs was evaluated with the
185 ELISPOT assay,[26] seeding 500 PCs by well in ELISPOT plates and culturing cells
186 for 18 hours. The number and size of IgM, IgA, and IgG elispots were assessed
187 using the Biosys Bioreader 5000 apparatus (Biosys, Miami, FL).
188 **Microarray hybridization and bioinformatics analysis**
189 RNA was extracted and hybridized to human genome U133 Plus 2.0 GeneChip
190 microarrays, according to the manufacturer's instructions (Affymetrix, Santa Clara,
191 CA). Gene expression data are deposited in the ArrayExpress public database
192 (http://www.ebi.ac.uk/microarray-as/ae/). The accession numbers are E-MEXP-3034
193 for prePBs, E-MEXP-2360 for PBs and BMPCs, E-MEXP-3945 for day 10 (D10) early
194 PCs and D30 PCs, and E-MTAB-2118 for Resto-6 SCs. Gene expression data were
195 analyzed with our bioinformatics platforms (RAGE, http://rage.montp.inserm.fr/)[27] and
196 Amazonia (http://amazonia.transcriptome.eu/).[28] Genes differentially expressed
197 between cell populations were determined with the SAM statistical microarray
198 analysis software.[29] The clustering was performed and visualized with the Cluster
199 and TreeView softwares.[30] Gene annotation and networks were generated with the
200 Reactome Functional Interaction Cytoscape plugin (http://www.cytoscape.org/).
201 **Statistical analysis**
202 Statistical comparisons were made with the non-parametric Mann-Whitney test,
203 unpaired or paired Student's *t*-test using SPSS software. *P*-values $\leq$ .05 were
204 considered as significant.

205 **RESULTS**

206 **IL-6 in combination with APRIL or BAFF or APRIL/BAFF-unrelated stromal-cell**
207 **soluble factors supports the generation and survival of PCs *in vitro***

208 Starting from MBCs, early PCs can be generated within 10 days using a 3-step
209 culture. These early PCs could survive poorly in presence of IL-6 (Figure 1A). Adding
210 human tonsil SCs could promote PC survival with 28% surviving CD138 PCs at day
211 30 (Figure 1A). In the presence of SCs, the CD138 fluorescence staining index at
212 day 14 was increased 3 fold (*P* = .006) compared to day 14 PCs generated with IL-6
213 alone (Figure 1B and 1C). It progressively increased 2 fold from day 14 to day 30 (*P*
214 *= .032)* and then was stable up to day 60 (Figures 1B and 1C). Coculturing early PCs
215 separated from SCs in transwell culture plates promoted PC survival at the same
216 extent as coculture of PCs with SCs at day 14, 17, 24 and 30, showing a contact
217 between PCs and SCs is not mandatory (Figure 2A). In agreement, coculture
218 supernatant of PCs with SCs (PC/SC-CM) or of SCs alone (SC-CM) also promoted
219 PC survival at day 30 (Figure 2A). Various growth recombinant factors - APRIL,
220 BAFF, SDF-1, IGF-1 - known to sustain malignant PC survival[19] did not induce PC
221 survival when used alone (Figure 2B). IL-6 alone supported PC survival but at a 76%
222 lower level than that induced by the SC-CM (*P* = .003). Adding APRIL together with
223 IL-6 increased significantly 6.5 fold (*P* = .02) the IL-6 PC survival activity (Figure 2B).
224 Adding BAFF also increased the PC survival induced by IL-6 (*P* = .02) and SDF-1
225 and/or IGF-1 did not increase IL-6 activity (Figure 2B). The PC survival induced by
226 the SC-CM was fully abrogated by an anti-IL-6 mAb showing the critical role of IL-6
227 produced by SCs, but was unaffected by an APRIL/BAFF inhibitor, TACI-Fc (Figure
228 2C). The TACI-Fc APRIL/BAFF inhibitor fully blocked the ability of APRIL to increase
229 2.2 fold the PC generation induced by the SC-CM (Figure 2C). It also inhibited the

230 APRIL-induced growth of the XG1 myeloma cell line (supplementary Figure 1).[31] As
231 APRIL/BAFF activate NF-κB pathways,[32] we investigated whether these pathways
232 could be involved in PC generation by SCs. An inhibitor of both canonical and
233 alternative NF-κB pathways (IKK16) did not affect the generation of PCs supported
234 by SCs, whereas it impeded the additive effect of APRIL in PC generation promoted
235 by SCs (Figure 3). Thus, SCs produce communication signals, which cooperate with
236 IL-6 to promote PC survival and are not inhibited by a NF-κB inhibitor. This is in
237 agreement with the low expression of NF-κB-induced genes[33] in PCs generated with
238 SCs *in vitro* contrarily to BMPCs (supplementary Figure 2). These communication
239 signals are not SDF-1 or IGF-1 since the SC-CM activity was unaffected by their
240 specific inhibitors (Figure 2C). Adding these 3 inhibitors together (APRIL/BAFF, SDF-
241 1 and IGF-1 inhibitors) yielded to an apparent decrease in SC-CM induced PC
242 generation, which did not reach statistical significance (Figure 2C).
243 Of note, PCs could survive up to 120 days in these culture conditions adding fresh IL-
244 6, APRIL and culture medium weekly and cultures were stopped after 120 days for
245 convenience but not due to a decline in PC survival. These PCs were thus termed
246 LLPCs. These results indicate that IL-6 is mandatory to induce the long-term survival
247 of LLPCs in combination with either APRIL or BAFF or with APRIL/BAFF-unrelated
248 soluble factors produced by SCs.
249 **Characterization of D30 PCs**
250 D30 PCs had a more mature PC phenotype than D10 early PCs with a progressive
251 7.4-fold increase in CD138 density (Figure 1C, $P \leq .001$), a 2.2-fold increase in CD54
252 staining index, a 1.5-fold increase in $CD9^+$ PCs, and a decrease in HLA-DR, CD45,
253 CD62L, CCR10 expressing PCs and staining indexes ($P \leq .05$, Figure 4 and
254 supplementary Figure 3). D30 PCs did not proliferate (0.04% cells incorporating

255 BrdU), whereas a low fraction of D10 early PCs was in the S phase of the cell cycle
256 (4% BrdU+) (Figure 5A and 5B). D30 PCs displayed the cytology of mature PCs and
257 produced cytoplasmic kappa or lambda Ig light chains (supplementary Figure 4). Day
258 60 PCs comprise IgG PCs only, with a quick disappearance of IgM PCs (no more
259 detectable at day 30) and a progressive one of IgA PCs as assayed by FACS, ELISA
260 and ELISPOT assays (Figures 6 A-F). Of note, the rate of IgG production was similar
261 between day 30 and day 60 PCs (Figure 6F). The number of IgG and IgM producing
262 cells and the Ig isotype produced were not significantly different starting from the
263 same number of LLPCs generated with either SC coculture, IL-6+APRIL, SC-CM or
264 APRIL+SC-CM (Figure 6G). There was a trend in increasing IgA secreting cells in the
265 two experiments adding APRIL compared to the culture groups without APRIL ($P <$
266 .1). Combining data of these two experiments show APRIL increased 3.4 fold the
267 number of IgA secreting cells (47 vs. 14 IgA secreting cells/500 PCs, $P < .001$, Figure
268 6G).

