Original Article

Platelet-Activating Factor Antagonists Decrease Follicular Dendritic-Cell Stimulation of Human B Lymphocytes

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Abstract

Both B-lymphoblastoid cell lines and tonsillar B lymphocytes express receptors for platelet-activating factor (PAF). In lymph node germinal centres, B lymphocytes interact with follicular dendritic cells (FDCs), which present antigen-containing immune complexes to B lymphocytes. FDCs have phenotypic features that are similar to those of stromal cells and monocytes and may therefore be a source of lipid mediators. In this study, we evaluated the effects of the PAF antagonist WEB 2170 on the activation of tonsillar B lymphocytes by FDCs. FDCs were isolated from tonsils by Bovine Serum Albumin (BSA) gradient centrifugation. After being cultured for 6 to 10 days, they were incubated with freshly isolated B cells in the presence or absence of the specific PAF receptor antagonist WEB 2170. B-lymphocyte proliferation was assessed by [^{3}H]-thymidine incorporation, and immunoglobulin (Ig) G and IgM secretion was assessed by enzyme-linked immunosorbent assay (ELISA). WEB 2170 (10^{-6} to 10^{-8} M) inhibited [^{3}H]-thymidine incorporation by up to 35% ± 3%. Moreover, the secretion of IgG and IgM was inhibited by up to 50% by WEB 2170 concentrations ranging from 10^{-6} to 10^{-8} M. There was no evidence of toxicity by trypan blue staining, and the addition of WEB 2170 to B cells in the absence of FDCs did not inhibit the spontaneous production of IgG or IgM. The effect of the PAF antagonist is primarily on B lymphocytes, as reverse transcription polymerase chain reaction detected little PAF receptor messenger ribonucleic acid (mRNA) from FDCs. These data suggest that endogenous production of PAF may be important in the interaction of B lymphocytes with FDCs.

Antigen presentation is a crucial part of any immune response. Antigen-presenting cells coordinate the interaction between antigens and effector cells such as T lymphocytes and B lymphocytes. Follicular dendritic cells (FDCs) are specific antigen-presenting cells that interact with B lymphocytes. These cells, found in lymph node germinal centres (GCs), trap antigens in immune complexes and present them to surface immunoglobulin receptors on B lymphocytes. This leads to the interaction of B lymphocytes with antigens and is a crucial step in the generation of long-lasting antibody responses and memory B lymphocytes.[1] However, FDCs provide additional signals via adhesion receptors and through a network of channels that rescue B lymphocytes from apoptosis, allowing them to proliferate and ultimately secrete immunoglobulin. These points of attachment include adhesion molecules such as VLA-4, the complement receptor CR2, and other molecules potentially.[2] There are also multiple tight conjunction links between the B lymphocytes and

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the FDCs, and it is presumed that molecules such as soluble mediators or lipids pass through these tight junctions and enhance the communication between B lymphocytes and the FDCs.[3]

The lineage of FDCs is unclear. They may arise from bone marrow stem cells similar to those that interact with B lymphocytes in their early development. However, a second possible lineage is monocyte or macrophage lineage, similar to the lineage of dendritic cells that interact with T lymphocytes.[4] This confusion persists because FDCs have both features of stromal cells and features of monocytes such as CD14 and adhesion molecules such as VLA-4.[5,6]

We have determined that platelet-activating factor (PAF), a potent lipid mediator, can abrogate apoptosis and elevate immunoglobulin levels in B-lymphoblastoid cell lines.[7,8] More recently, we demonstrated that GC-like B lymphocytes isolated from tonsils had a high level of PAF receptor (PAFR) messenger ribonucleic acid (mRNA) expression when compared to more mature mantle-zone B lymphocytes and that PAF induced tonsillar B lymphocytes to produce the cytokine interleukin-4 (IL-4).[9] Finally, following antigen receptor ligation, PAFR was irreversibly down-regulated on immortalized B lymphocytes, suggesting that the optimal time for a B lymphocyte to respond to PAF is upon entering the GC.[10] The source for PAF in the lymph node that might stimulate GC B lymphocytes is unknown. Because both cells of monocyte or stromal cell origin have been shown to produce lipid mediators,[11–14] it is possible that mediators such as PAF may assist FDCs in attracting or activating B lymphocytes. In these studies, we determined that a pharmacologic antagonist of PAF, WEB 2170, could alter the ability of FDCs to stimulate proliferation and immunoglobulin secretion in B lymphocytes.

