

# Dendritic Cells Enhance Growth and Differentiation of CD40-activated B Lymphocytes

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## Summary

After antigen capture, dendritic cells (DC) migrate into T cell-rich areas of secondary lymphoid organs, where they induce T cell activation, that subsequently drives B cell activation. Here, we investigate whether DC, generated *in vitro*, can directly modulate B cell responses, using CD40L-transfected L cells as surrogate activated T cells. DC, through the production of soluble mediators, stimulated by 3- to 6-fold the proliferation and subsequent recovery of B cells. Furthermore, after CD40 ligation, DC enhanced by 30–300-fold the secretion of IgG and IgA by sIgD<sup>-</sup> B cells (essentially memory B cells). In the presence of DC, naive sIgD<sup>+</sup> B cells produced, in response to interleukin-2, large amounts of IgM. Thus, in addition to activating naive T cells in the extrafollicular areas of secondary lymphoid organs, DC may directly modulate B cell growth and differentiation.

At the antigen/pathogen port of entry (e.g., mucosa, epidermis), dendritic cells (DC)<sup>1</sup>, such as Langerhans cells (LC), capture the antigen and then migrate via the afferent lymphatics into the T cell-rich areas of regional lymph nodes, where they are called interdigitating dendritic cells (IDC) (for review see reference 1). There, they present processed antigen to naive T cells and generate an antigen-specific primary T cell response. Once primed by DC, T cells can promote B cell activation, both by releasing T cell-derived cytokines such as IL-2, IL-4, and IL-5, and by direct intercellular contacts (for review see reference 2). Among the signals involved in T/B cell cooperation, the interaction between CD40 (on B lymphocytes) and its ligand (CD40L) expressed on activated T cells, appears to be of critical importance (for review see reference 3). CD40, a molecule related to the TNF receptor family, is expressed on multiple cell types, including mature B cells and bone marrow-derived DC (4). Cross-linking of CD40 promotes B cell survival (5) proliferation (6) as well as B cell differentiation and immunoglobulin class switching (7, 8). The ligand for CD40, CD40L, is a TNF family member expressed on activated but not resting T cells (9). The importance of CD40/CD40L pathway in B cell immunopoiesis has been demonstrated *in vivo* in patients with X-linked hyper IgM syndrome (for review see reference 10).

The role of DC in humoral responses has been documented *in vitro* (11) and *in vivo* (12–15). Notably, DC in-

cubated *in vitro* with antigen can induce, upon reinjection into mice, a protective humoral response (15). The critical role of DC in induction of humoral responses is viewed as a consequence of T cell priming, required for cognate interaction between T cells and B cells. However, as the primary B cell activation occurs within the extrafollicular T cell-rich areas (16), we wondered whether, in addition to priming T cells, DC were able to interact directly with B cells. Accordingly, we set up a system in which a CD40L-transfected murine cell line (CD40L L cells) was used as surrogate activated T cells, to study the effects of DC on B cell activation. Recent studies have indicated the possibility of generating large numbers of DC *in vitro* starting either from unseparated blood or bone marrow populations or from purified CD34<sup>+</sup> hematopoietic progenitors (17). DC, generated by culturing human CD34<sup>+</sup> hematopoietic progenitors with GM-CSF and TNF $\alpha$  have been shown earlier to induce a strong proliferation of allogeneic T cells (18, 19) and to express a functional CD40, the triggering of which induces their maturation into cells with characteristics of IDC (20). These *in vitro*-generated DC have been shown to contain LC as well as other DC related to dermal DC and thus were termed dendritic-Langerhans cells (D-Lc) (21).

Here, we demonstrate that D-Lc can strongly enhance both proliferation and Ig production of CD40-activated naive and memory B cells. These results suggest that DC might directly be involved in the extrafollicular plasma cell formation during induction of primary naive B cell responses or reactivation of secondary memory B cell responses.

<sup>1</sup>Abbreviations used in this paper: DC, dendritic cells; D-Lc, dendritic-Langerhans cells; GC, germinal center; IDC, interdigitating dendritic cells.

## Materials and Methods

**Reagents and Cell Lines.** Cultures of CD34<sup>+</sup> progenitors were established in RPMI-1640 (GIBCO BRL, Gaithersburg, MD) supplemented with 10% (vol/vol) FCS, 10 mM HEPES, 2 mM l-glutamine,  $5 \times 10^{-2}$  M 2-mercaptoethanol, and 0.08  $\mu$ g/ml gentamycin (gentalline; Schering Plough, Levallois Perret, France), which will be referred to as complete RPMI. Cultures of B lymphocytes were carried out in modified Iscove's medium, supplemented with 10% inactivated FCS (MultiSer, Castell Hill, NSW), 2 mM l-glutamine (GIBCO BRL, Gaithersburg, MD) and 0.08  $\mu$ g/ml gentamycin.

The murine CD40 L-transfected cell line (CD40L L cells) was produced in the laboratory as described earlier (22) and used as stimulator of B cell proliferation and differentiation. Mouse fibroblastic L cells stably transfected with the human CD32/Fc $\gamma$ RII have been previously described (23). A chimeric fusion protein between the mouse CD8- $\alpha$  and the human CD40 T cell ligand was constructed in our laboratory as previously reported (22) and produced in COS cells.

rhGM-CSF (specific activity:  $2 \times 10^6$  U/mg; Schering Plough Research Institute, Kenilworth, NJ) was used at a saturating concentration of 100 ng/ml (200 U/ml). rhTNF- $\alpha$  (specific activity,  $2 \times 10^6$  U/mg; Genzyme, Boston, MA) was used at an optimal concentration of 2.5 ng/ml (50 U/ml). Purified rhIL-2 ( $5 \times 10^4$  U/mg) from Amgen (Thousand Oaks, CA) was used at 20 U/ml. Purified rhIL-4 and rhIL-10 ( $10^7$  U/mg each; Schering Plough Research Institute, Kenilworth NJ) were used at 50 U/ml and 20 ng/ml, respectively.

**Generation of D-Lc.** Umbilical cord blood samples were obtained according to institutional guidelines. Cells bearing CD34 antigen were isolated from mononuclear fractions through positive selection, using anti-CD34 mAb (10  $\mu$ g/ml; Immuno-133.3; Immunotech Marseille, France) and goat anti-mouse IgG-coated microbeads (Miltenyi Biotec GmbH, Bergish Gladbach, Germany). Isolation of CD34<sup>+</sup> progenitors was achieved using Mini-macs separation columns (Miltenyi Biotec) (21).

Cultures were established in the presence of GM-CSF and TNF- $\alpha$  in complete RPMI. CD34<sup>+</sup> cells were seeded for expansion in 24-well culture plates (Limbro, Flow Laboratories, McLean, VA) at  $2 \times 10^4$  cells/ml. Optimal conditions were maintained by splitting these cultures every 4–5 d. Cells were routinely collected at day 12 when the cultures contained between 70–90% of CD1a<sup>+</sup> DC (18, 21). These CD1a<sup>+</sup> cells includes LC that express Birbeck granules as well as other DC and thus will be referred to as D-Lc throughout the text (21). The CD1a<sup>-</sup> population includes granulocytes, monocytes, undifferentiated precursors and, in some instances, mature DC that have lost CD1a.

