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CD40 Stimulation of Human Peripheral B Lymphocytes: Distinct Response from Naive and Memory Cells

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During secondary immune response, memory B lymphocytes proliferate and differentiate into Ig-secreting cells. In mice, the binding of CD40 by CD154 clearly enhances the activation and differentiation of memory B lymphocytes. In humans, the role of CD40-CD154 in the stimulation of memory B lymphocytes is not as obvious since in vitro studies reported positive and negative effects on their proliferation and differentiation in Ig-secreting cells. In this study, we examine the response of peripheral memory and naive cells in relation to the duration of CD40-CD154 interaction. We measured the proliferation and differentiation of both subsets stimulated with CD154 and IL-4 for short- (4–5 days) and long-term (>7 days) periods. Following short-term stimulation, memory B lymphocytes did not expand but represented the only subset differentiating into IgG- and IgM-secreting cells. A longer stimulation of this population led to cell death, while promoting naive B lymphocyte proliferation, expansion, and differentiation into IgM- or IgG-secreting cells. This prolonged CD40 stimulation also triggered naive B lymphocytes to switch to IgG and to express CD27 even in absence of somatic hypermutation, suggesting that these latter events could be independent. This study suggests that naive and memory B lymphocytes have distinct requirements to engage an immune response, reflecting their different roles in humoral immunity. The Journal of Immunology, 2003, 171: 4621–4629.

Human peripheral blood B lymphocytes can be divided in two distinct populations: naive B lymphocytes (60%) and memory B lymphocytes (40%) (1). Previous studies have used membrane IgD expression to distinguish naive and memory B lymphocytes, considering that only IgD- cells were memory B lymphocytes (2, 3). The recent finding that the expression of CD27 is restricted to memory B lymphocytes has facilitated distinction between these two populations (1, 4, 5). CD27, belonging to the TNFR family (6), is a surface Ag expressed on a T lymphocyte subset (7) and on B lymphocytes carrying somatic hypermutation in their Ig V region genes, known as memory B lymphocytes (1). Both subsets can be further separated according to the expression of IgA, IgD, IgG, and IgM. All naive B lymphocytes are IgD+ IgM- CD27+, while memory B lymphocytes can be IgD- IgM+ CD27+, IgD- IgM- CD27-, or IgG+ IgA+ CD27+, representing, respectively, 15, 10, and 15% of circulating B lymphocytes (1). Following antigenic challenge, responding B lymphocytes proliferate and form germinal centers (8, 9) while interacting with activated T lymphocytes by direct cell-cell contact and soluble factors (10, 11). This response requires the stimulation of CD40 expressed on B lymphocytes by its ligand, CD154, expressed on activated T lymphocytes (12). The binding of CD154 induces CD40 trimerization, triggering a signaling cascade leading to maturational events (13).

The importance of CD40 stimulation has been demonstrated in patients suffering from X-linked hyper-IgM syndrome, in which CD154 mutations block CD40-CD154 interaction (14, 15). In these cases, B lymphocyte activation and Ig production are impaired, resulting in a nonfunctional immune response to T-dependent Ags (14). Studies using CD40/CD154 knockout mice pointed out the importance of CD40 signaling in B lymphocyte activation, proliferation, and differentiation (reviewed in Refs. 12 and 15). The failure of CD40-CD154 interaction shows that it is essential for isotype switching, somatic hypermutation, generation of memory B lymphocytes, and Ig production (14, 16). However, most of these studies were focusing on naive B lymphocytes and primary immune response. The involvement of CD40-CD154 interaction is more difficult to establish for memory B lymphocytes in relation to secondary immune response, since the disruption of CD40/CD154 blocks the generation of these cells in vivo models (14, 16). Nevertheless, immunohistochemical studies revealed that CD154-expressing T lymphocytes are found in close proximity to Ag-specific B lymphocytes after secondary immunization (17). In addition, the injection of neutralizing anti-CD154 Abs in mice inhibits the secondary humoral response to T-dependent Ags (15, 18), supporting that CD40 signaling is essential in memory B lymphocyte response.

