
Michel Jourdan, Anouk Carauch, Gersende Caron, Nicolas Robert, Geneviève Fiol, Thierry Rème, Karine Bolloré, Jean-Pierre Vendrell, Simon Le Gallou, Frédéric Mourcin, John De Vos, Aloukadel Kassambara, Christophe Duperray, Dirk Hose, Thierry Fest, Karin Tarte and Bernard Klein

J Immunol 2011; 187:3931-3941; Prepublished online 14 September 2011; doi: 10.4049/jimmunol.1101230
http://www.jimmunol.org/content/187/8/3931

Supplementary Material http://www.jimmunol.org/content/suppl/2011/09/14/jimmunol.1101230.DC1

References This article cites 38 articles, 19 of which you can access for free at:
http://www.jimmunol.org/content/187/8/3931.full#ref-list-1

Subscription Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

Michel Jourdan,* Anouk Caraux,* Gersende Caron,†‡ Nicolas Robert,§ Geneviève Fiol,* Thierry Rème,*§ Karine Bolloré,*§ Jean-Pierre Vendrell,*§ Simon Le Gallou,† Freédéric Mourcin,† John De Vos,*‡§ Alboukadel Kassambara,*,§ Christophe Duperray,*§ Dirk Hose,‖§ Thierry Fest,‡ Karin Tarte,‡* and Bernard Klein*§*

The early steps of differentiation of human B cells into plasma cells are poorly known. We report a transitional population of CD20low/CD38+ preplasmablasts along differentiation of human memory B cells into plasma cells in vitro. Preplasmablasts lack documented B cell or plasma cell (CD20, CD38, and CD138) markers, express CD30 and IL-6R, and secrete Igs at a weaker level than do plasmablasts or plasma cells. These preplasmablasts further differentiate into CD20low/CD38highCD138+ plasmablasts and then CD20 low/CD38 high/CD138+ plasma cells. Preplasmablasts were fully characterized in terms of whole genome transcriptome profiling and phenotype. Preplasmablasts coexpress B and plasma cell transcription factors, but at a reduced level compared with B cells, plasmablasts, or plasma cells. They express the unspliced form of XBP1 mRNA mainly, whereas plasmablasts and plasma cells express essentially the spliced form. An in vivo counterpart (CD19+CD20low/CD38+IL-6R+) of in vitro-generated preplasmablasts could be detected in human lymph nodes (0.06% of CD19+ cells) and tonsils (0.05% of CD19+ cells). An open access “B to Plasma Cell Atlas,” which makes it possible to interrogate gene expression in the process of B cell to plasma cell differentiation, is provided. Taken together, our findings show the existence of a transitional preplasmablast population using an in vitro model of plasma cell generation and of its in vivo counterpart in various lymphoid tissues. The Journal of Immunology, 2011, 187: 3931–3941.

The production of high-affinity Ig-producing plasma cells (PCs) is the end product of a complex network of cell interaction, gene rearrangements, and mutations. Ag encounter induces both B and T cells to move to the outer T cell zone, in which they interact each other. Activated B cells then migrate to follicles and initiate a germinal center (GC) reaction characterized by an extensive centroblastic proliferation associated with random somatic hypermutation in Ig genes (1) that are dependent on activation-induced deaminase (AID). CXCR4+ centroblasts are found within the dark zone of the GC, in close contact with poorly characterized dark zone follicular dendritic cells (FDCs) that do not produce CXCL13 (CXCR5 ligand) but express high levels of CXCL12 (CXCR4 ligand) (2, 3). The internalization of CXCR4 after CXCL12 exposure allows the rapid CXCR5-dependent migration of centroblasts to the light zone, acquiring a centrocyte phenotype, and these centrocytes compete with each other for the capture of Ag presented as immune complexes by CXCL13+ light zone FDCs. B cells with high-affinity Ig will pick up greater amounts of Ag, thus receiving a BCR-mediated signal, together with a complex set of FDC-derived growth factors and adhesion molecules (3–5). FDC/B cell interaction is short-lived but allows the capture of Ag for further presentation to a specific subset of GC-restricted follicular helper T cells (TFH). TFH provide them with essential survival, class switch recombination, and differentiation signals, in particular CD40L and IL-21, that will contribute to the NF-κB/IRF4-dependent downregulation of BCL6 and the IRF4/STAT3-dependent upregulation of Blimp1. Owing to their lower affinity for Ag, the vast majority of centrocytes are not rescued by this FDC/TFH system and are engulfed by tingible body macrophages (6). The GC is a dynamic structure, with GC B cells moving cyclically between the dark and light zones to undergo repeated rounds of mutation and selection (2, 7). A successful selection will eventually promote GC B cells to give rise to early Ig secreting plasmablasts (PBs) or memory B cells (MBCs) under presently unknown signals. MBCs persist after immunization both as Ag-dependent memory within residual GC-like...
structures, in close contact to FDCs and T cells, and Ag-independent memory dispersed outside B cell follicles (8). In case of secondary immunization, MBCs can be restimulated to highly proliferate and differentiate into PBs and PCs.

The migration pattern of PBs has been recently documented in mice (9). PBs travel through the T cell zone toward the medullary cords. They are first found in the outer T cell zone close to CD11c+/CD8a− dendritic cells, which highly produce IL-6. In vivo imaging has shown that PBs have a unique pattern of migration in T cell zone characterized by long linear paths that are randomly oriented (10). This migration pattern is dependent on an integrin ICAM1/2 axis and independent on a chemokine gradient. Once arrived in the medullary zone, PBs further differentiate in contact with Gr1+/CD11b+F4/80+ monocytes/macrophages that highly produce CXCL12, APRIL, and IL-6 (9). PBs are retained in the medullary zone by the CXCL12 gradient but still keep moving. One putative reason of this continuous moving is to be able to share the limiting PC niche. Most PBs will die in medullary cords, and the surviving ones will exit the lymph node into lymphatic vessels through a SP1-dependent gradient (11) and then mature PCs (reviewed in Ref. 13). The usual features of PBs are cell cycling, Ig secretion, high CD38 expression, and loss of B cell markers unlike CD19 (14–16). Those of mature PCs are highly proliferate and differentiate into PBs and PCs.

