

# AN IN VITRO MODEL OF DIFFERENTIATION OF MEMORY B CELLS INTO PLASMABLASTS AND PLASMA CELLS INCLUDING DETAILED PHENOTYPIC AND MOLECULAR CHARACTERIZATION.

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## Abstract.

Human plasma cells (PCs) and their precursors play an essential role in humoral immune response, but are rare and difficult to harvest. We report here i) the generation of human syndecan-1<sup>+</sup> and immunoglobulin secreting PCs starting from memory B cells (MBCs) in a 3-step- and 10-day (D) culture, including a 6-fold cell amplification. ii) We report the detailed phenotypic and Affymetrix gene expression profiles of these *in vitro* PCs as well as of intermediate cells - activated B cells (actBCs) and plasmablasts (PBs) - compared to MBCs and bone marrow PCs, which is accessible through an open web ATLAS (<http://amazonia.transcriptome.eu/>). iii) We show this B cell to PC differentiation to involve *IRF4* and *AICDA* expressions in D4 actBCs, decrease of *PAX5* and *BCL6* expressions and increase in *PRDM1* and *XBP1* expressions in D7 PBs and D10 PCs. It involves downregulation of genes controlled by Pax5, induction of genes controlled by Blimp-1 and XBP1 (unfold protein response). iv) The detailed phenotype of D10 PCs resembles that of peripheral blood PCs detected after immunization of healthy donors. This *in vitro* model will facilitate further studies in PC biology. It will likewise be helpful to study plasma-cell dyscrasias, including multiple myeloma.

## Introduction

Human plasma cells (PCs) and their precursors play an essential role in humoral immune response, but likewise give rise to a variety of malignant B-cell disorders, including multiple myeloma. The final steps of B cell differentiation have been extensively studied during the last 10 years.<sup>1-3</sup> Naïve B cells entering into lymph node through high endothelial venules are selected by the Ag in the germinal center reaction, yielding selection of B cells with high affinity immunoglobulins (Igs) and differentiation into memory B cells (MBCs, CD20<sup>+</sup>CD19<sup>+</sup>CD27<sup>+</sup>CD38<sup>-</sup>) and early plasmablasts (PBs, CD20<sup>-</sup>CD19<sup>+</sup>CD27<sup>++</sup>CD38<sup>++</sup>). PBs exit into peripheral blood and may survive for a short period only unless they are recruited into mucosa or bone marrow niches, depending on their chemokine receptor expression.<sup>4-6</sup> These niches provide these PBs the factors to survive and further differentiate into long-living mature PCs.<sup>7</sup> CCR10 expressing IgA<sup>+</sup> PBs are mainly recruited to mucosa niche by the CCL28 chemokine.<sup>8</sup> In the bone marrow, the PC niche involves SDF-1 producing cells recruiting CXCR4<sup>+</sup> PBs and is shared by hematopoietic stem cells and Pre-pro-B cells.<sup>9</sup> The rarity of this niche explains the low amount of bone marrow PCs (0.5% of BM cells) and is a matter of regulation of normal Ig production.<sup>10</sup>

The differentiation of B cells into PCs involves profound molecular changes yielding a cell able to produce large amounts of Igs for a long-term period. Two sets of transcription factors (TFs) that repress each other are involved in this process (see Cobaleda *et al.*<sup>11</sup> and Calame<sup>12</sup> for review). The guardian of B cell phenotype is the PAX5 TF, which induces B cell genes and represses genes as *PRDM1* and *XBP1*, whose gene products – Blimp-1 and XBP1 - are critical for PC generation and survival. The BCL6 TF in association with MTA3 maintains B cell phenotype and proliferation, downregulating *PRDM1* expression. In germinal centre, activation of B cells through BCR, CD40 and/or Toll like receptor (TLR) results in upregulation of

IRF4, downregulation of BCL6 protein and loss of *PRDM1* gene repression. This results in downregulation of *PAX5* gene and then upregulation of *XBP1*. In the centrocyte region, stimulation by IL-10, IL-21 or IL-6 results in STAT3 activation, yielding to *PRDM1* overexpression.<sup>13,14</sup> This results in the full engagement of B cell differentiation into PBs, in particular with the switch from surface to cytoplasmic Igs, and induction of the unfold protein response driven by XBP1. The detailed hierarchy of this molecular regulation is not fully understood and is still a challenging issue. Recent data suggest that a PAX5 downregulation and consecutive XBP1 upregulation are the initial driving events in PC generation independently of Blimp-1 expression.<sup>15</sup> Other data indicate a major role of IRF4 whose expression is triggered by NF-κB signaling.<sup>16</sup> In humans, research in PC differentiation mechanisms is hampered by the rarity and lack of availability of PCs, *i.e.* due to the necessity of bone marrow aspiration.

In current *in vitro* models of B cell differentiation,<sup>17-21</sup> mainly CD20<sup>-</sup> CD38<sup>++</sup>CD138<sup>+/-</sup> PBs have been obtained. In a recent work, Huggins *et al.*<sup>22</sup> have reported the possibility to obtain syndecan-1<sup>+</sup> PCs through a 3-step culture but a detailed phenotypic and molecular characterization of these *in vitro* generated cells are not available. In the current study, we first aim to design an easy culture process making it possible to reproducibly obtain syndecan-1<sup>+</sup> PCs. The second aim was to extensively characterize these in-vitro generated PBs and PCs using Affymetrix gene expression profiling and multicolor cytometry and to make accessible an open web atlas of the respective gene expression data.

## **Materials and Methods.**

### **Reagents**

Human recombinant interleukin (IL)-2, IL-12 and interferon- $\alpha$  (IFN- $\alpha$ ) were purchased from R&D Systems, Lille, France), IL-4, IL-6 and IL-15 from AbCys SA (Paris, France) and IL-10 and hepatocyte growth factor (HGF) from Peprotech (Rocky Hill, NJ, USA). Hyaluronic acid (HA) was purchased from Sigma (St Louis, MO, USA). The list of mAbs used for phenotype study are detailed in supplemental Methods.

### **Cell samples**

Peripheral blood cells from healthy volunteers were purchased from the French Blood Center (Toulouse, France). After removal of CD2<sup>+</sup> cells using anti-CD2 magnetic beads (Invitrogen, Cergy Pontoise, France), CD19<sup>+</sup> CD27<sup>+</sup> MBCs were sorted by FACS Aria with a 95% purity. Bone marrow PCs (BMPCs) from healthy volunteers were purified (cell purity  $\geq$  80% assayed by cytometry) using anti-CD138 magnetic microbeads sorting (Miltenyi-Biotec, Paris, France), after approval by the ethics committee and written informed consent as described.<sup>23</sup> Cells produced in the culture system were purified by multi-color fluorescence activated cell sorting (FACS) using FITC-conjugated anti-CD20 mAb and PE-conjugated anti-CD38 mAb for Day 4 activated B cells (CD20<sup>+</sup>CD38<sup>-</sup> cells), day 4 and day 7 plasmablasts (CD20<sup>-</sup>CD38<sup>+</sup>). Day 10 PCs (CD20<sup>-</sup>CD138<sup>+</sup>) were FACS-sorted using FITC-conjugated anti-CD20 mAb and PE-conjugated anti-CD138 mAb. The purity of FACS-sorted cell populations was  $\geq$  95% as assayed by cytometry.

### **Cell cultures**

#### **B-cell activation**

All cultures were performed in Iscove's modified Dulbecco medium (IMDM, Invitrogen) and 10% fetal calf serum (FCS), supplemented with 50 µg/ml human transferrin and 5 µg/ml human insulin (Sigma). Purified B cells were plated at  $1.5 \times 10^5$ /ml and cultured with various combinations of cytokines as indicated: IL-2 (20 U/ml), IL-4 (50 ng/ml), IL-10 (50 ng/ml) and IL-12 (2 ng/ml) or IL-2 (20 U/ml), IL-10 (50 ng/ml) and IL-15 (10 ng/ml) or IL-2 (20 U/ml) and IL-4 (50 ng/ml). Cells were cultured in 5 ml/well in 6 well flat-bottomed culture plates. In respective cultures groups, 10 µg/ml of phosphorothioate CpG oligodeoxynucleotide 2006<sup>24</sup> (Sigma) and/or histidine tagged soluble recombinant human CD40L (50 ng/ml) and anti-poly-histidine mAb (5 µg/ml) (R&D Systems) were added at culture start. In respective experiments, soluble CD40L was replaced by  $3.75 \times 10^4$ /ml mitomycin-treated CD40L transfectant (a generous gift from S. Saeland, Schering-Plough, France).