In humans, the study of B lymphocyte activation via CD40 is limited to in vitro models. Until now, the impact of CD40 stimulation on B lymphocytes from peripheral blood or tonsils has been studied using different tools to stimulate CD40: cells transfected with CD154, anti-CD40 Abs (19–22), or soluble CD154 (23) in the presence of IL-4 (24, 25). A brief overview of the previous works suggested different effects of CD40 stimulation in memory and naive B lymphocyte responses. Some studies reported that the proliferation of memory and naive B lymphocytes was equivalent following short-term (21, 22) or long-term CD40 stimulation (26). In contrast, others have shown the predominance of IgD+ naive B lymphocyte proliferation over IgD- memory cells (19, 20, 27). Nevertheless, Ig secretion was restricted to differentiated memory B lymphocytes (19, 21–23, 27). Even though in vivo studies with mouse models demonstrated the requirement of CD40 stimulation for memory B lymphocyte response, the outcome of this population remains unclear in humans.

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To help clarify this matter, peripheral human B lymphocytes were separated into CD27⁻ memory and CD27⁺ naive B lymphocyte populations. Both subsets were stimulated in vitro through CD40 with a cell line expressing CD154 in the presence of IL-4. Proliferation and Ig secretion were monitored during short (4–5 days)- and long-term stimulations (>7 days). We observed a distinct response from naive and memory cells related to the duration of CD40 stimulation. Memory B lymphocytes differentiated rapidly to produce IgG and IgM with no expansion, followed by cell death. In contrast, naive CD27⁻ cells expanded rapidly and differentiated to secrete Ig only after a longer stimulation. This population also performed isotype switching and acquired CD27 expression in the absence of significant somatic hypermutation, suggesting that these events could be independent in the generation of memory B lymphocytes. These observations are in accordance with the role of both subsets in primary and secondary immune responses.

Materials and Methods

Isolation of human peripheral B lymphocytes

Blood samples were collected from healthy individuals after informed consent in heparinized tubes (Vacutainer; BD Labware, Franklin Lakes, NJ), pooled, and diluted in 1 vol of PBS (10 mM potassium/phosphate buffer with 136 mM NaCl (pH 7.4), Dulbecco’s PBS; Life Technologies, Grand Island, NY). PBMC were prepared by density centrifugation over Ficoll-Paque (Amersham Pharmacia Biotech, Baie D’Urfe, Canada). RBC were removed by lysis with 0.83% (v/v) NH₄Cl and platelets by a second centrifugation over Ficoll-Paque diluted 1/2 with PBS. B lymphocytes were purified by negative selection using the StemSep CD19 mixture according to the manufacturer’s instructions (StemCell Technologies, Vancouver, Canada). Purified human B lymphocytes were >90% CD19⁺ as determined by flow cytometry analysis. CD27⁻ and CD27⁺ B cells were separated by cell sorting, using an Epic Coulter (Beckman Coulter, Burlington, Canada), under sterile conditions. Both purified populations were >90% pure, as determined by flow cytometry.

Culture of human B lymphocytes

Purified B lymphocytes were seeded at 3.75 × 10⁶ cells/ml in Primaria plates (BD Labware) in the presence of 0.5 × 10⁻⁶ cells/cm² gamma-irradiated with 75 Gy (7500 rad) L4.5 expressing CD154 (28). The specificity of the response induced by CD154 expressed on L.929 (L4.5 cell line) has been previously confirmed using mock-transfected L929 cells and cytokines as described elsewhere (28). Human B lymphocytes were cultured in IMDM supplemented with 10% heat-inactivated ultra-low IgG FBS (Life Technologies as described elsewhere (28). Human B lymphocytes were cultured in irradiated mock-transfected L929 cells and cytokines as described elsewhere (28). Human B lymphocytes were cultured in IRBol mouse mAbs obtained from BD PharMingen (Oakville, Ontario, Canada). All stimulations were done with 1 μg of each Ab for 1 × 10⁶ cells at 4°C. Cells were fixed with 2% paraformaldehyde. In all analyses, over 95% of cells were double negative with markers set according to isotype-matched negative control staining. The regions containing dead cells were determined using 7-aminactinomycin D staining, following the manufacturer’s instructions (BD PharMingen). Analyses were done by gating 5000–10,000 cells with a FACSCalibur flow cytometer and the CellQuest software (BD Biosciences, Mountain View, CA).