269 **B and PC transcription factors**

270 D30 PCs had a higher expression of genes coding for IRF4 and BLIMP1 PC
271 transcription factors than D10 early PCs and failed to express *PAX5* ($P \leq .02$, Figure
272 7). D30 PCs expressed 3-fold more the spliced form of *XBP1* mRNA than D10 early
273 PCs and 2-fold more the unspliced mRNA form than early PCs, resulting in an
274 increased *XBP1s*/*XBP1u* mRNA ratio ($P \leq .009$, Figure 7). Of note, *BCL6* gene
275 expression in D30 PCs was low compared to that in B lymphocytes but significantly
276 higher than that in early PCs ($P = .04$, Figure 7).

277 **Gene expression profile (GEP) of D30 PCs**

278 *In vitro* generated D4 prePBs, D7 PBs, D10 early PCs, D30 PCs, and BMPCs
279 purified from healthy individuals were profiled using Affymetrix U133 plus 2.0

280 microarrays. D30 PCs were generated using cocultures with SCs. The 5 populations
281 are classified into 2 major clusters, a PC cluster comprising D10 early PCs, D30 PCs,
282 and BMPCs and a PB cluster comprising prePBs and PBs (Figure 8). To look for
283 genes indicator of LLPCs, we ran a SAM supervised analysis comparing D10 early
284 PCs to D30PCs+BMPCs starting from the 5000 genes with the highest variance. 160
285 probe sets (141 unique genes) were overexpressed in D30PCs+BMPCs compared to
286 D10 early PCs (Wilcoxon statistic, fold change ≥ 2, FDR $\leq 1\%$) and 490 (427 unique
287 genes) in D10 early PCs versus D30PCs+BMPCs (supplementary Table 1). Genes
288 coding for translation, focal adhesion, IL-6 signalling, and integrin signalling pathways
289 were enriched in D30PC+BMPC genes (supplementary Table 2), and genes coding
290 for DNA replication and mitosis in D10 early PCs (supplementary Table 3). The gene
291 expression profiles of LLPCs harvested from the spleen of patients with primary
292 immune thrombocytopenia treated with rituximab anti-CD20 mAb was recently
293 documented and compared to that of PBs harvested from the spleen of untreated
294 patients.[34] Similarly to the current *in vitro* generated LLPCs, LLPCs from these
295 patients overexpress genes coding for PC transcription factors (*JUN, FOS, EGR1*),
296 negative regulators of the cell cycle (*KLF4, KLF2, PPP1R15A*) and cell
297 surface/cytokine receptors (*CD9, SDC1, FCRL5*). Conversely, similarly to *in vitro*-
298 generated D10 early PCs, patients' PBs overexpress genes coding for positive cell
299 cycle regulators (*CCND2, BUB1B, BUB1, TIMELESS, CENPF, MAD2L1, BIRC5,*
300 *ZWINT, MKI67, MCM4, CCNB2*) and surface/cytokine receptors (*ITGB1, TNFSF10*)
301 (supplementary Table 1). Comparing gene expression between BMPCs and D30
302 PCs, 198 unique genes were overexpressed in BMPCs and 555 in D30 PCs (SAM
303 supervised analysis, Wilcoxon statistic, fold change ≥ 2, FDR $\leq 1\%$, supplementary
304 Table 4). Genes coding for protein metabolism, translation, antigen processing and

305 presentation, and CXCR4 signalling were enriched in BMPC genes (supplementary
306 Table 5). Genes coding for glypican pathway, TGFβ receptor and Smad2/3
307 signalling, protein export and proteasome were enriched in D30 PC genes
308 (supplementary Table 6). GEP were done using D30 PCs generated with SCs and
309 IL-6. It could be of interest to investigate further whether adding APRIL together with
310 IL-6 or SC-CM could change the gene expression profiling, making it closer to that of
311 BMPCs.

312 **DISCUSSION**

313 This study shows i) the feasibility to generate human mature PCs *in vitro*, ii) the long-
314 term survival of these PCs does not require a contact with niche cells, but only cell
315 communication factors, in particular IL-6 and APRIL.

316 These PCs are called long-lived PCs because they are non-cycling PCs surviving
317 and producing Igs for months *in vitro* as their counterpart *in vivo*.[1,35,36] In addition,
318 they have a phenotype similar to that of LLPCs *in vivo*: high expression of CD138,
319 increased expression of CD9, weak expression of CD62L, CD45 and HLA-DR
320 compared to PBs and early PCs.[22,37] These *in vitro* generated LLPCs expressed
321 *IRF4 and PRDM1* genes coding for PC transcription factors at a higher level than
322 early PCs. Murine LLPCs also highly express Blimp1 compared to early PCs in the
323 BM.[38] XBP1 is a master regulator of unfold protein response critical to protect PCs
324 from stress induced by high Ig production.[39] *XBP1* mRNA has to be spliced to
325 encode for an active protein and, in agreement, we found that LLPCs had an
326 increased ratio of spliced to unspliced *XBP1* mRNAs compared to early PCs. LLPCs
327 expressed weakly but significantly *BCL6* gene compared to early PCs. This deserves
328 further study since Bcl6 is also inducible in malignant PCs in response to SC-derived
329 factors, conferring a survival advantage on them.[2]