#### Plasmablast generation.

At day 4 of culture, the cells were harvested, washed and seeded at  $2.5 \times 10^5$ /ml with various combinations of cytokines: IL-2 (20 U/ml), IL-6 (50 ng/ml), IL-10 (50 ng/ml), and IL-12 (2 ng/ml) or IL-2 (20 U/ml), IL-6 (50 ng/ml), IL-10 (50 ng/ml) and IL-15 (10 ng/ml).

#### Plasma cell generation

At day 7 of culture, cells were washed and cultured with IL-6 (50 ng/ml), IL-15 (10 ng/ml) and IFN- $\alpha$  (500 U/ml) for 3 days. In some cultures, HGF (20 ng/ml) and/or HA (100 µg/ml) were also added.

#### **Flow cytometry analysis, cytology and immunoglobulin production**

Cells were stained with FITC-anti-CD20, PE-anti-CD138 (Beckman Coulter) or PE-anti-CD38 (Becton Dickinson) mAbs. Isotype-matched mouse mAbs were used as control. Cytospin smears of purified CD20<sup>+</sup>CD38<sup>-</sup> cells harvested at day 4 of culture, CD20<sup>-</sup>CD38<sup>bright</sup> cells at day 7, and CD20<sup>-</sup>CD138<sup>+</sup> cells at day 10 were stained with

May-Grünwald-Giemsa. The percentage of cells in the S-phase of the cell cycle was determined using propidium iodide and data were analyzed with the ModFit LT software (Verity Software House, Topsham, ME).<sup>25</sup> Immunoglobulin (Ig) production was measured in culture supernatants harvested at the end of each culture step: day 4, day 7 and day 10. IgM, IgA and IgG levels were evaluated by nephelometry with an automated Behring Nephelometer analyser II (Siemens, Paris, France). The sensitivity of the assay was 2 µg/ml for IgM, 3 µg/ml for IgA and 4 µg/ml for IgG. Ig production (pg/cell/day) was estimated dividing Ig amount in the culture supernatant by the number of living cells and the duration of the culture period.

### **Immunophenotypic analysis**

Cells were stained using 4- to 7-colour direct immunofluorescence stain. Surface staining was performed prior to cell fixation and permeabilization. The Cytotfix/Cytoperm kit (BD Biosciences) was used for intracellular staining of IgM, IgA, IgG and Ki67 antigen, according to manufacturer's recommendations. Flow cytometry analysis was performed with a FACSAria cytometer using FACSDiva 6.1 (Becton Dickinson, San Jose, CA). For data analysis, Cell Quest (Becton Dickinson) and Infinicit 1.3 (Cytognos SL, Salamanca, Spain) softwares were used. The fluorescence intensity of the cell populations was compared using the stain index (SI) provided by the formula: [mean fluorescence intensity (MFI) obtained from the given mAb minus MFI obtained with a control mAb]/[2 times the standard deviation of the MFI obtained with the same control mAb].<sup>26</sup>

### **Real-time RT-PCR analysis**

Total RNA was extracted using the RNeasy Kit (Qiagen, Valencia, CA) and reverse transcribed with the Reverse Transcription Kit (Qiagen). The assays-on-demand primers and probes and the TaqMan Universal Master Mix were used according to the manufacturer's instructions (Applied Biosystems). Real-time RT-PCR was

performed using the ABI Prism 7000 Sequence Detection System and normalized to *β2-microglobulin* for each sample, and compared with the values obtained for a known positive control using the following formula  $100/2^{\Delta\Delta Ct}$  where  $\Delta\Delta Ct = \Delta Ct$  unknown  $-\Delta Ct$  positive control as described.<sup>27</sup>

### **Microarray hybridization and bioinformatic analysis**

RNA was extracted and hybridized to human genome U133 Plus 2.0 GeneChip microarrays, according to the manufacturer's instructions (Affymetrix, Santa Clara, CA). Gene expression data are deposited in the ArrayExpress public database (<http://www.ebi.ac.uk/microarray-as/ae/>, accession number n° E-MEXP-2360). Gene expression data were analyzed with our bioinformatics platforms (RAGE, <http://rage.montp.inserm.fr/>)<sup>28</sup> and Amazonia (<http://amazonia.transcriptome.eu/>).<sup>29</sup> The clustering was performed and visualized with the Cluster and TreeView softwares.<sup>30</sup> Gene differentially expressed between cell populations were determined with the SAM statistical microarray analysis software.<sup>31</sup> The biological pathways encode by these genes were analyzed with Ingenuity software (Ingenuity, CA, USA)

### **Statistical analysis**

Statistical comparisons were made with the non-parametric Mann-Whitney test, unpaired or paired Student's *t*-test using SPSS software. *P*-values  $\leq .05$  were considered as significant.

## Results

### Obtaining of plasma cells through a 3-step culture process *in vitro*

#### Step 1. Optimization of B cell amplification and differentiation

Starting from purified CD19<sup>+</sup>CD27<sup>+</sup> MBCs, we first investigated which combination of activation signals allowed obtaining a maximum number of viable activated B cells. The best result, *i.e.* a 6.1-fold amplification, was achieved using activations by soluble recombinant CD40L (sCD40L) and CpG oligodeoxynucleotide 2006 (ODN) and the IL-2+IL-10+IL-15 cytokine combination (Table 1). Comparable data were obtained using either a CD40L transfectant or sCD40L to trigger CD40 activation (results not shown). Activation by either sCD40L or ODN only plus in each case the same additional cytokine combination yielded a 46% or 68% lower amplification (3.3- and 2-fold stimulation respectively,  $P \leq .008$ ) (supplemental Table 1), indicating an additive effect if sCD40L and ODN are simultaneously used. Other cytokine combinations were reported to trigger B cell activation together with CD40 activation. Using sCD40L activation and IL-2+IL-4 alone resulted in no cell amplification (supplemental Table 1). Adding IL-2+IL-4+IL-10+IL-12 as we reported initially<sup>20</sup> resulted in 3.4-fold amplification as with IL-2+IL-10+IL-15. In all culture conditions, except with sCD40L+IL-2+IL-4, cells were  $\geq 87\%$  viable. Using the optimized activation combination (sCD40L+ODN+IL-2+IL-10+IL-15), the expanded cells at day 4 comprised 42.3% of CD20<sup>+</sup>CD38<sup>-</sup> cells, 16.4% of CD20<sup>+</sup>CD38<sup>+</sup> cells and 19.5% of CD20<sup>-</sup>CD38<sup>++</sup> cells (Table 1). CD20<sup>+</sup>CD38<sup>-</sup> cells have an activated B cell cytology and were cycling (38%  $\pm$  3% in the S phase) unlike MBCs (0.5%  $\pm$  0.3% in the S phase) (Figure 1). CD20<sup>-</sup>CD38<sup>++</sup> cells showed a typical plasmablast morphology, with an eccentric nucleus, relatively abundant basophilic cytoplasm with archoplasm (Figure 1). They were also highly cell cycling (50%  $\pm$  5% in the S

phase). CD20<sup>+</sup>CD38<sup>-</sup> cells were termed Day 4 activated B cells (D4 actBCs) and CD20<sup>-</sup>CD38<sup>++</sup> cells plasmablasts (PBs).

### Step 2. Cell amplification and plasmablastic differentiation

Step 2 aims to promote further PC differentiation. Soluble CD40L was removed since it partially blocks plasma cell differentiation.<sup>20</sup> We also found that presence of ODN blocked PC differentiation (results not shown). IL-6 was added together with IL-2+IL-10+IL-15 since it promotes PC differentiation and survival,<sup>18</sup> in particular through STAT3 activation and Blimp-1 induction.<sup>14</sup> After 3 days of culture, a 3.7-fold cell expansion with  $\geq 80\%$  viable cells could be found if cells were cultured in step 1 with sCD40L and ODN (Table 1). The expansion in step 2 was 57% and 32% lower ( $P \leq .05$ ) respectively, if cells were cultured with ODN or sCD40L only in step 1 (supplemental Table 2). At day 3 of step 2 culture (day 7 of the whole culture), the percentage of CD20<sup>+</sup>CD38<sup>-</sup> cells decreased from 42.3% at day 4 to 11.7% ( $P = 2.10^{-8}$ ,  $n = 13$ ) with an increase in the percentage of CD20<sup>-</sup>CD38<sup>++</sup> cells (56.6%,  $P = 8.10^{-12}$ ,  $n = 13$ ). In addition, 15.9% of CD20<sup>-</sup>CD38<sup>++</sup>CD138<sup>+</sup> cells were detected. Day 7 CD20<sup>-</sup>CD38<sup>++</sup> were sorted and show the same plasmablastic morphology as day 4 CD20<sup>-</sup>CD38<sup>++</sup> cells and were termed day 7 plasmablasts (D7 PBs) (Figure 1). They had a reduced number of cycling cells compared to day 4 plasmablasts (13%  $\pm$  4% versus 50%  $\pm$  5% in the S phase, paired t-test,  $n=3$ , Figure 1) and an increased CD38 density (anti-CD38 stain index (SI), 125 versus 20,  $P = 5.10^{-4}$ ,  $n = 5$ ). Thus, starting from one memory B cells, 12.3  $\pm$  6.1 CD20<sup>-</sup>CD38<sup>++</sup> D7 PBs could be generated using the optimal step 1 and 2 culture conditions (Table 1). This step-2 culture could not be extended longer than 3 days as a rapid plasmablast death occurred on days 4-5, despite addition of fresh cytokines. Prolonging the first step 1 culture for additional 4 days with fresh sCD40L+ODN+IL-2+IL-10+IL-15 yielded to a

further B cell amplification, but to a rapid cell death in step 2 and a lower number of overall generated PCs (results not shown).