RT-PCR amplification of activation-induced cytidine deaminase (AID) mRNA

Total RNA from L4.5, Ramos cell line (CRL-1596; American Type Culture Collection, Manassas, VA) (30), human B lymphocytes, and CD19⁺ and CD27⁻ B lymphocytes cultured for 28 days with L4.5 and IL-4 was isolated using TRIzol reagent (Invitrogen, Burlington, Ontario, Canada). RT-PCR amplification of AID using primer 5′-GAG GCA AGA AGA CAC TCT GG-3′ and 3′-CAA AAG GAT CCG CCG AAG CTG TCT GGA G-3′ generated a unique 569-bp fragment. RT-PCR was performed starting from 150 ng total RNA, using the Titan One Tube RT-PCR System according to the manufacturer’s instructions (Roche Diagnostics Canada, Laval, Quebec, Canada). Following a 30-min reverse transcription at 50°C, a PCR composed of 33 cycles of 45 s at 94°C, 45 s at 57°C, and 45 s at 72°C was applied. Cycling was followed by a final elongation period of 10 min at 72°C. No AID mRNA was detected in L4.5 cells. β-Actin amplification was used as internal control in RT-PCR, starting from 150 ng total RNA. The primers used in 5′-GGT GGC CCC CCC AGG CAG CA-3′ and 3′-CTC CTT AAT GTC ACG CAC GAG CTC GTA CAC-3′ generated a single 540-bp fragment. Negative control without RNA confirmed the absence of cross-contamination.

PCR amplification of human IgG1 V₄ regions

Total RNA from human B lymphocytes and CD19⁺ and CD27⁻ B lymphocytes cultured for 28 days with L4.5 and IL-4 was prepared and 750 ng was used for RT-PCR as described above. For amplification, a nondegenerated primer (D075: 5′-TGG TGG TAC CCA CTG AAC ACA GAG GAC TCA-3′) was used to hybridize to the cDNA 5′ untranslated region of all human V₄ family members, paired with a primer specific for the C₄ region of human IgG1 (βC₄): 5′-GCA GTG GTG AGA CTA GTC ACA AGA TTT GGG-3′. Following a 30-min reverse transcription at 50°C, a touchdown PCR cycling protocol was done as follow: 35 cycles of 30 s at 94°C, 60 s at 65°C (1°C decrease per cycle for the first 10 cycles, and the last 25 cycles at 55°C, and 60 s at 72°C. Cycling was followed by a final elongation period of 10 min at 72°C. Negative controls without RNA or with RNA isolated from the IgM-producing Ramos cell line confirmed the absence of cross-contamination.
Subcloning and sequencing of human IgG1 V_H PCR products

Human IgG1 V_H amplicons were resolved on 1% agarose gel electrophoresis and the band corresponding to amplicon size ranging between 730 and 830 bp was excised. DNA was purified using the QIAquick Gel Extraction kit (Qiagen, Mississauga, Ontario, Canada) and subcloned into the pCRII-TOPO vector (TOPO TA Cloning kit; Invitrogen) according to the manufacturer’s instructions. Plasmid DNA was isolated and purified by standard procedures (31) and inserts were sequenced by automated fluorescent DNA sequencing (ABI 373; PerkinElmer Applied Biosystems, Foster City, CA). From two donors, a total of 18 clones from cultured CD19+ and 16 from sorted CD27+ B lymphocytes were characterized. To establish the error rate of Taq DNA polymerase mix (Titan One Tube RT-PCR System), the C_H1 and C_H2 domains of the IgG constant region from 10 different clones were compared with sequences from GenBank. From this analysis, the Taq DNA polymerase error frequency was estimated to be 0.34%, which corresponds to 1 bp over 294.

Mutation analysis of IgG1 V_H genes

Mutation analysis of the V_H clones was done using the ImMunoGeneTics database available on the internet (the international ImMunoGeneTics database http://imgt.cines.fr:8104 (32–34)). The number of mutations was determined after the alignment of each sequenced amplicon with the germ-line gene presenting the highest homology from framework 1 to framework 3 regions, for a total of 312 bp. Mutation proportions were established from the ratio mutated bp/total number of bp.

Results

High level of IgM-secreting cells are generated following CD40 stimulation

In vitro proliferation and differentiation of human B lymphocytes can be achieved by stimulation with CD154 and IL-4 (25). As reported previously, in this system the cell growth is characterized by three phases (35): the initiation period (<7 days) followed by an exponential growth during 1 or 2 wk and a plateau phase where proliferation is stopped. We have evaluated the viability and cellular expansion of human peripheral blood B lymphocytes when stimulated through CD40 in the presence of IL-4 for 37 days (Fig. 1a). We observed that viability was stable (91 ± 5%) as long as the cellular expansion was high, but decreased rapidly from days 28 to 37 (81–28%) after expansion has reached its maximum (1000-fold) on day 23.

