

# Follicular Dendritic Cell Secreted Protein (FDC-SP) Regulates Germinal Center and Antibody Responses<sup>1</sup>

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We previously identified follicular dendritic cell secreted protein (FDC-SP), a small secreted protein of unknown function expressed in human tonsillar germinal centers (GC). To assess potential *in vivo* activities of FDC-SP, transgenic mice were generated to constitutively express FDC-SP in lymphoid tissues. FDC-SP transgenic mice show relatively normal development of immune cell populations, with the exception of a small increase in mature follicular B cells, and normal lymphoid tissue architecture. Upon immunization with a T-dependent Ag, FDC-SP transgenic mice were capable of producing an Ag-specific Ab; however, the titers of Ag-specific IgG2a and IgE were significantly reduced. GC responses after immunization were markedly diminished, with transgenic mice showing decreased numbers and sizes of GCs but normal development of follicular dendritic cell networks and normal positioning of GCs. FDC-SP transgenic mice also showed reduced production of Ag-specific IgG3 Ab after immunization with a type II T-independent Ag, suggesting that the FDC-SP can also regulate the induction of B cell responses outside the GC. Purified FDC-SP transgenic B cells function normally *in vitro*, with the exception of blunted chemotaxis responses to CXCL12 and CXCL13. FDC-SP can induce the chemotaxis of CD40-stimulated nontransgenic B cells and can significantly enhance B cell migration in combination with chemokines, indicating that FDC-SP may function in part by regulating B cell chemotaxis. These results provide the first evidence for immunomodulatory activities of FDC-SP and implicate this molecule as a regulator of B cell responses. *The Journal of Immunology*, 2007, 178: 7859–7867.

Induction of humoral immunity occurs within the context of discrete microenvironments within peripheral lymphoid organs. Proper compartmentalization within lymphoid tissues is essential for generating a specific and effective response. The structure of B cell follicles changes dramatically during the course of a T-dependent Ab response, with rapidly proliferating B cells generating structures known as secondary follicles or germinal centers (GCs).<sup>3</sup> As follicles become filled with B lymphoblasts they take on the characteristically organized GC structure that has been well defined by immunohistology (1–3). GC B cells have unique functional characteristics and phenotype associated with their remarkable proliferation and differentiation program, the mo-

lecular basis of which is not completely understood. Within the GC environment B cells undergo critical functional processes including proliferation, apoptosis, somatic hypermutation, selection for high-affinity Ag binding, isotype switch, and differentiation into plasma cells or memory cells (2, 4, 5). Within primary follicles and GCs, B cells functionally interact with resident stromal cells often referred to as follicular dendritic cells (FDCs) (1, 6, 7), as well as Ag-specific follicular T cells (8, 9).

The molecular basis of the interaction between FDCs and B cells is not well understood but likely involves interactions mediated by trapped immune complexes and chemokines. FDCs efficiently trap immune complexes on their surface via abundantly expressed Fc receptors and complement receptors (7, 10–12) and are widely believed to represent the central APC for B lymphocytes that drives efficient affinity maturation of the Ab response and the generation of memory B cells (13–15). *In vitro* experiments indicate that FDC-like cells can support B cell survival, proliferation, Ig secretion, and the expression of costimulatory molecules (7, 16, 17). In addition, FDCs are a source of B cell chemoattractants, including CXCL12 and CXCL13 (18–20). CXCL13 and CXCL12 are potent chemoattractants for B cells *in vitro* and use CXCR5 and CXCR4, respectively, as receptors.

To identify the molecules underlying FDC-B cell interactions, we undertook a study to isolate genes expressed in enriched primary human FDCs isolated from tonsils. This resulted in the discovery of several novel genes, including Bam32, DCAL-1, and FDC secreted protein (FDC-SP) (21–23). Our previous work showed that FDC-SP mRNA has a striking and restricted expression pattern, with strong expression in tonsillar GCs and FDC-like cell lines but not in resting or GC B cells. The FDC-SP gene encodes a small secreted protein of unknown function. In this report we have characterized murine FDC-SP

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<sup>3</sup> Abbreviations used in this paper: GC, germinal center; FDC, follicular dendritic cell; FDC-SP, FDC secreted protein; NP, 4-hydroxy-3-nitrophenylacetyl; PNA, peanut hemagglutinin.

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expression and function to assess potential immunomodulatory activity of this molecule.

## Materials and Methods

### Animals

C57BL/6 and CD1 mice were purchased from Charles River Canada and were used between 8 and 12 wk of age. All animals were housed at the Central Animal Care Facility (University of Manitoba, Winnipeg) in compliance with the guidelines established by the Canadian Council on Animal Care.