### Step 3. Plasma cell differentiation

To avoid the rapid cell death occurring after 3 days in step 2, cells were washed and cultured with IL-6+IL-15+IFN- $\alpha$  for 3 days. 60% of the cells died at this stage. Adding hepatocyte growth factor and/or hyaluronic acid as suggested<sup>22</sup> did not improve cell survival (results not shown). Differentiation within this last step 3 was independent on the initial step 1 conditions (supplemental Table 3). Surviving cells comprised mostly CD20<sup>-</sup>CD38<sup>++</sup> cells (79%), including 54.8% CD20<sup>-</sup>CD38<sup>++</sup>CD138<sup>+</sup> (Table 1). FACS-sorted CD138<sup>+</sup> cells had PC cytology and were termed day 10 PCs (D10 PCs). These cells were rarely cell cycling with 2% of cells in the S phase, compared to D7 or D4 PBs (13% and 50% respectively). Thus, this 3-step culture process made it possible to generate 12.3 CD20<sup>-</sup>CD38<sup>++</sup> D7 PBs (at step 2) and 6.3 CD20<sup>-</sup>CD38<sup>++</sup>CD138<sup>+</sup> D10 PCs (at step 3) starting from one memory B cells (Table 1). The density of CD38 expression was increased in D10 PCs compared to D7 PCs (SI 276 versus 125,  $P = .006$ ,  $n = 5$ ).

### **Expression of surface, cytoplasmic IgM, IgG, IgA and immunoglobulin production**

Surface (s) Igs were detected by labeling cells with anti-Ig heavy chain antibodies (IgM, IgA, and IgG) without permeabilization and cytoplasmic (cy) and surface Igs after cell permeabilization. MBCs used to start culture comprised 43% $\pm$ 12% sIgM<sup>+</sup>, 27% $\pm$ 6% sIgA<sup>+</sup> and 26% $\pm$ 5% sIgG<sup>+</sup> cells ( $n = 5$ , Figure 2). Permeabilization of MBCs yielded similar percentages of cyIgM<sup>+</sup>, cyIgA<sup>+</sup> and cyIgG<sup>+</sup> cells with similar mean fluorescence intensities (MFI). CD20<sup>+</sup>CD38<sup>-</sup> D4 actBCs comprised 61 $\pm$ 7% sIgM<sup>+</sup> and 18% $\pm$ 3 sIgA<sup>+</sup> cells (not significantly different from MBCs) and a 3 fold-reduced percentage of sIgG<sup>+</sup> cells (8% versus 26%,  $P \leq .05$ ,  $n = 5$ ) (Figure 2). D4 actBCs

cells were preparing to secrete Igs, as permeabilization resulted in detection of 22%  $\text{cyIgG}^+$  actBCs and a respectively 20 fold and 2.5 fold significantly increased MFI ( $P \leq .05$ ,  $n = 5$ ) for  $\text{cyIgM}$  and  $\text{cyIgA}$  labeling (Figure 2). The differentiation of D4 actBCs into D7 PBs and consecutively day 10 PCs was associated with a loss of  $\text{cyIgM}^+$  cells (from 54% in D4 actBCs to 18% in D7 PBs and 8% in D10 PCs,  $P \leq .05$ ,  $n = 5$ ), an increase in  $\text{cyIgG}^+$  cells (from 22% in D4 actBCs to 56% in D7 PBs and 73% in D10 PCs,  $P \leq .05$ ,  $n = 5$ ), with no significant difference in the percentage for  $\text{cyIgA}^+$  cells. In agreement with detection of cytoplasmic Igs and expression of PC markers by flow cytometry, the rate of IgG production/cell/day increased 8 fold at day 10 compared to day 4 ( $P = .003$ ,  $n = 5$ , Figure 3A). The rates of IgA and IgM production also significantly increased ( $P \leq .005$ ,  $n = 5$ , Figure 3A).

#### **Phenotype of B cells, D7 plasmablasts and D10 plasma cells**

D4 actBCs expressed CD19, C27, CD45, and HLA class II (Figure 4A). D7 PBs and D10 PCs were  $\text{CD19}^+$ ,  $\text{CD45}^+$  and  $\text{HLA-class II}^+$  but with a respectively 2.5-, 3.0- and 5-fold lower expression for D7 PBs and 2.5-, 3.4- and 12-fold for D10 PCs ( $P \leq .05$ ,  $n = 5$ ) compared to D4 actBCs. CD27 expression was increased 2.5- and 3-fold in D7 PBs and D10 PCs respectively, compared to D4 actBCs ( $P \leq .05$ ,  $n = 5$ ). In agreement with S phase data in Figure 1, only D4 actBCs and D7 PBs were  $\text{Ki-67}^+$ . CD43 was expressed in  $66\% \pm 8\%$  of D7 PBs and  $57\% \pm 9\%$  of D10 PCs. Regarding homing molecules, plasma cell differentiation was characterized by a disappearance of CXCR5, a progressive reduction in CXCR4 (respectively 1.8- and 3-fold decrease in D7 PBs and D10 PCs compared to D4 actBCs,  $P \leq .05$ , paired t-test,  $n = 3$ ), induction of CCR10 and increased CD62L/L-selectin (Figure 4B).

#### **B cell and plasma cell transcription factors**

In Figure 3B the gene expression of 5 major transcription factors that control B cell to plasma cell differentiation is shown. A clear-cut difference was the lack of

expression of *PAX5*, the guardian of B cell phenotype<sup>11</sup> in D7 PBs and D10 PCs, unlike D0 MBCs and D4 actBCs. *BCL6* and *PRDM1*, whose gene products mutually repress gene expression of the other,<sup>12</sup> showed a correlated inverse pattern. *BCL6* expression progressively decreased and *PRDM1* progressively increased from D0 MBCs to D10 PCs. *PRDM1*, *XBP1* and high *IFR4* expressions were already found in D4 actBCs, suggesting these cells, with a B cell phenotype (Figure 1 and 3B), were already on the way towards plasmablastic differentiation.

### **Gene expression atlas of B cell to plasma cell differentiation**

Genome wide gene expression profiling of the 4 cell populations identified above and of purified bone marrow PCs (BMPCs) were performed using Affymetrix U133 Plus 2.0 microarrays. First an unsupervised clustering with 2000 probe sets after filtering with a standard deviation  $\geq 520.8$  defined 5 clusters grouping the samples of 5 populations with a strong correlation: D0 MBCs ( $r = 0.74$ ), D4 actBCs ( $r = 0.76$ ), D7 PBs ( $r = 0.41$ ), D10 PCs ( $r = 0.59$ ) and BMPCs ( $r = 0.75$ ) (Figure 5A). D7 PBs and D10 PCs clusters were correlated together ( $r = 0.27$ ,  $P = .05$ ), unlike other clusters (Figure 5A). About one third of these genes delineated a plasma cell cluster (D7 PBs, D10 PCs and BMPCs) versus a B cell cluster (MBCs and D4 actBCs). To extract optimally this plasma cell versus B cell gene signature, a supervised analysis was run comparing D0 MBCs + D4 actBCs and D7 PBs + D10 PCs + BMPCs (Wilcoxon statistic, 1000 permutations, 2 fold ratio) yielding to 676 probe sets on the basis of an 0% FDR. They corresponded to 459 unique genes (202 PC and 257 BC genes) using Ingenuity analysis, separating B cells from PCs (Figure 5B and supplemental Table 4). The resulting networks encoded by these PC and BC genes were analyzed and scored with Ingenuity. The PC and BC networks and detailed data including gene lists associated with networks are shown in supplemental Table

