Follicular Dendritic Cells Inhibit Human B-Lymphocyte Proliferation


In germinal centers, B lymphocytes are intimately associated with follicular dendritic cells (FDCs). It has been hypothesized that FDCs are involved in the regulation of B-cell growth and differentiation through cell-cell interactions. In this study, highly enriched preparations of FDCs were isolated by cell sorting using the FDC restricted monoclonal antibody DRC-1. When irradiated FDCs were cultured with mitogen stimulated B cells, B cell 3H-TdR uptake was inhibited by up to 80%. This inhibitory effect was not seen when paraformaldehyde fixed FDCs were added to B-cell cultures, suggesting that the FDCs needed to be metabolically active. Moreover, supernatants from cultured FDCs were similarly able to inhibit B-cell proliferation. These results demonstrate that FDCs may downregulate the clonal expansion of B cells that occurs within lymphoid follicles as part of the normal physiologic immune response. Potentially, the loss of the inhibitory role of FDCs in vivo may be of importance in certain infectious and neoplastic processes in which germinal centers are affected. © 1992 by The American Society of Hematology.

The germinal center of the secondary lymphoid follicle is a complex microenvironment that plays a central role in the generation of the secondary immune response. Aside from being a major site of B-cell proliferation and clonal expansion, it is hypothesized that the generation of memory B cells, antibody heavy chain switching, and antibody affinity selection occurs in germinal centers (GCs). A recent report suggests that B-cell survival within GCs is maintained through specific cell surface signals. It is likely that there is also a requirement for the downregulation of proliferation. Considering the different cellular constituents of GCs, specific interactions between B cells and the other components may be important in the positive and negative regulation of B-cell growth.

Activated B lymphocytes are the major cell population within GCs, although small numbers of T cells, predominantly CD4+, and macrophages are located there. In addition to these cells, follicular dendritic cells (FDCs) are uniquely located in follicles. Morphologic and immunohistochemical studies have divided the GC into anatomic divisions based in part on the degree of B-cell proliferation that is present. The dark zone of the GC is the site of the most active B-cell proliferation, whereas within the light zone there is less active mitosis and B-cell death. Within the light zone of the GC, it is hypothesized that the differentiation of B cells into memory cells and Ig-secreting cells is accompanied by a decrease in B-cell proliferation. In addition to being intimately associated with the cellular processes of FDCs, murine and human B-cell proliferation in vitro has been reported to be enhanced by FDC-enriched preparations.

Studies of the interaction of FDCs with B cells have been limited by the low numbers in which FDCs are present in lymphoid tissues, generally less than 0.1% of the total cells. In the present report we have prepared enriched preparations of human FDCs to examine their effects on B-cell proliferation. These FDCs were found to inhibit B-cell proliferation largely through the production of a soluble factor. These studies provide a foundation to examine the soluble factor(s) that mediate interactions between FDCs and B cells that occur in GCs.

MATERIALS AND METHODS

FDC isolation and enrichment. Human tonsils were obtained immediately after elective tonsillectomy after appropriate Human Protection Committee validation and informed consent. The tissue was placed in a tissue culture dish and minced with scalpel and forceps in RPMI 1640 (GIBCO Laboratories, Grand Island, NY) contained 10% normal human AB serum, 2 mg/mL collagenase (Type IV; Sigma, St Louis, MO), and DNase 0.1 mg/mL (Type 1; Sigma). The minced tissue was incubated for 15 minutes at 37°C, and the supernatant collected and passed through nylon mesh. The mincing, digestion, incubation was repeated three times. The cell suspension was centrifuged at 1,800 rpm for 30 minutes over a 35% Percoll gradient (Pharmacia, Uppsala, Sweden). The interface that was enriched for FDCs was then collected and used fresh or stored in 10% dimethylsulfoxide and 20% FCS at −196°C in the vapor phase of liquid nitrogen.