330 A second major finding is that the generation and survival of human LLPCs do not
331 require a contact with niche cells *in vitro*, but can be obtained with 2 recombinant
332 growth factors only, IL-6 and APRIL. APRIL can be replaced by BAFF, which
333 activates the same receptors. In addition, APRIL can be replaced by APRIL/BAFF-
334 unrelated soluble factors produced by SCs. Of note, an inhibitor of both the canonical
335 and alternative NF-κB pathways did not affect the generation of LLPCs supported by
336 SCs, whereas it abrogated the additive effect of APRIL in getting LLPCs with SCs. In

337 addition, a set of genes, whose expression is induced by NF-κB pathway activation in
338 malignant PCs,[33] is poorly expressed in LLPCs generated with SCs *in vitro* compared
339 to BMPCs as previously mentioned by Cocco *et al*.[24] Thus the activation of the NF-
340 κB pathway is not mandatory to generate LLPCs *in vitro*, but could enhance it. The
341 current data are in line with recent findings showing that a combination of APRIL,
342 BAFF, IGF-1, SDF-1 and VEGF can support modestly the *in vitro* 14-day survival of
343 human PCs harvested from the BM, whereas STAT3 activating cytokines, in
344 particular IL-6, are critical.[40]

345 Using more complex culture conditions, including IL-6, IL-21, IFN-α and SC-CM, a
346 recent study has shown human LLPCs can be generated *in vitro* also.[24] That the *in*
347 *vitro* long-term survival of human PCs can be supported by 2 growth factors (IL-6 and
348 APRIL) only is quite surprising regarding the current view of the complexity of the PC
349 niche, comprising SCs and various hematopoietic cells (eosinophils, dendritic cells,
350 megakaryocytes, neutrophils, basophils).[6,14,41] SCs are thought to serve as docking
351 cells bringing close together PCs and hematopoietic cells,[14] but also producing
352 soluble factors promoting PC survival, in particular IL-6 and galectin.[42,43] The ability
353 of hematopoietic cells to sustain PC survival is due mainly by their ability to produce
354 APRIL.[9,11]

355 The fact that soluble growth factors can replace niche cells for the generation and
356 survival of LLPCs *in vitro* suggests it could be the case *in vivo* and questions about
357 the regulation of LLPC count *in vivo*. It is generally assumed that the tiny PC count in
358 the BM is regulated by the rarity of BM niche cells[5], new PCs being in competition
359 with old ones for the availability of niche cells.[44] The current finding suggests the PC
360 niche is mainly a liquid niche comprising a life-sustaining mixture and concentration
361 of chemokines and growth factors, which is likely the case close to the docking SCs

362 attracting both PCs and hematopoietic cells.[14] When entering the BM, if a PC cannot
363 migrate close to a SC, it will not encounter the life-sustaining concentrations of
364 soluble factors and die. But in case of deregulated production of these cell
365 communication signals such as in inflammatory conditions, one can expect many
366 PCs may survive *in vivo*. This may explain the accumulation of LLPCs in the spleen
367 of patients with primary immune thrombocytopenia treated with Rituximab anti-CD20
368 mAb, in association with a 2-fold increase in BAFF concentration in the spleen
369 compared to Rituximab untreated patients.[34] Besides genomic abnormalities, this
370 could also explain the progressive accumulation of premalignant PCs and then
371 malignant PCs in patients with malignant PC disorders who display increased plasma
372 concentrations of IL-6,[45] APRIL or BAFF.[46]

373 The current finding of a mandatory role of IL-6 to promote the survival of human
374 LLPCs *in vitro* questions the role of IL-6 for LLPC maintenance. In mice, whereas IL-
375 6 produced by SCs is mandatory to get the survival of BMPCs *in vitro*,[16,17] the
376 survival of transferred LLPCs is not impaired in *IL-6*$^{-/-}$ mice unlike *APRIL*$^{-/-}$ ones.[14]
377 This is likely due to bias in the murine or human models used. In particular, whereas
378 LLPCs can not be transferred in *APRIL*$^{-/-}$ mice[14], Ag-specific PCs can be generated in
379 *APRIL*$^{-/-}$ mice using repeated Ag boosts indicating additional factors can replace
380 APRIL.[14] In *IL-6*$^{-/-}$ mice, a role of the other cytokines able to trigger gp130 IL-6
381 transducer chain and/or STAT3 activation and to supplement for a deficit in IL-6
382 induced signalling to support PC survival has not been evaluated. This is the case in
383 humans since a recent study has emphasized that inhibition of STAT3 activation by
384 small compounds can fully block the *in vitro* 14-day survival of human PCs harvested
385 from the peripheral blood or BM of healthy individuals. STAT3 activation in these PCs
386 could be driven by either IL-6, IL-10 or IL-21.[40]

387 These *in vitro* models to get human PCs likely introduce some bias, in particular by
388 the method to activate B cells (through BCR, CD40, or TLR), the combination of
389 cytokines used to generate prePBs, PBs, early PCs and then LLPCs, the origin of
390 stromal cells and the culture conditions influencing PC metabolism (nutrients,
391 glucose, O_2 concentration).[10,21,24,26] In the current model, we used a stromal cell line
392 obtained from tonsils because it grow easily until confluence and at confluence, can
393 survive for several months without proliferating but providing a continuous SC
394 support. In initial experiments, similar data were obtained in term of phenotype and
395 long-term survival with BM SCs. But it is of major interest to investigate further
396 whether the use of SCs from different tissue origins could change the phenotype and
397 gene expression profiling of LLPCs, in particular their proximity with BMPCs. For
398 example, a progressive loss of IgA secreting PCs occurred in cultures with tonsil SCs
399 or SC-CM likely due to the lack of a critical survival factor for IgA PC survival *in vitro*.
400 Adding APRIL can revert this loss, increasing 3.4 fold the survival of IgA secreting
401 PCs and this could eventually also occur with BM SCs. The ease of the current
402 model to get PCs *in vitro* will make possible further identification of these possible
403 biases.