For certain experiments, after 12 d of culture in the presence of GM-CSF and TNF- $\alpha$ , cells generated from CD34<sup>+</sup> progenitors were collected and labeled with FITC-conjugated OKT6 (CD1a; Ortho Diagnostic). Cells were separated according to CD1a expression into CD1a<sup>-</sup> and CD1a<sup>+</sup> fractions using a FAC-Star<sup>®</sup> (Becton Dickinson, Mountain View, CA). The procedure of staining and sorting was performed in the presence of 5 mM EDTA in order to avoid cell aggregation. Reanalysis of the sorted populations showed a purity higher than 98%.

**Isolation of Tonsillar B Cells.** Mononuclear cells from tonsils were isolated by a standard Ficoll-HyPaque (dose = 1,077 g/ml) gradient method. Tonsillar B cells were first enriched in the E<sup>-</sup> fraction and submitted to anti-CD2, anti-CD4, anti-CD8, anti-CD14, anti-CD16 mAb negative selection with magnetic beads coated with anti-mouse IgG (Dynabeads; Dynal, Oslo, Norway). In the isolated population, >99% expressed CD19 and CD20 and

<1% expressed CD2 or CD14 antigens. Isolation of sIgD<sup>+</sup> and sIgD<sup>-</sup> B cells subpopulations was performed using a preparative magnetic cell sorter (MACS; Becton Dickinson) (24). IgD was expressed on >99% of the sIgD<sup>+</sup> B cell subpopulation and <1% of sIgD<sup>-</sup> B cell subpopulation, as assessed by fluorescence analysis using a FACScan<sup>®</sup> (Becton Dickinson). For certain experiments, sIgD<sup>-</sup> B cells were further separated according to CD38 and CD39 expression into CD38<sup>+</sup>CD39<sup>-</sup> germinal center (GC) cells and CD38<sup>-</sup>CD39<sup>+</sup> memory B cells using anti-CD38 and anti-CD39 mAbs and bead depletion as described earlier (25).

In some experiments, total B cells were separated according to their size using a discontinuous gradient of Percoll (Pharmacia, Uppsala, Sweden) as described earlier (22). B cells recovered at the interface of 35–55% layers were referred to as low density B cells, and cells recovered at the interface of 65–55% Percoll solutions were referred to as high density or resting B cells. In some cases, resting B cells were further labeled with FITC-conjugated anti-CD20, PE-conjugated anti-CD38 (Becton Dickinson) and biotinylated anti-IgD (Sigma), followed by incubation with Streptavidin-Tricolor (Kallestad, Austin, TX) and sorted into CD20<sup>+</sup>IgD<sup>+</sup>CD38<sup>-</sup> naive B cells and CD20<sup>+</sup>IgD<sup>-</sup>CD38<sup>-</sup> memory B cells (25). Reanalysis of the sorted populations showed a purity higher than 99%.

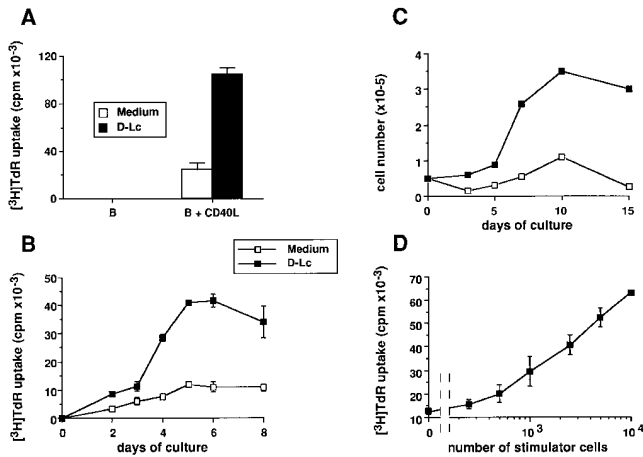
**Isolation of Human Monocytes.** Total PBMC were isolated from healthy donors by Ficoll-HyPaque centrifugation; monocytes were isolated by elutriation centrifugation as described elsewhere (26). The preparation was >90% pure as controlled by flow cytometry with FITC-conjugated anti CD14 (Leu-M3; Becton Dickinson).

**Coculture of B Cells and D-Lc.**  $2.5 \times 10^3$  irradiated CD40L L cells (7,500 rads) were seeded together with  $10^4$  B lymphocytes (either total B cells or B cells subpopulations) in the presence or absence of in vitro generated D-Lc ( $10^4$ ) in 96-well culture plate (Nunc, Roskilde, Denmark). B cell proliferation was monitored by tritiated thymidine (<sup>3</sup>H]TdR) incorporation after 6 d of coculture, except for kinetic experiments. Cells were incubated for the last 16 h with 1  $\mu$ Ci of [<sup>3</sup>H]TdR. Tests were carried out in triplicate, and results were expressed as cpm  $\pm$  SD. For determination of Ig production, supernatants were recovered after 15 d and used for indirect ELISA (27). For Giemsa stainings and immunostainings,  $10^5$  B cells were cultured together with  $10^5$  irradiated D-Lc over  $2.5 \times 10^4$  irradiated CD40L L cells in a final volume of 1 ml in 24-well culture plates. After 6 d of culture, cells were gently harvested and used for staining experiments.

In other experiments, B cells and D-Lc were cultured in separate compartments using transwells (Costar, Wilmington, MA).  $10^5$  D-Lc cultured in the presence or absence of CD40 triggering ( $2.5 \times 10^4$  CD40L L cells or CD32 L cells used as control) in the lower compartment (in a total volume of 0.8 ml) were assayed for their ability to stimulate growth and differentiation of  $1.5 \times 10^4$  B cells activated by  $3.75 \times 10^3$  CD40L L cells or control CD32 L cells in the top of the transwells (in a total volume of 0.2 ml). DNA synthesis of B cells was performed by transferring, at day 6, the cells present in the top of the transwells into flat-bottomed 96-well plates and pulsing them with [<sup>3</sup>H]TdR for the last 16 h of the culture period.

Control phenotype of the cultures was routinely performed using FITC-labeled anti-CD3 and anti-CD19 (Immunotech) and FITC-labeled IgG1 (Kallestad).

