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Toward a Role of Dendritic Cells in the Germinal Center Reaction: Triggering of B Cell Proliferation and Isotype Switching[1]

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We have reported previously that in vitro generated dendritic cells (DC) can directly regulate B cell responses. Recently, germinal center DC (GCDC) were identified within B cell follicles. Due to their particular localization, we have tested in the present study whether GCDC could contribute to key events characteristic of the GC reaction. Our present results demonstrate that 1) ex vivo GCDC induce a dramatic GC B cell expansion upon CD40 and IL-2 activation and drive plasma cell differentiation, 2) this property is shared by GCDC and blood DC, but not by Langerhans cells, 3) IL-12 production by GCDC is critical in GC B cell expansion and differentiation, and 4) importantly, GCDC also induce IL-10-independent isotype switching toward IgG1. These observations support the novel concept that GCDC directly contribute to the germinal center reaction. ***The Journal of Immunology,* 1999, 162: 3428–3436.**

During a T cell-dependent humoral response, B lymphocytes first proliferate in T cell-rich areas and develop locally into plasmablasts, whereas a minority colonize primary follicles to form germinal centers (GC)[4] (1, 2). These particular structures of secondary lymphoid organs can be morphologically divided into a dark zone composed of highly proliferating centroblasts and a light zone in which nondividing centrocytes are submitted to a selection process according to their affinity for Ag (3). GC were shown to be the site of affinity maturation based on somatic mutations of Ig V regions (4) and subsequent positive selection of high affinity B cell clones. Ig class switching is also an important feature of the GC reaction (5–7). Selected centrocytes may further differentiate into memory B cells or plasmablasts expressing mutated Ig with increased affinity for the immunizing Ag. The molecular and cellular mechanisms governing the GC reaction are not fully elucidated, but probably involve cellular components of the GC. Follicular dendritic cells (FDC) may have a role in GC B cell clonal expansion (8–12), and through their capacity to retain native Ag in the form of immune complexes, may serve as APC for mutated centrocytes, allowing initial rescue from apoptosis (13). GC also contain some T cells, essentially of the memory phenotype, containing preformed CD40L (14) most likely to be critical for rescuing GC B cells from apoptosis and for subsequent differentiation. Nonselected centrocytes die by apoptosis and are engulfed by tingible body macrophages (TBM).

Another population of DC, termed germinal center DC (GCDC), has recently been identified within human GC (15). This population is clearly distinct from FDC and TBM and was demonstrated to stimulate T cell proliferation in vitro (15). The principles of DC-B cell collaboration have been shown in our previous studies using in vitro generated DC (16–18). Their localization into the GC and their accessibility prompted us to investigate the possible contribution of GCDC in the follicular B cell response. We show that GCDC induce a strong expansion of GC B cells and skew their differentiation into plasmablasts in the presence of CD40 activation and cytokines such as IL-2 or IL-15. In addition, they were found to induce CD40-dependent isotype switching of naive B cells preferentially toward IgG1. Comparison of various DC subsets supports the idea that GCDC are specialized DC for the induction of B cell responses and identifies GCDC as a critical element of the GC reaction.

Materials and Methods

Hemopoietic factors, cytokines, Abs, and cell lines

rhGM-CSF (sp. act., 2.10^6 U/mg; Schering-Plough Research Institute, Kenilworth, NJ) was used at a saturating concentration of 100 ng/ml (200 U/ml). rhTNF-α (sp. act., 2×10^7 U/mg; Genzyme, Boston, MA) was used at an optimal concentration of 2.5 ng/ml (50 U/ml). rhSCF (sp. act., 4×10^5 U/mg; R&D Systems, Abington, U.K.) was used at an optimal concentration of 25 ng/ml. rhIL-2 (sp. act., 3×10^6 U/mg; Amgen, Thousand Oaks, CA) was used at 50 U/ml. rhIL-15 (1×10^7 U/mg) was purchased from R&D Systems and used at 10 ng/ml.

The following blocking Abs were used at 10 μg/ml: mouse anti-gp80 mAb (Diaclone, Besançon, France), goat anti-IL-12 (R&D Systems), mouse IgG1 anti-IL-12 mAb (clone C8.6, kindly provided by A. O'Garra, DNAX, Palo Alto, CA), rat anti-hIL-10R mAb (clone 3F9), anti-hIL-10 mAb (clone 12G8, kindly provided by K. Moore, DNAX), mouse IgG1, and goat IgG isotype control Abs (R&D Systems).

The murine fibroblastic cell line stably transfected with human CD40 ligand (CD40-L L cells) was produced in the laboratory, as described previously (19). Mouse fibroblastic L cells transfected with the human CD32/FcγRII (20) were used as control-L cells.

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[4] Abbreviations used in this paper: GC, germinal center; CD40L, CD40 ligand; DC, dendritic cell; FDC, follicular DC; GM-CSF, granulocyte-macrophage CSF; h, human; LC, Langerhans cell; LCL, lymphoblastoid cell line; PE, phycoerythrin; TBM, tingible body macrophages.

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In vitro generation of DC

CD34-derived DC were obtained as described earlier (18, 21, 22). For most experiments, cells were routinely collected after 12 days of culture and labeled with FITC-conjugated anti-CD1a mAb (Ortho Diagnostic Systems, Raritan, NJ), and $CD1a^+$ DC were isolated using a $FACStar^{Plus}$ cytometer (Becton Dickinson, Mountain View, CA). The procedure of staining and sorting was performed in the presence of 0.5 mM EDTA to avoid cell aggregation. Reanalysis of the sorted population showed a purity higher than 98%.

Monocyte-derived DC. Monocytes were purified from PBMC by centrifigation over a 50% Percoll and depletion of $CD14^-$ cells using anti-CD3 (OKT3), anti-CD8 (OKT8), anti-CD16 (Immunotech, Luminy, France), anti-CD19 (4G7), and anti-NKH1 (Coulter) Abs and bead depletion. Cells were routinely >90% $CD14^+$. Monocytes were then cultured in presence of GM-CSF + IL-4 and used after 6–7 days to stimulate B cell proliferation. Monocyte-derived DC were generally >80% $CD1a^+$.