### Antibodies and reagents

FITC-labeled peanut hemagglutinin (PNA) was from Vector Laboratories. Biotin-labeled rat anti-mouse metallophilic macrophage marker 1 (MOMA-1) was from BMA Biomedicals. Biotin-labeled rat anti-mouse follicular dendritic cell (FDC-M2) was from BioCan Scientific. Biotin-labeled rat anti-mouse IgD (11–26) was from Southern Biotechnology. The PE-streptavidin-Alexa Fluor 647 conjugate was from Molecular Probes. F(ab')<sub>2</sub> goat anti-mouse IgM and rat anti-mouse CD40 (clone 1C10) stimulating Abs were from Jackson ImmunoResearch Laboratories and Southern Biotechnology, respectively. All other Abs were from BD Pharmingen. The alkaline phosphatase-conjugated streptavidin was from Jackson ImmunoResearch Laboratories. Alkaline phosphatase substrate tablets and LPS were from Sigma-Aldrich. The 2.4G2 hybridoma producing anti-mouse FcγRII/III mAb was a gift of Dr. E. Clark (University of Washington, Seattle, WA).

### Generation of mouse FDC-SP reagents

The mouse FDC-SP cDNA was subcloned into the bacterial expression vector pGEX-5x-2 (Amersham Biosciences) in-frame with the GST tag and the mammalian expression vector pcDNA3 (Invitrogen Life Technologies). The GST fusion protein does not include the putative secretion signal (aa 1–17) and contains a factor Xa protease cleavage site between the GST tag and FDC-SP. The expression of FDC-SP cDNA in the resulting constructs was confirmed by sequencing. The FDC-SP pGEX-5x-2 vector was transformed into *Escherichia coli* BL21 cells for protein expression, which was induced by adding isopropyl-β-D-thiogalactoside to a final concentration of 0.2 mM and incubating at 30°C for 3 h with shaking. The GST fusion protein was purified from sonicated cell lysates using glutathione Sepharose beads and then cleaved with factor Xa to remove the GST tag according to the manufacturer's protocols (Amersham Biosciences). The FDC-SP pcDNA3 vector was linearized by digestion with *ScaI* and transfected into L929 murine fibroblast cells (L cells) using Lipofectamine (Invitrogen Life Technologies). Transfected cells were cultured for 2 wk in the presence of 2 mg/ml G418 and then cloned by limiting dilution. G418-resistant clones were screened for FDC-SP expression by RT-PCR and high-expressing clones were selected for use as a source of FDC-SP in the experiments. Serum-free supernatants of L cell transfectants were prepared by extensively washing and culturing confluent cells in medium containing 0.5% BSA for overnight. Supernatants were harvested and used immediately or aliquoted and stored frozen at –80°C.

### Cell preparations

Spleens were harvested from C57BL/6 mice and cell suspensions were generated by grinding up a spleen with a frosted glass tissue grinder or cutting the spleen into small pieces and digesting it with collagenase (200 U/ml; Sigma-Aldrich) for 1 h at 37°C followed by gently pipetting up and down. B cells were purified by negative selection using anti-CD43 microbeads according to the manufacturer's protocol (Miltenyi Biotec). This method routinely resulted in B cell purity in excess of 98% as determined by anti-CD19 staining. Cells were cultured in RPMI 1640 medium containing 50 μM 2-ME, 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

### RT-PCR analysis

Total RNA was prepared from 2 × 10<sup>6</sup> cells per group using TRIzol (Invitrogen Life Technologies) according to the manufacturer's protocol. From each RNA sample 12.5 μl were used for a cDNA synthesis reaction and the resulting cDNA was used at 1/20 for PCR amplification with the following primer pair specific for mouse FDC-SP: GGAGCAGAGTG GAGAGTTTCA (5') and CCACGGGTAGCCTTGATTTA (3'). The PCR was heated to 94°C for 2 min and then cycled 35 times as follows: 58°C for 30 s, 72°C for 30 s, and 94°C for 30 s. The resulting PCR product was confirmed to be murine FDC-SP by cloning and sequencing.

### Chemotaxis assay

Chemotaxis assays were conducted in 24-well plates containing Transwell inserts with 5-μm pore size (Corning). Purified B cells were stimulated with anti-CD40 (2.5 μg/ml) plus IL-4 (5 ng/ml) for 48 h, washed three times with migration medium (RPMI 1640 containing 0.5% BSA), and resuspended at 10 × 10<sup>6</sup> per ml in the same medium. Migration medium (0.6 ml) containing, where indicated, FDC-SP, CXCL12, or CXCL13 (Peprotech) were added to the lower chamber. Washed B cell suspension (0.1 ml) was added to the upper chamber and the Transwell plate was incubated at 37°C for 3–4 h. Cells in the lower and upper chambers were then collected, diluted to an equal volume of 0.6 ml, and counted on a FACSCalibur flow cytometer (BD Biosciences) or a Guava personal cell analysis system (Guava Technologies). The percentage of migration was calculated as follows: lower chamber cell number/(lower chamber cell number + upper chamber cell number) × 100%.

### Generation of FDC-SP transgenic mice

The murine FDC-SP cDNA was cloned into the *HindIII* and *EcoRV* sites of the transgenic expression vector pEμSR. This vector drives the expression of the transgene in lymphoid tissues, predominantly in B cells under the control of the SRα promoter and Eμ enhancer (24, 25). The *NotI* fragment containing the transgene was microinjected into the pronucleus of fertilized mouse eggs and the eggs were implanted into CD1 foster mothers at the University of Manitoba transgenic core facility. Offspring were screened for transgene integration by PCR of tail DNA. Three independent founder lines were generated; the data shown are from the no.5104 line, but all of the results have been confirmed in one other line.