Several studies have contributed to better understand the process of differentiation of human centrocytes or MBCs into PBs and then mature PCs (reviewed in Ref. 13). The usual features of PBs are cell cycling, Ig secretion, high CD38 expression, and CD138 proteoglycan expression (17). We have shown that tonsil-purified CD20+/CXCR4+/CXCR5+ centrocytes already express PC transcription factors (IRF4, Blimp-1, XPB1) together with B cell transcription factors (BCL6, PAX5), although it is not clear whether centrocytes are a heterogeneous cell population containing centrocytes and cells already committed to PC differentiation (18). In an elegant study using cellular affinity matrix technology to capture secreted Igs, Arce et al. (19) have shown the existence of a minor population of CD38−/low Ig-secreting cells in tonsils together with classical CD38high Ig-secreting cells, CD38−/low cells weakly expressed CD27 compared with CD38high cells. The CD38−/low Ig-secreting cells were not detected in the peripheral blood or BM. They were thought to be the precursors of CD38high Ig-secreting cells in an in vitro model of B cell differentiation using PBMCs and tetanus toxoid activation. CD38−/low Ig-secreting cells were also reported in another model of B cell differentiation mimicking T cell help deploying CD40L, IL-2, and IL-10 activation of MBCs (20). Given their poor ability to survive upon CD40 activation removal, these CD38−/low Ig-secreting cells were initially assumed to be early precursors of short-term surviving plasma cells (20). In a following study, this group has shown that the ability to secrete Igs was mainly restricted to CD27high cells, independently of CD38 expression (21).

Using a similar model of PC generation based on initial CD40L and oligodeoxynucleotide (ODN) activation of MBCs, we show in this study that CD38−/low CD20−/low CD27−/low Ig-secreting cells (termed preplasmablasts, prePBs) precedes the generation of CD38high/CD27high PBs and then PCs. PrePBs fully survive and differentiate into PBs and PCs upon removal of CD40 activation. A full molecular and phenotypic characterization of prePBs is provided, showing that these cells express specifically CD30, IL-6R, and cyttoplasmic Igs, express weakly CD27, do not express B cell or PC markers (CD38 and CD138), and secrete Igs at a lower level than do PBs or PCs. The in vivo counterpart of these prePBs could be found in various lymphoid tissues.

Materials and Methods

Reagents

Human recombinant IL-2 and IFN-α were purchased from R&D Systems (Minneapolis, MN), IL-6 and IL-15 were from AbCys (Paris, France), and IL-10 was from PropeTech (Rocky Hill, NJ). Mouse (or rat when indicated) mAbs conjugated to Alexa Fluor 488, Alexa Fluor 647, allophycocyanin, allophycocyanin-H7, allophycocyanin-Cy7, FITC, PerCP-Cy5.5, PE, and PE-Cy7 specific for human CD19 (clones HIB19 and R2/15), CD24 (clone ML5), CD27 (clones L28 and M-T271), CD30 (clone BerH8), CD38 (clone HIT2), CD43 (clone I9G10), CD45 (clone HI30), CD138 (clone MI15), CXCR5 (clone RFB9B2), IgG1 (clone G18-145), IgM (clone G20-127), and Ki67 (clone B56) were purchased from BD Biosciences (Le Pont De Claix, France); CD20 (clone B9E9), CD126 (IL-6R, clone M91), and CD138 (clone B-AB38) from were Beckman Coulter (Fullerton, CA); CCR10 (rat, clone 314305) was from R&D Systems and IgG (polyclonal goat Ab) was from SouthernBiotech (Birmingham, AL).

Cell samples

Peripheral blood cells from healthy volunteers were purchased from the French Blood Center (Etablissement Français du Sang Pyrénées-Méditerranée, Toulouse, France). After removal of CD2+ cells using anti-CD2 magnetic beads (Invitrogen, Cergy Pontoise, France), CD19+/CD27− MBCs were sorted with a multicolor fluorescence FACSaria device with a purity ≥ 95% (see Fig. 1A). Cells produced in the culture system were FACSSorted using FITC-conjugated anti-CD20 mAb and PE-conjugated anti-CD38 mAb for day (D) 4 CD20highCD38− cells, D4 CD20−CD38− cells, and D4 CD20+CD38− cells, as well as D7 PBs (CD20−/CD38+). D10 PCs (CD20−/CD38+) were FACSSorted using FITC-conjugated anti-CD20 mAb and PE-conjugated anti-CD38 mAb. The purity of FACSSorted populations was ≥ 95% as assayed by cytometry. We looked for prePBs in tonsil, lymph node, BM, and peripheral blood samples. Tonsils were obtained from routine tonsillectomies performed at Children’s Clinic La Sagesse at Rennes (France). Reactive nonmalignant lymph node biopsies were collected at Centre Hospitalier Universitaire de Rennes. After mincing, tonsillar and lymph node mononuclear cells were isolated by Ficoll-density gradient centrifugation. BM aspirates were collected from adult patients undergoing thoracic surgery (Centre Hospitalier Universitaire de Rennes) and BM mononuclear cells were isolated by Ficoll density gradient centrifugation. Tonsils, lymph node biopsies, and BM aspirates were all collected after subject recruitment followed Institutional Review Board approval and written informed consent process according to the Declaration of Helsinki.