Purification of GCDC and TBM from tonsils

GCDC were prepared as previously described (15), with some modifications. Briefly, tonsils obtained from children undergoing tonsillectomy were finely minced and digested with collagenase IV and DNase. Cells collected after two rounds of enzymatic digestion were centrifuged through Ficoll-Hypaque with SRBC for 15 min at 500 rpm, then for 30 min at 2000 rpm. $CD3^+$ T cells, $CD19^+$ B cells, and $CD14^+$ monocytes were removed from the resulting population by magnetic beads (anti-mouse Ig-coated Dynabeads; Dynal, Oslo, Norway). Anti-CD3 (OKT3), anti-CD19 (4G7), and anti-CD14 (MOP9) mAb were purified from ascites. A second depletion was performed with anti-NKH1 (Coulter, Hialeah, FL), anti-glycophorin A (Immunotech), and anti-CD20 (purified from ascites). The remaining cells were stained with the following mAbs: anti-CD1a FITC (Ortho Diagnostics Systems), anti-CD14 FITC, anti-CD57 FITC, anti-CD16 FITC, anti-CD7 FITC, anti-CD20 FITC, anti-CD3 FITC (Becton Dickinson), anti-CD11c PE (Becton Dickinson), and anti-CD4 PE-Cy5 (Immunotech). $CD4^+CD11c^+CD3^-CD20^-CD1a^-$ were isolated by cell sorting using a $FACStar^{Plus}$ (Becton Dickinson). An average of 2.10^5–8.10^5 cells with >97% purity was obtained by this method.

TBM were isolated by cell sorting of large cells with strong autofluorescence. Cell purity was approximately 50–80%, as judged by staining for nonspecific esterase and CD71, which expression on TBM is higher than on GCDC (15).

Isolation of skin Langerhans cells (LC)

Epidermal cell suspensions were obtained from normal skin of patients undergoing reconstitutive plastic surgery of breast or abdomen, as described elsewhere (23). LC enrichment was achieved by successive density-gradient centrifugation steps and depletion of basal keratinocytes. Further purification was obtained by positive selection of $CD1a^+$ cells using anti-CD1a mAb (OKT6; Ortho Diagnostics), goat anti-mouse IgG-coated microbeads, and Minimacs separation columns (Miltenyi Biotec, Auburn, CA). The isolated cells contained 95–99% LC (24). LC were then matured for 48 h in presence of GM-CSF + TNF-α and used for cocultures after irradiation (3000 rad).

Isolation of B cells

Mononuclear cells from tonsils were isolated by a standard Ficoll-Hypaque (density = .077 g/ml) gradient method. Tonsillar B cells were first enriched in the E^- fraction and then 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). In the isolated population (total B cells), >99% expressed CD19 and CD20 and <1% expressed CD2 or CD14. Isolation of $sIgD^+$ naive B cells was performed using a preparative magnetic cell sorter (MACS; Miltenyi Biotec GmBH), as described elsewhere (25), allowing >98% purity. GC B cells were first enriched by centrifigution over a 55% Percoll and depleted of IgD^+ and $CD39^+$ B cells using biotinylated anti-IgD (Sigma, St. Louis, MO) and anti-CD39 Abs. This population was routinely >98%, as assessed by anti-CD38 or anti-CD10 FACS staining.

Cocultures of B cells and DC

Cultures were conducted in modified Iscove's medium, supplemented with 5% inactivated FBS, 2 mM L-glutamine, and 0.08 μg/ml gentamicin (Schering-Plough, Levallois Perret, France). A total of 2.5×10^3 irradiated CD40L-L cells (7500 rad) was seeded together with 10^4 B lymphocytes (either GC or $sIgD^+$ B cells) in the presence or the absence of 10^4 DC (irradiated 3000 rad) in 96-well culture plates (16). B cell proliferation was monitored by tritiated thymidine ([^{3}H]TdR) incorporation after 6 days of coculture, except for kinetic experiments. Cells were incubated for the last 16 h with 1 μCi of [^{3}H]TdR. Experiments were conducted in triplicate, and results were expressed as cpm ± SD. For determination of Ig production, supernatants were recovered after 13 days and used for indirect ELISA (26). ELISA specific for IgG subclasses was used as described in detail elsewhere (27). The limits of sensitivity were 50, 90, 65, and 30 ng/ml for IgG1, IgG2, IgG3, and IgG4, respectively. Phenotyping of the cultured cells was routinely performed using FITC-labeled anti-CD3, anti-CD19 (Immunotech), and FITC-labeled IgG1 (Kallestad, Austin, TX), and showed the absence of detectable contaminating T cells throughout the culture.

In other experiments, B cells and DC were cultured in separate compartments using transwells (Costar, Wilmington, MA). A total of 10^5 irradiated CD34-derived DC cultured in the presence or the absence of CD40 triggering (2.5×10^4 irradiated CD40L-L cells or CD32-L cells used as control) in the lower compartment (in a total volume of 0.8 ml) was assayed for their ability to stimulate growth and differentiation of 1.5×10^4 GC B cells activated by 3.75×10^3 CD40L-L cells in the upper compartment of the transwells (in a total volume of 0.15 ml). Measurement of DNA synthesis of B cells was performed by transferring, at day 6, the cells present in the top of the transwells into flat-bottom 96-well plates and pulsing them with [^{3}H]TdR for the last 16 h of the culture period (18).

For phenotypic studies, 10^5 GC B cells were cultured over 2.5×10^4 CD40L-L cells and IL-2, with or without 5×10^4 GCDC. Cultures were performed in 24-well culture plate in a total volume of 1 ml. For plasma cell formation study, cells were recovered after 8 days and processed for FACS staining using anti-CD20 FITC, anti-CD38 PE (Becton Dickinson), and anti-CD19 PE-Cy5 (Immunotech). The percentage of $CD20^-CD38^{high}$ cells was analyzed on a FACS Calibur (Becton Dickinson) by gating on $CD19^+$ B cells.

Giemsa and immunostainings

Cells recovered from 8 days coculture were cytocentrifuged for 5 min at 400 rpm on microscope slides and used for May-Gründwald-Giemsa staining. Double staining was performed using mouse IgG1 anti-CD11c, Ki67 (Dako, Carpenteria, CA), mouse IgG2a anti-CD71, and mouse IgG2b anti-CD11c (Becton Dickinson). Binding of mouse IgG1 was revealed by sheep anti-mouse IgG1 (The Binding Site, Birmingham, U.K.), followed by mouse antialkaline phosphatase-alkaline phosphatase complexes (Dako; APAAP technique). The binding of mouse IgG2a and IgG2b was revealed by sheep anti-mouse IgG2a-biotin or sheep anti-mouse IgG2b-biotin (The Binding Site), followed by Extravidin peroxidase (Sigma). Revelation of alkaline phosphatase activity was performed using Fast Blue substrate; peroxidase activity was indicated by 3-amino-ethylcarbazole.