Methods

Media and Reagents

RPMI-1640 was purchased from Life Technologies (Burlington, ON) and was supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and with penicillin (50 U/mL), streptomycin (50 μg/mL), L-glutamine (10 μg/mL), and sodium pyruvate (1 μg/mL) (all purchased from Life Technologies). PAF (1-alkyl-2-acetyl-sn-3-glycerophosphocholine, C-16) was purchased from BIOMOL International (Plymouth Meeting, PA). The specific PAFR antagonist, WEB 2170, was courtesy of Boehringer-Ingelheim (Ingelheim-am-Rhein, Germany).

Fractionation of B Lymphocytes and FDCs from Tonsils

Human FDCs were isolated from tonsils excised surgically for routine indications. After mincing, the mononuclear cell fraction was isolated by Ficoll-Paque density centrifugation (Pharmacia, Toronto, ON). Tonsillar mononuclear cells were then separated into T- and B-lymphocyte fractions by rosetting once with neuraminidase-treated sheep red blood cells, followed by a second Ficoll-Paque gradient. Monocytes were removed by adherence depletion. The B-lymphocyte fraction was subsequently applied to a 1.5% albumin gradient and centrifuged for 5 minutes at 400 rpm at 4°C, with no brake. The pellet contained primarily FDCs and some associated B lymphocytes. FDCs were plated in six well plates in complete medium; after 3 days, the nonadherent B cells were removed, and the remaining FDCs were kept in culture. They were fed by the addition of complete medium twice weekly prior to use.

Cell Culture

FDCs were maintained in six well plates until use, trypsinized, counted, and resuspended in complete medium at 4×10^5 per millilitre. B lymphocytes were isolated from tonsils as described above and were resuspended in complete medium at 0.5×10^6 per millilitre. To obtain low-density GC-like cells, mixed B lymphocytes were applied to Percoll (Amersham Biotec, Piscattawa, NJ) gradients, and the fraction obtained between 30% to 50% Percoll was collected. B lymphocytes and FDCs were cultured together in complete medium, with or without the addition of the PAF antagonist WEB 2170 in 96 well plates.

[3H]-Thymidine Incorporation

B lymphocytes and FDCs were cultured together for 90 to 114 hours at 37°C, in 5% carbon dioxide. [^{3}H]-Thymidine was then added (1 μCU per well), and the cells were incubated an additional 6 hours. The cells were harvested by water lysis (PHD Cell Harvester, Cambridge Technology, Cambridge MA), and [^{3}H]-thymidine incorporation was measured by a liquid scintillation beta-counter (Wallac, Gaithersburg, MD).

Measurement of Immunoglobulins M and G

Cell culture supernatants were harvested after 7 days, and immunoglobulin (Ig) G and IgM were measured by enzyme-linked immunosorbent assay (ELISA), as described.[7] Briefly, supernatants from cell culture or standard sera (Binding Site, Birmingham, UK) were applied at appropriate dilutions on Nunclon round-bottom ELISA plates (Nunc A/S, Roskilde, Denmark) precoated with goat antihuman IgM or IgG (Biosource, Camarillo, CA) and blocked with 1% Bovine serum albumin (BSA) in a tris(hydroxymethyl)aminomethane (TRIS)–based buffer (pH, 7.2), then incubated overnight at 4°C. After extensive washing with water, the appropriate dilution of the alkaline phosphatase conjugated goat antihuman second antibody (Biosource) was added, and the plates were incubated for 1 hour at 37°C and then equilibrated to room temperature. After extensive washing, the plates were developed with alkaline phosphatase substrate 105 (Sigma Corp., St. Louis, MO) and read at wavelength 405 (Anthos 2001 ELISA Reader, Anthos Labtec Instruments, Salzburg, Germany).

Detection of PAFR by Polymerase Chain Reaction

Total cellular ribonucleic acid (RNA) was extracted from 15×10^6 cells with TRIzol (Life Technologies) with the modifications for reverse transcriptase polymerase chain reaction (RT-PCR). RNA was dissolved in DEPC H_2O and stored at −80°C until use. RT-PCR was performed for 30 cycles in a Hybaid Omnigene thermal cycler (Hybaid Ltd., Middlesex, UK) using the PAFR-specific primers 5′-CGGACATGCTCTTCTTGATCA-3′ (sense) and 5′-GTCTAAGACACAGTTGGTGCTA-3′ (antisense), as described.[11] Polymerase chain reaction (PCR) products were applied to a 2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet (UV) light.

