Generation of Memory B Cells and Plasma Cells in Vitro

Christophe Arpin, Julie Déchanet, Cees Van Kooten, Pierre Merville, Géraldine Grouard, Francine Brière, Jacques Banchereau, Yong-Jun Liu*

After germinal center B cells undergo somatic mutation and antigen selection, they become either memory B cells or plasma cells, but the signal requirements that control entry into either pathway have been unclear. When purified human germinal center cells were cultured with interleukin-2, interleukin-10, and cells expressing CD40 ligand, cells with characteristics of memory B cells were generated. Removal of CD40 ligand from the system resulted in terminal differentiation of germinal center B cells into cells with the characteristics of plasma cells. These results indicate that CD40 ligand directs the differentiation of germinal center B cells toward memory B cells rather than toward plasma cells.

B cells produce antibodies that eliminate certain pathogens. T cell–dependent humoral (antibody) immune responses are initiated by activation of naïve and memory B cells in the T cell–rich extrafollicular areas of the secondary lymphoid organs (*1*). This initial B cell activation generates short-lived plasma cells and recruits germinal center (GC) precursors into B cell follicles (*1*). GCs develop by proliferation, mutation (*2*), and antigen-driven selection, and the resulting population differentiates into either memory B cells (3) or plasma cells (*4*). Here, we analyze the molecular mechanism that controls the differentiation of GC B cells into memory B cells and plasma cells.

Human GC B cells, memory B cells, and plasma cells can be distinguished by their expression of antigens CD38 and CD20 (Fig. 1A): GC B cells are $CD38^+CD20^+$ (*5*, 6), memory B cells are $CD38^-CD20^+$ (*7*), and plasma cells are $CD38^+CD20^-$ (6, *8*). Accordingly, we used these markers to follow the differentiation of GC B cells into either plasma cells or memory B cells in vitro. A primary culture was set up to mimic the stage at which high-affinity GC B cells pick up antigen from follicular dendritic cells and present it to GC T cells that express CD40 ligand (CD40L) (9). Inter-