Results

GCDC stimulate a high rate of proliferation of GC B cells activated through CD40

A population of hemopoietic-derived DC, stimulating allogeneic T cells, has recently been identified within B cell follicles (15). As illustrated in Fig. 1*A*, GCDC are evenly distributed in the GC and can be distinguished from TBM by their weaker expression of CD71. To determine whether these DC could have a direct role in GC B cell proliferation, GCDC were purified by FACS sorting (Fig. 1, *B* and *C*), according to CD11c and CD4 expression (15), and cultured with freshly isolated GC B cells. In the absence of activation, no thymidine uptake by GC B cells was detected whether or not GCDC were added to culture (data not shown). Engagement of CD40 (by the use of CD40L-transfected L cells) rescued GC B cells from apoptotic cell death and induced short-term low DNA synthesis (Fig. 2*B*). In the various cytokines (IL-1 to IL-15) and combinations used, IL-4 + IL-10, and to a lesser extent IL-2 + IL-10, gave the highest stimulation of DNA synthesis by GC B cells cultured in presence of CD40L-L cells for 6 days (Fig. 2*A*). Addition of irradiated GCDC modestly increased cytokine-independent proliferation of GC B cells (mean increase 3.4, range 1.9–4.5, n = 10), but their stimulatory effect was dramatically potentiated in the presence of IL-2 or IL-15 (mean increase 13.5, range 5.9–24.1, n = 10), two cytokines that have limited effects in the absence of GCDC (Fig. 2*A*). The combination

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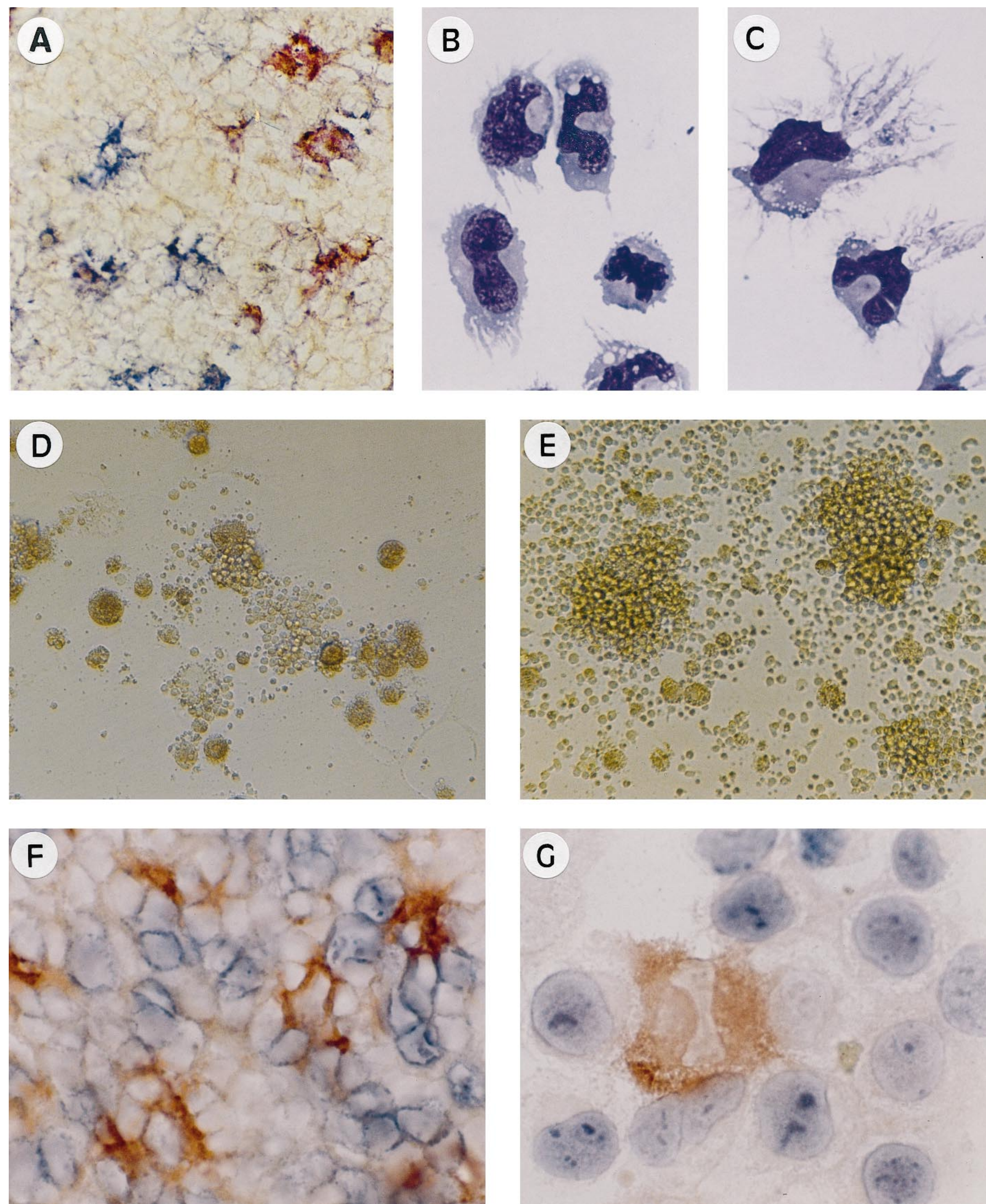

FIGURE 1. $CD11c^+$ GCDC displayed B cell stimulatory capacity. *A*, Immunoenzymatic staining of a tonsil section with anti-CD11c (blue) and anti-CD71 (red) mAbs, showing a GC containing $CD11c^+CD71^{low}$ GCDC and $CD11c^+CD71^{high}$ TBM. Original magnification ×400. *B* and *C*, Morphology of GCDC either freshly isolated (*B*) or stimulated with CD40L-L cells for 48 h (*C*). *D* and *E*, After 6 days of culture with CD40L-L cells and IL-2, few viable cell clusters are observed in cultures of GC B cells (*D*), in contrast to the numerous large cell aggregated that appeared when GCDC and GC B lymphocytes are cultured together (*E*). *F* and *G*, Immunoenzymatic staining of a tonsil section (GC, *F*) and cells isolated from 6 days cocultures of GCDC and GC B cells (*G*), with anti-Ki67 (blue) and anti-CD11c (red) showing clusters of GCDC and proliferating GC B cells. Original magnification ×1000.