### Immunizations, flow cytometry, and immunofluorescence analysis

Chicken OVA (Sigma-Aldrich) was precipitated in alum (Pierce) before immunization. FDC-SP transgenic mice and age- and sex-matched CD1 wild-type mice were immunized i.p. with 2 μg of OVA in alum. 4-Hydroxy-3-nitrophenylacetyl (NP)-LPS or NP-Ficoll (Biosearch Technologies) was injected i.p. without alum (12.5 μg/mouse). Mice were sacrificed on the indicated day by cardiac puncture and serum and spleens were collected.

For immunofluorescence staining, spleens were embedded in OCT compound (Sakura) and snap frozen in a liquid nitrogen bath. Cryosections were prepared, mounted on Superfrost Plus slides (Fisher Scientific), and stored at –20°C until use. Sections were fixed in –20°C acetone for 2 min and blocked in PBS containing 5% goat serum (Sigma-Aldrich) for 30 min. Primary biotin-labeled Ab was added and slides were incubated at 4°C for overnight. Slides were then washed before streptavidin-Alexa 647 Fluor conjugate was added for 1 h at room temperature. FITC- and tetramethylrhodamine isothiocyanate- or PE-labeled Abs were then added for 1 h at room temperature. Sections were mounted in Prolong Gold anti-fade reagent (Molecular Probes) and visualized on a confocal microscope (Ultra-view LCI; PerkinElmer). The size (in micrometers) and number of GCs were analyzed per ×40 field on each section using Ultraview image analysis software. Two sections that are at least 20–30 slices apart were analyzed per group to eliminate sectioning artifacts.

For FACS analysis, single cell suspensions were preincubated with a 2.4G2 Ab to block Fc receptors (15 min on ice) before staining with biotin-, allophycocyanin-, FITC-, and PE-labeled Abs for 15 min on ice. The PE-streptavidin-Alexa Fluor 647 conjugate was then added for 15 min on ice to detect the biotin-labeled Ab. Cells were then washed and fluorescence was analyzed by collecting a total of 50 × 10<sup>3</sup> cells per sample using a FACSCalibur instrument (BD ImmunoCytometry Systems).

### ELISA

Blood was collected from naive or OVA-alum immunized wild type and FDC-SP transgenic mice at the indicated times. OVA-specific IgM, IgG1, IgG2a, and IgE levels in serum were evaluated by ELISA as previously described (26). The plates were coated with OVA (20 μg/ml) to detect the OVA-specific IgM, IgG1, IgG2b, and IgG2a and data are expressed at endpoint Ab titers. For OVA-specific IgE, the plates were coated with rat anti-mouse IgE, developed using biotin-OVA, and calibrated against a murine anti-OVA standard. NP-specific IgM and IgG3 were measured using plates coated with NP20-BSA (Biosearch Technologies).

For the analysis of cytokine production, splenocytes were isolated at day 5 after OVA immunization and restimulated in vitro with 300 μg/ml OVA. Supernatants were collected from cultures at 24 and 48 h for IL-4 and IFN-γ, respectively and cytokine levels were assessed by ELISA as previously described (26).

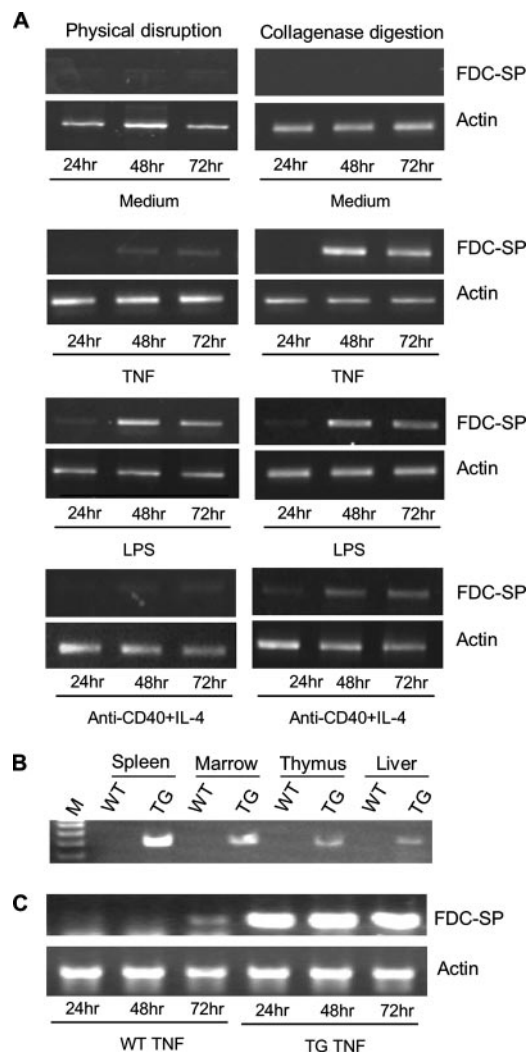


**FIGURE 1.** Inducible expression of FDC-SP in mouse spleen. *A*, Induction of FDC-SP expression in response to TNF and LPS. Mouse spleen cell suspensions were prepared by two methods and cultured in the presence of the indicated stimuli for the indicated times and then analyzed by RT-PCR. This figure is representative of three independent experiments. *B*, Expression of FDC-SP in tissues of transgenic mice. RT-PCR was performed on RNA extracted from the indicated tissues. M, Molecular marker; WT, wild type; TG, transgenic. *C*, Constitutive overexpression of FDC-SP in the spleens of transgenic mice. Spleen cells from wild-type or transgenic mice were stimulated with TNF and RNAs were isolated at the indicated times to detect FDC-SP expression by RT-PCR.