Results

Isolation and Identification of FDCs

FDCs were purified from tonsils and maintained in tissue culture for at least 6 days prior to use. This ensured that most FDC-associated B lymphocytes from the original tonsil were removed. The FDCs exhibited the typical morphologic appearance of spindle-shaped adherent cells in culture and stained negatively for CD20 and CD3 and positively for CD14 and VLA-4 by flow cytometry (data not shown). In addition, viability testing indicated that by day 6, all B cells had died in culture, without added B-cell mitogens, but the FDCs remained viable and multiplied steadily.

Effect of PAF Antagonists on Cell Proliferation

FDCs maintain B-cell growth in culture, even without the addition of mitogens.[15] We cultured purified FDCs with freshly isolated B lymphocytes and assessed cellular proliferation by [^{3}H]-thymidine incorporation following 120 hours of culture. In the absence of FDCs, B lymphocytes alone did not incorporate [^{3}H]-thymidine significantly whereas FDCs alone did show some baseline [^{3}H]-thymidine incorporation (Figure 1, upper panel). Treatment with mitomycin C (15 μg/mL for 2 hours) did not significantly decrease background thymidine incorporation by FDCs. The combination of mixed tonsillar B lymphocytes and FDCs caused morphologically larger B-lymphocyte clusters (data not shown)

and significantly greater uptake of [^{3}H]-thymidine than either of the two cell types alone (see Figure 1, upper panel). Addition of the PAF antagonist WEB 2170 (10^{-6} to 10^{-8} M) to mixed B lymphocytes consistently diminished cell proliferation (see Figure 1, upper panel). Significant inhibition was also achieved when we separated tonsillar B lymphocytes into characterized fractions by Percoll density centrifugation. The low-density Percoll fraction contains cells that have the highest PAFR expression, based on mRNA and functional studies.[9] Culturing of the low-density fraction and FDCs also led to significant increases in [^{3}H]-thymidine incorporation, which was inhibited 35% ± 3% by WEB 2170 (see Figure 1, lower panel). However, the other fractions resulting from Percoll density separation (medium and high density) were not significantly affected by the addition of WEB 2170 (data not shown). No toxicity resulted from WEB 2170 administration, as was demonstrated by direct observation of the cultures and by trypan blue staining. Inhibition was not found with WEB 2170 doses ≤ 10^{-10} M (data not shown).

Effect of PAF Antagonists on Production of Immunoglobulin

Maintenance of B lymphocytes in culture by FDCs leads to an increase in Ig production.[16] To assess if PAF antagonists would decrease the ability of FDCs to sustain Ig production, we added WEB 2170 to B lymphocytes, FDCs, or the combination of B lymphocytes and FDCs at the initiation of culture, and supernatants were harvested after 7 days of incubation. In keeping with the purity of our FDCs, no IgG or IgM was detected from these cells in culture alone (data not shown). B lymphocytes alone made detectable amounts of Ig, but the combination of FDCs and B lymphocytes produced three to five times more IgM (91 ± 68 ng/mL vs 308 ± 175 ng/mL) and IgG (220 ± 72 ng/mL vs 875 ± 448 ng/mL). The addition of WEB 2170 at doses of 10^{-8} to 10^{-6} M led to a significant inhibition of Ig production, from 44 to 75% of the baseline IgM or IgG production (Figure 2).

Detection of PAFR mRNA from FDCs, by PCR

We have demonstrated that both B-lymphocyte cell lines and freshly isolated tonsillar B lymphocytes express high levels of PAFR mRNA[9] and that stimulation of the PAFR leads to demonstrable intracellular signalling and increased Ig production.[8,9] In addition, PAF rescued B-lymphocyte cell lines from apoptosis[7,17] and induced expression of IL-4 and IL-13 mRNA in freshly isolated B lymphocytes.[9] Radiolabelled WEB 2170 was taken up on PAFR expressed by B lymphocytes and B-lymphocyte cell lines.[10] However, it is unknown whether FDCs express PAFR and would thus be directly affected by the PAF antagonist. RNA was extracted from cultured B lymphocytes and cultured FDCs that were morphologically free of contaminating B lymphocytes, as detailed above. The results of the PCR analysis are shown in Figure 3. Although PAFR mRNA was detectable from FDCs (see Figure 3, lane 3), only a small amount was present as compared with that detected from B lymphocytes (see Figure 3, lane 4). This suggests that the predominant action of WEB 2170 is on B-lymphocyte PAFR.