of IL-2/IL-15 + GCDC was as efficient as IL-4 + IL-10 in stimulating high rate of DNA synthesis by GC B cells. Whatever the cytokine combination, addition of GCDC induced a >threefold increase of GC B cell proliferation. As illustrated in Fig. 2*C*, the stimulatory effect of GCDC was potentiated by IL-2 or IL-15 in a dose-dependent manner, maximal stimulation being reached at 3 and 10 ng/ml for IL-2 and IL-15, respectively. Kinetic studies showed that the stimulatory effect of GCDC became detectable between day 2 and day 3 and was long lasting, with a peak of B cell proliferation occurring after 6 to 8 days (Fig. 2*B*). CD40 triggering, alone or in combination with IL-2 or GCDC, did not allow significant B cell expansion, as illustrated by the presence of sparse clusters of viable B cells after 6 days (Fig. 1*D*) or direct enumeration using trypan blue dye exclusion (Fig. 2*D*). In contrast, addition of GCDC to IL-2-supplemented cocultures dramatically enhanced both size and number of clusters of viable cells (Fig. 1*E*). In such clusters, the majority of B cells in contact with GCDC express the proliferation-associated nuclear Ag Ki67 (Fig. 1*G*), as observed in vivo in the GC dark zone (Fig. 1*F*). A net increase in viable cell numbers could be detected after 5 days in GC B cells cultured with GCDC + IL-2 and reached maximum increase of 15-fold at day 9 (Fig. 2*D*).

Altogether, these results demonstrate that ex vivo purified GCDC display a high capacity to promote CD40-induced GC B cell growth, an effect mainly dependent on the presence of cytokines such as IL-2 or IL-15.

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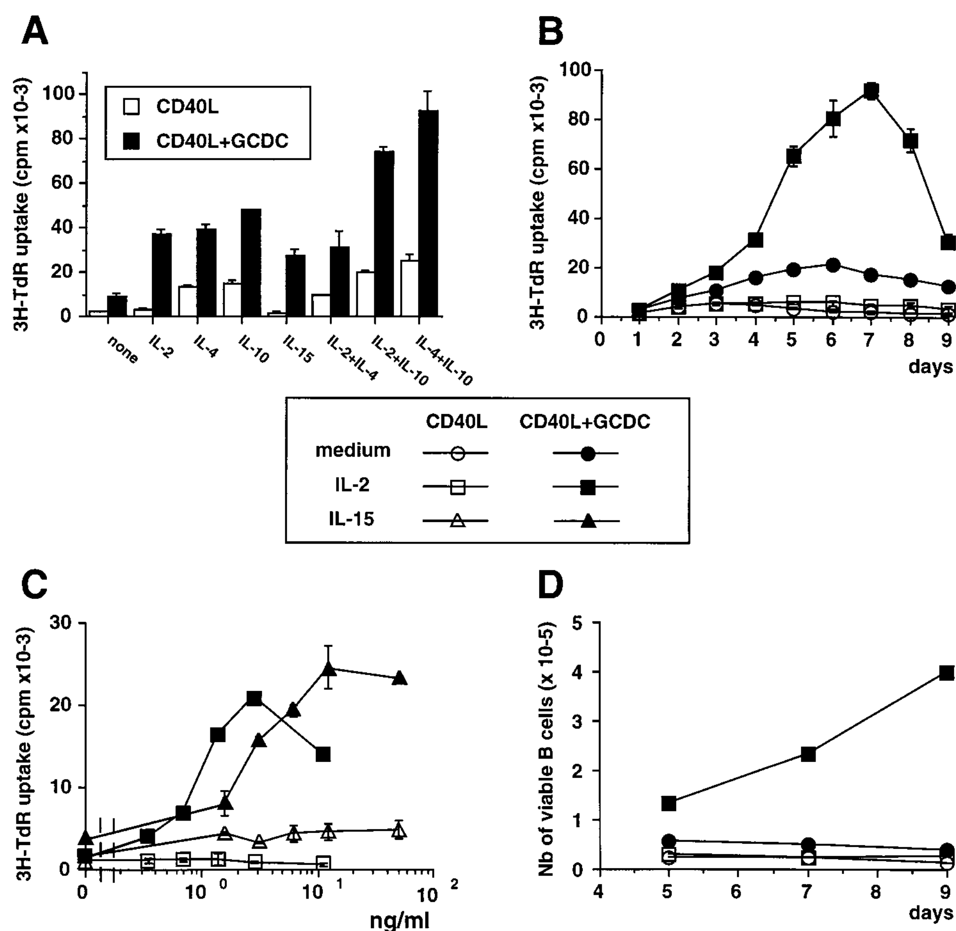

FIGURE 2. GCDC potentiated CD40-dependent growth of GC B cells. *A*, *B*, and *C*, Cultures were established in the presence of 2.5×10^3 irradiated CD40L-L cells (7500 rad) and 10^4 freshly isolated GC B cells. A total of 10^4 ex vivo purified GCDC was added with or without exogenous cytokines at the concentration indicated in *Materials and Methods*. *A*, Thymidine uptake after 6 days of coculture in the presence of various cytokines. *B*, Kinetics of thymidine uptake in cocultures performed with or without exogenous IL-2. *C*, Increasing doses of IL-2 or IL-15 were added to coculture of GC B cells, GCDC, and irradiated CD40L-L cells. Thymidine uptake was determined after 6 days. *D*, 10^5 GC B cells were cultured in the presence of 2.5×10^4 irradiated CD40L-L cells in the presence or absence of 5×10^4 GCDC, with or without exogenous IL-2. Cultures were performed in 24-well plates in a total volume of 1 ml. The number of viable B cells was determined at various time points using trypan blue dye exclusion. (One experiment representative of three.)