Cell cultures

In step one, B cell activation, all cultures were performed in IMDM (Invitrogen) and 10% FCS (Invitrogen), supplemented with 50 μg/ml human transferrin and 5 μg/ml human insulin (Sigma-Aldrich, St Louis, MO). Purified MBCs were cultured with IL-2 (20 U/ml), IL-10 (50 ng/ml), and IL-15 (10 ng/ml) in six-well culture plates (1.5 × 107/ml in 5 ml/well), Phosphorothioate CpG ODN 2006 (10 μg/ml; Sigma-Aldrich) (22), histidine-tagged recombinant human soluble CD40L (scd40L, 50 ng/ml) and anti-polylisine mAb (5 μg/ml) (R&D Systems) were added at culture start. In step two, PB generation, at D4 culture, the cells were harvested, washed, and seeded at 2.5 × 105/ml with IL-2 (20 U/ml), IL-6 (50 ng/ml), IL-10 (50 ng/ml), and IL-15 (10 ng/ml). In step three, PC generation, at D7 culture, cells were washed and cultured at 5 × 105/ml with IL-6 (50 ng/ml), IL-15 (10 ng/ml), and IFN-α (500 U/ml) for 3 d.

Cell cycle analysis and immunophenotypic analysis

The percentage of cells in the S phase of the cell cycle was determined using propidium iodide, and data were analyzed with ModFit LT software (Verity Software House, Topsham, ME) (23). Cells were stained with combination of four to seven mAbs conjugated to different fluorochromes. Surface staining was performed prior to cell fixation and permeabilization. The Cytofix/Cytperm kit (BD Biosciences) was used for intracellular staining of IgM, IgA, IgG, and Ki67 Ag, according to the manufacturer’s recommendations. Flow cytometry analysis was performed with a FACSaria cytometer using FACSDiva 6.1 (Becton Dickinson, San Jose, CA) and with a Cyan ADP cytomter driven by Summit software (Beckman Couter). For data analysis, CellQuest (Becton Dickinson), Summit, Kaluza (Beckman Couter), and Infinicit 1.3 (Cyognos, Salamanca, Spain) softwares...
were used. The fluorescence intensity of the cell populations was quantified using the mean fluorescence intensity obtained from the given mAb minus mean fluorescence intensity obtained with a control mAb/(2 × SD mean fluorescence intensity obtained with the same control mAb) (24). The extensive phenotypic study of tonsil cells was carried out using the multicolor flow cytometry methodology and Abs we previously reported in detail (25).

Analysis of Ig secretion

ELISPsOT. D4 CD20⁺ CD38⁻, D7 CD20⁺ CD38⁺, and D10 CD20⁺ CD38⁺ cells were purified using cytometry cell sorting and cultured for 18 h in ELISpots (Millipore, Bedford, MA), which were precoated with goat anti-human IgM, IgA, or IgG polyclonal Abs (Caltag Laboratories, Burlingame, CA). After nine PBS washings, alkaline phosphatase-conjugated goat anti-human IgM, IgA, or IgG Abs (R&D Systems, Minneapolis, MN) were added and reaction was stopped with distilled water. IgM-, IgA-, and IgG-secreting cells were enumerated and immunospot analyses were performed and visualized with the Clustering and TreeView softwares (26).

Real-time RT-PCR analysis

Total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA) and reverse transcribed with a 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, Courtaboeuf, France). The primers used for assessing specific expression of spliced or unspliced forms of XBP1 mRNA were from Applied Biosystems (Hs0329085 and Hs02856596 primers). 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 equation: 100/(2ⁿΔCt), where ΔΔCt = ΔCt unknown — ΔCt positive control as described (26).

Microarray hybridization and bioinformatic analysis

RNA was extracted and hybridized to human genome U133 Plus 2.0 (Affymetrix, Santa Clara, CA). Gene expression data from D4 CD20⁺CD38⁻, D7 CD20⁺CD38⁺, and D10 CD20⁺CD38⁺ cells were deposited in the ArrayExpress public database (http://www.ebi.ac.uk/microarray-as/aef, accession no. E-MEXP-3034). Gene expression data from D0 MBCs, D7 PBs, D10 PCs and purified BM PCs were from the ArrayExpress public database (http://www.ebi.ac.uk/microarray-as/aef, accession number E-MEXP-2360). Gene expression data were analyzed with our bioinformatics platforms (RAGE, http://rage.montp.inserm.fr/) (27) and Amazonia (http://amazonia.transcriptome.eu/) (28). The clustering was performed and visualized with the Cluster and TreeView softwares (29). Gene differentially expressed between cell populations was determined with the significance analysis of microarray (SAM) statistical microarray analysis software (30).

Statistical analysis

Statistical comparisons were made with the nonparametric Mann–Whitney U test and the unpaired or paired Student t test using SPSS software. A p value of ≤0.05 was considered as significant.

Table I. Expression of surface and cytoplasmic IgH isotypes by MBCs and D4 activated cells