5. The highest scoring PC network mainly comprises genes induced by XBP1 transcription factor (supplemental Figure 1).

The changes in gene expression at different stages of B cell to plasma cell differentiation can be quickly visualized using our Amazonia “B to plasma cell” Atlas (<http://amazonia.transcriptome.eu/>). Regarding genes coding for membrane markers of B cells and PCs, the plasma cell Atlas is in agreement with FACS data of Figure 4A and 4B (supplemental Figure 2A and 2B). The 3 PC populations did not express *CD20* and *CD22* genes and expressed weakly *HLA-class II* genes in agreement with decreased *CIITA* compared to D0 MBCs and D4 actBCs. The 3 PC still expressed *CD19*, although at a lower level and *CD45* gene expression progressively decreased from MBCs to BMPCs. *CD24* expression was lost on D7 PBs and D10 PCs, but expressed in BMPCs again. PC differentiation is evidenced by increased *Ig heavy chain (IgH)* gene expression, increased expression of *CD27*, expression of *CD38* and its ligand *CD31/PECAM1* and of *CD138*. *CD9* gene was highly expressed only on BMPCs and *CD40* was highly expressed in BMPCs. *Fas/CD95* expression was increased in MBCs, D4 actBCs, D7 PBs and D10 PCs compared to BMPCs. *CD23* expression was rapidly lost on D4 actBCs. Of interest, only D4 actBCs highly expressed *AICDA* gene suggesting these cells could be in a process of Ig hypermutations and/or switch. Both D4 actBCs and D7 PCs expressed *MKi67* gene, in agreement with cell cycle cytometry data. Among transcription factors, which have been shown to control the B cell and plasma cell phenotype, the expression of 13 of these could be investigated with Affymetrix U133 Plus 2.0 microarrays. The current knowledge of the mechanisms of action of these transcription factors are shown in Figure 6A. Affymetrix data for the main transcription factor genes – *Pax 5*, *IRF4*, *PRDM1*, *XBP1* – are confirmed by real-time RT-PCR data (Figure 3B and Figure 6B). In agreement with *PAX5* downregulation in D7 PBs, D10 PCs and BMPCs, the

following PAX5-regulated genes<sup>11</sup> were downregulated in these cells: *IRF8*, *SPIB*, *BACH2*, *EBF*, *ID3*, *CIITA*, in association with increased expression of PAX5-inhibited genes<sup>11</sup>, *PRDM1* and *XBP1*. Regarding genes coding for homing molecules (supplemental Figure 2B), PC differentiation was associated with loss of expression of genes coding for lymph node chemokine receptors (*CCR7*, *CXCR5*). *CXCR4* expression was decreased and *CCR10* expression increased in D7 PBs and D10 PCs in agreement with flow cytometry data. *CCR2* gene, which is downregulated by PAX5, was not expressed in MBCs but highly in BMPCs. *CD62L* was highly expressed in D7 PBs and D10 PCs unlike BMPCs. PC differentiation is associated with increased expression of *ITGA4* and *ITGB1*, coding for the VLA4 heterodimer, increased expression of *ICAM2*, coding for a VLA4 ligand. The gene coding for sphingosine phosphate receptor (*EDG1/S1PR1*), which is involved in cell exit from tissues<sup>32</sup>, was decreased in D10 PCs and BMPCs. *ITGAL*, a gene coding for CD11a is increased in D4 actBCs, D7 PBs and D10 PCs. Finally, the gene coding for ERN1, that induces *XBP1* mRNA splicing, and the genes coding for XBP1-driven unfold protein response were upregulated throughout B to PC differentiation (supplementary Figure 3).

## Discussion

Human PCs and their precursors play an essential role in humoral immune response, but likewise give rise to a variety of malignant B-cell neoplasias. They are difficult to obtain, as they are rare cells located in specific niches in the bone marrow and mucosa,<sup>10</sup> hindering the understanding of their physiology and pathophysiology. The aim of the current study was to provide a full phenotypic and molecular characterization of *in vitro* generated PCs and of the intermediate cells. We have first compared the activation signals and cytokine combinations reported in various methodologies for *in vitro* PB and PC generation<sup>17,20,22</sup> in order to get a maximum of PCs, while limiting the number of activation signals and cytokines. Here, a mean number of 6.3 viable PCs could be generated in a 3-step culture system starting from one MBC. PCs show a plasma cell morphology, secrete Igs, express plasma cell markers (CD38, CD31, CD138), and lack B cell markers (CD20, CD21, CD22, CD23). This PC phenotype was associated with expression of plasma cell transcription factor genes (*PRDM1*, *XBP1*) and decreased expression of B cell ones (*PAX5*, *BCL6*).

The current strategy mimics the activation and differentiation process occurring in the germinal center reaction using activation of CD40 (mimicking T cell help) and TLR activation (mimicking Ag activation) with a combination of cytokines produced by T helper cell, dendritic cells and macrophages.<sup>2</sup> These activation signals trigger NF- $\kappa$ B signalling that induces *IRF4* expression, resulting in downregulation of *BCL6*, being critical to maintain the centroblast phenotype.<sup>2</sup> This is what was observed in D4 actBCs, activated by sCD40L and CpG ODN, that express highly *IRF4* and a lower level of *BCL6* compared to MBCs. The *IRF4* expression is associated with high *AICDA* expression in D4 actBCs in agreement with data showing *IRF4* to control *AICDA* gene expression.<sup>33</sup> AID controls the process of Ig variable gene

mutation. AID also controls heavy chain isotype switching, which may explain the progressive loss of IgM<sup>+</sup> cells and appearance of IgG<sup>+</sup> cells in this *in vitro* model. Alternatively, the shift from B cells expressing IgM to PCs expressing mainly IgG could be due to a selective proliferation of IgG<sup>+</sup> starting B cells. IRF4, when expressed at high level, also induces *PRDM1* and *XBP1* expression.<sup>33</sup> The high *IRF4* expression in D4 actBCs may explain why these cells are already in the way towards PC differentiation, expressing weakly *PRDM1* and *XBP1* and cytoplasmic Igs, while still highly proliferating and expressing B cell markers. The second step of culture consists of removing CD40 activation and CpG ODN that block the full process of plasma cell generation and adding IL-6 to further promote STAT3 activation. STAT3 induces *PRDM1* expression<sup>34</sup> together with IRF4<sup>33</sup> and also further downregulates *BCL6* expression. In particular, a knock out of STAT3 abrogates PC differentiation.<sup>35</sup> The final step consists of removing cytokines inducing proliferation (IL-2 and IL-10), and adding IFN- $\alpha$ , IL-6 and IL-15 yielding to PCs that express syndecan-1 and secrete higher amounts of Igs, as measured in the culture supernatants. Both IFN- $\alpha$  and IL-6 highly stimulate STAT3 pathway resulting in the observed increased *PRDM1* expression in D10 PCs (Figure 5). This likely explains syndecan-1 expression and increased Ig secretion in D10 PCs as the *PRDM1* gene product – Blimp-1 - induces *syndecan-1* gene expression and splicing of Ig RNA yielding to Ig secretion in B cells.<sup>36</sup> Huggins et al.<sup>22</sup> have reported that addition of hyaluronic acid, to stimulate CD44, and hepatocyte growth factor further improved differentiation of PBs into PCs, but we found no benefit of adding hyaluronic acid or HGF. Adding IL-21 and/or APRIL did not result also in improvement of PC generation and survival (results not shown) and these *in vitro* generated PCs progressively died in culture. The identification of signals promoting long-term PC survival is a major unresolved issue. PC long-term survival and

differentiation may require cell to cell contacts, mimicking what is occurring in the putative PC niches. Tokoyoda *et al.*<sup>9</sup> reported that murine PCs home in contact to SDF-1 producing cells in the bone marrow, sharing the same niche with hematopoietic stem cells and Pre-pro-B cells. In mucosa, a recent report has shown that tissue PCs are located in APRIL-rich niches, comprised myeloid cells.<sup>37</sup> Thus, the current model will make it possible to further identify the niche that can promote long-term survival of human PCs.