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Stimulation of B cell proliferation is restricted to specific DC subsets

Another set of experiments was then designed to determine whether the strong stimulation of GC B cell proliferation observed with GCDC was shared by other APC or was a particular feature of certain populations of DC. For that purpose, increasing numbers of irradiated APC, including DC either purified ex vivo or propagated in vitro (freshly isolated GCDC, skin LC, monocyte-derived DC, and CD34$^+$ progenitor-derived DC), as well as non-DC cell types (peripheral blood monocytes, EBV-transformed lymphoblastoid cell lines (EBV-LCL), and a population enriched in TBM) were added to cocultures of GC B cells and CD40L-L cells in the presence of IL-2 (Fig. 3). Neither EBV-LCL nor peripheral blood monocytes, nor TBM, even at high cell density, were able to induce significant B cell proliferation (Fig. 3, *A* and *B*). In contrast, CD34-derived DC and monocyte-derived DC shared with GCDC the capacity to trigger the proliferation of GC B cells (Fig. 3, *A* and *C*). Indeed, as few as 370 monocyte-derived DC induced a 4.4-fold increase in thymidine uptake, and maximal stimulatory effect was reached with 3000 DC (12-fold increase). Higher numbers of CD34-derived DC were usually required to reach comparable levels of stimulation. Altogether, the above results suggest that the stimulation of GC B cell growth is mainly restricted to cells of the DC lineage.

In contrast, LC isolated from skin (Fig. 3*C*) did not induce any significant B cell proliferation when compared with CD34-derived DC, suggesting a restriction to certain populations of DC. Along with this idea, in vitro propagation of DC from CD34$^+$ progenitors gives rise to a LC-related population (CD1a$^+$-derived DC) and an interstitial type DC (CD14$^+$-derived DC) (21), and only the latter subset was efficient in sustaining GC B cell proliferation (data not shown).

Taken together, these results demonstrate that the capacity to induce the proliferation of GC B cells is restricted to GCDC and related populations of monocyte-derived DC (and CD14$^+$-derived DC), and support the novel concept that GCDC directly participate in the GC reaction.

GCDC-biased GC B cell differentiation into plasma cells

The levels of IgG, IgA, and IgM in supernatants from coculture of GC B cells and GCDC were analyzed at day 13 by ELISA. Results of Fig. 4*A* show that GC B cells activated solely by their CD40 without or with IL-2 produced marginal amounts of Ig. Addition of GCDC to cultures significantly promoted IgG secretion. Addition of IL-2 dramatically potentiated this secretion, and to a much lesser extent that of IgA and IgM (Fig. 4*A*). The phenotype of B cells, cultured in presence or absence of GCDC, was determined after 10 days, by triple-color staining using anti-CD20 FITC, anti-CD38 PE, and anti-CD19 PE-Cy5 Abs. As shown in Fig. 4*B*, >40% of B cells recovered from GCDC-supplemented cultures expressed the phenotype of plasma cells, e.g., high levels of CD38 and down-regulated expression of CD20 (28). In contrast, in the absence of GCDC, the majority of B cells still expressed CD20, but had down-regulated CD38, a phenotype that has been ascribed to memory B cells (29, 30). Giemsa staining on isolated cells from 8 days of coculture further confirms the classical morphology of plasma cells (data not shown).

Within secondary follicles, positively selected B cells differentiate into either memory cells or plasma cells. The present data suggest that, in addition to the stimulation of a high rate of B cell

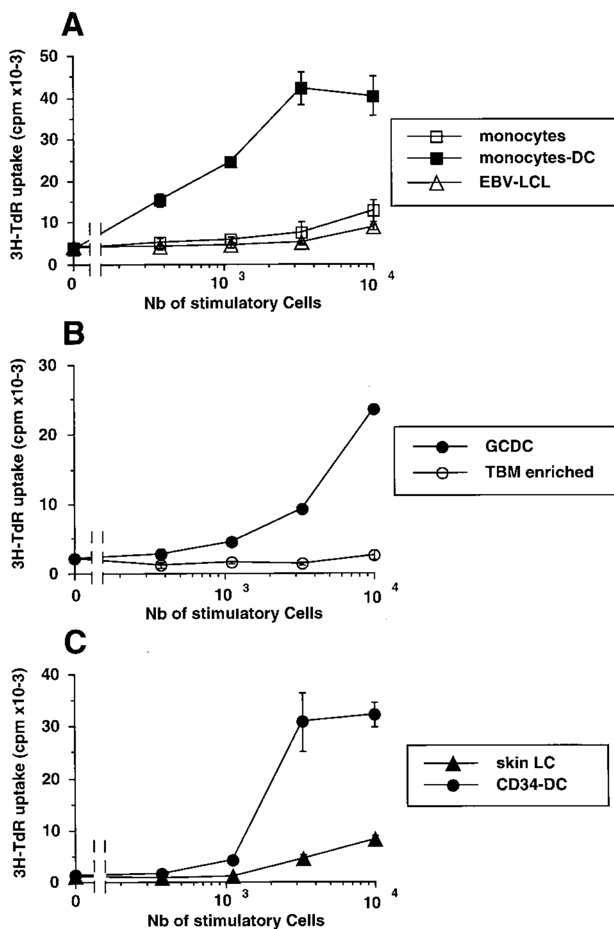

FIGURE 3. Stimulation of GC B cell proliferation is restricted to GCDC and related subsets of DC. Freshly purified GC B cells were cultured in presence of irradiated CD40L-L cells and increasing numbers of irradiated stimulatory cells. *A*, Peripheral blood monocytes, DC derived from monocytes in presence of GM-CSF + IL-4, and an EBV-transformed B cell line (EBV-LCL). *B*, Ex vivo purified GCDC and an enriched population of TBM isolated from the same tonsil. *C*, LC isolated from skin, matured in the presence of GM-CSF during 48 h, and DC derived from $CD34^+$ progenitors in the presence of GM-CSF + TNF-α. Thymidine uptake was determined after 6 days of coculture. (One experiment representative of three.)

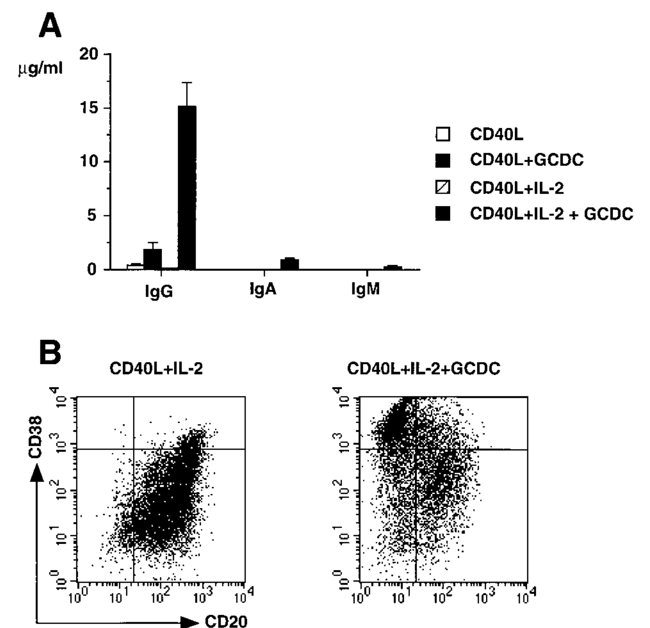

FIGURE 4. GCDC-induced differentiation of GC B cells into Ab-secreting plasma cells. *A*, GC B cells were cultured in the presence of CD40L-L cells in the presence or absence of GCDC and exogenous IL-2. Ig production was measured by ELISA after 13 days of coculture. *B*, Analysis of the phenotype of B cells was performed after 8 days of coculture by triple-color staining using anti-CD20 FITC, anti-CD38 PE, and anti-CD19 PE-Cy5. The results shown were obtained by gating on $CD19^+$ cells (B cells). (One experiment representative of five.)

proliferation, GCDC bias differentiation of GC B cells toward the latter pathway.