In parallel, the proportion of IgA-, IgG-, and IgM-secreting cells was evaluated on days 1, 6, 14, 22, 28, and 36 using ELISPOT assays (Fig. 1b). No Ig-secreting cells were detected on days 1 and 6. After 14 days, IgG- and IgM-secreting cells represented 2 and 8% of the total cells, respectively. The proportion of IgA-, IgG-, and IgM-secreting cells increased more rapidly after day 22, whereas expansion was maximum, reaching 7, 16, and 28% of the total cell number, respectively. Until day 28, IgM-secreting cells represented >70% of total Ig-secreting cells. IgA- and IgG-secretting cells increased more rapidly after 28 days, representing ~50% of total secreting cells. These results could be interpreted in three ways: long-term CD40 stimulation allows the expansion and differentiation of both memory and naive cells with or without isotype switching or no expansion of memory cells but expansion, differentiation, and isotype switching by naive cells.

CD27+ B lymphocyte proportion decreases rapidly following CD40 stimulation

In peripheral blood, IgA- and IgG-expressing cells are CD27+ memory B lymphocytes, whereas IgM-expressing cells are mainly (75%) CD27- naive B lymphocytes (4, 36). The previous observations showing that the balance between IgM- and isotype-switched Ig-secreting cells changed over time (Fig. 1) led us to analyze the progression of CD27+ memory and CD27- naive B lymphocyte populations in the presence of CD154 and IL-4. The expression of CD27 on CD19+ B lymphocytes was then evaluated by flow cytometry during a long-term stimulation (28 days; Fig. 2).

We observed that the proportion of CD27+ memory cells rapidly decreased from 23% on day 0 to 5% on days 6 and 9. Unexpectedly, a CD27+ population (25%) reappeared on day 14. This population kept increasing, reaching 46% on day 23 and remained around 40% until day 28. However, the expression level of CD27 on this emerging CD19+CD27+ population was lower (mean fluorescence intensity (MFI) = 26) than the expression level on peripheral B lymphocytes on day 0 (MFI = 80). These results indicate that memory cells could be able to modulate CD27 expression and to expand or that only CD27- naive B lymphocytes could be able to expand and acquire CD27 expression following long-term stimulation with CD154 and IL-4.

Proliferation of CD27+ memory B lymphocytes is engaged following CD40 stimulation, but does not lead to expansion

The decrease of CD27+ memory B lymphocytes (Fig. 2) could be caused by a modulation of CD27 expression or a loss of this population. To discriminate between these possibilities, the expansion of sorted CD27+ (92%) and CD27- (97%) B lymphocytes was evaluated in parallel with total CD19+ cells (93%) as control by viable cell counts (Fig. 3, a and b). The total cell number of all populations was stable during the first 5 days, with a viability of 92 ± 1%. On day 9, total CD19+ and CD27- naive cells started...
to expand at a similar rate with a high viability level (94 ± 2%). In contrast, the number of CD27+ cells decreased by almost 10-fold after 9 days and mortality was increased to 52%. These results suggest that CD27+ memory B lymphocytes are lost following CD40 stimulation as a result of cell death.

To further characterize the outcome of these B lymphocyte populations during the initiation phase, their proliferation was evaluated using BrdU incorporation after 1, 4, and 7 days of CD40 stimulation (Fig. 3c). As observed above (Fig. 3b), total CD19+, CD27 naïve, and CD27+ memory B lymphocytes showed similar proliferation levels during the first 4 days. After 7 days, the proliferation rate was increased in total and naive cells, while CD27+ cell proliferation remained as low as that of day 4. Then, the dramatic decrease observed in the CD27+ population can be attributed to a low proliferation combined with an increased cell death and not to the modulation of CD27 expression. These results suggest that CD27+ naïve B lymphocytes were disadvantaged over CD27+ memory B lymphocytes and represented the only subset able to expand in these culture conditions.

Memory B lymphocytes differentiate into Ig-secreting cells earlier than naive cells following CD40 stimulation

To evaluate the capacity of total CD19+, CD27+, and CD27+ B lymphocytes to differentiate into Ig-secreting cells following CD40 stimulation, we used standard ELISA. IgG and IgM secretion was monitored after short (5 days)- and long-term (14 and 22 days) stimulation of the sorted populations (Table I). After 5 days, only CD27+ memory B lymphocytes differentiated into IgG- and IgM-secreting cells. After 14 days, while sorted CD27+ cells were all dead, CD27+ cells started to differentiate into IgM- and IgG-secreting cells. On day 22, as indicated by IgG and IgM secretion, the differentiation level was indistinguishable in sorted CD27+ and total CD19+ B lymphocytes. Consequently, CD27+ cells were the only subset able to respond to the long-term CD40 stimulation by expansion and differentiation. In addition, these results show that memory B lymphocytes differentiate more rapidly than naive cells following CD40 stimulation. Also, it suggests the capacity of naive cells to perform isotype switching.