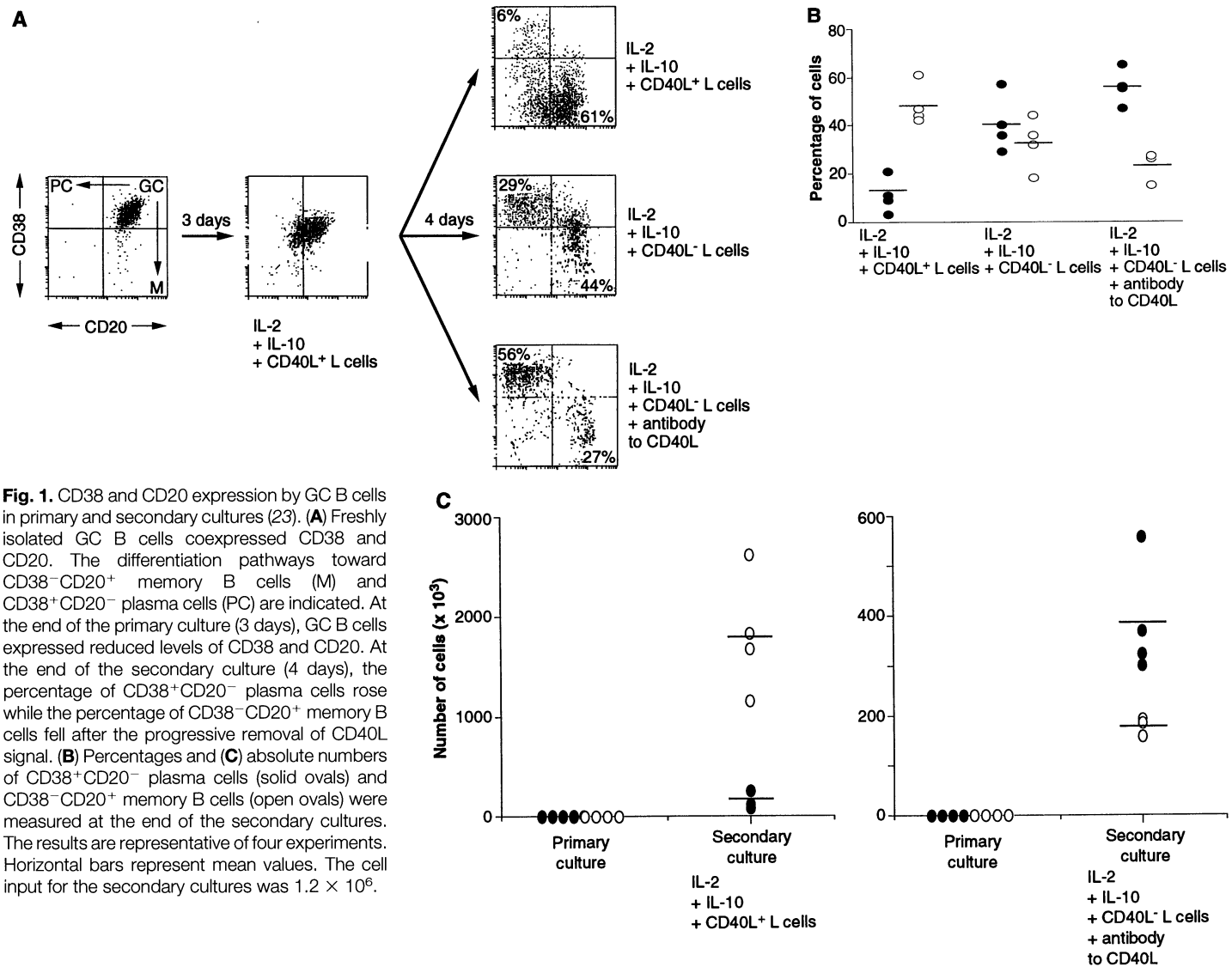

Fig. 1. CD38 and CD20 expression by GC B cells in primary and secondary cultures (*23*). (**A**) Freshly isolated GC B cells coexpressed CD38 and CD20. The differentiation pathways toward $CD38^-CD20^+$ memory B cells (M) and $CD38^+CD20^-$ plasma cells (PC) are indicated. At the end of the primary culture (3 days), GC B cells expressed reduced levels of CD38 and CD20. At the end of the secondary culture (4 days), the percentage of $CD38^+CD20^-$ plasma cells rose while the percentage of $CD38^-CD20^+$ memory B cells fell after the progressive removal of CD40L signal. (**B**) Percentages and (**C**) absolute numbers of $CD38^+CD20^-$ plasma cells (solid ovals) and $CD38^-CD20^+$ memory B cells (open ovals) were measured at the end of the secondary cultures. The results are representative of four experiments. Horizontal bars represent mean values. The cell input for the secondary cultures was 1.2×10^6.

leukin-2 (IL-2) plus IL-10 induced strong proliferation of GC B cells cultured on L cells transfected with CD40L, as previously reported for total tonsillar B cells (*10*). After 3 days in culture, the cells were in an exponential growth phase, produced little immunoglobulin (Ig), expressed reduced amounts of CD38 and CD20 (Fig. 1A), displayed blastic morphology, and contained small amounts of intracytoplasmic Ig. Thus, GC B cells were rescued from apoptosis and underwent proliferation but had not differentiated into plasma cells and memory B cells.

The secondary culture conditions were set up on the basis of two considerations. First, it has been shown that CD40L expression on T cells is transient (*11*). Second, proliferation and differentiation are mutually exclusive events controlled by a balance of opposing cellular signals. For example, removal of IL-7 was shown to lead to maturation of pre-B cells into surface immunoglobulin (sIg)–positive B cells (*12*). Thus, in the secondary culture, CD40L—a key molecule for the survival (*5*) and proliferation (*13*) of GC B cells—was removed. Cells from the primary culture were recultured for 4 days under three conditions: with fresh CD40L-transfected L cells, IL-2, and IL-10; with nontransfected L cells, IL-2, and IL-10; and with nontransfected L cells, IL-2, and IL-10 plus antibody to CD40L. The antibody was added to block the activity of the remaining CD40L-positive L cells harvested from the primary culture.