Role of soluble factors in GCDC-induced B cell proliferation and differentiation

The low number of GCDC recovered after purification from tonsils was limiting for experiments aimed at elucidating the mechanism. As an alternative, we used in vitro generated CD34-derived DC to perform transwell experiments. Freshly isolated GC B cells were cultured in the top well in the presence of CD40L-L cells. As expected, addition of DC in the same compartment in the presence of IL-2 strongly stimulated B cell proliferation (Fig. 5*A*). Culture of DC in the bottom well, in presence of CD40L-L cells or CD32 L cells, induced low but significant cytokine-independent B cell proliferation (Fig. 5*A*). In contrast, only CD40-activated DC were able to reconstitute high levels of B cell proliferation in the presence of IL-2. Thus, the strong capacity of DC to trigger B cell proliferation is due to soluble molecules produced by DC upon CD40 stimulation. Analysis of Ig production in such transwell experiments indicated a similar mechanism for DC-induced differentiation of B cells (data not shown).

We then tested various anti-cytokine-blocking Abs for their capacity to inhibit GCDC-dependent B cell proliferation and differentiation. Fig. 5*B* illustrates the results obtained with the most relevant Abs that were tested. None of these Abs inhibited GC B cell proliferation in the absence of DC. In IL-2-supplemented cultures, anti-IL-12 Abs inhibited 59% of the DNA synthesis of B cells in response to GCDC stimulation, whereas blocking endogenous IL-10 or IL-6Rα-chain only resulted in a modest inhibition (6 and 19%, respectively). Furthermore, blocking IL-12 resulted in 80% inhibition of GCDC-induced GC B cell expansion (data not shown). Indeed, as observed with CD34-derived DC (16), CD40-activated GCDC produced low but significant levels of IL-12 p70 (17.8 ± 6 pg/ml, range 10.4–25.7, $n = 3$). Nevertheless, a combination of IL-2 + IL-12 was not sufficient to stimulate CD40-induced proliferation of GC B cells (data not shown), suggesting the participation of other GCDC-derived molecules, as yet not identified.

GCDC induce isotype switching of naive B lymphocytes

In addition to the high rate of B cell proliferation, GC are the major site of isotype switch in human tonsils (7). To analyze the possible contribution of GCDC to such events, highly purified tonsillar naive B cells were cultured in presence of CD40L-L cells with or without GCDC, and Ig productions were determined after 13 days of coculture. As illustrated in Fig. 6, CD40-activated naive B cells produced only marginal amounts of Ig, whether or not IL-2 was added. Addition of IL-10 to such cultures induced naive B cells to produce high levels of IgM (9.2 μg/ml), IgG (8.0 μg/ml), and to a lesser extent IgA, in agreement with previous reports on the role of IL-10 as a switch factor (31, 32). In addition to triggering IgM secretion, GCDC induced secretion of significant amounts of IgG

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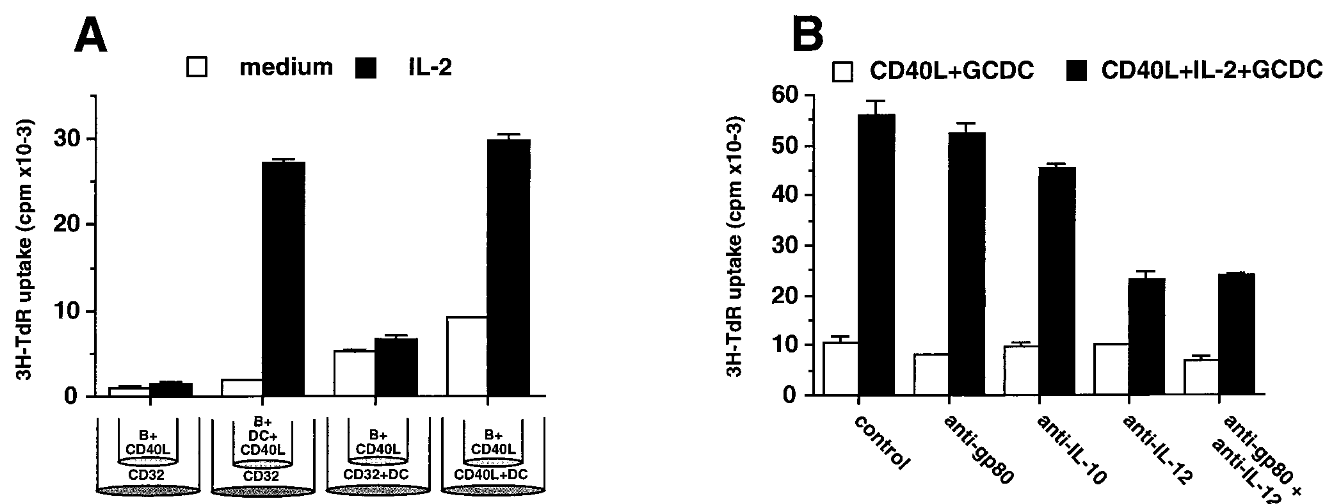

FIGURE 5. GCDC-stimulated GC B cell proliferation through the production of soluble factors. *A*, GC B cells (1.5×10^4) were cultured with 3.75×10^3 irradiated CD40L-L cells in the top of the transwell. A total of 10^5 CD34-derived DC was added in the bottom well in the presence of 2.5×10^4 irradiated CD40L-L cells or CD32-transfected L cells with or without exogenous IL-2. Thymidine uptake by GC B cells was determined after 6 days of culture. *B*, GC B cells (10^4) were cultured with CD40L-L cells (2.5×10^3) and GCDC (10^4) in the presence or absence of exogenous IL-2. Various blocking Abs or their respective isotype-matched controls were added at the start of the culture (10 μg/ml), and thymidine uptake was determined after 6 days. (One experiment representative of three.)

