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⁺ B cells (essentially memory B cells). In the presence of DC, naïve sIgD⁺ B cells produced, in response to interleukin-2, large amounts of IgM. Thus, in addition to activating naïve T cells in the extrafollicular areas of secondary lymphoid organs, DC may directly modulate B cell growth and differentiation.

A t the antigen/pathogen port of entry (e.g., mucosa, epidermis), dendritic cells (DC), 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 naïve 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 (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, 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 incubated 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⁺ hematopoietic progenitors (17). DC, generated by culturing human CD34⁺ hematopoietic progenitors with GM-CSF and TNFα have been shown earlier to induce a strong proliferation of allogenic 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 naïve and memory B cells. These results suggest that DC might directly be involved in the extrafollicular plasma cell formation during induction of primary naïve B cell responses or reactivation of secondary memory B cell responses.

Abbreviations used in this paper: DC, dendritic cells; D–Lc, dendritic–Langerhans cells; GC, germinal center; IDC, interdigitating dendritic cells.

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Materials and Methods

Reagents and Cell Lines. Cultures of CD34+ progenitors were established in RPMI-1640 (GIBCO BRL, Gaithersburg, MD) supplemented with 10% (vol/vol) FCS, 10 mM Hepes, 2 mM l-glutamine, 5 × 10^{-2} M 2-mercaptoethanol, and 0.08 µg/ml gentamicine (gentamcline; 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, N SW), 2 mM l-glutamine (GIBCO BRL, Gaithersburg, MD) and 0.08 µg/ml gentamicine.

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/FcγRII have been previously described (23). A chimeric fusion protein between the mouse CD8-α and the human CD40 L T cell ligand was constructed in our laboratory as previously reported (22) and produced in COS cells.

rhGM-CSF (specific activity: 2 × 10^{9} U/mg; Schering Plough Research Institute, Kenilworth, NJ) was used at a saturating concentration of 100 ng/ml (200 U/ml). rhTNF-α (R&D Systems, Minneapolis, MN) was used at a saturating concentration of 100 ng/ml (200 U/ml). rhIL-4 (10^{6} U/mg) from Amgen (Thousand Oaks, CA) was used at 20 U/ml. Purified rhIL-10 (10^{3} U/mg; Schering Plough) was used at 20 U/ml.

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 µg/ml; Immunotech Marseille, France) and goat anti–mouse IgG-coated microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Isolation of CD34+ progenitors was achieved using MiniMACS separation columns (Miltenyi Biotec) (21).

Cultures were established in the presence of rhGM-CSF and TNF-α in complete RPMI. CD34+ cells were seeded for expansion in 24-well culture plates (Limbro, Flow Laboratories, McLean, VA) at 2 × 10^{5} 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+ DC (18, 21). These CD1a+ 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+ 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-α, cells generated from CD34+ progenitors were collected and labeled with FITC-conjugated CD1a (Ortho Diagnostic). Cells were separated according to CD1a expression into CD1a+ and CD1a- fractions using a FACStar (Becton Dickinson). 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 standard ficoll–hyphaque (dose = 1,077 g/ml) gradient method. Tonsillar B cells were first enriched in the E+ 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, Oсло, Norway). In the isolated population, >99% expressed CD19 and CD20 and <1% expressed CD2 and CD14 antigens. Isolation of sIgD+ and sIgD+ B cells subpopulations was performed using a preparative magnetic cell sorter (MACS; Becton Dickinson) (24). IgD was identified on >99% of the sIgD+ B cell subpopulation and <1% of sIgD− B cell subpopulation, as assessed by fluorescence analysis using a FACScan® (Becton Dickinson). For some experiments, sIgD− B cells were further separated according to CD38 and CD39 expression into CD38−CD39− germinal center (GC) cells and CD38+CD39+ 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+IgD−CD38− naive B cells and CD20+IgD−CD38− 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–Hyphaque 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)

Cultures of B cells and D–Lc. 2.5 × 10^{9} irradiated CD40L L cells (7,500 rads) were seeded together with 10^{5} B lymphocytes (either total B cells or B cell subpopulations) in the presence or absence of in vitro generated D–Lc (10^{5}) in 96-well culture plate (Nunc, Roskilde, Denmark). B cell proliferation was monitored by tritiated thymidine ([3H]Tdr) incorporation after 6 d of coculture, except for kinetic experiments. Cells were incubated for the last 16 h with 1 µCi of [3H]Tdr. Tests were carried out in triplicate, and results were expressed as cpm ± 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 × 10^{5} 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, Wlimington, MA). 10^{5} D–Lc cultured in the presence or absence of CD40 triggering (2.5 × 10^{5} 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 × 10^{5} B cells activated by 3.75 × 10^{5} CD40L L cells or CD32 L cells used as control in the lower compartment (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 [3H]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–Grü namwald-Giemsa staining and the others were fixed either in cold acetone or 4% paraformaldehyde during 10 min for immunocy-
by culturing cord blood CD34+ cells, we wondered whether D–Lc generated in vitro could differentiate B cells as measured by Ig production (Caux, C., B. Vanbervliet, and J. Banchereau, unpublished results). Activation of T cells, subsequently resulting in the differentiation of B cells and DC were found to induce the activation of T cells, as well as CD1a+ cells including granulocytes and monocytes (see Materials and Methods). The role of the latter cells will be discussed later on (Fig. 4). As shown in Fig. 1A, D–Lc strongly enhanced CD40-induced B cell proliferation as measured by [3H]TdR uptake after 6 d of coculture (three to eight-fold enhancement of [3H]TdR uptake). Note that in the absence of CD40L L cells, D–Lc cannot induce B cell proliferation (Fig. 1A, left). Kinetics analysis shows that addition of 10^4 D–Lc to CD40 trigger resulted in a twofold increase of [3H]TdR incorporation at day 2, which reached fourfold at day 4, when a plateau is reached (Fig. 1B). This enhancement of B cell DNA synthesis by D–Lc is associated to an important increase in number of viable B cells (Fig. 1C) that reaches a plateau at day 10, a time when D–Lc increased B cell num-