Further enrichment of FDCs was subsequently performed by incubating the cells from the 35% Percoll interface with the FDC-restricted monoclonal antibody (MoAb) DRC-1 (IgM isotype) (Dako Corp, Carpenteria, CA) followed by fluorescein conjugated goat anti-mouse Ig (Tago, Burlingame, CA). An aliquot of cells was also stained with isotype-identical irrelevant mouse IgM MoAb. All antibodies were previously ultracentrifuged at 15,000 rpm at 4°C to remove aggregates. To block nonspecific binding of MoAbs to Fc receptors on FDCs, all incubations and washes were performed with 10% human AB serum in SMEM. Cells were then subjected to fluorescence-activated cell sorting (FACS) using either an EPICS C or EPICS Elite (Coulter Electronics, Hialeah, FL) flow cytometer with selection of large cells, which densely expressed the DRC-1 antigen. The purity of the FDCs after sorting was confirmed by reanalysis of the sorted population, with greater than 80% DRC-1+ cells consistently seen following enrichment. Cytospin preparations of sorted cells confirmed that the cells had the morphology of FDCs and immunohistochemical studies using standard techniques demonstrated small numbers of contaminating B cells, but essentially no monocytes. Further phenotypic characteristics of these sorted cells was performed with anti-CD23 (Blast 2, IgG1), anti-CD54 (RR1/1, IgG1), anti-human IgG (no. 16, IgG2a), anti-CD21 (HB5, IgG2a)
followed by goat anti-mouse IgG coupled to horseradish peroxidase (FisherBiotech, Pittsburgh, PA) as described.23 Supernatants from FDCs were prepared by culturing purified isolated FDCs at 5 × 10⁴/mL in tissue culture medium for 24 hours at 37°C. Supernatants were also prepared from equal numbers of the DRC-1- cell populations. Supernatants were harvested and filtered through 0.22-μm filters (Millipore, Bedford, MA) before their addition to culture wells with B cells.

**B-cell preparation.** Single cell suspensions of tonsil were prepared as described above. The mononuclear fraction of tonsil cells was isolated by Ficoll-Hypaque density gradient centrifugation. After sheep red blood cell (E) resuspending the E population contained greater than 90% CD20⁺ (B1, IgG2a, isotype)²⁴ cells, less than 5% CD14⁺ (MY4, IgG1 isotype)²⁵ and less than 5% CD2⁺ (T11, IgG1 isotype),²⁶ as determined by indirect immunofluorescence and flow cytometry (IF). Cells were then cryopreserved until use. After culture with Staphylococcus aureus Cowan strain I (SAC) at a 1:10,000 final concentration for 24 hours cells were harvested and examined for expression of the following cell surface antigens by IF: IgD (no. 17, IgG1); IgG (no. 16, IgG2a); CD38 (HB7, IgG1); CD39 (AC2, IgG); CD77 (38.13, IgM).