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by naive B cells (Fig. 6*A*) that were enhanced in IL-2-supplemented cultures (1.2 μg/ml versus 4.77 μg/ml in the presence of IL-2). These levels of IgG production by CD40-activated naive B cells are comparable with that induced by the known switch factor IL-10 (Fig. 6*A*). In contrast, IgA production induced by IL-2 + GCDC remained marginal (Fig. 6*B*). Analysis of IgG subclasses indicates that GCDC induced naive B cells to produce high amounts of IgG1 and low levels of IgG3, whereas IgG2 and IgG4 were never detected (Table I). While exogenous IL-10 induced naive B cells to secrete comparable amounts of IgG1 and IgG3 (Table I and (31)), GCDC preferentially induce naive B cells to switch toward IgG1 (93 $\pm$ 6% of total IgG secretion, range 84–100%, $n = 7$). Furthermore, the possible contribution of endogenous IL-10, produced by both DC (33) and B cells (34), was excluded by the fact that blocking anti-IL-10R Abs did not result in any significant inhibition of DC-dependent Ig production by naive B cells (Table I).

Taken together, these results demonstrate the capacity of GCDC to induce naive B cells to produce secondary isotypes, primarily IgG1, and identify IL-2 as an important cytokine that potentiates this DC-dependent isotype switching. Deciphering the respective roles of IL-2 and GCDC in this process will require further analysis at the molecular level.

Discussion

Cardinal features of GC include a rapid rate of B cell proliferation, somatic mutation, isotype switching, affinity selection, and differentiation into memory B cells or Ab-producing cells. The mechanisms controlling these events are not fully elucidated, but probably involve cellular components of the GC, as reported for follicular DC and T cells (13, 35). In the present study, we propose the novel concept that the recently identified GC population of hemopoietic derived DC localized within human B cell follicles (GCDC) (15) plays a central role during the GC reaction. Ex vivo purified tonsillar GCDC stimulated a high rate of DNA synthesis by CD40-activated GC B cells, in particular in the presence of cytokine such as IL-2 or IL-15. The present results demonstrate that GCDC provide important complementary signals to CD40 stimulation, allowing >15-fold expansion of GC B cells in the presence of IL-2 or IL-15 and subsequent differentiation of GC B cells into plasma cells. Furthermore, in the presence of CD40L and IL-2, GCDC induced isotype switching of naive B cells preferentially toward IgG1.

The dramatic effect observed on B cell growth leads to the hypothesis that GCDC may have an important role in vivo in sustaining the proliferation of centroblasts within the dark zone. The presence of GCDC within the dark zone in contact with $Ki67^+$ GC B cells (15) would support this hypothesis. This study underlines the critical role of IL-2 for DC-dependent B cell growth and differentiation. This observation is consistent with two previous studies indicating that GC-T cells contained detectable IL-2 mRNA (36, 37). Along with this, an IL-2-dependent pathway of development for GC B cells has been described (38, 39). Interestingly, CD40-activated naive B cells as well as GC B cells express transiently CD25, whereas addition of DC was found to maintain CD25 expression at high levels throughout the culture period. In the presence of IL-2, those effects are further enhanced (unpublished observations). The present results, together with the fact that GCDC are found in close contact with T cells in situ (15), would be consistent with this hypothesis and provide a cellular basis for stimulation of centroblast growth during the GC reaction. Furthermore, the requirement for CD40 activation is consistent with studies performed in mice showing that Abs to CD40L can abrogate an ongoing GC reaction (40, 41). Nevertheless, the rare T cells present within GC were shown to be mainly restricted to the light zone (14), challenging the hypothesis that GCDC and B cells receive activation from surrounding T cells. Several hypotheses can be advanced to reconcile our present culture system with the signals GCDC and B cells may encounter in vivo. 1) As GC B cells originate from precursors activated in the T zone, it is not unlikely that CD40 signaling may occur in vivo before entry into the follicle. 2) Besides activated T cells, the source of CD40L could be provided by B lymphocytes or DC, as proposed by others (42–44). 3) It is also possible that in vivo, signals other than CD40-CD40L engagement may confer the B cell stimulatory capacity to GCDC. Indeed, distinct cytokines are secreted by DC activated either

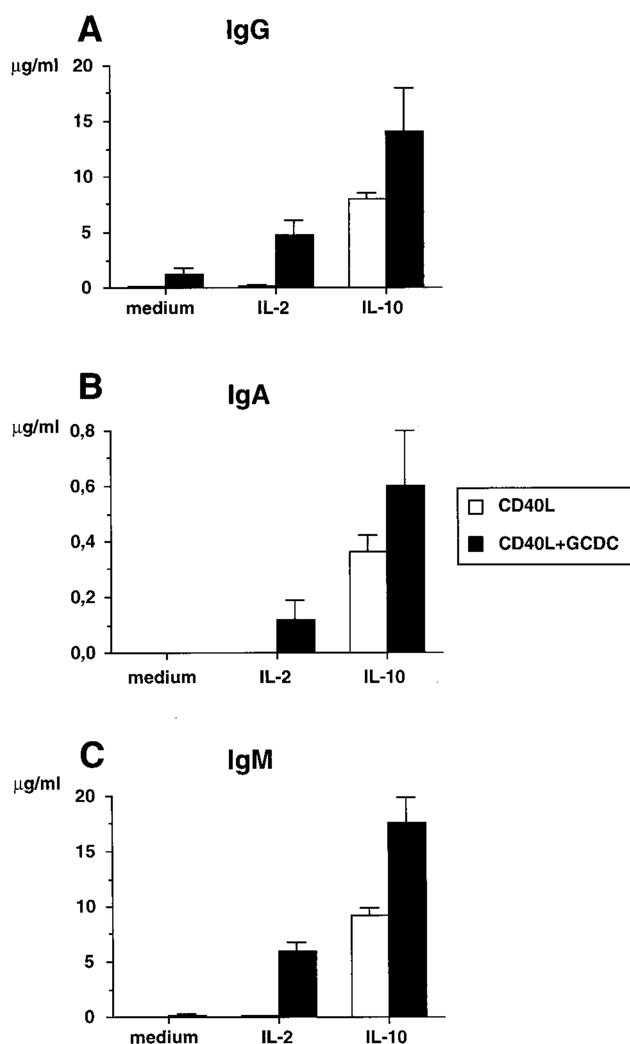

FIGURE 6. GCDC-induced CD40-dependent isotype switching of naive B cells toward IgG. Highly purified $sIgD^+$ naive B cells (10^4) were cultured in the presence of irradiated CD40L-L cells (2.5×10^3) with or without ex vivo purified GCDC (10^4). Cultures were performed either without exogenous cytokines or with IL-2 (50 U/ml) or IL-10 (100 ng/ml). IgG, IgA, and IgM were quantified by ELISA after 13 days of coculture. (One experiment representative of three.)

through CD40 or PMA and ionomycin (33, 45–47), suggesting alternative signals that activate DC and that remain to be identified. In this context, members of the TNF/TNFR family, such as OX40 ligand or the recently identified RANK/TRANCE-R (48, 49), are potential candidates.