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 IgG2 anti–HLA-DR from Becton Dickinson) and the APAAP technique (mouse IgG 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+ cells for 12 d in the presence of GM-CSF and TNF-α (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+ as well as CD1a− cells including granulocytes and monocytes (see Materials and Methods). The role of
Table 1. D-Lc Enhance Growth and Ig Secretion of Resting B Lymphocytes Activated Through CD40

|                  | [3H] Tdr uptake (cpm × 10^-3) | IgG (μg/ml) | IgM (μg/ml) |
|------------------|--------------------------------|-------------|-------------|
|                  | Medium D-Lc                   | Medium D-Lc | IL-2        | IL-2 + D-Lc |
| Exp. 1 Total B cells | 7.3 ± 0.02                    | 0.1 ± 0.05  | 0.1 ± 0.02  | 26.8 ± 2.6  |
| Low density      | 5.6 ± 0.06                    | 0.2 ± 0.05  | 0.1 ± 0.02  | 16.6 ± 1.5  |
| High density     | 10.0 ± 1.6                    | 0.7 ± 0.01  | 0.2 ± 0.06  | 41.9 ± 5.1  |
| Exp. 2 Total B cells | 3.5 ± 0.6                    | 0.5 ± 0.01  | 0.1 ± 0.02  | 6.0 ± 2.0   |
| High density IgD^+CD38^+ | 3.1 ± 0.6                | 0.1 ± 0.01  | 0.3 ± 0.1   | 21.5 ± 5.8  |
| High density IgD^-CD38^-  | 3.5 ± 0.2                 | 0.1 ± 0.05  | 0.1 ± 0.05  | 0.6 ± 0.2   |

D-Lc stimulate differentiation of resting naive and memory B cells activated through CD40. Highly purified B cells were isolated 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 DC40 L cells in the presence or absence of D-Lc. For experiment 2, CD20^- resting B cells (high density) were further separated into IgD^-CD38^- naive B cells and IgD^-CD38^- memory B cells, using three-color FACs^®^ sorting (as described in Materials and Methods). These cells were dispensed at 5 × 10^3/ml together with 2.5 × 10^6 CD40 L cells with or without 10^4 D-Lc. DNA synthesis was assayed after 6 d of coculture, and levels of IgG (in the presence 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 CD40 L cells.

The increased proliferation of CD40-activated B cells in the presence of D-Lc, and the enhanced secretion of cytokines, cytokine receptor expression, and cell surface markers, suggest that CD40-Lc interaction enhances CD40-induced B cell responses. D-Lc enhance the CD40-induced proliferation of all B cell subsets (naive and memory, B cells) and the production of both resting and activated B cells (high density). CD20^- small resting B cells FACs^®^ sorted into IgD^-CD38^- cells (resting memory B cells) produced high levels of IgG, while GC cells produced <0.5 μg/ml IgG and 4 μg/ml IgM, 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^- small resting B cells FACs^®^ sorted into IgD^-CD38^- cells (resting memory B cells) produced high levels of IgG, while GC cells produced <0.5 μg/ml IgG and 4 μg/ml IgM, respectively, under such culture conditions.

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

D-Lc activated naive B cells cultured in the presence of CD40-Lc secrete IgM in response to IL-2. In view of the weak stimulation of IgM production, we wondered whether IgD^- 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 (IgD^+, IgD^−, 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-γ 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.
of CD40L L cells. Significant B cell proliferation and differentiation was observed in absence of IL-2 (Fig. 4 A c). In contrast, even at high cell density (10^6 cells), CD1a^+ 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^+ D–Lc.

Although highly purified B cells (>99% CD20^+ 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^+ cells can be detected at any timepoint tested, and >98% of the small cells are CD19^+ B cells. Similar results were observed in cultures set up with sIgD^+ 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^+ 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-activated 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 × 10^4 D–Lc, whereas more than 5 × 10^4 monocytes were required to observe the same effect. At higher cell density (10^6 cells), monocytes induced levels of IgG in the order of 5 μg/ml compared with 17 μg/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 × 10^3 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 that monocytes in inducing B cells to secrete IgG and IgM.

The Effect of D–Lc Can Be Observed 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 CD8α and the human CD40L. The soluble protein was a less efficient activator than the human CD40L. The soluble protein was a less efficient activator than the human CD40L.
The effects of D–Lc on CD40-activated B cell are restricted to CD1a⁺ dendritic cells and are not due to potentially contaminating T cells. In vitro generated D-Lc were FACS® sorted into CD1a⁺ cells and CD1a⁻ DC and used as stimulators of 10⁶ CD40-activated B cells. Increasing numbers of either total D-Lc or FACS® 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.

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 cytospins shows close contacts between CD40-activated B cells and D-Lc (Fig. 7 A). Immunostainings on such cytospins showed large HLA-DR²⁺ CD8₀high cells with dendritic morphology surrounded by HLA-DR²⁺ CD8₀low 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⁺/HLA-DR²⁺/CD8₀high), 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 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.

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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®. 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 ± 0.8 μg/ml versus 15.5 ± 2.5 μg/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+ hematopoietic progenitors cultured for 12 d in the presence of GM-CSF plus TNF-α (D–Lc) (18, 21). CD1a+ 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+ 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+ 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); (d) 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+ IL-2 producing CD4+ 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+ 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).
responses is not yet totally elucidated. However, the fact that D–Lc stimulate the proliferation of IgD+ 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® 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 naïve 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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