**B-cell cultures.** Purified tonsillar B cells were cultured at 1.5 × 10⁵/mL in tissue culture medium (RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 10% fetal calf serum (FCS), 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 5 μg/mL gentamycin) for 24 hours with SAC at a 1:10,000 final concentration (Calbiochem, La Jolla, CA). These activated B cells were cultured at 50,000 cells/well in 96-well round-bottom plates (Costar, Cambridge, MA) in the presence or absence of purified FDCs obtained by cell sorting for 48 hours. The DRC-1-negative cells isolated by cell sorting were used as negative controls. DRC-1⁺ or DRC-1⁻ populations were treated with 4,000 cGy of gamma irradiation using a 137Cs source (Gammacell 1000; Atomic Energy of Canada LTD) at 2,000 cGy/1.5 min or fixed in 1% paraformaldehyde before addition to the culture wells. For paraformaldehyde fixation, cells were washed twice with phosphate-buffered saline (PBS), then incubated with 1% paraformaldehyde (Fisher, Fair Lawn, NJ) in PBS at 37°C for 5 minutes. Cold glycyglycine (0.6%) (Sigma) was then added and cells were washed three times with PBS and resuspended in culture medium. Unstimulated tonsillar B cells were cultured with combinations of SAC (1:10,000) in the presence or absence of purified FDCs for 72 hours. A neutralizing mouse monoclonal antihuman tumor necrosis factor-α (TNF-α) antibody (generously provided by Dr C. Benjamin, Biogen, Cambridge, MA) was used at 10 μg/mL, a fourfold concentration above which 50 to 100 ng/mL of TNF-α would be neutralized. A rabbit neutralizing polyclonal antibody against transforming growth factor-β (TGF-β) was used at 20 μg/mL, which is fivefold above the ND₅₀ of TGF-β inhibition of the interleukin-4 (IL-4)–dependent ³H-TdR uptake by the HT-2 cell line (R&D Systems, Minneapolis, MN). Indomethacin (Sigma) was used at 10 μg/mL.

Thymidine uptake assay. Thymidine uptake was used as an index of mitogenic activity and measured 72 hours after cultures of B cells and FDCs were initiated. Microcultures were pulsed with 1 μCi of ³H-thymidine (Amersham Corp, Eastbourne, England) per well and were harvested 15 to 18 hours later. Dried filters were counted on a Packard Tri-carb scintillation counter (Downers Grove, IL).

**RESULTS**

The effect of FDCs on B-cell proliferation was examined using enriched populations of FDCs isolated from the low-density fraction of single cell suspensions of hyperplastic normal tonsil. The FDCs were further purified by cell sorting using the MoAb DRC-1 that specifically identifies FDCs, and does not react with B cells, T cells, monocyte/macrophages, or other stromal cells.²¹ The cell surface antigen expression of these cells was further examined by performing immunohistochemistry on cytospin preparations of DRC-1⁺ cells. Essentially all the DRC-1⁺ cells, which also had the morphology of FDCs, expressed CD21 (C3d receptor), CD54 (ICAM-1), surface IgG, and more than 50% expressed CD23. These results suggest that these FDCs were bearing immune complexes. These enriched FDCs were cultured with tonsillar B cells that were previously activated for 24 hours with SAC. These cells had surface antigen characteristics of a mixture of GC and non-GC B cells (IgD, 41%; IgG, 57%; CD38, 89%; CD39, 54%; CD77, 53%, (median percentage of positive cells from three experiments). As seen in Table 1, the addition of irradiated FDCs (DRC-1⁺ cells) to tonsillar B cells that had been previously activated for 24 hours with SAC consistently inhibited ³H-TdR uptake by over 80% after an additional 72 hours of culture. In contrast, the negatively sorted population (DRC-1⁻ cells) had no significant effect on B-cell ³H-TdR incorporation. When lower dilutions of SAC (1:80,000 and 1:160,000) were used to activate B cells for 24 hours before the addition of FDCs, a stimulation index of 1.5 to 2.5 was observed (data not shown). The addition of FDCs to minimally stimulated B cells did not enhance ³H-TdR uptake but inhibited up to 60%. Similarly, when unstimulated B cells were cocultured for 72 hours with both SAC and purified FDCs, the ³H-TdR uptake was also significantly inhibited. These results indicate that isolated FDCs can inhibit B-cell proliferation.