The results of the secondary culture indicate that the differentiation of $CD38^+CD20^+$ GC B cells into either $CD38^-CD20^+$ cells or $CD38^+CD20^-$ cells is directed by CD40L. Both the absolute number and the percentage of cells with the $CD38^+CD20^-$ plasma cell phenotype increased after the progressive removal and inhibition of CD40L (Fig. 1), whereas the absolute number and percentage of cells with the $CD38^-CD20^+$ memory B cell phenotype decreased. The amounts of Ig secretion at the end of the secondary culture correlated with cell phenotype (Table 1). The relatively small amounts of immunoglobulin M (IgM) rule out an expansion of CD5-positive contaminating IgM plasmablasts (*14*). After all cultures, cells had lost CD10 (a GC marker) but maintained CD40 expression, while CD39 and CD44 (memory and plasma cell markers) were appropriately induced.

The morphology and intracellular Ig content of purified $CD38^-CD20^+$ and $CD38^+CD20^-$ cells were analyzed and compared with those of the starting $CD38^+CD20^+$ GC B cells. Freshly isolated $CD38^+CD20^+$ GC B cells are medium to large lymphocytes showing typical nuclei with a cleft or multiple nucleoli (Fig. 2A). They have weak intracellular staining with antibody to Ig (anti-Ig) (Fig. 2B). Sorted $CD38^-CD20^+$ cells are blastic cells with very weak intracellular anti-Ig staining (Fig. 2, C and D). Sorted $CD38^+CD20^-$ cells have the morphology typical of terminally differentiated plasma cells: They are oval cells with compact, dense, eccentric nuclei; the heterochromatin is organized in a patchy, wheel-like pattern; and they have basophilic cytoplasms with pale Golgi zones (Fig. 2E). The strong intracellular anti-Ig staining further confirms that these cells are plasma cells (Fig. 2F).

To demonstrate that the $CD38^+CD20^-$ plasma cells were terminally differentiated, we determined their proliferative capacity in a tertiary culture with CD40L-transfected L cells, IL-2, and IL-10 or with anti-Ig, IL-2, and IL-10. The reactivity of the $CD38^-CD20^+$ memory B cells was assessed in parallel. The $CD38^-CD20^+$ memory cells, but not the $CD38^+CD20^-$ plasma cells, proliferated strongly when cultured with CD40L-transfected L cells, IL-2, and IL-10 (Fig. 3A) and underwent moderate DNA synthesis when cultured with anti-Ig, IL-2, and IL-10 (Fig. 3B).

Table 1. Production of immunoglobulins IgG, IgA, and IgM at the end of the secondary cultures. Cells were purified and cultured as described (Fig. 1), and immunoglobulins were quantitated by enzyme-linked immunosorbent assay (*10*). The results are representative of four experiments.

Culture conditions	Isotype (picograms per cell)			
	IgG	IgA	IgM	Total
IL-2 + IL-10 + $CD40L^+$ L cells	7.6	2.3	6.3	16.2
IL-2 + IL-10 + $CD40L^-$ L cells	29.8	5.3	14.6	49.7
IL-2 + IL-10 + $CD40L^-$ L cells + antibody to CD40L	43.7	9.2	22.6	75.5