Discussion

The interaction between FDCs and B lymphocytes is a crucial one for the generation of high-affinity antibody secreting memory B lymphocytes.[18–21] FDCs provide crucial elements for B-lymphocyte survival in the GC of lymph nodes, such as antigen and accessory molecules. As antigen-presenting cells, FDCs trap IgG-coated antigens, primarily in immune complexes, and present them on their surfaces for long durations.[1] B lymphocytes that have a high affinity for the antigen are selected, and these are allowed to further develop in a process known as affinity maturation. The surfaces of FDCs display other accessory molecules, including complement receptors and adhesion molecules such as VLA-4, that provide stimulatory signals for B-cell proliferation and Ig production. In addition, FDCs can rescue B lymphocytes from apoptosis, independently of adhesion molecules or CD40 ligation.[22,23]

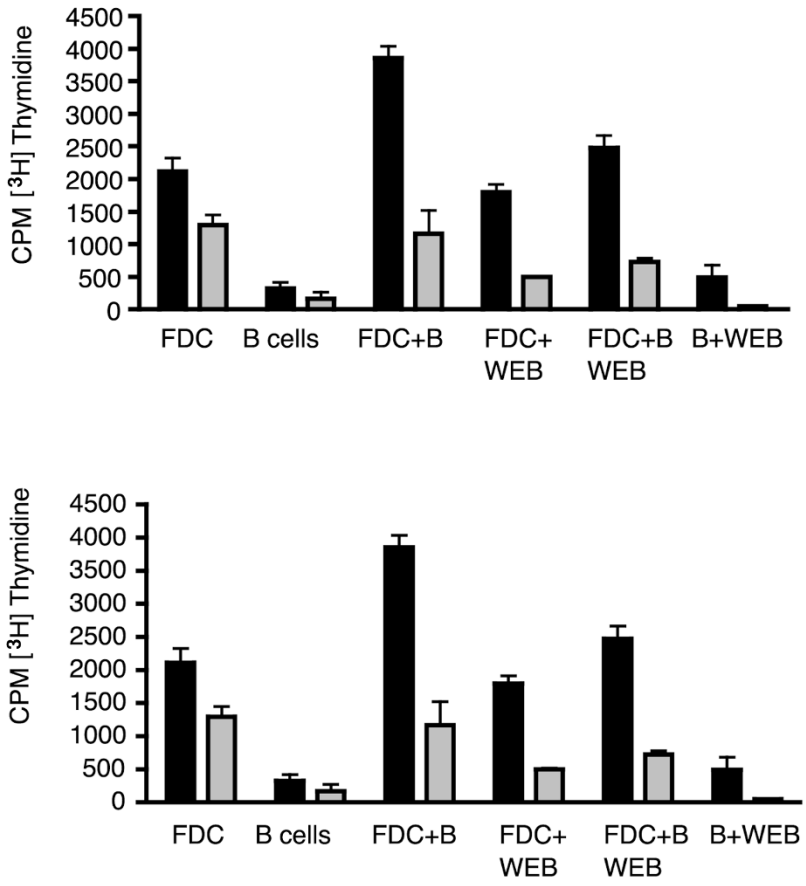

Figure 1 WEB 2170 diminished the proliferation of B lymphocytes. Tonsillar B lymphocytes were cultured with isolated follicular dendritic cells for 120 hours with and without WEB 2170 (10^{-8} M). After 114 to 116 hours, [^{3}H]-thymidine (1.0 μCU per well) was added, and the cells were harvested 4 to 6 hours later. The histograms represent cell proliferation as expressed by incorporation of [^{3}H]-thymidine. *Upper panel* shows assessment of mixed tonsillar B lymphocytes($n = 4$); *lower panel* shows assessment of low-density tonsillar B lymphocytes The *gray and black* bars represent two separate experiments performed in triplicate ($n = 2$).