<table>
<thead>
<tr>
<th></th>
<th>D0</th>
<th></th>
<th>D4</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MBCs</td>
<td>CD20⁺CD38⁻</td>
<td>CD20⁺CD38⁺</td>
<td>CD20⁺CD38⁻</td>
<td>CD20⁺CD38⁺</td>
<td>CD20⁺CD38⁺</td>
</tr>
<tr>
<td>% SI</td>
<td>% SI</td>
<td>% SI</td>
<td>% SI</td>
<td>% SI</td>
<td>% SI</td>
<td>% SI</td>
</tr>
<tr>
<td>sIgM</td>
<td>43 ± 12</td>
<td>36 ± 8</td>
<td>65 ± 3</td>
<td>10 ± 3</td>
<td>65 ± 5</td>
<td>9 ± 3</td>
</tr>
<tr>
<td>cyIgM</td>
<td>45 ± 10</td>
<td>9 ± 2</td>
<td>60 ± 11</td>
<td>30 ± 8</td>
<td>44 ± 14</td>
<td>115 ± 41</td>
</tr>
<tr>
<td>sIgA</td>
<td>27 ± 6</td>
<td>25 ± 10</td>
<td>20 ± 5</td>
<td>11 ± 1</td>
<td>14 ± 2</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>cyIgA</td>
<td>25 ± 6</td>
<td>48 ± 15</td>
<td>25 ± 5</td>
<td>37 ± 8</td>
<td>25 ± 9</td>
<td>154 ± 38</td>
</tr>
<tr>
<td>sIgG</td>
<td>26 ± 5</td>
<td>33 ± 9</td>
<td>9 ± 2</td>
<td>7 ± 3</td>
<td>15 ± 4</td>
<td>12 ± 4</td>
</tr>
<tr>
<td>cyIgG</td>
<td>27 ± 5</td>
<td>10 ± 4</td>
<td>20 ± 6</td>
<td>11 ± 4</td>
<td>29 ± 6</td>
<td>24 ± 7</td>
</tr>
<tr>
<td>Ki67</td>
<td>2 ± 1</td>
<td>NA</td>
<td>93 ± 1</td>
<td>24 ± 2</td>
<td>96 ± 1</td>
<td>54 ± 6</td>
</tr>
<tr>
<td>S-phase</td>
<td>0.5 ± 0</td>
<td>NA</td>
<td>25 ± 8</td>
<td>NA</td>
<td>56 ± 4</td>
<td>NA</td>
</tr>
</tbody>
</table>

MBCs were cultured as described in Fig. 1. Starting MBCs and D4-activated cells were labeled with fluorochrome-conjugated anti-CD20 and anti-IgM Abs, and with fluorochrome-conjugated anti-CD20, IgM, IgA, and Ki67 mAbs or isotype-controlled mAbs before or after cell permeabilization or with propidium iodide. For each cell population, data are the mean percentage ± SD of positive cells and the mean staining indexes ± SD from three to five separate experiments.

Mean expression is significantly different from that in D4 CD20⁺CD38⁻ cells. NA, not applicable.
D7 CD20 experiments. *The mean is significantly different from that in D4 CD20 PCs. D4 CD20 expansions are the mean values representative of four, and the fold device. Results are those of one experience was determined with a FACScan chrome-conjugated anti-CD20, anti-cell phenotype was assayed with fluoro plus IFN- cell culture conditions (IL-6 plus IL-15 cultured for an additional 3 d with plasma IL-15). D7 cells were washed and cultured for 3 d with PB culture conditions (IL-2 plus IL-6 plus IL-10 plus IL-15 cytokines. At D4, CD20highCD38, CD20highCD38+, and CD20lowCD38+ cells were FACS sorted and cultured for 18 h in ELISPOT plates or cultured for 24 h and culture supernatants were harvested. The number of IgM-, IgA-, and IgG-secreting cells was assessed by ELISA and results are the mean Ig production (Table I, Supplemental Fig. 1). The level of Ig secretion by the D4 CD20highCD38+ cells compared with CD20lowCD38+ cells increased 10- to 20-fold after cell permeabilization (p < 0.05), indicating that these cells were strongly involved in cyIg production (Table I, Supplemental Fig. 1). The increase in SI with the anti-IgG mAb was significant (p < 0.05) but less pronounced (2- to 4-fold) due to the use of allophycocyanin- and PerCP- Cy5.5-fluorochrome-conjugated Abs, resulting in a lower SI as reported (32). Of note, CD20lowCD38+ cells comprised increased IgG + and decreased IgM + cell numbers compared with CD20lowCD38+ cells (p < 0.01), unlike IgG. Additionally, CD20lowCD38+ cells had a lower cytoplasmic Ig content compared with CD20- CD38+ cells as indicated by fluorescence intensity (Table I, Supplemental Fig. 1). The level of Ig secretion by the D4 CD20lowCD38+ cell subset was further compared with that of in vitro-generated D7 PBs or D10 PCs using ELISPOT and ELISA. There were 8% IgG-secreting cells in D4 CD20lowCD38+ cells, based on CD20 and CD38 expressions at the end of a 4-d activation by sCD40L and CpG ODN (step one). These two populations were CD20highCD38 cells and CD20low CD38 cells comprising, respectively, 18 ± 7 and 82 ± 7% of D4 cells (Fig. 1B). The CD20 and CD38 FACS dot plot data suggest that the CD20low CD38 cell population may account for a continuous wave of differentiation of CD20lowCD38 cells (27 ± 7% of D4 cells) into CD20 basCD38 cells (17 ± 7% of D4 cells) passing through a transitional CD20lowCD38 stage (30 ± 10% of D4 cells) (Fig. 1B). To demonstrate this, we further investigated the characteristics of D4 CD20lowCD38, CD20basCD38, and CD20CD38+ subsets (referred to below as CD20low− subsets).

**Results**

**Activation of MBCs by sCD40L and CpG ODN yields two distinct cell populations**

In the first step of differentiation of MBCs into PCs, two distinct cell populations could be identified in 17 separate experiments.

**FIGURE 2.** Ig secretion by D4 CD20−CD38− cells, D7 PBs, and D10 PCs. D4 CD20−CD38− cells, D7 CD20−CD38− PBs, and D10 CD20−CD38− PCs were flow cytometry cell sorted and cultured for 18 h in ELISPOT plates or cultured for 24 h and culture supernatants were harvested. The number of IgM-, IgA-, and IgG-secreting cells was assessed by ELISA and results are the mean Ig production (Table I, Supplemental Fig. 1). The level of Ig secretion by the D4 CD20−CD38− cells compared with CD20−CD38− cells increased 10- to 20-fold after cell permeabilization (p < 0.05), indicating that these cells were strongly involved in cyIg production (Table I, Supplemental Fig. 1). The increase in SI with the anti-IgG mAb was significant (p < 0.05) but less pronounced (2- to 4-fold) due to the use of allophycocyanin- and PerCP- Cy5.5-fluorochrome-conjugated Abs, resulting in a lower SI as reported (32). Of note, CD20−CD38− cells comprised increased IgG + and decreased IgM + cell numbers compared with CD20−CD38− cells (p < 0.01), unlike IgG. Additionally, CD20−CD38− cells had a lower cytoplasmic Ig content compared with CD20− CD38− cells as indicated by fluorescence intensity (Table I, Supplemental Fig. 1). The level of Ig secretion by the D4 CD20−CD38− cell subset was further compared with that of in vitro-generated D7 PBs or D10 PCs using ELISPOT and ELISA. There were 8% IgG-secreting cells in D4 CD20−CD38− cells.