## Results

### Generation of FDC-SP transgenic mice

We originally cloned FDC-SP from enriched human tonsillar FDC cells and showed the expression of FDC-SP in the light zone of tonsillar GCs and FDC-like cell lines, but not GC B cells (21). Like human FDC-SP, its murine homologue encodes a secreted peptide of 84 aa with no readily apparent homology to other secreted proteins (21). We found that murine FDC-SP mRNA was not detectable in spleen, mesenteric lymph node, thymus, or bone marrow; however, mRNA expression could be induced by stimulating spleen cells with TNF- $\alpha$ , LPS, or anti-CD40 plus IL-4 (Fig. 1*A*). TNF- $\alpha$ - or anti-CD40 plus IL-4-induced FDC-SP expression was strongest under conditions where collagenase digestion was used to gently dissociate splenocytes, consistent with expression by fragile FDCs (27). To assess the *in vivo* biological activities of

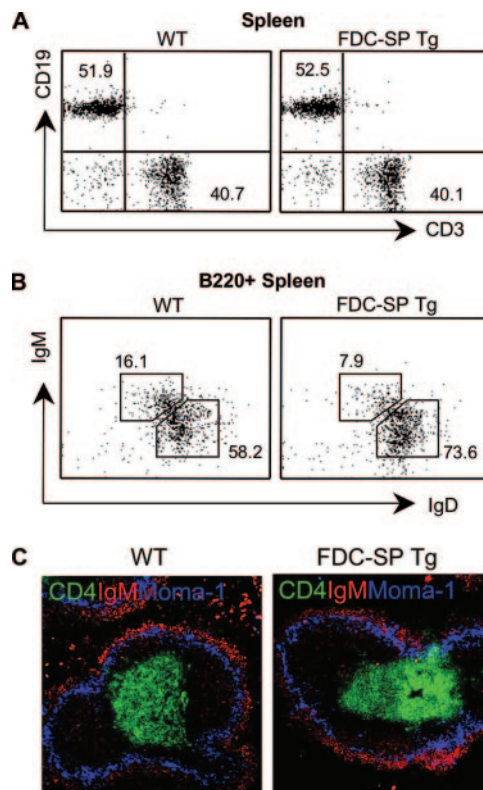


**FIGURE 2.** Characterization of FDC-SP transgenic mice. FDC-SP transgenic mice were generated as indicated in *Materials and Methods*. *A*, FACS analysis of spleen cells showing comparable numbers of T cells (CD3<sup>+</sup>) and B cells (CD19<sup>+</sup>) in wild-type (WT) and transgenic (Tg) mice. *B*, FACS analysis of cells from spleen showing immature (B220<sup>+</sup>IgM<sup>high</sup>IgD<sup>low</sup>) and mature (B220<sup>+</sup>IgM<sup>low</sup>IgD<sup>high</sup>) B cells. Cells within the lymphocyte and B220<sup>+</sup> gate are depicted and the numbers show the relative percentage of the cells within the indicated gates. The dot plots in *A* and *B* are representative of six mice per group. *C*, Frozen spleen sections from naive wild-type or FDC-SP transgenic mice were stained with anti-CD4 (green), anti-IgM (red), and anti-Moma-1 (blue) and imaged using a confocal microscope.

FDC-SP, we generated transgenic mice designed to constitutively express FDC-SP in lymphoid tissues. FDC-SP was expressed in multiple tissues of the transgenic mice (Fig. 1*B*), with strong constitutive expression in the spleen (Fig. 1*C*).

### Analysis of lymphoid tissues in FDC-SP transgenic mice

The cellular compositions of the spleen, bone marrow, blood, thymus, lymph node, and the peritoneal cavity of FDC-SP transgenic mice were grossly normal in the overall cellularity and proportions of different cell types (Fig. 2*A* and Table I). Within the splenic B cell compartment of FDC-SP transgenic mice there was a small but significant increase in the proportion of mature IgM<sup>low</sup>IgD<sup>high</sup> follicular B cells with a corresponding decrease in immature IgM<sup>high</sup>IgD<sup>low</sup> B cells (Fig. 2*B*). Consistent with the increase in the proportion of mature B cells in the spleen, increased frequencies of mature B220<sup>high</sup> recirculating B cells were found in the bone marrow (Table I). The overall splenic structure appeared grossly normal based on H&E staining (data not shown), as was the white pulp organization in terms of B zone, T zone, and marginal zone partitioning (Fig. 2*C*). These data suggest that constitutive FDC-SP expression does not impair lymphocyte development or substantially affect lymphoid tissue structure.