An important question is what the differences are between these *in vitro* D10 PCs compared to the most studied human PCs *in vivo*, *i.e.* tonsil PCs, bone marrow PCs and peripheral blood PCs? Tonsil PCs, either present in germinal centers or follicular and parafollicular zones, express CD20, CD19, HLA-class II, CD45, CD22, CD9 and highly CD38 and do not express CD62L and CD138.<sup>38,39</sup> Peripheral blood PCs detected in healthy individuals after tetanus toxoid immunization are CD20<sup>-</sup>CD19<sup>+</sup>CD45<sup>+</sup>CD62L<sup>+</sup>HLA-class II<sup>+</sup>CD9<sup>-</sup>CD38<sup>high</sup> and half of them express CD138.<sup>38</sup> Bone marrow PCs express CD138, highly CD38 and CD31, lack CD20, express weakly CD19 and half of them express CD45 and HLA-class II.<sup>38</sup> The phenotype of *in vitro* generated D10 PCs is different from that of tonsil PCs that are more immature through the expression of CD20, CD22 and lack of expression of CD138. It could correspond to the phenotype of the fraction of CD45<sup>+</sup> HLA-class II<sup>+</sup> bone marrow PCs since they all express these 2 molecules, but at reduced levels compared to B cells. A difference is that D10 PCs express CD62L, weakly CXCR4 and CCR2 and did not express CD9. Actually the phenotype of these D10 PCs fits well with that of peripheral blood PCs induced by tetanus toxoid immunization of healthy individuals.<sup>38</sup> These circulating PCs do express CD62L, intermediate levels of CD138 and weakly CXCR4 and CCR2, and do not express CD9, unlike BMPCs. They are considered to be newly-generated PCs, leaving the lymphoid organs to

home to bone marrow or tissue, as it is the case for *in vitro* generated D10 PCs. As both these circulating PCs and *in vitro* generated D10 PCs express weakly CXCR4, other molecules could be involved in their homing to bone marrow or mucosa. VLA4 could be this homing molecule, since hematopoietic stem cells can home to BM through VLA4 in a CXCR4 independent manner.<sup>40</sup> Of interest, both peripheral blood PCs and D10 PCs highly express VLA4 making it possible their homing to BM or tissues.

Besides studying B-cell differentiation, this *in vitro* model is likewise of major interest to further understand the biology of multiple myeloma by introducing genes deregulated in MMCs and looking for their ability to induce long term survival and proliferation of (malignant) PCs. It would be also of interest to look for whether the MM bone marrow environment could trigger the survival of these PCs *in vitro*.

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## **Authorship**

MJ designed research, performed the experiments and wrote the paper.

GF provided technical assistance.

JDV designed the Amazonia web site.

AC and ML provided assistance for cytometry experiments.

CB performed the cytology analysis.

CC performed the Ig production determination.

DH provided GEP data for BMPCs and DH and AC participated in the writing of the paper.

BK is the senior investigator who designed research and wrote the paper.

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	B cell amplification and differentiation. D0-D4. Step1	Plasmablastic differentiation. D4-D7. Step 2	Plasma cell differentiation. D7-D10. Step 3
Activation	sCD40L + ODN	-	-
Cytokines	IL-2 +IL-10+IL-15	IL-2+IL-6 +IL-10+IL-15	IL6+IL-15 +IFN- $\alpha$
Mean cell amplification	6.1 $\pm$ 1.8 n=19	3.7 $\pm$ 1.3 n=18	0.51 $\pm$ 0.09 n=17
Cell viability	94%	81%	38%
CD20 <sup>+</sup> CD38 <sup>-</sup> activated B cells (%)	42.3 $\pm$ 12.2 n=13	11.7 $\pm$ 5.1 n=13	2.2 $\pm$ 1.2 n=13
CD20 <sup>+</sup> CD38 <sup>+</sup> intermediate cells (%)	16.4 $\pm$ 12.5 n=13	20.1 $\pm$ 8.8 n=13	13.6 $\pm$ 8.5 n=13
CD20 <sup>-</sup> CD38 <sup>++</sup> plasmablasts/plasma cells (%)	19.5 $\pm$ 5.5 n=13	56.6 $\pm$ 7.7 n=13	79.0 $\pm$ 8.8 n =13
CD20 <sup>-</sup> CD38 <sup>+</sup> CD138 <sup>+</sup> plasma cells (%)	2.1 $\pm$ 0,9 n=13	15.9 $\pm$ 6;2 n=13	54.8 $\pm$ 8.7 n=13
Yield of plasmablast/plasma cell generation for one starting memory B cells		Plasmablasts /plasma cells: 12.3 $\pm$ 6.1 n=13	Plasma cells:  6.3 $\pm$ 3.3 n=13

**Table 1: Generation of plasma cells from memory B cells.**

Purified memory B cells were cultured for 10 days using a 3 step culture system. In step 1, B cells were activated for 4 days with sCD40L and ODN and IL-2 + IL-10 + IL-15. In step 2, plasmablast differentiation was further promoted removing sCD40L and ODN and adding IL-6 together with IL-2 + IL-10 + IL-15. In step 3, plasma cell differentiation was induced for 3 days, removing IL-2 and IL-10 and adding IFN- $\alpha$  together with IL-6 + IL-15. At the end of every step, cell counts and viability were determined and cell phenotype was assayed with fluorochrome-conjugated anti-CD20, CD38, or CD138 mAbs, or isotype-matched control mAbs. Flow cytometry

was performed with a FACScan device. Results are shown as the mean  $\pm$  SD of n experiments (n is indicated in the table).

## Legends of figures

### **Figure 1. Three-step *in vitro* model of plasma cell generation.**

Peripheral blood human memory B cells (MBCs) were purified and cultured with sCD40L, ODN and IL-2+IL-10+IL-15, then with IL-2+IL-6+IL-10+IL-15+ for 3 days and then with IFN- $\alpha$ +IL-6+IL-15 for 3 days. Cells were labeled with anti-CD20, CD38 and anti-CD138 mAbs, CD20<sup>+</sup>CD38<sup>-</sup> D4 actBCs, CD20<sup>-</sup>CD38<sup>++</sup> D4 or D7 PBs, and CD20<sup>-</sup>CD38<sup>++</sup>CD138<sup>+</sup> D10 PCs were FACS sorted and stained with May-Grünwald-Giemsa (x1000 magnification). The percentage of cells in the S-phase of the cell cycle was determined using propidium iodide and data were analyzed with the ModFit LT software. Histograms are those of one experiment representative of 3.

### **Figure 2. Expression of surface and cytoplasmic immunoglobulin heavy chain isotypes by B cells and plasma cells generated in the 3-step culture system.**

MBCs were cultured as described in Figure 1. Starting MBCs, D4 actBCs, D7 PBs and D10 PCs were labeled with fluorochrome-conjugated anti-CD20, CD38 and CD138 mAbs and with fluorochrome-conjugated anti-human IgM, IgA, IgG mAbs or isotype-controlled mAbs before or after cell permeabilization. The bold histograms represent labeling with anti-IgM, IgA or IgG mAb and the light ones with the control mAb. Histograms are those of one experiment representative of five. The numbers in the panels are the means  $\pm$  SD of the percentage of labeled cells (*i.e.*  $\geq$  MFI + 1SD of the control mAb). \* indicates that the mean percentage of labeled cells is different from that in D0 MBCs, \*\* from that in D4 actBCs, and \*\*\* from that in D7 PBs.

### **Figure 3. Immunoglobulin production and gene expression of transcription factors involved in B cells to plasma cell differentiation.**

A. MBCs were cultured as described in Figure 1 and culture supernatants were harvested at day 4, day 7 and day 10 to assay for IgM, IgA and IgG concentrations

using nephelometry. The rate of Ig production per cell and per day was calculated dividing the amount of Igs in the culture supernatant by the number of viable cells at the time of culture supernatant harvesting and by the number of days of culture. Data are the means  $\pm$  SD of the rates of Ig production determined in 5 separate experiments. \* indicates that the rate of Ig productions are different from those at day 4 and \*\* from those at day 7.

B. D0 MBCs, D4 actBCs, D7 PBs and D10 PCs were FACS sorted and the expression of *PAX5*, *BCL6*, *IRF4*, *PRDM1* and *XBP1* genes was evaluated by real time RT-PCR. The gene expression in the different cell populations was compared assigning the arbitrary value 1 to the maximal expression. Data are the mean value  $\pm$  SD of gene expression determined in 5 separate experiments. \* indicates that the mean expression is different from that in D0 MBCs, \*\* from that in D4 actBCs, and \*\*\* from that in D7 PBs.

**Figure 4. Phenotype and expression of homing molecules of B cells and plasma cells generated in the 3-step culture system.**

MBCs were cultured as described in Figure 1. Cells were stained for CD20, CD38, and CD138. The cell phenotype was analyzed by gating on CD20<sup>+</sup>CD38<sup>-</sup> lymphocytes, CD20<sup>-</sup>CD38<sup>++</sup>CD138<sup>-</sup> D7 PBs and CD20<sup>-</sup>CD38<sup>++</sup>CD138<sup>+</sup> D10 PCs. A. Black histograms show FACS labeling with anti-CD19, CD27, CD45, HLA class II, Ki-67 (after cell permeabilization), and CD43. Gray histograms display the corresponding negative control mAbs. Data from one experiment representative of 3 are shown. Numbers in panels indicate mean values  $\pm$  SD of the percentage of positive cells of 3 separate experiments and numbers in brackets the mean staining indexes  $\pm$  SD. B. Black histograms show FACS labeling with anti-CXCR5, CXCR4, CCR10, and CD62L mAbs. Gray histograms display the corresponding negative

control mAbs. Data from one experiment representative of 3 are shown. Numbers in panels indicate mean values  $\pm$  SD of the percentage of positive cells and numbers in brackets the mean staining indexes  $\pm$  SD of 3 separate experiments.