404 All prePBs and PBs generated in this model express CD19 whereas Chaidos *et al*.[47]
405 reported recently the existence of $CD19^-CD38^+CD138^-$ plasmablasts (called Pre-
406 PCs) in healthy individuals, together with the known $CD19^+CD38^+CD138^-$ PBs and
407 $CD19^+CD38^+CD138^+$ PCs.[22] The malignant counterpart of these Pre-PCs are found
408 in patients with MM. Running a supervised analysis of gene expression profiling of
409 malignant Pre-PCs and PCs, Chaidos *et al*. found enrichment of genes coding for
410 epigenetic pathways.[47] The *in vitro* model we used likely failed to generate these
411 $CD19^-CD38^+CD138^-$ since all prePBs generated at day 4, PBs at day 7 and PCs at

412 day 10 express CD19.[10,26] In addition, the epigenetic genes differentially expressed
413 between malignant Pre-PCs and PCs could not classify the current *in vitro* generated
414 PBs, early PCs and LLPCs (data not shown).
415 Besides its interest for understanding the fine pathways controlling PC generation
416 and survival in humans, the current model should be promising to study the
417 mechanisms involved in malignant PC disorders and controlling the activity of drugs
418 used to treat patients with these disorders. As several genes whose expression or
419 abnormalities are associated with disease activity have been identified,[48-55] their
420 modulation throughout the different stages of PC generation (prePBs, PBs, early
421 PCs, LLPCs) could help to understand better their function. Of note, it is now feasible
422 to force or repress the expression of a given gene in these PCs using measles
423 envelop pseudotyped lentiviral delivery.[56] The same holds true for drugs used to treat
424 patients with MM, in particular to identify if these drugs could target a specific PB or
425 PC stage and the underlying mechanism.

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435

436 **AUTHOR CONTRIBUTIONS**

437 MJ designed research, performed the experiments and wrote the paper.
438 KT, TF and FG, provided stromal cells and corrected the paper.
439 MC, NR, and KB performed the experiments.
440 CD provided assistance for cytometry experiments.
441 DH provided data of gene expression profiling of BMPCs.
442 BK is the senior investigator who designed research and wrote the paper.

443

444 **CONFLICT OF INTEREST**

445 The authors have no conflict of interest to declare.

446

447 Supplementary information is available at Leukemia's website.

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626 **FIGURE LEGENDS**

627 **Figure 1. Generation and survival of mature CD138^{bright} PCs *in vitro*.**

628 (A) PCs (CD38^{+}CD138^{+} cells) were generated with or without stromal cells (SCs) in
629 the presence of IL-6 (10 ng/ml) and cell count and viability assayed at day (D) 10, 14,
630 24, 30, and 60 using trypan blue dye exclusion. Data are the mean concentration of
631 viable cells ± SD determined in six separate experiments. *The mean value is
632 significantly higher than that in culture without SCs at the same culture day using a
633 paired *t*-test ($P \leq .05$). (B) Expression of CD138. The histograms show FACS
634 labelling with an anti-CD138 (black) or an isotype-matched control mAb (white) of
635 one experiment representative of four. The percentages of CD138 positive cells and
636 CD138 staining index (SI) are indicated in the panels. (C) CD138 fluorescence
637 staining index. Data are the mean CD138 staining index ± SD of four separate
638 experiments. Statistical analysis was done using a paired *t*-test. ns = not significant.

639 **Figure 2. IL-6 in combination with APRIL or BAFF or SC-CM supports the**
640 **generation and survival of PCs *in vitro*.**

641 (A) D10 early PCs were FACS sorted and cultured from D10 to D30 either in contact
642 with SCs (culture with SCs), or with SCs placed in a lower chamber of a transwell
643 culture plate (SCs+PCs in transwells), or with supernatant of PC and SC coculture
644 (PC/SC-CM), or culture supernatant of SCs (SC-CM). Results are the mean of viable
645 cell counts ± SD of 3-paired experiments. ns: the mean count of PCs at a given
646 culture day is not significantly different from that in the SC group at the same culture
647 day using a paired *t*-test (B) FACS-sorted early PCs were cultured from D10 to D30
648 with culture medium and 10% FCS (Co) or in the presence of recombinant cytokines
649 and/or the SC-CM. The concentrations of cytokines used were 10 ng/ml for IL-6 and
650 IGF-1 and 200 ng/ml for APRIL, BAFF, and SDF-1. Counts of metabolic active cells

651 were assayed quantifying intracellular ATP amount with a Cell Titer Glo Luminescent
652 Assay. Results are the mean ± SD of the luminescent signals expressed as the
653 percentage of that in the group cultured with the SC-CM, determined in 3- to 7-paired
654 experiments. *The mean value is significantly different from that in the SC-CM group
655 using a paired *t*-test. **The mean value is significantly different from that in the IL-6
656 group using a paired *t*-test ($P \leq .05$). (C) Effect of various inhibitors of cytokines on
657 the generation of plasma cells induced by the SC-CM. The anti-IL-6 mAb was used
658 at a concentration of 10 µg/ml, the TACI-Fc at 10 µg/ml, the AMD3100 (SDF-1
659 inhibitor) at 10 µM and the IGF-1R inhibitor (NVP-AEW541) at 1 µM. Metabolic active
660 cells were assayed quantifying intracellular ATP amount with a Cell Titer Glo
661 Luminescent Assay. Results are the mean ± SD of the luminescent signals
662 expressed as the percentage of that in the group cultured with the SC-CM,
663 determined in 3- to 10-paired experiments. *The mean value is significantly different
664 from that in the SC-CM group using a paired *t*-test.

665 **Figure 3. A NF-κB inhibitor does not affect the generation of PCs by SCs but**
666 **inhibited the additive effect of APRIL.**

667 PCs were generated with SCs only or with SCs and 200 ng/ml recombinant APRIL.
668 The IKK16 NF-κB inhibitor (1 or 3 µM) was added for 4 days in 2 culture groups and
669 PCs counted at the end of the culture. For each experiment the PC count was
670 expressed as the percentage of the PC count obtained with the control group of the
671 same experiment (SCs or SCs + APRIL). Results are the mean percentages ± SD of
672 five separate experiments. *The mean percentage is significantly decreased
673 compared to that in the SCs + APRIL group using a paired *t*-test.

674 **Figure 4. D30 PCs have a more mature phenotype than D10 early PCs.**

675 PC phenotype was assessed by flow cytometry. Results are the mean percentage ±
676 SD of positive cells and the mean staining index ± SD determined in 4 to 6 separate
677 experiments. *The mean value is significantly different from that in D10 PCs using a
678 paired *t*-test ($P \leq .05$).

679 **Figure 5. D30 PCs do not cell cycle.**

680 The cell cycle was assessed using DAPI staining and quantification of cells in the S
681 phase using bromodeoxyuridine (BrdU) incorporation and labelling with an anti-BrdU
682 antibody. (A) Dot plots show a representative experiment out of three. The
683 percentages of cells in the G0/G1, S, and G2/M phases are indicated. (B) Results are
684 the mean percentage ± SD of cells in the S phase of three separate experiments.