**Giemsa and Immunostainings.** Cells from cocultures of B cells and D-Lc over CD40L L cells were cytocentrifuged for 5 min at 500 rpm on microscope slides. Some slides were used for May-Grünwald-Giemsa staining and the others were fixed either in cold acetone or 4% paraformaldehyde during 10 min for immunocy-



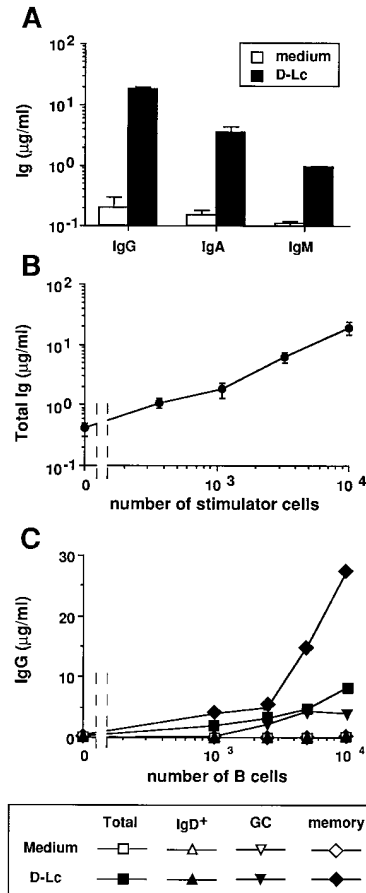
**Figure 1.** In vitro generated D-Lc enhance DNA synthesis and viable cell number of B lymphocytes activated through their CD40. (A)  $10^4$  highly purified B cells were cultured over  $2.5 \times 10^3$  irradiated CD40L L cells (*right*) or in medium alone (*left*) in the presence or absence of  $10^4$  irradiated D-Lc, as detailed in Materials and Methods. Thymidine uptake, determined after 6 d, is expressed as mean  $\pm$  SD of triplicate cultures. (B) Kinetic of DNA synthesis of  $10^4$  CD40-activated B cells cultured in the presence or absence of  $10^4$  D-Lc was monitored between day 0 and day 8. (C) For numeration experiments,  $5 \times 10^4$  highly purified B cells were cultured over  $2.5 \times 10^4$  irradiated CD40L L cells in the presence or absence of  $5 \times 10^4$  D-Lc in 24-well plates. Viable cell recovery was determined at many timepoints using Trypan blue dye exclusion between day 0 and day 15. (D) Increasing D-Lc numbers were added to  $10^4$  CD40-activated B cells, and thymidine uptake was determined after 6 d of coculture (results from experiments representative of 15).

tology. Single stainings were performed using the alkaline phosphatase-anti-alkaline phosphatase system (APAAP technique) with mouse anti-HLA-DR (IgG1 from Immunotech) or anti-CD80 (IgG1 from Becton Dickinson) revealed by the Fast Red substrate (DAKO). Double stainings were performed using the biotin-avidin-peroxidase system (mouse IgG2a anti-HLA-DR from Becton Dickinson) and the APAAP technique (mouse IgG1 anti-CD20 from Becton Dickinson) as described elsewhere (30). Peroxidase activity was developed by 3-amino-9-ethylcarbazole, which gives a red color, and alkaline phosphatase activity was developed by Fast Blue substrate, which gives a blue color.

## Results

**D-Lc Enhance the Growth of CD40-activated B Cells.** In early experiments, using allogeneic cord blood DC and resting tonsillar T cells and B cells, DC were found to induce the activation of T cells, subsequently resulting in the differentiation of B cells as measured by Ig production (Caux, C., B. Vanbervliet, and J. Banchereau, unpublished results). Accordingly, we wondered whether D-Lc generated in vitro by culturing cord blood CD34<sup>+</sup> cells for 12 d in the presence of GM-CSF and TNF- $\alpha$  (later on referred to as D-Lc) (18, 21) could directly modulate B cell responses. Inasmuch as CD40L on activated T cells appears to signal both B cells and DC, CD40L-transfected L cells (CD40L L cells) were used as surrogate activated T cells.

The D-Lc preparation used in this study contains 70–90% CD1a<sup>+</sup> as well as CD1a<sup>-</sup> cells including granulocytes and monocytes (see Materials and Methods). The role of



**Figure 2.** D-Lc strongly enhance Ig production by CD40-activated memory B cells. B cells were cultured over CD40L L cells in the presence or absence of D-Lc and their supernatants were harvested after 15 d and assayed for presence of (A) IgG, IgA, and IgM. (B) Increasing numbers of D-Lc were added to  $10^4$  CD40-activated B cells and total Ig production was measured after 15 d of coculture. Igs levels are expressed as mean  $\pm$  SD of triplicate cultures (results from 1 experiment representative of 10). (C) B cells were purified into IgD<sup>+</sup> B cells and IgD<sup>-</sup> B cells using the MACS system (purification detailed in Materials and Methods). IgD<sup>-</sup> B cells were further separated into CD38<sup>+</sup>CD39<sup>-</sup> germinal center B cells (GC) and CD38<sup>-</sup>CD39<sup>+</sup> memory B cells using mAbs and bead depletion as detailed in Materials and Methods. Increasing numbers of either total B cells, MACS purified sIgD<sup>+</sup> B cells, GC cells, or memory B cells were cultured over  $2.5 \times 10^3$  irradiated CD40L L cells in medium alone or in the presence of  $10^4$  irradiated D-Lc. (SD  $\leq$  10%). (results from 1 of 3 experiments). No significant B cell proliferation and differentiation was observed in absence of CD40L L cells.

the latter cells will be discussed later on (Fig. 4). As shown in Fig. 1 A, D-Lc strongly enhanced CD40-induced B cell proliferation as measured by [<sup>3</sup>H]TdR uptake after 6 d of coculture (three to eight-fold enhancement of [<sup>3</sup>H]TdR uptake). Note that in the absence of CD40L L cells, D-Lc can not induce B cell proliferation (Fig. 1 A, *left*). Kinetics analysis shows that addition of  $10^4$  D-Lc to CD40 triggering resulted in a twofold increase of [<sup>3</sup>H]TdR incorporation at day 2, which reached fourfold at day 4, when a plateau is reached (Fig. 1 B). This enhancement of B cell DNA synthesis by D-Lc is associated to an important increase in number of viable B cells (Fig. 1 C) that reaches a plateau at day 10, a time when D-Lc increased B cell num-

**Table 1.** D-Lc Enhance Growth and Ig Secretion of Resting B Lymphocytes Activated Through CD40

		$^3\text{H}$ ]TdR uptake (cpm $\times 10^{-3}$ )		IgG ( $\mu\text{g}/\text{ml}$ )		IgM ( $\mu\text{g}/\text{ml}$ )	
		Medium	D-Lc	Medium	D-Lc	IL-2	IL-2 + D-Lc
Exp. 1	Total B cells	7.3 $\pm$ 0.02	52.4 $\pm$ 0.1	0.1 $\pm$ 0.05	23.9 $\pm$ 0.9	0.1 $\pm$ 0.02	26.8 $\pm$ 2.6
	Low density	5.6 $\pm$ 0.06	39.0 $\pm$ 2.5	0.2 $\pm$ 0.05	10.9 $\pm$ 1.2	0.1 $\pm$ 0.02	16.6 $\pm$ 1.5
	High density	10.0 $\pm$ 1.6	56.2 $\pm$ 1.7	0.7 $\pm$ 0.01	27.5 $\pm$ 1.0	0.2 $\pm$ 0.06	41.9 $\pm$ 5.1
Exp. 2	Total B cells	3.5 $\pm$ 0.6	17.3 $\pm$ 0.2	0.5 $\pm$ 0.01	6.5 $\pm$ 0.9	0.1 $\pm$ 0.02	6.0 $\pm$ 2.0
	High density IgD <sup>+</sup> CD38 <sup>+</sup>	3.1 $\pm$ 0.6	15.4 $\pm$ 1.7	0.1 $\pm$ 0.01	0.5 $\pm$ 0.2	0.3 $\pm$ 0.1	21.5 $\pm$ 5.8
	High density IgD <sup>-</sup> CD38 <sup>-</sup>	3.5 $\pm$ 0.2	18.9 $\pm$ 4.7	0.1 $\pm$ 0.05	9.0 $\pm$ 1.2	0.1 $\pm$ 0.05	0.6 $\pm$ 0.2