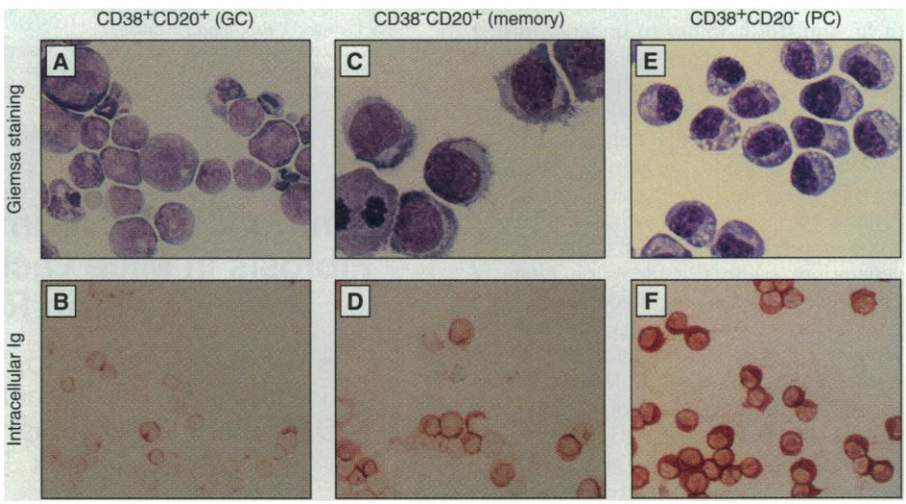

Fig. 2. Morphology and intracellular Ig content. (**A** and **B**) $CD38^+CD20^+$ fresh GC B cells. (**C** and **D**) $CD38^-CD20^+$ memory B cells and (**E** and **F**) $CD38^+CD20^-$ plasma cells (PC) were isolated by fluorescence-activated cell sorting (FACS). Staining was done with Giemsa stain (original magnification, ×1000) and red anti-Igκ + λ light chain alkaline phosphatase (original magnification, ×400).

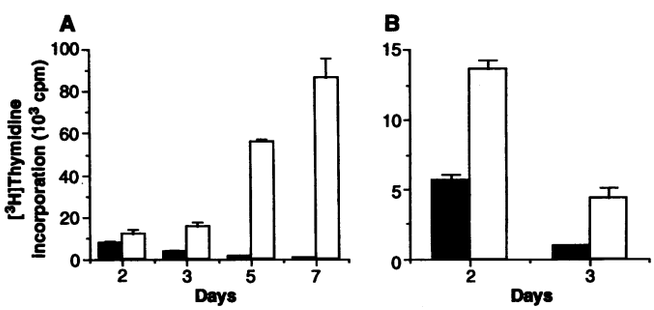

Fig. 3. Proliferation of $CD38^-CD20^+$ memory B cells (white bars) and $CD38^+CD20^-$ plasma cells (black bars) in tertiary cultures. In (**A**), 25,000 cells were cultured with IL-2 and IL-10 on 5000 irradiated CD40L-transfected L cells for 7 days in 200 μl of medium in 96 flat-bottomed well plates (*10*). In (**B**), 200,000 cells were cultured with IL-2, IL-10, and anti-Igκ + λ light chain antibodies (2 μg/ml) fixed on 10,000 irradiated CD32-transfected L cells for 3 days (*10*). [^{3}H]Thymidine (1 μCi) was added to each well for a 10-hour pulse. Values are mean ± SD of triplicates.

Schering-Plough, Laboratory for Immunological Research, 27 chemin des Peupliers, BP 11, 69571 Dardilly Cedex, France.

*To whom correspondence should be addressed.

The $CD38^-CD20^+$ cells generated in our culture system have all the features of memory B cells: phenotype, ability to respond to proliferative signals, and low levels of intracytoplasmic and secreted Ig. Although most memory B cells isolated from human tonsils are medium-size resting B cells (*7*), the memory B cells generated here are large cells. Large memory B blasts have been identified in vivo in the B cell follicles, where they have been construed as long-term memory B cells undergoing chronic stimulation by immune complexes on follicular dendritic cells (*1*), and among the recirculating thoracic duct lymphocytes, where they have been construed as memory B cells recently generated from GCs (*15*). The generation of $CD38^-CD20^+$ memory B blasts from GC B cells described here will provide a model to identify the signals that allow such blasts to revert to small resting cells.