685 **Figure 6. D30 and D60 PCs are functional PCs that produce Igs continuously.**

686 FACS-sorted D10 early PCs were cultured with SCs (A-F) or growth factors as
687 indicated (G). (A-C) Cytoplasmic (cy) Igs (IgG, IgA, and IgM) (A), cy-light chains (B),
688 and surface (s) Igs (C) were assessed by flow cytometry. Results are the mean
689 percentage ± SD of positive cells from 5 separate experiments. (D) IgG, IgA, and IgM
690 production was assessed by ELISA. Results are the mean ± SD of Ig production in
691 pg per cell and per day determined in 3 separate experiments. (E) The number of
692 IgG-, IgA-, and IgM-secreting cells was assessed by ELISPOT. Results are the mean
693 Ig-secreting cell number ± SD from 4 separate experiments. (F) ELISPOTs from a
694 representative experiment are shown. (G) FACS sorted D10 early PCs were cultured
695 with SCs or with a combination of cytokines and/or SC-CM as indicated. The number
696 of Ig-secreting cells was determined by ELISPOT at D30 of culture. Results are the
697 mean Ig-secreting cell number ± SD from 3 separate experiments. *The mean value
698 is significantly different from that in D10 PCs using a paired *t*-test.

699 **Figure 7. Gene expression of transcription factors involved in PC**
700 **differentiation.**
701 Naive B cells (BCs), D10 early PCs, and D30 PCs were FACS sorted. Gene
702 expression of *IRF4, PRDM1, XBP1u, XBP1s, PAX5,* and *BCL6* assayed by real-time
703 RT-PCR. The mRNA level in the different cell populations was compared assigning
704 the arbitrary value 1 to gene expression in BCs. Data are the mean value ± SD of
705 gene expression determined in 5 separate experiments. The ratio *XBP1s/XBP1u* in
706 D10 early PCs and D30 PCs is shown. *The mean value is significantly different from
707 that in D10 PCs using a paired *t*-test ($P \leq .05$).
708 **Figure 8. Unsupervised clustering of gene expression profile of purified D4**
709 **prePBs, D7 PBs, D10 early PCs, D30 PCs, and BMPCs.**
710 D4 prePBs, D7 PBs, D10 early PCs, D30 PCs and BMPCs were profiled using
711 Affymetrix U133 plus 2.0 microarray and an unsupervised hierarchical clustering was
712 run with the 5000 probe sets with the highest variance (log transform, center genes
713 and arrays, uncentered correlation and average linkage). The 5 populations are
714 classified into 2 major clusters, a PC cluster comprising D10 early PCs, D30 PCs and
715 BMPCs (r = 0.11) and a plasmablast cluster comprising prePBs and PBs (r = 0.20).
716 The horizontal lines represent the normalized and centered expression of each of the
717 5000 genes in the samples and are depicted according to the color scale shown at
718 the bottom (-1.5 to 1.5 on a log base 2 scale).