Table I. Summary table for FACS analysis of the different cell population percentages in spleen, bone marrow, mesenteric lymph node, blood, peritoneal wash, and thymus<sup>a</sup>

| Phenotype                                                             | Wild Type   | FDC-SP Transgenic |
|-----------------------------------------------------------------------|-------------|-------------------|
| <b>Spleen</b>                                                         |             |                   |
| B cell (CD19 <sup>+</sup> )                                           | 47.7 ± 3.7  | 46.8 ± 4.2        |
| T cell (CD3 <sup>+</sup> )                                            | 40.5 ± 5.9  | 40.7 ± 0.9        |
| Macrophage (CD11b <sup>+</sup> )                                      | 2.5 ± 0.93  | 3.9 ± 1.6         |
| Marginal Zone (CD23 <sup>+</sup> CD21 <sup>+</sup> IgM <sup>+</sup> ) | 7.9 ± 2.2   | 7.0 ± 3.5         |
| T1 (CD23 <sup>+</sup> CD21 <sup>+</sup> IgM <sup>+</sup> )            | 6.2 ± 2.5   | 4.0 ± 2.5         |
| T1 (CD23 <sup>+</sup> CD21 <sup>+</sup> IgM <sup>+</sup> )            | 4.6 ± 2.1   | 3.4 ± 0.8         |
| Follicular (CD23 <sup>+</sup> CD21 <sup>low</sup> IgM <sup>+</sup> )  | 62.9 ± 4.3  | 74.9 ± 4.3**      |
| Immature (IgM <sup>high</sup> IgD <sup>low</sup> )                    | 16.8 ± 2.0  | 8.9 ± 3.2***      |
| Mature (IgM <sup>low</sup> IgD <sup>high</sup> )                      | 59.5 ± 7.8  | 76.3 ± 3.3**      |
| <b>Mesenteric lymph node</b>                                          |             |                   |
| B cell (CD19 <sup>+</sup> )                                           | 16.8 ± 7.1  | 10.8 ± 3.0        |
| T cell (CD3 <sup>+</sup> )                                            | 87.4 ± 1.9  | 86.9 ± 3.4        |
| Macrophage (CD11b <sup>+</sup> )                                      | 0.95 ± 0.18 | 0.98 ± 0.12       |
| <b>Bone marrow</b>                                                    |             |                   |
| Pro-/Pre-B (B220 <sup>low</sup> IgM <sup>-</sup> )                    | 35.1 ± 4.2  | 35.8 ± 5.2        |
| Immature (B220 <sup>low</sup> IgM <sup>+</sup> )                      | 4.7 ± 1.7   | 6.2 ± 2.5         |
| Mature (B220 <sup>high</sup> IgM <sup>+</sup> )                       | 8.6 ± 3.2   | 19.3 ± 2.8***     |
| <b>Peritoneal wash</b>                                                |             |                   |
| IgM <sup>+</sup> CD5 <sup>+</sup>                                     | 28.4 ± 5.0  | 27.1 ± 7.2        |
| <b>Blood</b>                                                          |             |                   |
| B cell (CD19 <sup>+</sup> )                                           | 27.6 ± 3.7  | 23.1 ± 12.1       |
| T cell (CD3 <sup>+</sup> )                                            | 21.4 ± 2.7  | 18.0 ± 11.2       |
| Macrophage (CD11b <sup>+</sup> )                                      | 14.6 ± 4.3  | 16.0 ± 8.5        |
| <b>Thymus</b>                                                         |             |                   |
| CD4 <sup>+</sup>                                                      | 12.4 ± 3.4  | 15.6 ± 3.0        |
| CD8 <sup>+</sup>                                                      | 2.4 ± 0.8   | 3.4 ± 0.6         |
| CD4 <sup>+</sup> CD8 <sup>+</sup>                                     | 81.1 ± 4.6  | 77.1 ± 8.8        |
| CD4 <sup>-</sup> CD8 <sup>-</sup>                                     | 4.3 ± 0.7   | 3.7 ± 0.5         |

<sup>a</sup> The results (based on 10 × 103 events collected) are expressed as the mean ± SD percentage of the indicated staining combinations of six different mice per group for spleen and peritoneal wash and three mice for other tissues. No significant differences were observed in overall cellularity of lymphoid tissues. \*\*\*,  $p < 0.0005$  by Student's *T* test; \*\*,  $p < 0.005$  by Student's *T* test; differences for values without asterisks are nonsignificant.

#### Altered Ab responses in FDC-SP transgenic mice

We next determined whether deregulated FDC-SP expression leads to global alterations in Ab responses. The basal serum Ig levels in FDC-SP transgenic mice were comparable to those in the wild-type controls (data not shown). After immunization with chicken OVA, Ag-specific Ab responses were assessed (Fig. 3A). The generation of OVA-specific IgM and IgG1 appears normal in FDC-SP transgenic mice; however, the levels of OVA-specific IgG2a and IgE were significantly reduced by 4.6- and 3.4-fold, respectively. These alterations did not appear to be due to the effects of FDC-SP on T cell priming, because no differences were observed in OVA-induced T cell cytokine production (Fig. 3, B and C) or proliferation postimmunization (Fig. 3D). To further assess whether B cell function might be intrinsically defective, we examined the ability of FDC-SP transgenic mice to mount T-independent Ab responses. Although the production of anti-NP IgM was unaffected, transgenics showed selective impairment of anti-NP IgG3 production after immunization with the type II T-independent Ag NP-Ficoll, but not the type I T-independent Ag NP-LPS (Fig. 4). This result confirms that FDC-SP can selectively alter B cell function in vivo.