D-Lc stimulate differentiation of resting naive and memory B cells activated through their CD40. Highly purified B cells were separated according to their size, using Percoll gradients, into low density B cells (activated B cells) and high density B cells (resting B cells). In experiment 1,  $10^4$  B cells were cultured over DC40L L cells in the presence or absence of D-Lc. For experiment 2, CD20<sup>+</sup> resting B cells (high density) were further separated into IgD<sup>+</sup>CD38<sup>-</sup> naive B cells and IgD<sup>-</sup>CD38<sup>-</sup> memory B cells, using three-color FACS<sup>®</sup> sorting (as described in Materials and Methods). These cells were dispensed at  $5 \times 10^3$ /well together with  $2.5 \times 10^3$  CD40L L cells with or without  $10^4$  D-Lc. DNA synthesis was assayed after 6 d of coculture, and levels of IgG (in the absence of cytokine) and IgM (in the presence of IL-2) were determined at 15 d (1 experiment representative of 3). No significant B cell proliferation and differentiation was observed in absence of CD40L L cells.

ber by fivefold. As few as 500 D-Lc are sufficient to induce a two- to threefold increase of CD40-induced B cell proliferation (Fig. 1 D), a maximal effect being obtained with  $10^4$  D-Lc (ratio 1:1). Thus, D-Lc enhance the proliferation of CD40-activated B cells in absence of any exogenous cytokine.

**D-Lc Induce CD40-activated Memory B Cells to Secrete Igs.** To investigate the effect of D-Lc on the differentiation of CD40-activated B cells into Ig-secreting cells, tonsillar B cells were cultured over CD40L L cells with or without D-Lc. In the absence of D-Lc,  $10^4$  B cells produced only marginal amounts of Igs (<0.2  $\mu\text{g}/\text{ml}$ ; Fig. 2 A). Addition of  $10^4$  D-Lc to CD40-activated B cells results in the production of microgram amounts of IgG (range, 4–70  $\mu\text{g}/\text{ml}$ ; mean increase, 125;  $n = 12$ ) and IgA (range, 0.8–6  $\mu\text{g}/\text{ml}$ ; mean increase, 33;  $n = 12$ ). In contrast, IgM secretion was increased only 5- to 15-fold (range, 0.5–2  $\mu\text{g}/\text{ml}$ ; mean increase, 10;  $n = 12$ ). As few as 330 D-Lc induced an eightfold increase in total Ig secretion, maximal effect being obtained with  $10^4$  D-Lc per well (40-fold increase) (Fig. 2 B).

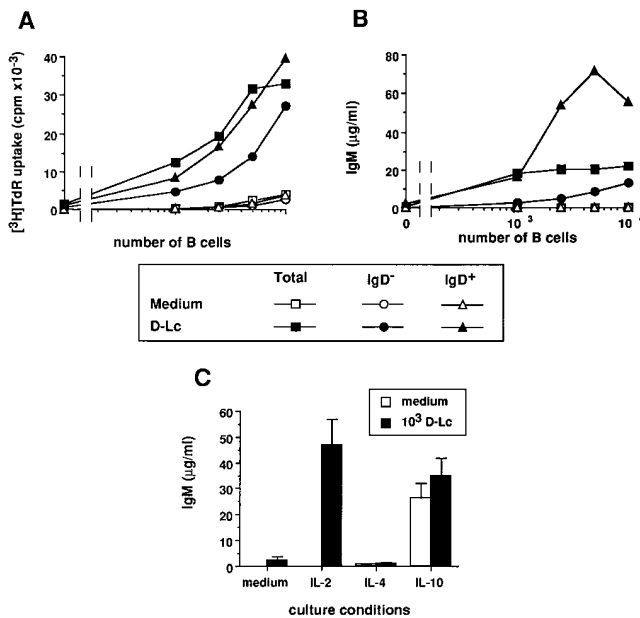
Tonsillar B cells consist of naive B cells, expressing surface IgM and IgD as well as GC B cells and memory B cells that have mostly undergone isotype switch, thus resulting in a loss of IgD expression (28, 29). Therefore, we analyzed whether either purified sIgD<sup>+</sup> B cells (naive B cells) or sIgD<sup>-</sup> B cells (GC and memory B cells) were preferential targets for D-Lc effect on IgG and IgA secretions. As shown in Fig. 2 C, CD40-activated naive sIgD<sup>+</sup> B cells did not produce IgG whether or not D-Lc are added. Separation of sIgD<sup>-</sup> B cells into CD38<sup>+</sup>CD39<sup>-</sup> GC cells and CD38<sup>-</sup>CD39<sup>+</sup> memory B cells (using mAbs and bead depletion) (25), allowed identification of the responding cell population. As shown in Fig. 2 C, memory B cells repre-

sent the main source of IgG as, when cultured in the presence of D-Lc,  $10^3$  memory B cells produced 5  $\mu\text{g}/\text{ml}$  IgG and  $10^4$  27  $\mu\text{g}/\text{ml}$ , while GC cells produced <0.5  $\mu\text{g}/\text{ml}$  and 4  $\mu\text{g}/\text{ml}$ , respectively, under such culture conditions.

To exclude that these observations could be the result of expansion of in vivo-activated B cells, resting and activated B cells were separated according to their density by Percoll gradients. As shown in Table 1 (experiment 1), in the absence of exogenous cytokine, D-Lc enhanced IgG production of both resting (high density) and activated (low density) B cells. Finally, CD20<sup>+</sup> small resting B cells FACS<sup>®</sup> sorted into IgD<sup>-</sup>CD38<sup>-</sup> cells (resting memory B cells) produced high levels of IgG, when stimulated with D-Lc (90-fold enhancement) in the absence of cytokine (Table 1, experiment 2). Similar results were obtained for IgA (data not shown).

Taken together, these data indicate that, D-Lc induce CD40-activated memory B cells to secrete considerable amounts of IgG and IgA.

**CD40-activated Naive B Cells Cultured in the Presence of D-Lc Secrete IgM in Response to IL-2.** In view of the weak stimulation of IgM production, we wondered whether sIgD<sup>+</sup> B cells were sensitive to the stimulatory effect of D-Lc. As shown in Fig. 3 A and Table 1, D-Lc equally enhanced the CD40-induced proliferation of all B cell subsets (sIgD<sup>+</sup>, sIgD<sup>-</sup>, resting, and in vivo-activated B cells) in absence of any exogenous cytokines. As D-Lc enhanced growth of naive B cells, we wondered whether addition of exogenous cytokines could enhance the IgM production induced by D-Lc. Among the tested cytokines (IL-2, IL-3, IL-4, IL-6, IL-10, IL-12, IFN- $\gamma$  and their combinations), only IL-2 yielded significant results. D-Lc did not significantly affect the low levels of IgM obtained in response to IL-4 and the high levels obtained in response to IL-10 (Fig.