Naïve B lymphocytes can acquire CD27 expression following CD40 stimulation

Based on the results presented in Fig. 3 and Table I, we concluded that CD27+ naïve B lymphocytes were the only cell subset responding to prolonged CD40 stimulation. Since CD27+ cells emerged in high proportion (>40%) following a sustained stimulation (>23 days; Fig. 2), we assessed whether CD27 expression could be induced on naïve B lymphocytes. Total CD19+ and sorted CD27+ B lymphocytes were stimulated with CD154 and IL-4, and the expression of CD27 was evaluated by flow cytometry (Fig. 4). Total CD19+ and CD27+ B lymphocytes showed a similar progression of CD27 expression on days 9, 19, and 28 (Fig. 4). On day 28, populations issued from total CD19+ and naïve CD27+ B lymphocytes showed 31 and 37% CD19+CD27+ cells, respectively. The MFI was similar in both populations, but lower in emerging CD27+ B lymphocytes (MFI = 29) than in the initial peripheral populations (MFI = 61). In human tonsils, low expression of CD27 has been observed on a B cell subset (CD20+CD38low/−) in germinal center (37). We have also observed that these newly formed CD27+ B lymphocytes expressed a normal level of CD19 and a low level of CD38 (data not shown). In addition, most (>98%) of the cells were CD138− (data not shown). These results indicate that CD27 expression can be induced on naïve B lymphocytes, confirming that the newly formed CD27+ population can emerge from CD27− cells following long-term CD40 stimulation.

Long-term CD40 stimulation of naïve B lymphocytes can induce isotype switching

Following long-term stimulation, we observed a high level of IgG secretion from remaining naïve B lymphocytes (Table I). To verify the occurrence of isotype switching, the proportion of IgG+ cells was monitored in total CD19+ and sorted CD27− populations following CD40 stimulation (Fig. 5). As observed for CD27+ cells (Fig. 4), the IgG+ population decreased rapidly within the total CD19+ population and reappeared gradually during the culture. Total CD19+ and naïve CD27− B lymphocytes showed a similar

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FIGURE 2. The proportion of CD27+ memory B lymphocytes decreases rapidly following CD40 stimulation. Purified human B lymphocytes were cultured as described in Fig. 1, for 28 days (d). The expression of CD19 and CD27 was evaluated by flow cytometry using direct double staining with PE-conjugated anti-CD27 and allophycocyanin-conjugated anti-CD19 Abs. These results are representative of experiments done with cells from three donors.
CD40 stimulation. Total CD19/H11001 was measured by BrdU incorporation, as described in Materials and Methods. Proliferation (samples used in these experiments were representative of the two donors used). These naive B lymphocytes were cultured, as described in Fig. 1, for 22 days.

FIGURE 3. CD27+ memory B lymphocytes do not expand following CD40 stimulation. Total CD19+ and sorted CD27+ memory and CD27− naive B lymphocytes were cultured, as described in Fig. 1, for 22 (b) or 7 (c) days. The expression of CD19 and CD27 (a) was evaluated on total CD19+ and on sorted CD27+ and CD27− B lymphocytes by flow cytometry using direct double staining with PE-conjugated anti-CD27 and FITC-conjugated anti-CD19 Abs. These profiles are representative of the two samples used in b and c. Expansion factors (b) were determined as described in Fig. 1 for total CD19+, CD27−, and CD27+ B lymphocytes. Proliferation (c) was measured by BrdU incorporation, as described in Materials and Methods, on days 1, 4, and 7 for all subsets. Error bars can be smaller than symbols. These results are representative of experiments done with cells from three donors.

progression of IgG+ cells, reaching 7 and 9% on day 28, respectively, indicating that naive B lymphocytes have been able to switch.