Our results show that interruption of the CD40 signal after 3 days of primary culture results in the terminal differentiation of proliferating B blasts into plasma cells. They are characterized by typical phenotype and morphology, large amounts of intracellular Ig and secretion of large amounts of Ig, and the inability to undergo further proliferation. During humoral immune responses, responding B cells differentiate into either memory B cells or plasma cells. These two facets of the response must be tightly integrated to ensure adequate amounts of antibody production and the generation of memory B cells. CD40L plays a critical role at two stages of memory B cell generation: (i) in the induction phase of GC reaction, as demonstrated by the lack of GCs in hyper-IgM patients (*16*) and in mouse models where CD40-CD40L interactions were interrupted (*17, 18*), and (ii) in the differentiation phase of high-affinity GC B cells toward memory B cells, as demonstrated here. This second phase (CD40L-dependent GC B cell differentiation) was also suggested by an interesting in vivo observation: Mice receiving soluble CD40-IgG chimeric molecules have normal GCs but no memory B cell generation. Because soluble CD40-IgG chimeric molecules have a lower affinity for CD40L than do antibodies to CD40L, this observation suggests that the GC differentiation phase is more easily blocked than the GC induction phase (*19*). The recent identification of T cells that express CD40L in the light zone of GCs from human tonsils (9) supports the hypothesis that the GC differentiation phase occurs here (*20*). After somatic mutation and positive selection, high-affinity GC B cells pick up antigen from follicular dendritic cells and present it to GC T cells (*20*). During this cognate T-B cell interaction, T cells may be induced to secrete cytokines and to express CD40L (*21*), resulting in the generation of $CD38^-CD20^+$ memory B blasts. Because CD40L expression on T cells can be rapidly down-regulated by CD40 antigen on B cells (*11*), a proportion of proliferating B blasts will differentiate into $CD38^+CD20^-$ plasma cells in the absence of CD40L signaling.

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23. GC B cells were isolated from total tonsillar B cells as described (*6, 22*). The purity of isolated GC B cells (Fig. 1A) was greater than 98% in all experiments. Their GC nature was confirmed by their mutated immunoglobulin V genes (*7*). For the primary culture, cells (10^6/ml) were cultured with IL-10 (100 ng/ml) and IL-2 (10 U/ml) on CD40L-transfected murine fibroblasts (2×10^5 fibroblasts per milliliter) irradiated with 75 Gy (where 1 Gy equals 100 rads) in Iscove medium containing 5% fetal calf serum for 3 days (*10*). For the secondary culture, the cells were washed and recultured for 4 days under three conditions, as described in the text. The antibody to CD40L used in the third condition was LL2 (C. Van Kooten, in preparation).

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Immune System Impairment and Hepatic Fibrosis in Mice Lacking the Dioxin-Binding Ah Receptor

Pedro Fernandez-Salguero, Thierry Pineau, David M. Hilbert, Timothy McPhail, Susanna S. T. Lee, Shioko Kimura, Daniel W. Nebert, Stuart Rudikoff, Jerrold M. Ward, Frank J. Gonzalez*

The aryl hydrocarbon (Ah) receptor (AHR) mediates many carcinogenic and teratogenic effects of environmentally toxic chemicals such as dioxin. An AHR-deficient ($Ahr^{-/-}$) mouse line was constructed by homologous recombination in embryonic stem cells. Almost half of the mice died shortly after birth, whereas survivors reached maturity and were fertile. The $Ahr^{-/-}$ mice showed decreased accumulation of lymphocytes in the spleen and lymph nodes, but not in the thymus. The livers of $Ahr^{-/-}$ mice were reduced in size by 50 percent and showed bile duct fibrosis. $Ahr^{-/-}$ mice were also nonresponsive with regard to dioxin-mediated induction of genes encoding enzymes that catalyze the metabolism of foreign compounds. Thus, the AHR plays an important role in the development of the liver and the immune system.

The AHR is a ligand-activated transcription factor that is distinct from members of the steroid receptor superfamily (*1*). It is a member of the basic helix-loop-helix (bHLH) superfamily of DNA binding proteins and is activated by ligand binding and by dimerization with the AHR nuclear translocator (Arnt) (*1, 2*). A functional AHR is required in laboratory animals to mediate the harmful effects of toxic environmental chemicals such as dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin, or TCDD), benzo[*a*]pyrene in cigarette smoke and the products of other combustion processes, poly-