**FIGURE 3.** Plasmablast and plasma cell differentiation potential of D4 CD20low−CD38− prePBs and of D4 CD20high−CD38− cells. Purified MBCs were cultured for 4 d using sCD40L and CpG ODN activation and IL-2 plus IL-10 plus IL-15 cytokines. At D4, CD20high−CD38+, CD20high−CD38−, and CD20low−CD38− cells were FACS sorted and cultured for 3 d with PB culture conditions (IL-2 plus IL-6 plus IL-10 plus IL-15). D7 cells were washed and cultured for an additional 3 d with PB culture cell conditions (IL-6 plus IL-15 plus IFN-α). At each culture step, the cell phenotype was assayed with fluoro chrome-conjugated anti-CD20, anti-CD38, and anti-CD138 mAbs, or with isotype-matched control mAbs. Fluorescence was determined with a FACSscan device. Results are those of one experiment representative of four, and the fold expansions are the mean values ± SD of those in four experiments.
that is, 2.5- and 3.7-fold less than in D7 PBs or D10 PCs ($p < 0.02$; Fig. 2A), and with a 3- to 7-fold smaller immunospot size ($\geq 200 \mu m$; Fig. 2B). The number of IgA-secreting cells in D4 CD20$^{+}$CD38$^{-}$ cells was not significantly different from those in D7 PBs or D10 PCs (Fig. 2A). There were 3–5% IgM-secreting cells in the D4 CD20$^{+}$CD38$^{-}$ cell subset as in D7 PBs and D10 PCs (Fig. 2A), with no difference in IgM immunospot size. The measurement of Ig production in culture supernatants confirmed ELISPOT data. D4 CD20$^{+}$CD38$^{-}$ cells secreted 5- to 7-fold less IgG, 1.5- to 2-fold less IgA, and similar IgM levels than did D7 PBs or D10 PCs (Fig. 2C).

Cell cycling was looked for with anti-Ki67 and propidium iodide staining (Table I). Starting MBCs were quiescent and all D4 cell populations were cycling with 93–100% Ki67$^+$ cells. CD20low CD38$^-$, CD20$^{+}$CD38$^{-}$, and CD20$^{-}$CD38$^+$ cells had a higher proportion of cells in the S phase (46–56%) than did CD20$^{+}$CD38$^{-}$ cells (25%, $p < 0.05$).

These results indicate that the D4 CD20$^{low+}$ cell population comprised mostly CD20$^{low+}$CD38$^{-}$ cells that expressed cyIgs and secreted Igs, but at a weaker level than D7 PBs and D10 PCs. Given the Ig secretion, the lack of CD38 and the lack or weak expression of CD20 B cell marker, these CD20$^{low+}$CD38$^{-}$ cells are termed prePBs in the following.

**D4 CD20$^{low+}$CD38$^{-}$ prePBs generate CD20$^{+}$CD38$^{high+}$ PBs and then CD138$^+$ plasma cells, unlike D4 CD20$^{high+}$CD38$^{-}$ cells**

The PC differentiation potential of D4 CD20$^{low+}$CD38$^{-}$ prePBs was assayed culturing these cells for three additional days in PB step two culture conditions (Fig. 3). CD20$^{low+}$CD38$^{-}$ cells were sorted into CD20$^{low+}$CD38$^{-}$ and CD20$^{+}$CD38$^{-}$ cells to evaluate whether these two populations had a similar differentiation potential and thus could actually correspond to a unique cell subset. This was the case since a vast majority of both D4 CD20$^{low+}$CD38$^{-}$ and D4 CD20$^{+}$CD38$^{-}$ cells differentiated into D7 CD20$^{+}$CD38$^{high+}$ PBs in four separate experiments (73 ± 15 and 87 ± 7%, respectively) in association with a 4.2- or 3.3-fold cell expansion, respectively (Fig. 3). When further put into step three plasma cell culture conditions for 3 d (D7 to D10), the sorted CD20$^{low+}$CD38$^{-}$ or CD20$^{+}$CD38$^{-}$ cells generated CD20$^{low+}$CD38$^{high+}$CD138$^+$ PCs (51 ± 7 and 72 ± 5%, respectively; $n = 4$; Fig. 3). Conversely, D4 CD20$^{high+}$CD38$^{-}$ cells gave rise to few CD38$^{+}$CD20$^{-}$ cells (23 ± 13%, $n = 4$) in step two culture conditions, and did not survive when further put in step three plasma cell culture conditions (Fig. 3). Thus, these in vitro differentiation data reveal the presence of only two populations of CD38$^{-}$ cells at D4 of culture: first, actively cell cycling CD20$^{low+}$CD38$^{-}$ prePBs that efficiently differentiated into CD20$^{+}$CD38$^{high+}$ PBs and then CD20$^{+}$CD38$^{-}$CD138$^+$ PCs; and second, CD20$^{high+}$CD38$^{-}$ cells that poorly differentiated and survived into PB and then PC culture conditions.