To further investigate, the expression of AID mRNA, essential for isotype switching and somatic hypermutation (38), was monitored in comparison to β-actin mRNA in resting and CD40-activated B lymphocytes (Fig. 6). Amplification conditions allowed the detection of AID mRNA in the Ramos cell line, which expresses low levels of AID (30). In comparison to the low level observed in Ramos, the expression of AID was high and similarly induced in both sorted naive and total CD19+ cells. This further supports that naive B lymphocytes can carry out isotype switching.

In addition, triple staining was used to monitor CD27 and IgG expression within the CD27− population (Fig. 7). The first IgG+ cells detected were not expressing CD27. This IgG+CD27− population represented 77 and 68% of all IgG+ cells on days 9 and 19, respectively. In contrast, on day 28, the majority (69%) of the IgG+ population was expressing CD27. Similar results were obtained with total CD19+ B lymphocytes following long-term stimulation (data not shown). This last observation indicates that isotype switching and CD27 expression in naive B lymphocytes can occur independently on different populations or that IgG switching occurs before CD27 expression following long-term stimulation.

Emerging IgG+ B lymphocytes are characterized by the absence of somatic mutations in their V H genes

To rule out the possibility that residual CD27+ memory B lymphocytes could be responsible for the emerging CD27+ population, the presence of somatic mutations in IgG1 V H genes was evaluated for total CD19+ and sorted CD27− naive B lymphocytes after a 28-day CD40 stimulation (Table II). All sequences amplified were part of the larger V H family, V H 3. The number of mutations per clone (312 nt) ranged from 0 to 3 (≤0.96%) and 0 to 5 (≤1.6%) in total CD19+ and naive CD27− B lymphocytes, respectively. Considering a Taq DNA polymerase error frequency of 0.34%, the number of unmutated clones was 89 and 69% for total CD19+ and CD27− B lymphocytes, respectively. The remaining mutated clones showed 5 or less mutations, which can be expected for this in vitro stimulation (39, 40) but is not significant when compared with 13–34 mutations obtained from peripheral memory B lymphocytes. Despite the fact that AID mRNA was expressed in both populations (Fig. 6), it was not sufficient to achieve somatic hypermutation. Overall, the low frequency of somatic mutations in IgG+ cells confirmed the disappearance of memory B lymphocytes and the switching to IgG by naive cells following long-term CD40 stimulation. It also suggests that CD27 expression can be acquired without somatic hypermutation and that the rapid loss of the CD27+ population corresponds to the loss of memory B lymphocytes.

Table I. Memory B lymphocytes differentiate more rapidly than naive cells after CD40 stimulation

<table>
<thead>
<tr>
<th>Days</th>
<th>Populations</th>
<th>IgG+ (ng/ml)</th>
<th>IgM+ (ng/ml)</th>
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<tbody>
<tr>
<td>5</td>
<td>Total CD19+</td>
<td>188 ± 4</td>
<td>19 ± 1</td>
</tr>
<tr>
<td></td>
<td>CD27</td>
<td>&lt;10</td>
<td>&lt;10</td>
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<td>CD27+</td>
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<td>Total CD19+</td>
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<td>388 ± 17</td>
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<td></td>
<td>CD27+</td>
<td>800 ± 57</td>
<td>2000 ± 221</td>
</tr>
<tr>
<td>22</td>
<td>Total CD19+</td>
<td>860 ± 28</td>
<td>1800 ± 128</td>
</tr>
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* Total CD19+ and sorted CD27+ and CD27− B lymphocytes from two donors were cultured, as described in Fig. 1, for 5 or 22 days as indicated.

* IgG+ and IgM secretion was determined by standard ELISA as described in Materials and Methods.
Naive B lymphocytes could be advantaged over memory cells by L4.5

In vivo, CD40 stimulation of memory and naive B lymphocytes occurs in a complex environment involving Ag, cytokines, and accessory cells. In vitro, simple signals such as IL-4 and CD154 (20, 24, 25) and more recently IL-10 and CD154 (41) have been reported to induce proliferation in tonsillar and splenic naive and memory B lymphocytes. To verify that CD154 and IL-4 were not limiting for memory B lymphocyte response, we used additional signals, including IL-2, IL-10, and SAC, for 3–5 days. Based on the relationship observed between the rapid disappearance of CD27⁺ cells and the absence of somatically mutated cells (Fig. 4 and Table II), we used as an indicator the expression of CD27 and IgG to follow memory cells (Table III). SAC and IL-2, known to activate memory B lymphocytes (42), allowed their proliferation and maintenance. Similar results were obtained with SAC and IL-4 stimulation, suggesting that IL-4 was not limiting for memory cell response. However, the addition of L4.5 and IL-10 to SAC and IL-2, as well as L4.5 and IL-10 signals did not allow the maintenance of CD27⁺ and IgG⁺ cells. These results suggest that stimulation through the B cell receptor (SAC) without L4.5 activated significantly memory B lymphocytes, while L4.5 addition to these signals seemed to favor naive cell expansion. This advantage of naive cells over memory cells could result from the nature of the CD154 signal provided by the L4.5 cell line.