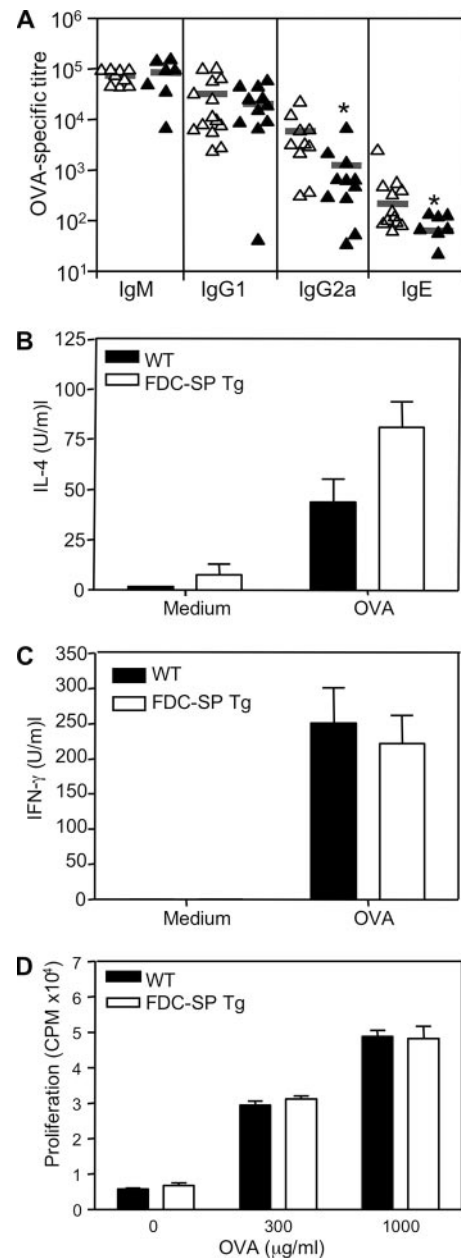
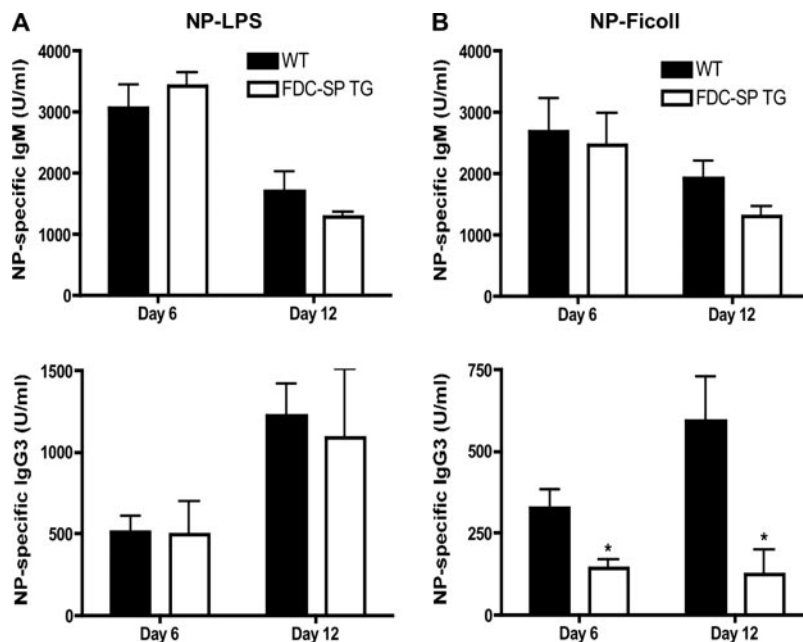


**FIGURE 3.** Altered T-dependent Ab responses in FDC-SP transgenic mice. *A*, OVA-specific IgM, IgG1, IgG2a, and IgE in serum collected from wild-type (WT) or FDC-SP transgenic (Tg) mice at day 14 after OVA-alum immunization. Results for individual mice are shown with means indicated by the gray bars. Ab responses that are significantly reduced in FDC-SP transgenics are indicated with asterisks (\*,  $p < 0.05$  by Student's *t* test). *B* and *C*, Splenocytes from OVA-immunized mice were isolated at day 5 and restimulated in vitro with 300 μg/ml OVA, and supernatants were collected at 24 h for IL-4 (*B*) or 48 h for IFN-γ (*C*). The cytokine results are expressed as the mean ± SD (U/ml) for five mice per group. *D*, Splenocytes from OVA-immunized mice were restimulated in vitro with OVA for 72 h and the [<sup>3</sup>H]thymidine uptake was measured as the index of proliferation. The proliferation results are expressed as the mean ± SD (cpm) and are representative of two independent experiments.