We next sought to determine whether the inhibition of B-cell proliferation by irradiated FDCs was mediated via cell–cell contact through a surface molecule or via a soluble factor, the release of which requires metabolic activity. FDCs that were paraformaldehyde-fixed were compared with irradiated FDCs for their effects on B-cell ³H-TdR uptake. As seen in Table 2, whereas irradiated FDCs inhibited B-cell ³H-TdR uptake by 50% to 70%, the addition of fixed FDCs had no significant effect on B-cell

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**Table 1. Effect of Purified FDCs on B-Cell Proliferation**

<table>
<thead>
<tr>
<th></th>
<th>Exp 1</th>
<th>Exp 2</th>
<th>Exp 3</th>
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<tbody>
<tr>
<td>Pre-activated B cells cultured with:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Media</td>
<td>2,945 ± 448*</td>
<td>3,930 ± 409</td>
<td>5,207 ± 631</td>
</tr>
<tr>
<td>DRC-1⁺ cells</td>
<td>378 ± 67</td>
<td>724 ± 102</td>
<td>828 ± 194</td>
</tr>
<tr>
<td>DRC-1⁻ cells</td>
<td>2,113 ± 157</td>
<td>2,294 ± 290</td>
<td>4,560 ± 373</td>
</tr>
<tr>
<td>B cells cocultured with SAC and:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Media</td>
<td>2,819 ± 178</td>
<td>9,347 ± 1,033</td>
<td>7,433 ± 987</td>
</tr>
<tr>
<td>DRC-1⁺ cells</td>
<td>1,464 ± 253</td>
<td>2,806 ± 170</td>
<td>1,429 ± 448</td>
</tr>
<tr>
<td>DRC-1⁻ cells</td>
<td>2,994 ± 244</td>
<td>8,916 ± 1,182</td>
<td>7,460 ± 1,016</td>
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</tbody>
</table>

*Mean cpm ± SEM from triplicate wells.

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1 × 10⁴ purified irradiated FDCs (DRC-1⁺ cells) or DRC-1⁻ cells from the 25% Parcoll interface were cultured with 5 × 10⁴ tonsillar B cells. B cells were either pre-activated with SAC for 24 hours or unactivated B cells were cultured with SAC just before the addition of DRC-1⁺ or DRC-1⁻ cells, then cultured for an additional 72 hours. Results are shown from three of five experiments using cells derived from different individuals.
proliferation. This suggests that the inhibition of B-cell DNA synthesis requires metabolically active FDCs and not simply cell surface contact signals that could be mediated by fixed cells.

Both cell–cell contact signals as well as soluble mediators have been shown to modulate B-cell proliferation. To further distinguish between the effects of cell contact signals and soluble mediators released by FDC on B-cell proliferation, culture supernatant from FDCs was examined for effects on B-cell 3H-TdR incorporation. As seen in Table 3, supernatants derived from DRC-1+ cells inhibited B-cell 3H-TdR incorporation by approximately 50%, whereas supernatants from the negatively sorted (DRC-1−) cells had no effect. In addition to the effect on 3H-TdR uptake, the addition of FDC supernatants to pre-activated B cells led to a decrease in cell numbers when examined after 72 hours of culture (data not shown). These results demonstrate that a soluble product derived from FDCs is capable of inhibiting B-cell proliferation.

Several soluble factors, including prostaglandin E2,28 TNF-α,29,30 and TGF-β31 have been reported to inhibit B-cell activation and proliferation. Indomethacin or neutralizing antibodies against TNF-α or TGF-β were added to the cultures of FDCs and SAC-activated B cells. No significant effect of these antibodies on the inhibitory effect of FDCs was observed (Table 4). Similarly, we have been unable to prevent the antiproliferative effect using neutralizing antibodies against IL-1, IL-6, interferon (IFN)-γ, and IFN-α (data not shown). In addition, FDC supernatants to which anti–TNF-α or anti–TGF-β were added were still capable of inhibiting 3H-TdR uptake of SAC-activated B cells (data not shown).

From these studies, we have not as yet identified the soluble factor(s) produced by FDCs that is responsible for inhibition of B-cell proliferation.