**Molecular characterization of D4 CD20$^{low+}$CD38$^{-}$ prePBs**

The gene expression of sorted D4 CD20$^{high+}$CD38$^{-}$, CD20$^{low+}$ CD38$^{-}$, and CD20$^{-}$CD38$^{+}$ cells obtained from MBCs from five healthy donors was profiled using Affymetrix U133 Plus 2.0 microarrays. An unsupervised hierarchical clustering using the 5000 probe sets with the highest SD was run on this 495 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. The dendogram shows that CD20$^{high+}$CD38$^{-}$ samples and CD20$^{low+}$CD38$^{-}$ samples are grouped in two separate clades ($r = 0.44$). In the CD20$^{low+}$CD38$^{-}$ clade, CD20$^{high+}$CD38$^{-}$ and CD20$^{-}$CD38$^{+}$ cells originating from the same donor cluster together for three of five donors, indicating that these two populations are close. The probe sets differentially expressed between D4 CD20$^{high+}$CD38$^{-}$ and CD20$^{-}$CD38$^{+}$ cells were determined with a SAM supervised analysis for pairs (Wilcoxon statistic, 2-fold ratio, 0% false discovery rate), identifying 495 unique genes. 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 495 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.
Phenotype of D4 CD20low/CD20high cells

D4 CD20low/CD20high cells expressed higher levels of IL-6R, CD30, CCR10, and CD43 than did D4 CD20low/CD20low cells (Fig. 5, Table III). Conversely, D4 CD20low/CD20high cells expressed higher levels of CD24, CD19, CD45, CXCR5, and CD27 than did D4 CD20low/CD20low cells (Fig. 5, Table III). Of note, CD27 dramatically increased again in CD20low/CD20low cells differentiated into D7 PBs and D10 PCs as documented previously (31). D4 CD20low/CD20high cells, D4 CD20low/CD20low cells, and D4 CD20high/CD20high cells are termed CD20low prePBs, CD20+ prePBs, and CD20high B cells. This open access atlas can be visualized on our Amazonia Web site (http://amazonia.transcriptome.eu/index.php?zone=PlasmaCell).

Additionally, they expressed CD30 (TNFRSF8) and EMP1 (Table II, Supplemental Table I). Conversely, CD20high/CD38low cells highly expressed genes for B cell Ags, that is, CD19, CD20 (MS4A1), CD22, and CD42 (Table II, Supplemental Table I).

**Phenotype of D4 CD20low/CD20low cells**

The FACS analysis confirmed Affymetrix data. D4 CD20low/CD20low cells expressed higher levels of IL-6R, CD30, CCR10, and CD43 than did D4 CD20low/CD20low cells (Fig. 5, Table III). Conversely, D4 CD20low/CD20low cells expressed higher levels of CD24, CD19, CD45, CXCR5, and CD27 than did D4 CD20low/CD20low cells (Fig. 5, Table III). Of note, CD27 dramatically increased again when D4 CD20low/CD20low cells differentiated into D7 PBs and D10 PCs as documented previously (31). D4 CD20low/CD20low cells had a phenotype close to that of CD20low/CD20low cells, but with a significantly weaker expression of prePB and PB markers (CD30, IL-6R, and CD43), in agreement with gene expression data.

**B to plasma cell atlas**

An open access “B to Plasma Cell Atlas” was developed allowing interrogating gene expression of D4 CD20low/CD20low prePBs and CD20high/CD20high B cells together with those of MBCs, PBs, early PCs, and BMPCs (31). For convenient atlas use, D4 CD20low/CD20low cells, D4 CD20low/CD20low cells, and D4 CD20high/CD20high cells are termed CD20low prePBs, CD20+ prePBs, and CD20high B cells. This open access atlas can be visualized on our Amazonia Web site (http://amazonia.transcriptome.eu/index.php?zone=PlasmaCell).

**Fig. 6A** displays the expression of genes coding for transcription factors controlling B cell or PC fates, and **Fig. 6B** displays the validation of these gene expressions by real-time RT-PCR. CD20low prePBs weakly expressed PAX5 B cell transcrip...
MBCs were cultured as described in Fig. 1. Cells were stained for CD20 and CD38 and the cell phenotype was analyzed by gating on D4 CD20<sup>high</sup>CD38<sup>active</sup> XBP1 protein (33), spliced mRNA has to be spliced by IRE1 endonuclease to encode for an expressed PC transcription factor genes PRDM1<sup>a</sup> to a weaker level than PBs or early PCs (Fig. 6). CD20<sup>low</sup>/CD38<sup>pos</sup> cells, D4 CD20<sup>low</sup>CD38<sup>low</sup> are shown. CD27, CCR10, and CD43. Gray histograms display the corresponding negative control mAbs. Data from one experiment representative of three to five are shown.

factor and PAX5 target genes (IRF8, BACH2, EBF1, SPIB) (Fig. 6A, 6B) as well as BCL6 gene (Fig. 6B). CD20<sup>bowl</sup> prePBs expressed PC transcription factor genes PRDM1 and XBP1 at a weaker level than PBs or early PCs (Fig. 6A). Because XBP1 mRNA has to be spliced by IRE1 endonuclease to encode for an active XBP1 protein (33), spliced XBP1 (XBP1<sub>s</sub>)s and unspliced (XBP1<sub>u</sub>)mRNAs were quantified. D4 prePBs expressed high levels of XBP1<sub>s</sub> mRNA, mainly XBP1<sub>s</sub> mRNA. The XBP1<sub>s</sub>/XBP1<sub>u</sub> mRNA ratio was 0.3 in prePBs, and this ratio was increased 3.5- to 5-fold in D7 PBs and D10 PCs that expressed XBP1<sub>s</sub> mRNA mainly (Fig. 6C). The expression of genes coding for B cell and PC surface markers or homing molecules are displayed in Supplementary Figs. 2 and 3. Affymetrix data are in agreement with the phenotype of prePBs reported above: lack of B cell Ags and of CD38. Of interest, prePBs specifically expressed CD30 and EMP1 genes, unlike B cells, PBs, early PCs, or BMPCs. PrePB genes were significantly higher and B cell genes lower in CD20<sup>pos</sup> prePBs than in CD20<sup>bowl</sup> prePBs.