Discussion

Immune responses to T-dependent Ags are distinct whether or not it is the first encounter with this Ag. During the primary responses, naive B lymphocytes proliferate, differentiate into Ig-secreting cells, and form in parallel a pool of memory B lymphocytes. These memory B lymphocytes are then responsible for the secondary response.
immune responses in which memory cells proliferate and differentiate to produce Ig of higher affinity. Considering the roles of naive and memory B lymphocytes within humoral response kinetics, we could expect that these populations have distinct activation requirements (43, 44). We showed here that naive and memory B lymphocytes isolated from peripheral blood did not respond in the same manner to the same CD154 stimulation. In addition, the short- and long-term periods used to stimulate both populations seemed to amplify these differences. In fact, memory B lymphocytes did not expand, but rapidly differentiated to secrete Ig and started to die (Fig. 3 and Table I), while naive B lymphocytes proliferated exponentially, then differentiated to secrete IgM and IgG and started to express CD27 (Figs. 4, 5, and Table I). Overall, short-term stimulations favored the differentiation of memory B lymphocytes, whereas long-term stimulations favored naive B lymphocyte expansion and differentiation.

Previous studies using peripheral or tonsillar B lymphocytes reported contrasting behavior of naive and memory cells following CD40 stimulation. In this study, we showed that these discrepancies could be related to the duration of the CD154 signal. Interestingly, the studies showing that memory B lymphocyte proliferation was equivalent to naive cells (21, 22) used a 3-day CD40 stimulation and those reporting a lower proliferation for memory cells than naive cells (19, 27) used a 5- to 7-day stimulation. Our results confirm both of these findings and underline the long-term predominance of naive cells. Until now, Ig secretion has been restricted to differentiated memory B lymphocytes (19, 21–23, 27), but the duration of CD40 stimulation in each case has never exceeded 14 days. This CD40 stimulation period was probably too short to allow Ig secretion from differentiated naive cells as we report here.

Table II. Few somatic mutations are detected in IgG V region genes from IgG\(^+\) cells emerging from total CD19\(^+\) and CD27\(^+\) naive B lymphocytes after 28 days of CD40 stimulation

<table>
<thead>
<tr>
<th>Total Clones(^a)</th>
<th>Mutations/Clone(^b)</th>
<th>Mean of Mutations (%)</th>
<th>Unmutated Clones(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD19(^+)</td>
<td>18</td>
<td>0</td>
<td>8/18</td>
</tr>
<tr>
<td>1</td>
<td>0.32</td>
<td>8/18</td>
<td>0.23 ± 0.26</td>
</tr>
<tr>
<td>2</td>
<td>0.64</td>
<td>1/18</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.96</td>
<td>1/18</td>
<td></td>
</tr>
<tr>
<td>CD27(^+)</td>
<td>16</td>
<td>0</td>
<td>7/16</td>
</tr>
<tr>
<td>1</td>
<td>0.32</td>
<td>4/16</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.64</td>
<td>2/16</td>
<td>0.44 ± 0.57</td>
</tr>
<tr>
<td>3</td>
<td>1.3</td>
<td>1/16</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1.6</td>
<td>2/16</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>9</td>
<td>13–20</td>
<td>39</td>
</tr>
<tr>
<td>CD19(^+)</td>
<td>21–30</td>
<td>6.7–9.6</td>
<td>49</td>
</tr>
<tr>
<td>31–34</td>
<td>9.9–10.9</td>
<td>29</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Total number of V\(_H\) clones analyzed per population from two donor samples.

\(^b\) Number of point mutations detected per V\(_H\) clone of 312 bp, after sequence alignment with germline V\(_H\) genes, as described in Materials and Methods.

\(^c\) The proportion of unmutated clones is obtained by the addition of the number of clones with 0 and 0.32% of mutations, which is under the error frequency related to the amplification (0.34%).