### Table 2. Effect of Fixation of FDCs on B-Cell Proliferation

<table>
<thead>
<tr>
<th>Exp 1</th>
<th>Exp 2</th>
<th>Exp 3</th>
</tr>
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<tbody>
<tr>
<td>Media</td>
<td>7,862 ± 813*</td>
<td>2,301 ± 503</td>
</tr>
<tr>
<td>Irradiated DRC-1+ cells</td>
<td>2,331 ± 687</td>
<td>1,219 ± 64</td>
</tr>
<tr>
<td>Fixed DRC-1+ cells</td>
<td>6,509 ± 672</td>
<td>2,484 ± 233</td>
</tr>
</tbody>
</table>

1 × 10⁴ purified irradiated or parafomaldehyde-fixed FDCs (DRC-1+ cells) were cultured with 5 × 10⁴ pre-activated tonsillar B cells for 72 hours. Results are shown from three of four experiments using cells derived from different individuals.

*Mean cpm ± SEM from triplicate wells.

### Table 3. Effect of FDC Supernatants on B-Cell Proliferation

<table>
<thead>
<tr>
<th>Exp 1</th>
<th>Exp 2</th>
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<tbody>
<tr>
<td>Media</td>
<td>8,923 ± 755*</td>
</tr>
<tr>
<td>DRC-1+ cells sup.</td>
<td>3,844 ± 326</td>
</tr>
<tr>
<td>DRC-1+ cells sup.</td>
<td>8,759 ± 1,522</td>
</tr>
</tbody>
</table>

Supernatants (1:2 dilution) from purified irradiated FDCs (DRC-1+ cells) or DRC-1− cells from the 35% Percoll interface were cultured with 5 × 10⁴ pre-activated tonsillar B cells. Results are shown from two of four experiments using cells derived from different individuals.

*Mean cpm ± SEM from triplicate wells.

### Table 4. Effect of Anti–TNF-α, Anti–TGF-β, and Indomethacin on FDC/B-Cell Cultures

<table>
<thead>
<tr>
<th>Exp 1</th>
<th>Exp 2</th>
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<tbody>
<tr>
<td>Media</td>
<td>1,612 ± 142*</td>
</tr>
<tr>
<td>Anti–TGF-β</td>
<td>2,218 ± 283</td>
</tr>
<tr>
<td>Anti–TNF-α</td>
<td>1,640 ± 124</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>1,567 ± 65</td>
</tr>
<tr>
<td>DRC-1+ cells</td>
<td>374 ± 70</td>
</tr>
<tr>
<td>DRC-1+ cells + anti–TGF-β</td>
<td>579 ± 45</td>
</tr>
<tr>
<td>DRC-1+ cells + anti–TNF-α</td>
<td>406 ± 95</td>
</tr>
<tr>
<td>DRC-1+ cells + indomethacin</td>
<td>649 ± 185</td>
</tr>
</tbody>
</table>

1 × 10⁴ purified irradiated FDCs (DRC-1+ cells) were cultured with 5 × 10⁴ pre-activated tonsillar B cells for 72 hours. The following were added to the cultures (final concentration): anti–TGF-β (10 μg/mL); anti–TNF-α (10 μg/mL); indomethacin (10 μg/mL). Results are shown from two of four experiments using cells derived from different individuals.

*Mean cpm ± SEM from triplicate wells.

**DISCUSSION**

In the present report we show that purified FDCs inhibited normal B-cell proliferation in vitro. We observed that the FDCs were required to be metabolically active to exert this effect. Moreover, the inhibitory effect of FDCs could be similarly observed with cell-free supernatants derived from FDCs. Within certain areas of the GC of lymphoid follicles, B-cell proliferation is very active, whereas in other areas B-cell proliferation appears to be more tightly regulated. The findings in this study provide a potential mechanism by which FDCs regulate B-cell growth within GCs. This effect of FDCs could also play a role in the physiologic regulation of immune responses in vivo and potentially in infectious or neoplastic diseases that involve lymphoid follicles.