In addition, GCDC may play a role in B cell activation in the light zone of GC. After proliferating in the dark zone, centroblasts give rise to noncycling centrocytes that are subjected to selection based on their affinity for Ag held on FDC and then develop into either memory cells or plasma cells. The present study demonstrates that GCDC strongly potentiate Ig synthesis by GC B cells, corresponding to a massive differentiation of B cells into plasma cells. This observation is consistent with the in vivo detection that some GC B cells directly differentiate into plasmablasts within GC (35, 50). Several considerations also suggest reentry of selected centrocytes in the dark zone for further rounds of proliferation, ensuring emergence of high affinity cell clones (40, 51). Altogether, these results suggest that within the light zone of GC, DC may directly contribute to further expansion of positively selected centrocytes and orient their differentiation toward plasma cells, thus allowing a rapid and efficient production of Abs required to neutralize invading pathogens.

Our present data suggest that GCDC may not only be involved in the proliferation of GC B cells, but also in the activation of naive B cells, as was initially observed with in vitro generated DC (16, 17). Indeed, GCDC as well as blood $CD11c^+$ DC can induce naive B cells to differentiate into plasma cells secreting high levels of IgM in the presence of IL-2. Strikingly, these DC also triggered preferential isotype switching of naive B cells toward IgG1 in the presence of CD40L and IL-2. Our results together with a recent report in relB-deficient mice (52) suggest that DC may provide key signals to naive B cells, allowing isotype switching toward IgG1. Interestingly, this GCDC-dependent isotype switching was not inhibited by blocking endogenous IL-10, suggesting that GCDC might produce alternative IgG1 switch factors.

The identification of the molecules involved in GCDC-dependent B cell activation could give key information as to the factors that control B cell proliferation within GC. Characterization of GCDC-stimulatory activity using transwells indicated that the effect is caused by soluble molecules, the production of which requires CD40 triggering. Ex vivo purified GCDC were recently shown to produce mRNA for IL-7, IL-10, and IL-13 (33), each of those cytokines being able to stimulate B cells (53–55). Nevertheless, their possible contribution to GCDC-dependent B cell activation was ruled out by the use of blocking Abs (data not shown). Interestingly, anti-IL-12 Abs strongly inhibited GCDC-dependent GC B cell growth (59% inhibition) and Ig secretion (>80%). Indeed, CD40-activated GCDC produced detectable bioactive IL-12. In line with this, in situ hybridization for IL-12 p35 and p40 in mice suggests the existence of rare GC cells expressing mRNA for both chains of IL-12 (56). Nevertheless, a combination of IL-2 + IL-12 was not sufficient to reconstitute the stimulatory activity of GCDC, suggesting the contribution of other DC products, the identification of which could contribute to a better understanding of the regulation of the follicular B cell response.

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Table I. *DC-biased IL-2-dependent isotype switching of naive B cells toward IgG1*[a]

Expt.	CD40L + IL-2	IgG1	IgG2	IgG3	IgG4
1	DC	4.26 ± 0.78	<0.09	0.65 ± 0.14	<0.03
2	DC	10.65 ± 0.14	<0.09	0.48 ± 0.01	<0.03
3	IL-10	1.64 ± 0.28	<0.09	2.02 ± 0.81	<0.03
	IL-10 + anti-IL-10	0.15 ± 0.03	<0.09	0.10 ± 0.02	<0.03
	DC	2.52 ± 1.10	<0.09	0.48 ± 0.07	<0.03
	DC + anti-IL-10	2.55 ± 1.22	<0.09	0.30 ± 0.10	<0.03

Cultures were performed as indicated in Fig. 6; for experiment 3 a blocking anti-IL-10R Ab (10 µg/ml) was added at the start of the culture. IgG subclasses were determined after 13 days of coculture as indicated in *Materials and Methods*. Results are expressed in µg/ml.

Recent investigation indicates that DC are of diverse origin, as illustrated by the description of at least two types of myeloid precursors in humans (21, 57, 58) and the description of a lymphoid pathway of differentiation in mice (59) and possibly in humans (60). These observations, together with the identification of different populations of DC within secondary lymphoid organs, such as spleen (61), lymph nodes (62), Peyer's patches (63), and tonsils (15, 64), lead to the emerging idea that DC subsets may have different specialized functions. By comparing different ex vivo purified DC subsets as well as various populations of DC generated in vitro, we identified clear functional differences in their capacity to stimulate B cell responses. Tonsillar GCDC, blood $CD4^+CD11c^+$ DC, in vitro monocyte-derived DC, or the related CD14-intermediate derived DC had a strong capacity to trigger GC B cell proliferation, whereas LC or the in vitro related CD1a-intermediate derived DC displayed little, if any, B cell stimulatory activity. These observations suggest that the stimulation of B cell responses may be a particular feature of GCDC and related cells. The presence of GCDC within primary follicles in the spleen (15), and the fact that they display a phenotype similar to $CD4^+CD11c^+$ blood DC (65) suggest that DC originating from blood colonize primary follicles. In line with this, migration of cells with dendritic morphology from the subcapsular sinus into the B cell follicles has been described in mice, in the first few days after Ag injection (66). The identification of the migratory pathway of DC homing within B cell follicles will be of critical interest to better appreciate their potential role in the development of the humoral response. Because GCDC have been shown to carry immune complexes (15), it is tempting to speculate that they could correspond to the Ag-transporting cells described by Szakal et al. 15 yr ago (67). The above considerations together with the present data suggest that GCDC constitute a particular subset of DC specialized in the induction of humoral response.

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