\(^d\) Peripheral memory B lymphocytes were used as positive control.
Only a few studies evaluated the response of naive and memory B lymphocytes to long-term CD40 stimulation. Their results demonstrated the expansion of memory cells in vitro, but were obtained from tonsillar B cells (20, 26). Contrary to resting B lymphocytes in blood, tonsillar populations potentially contain activated cells (19) that could be responsible in part for the discrepancy between these results and ours. Overall, all of these studies and ours indicate that naive and memory B lymphocyte response to CD40 stimulation is composed of sequential events that are closely related to the duration of CD40 stimulation.

In vivo, CD40-CD154 interaction is involved in the early immune response of naive B lymphocytes, leading to the generation of memory cells in germinal centers (45). In vitro, CD154 and IL-4 signals stimulated naive B lymphocytes to secrete unmutated IgM and IgG and triggered the expression of AID, CD27, and CD38 but not CD138. These emerging cells were not fully differentiated plasma cells nor memory cells. Our in vitro model seems to allow the accumulation of intermediates that could be temporarily present in germinal centers following CD40 stimulation. The absence of a more complex environment could be responsible for this pause in the differentiation of naive B lymphocytes from the periphery. Interestingly, the generation of these intermediates suggests that isotype switching could occur before or independently of CD27 expression (Fig. 7) and that CD27 expression can be acquired in the absence of somatic hypermutation (Table II). In vivo, CD27+ B lymphocytes showing low somatic mutations can be found in patients suffering from common variable immunodeficiency (46) and persistent polyclonal B cell lymphocytosis (47). In normal germinal centers, the sequence in which CD27 expression, isotype switching, and somatic hypermutation occurs during memory B lymphocyte generation is unknown (48). Although isotype switching has already been described in CD40-stimulated naive B lymphocytes (39, 49, 50), our observations are new indications concerning the independence and the sequence of these events.

Following CD154 and IL-4 stimulation, we showed a distinct response from peripheral naive and memory B lymphocytes. Our results also suggest that even when positive signals for memory B lymphocytes (SAC + IL-2, and IL-10) are added to L4.5 (Table III), naive cells seem advantaged. Such predominance of naive B lymphocytes could be explained by competition mechanisms, resulting from the fact that both populations are receiving the same signals at the same time. Since both resting populations expressed the same level of CD40 (data not shown), the advantage of naive B lymphocytes could also depend on the cell line used, which expresses a heterogeneous level of CD154 (51). We believe that the level of CD154 expression could play a role in the response of naive and memory B lymphocytes, as both subsets seem to require help from naive and memory T cells, respectively (52–55). Since the level of CD154 expression on memory T lymphocytes is variable upon activation (56, 57), the intensity of the CD154 signal required to enhance memory B lymphocyte expansion remains unclear. We are currently investigating the effect of intensity variation in CD40-CD154 interaction on naive and memory B lymphocyte responses.

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References


Table III. Naïve B lymphocytes could be advantaged over memory cells by L4.5

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Conditions</th>
<th>Expansion Factor</th>
<th>% of Positive Cells</th>
<th>CD19</th>
<th>CD27</th>
<th>IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (3 days)</td>
<td>Resting</td>
<td>98.6</td>
<td>20.2</td>
<td>2.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAC + IL-2</td>
<td>1.6</td>
<td>98.0</td>
<td>21.9</td>
<td>3.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAC + IL-4</td>
<td>1.4</td>
<td>96.4</td>
<td>24.7</td>
<td>2.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 (4 days)</td>
<td>Resting</td>
<td>98.0</td>
<td>36.5</td>
<td>10.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L4.5 + IL-4</td>
<td>2.9</td>
<td>98.4</td>
<td>7.5</td>
<td>2.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L4.5 + IL-10</td>
<td>4.1</td>
<td>97.7</td>
<td>2.0</td>
<td>2.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 (5 days)</td>
<td>Resting</td>
<td>97.2</td>
<td>21.5</td>
<td>2.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L4.5 + IL-10</td>
<td>5.0</td>
<td>99.1</td>
<td>4.1</td>
<td>1.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAC + L4.5 + IL-2 + IL-10</td>
<td>5.7</td>
<td>99.7</td>
<td>2.7</td>
<td>1.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Total CD19+ B lymphocytes were cultured as described in Materials and Methods with L4.5 or SAC with cytokines as indicated.

b Expansion factor was calculated on the last day in function of seeding cell numbers.

c Proportion of cells expressing CD19, CD27, and IgG was determined by flow cytometry using triple staining as described in Materials and Methods.