#### FDC-SP regulates GC responses

We next examined whether deregulated FDC-SP expression affects GC responses. FDC-SP transgenic mice were immunized with OVA and the frequency of GC B cells was assessed by flow cytometry (Fig. 5A). GC responses induced in the spleen following

**FIGURE 4.** T-independent Ab response in FDC-SP transgenic mice. Mice were immunized i.p. with the NP-LPS (A) or NP-Ficoll (B) Ags and serum was collected on the indicated days. Anti-NP IgM or IgG3 levels were measured by ELISA. Levels of NP-specific IgG3 were significantly reduced in FDC-SP transgenic mice immunized with NP-Ficoll ( $p < 0.05$  by Student's  $t$  test). WT, Wild type; TG, transgenic.



OVA immunization were detectable at day 7 postimmunization, peaked at day 14, and declined substantially by day 21 (Fig. 5B). The frequencies of GC B cells by day 7 post immunization were substantially reduced (38%) in FDC-SP transgenic mice. On day 14 a significant reduction in GC B cells (68%) was observed in the transgenic mice. Reductions in the frequency of GC B cells were also observed after low-dose sheep RBC immunization (data not shown). To determine whether these reductions reflected reduced frequency and/or size of GC, we performed immunofluorescence

staining of frozen spleen sections (Fig. 6). Markedly fewer PNA<sup>+</sup> GC B cell clusters were observed per section in the transgenic mice and the GC sizes were also significantly reduced, with transgenic GCs having ~50% reduction in diameter on average (Fig. 6, A and B). The positioning of GCs at the interface between the T and B zones appeared normal in FDC-SP transgenic mice (Fig. 6C), and these mice had no obvious decrease in size or density of the FDC network as demonstrated by FDC-M2 staining (Fig. 6D). These data provide the first evidence for a function of FDC-SP in the regulation of GC responses.

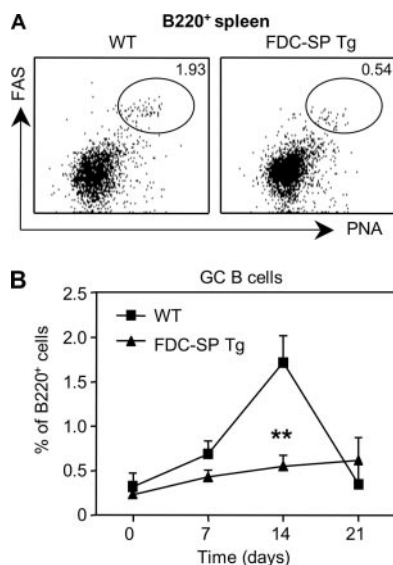


**FIGURE 5.** Reduced germinal center B cell responses in FDC-SP transgenic mice. A, FACS dot plot showing gating on GC B cells (PNA<sup>+</sup>Fas<sup>+</sup>) from day 14 OVA-immunized spleens of wild-type (WT) or FDC-SP transgenic (Tg) mice. The numbers show the relative percentage of the cells within the B220<sup>+</sup> gate. Similar results were observed using GL7/Fas gating to identify GC B cells (data not shown). B, FACS quantitation of the frequency of GC B cells (PNA<sup>+</sup>Fas<sup>+</sup>) from OVA-immunized spleens. The data show the relative percentage of the cells within the B220<sup>+</sup> gate at the indicated time and are representative of at least eight mice per group. Double asterisks denote a significantly reduced number of GC B cells (\*\*,  $p < 0.005$  by Student's  $t$  test).

#### *In vitro functional capacity of FDC-SP transgenic B cells*

To examine whether the constitutive expression of FDC-SP in the lymphoid tissues of the transgenic mice may provoke an intrinsic defect in B cells that accounts for altered GC and Ab responses, we tested the functional capacity of B cells from FDC-SP transgenic mice in vitro. FDC-SP splenic B cell proliferative responses to various mitogenic stimuli appeared normal (Fig. 7A). In addition, LPS plus IL-4 (Fig. 7, B and C) or anti-CD40 plus IL-4 (data not shown) activation induced isotype switching and plasma cell differentiation of FDC-SP splenic B cells at comparable levels to that of the control. These data suggest that the observed in vivo alterations were not due to intrinsic defects of FDC-SP transgenic B cells proliferation, isotype switching, or plasma cell differentiation. In contrast, the migration of transgenic B cells toward the chemokines CXCL12 and CXCL13 is consistently impaired by 25–35% (Fig. 7D), suggesting that the chronic exposure of B cells to high levels of FDC-SP may partially desensitize their chemotactic responsiveness. The expression of CXCR4 and CXCR5 were equivalent in transgenic and nontransgenic B cells (data not shown), consistent with a receptor desensitization mechanism acting at the intracellular level.