**Figure 3.** In the presence of IL-2, D-Lc strongly enhance IgM production by naive sIgD<sup>+</sup> B cells. B cells were purified into IgD<sup>+</sup> B cells and IgD<sup>-</sup> B cells using the MACS system (purification detailed in Materials and Methods). Increasing numbers of either total B cells, MACS-purified IgD<sup>+</sup> B cells or IgD<sup>-</sup> B cells were cultured under CD40 activation in medium alone or in the presence of irradiated D-Lc. (A) Cytokine-independent proliferation of B cells was measured after 6 d of coculture. (B) IL-2-dependent IgM production was measured after 15 d. Results are expressed as mean of triplicate cultures (SD  $\leq$  10%). (results from 1 experiment representative of 6). (C) 10<sup>4</sup> highly purified B cells were cultured over 2.5  $\times$  10<sup>3</sup> irradiated CD40L L cells in the presence or absence of 10<sup>3</sup> D-Lc, either in medium alone or with IL-2 (20 U/ml), IL-4 (50 U/ml), or IL-10 (20 ng/ml). Superposable results were obtained with IgD<sup>+</sup> B cells. Ig levels were determined after 15 d of culture. Results are expressed as mean  $\pm$  SD of triplicate cultures (1 experiment representative of 10). No significant B cell proliferation and differentiation was observed in absence of CD40L L cells.

3 C). However, in IL-2 supplemented cultures, addition of D-Lc induced CD40-activated B cells to secrete high levels of IgM, which reached 50  $\mu$ g/ml (mean increase, 161-fold;  $n = 16$ ). Separation of B cells into sIgD<sup>+</sup> and sIgD<sup>-</sup> B cells showed that production of IgM was mainly a property of the naive sIgD<sup>+</sup> B cell population (Fig. 3 B). Note, however, that sIgD<sup>-</sup> B cells can be induced to produce significant levels of IgM provided 10-fold more B cells (10<sup>4</sup>) are added to cultures. In addition, results presented in Table 1 show that D-Lc can induce small resting naive B cells (high density sIgD<sup>+</sup>CD38<sup>-</sup> cells) to produce high amounts of IgM in the presence of CD40 triggering and IL-2.

Thus, D-Lc induce CD40-activated naive B cells to secrete high amounts of IgM in response to IL-2.

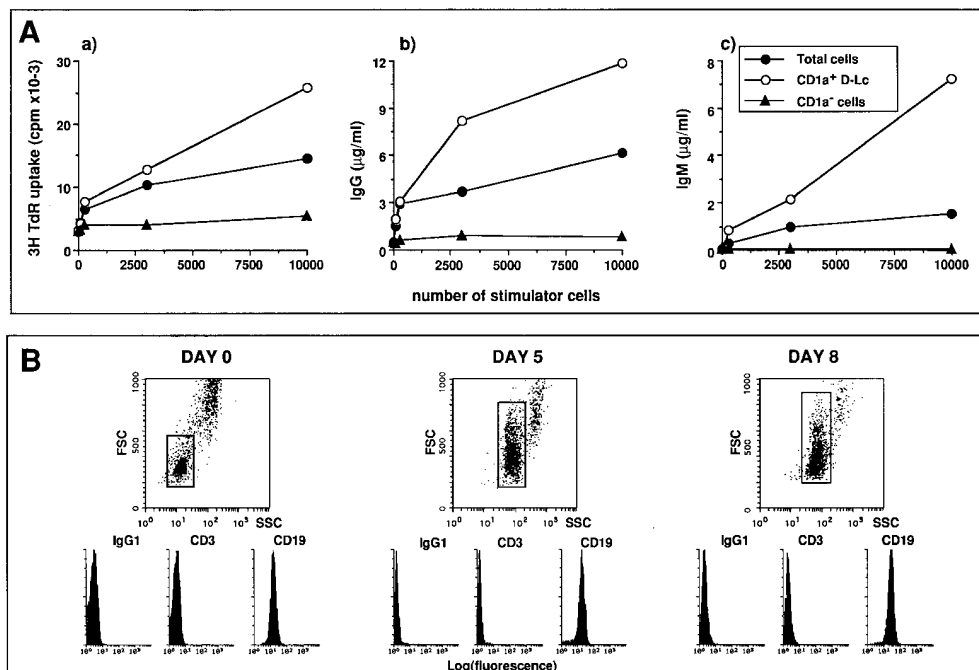
*The Effects of D-Lc on CD40-activated B Cells Are Borne by the CD1a<sup>+</sup> Population and Are Independent of the Outgrowth of Potentially Contaminating T Cells.* Because a population of D-Lc contains 70–90% CD1a<sup>+</sup> cells as well as other cell types, including granulocytes and monocytes, D-Lc were FACS<sup>®</sup> sorted into CD1a<sup>+</sup> and CD1a<sup>-</sup> cells. 10<sup>4</sup> CD1a<sup>+</sup> cells were found to induce a 5-fold increase of B cell prolifer-

ation, whereas CD1a<sup>-</sup> cells were ineffective (Fig. 4 Aa). In addition, as few as 250 CD1a<sup>+</sup> cells induced a 10-fold increase in IgG production, in absence of exogenous cytokines (Fig. 4 Ab), and in IgM production, in the presence of IL-2 (Fig. 4 Ac). In contrast, even at high cell density (10<sup>4</sup> cells), CD1a<sup>-</sup> cells remained ineffective in stimulating either IgG or IgM production, demonstrating that the described stimulatory effects of in vitro-generated D-Lc on B cells are restricted to CD1a<sup>+</sup> D-Lc.

Although highly purified B cells (>99% CD20<sup>+</sup> cells), isolated using either bead depletion or cell sorting (Table 1), were used in all experiments, the presence of IL-2 and D-Lc, known for their strong capacity to stimulate alloreactive T cells, renders it necessary to exclude a possible contribution of potentially contaminating T cells. For that purpose, phenotype of cultured cells was performed routinely. As an example, Fig. 4 B shows the phenotype of cells proliferating in cultures performed with total B cells, D-Lc, CD40L L cells, and IL-2 (the condition that would most likely allow the outgrowth of contaminating T cells). No significant outgrowth of CD3<sup>+</sup> cells can be detected at any timepoint tested, and >98% of the small cells are CD19<sup>+</sup> B cells. Similar results were observed in cultures set up with sIgD<sup>+</sup> B cells and in cultures set up in the absence of IL-2 (data not shown). D-Lc (high FSC/SSC) were detected during all the culture period (50% at day 0 to <10% at day 8). These results support the conclusion that CD1a<sup>+</sup> D-Lc can directly enhance the growth and differentiation of CD40-activated B cells.