\* indicates that the value is different from that in D0 MBCs using a paired t-test, \*\* from that in D4 actBCs, and \*\*\* from that in D7 PBs.

**Figure 5. Gene expression profiles of B cells and plasma cells generated in the 3-step culture system, of memory B cells and bone marrow plasma cells.**

The gene expression profile of purified B cells or PC populations (5 separate samples for each population) was determined with Affymetrix U133 Plus 2.0 microarrays. A. An unsupervised hierarchical clustering was run with the 2000 probe sets with the highest standard deviation (log transform, center genes and arrays, uncentered correlation and average linkage). The dendrogram shows that all samples of a given population (D0 MBCs, D4 actBCs, D7 PBs, D10 PCs, and BMPCs) strongly cluster together ( $r \geq .5$ ) and that D7 PBs and D10 PCs are correlated together unlike other populations. B. The probe sets differentially expressed between D0 MBCs+D4 actBCs and D7 PBS+D10 PCS+ BMPCs were determined with a SAM supervised analysis (Wilcoxon statistic, 2 fold ratio, 0% FDR), identifying 459 unique genes with Ingenuity software. When a gene was assayed by several probe sets, the probe set with the highest variance was used. An unsupervised hierarchical clustering was run on this 459 unique gene list. The normalized expression value for each gene is indicated by a color, with red representing high expression and green representing low expression.

**Figure 6. Visualization of gene expression of transcription factors using Amazonia “B cell to plasma cell Atlas”.**

The gene expression of the 54613 Affymetrix probe sets in MBCs, D4 actBCs, D7 PBs, D10 PCs and BMPCs can be visualized using the Amazonia web site (<http://amazonia.transcriptome.eu/>). The known interactions of these transcription factors are displayed in A. Data are the expression of genes coding for transcription factors controlling B cell and PC cell fate (B). \* indicates that the mean expression is different from that in D0 MBCs, \*\* from that in D4 actBCs, \*\*\* from that in D7 PBs and # between D10PCs and BMPCs.