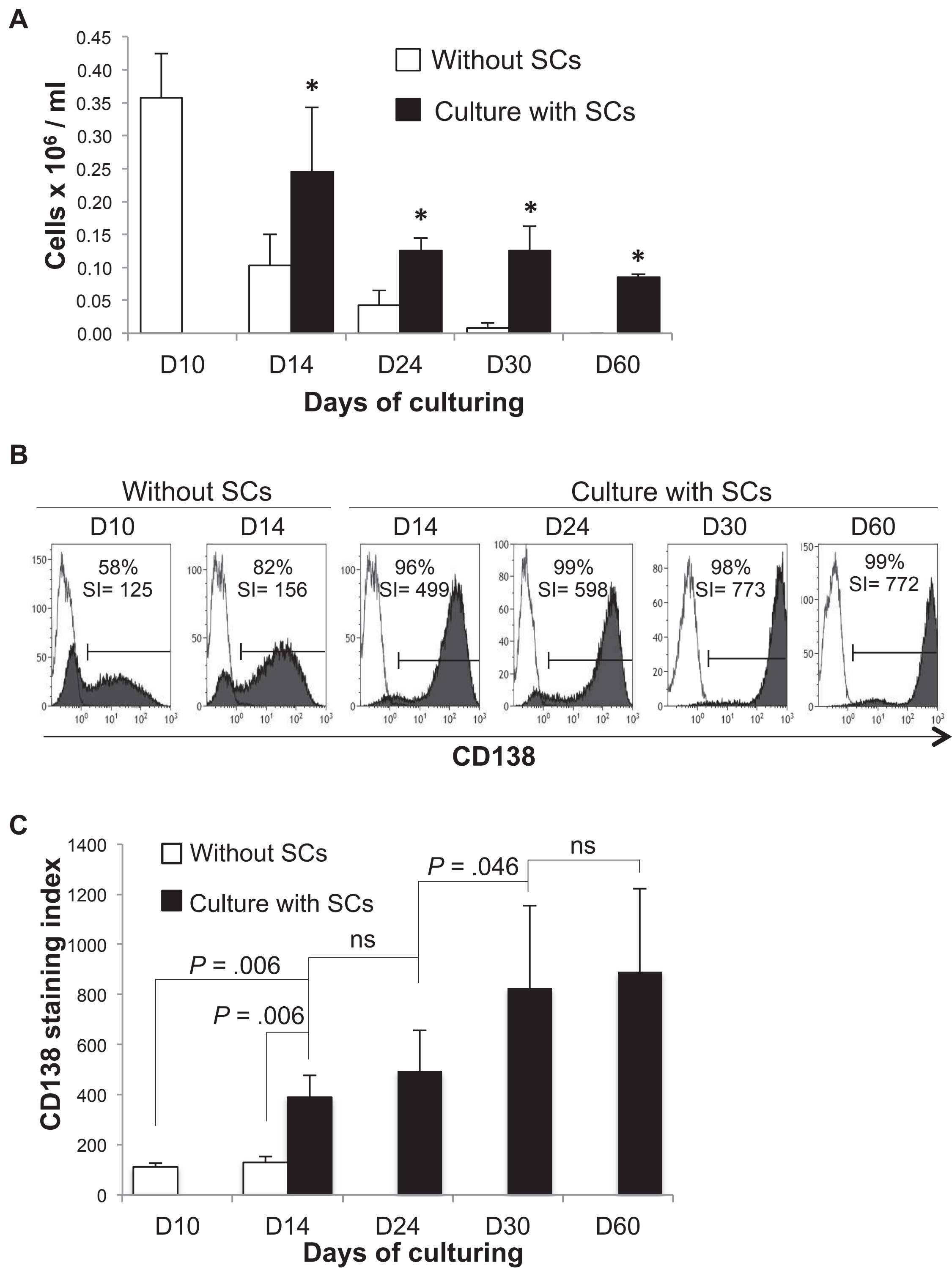

Figure 1

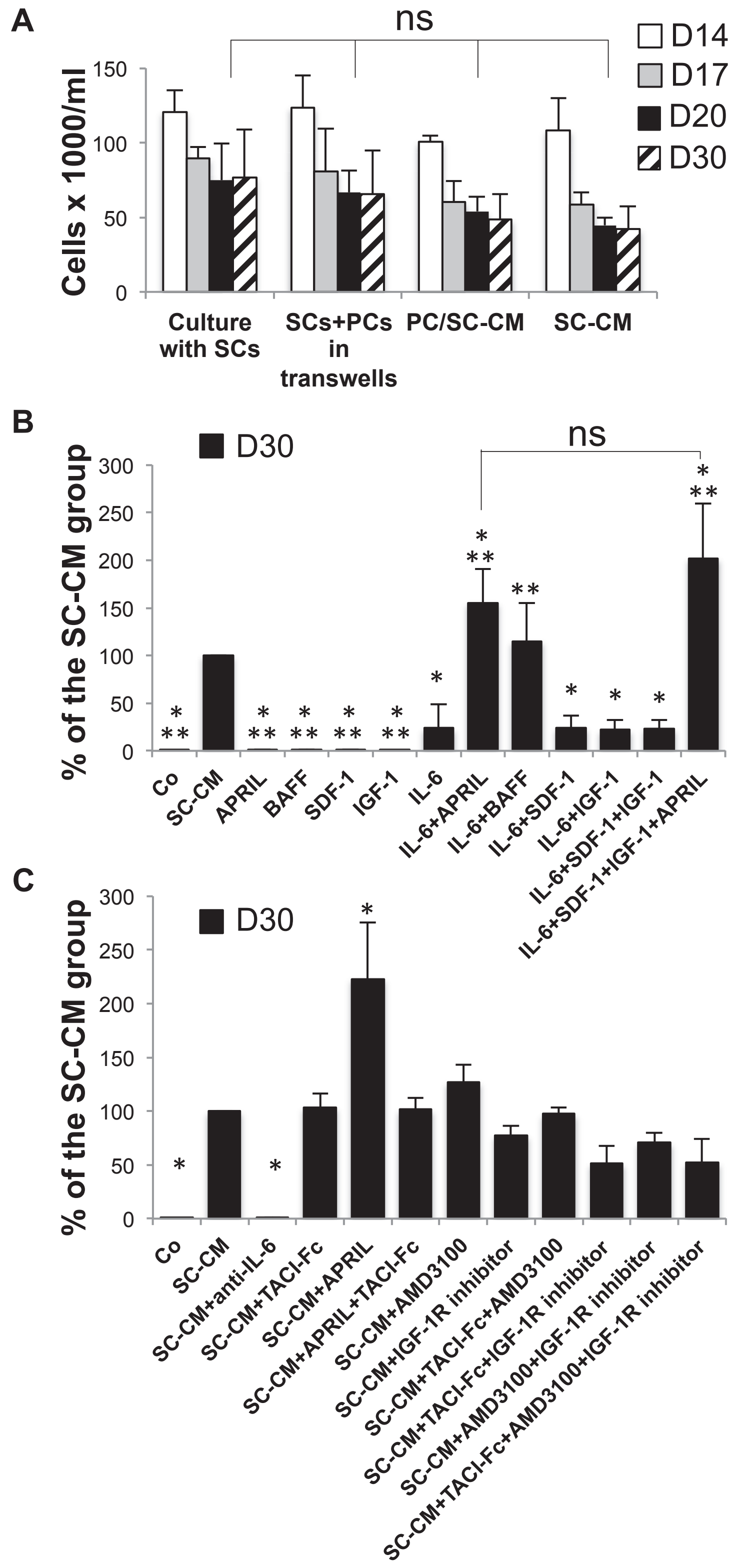

Figure 2

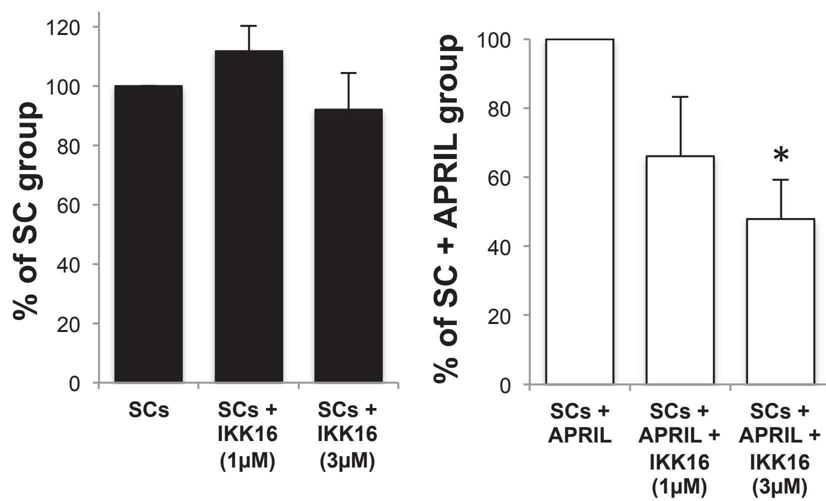