We have shown that B lymphocytes from tonsils express receptors for the potent ether lipid PAF. The highest level of PAFR mRNA expression was found in the low- and medium-density layers from Percoll-fractionated subsets of B lymphocytes, and the addition of PAF to the low-density fraction engendered the highest increases in intracellular calcium and in the production of Ig.[9] These two populations represent GC B lymphocytes as well as rapidly proliferating centroblasts and centrocytes. Because FDCs are potentially of hematopoietic-cell origin, related to monocytes or macrophages, we hypothesized that they might be sources of lipid mediators such as PAF.[24] Indeed, early work on FDCs indicated a possible role for prostaglandins in their function.[12] In these preliminary studies, we tested whether the PAF antagonist WEB 2170 could inhibit FDC-mediated stimulation of B lymphocytes.

FDCs support B-lymphocyte growth and the synthesis of Ig without additional mitogens.[19] This allowed direct observation of the role of the PAF antagonist. The high-affinity water-soluble PAF antagonist WEB 2170[25,26] clearly had an effect, decreasing cell proliferation (as measured by [^{3}H]-thymidine incorporation) by 35% and the production of IgM and IgG by the isolated tonsillar cells by 45 to 75%. This was seen over doses ranging from 10^{-6} to 10^{-8} M; doses exceeding this were ineffective. High exogenous doses of a pharmacologic PAF antagonist are probably needed to saturate the PAFR sites on B lymphocytes and to overcome the large local production known to occur where lipid mediators are synthesized. This dose is comparable to the effective dose for inhibiting PAF-mediated elevation of free cystolic calcium (Ca^{2+}) concentrations ([Ca^{2+}]i) or in vitro Ig production.[27,28] Although PAF antagonists had a consistent effect in FDC B-lymphocyte cultures, it is not surprising that they did not completely inhibit [^{3}H]-thymidine incorporation or Ig production. First, the source of Ig secretion from cultured ton-

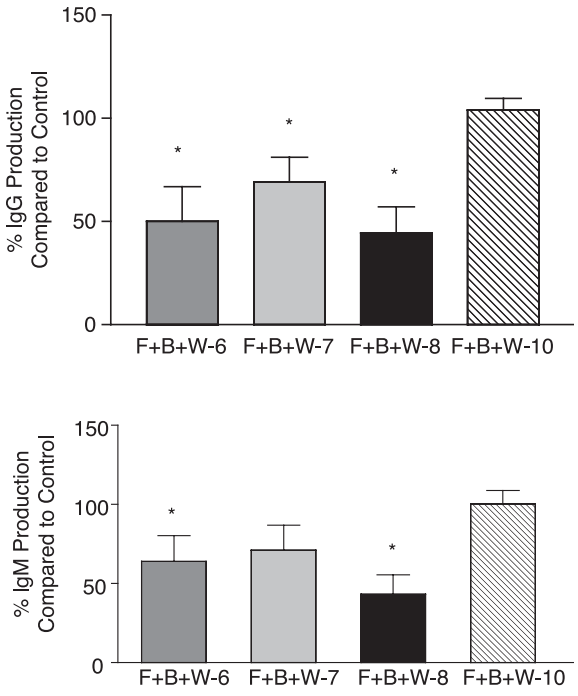

Figure 2 WEB 2170 diminished the production of immunoglobulin (Ig) in cultures of mixed follicular dendritic cells (FDCs) and B lymphocytes. Mixed tonsillar B lymphocytes and FDCs were cultured together with or without the indicated concentrations of WEB 2170 in 24 well plates for 7 days. On day 7, supernatants were harvested and used for measuring total IgG or IgM. The histograms indicate the percentage decrease in IgG (A) and IgM (B) from cultures untreated by WEB 2170 (values are indicated in the text) ($n = 5$). B = B lymphocytes; F = FDCs; W = WEB 2170. *$p < .05$.