Figure 1

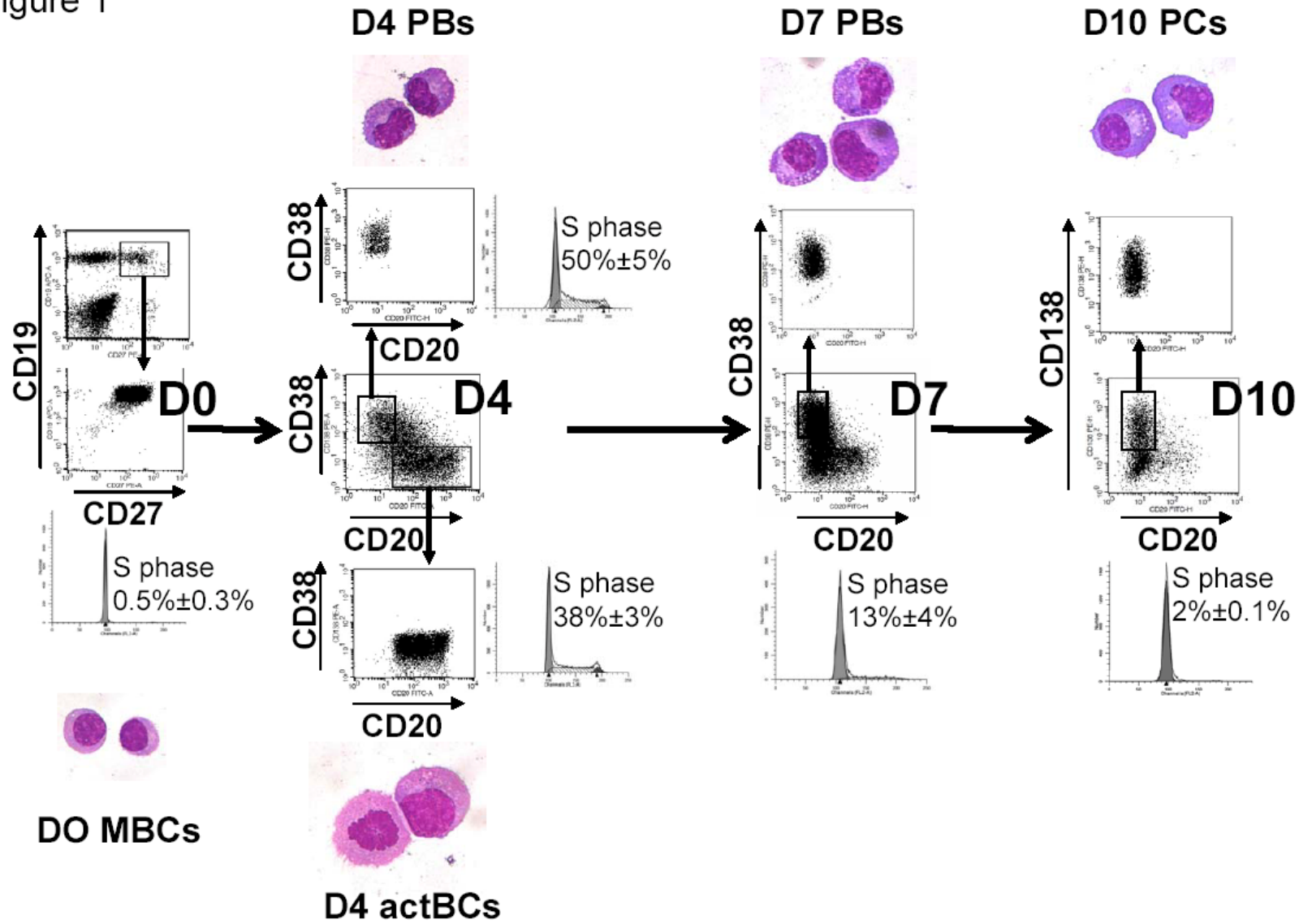


Figure 2

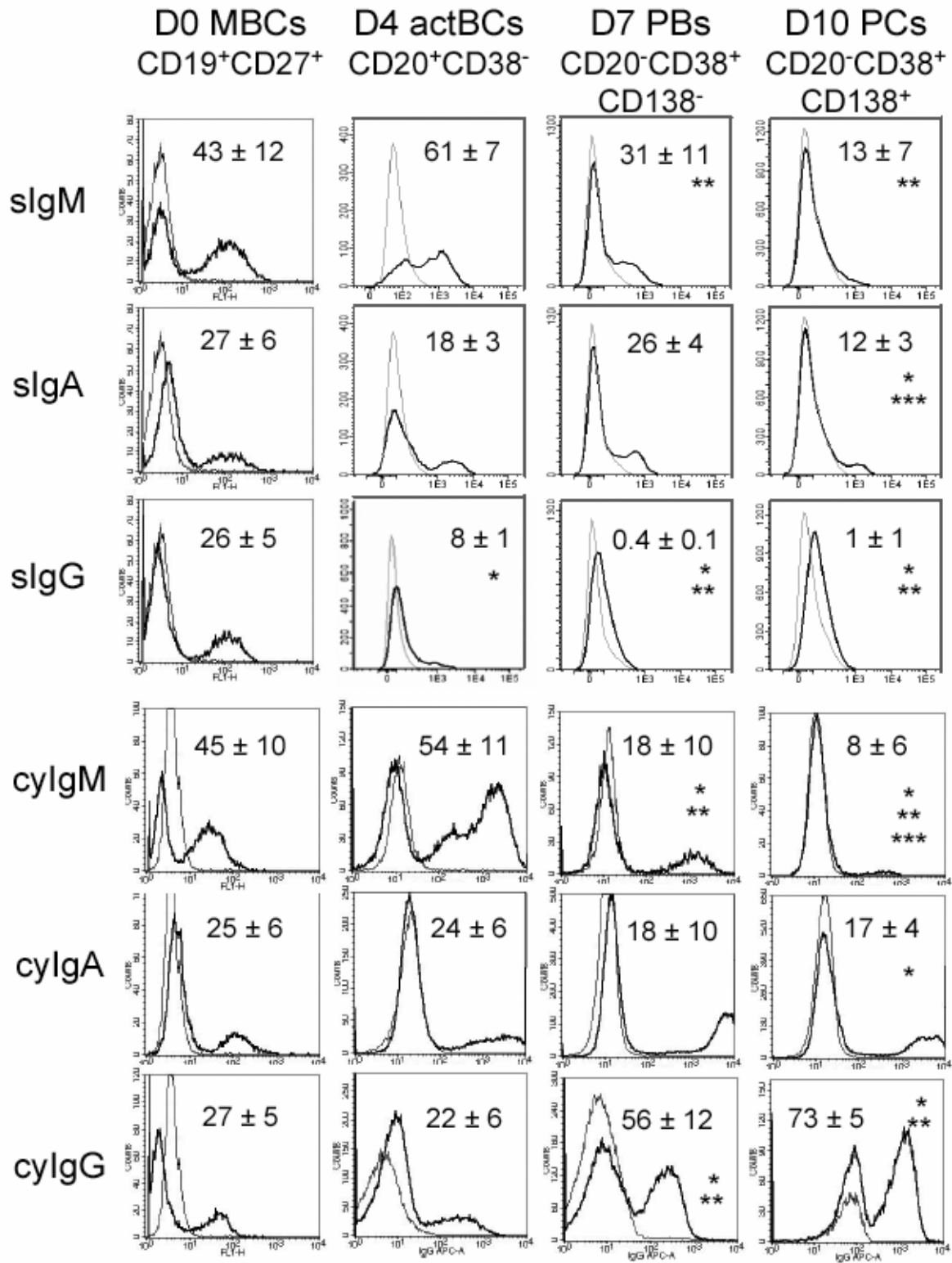
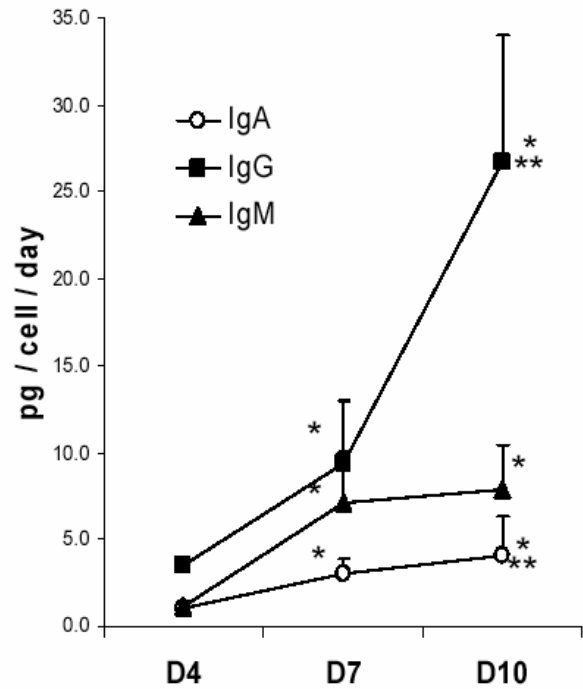