Figure 3

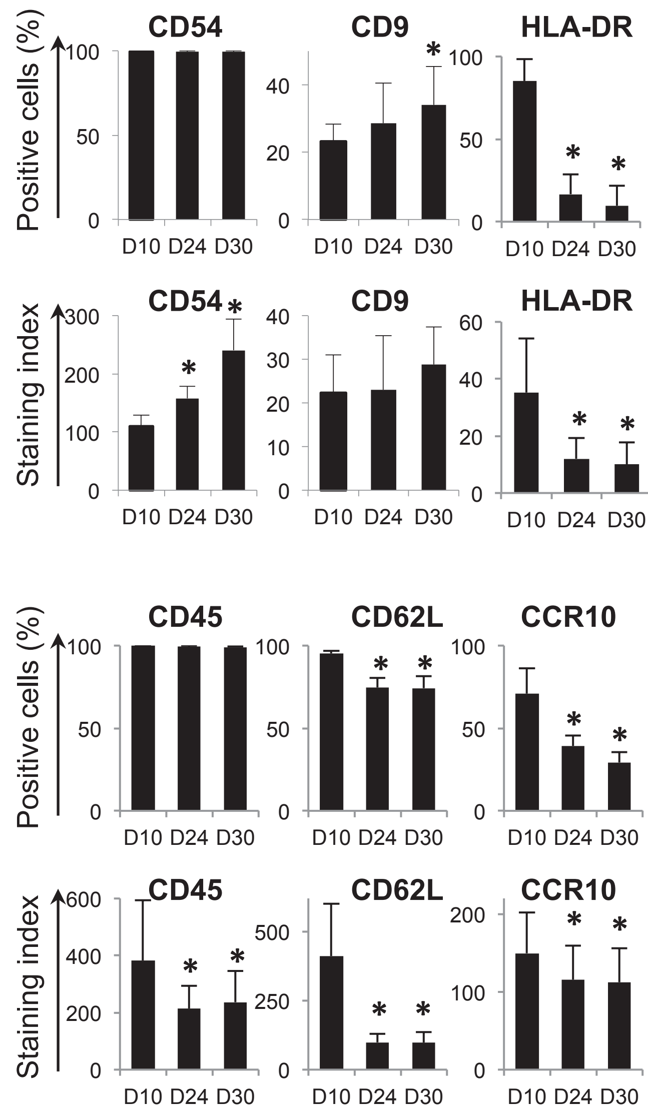

Figure 4

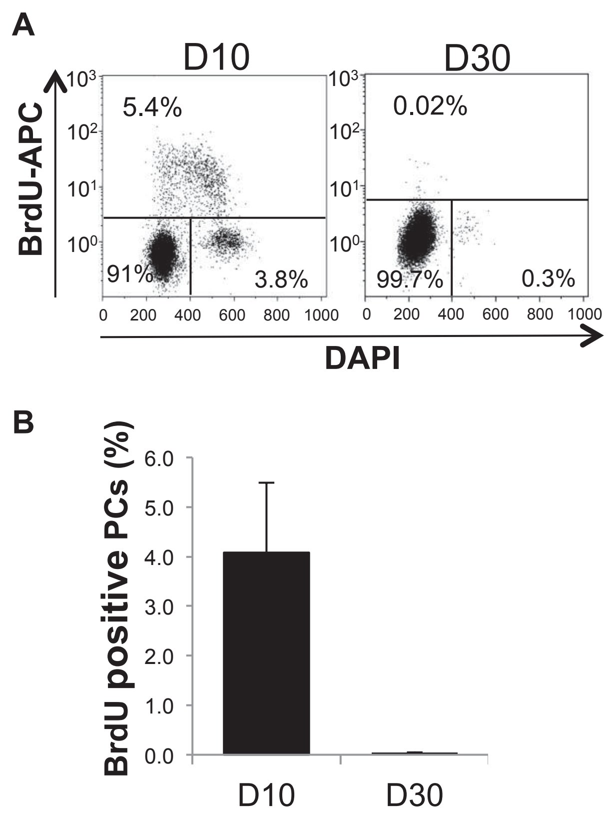

Figure 5