*D-Lc Are More Efficient than Monocytes in Enhancing CD40-induced B Cell Differentiation.* We compared the effects of various antigen-presenting cells including D-Lc, elutriated monocytes (>90% CD14), and lymphoblastoid cell lines (EBV-LCL) on the proliferation and differentiation of CD40-activated B cells. As shown in Fig. 5 A, monocytes were as potent as D-Lc in enhancing CD40-induced B cell proliferation, whereas EBV cell lines were totally inefficient (only one of the three tested is shown in Fig. 5). Significant IgG production, in absence of cytokine, was observed with 3.5  $\times$  10<sup>2</sup>–10<sup>3</sup> D-Lc, whereas more than 5  $\times$  10<sup>3</sup> monocytes were required to observe the same effects. At higher cell density (10<sup>4</sup> cells), monocytes induced levels of IgG in the order of 5  $\mu$ g/ml compared with 17  $\mu$ g/ml for D-Lc (Fig. 5 B). Concerning the IL-2-dependent IgM production, a significant effect was observed with less than 450 D-Lc, whereas more than 2.5  $\times$  10<sup>3</sup> monocytes were required to reach similar levels of IgM secretion. Thus, whereas D-Lc and monocytes shared an equal ability to enhance B cell proliferation, D-Lc are more efficient than monocytes in inducing B cells to secrete IgG and IgM.

*The Effect of D-Lc Can Be Obtained in the Presence of Soluble CD40L.* To analyze a possible contribution of the fibroblastic cell line (CD40L L cells), CD40 activation was carried out with a soluble form of CD40L using a fusion protein between the mouse CD8- $\alpha$  and the human CD40L. The soluble protein was a less efficient activator than the CD40L L cells (Fig. 6), particularly for proliferation of B cells, probably reflecting differences in the mode of CD40



**Figure 4.** The effects of D-Lc on CD40-activated B cell are restricted to CD1a<sup>+</sup> dendritic cells and are not due to potentially contaminating T cells. (A) In vitro generated D-Lc were FACS<sup>®</sup> sorted into CD1a<sup>-</sup> cells and CD1a<sup>+</sup> DC and used as stimulators of 10<sup>4</sup> CD40-activated B cells. Increasing numbers of either total D-Lc or FACS<sup>®</sup> sorted populations were added to the culture to study (a) the cytokine-independent proliferation of B cells, (b) the cytokine-independent IgG production, and (c) the IL-2-dependent IgM production. Results are expressed as mean of triplicate cultures (SD ≤ 10%). (results from 1 experiment representative of 3). No significant B cell proliferation and differentiation was observed in absence of CD40L L cells. (B) The phenotype of cultures consisting of total B cells, D-Lc, CD40L L cells, and IL-2 was routinely followed during

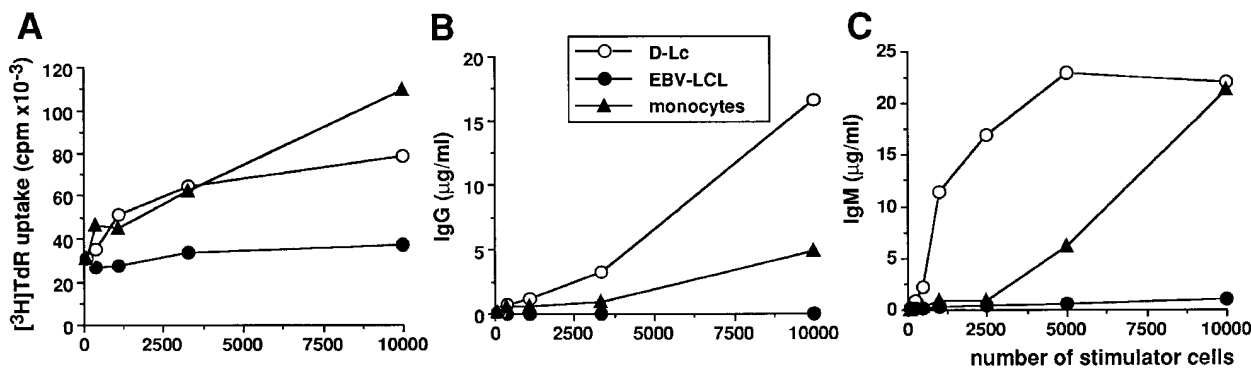
the culture period. Fluorescence histograms gated on small cells have been obtained with FITC-anti-CD3 and anti-CD19 after exclusion of dead cells using propidium iodide. High FSC/SSC cells correspond to the D-Lc population.

engagement. Yet, addition of D-Lc resulted in enhanced B cell proliferation (Fig. 6 A) and differentiation (Fig. 6 B). The effect was particularly remarkable when cytokines, such as IL-2 or IL-10, were added to the cultures. Thus, the presence of the fibroblastic line is not mandatory for the B cell to undergo growth and differentiation in response to surrogate activated T cells (in the form of soluble CD40L and recombinant cytokines) and D-Lc.

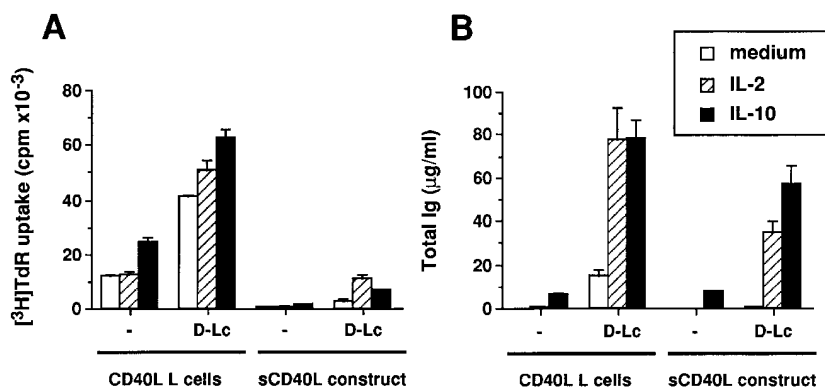
*The Effect of D-Lc on B Cell Activation Is Mediated by Soluble Factor(s): Partial Dependence on CD40 Ligation on D-Lc.* As previously described, B lymphocytes proliferate in clusters over CD40L L cells. Addition of D-Lc enhance the size and number of these clusters, which include L cells and D-Lc (data not shown). Giemsa staining on 6-d coculture

cytopins shows close contacts between CD40-activated B cells and D-Lc (Fig. 7 A). Immunostainings on such cytopins showed large HLA-DR<sup>2+</sup> CD80<sup>high</sup> cells with dendritic morphology surrounded by HLA-DR<sup>2+</sup> CD80<sup>low</sup> B cells (Fig. 7, B and C). Immunostainings using anti-CD3 mAb failed to detect any positive cells (data not shown). Double stainings definitely identifies CD40-activated B cells (CD20<sup>+</sup>/HLA-DR<sup>low</sup>) in close contacts with D-Lc (CD20<sup>-</sup>/HLA-DR<sup>high</sup>), suggesting that these interactions may contribute to the D-Lc-dependent stimulation of B cell growth and differentiation described in this study.