Figure 3

**A**



Immunoglobulin production

**B**

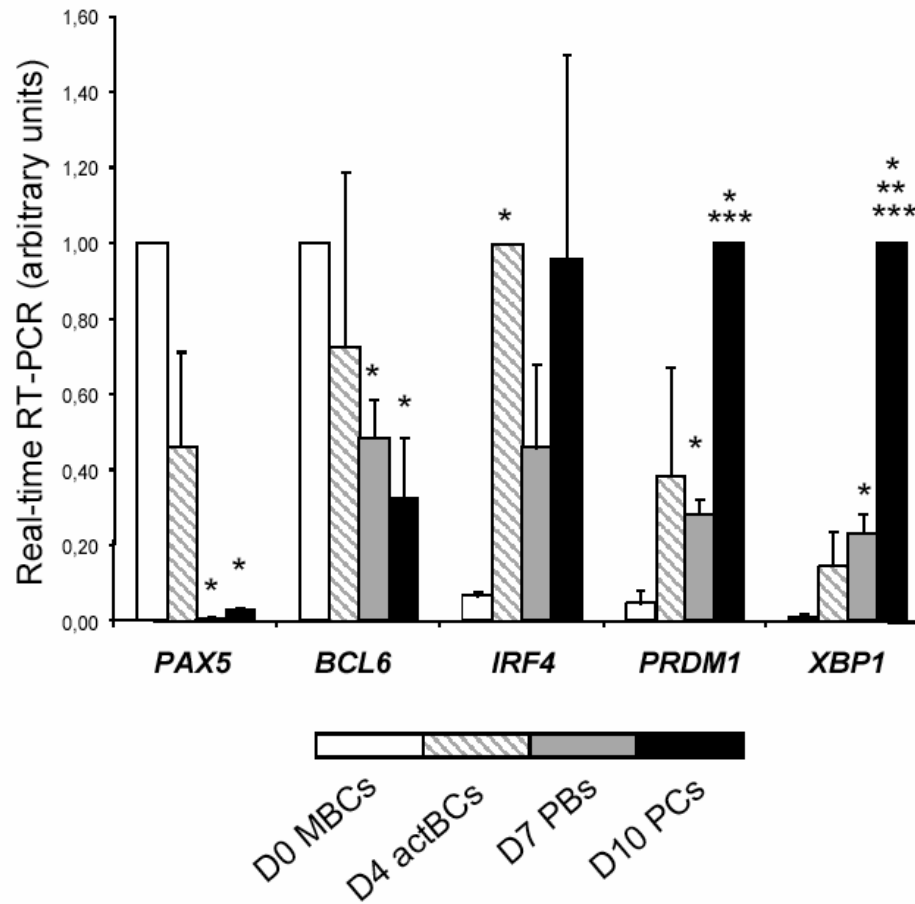


Figure 4A

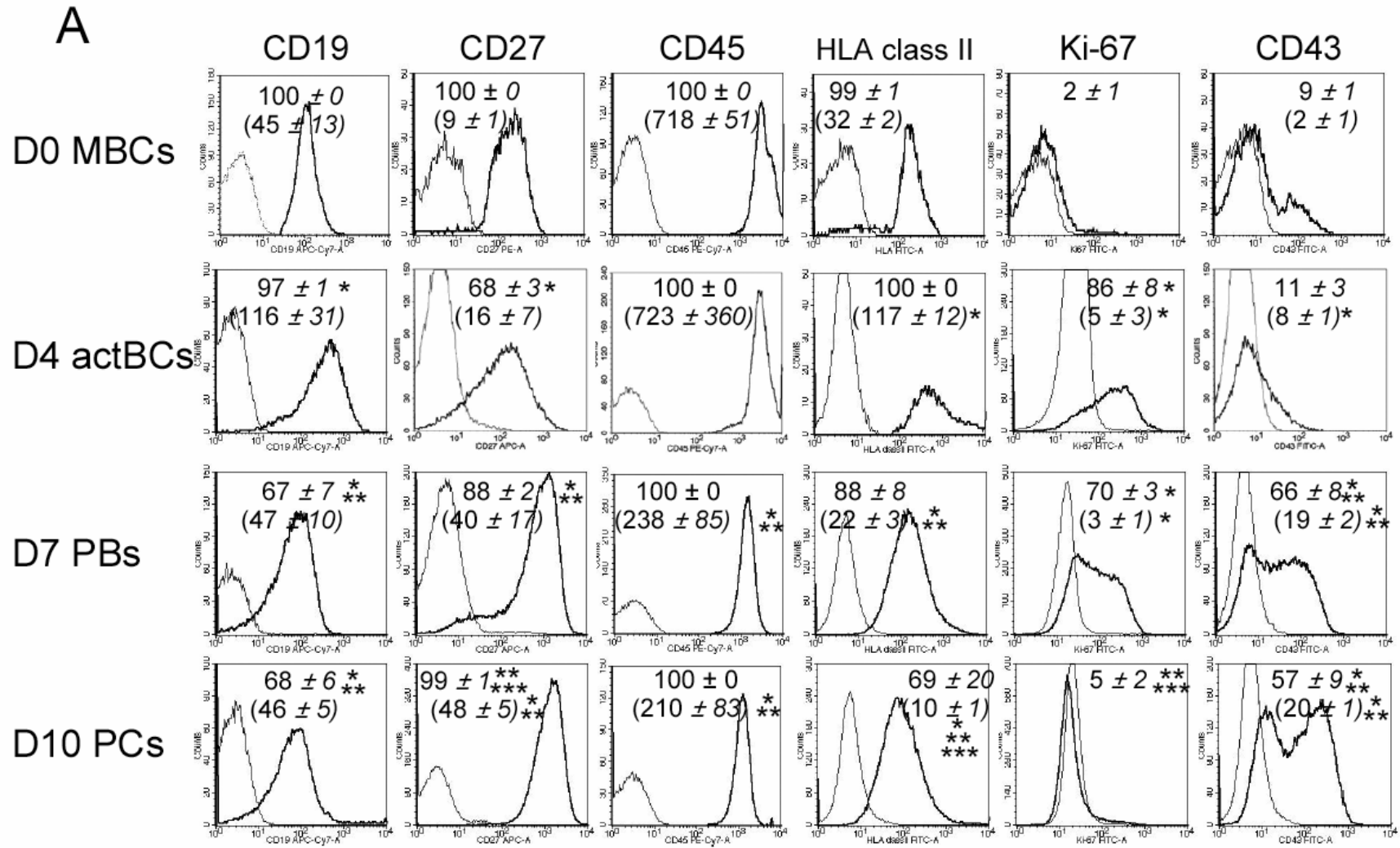


Figure 4B

B

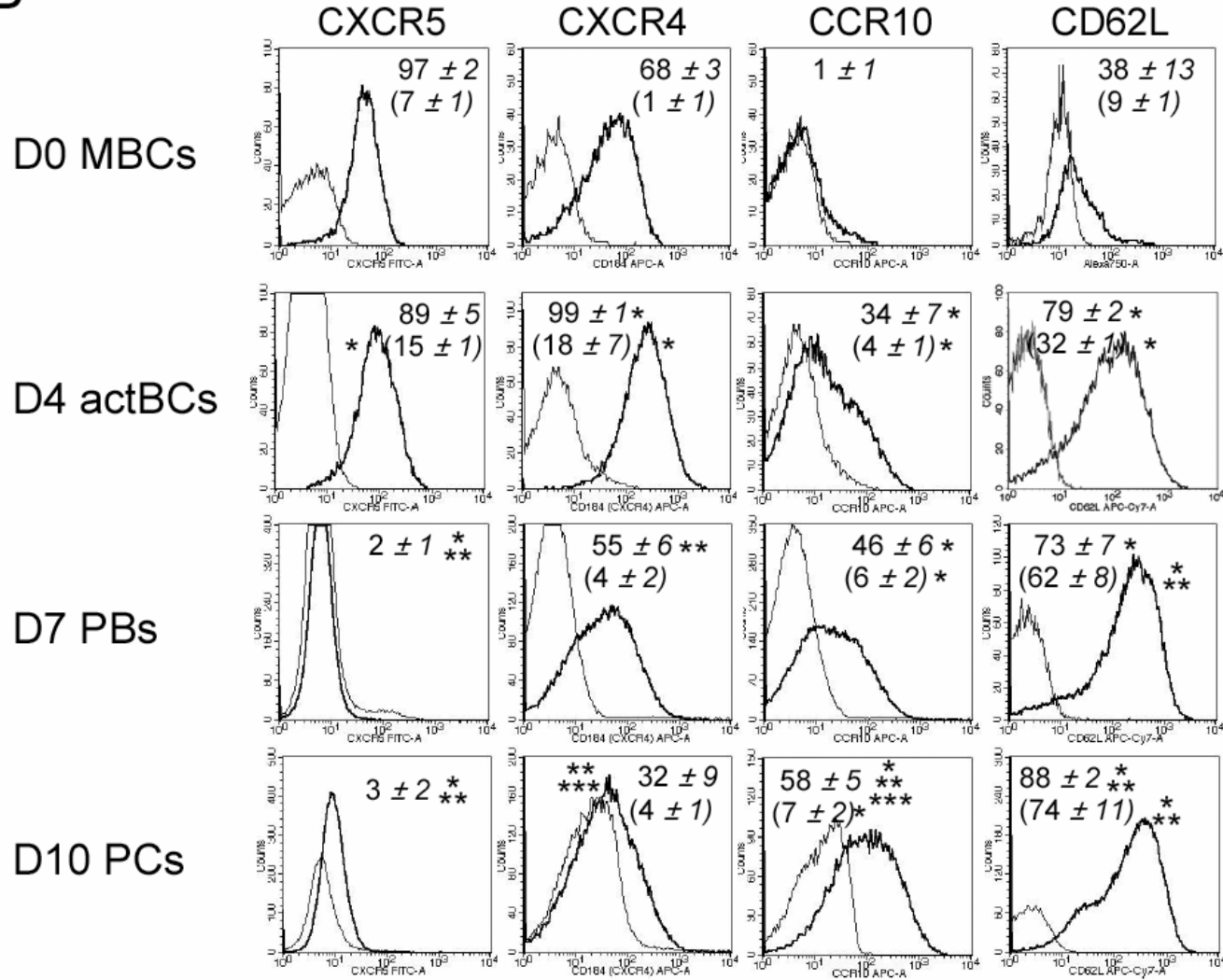


Figure 5

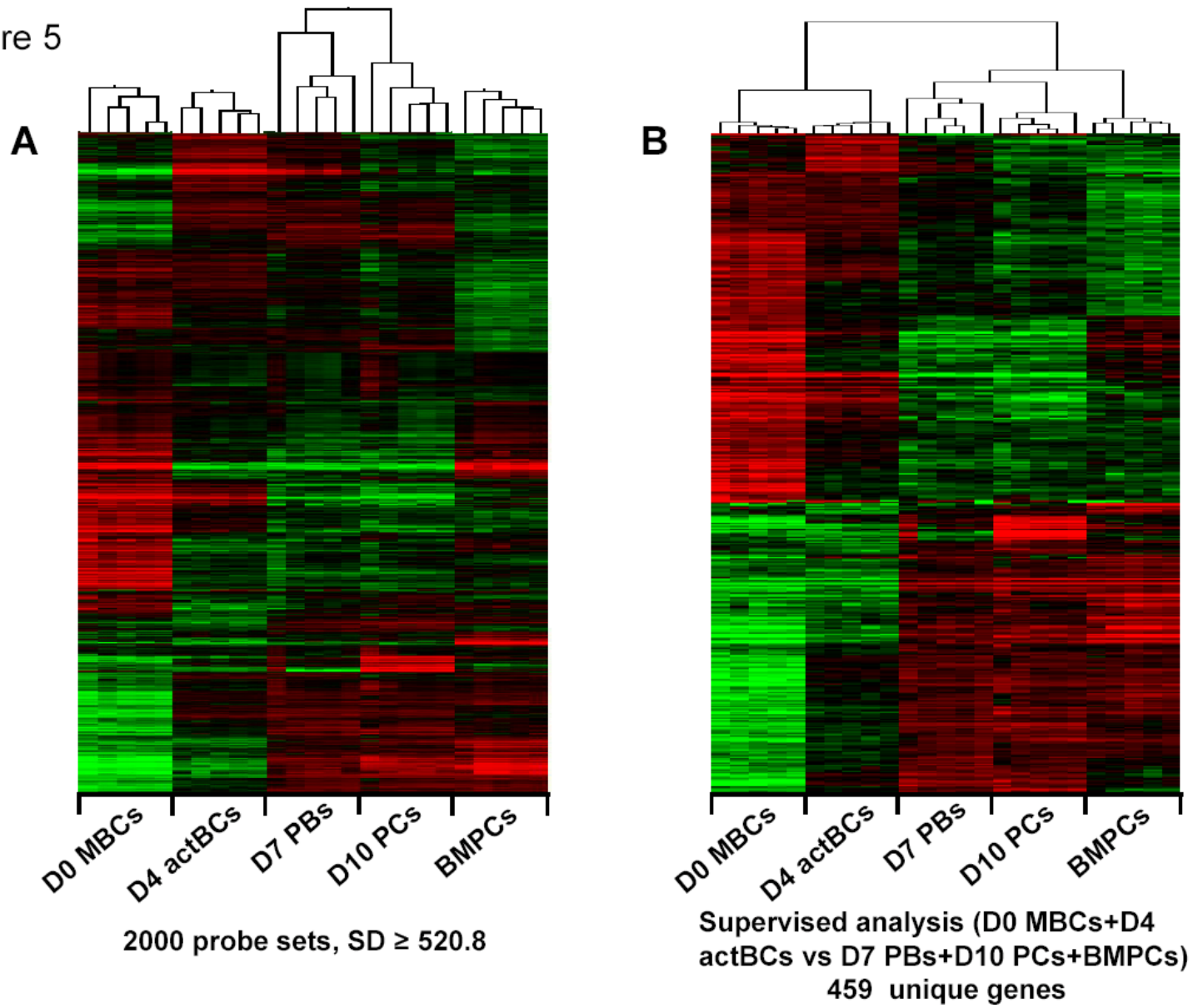


Figure 6