Table III. Expression of membrane markers by MBCs and D4 activated cells

<table>
<thead>
<tr>
<th>Membrane Markers</th>
<th>D0 MBCs</th>
<th>D4 CD20&lt;sup&gt;high&lt;/sup&gt;CD38&lt;sup&gt;+&lt;/sup&gt;</th>
<th>D4 CD20&lt;sup&gt;bowl&lt;/sup&gt;CD38&lt;sup&gt;−&lt;/sup&gt;</th>
<th>D4 CD20&lt;sup&gt;bowl&lt;/sup&gt;CD38&lt;sup&gt;−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>CD24</td>
<td>88 ± 4</td>
<td>82 ± 15</td>
<td>68 ± 21</td>
<td>25 ± 41</td>
</tr>
<tr>
<td>IL-6R</td>
<td>0.6 ± 0.9</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>CD30</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>CD27</td>
<td>100 ± 0</td>
<td>100 ± 0</td>
<td>225 ± 73</td>
<td>64 ± 12&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CXCR5</td>
<td>100 ± 0</td>
<td>718 ± 51</td>
<td>782 ± 497</td>
<td>425 ± 245&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD45</td>
<td>97 ± 2</td>
<td>7 ± 1</td>
<td>98 ± 0</td>
<td>20 ± 4</td>
</tr>
<tr>
<td>CCR10</td>
<td>1.3 ± 0.5</td>
<td>0 ± 0</td>
<td>21 ± 9</td>
<td>1.7 ± 1.1</td>
</tr>
<tr>
<td>CD43</td>
<td>9 ± 1</td>
<td>2.0 ± 0.4</td>
<td>18 ± 3</td>
<td>1.8 ± 0.6</td>
</tr>
<tr>
<td>CD27</td>
<td>100 ± 0</td>
<td>9 ± 1</td>
<td>84 ± 7</td>
<td>31 ± 9</td>
</tr>
</tbody>
</table>

MBCs were cultured as described in Fig. 1. Starting MBCs, D4-activated cells were labeled with fluorochrome-conjugated anti-CD20 and anti-CD38 mAbs and with indicated fluorochrome-conjugated mAbs or isotype-controlled mAbs. For each cell population, data are the mean percentage ± SD of positive cells and the mean staining indexes ± SD from three to five separate experiments.

<sup>a</sup>Mean expression is significantly different from that in D4 CD20<sup>high</sup>CD38<sup>−</sup> cells.

<sup>b</sup>Mean expression is significantly different from that in D4 CD20<sup>bowl</sup>CD38<sup>−</sup> cells.
Characterization of Human Preplasmablasts

The presence of prePBs, defined as CD19<sup>+</sup>CD20<sup>+</sup>CD38<sup>lo</sup>/CD38<sup>+</sup> cells, was looked for in human tonsils and reactive lymph nodes. Fig. 7A shows that gating consecutively on CD19<sup>+</sup> cells and then CD20<sup>+</sup> cells made it possible to detect a minor population of CD38<sup>-</sup> and CD138<sup>-</sup> cells in seven of seven tonsil samples and in seven of nine lymph node samples. This CD19<sup>+</sup> CD20<sup>+</sup>CD38<sup>lo</sup> CD38<sup>-</sup> population accounted for 0.06 ± 0.02 (range, 0.02–0.08%) and 0.05 ± 0.03% (range, 0.02–0.09%) of CD19<sup>+</sup> cells in tonsil and positive lymph node samples, respectively. CD19<sup>+</sup>CD38<sup>lo</sup>/CD38<sup>-</sup> cells could not be detected in BM (five samples) and peripheral blood (five samples, data not shown). Looking for cytoplasmic Ig expression, approximately two thirds of these CD19<sup>+</sup>CD38<sup>lo</sup>/CD38<sup>-</sup> cells expressed high levels of Ig L chains after cell permeabilization (Fig. 7B). The other third expressed one log weaker levels of Ig L chain after cell permeabilization and we previously showed these cells are B cells that express surface Ig L chains (25). Fig. 7C shows that a large part of in vivo CD19<sup>+</sup>CD20<sup>+</sup>/CD38<sup>-</sup> CD38<sup>-</sup> cells also expressed IL-6R, unlike CD19<sup>+</sup>CD38<sup>+</sup> cells. Thus, 45 ± 5% of CD19<sup>+</sup>CD20<sup>+</sup>/CD38<sup>-</sup> CD38<sup>-</sup> cells are IL-6R<sup>+</sup> in tonsils (range, 36–51%) and 36 ± 14% are IL-6R<sup>+</sup> in lymph nodes (range, 18–57%).

Discussion

Owing to the rarity of plasma cells and their anatomic location, the early stages of differentiation of B cells into plasma cells are not fully elucidated, in particular in humans. Using an in vitro model mimicking T cell help, we report a transitional prePB stage in the differentiation of human memory B lymphocytes into PBs, and then plasma cells. These prePBs are characterized by the loss of B cell markers, except CD19, a decrease in CD27, the lack of CD38, and the secretion of lgs at a lower level than CD38<sup>+</sup> PBs or CD138<sup>+</sup> plasma cells. Their prePB status is evidenced by their ability to fully differentiate into PBs that highly express CD38 within 3 d (CD19<sup>+</sup>CD20<sup>+</sup>CD38<sup>high</sup> cells) and then plasma cells that express CD138 within additional 3 d (CD19<sup>+</sup>CD20<sup>+</sup>CD38<sup>high</sup> cells). Human CD38<sup>-</sup>/CD38<sup>low</sup> Ig-secreting cells were already reported but with challenging results regarding their behavior and phenotype. With a model of MBC activation by CD40L and cytokines close to the current one, Tangye et al. (20) reported that CD38<sup>-</sup> B cells poorly survived upon removal of CD40L activation and were thought to be precursors of short-living plasma cells. In the current study, sorted CD20<sup>+</sup>/CD38<sup>-</sup> prePBs efficiently differentiated into PBs and then plasma cells. An explanation could be the use of whole CD38<sup>-</sup> B cells in the study by Tangye et al. comprising activated B cells (here CD20<sup>+</sup>/CD38<sup>-</sup> cells) and prePBs, avoiding an efficient differentiation of prePBs. In a following study published by Avery et al. (21) using a similar in vitro model, precursors of Ig-secreting cells were shown to be mainly CD27<sup>+</sup> cells, independently of CD38 expression, and CD27<sup>+</sup> cells had a reduced cell cycling. These data also do not fit with the current ones since prePBs have a reduced CD27 gene and protein expression compared with MBCs, PBs, or plasma cells. Additionally, the CD27<sup>+</sup> prePBs were highly proliferating with level in the different cell populations was compared assigning the arbitrary value 1 to the maximal expression. Data are the mean value ± SD of gene expression determined in five separate experiments. *The mean expression is significantly different from that in CD20<sup>+</sup> B cells (p < 0.05).