Experiments were designed to determine whether the functions of D-Lc on B cells were mediated through soluble factors and/or cell-cell interactions and whether D-Lc



**Figure 5.** D-Lc are more efficient than monocytes in enhancing CD40-induced B cell differentiation. D-Lc, one EBV cell line, and elutriated monocytes were compared, after irradiation, in their ability to enhance (A) cytokine-independent CD40-induced B cell proliferation, (B) cytokine-independent IgG production, and (C) IL-2-dependent IgM production. Increasing numbers of stimulator cells were added to 10<sup>4</sup> highly purified B cells cultured over 2.5 × 10<sup>3</sup> irradiated CD40L L cells. Results are expressed as mean of triplicate cultures (SD ≤ 10%). (results from 1 experiment representative of 3). No significant B cell proliferation and differentiation was observed in absence of CD40L L cells.



**Figure 6.** D-Lc enhance both proliferation and differentiation of B cells activated with a soluble form of CD40L. A soluble form of CD40L (sCD40L) was expressed as a dimeric fusion protein between the mouse CD8 $\alpha$  and the human CD40L. sCD40L was used as a supernatant of COS cells at a final concentration of 25% (right) to stimulate (A) B cell proliferation ( $10^4$  cells per well) and (B) B cell differentiation ( $10^4$  cells per well). IL-2 and IL-10 were added at concentration indicated in Materials and Methods. Results obtained with CD40L L cells are indicated for comparison (left). (results from 1 experiment representative of 3). No significant B cell proliferation and differentiation was observed in absence of CD40 engagement.

had to be activated through their CD40 antigen. Neither D-Lc supernatants nor fixed D-Lc significantly stimulated B cell growth and differentiation. As an alternative, D-Lc were separated from B cells by a permeable membrane using Transwells<sup>®</sup>. As shown in Fig. 8 A, D-Lc cultured in the lower compartment, with or without CD40 triggering, can enhance CD40-dependent B cell proliferation as much as if they were cocultured in direct contact. In the presence of IL-2, CD40-activated D-Lc, but not unactivated D-Lc, can support IgM production by B cells activated through CD40 (Fig. 8 B). However, IgM levels obtained were lower than those observed in direct contact coculture ( $6.1 \pm 0.8 \mu\text{g/ml}$  versus  $15.5 \pm 2.5 \mu\text{g/ml}$ ), suggesting that cell-cell contacts may also contribute. Note that D-Lc do not deliver any signal when B cells are not activated (Fig. 8; B plus CD32 L cells).

Thus, the effect of D-Lc on B cell proliferation appears to be mediated by soluble factor(s), the production of which is independent of CD40 engagement on the D-Lc. In contrast, the effect on B cell differentiation, also mediated through soluble mediator(s), requires CD40 ligation of the D-Lc.

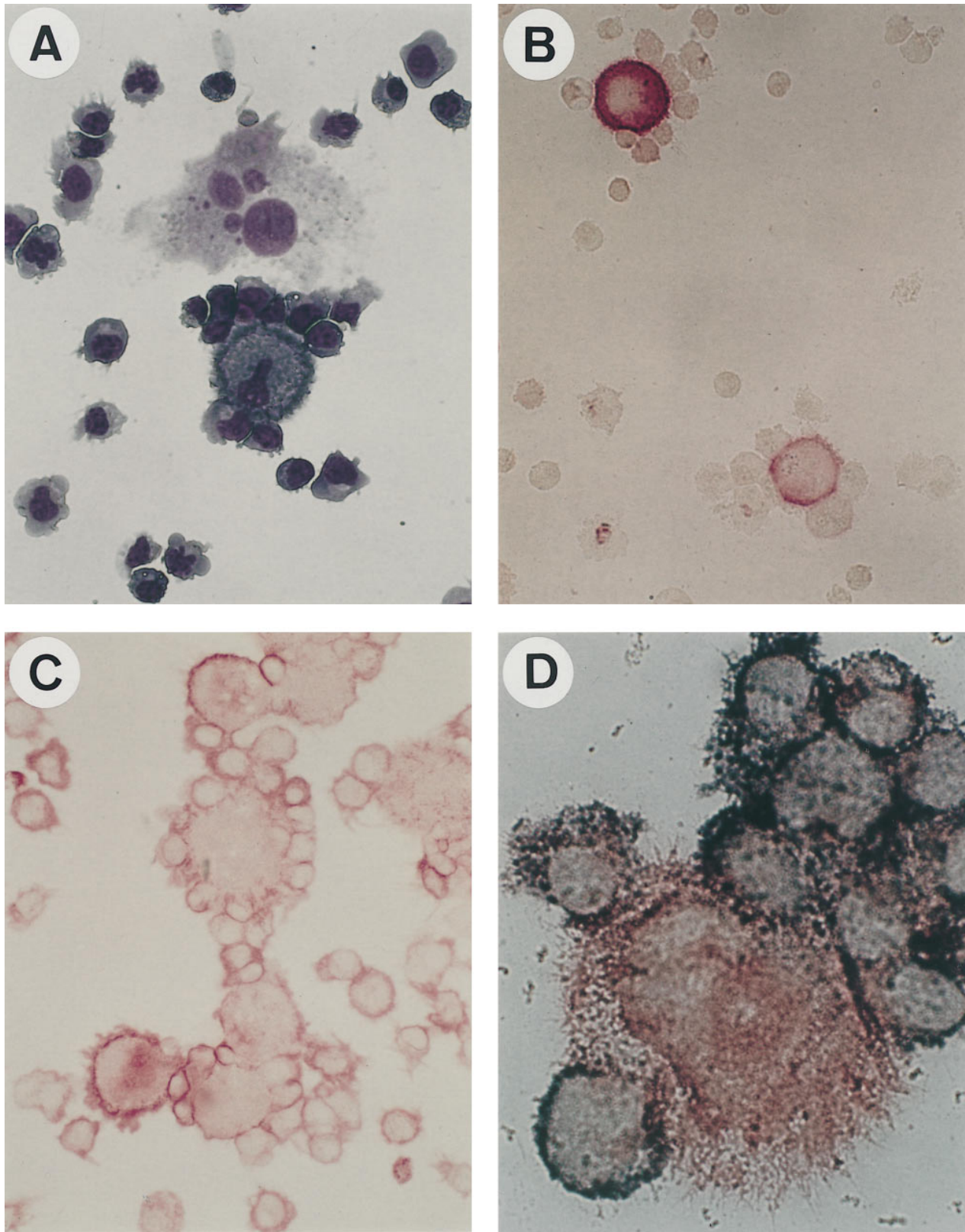
## Discussion

The present study demonstrates that DC can directly provide signals to CD40-activated B cells, leading to enhanced proliferation as well as differentiation. To our knowledge, this study represents the first study demonstrating a direct effect of DC on normal B lymphocytes. Such information represents an important complement to studies performed earlier with mouse B lymphocytes, pointing out the critical role of DC during the course of the humoral response, including the following: (a) the demonstration that clusters of helper T lymphocytes, histocompatible B cells, and DC represented the site in which antibody-producing cells develop during the response to thymus-dependent antigens *in vitro* (11); (b) the observation that DC-oriented switch towards IgA of normal mouse B lymphocytes or pre B cell lines, stimulated with activated T cells or LPS (30, 31). However, potential direct effects of DC on B cells (in addition to effects on activated T cells) have never been studied. To address this question, CD40L L cells were used as