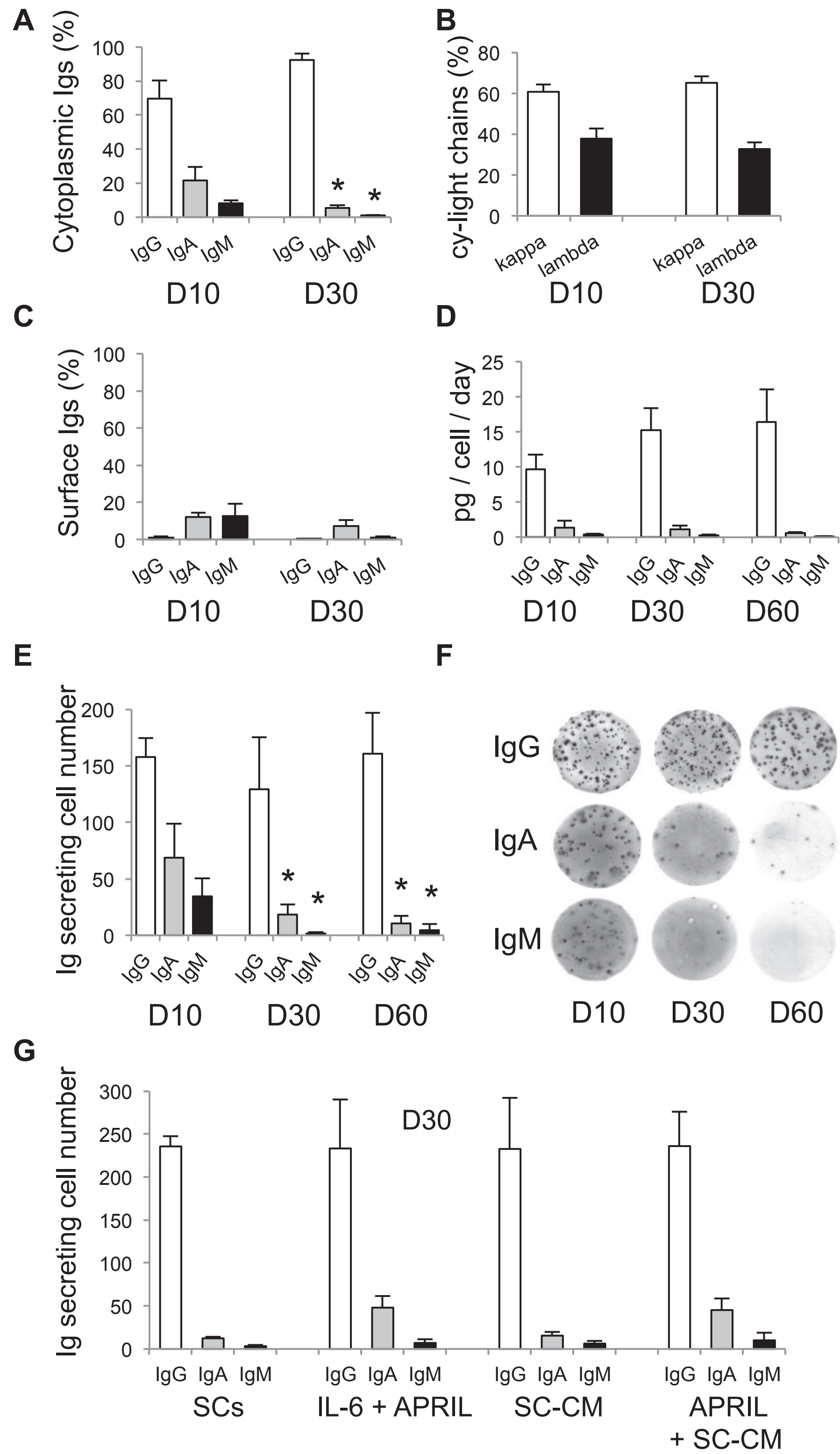

Figure 6

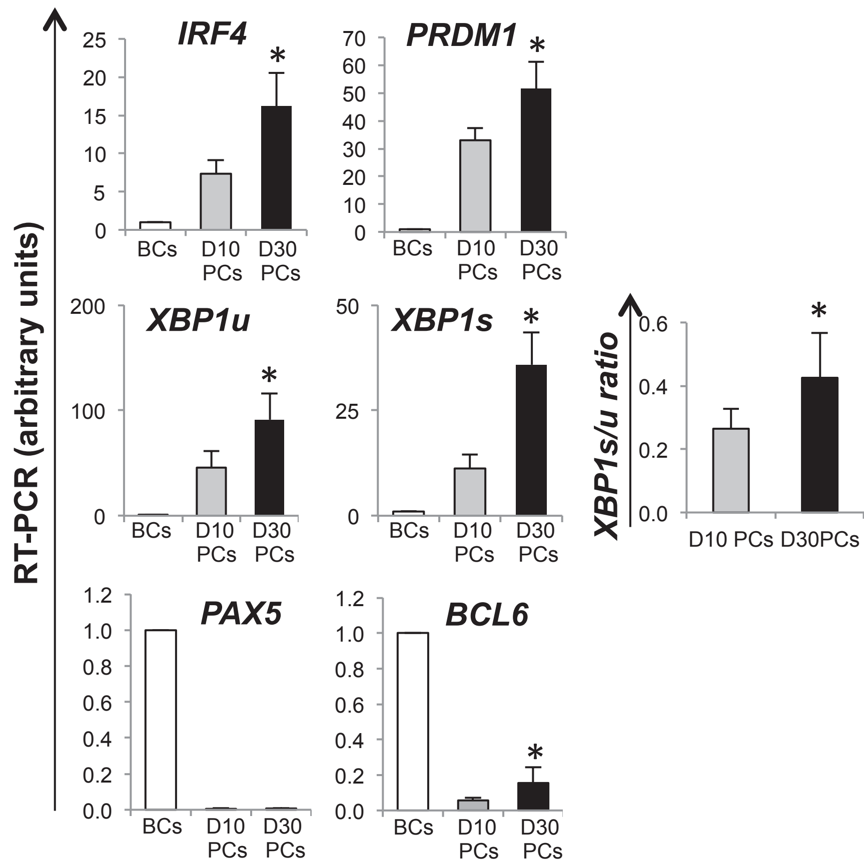

Figure 7

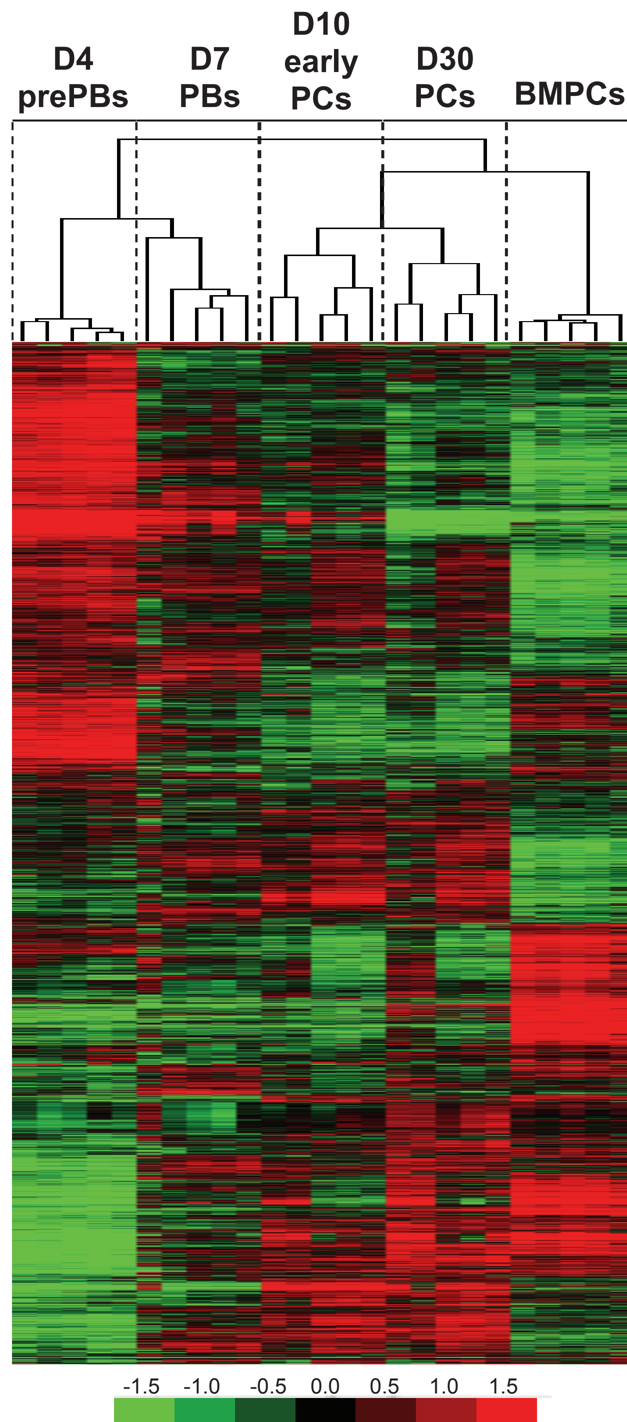

Figure 8