Cell surface molecules that mediate the adhesion of lymphoid cells with other cells or extracellular matrix components are involved in the transduction of signals to normal lymphoid cells which influence their activation and proliferation. In addition to cell–cell contact, the costimulatory signals delivered to lymphocytes can also be mediated by soluble factors. One method to differentiate cell–cell contact from soluble mediators involves cell fixation, which preserves cell–cell contact signals, but renders cells metabolically inactive. FDCs express a variety of adhesion receptors that may transmit growth and differentiative signals to other cells through cell–cell contact. At least two adhesion-receptor ligand pairs, LFA-1/ICAM-1 and VLA-4/VCAM-1, have been shown to be involved in the binding of normal B cells to FDCs. Although these adhesion receptors have been shown to be involved in upregulating T-cell proliferation, their role in B-cell proliferation is presently unknown. In the present study, FDCs were unable to inhibit B-cell proliferation after fixation. This suggests that cell–cell contact signals do not have a major role in the observed effect of FDCs on B cells. Another possibility is that surface-bound immune complexes on FDCs are inhibiting B-cell proliferation. Conflicting reports in the literature suggest that immune
complexes on their surfaces, and those complexes may be involved in the observed effect. In contrast to other adhesion molecules whose function is intact following fixation, paraformaldehyde fixation may render that candidate structure(s) or immune complexes on FDCs inoperative. Alternatively, the method of isolation of the FDCs or their state of activation may have functionally altered the candidate molecule(s).

FDC-enriched populations secrete a variety of cytokines, including TNF and IL-6. A secretory role for FDCs is consistent with an ultrastructural study which reported that FDCs in certain areas of the GC have extensive rough endoplasmic reticulum, Golgi apparatus, and cytoplasmic vesicles. In the present study supernatants from cultured FDCs inhibited in vitro B-cell proliferation, but less than seen with irradiated cells. Cell–cell interaction may in fact be necessary but not sufficient for a maximum effect on B-cell proliferation, either through adhesion or because of a higher local concentration of soluble factors achieved through proximity, or through a dynamic interaction. Alternatively, the soluble factor(s) may be short-lived and a greater effect of coculturing cells and FDCs may be caused by continued production affecting B cells over a longer period of time.

Previous studies in murine and human systems suggest that FDC preparations enhance B-cell proliferation in vitro, which is in contrast to the findings presented here. In the dark zone of the follicle FDCs are present among the "light zone" FDCs. Additional studies of these FDCs may have selected for the subset of cells that can have a downregulatory effect on B-cell growth, which might be "light zone" FDCs. Additional studies of these FDCs suggested that they were not from the dark zone by virtue of their expression of CD54 and surface Ig in the form of immune complexes.

Disturbed regulatory effects of FDCs on B cells may potentially be involved in diseases where GCs are affected. In human immunodeficiency virus (HIV) infection, FDCs may serve as reservoirs of virus, and the FDC network is disrupted. It is possible that damaged FDCs have impaired control of B-cell proliferation, which is consistent with the sustained polyclonal activation of normal B cells and the subsequent development of non-Hodgkin’s lymphomas seen in acquired immunodeficiency syndrome (AIDS).

Follicular NHLs recapitulate the structure and cellular composition of normal GCs. Disruption of the FDC network, and quantitative deficiencies of FDCs are also seen in follicular lymphomas unrelated to AIDS. Therefore, one can also be hypothesized that restricted FDC inhibition of neoplastic B-cell proliferation contributes to the evolution of these tumors. The FDCs in follicular NHLs are reported to be phenotypically similar to normal GC FDCs. However, studies from our laboratory have demonstrated that one function of FDCs in neoplastic follicles, the support of B-cell adhesion, is abnormal. Future studies of the in vitro function of FDCs from follicular NHLs may provide further insight into the role of FDCs in neoplastic B-cell growth. These studies may suggest new therapeutic approaches with which to control the growth of malignant B cells.

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Follicular dendritic cells inhibit human B-lymphocyte proliferation

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