**The mean expression is significantly different from that in CD20<sup>+</sup> prePBs (p < 0.05).
50% of cells in the S phase. A possible explanation for these differences is that we used CpG ODN and CD40L stimulation of memory B cells, whereas Tange et al. used CD40L stimulation only. CpG ODN is known to promote B cell differentiation activating TLRs. Thus, either CpG ODN can downregulate CD27 expression on prePBs that further differentiate into PBs, or the differentiation of CD27low prePBs may require both CD40L and TLR activation. Arce et al. (19) identified CD38low/Ig-secreting cells in tonsils. These CD38low/Ig-secreting cells were shown to weakly express CD27, in agreement with our current data. These cells were suggested to be precursors of CD38high Ig-secreting cells because they were detected earlier in culture of tetanus toxoid-activated B cells. However, this was not proven formally using cell sorting and culturing these CD38low cells.

The relevance of this transitional CD19+CD20lowCD38low prePB stage is highlighted by the peaked expression of CD30 gene and protein. Memory B cells, PBs, early plasma cells, and BM plasma cells do not express CD30. CD30 is a TNFR family member that is expressed on activated T cells, EBV-infected B cells, some large diffuse lymphoma, Reed–Sternberg cells, and anaplastic large cell lymphoma. As CD30 is an activation molecule, we looked for a modulation of prePB proliferation or differentiation using CD30 activation by histidine-bound CD30L and anti-histidine Abs. No modulation was found in our culture conditions (data not shown).

The loss of CD20 and most B cell markers in prePBs could be likely explained by the decrease in PAX5 that controls expression of B cell genes (34–36). PAX5 gene expression decrease in prePBs is associated with an upregulation of expression of IRF4 and PRDM1 genes and downregulation of BCL6. Thus, the usual crossregulation of B and plasma cell transcription factors should occur in prePBs: NF-κB induces upregulation of IRF4 that associates with STAT3 to trigger PRDM1 gene activation and induces downregulation of BCL6 gene expression, an inhibitor of PRDM1 gene activation. The resulting expression of PRDM1 gene product, BLIMP1, further represses BCL6 and PAX5 genes, leading to the release of XBP1 gene suppression by PAX5. XBP1 mRNA has to be spliced by the IRE1 endonuclease to encode for XBP1 that activates the unfolded protein response (33). Pre-PBs expressed mainly XBP1u mRNA, and PBs and PCs expressed mainly the XBP1s mRNA with a 3.5- to 5-fold increase of the XBP1s/XBP1u mRNA ratio compared with prePBs. These data again argue for the prePB status of D4 CD20lowCD38low cells that start to secrete Igs, but at a weaker level than PBs or PCs, and likely exhibit a less pronounced unfolded protein response. An
interesting point will be to identify the transcription factors controlling the transient CD30 gene expression in prePBs, and then the expression of CD38 and of its ligand, CD31, in PBs. Cells with the main characteristics of these in vitro PBs could be identified in tonsils and lymph nodules at a low frequency gating on CD19⁺ CD20low/CD38⁻ cells. They accounted for 0.06% and 0.05% of CD19⁺ cells in tonsils and positive lymph nodules, and they were not detected in peripheral blood and BM. We previously reported that PBs and B cells can be clearly identified by multicolor FACS analysis using cell permeabilization and anti-κ or λ Ig L chain staining (25). PBs highly express κ or λ Ig L chains, and B cells express intermediate levels. Using this technique, we found that two-thirds of these cells expressed highly cytoplasmic Ig, and the other third expressed intermediate levels characteristic of surface Ig B cells. Arce et al. (19) also detected CD38low/−/ Ig-secreting cells in tonsils, unlike peripheral blood and BM. These in vivo cells expressed cytoplasmic Ig and IL-6R, as did in vitro prePBs, unlike tonsil or lymph node B cells. CD30 could not be detected on these prePBs in vivo. This could be due to a rapid shedding of the protein in vivo following activation by CD30L (37).

Recent immunochemistry and in vivo imaging studies have documented the traffic of maturing plasma cells from GC to medullary cords in mice (9, 10). In these studies, plasma cells were documented as IgG1-producing cells, which exit the GC to the outer T cell zone in contact with CD11c⁺ CD80− dendritic cells producing IL-6. Then they migrate randomly along linear paths in the T cell zone and join the medullary cords in contact with APRIL-producing monocytes/macrophages. These data fit well with the molecular characterization and phenotype of prePBs reported in this study. The need of T cell mimicking signals to get them in vitro suggests they are generated in the GC light zone and join the medullary cords in contact with producing IL-6. They then migrate randomly though linear paths in the T cell zone. IL-6R expression by prePBs will prompt them to become Ig B cells by secreting Mfge8.

References