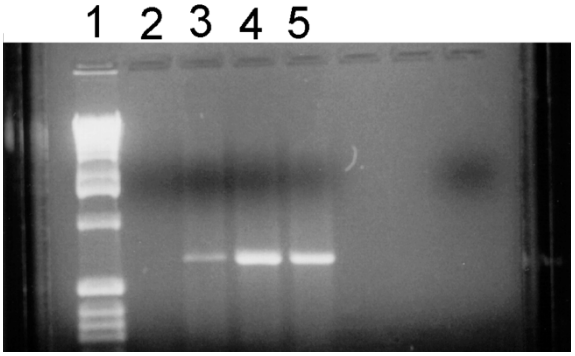

Figure 3 Results of reverse transcription polymerase chain reaction for the detection of platelet-activating factor receptor (PAFR) in follicular dendritic cells (FDCs) and B lymphocytes. Total RNA was extracted from purified FDCs or B lymphocytes, and PAFR messenger RNA (mRNA) was detected with specific primers (see "Methods"). The figure shows ethidium bromide–stained gel that is representative of two identical experiments. *Lane 1* shows DNA ladder; *lane 2*, negative control (B lymphocytes without reverse transcriptase); *lane 3*, FDCs; *lane 4*, human tonsillar B lymphocytes; *lane 5*, LA350 B-lymphocyte cell line. Both sources of B lymphocytes have a much stronger mRNA signal than the FDCs have. Equal lane loading was verified by using β-actin as a housekeeping gene (not shown).

sillar cells includes a small number of plasma cells that secrete immunoglobulins, as well as other mature B cells that may die in culture and release a basal level of IgG and IgM. Second, the interaction of FDCs and B lymphocytes is mediated by numerous adhesion molecules, chemokines, and other mediators[18,29]; these studies indicate that PAF may be an important contributor to this process.

The effect of the PAF antagonist WEB 2170 is most likely directly on B lymphocytes and not on the FDCs themselves. Whereas monocytes and other dendritic cells such as those derived from $CD34^+$ peripheral blood cells have been shown to express high levels of PAFR mRNA,[30] FDCs expressed little PAFR mRNA. Expression of PAFR mRNA was low as compared to B lymphocytes (see Figure 3), even when the mRNA was amplified to plateau by RT-PCR.[9,31] The RT-PCR used in this study has been validated[10] against a semiquantitatve RT-PCR and accurately reflects changes in the level of PAF mRNA.[9] Although no contaminating B lymphocytes were visible in our cultures, it is possible that the trace amounts of PAFR that were detected were due to cells other than the FDCs themselves. However, to date, no other populations (apart from FDCs) isolated from tonsils propagate under the culture conditions described.

In light of our recent observations regarding PAFR expression on GC B lymphocytes,[9] the action of the PAF antagonist in these studies suggests that bioactive lipid mediators may play a role in chemoattraction or GC cell organization, including the interaction of FDCs and B lymphocytes. The presence of the PAF antagonist did not diminish aggregation between B lymphocytes and FDCs (data not shown). Further investigation into the link

between FDCs and B lymphocytes may answer some heretofore unanswered questions. For example, although FDCs rescue B lymphocytes from apoptosis, the molecule that mediates this is still unknown. This effect is independent of CD40 and VLA-4 ligation,[22] and FDCs do not produce cytokines, such as IL-4, that rescue B lymphocytes from apoptosis.[32,33] A novel factor secreted by FDCs, 8D6, is not involved in rescue from apoptosis and is not solely responsible for maintaining the growth of B-lymphocyte lines cultured with FDCs.[2] We have demonstrated that PAF rescues the GC-like B-cell line Ramos from programmed cell death induced by B-cell receptor (BCR) ligation[7,17]; the role of PAF antagonists in the rescue of apoptosis is being explored in our laboratory.

We have undertaken to determine the source of PAF in these cultures. FDCs have features of cells of myeloid-cell origin[34] although some investigators have suggested an endothelial or fibroblast origin for FDCs.[35,5] FDCs also have gap junctions that are areas of molecular transport for intercellular communication.[36] B lymphocytes are also a source of bioactive ether lipids, including palmitoyl-2-acetoyl-sn-glycero-3-phosphocholine (PAGPC).[28] The presence of this PAF analogue, which is a potent mitogen for Ig synthesis by B lymphocytes, has to date made it difficult to purify PAF in mixed FDC/B-lymphocyte cultures. The rapidity of GC formation[19] predicts that rapidly produced or preformed chemoattractants, including chemokines or lipid mediators, are needed for direction of cellular traffic. Recent studies have established that FDCs produce monocyte chemoattractant protein-1[37] and B cell chemoattractant -1 (BCA).[38] Thus, a number of candidate molecules are available for assisting the assembly of GCs. Definition of the ability of FDCs to produce chemoattractant lipid mediators such as PAF or PAGPC is important for a full comprehension of interactions that lead to GC development in lymphoid tissue.

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