surrogate activated T cells and DC were derived from cord blood CD34<sup>+</sup> hematopoietic progenitors cultured for 12 d in the presence of GM-CSF plus TNF- $\alpha$  (D-Lc) (18, 21). CD1a<sup>+</sup> D-Lc strongly enhance, in absence of any exogenous cytokines, CD40-dependent B cell proliferation (2–6-fold increase of viable cells) as well as differentiation (10–125-fold increase of Ig secretion, depending on the isotype). This striking direct effect of CD1a<sup>+</sup> D-Lc on Ig secretion was restricted to memory B cells. Furthermore, in the presence of IL-2, which by itself has only limited effects on *in vitro* CD40-dependent B cell activation (8, 32, 33) D-Lc stimulate resting naive IgD<sup>+</sup> B cells to secrete large amounts of IgM.

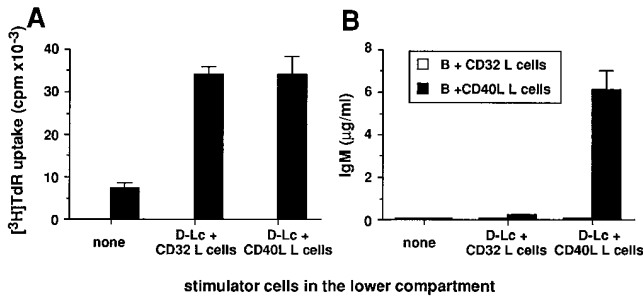
The direct modulation of B cell activation by D-Lc, under CD40 triggering, reported here, suggests an important role of the latter cells during secondary immune responses (reactivation of memory B cells) and in the initiation of primary humoral responses (activation of naive B cells) and should actually be discussed in the context of a series of pertinent *in vitro* and *in vivo* observations: (a) CD40 engagement of cultured DC (20) yields cells with features of IDC (including low CD1a as well as high CD25, CD80, and CD86), which are found in the extrafollicular areas of secondary lymphoid organs (34–36); (b) antigen-induced T cell-dependent B cell activation and differentiation into plasma cells occurs within extrafollicular areas during both primary (naive B cells) and secondary (memory B cells) humoral responses (16, 37, 38); (c) after antigenic challenge, CD40L-expressing T cells are mainly found within periarteriolar lymphatic sheaths (39), thus indicating that CD40-dependent activation of DC may occur within the extrafollicular areas of secondary lymphoid organs; (d) the IL-2 requirement for naive B cells to produce IgM is consistent with immunohistochemical studies showing, after immunization, the colocalization of CD40L<sup>+</sup> IL-2 producing CD4<sup>+</sup> T cells and specific antibody-secreting B cells in the extrafollicular areas of secondary lymphoid organs (39–41). In keeping with this, *in vitro* studies have shown IL-2 to be an important factor of primary humoral response (42–44). In particular, recently activated CD4<sup>+</sup> T cells generate effector T cells that secrete mainly IL-2 and induce B cells to secrete essentially IgM (44).

The mechanisms by which D-Lc regulate B cell re-



**Figure 7.** CD40-activated B cells are in close contact with D-Lc in coculture.  $10^5$  highly purified B cells were cultured in 24-well plates over  $2.5 \times 10^4$  irradiated CD40L L cells and  $5 \times 10^4$  D-Lc. After 8 d of coculture, cells were gently harvested, cytocentrifuged, and used for (A) MGG staining, (B) anti-HLA-DR staining, (C) anti-CD80 (B7-1) staining, and (D) double anti-HLA-DR (red) and anti-CD20 (blue) staining (as detailed in Materials and Methods). Magnification,  $\times 400$  (A, B, C);  $\times 1000$  (D).





**Figure 8.** The secretion of IgM by B cells is dependent on the production of soluble factor(s) by D-Lc after CD40 engagement.  $1.5 \times 10^4$  sIgD<sup>+</sup> B cells were cultured in the presence of  $3.75 \times 10^3$  CD40L L cells or CD32 control L cells in the top of the transwell.  $10^5$  D-Lc were cultured in the lower compartment with  $2.5 \times 10^4$  CD40L L cells or CD32 L cells used as controls. (A) DNA synthesis of B cells of the upper compartment, in absence of cytokine, was determined after 6 d of coculture. (B) IgM production in the presence of IL-2 was determined by ELISA after 15 d. Cultures were carried out in triplicates (one experiment representative of three).

sponses is not yet totally elucidated. However, the fact that D-Lc stimulate the proliferation of IgD<sup>+</sup> naive B cells without altering their Ig secretion indicate that D-Lc affect the growth and the differentiation of B cells through different mechanisms and suggest the contribution of several molecular entities. Furthermore, Transwells<sup>®</sup> experiments show that D-Lc enhance growth of CD40-activated B cells through the production of soluble factor(s) independently of CD40 triggering. In contrast, the effect on IgM secretion in the presence of IL-2 is partly dependent on the release by D-Lc of soluble mediator(s) after CD40 engagement. Among known soluble mediators, IL-10, which has been shown to act in synergy with IL-2 to induce strong B cell differentiation (45), was excluded using MAbs (data not shown). The mechanism of induction of IL-2 responsiveness is not yet

clear. As D-Lc expressed CD25 after CD40 engagement (20), IL-2 could signal D-Lc, which in turn induce B cells to produce IgM. Alternatively, such an effect might be due to the ability of D-Lc to upregulate CD25 expression on CD40-activated naive B cells (data not shown).

Together with the above considerations, our results emphasize the importance of a three-party cellular interaction, where DC allow the encounter and interaction of antigen-specific T cells with antigen-specific B cells during primary humoral responses. First, DC, homing into the T cell-rich areas, present processed antigen to naive T cells, through an MHC-TCR interaction, resulting in upregulation of CD40L on the T cell. In return, the activated T cell stimulates the DC through CD40, resulting in CD80 and CD86 upregulation and cytokine production (20). Then, the triggering of CD28 on the T cell, in the presence of DC-derived cofactors induces a full T cell activation followed by IL-2 production. Second, these reciprocal DC-T cell interactions are likely to occur concomitantly with cognate T-B cell interactions. Antigen-specific B cells, through CD40 engagement and T cell-derived IL-2 differentiate into IgM-secreting plasma cells, after close contact with DC previously activated through CD40. There is presently no evidence that DC present unprocessed antigen to B lymphocytes directly, though it is tempting to speculate that they may use surface Fc receptors to carry the antigens in the form of immune complexes or use lectins such as DEC 205 (46) or the mannose receptor (47) to present unprocessed antigen to specific B cells.

As a conclusion, the present results demonstrate that DC can directly modulate CD40-dependent B cell activation, suggesting that the critical role of DC in initiation of T-dependent humoral response might be related to direct modulation of B cell responses in the extrafollicular areas of lymphoid organs.

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