#### *Acute exposure to FDC-SP in vitro induces B cell chemotaxis*

In both human and mouse genomes the FDC-SP gene is located within 5 Mbp of a linked cluster of CXC chemokine genes, and FDC-SP protein is of similar m.w. and charge as chemokines. We thus asked whether FDC-SP could regulate B cell chemotaxis. Transwell migration assays were used to assess whether supernatants of FDC-SP transfected L cells can induce the migration of B

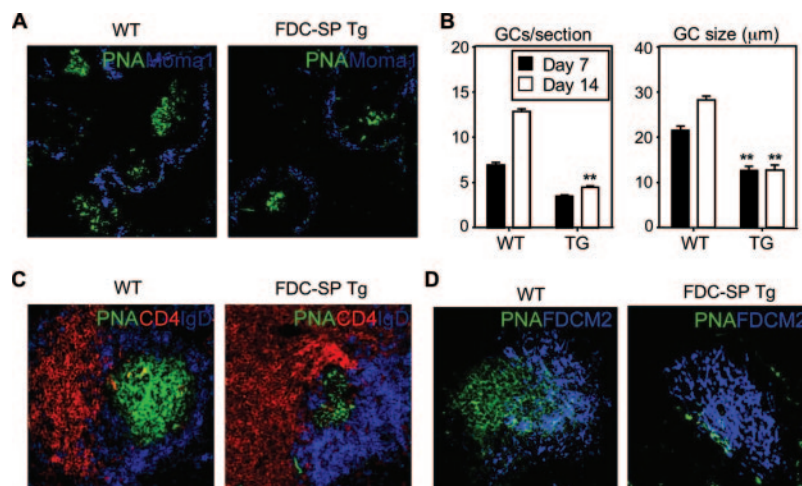


**FIGURE 6.** Immunofluorescence histology reveals that GCs formed in FDC-SP transgenic mice are fewer and smaller but are positioned normally at the T:B interface in association with FDC networks. *A*, Low magnification immunofluorescent images of spleen sections 14 days after OVA immunization. Staining shows PNA<sup>+</sup> GCs (green) within white pulp areas bounded by Moma-1-stained marginal zones (blue). *B*, *Left panel*, Quantitation of the number of GCs (PNA<sup>+</sup>IgD<sup>-</sup>) obtained by counting the number of GCs visible per transverse section. *Right panel*, GC size (PNA<sup>+</sup>IgD<sup>-</sup>) was measured using image analysis software and represents the maximum diameter. Data represent means obtained from the analysis of two sections per spleen and are representative of two mice per group. Double asterisks denote a significant reduction (\*\*,  $p < 0.005$  by Student's *t* test). *C*, Immunofluorescence staining of PNA (green), CD4 (red), and IgD (blue) showing the location of day 14 GCs at the interface between the B and T zones. *D*, Immunofluorescent staining of PNA (green) and FDC-M2 (blue), showing GCs associated with the FDC network. WT, wild type; Tg, transgenic.

cells (Fig. 8A). B cells indeed migrated toward FDC-SP-containing L cell supernatants; however, significantly increased migration to FDC-SP relative to the control supernatant was achieved only when B cells were stimulated with anti-CD40 plus IL-4 before

chemotaxis assay (Fig. 8A). Anti-CD40 plus IL-4 stimulation was used to induce an activated phenotype resembling GC B cells (28); the activation of B cells via BCR cross-linking or LPS stimulation did not effectively induce responsiveness to FDC-SP (data not

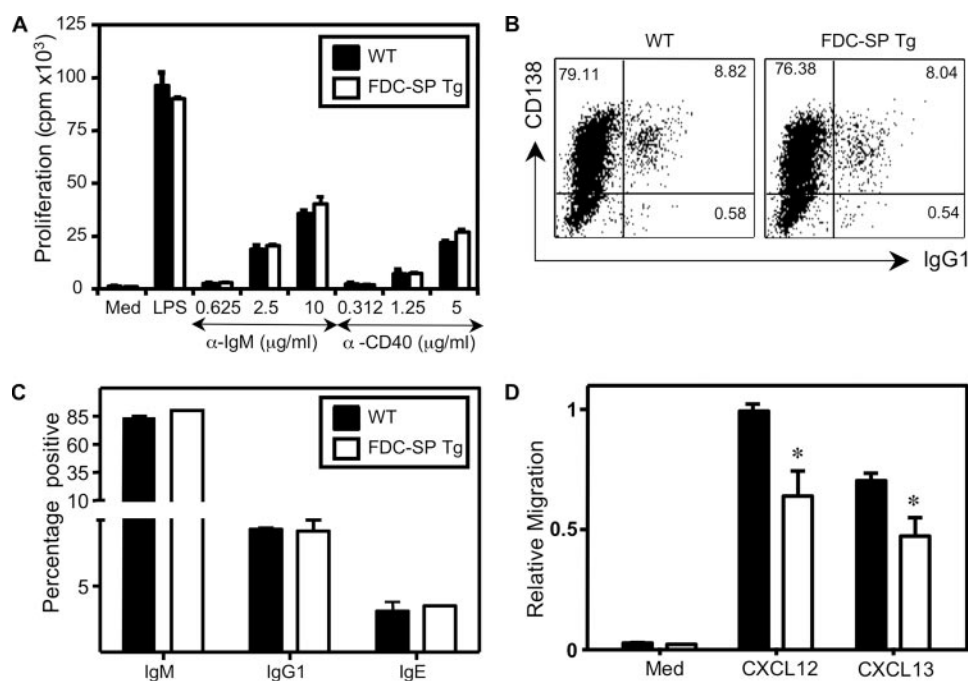


**FIGURE 7.** In vitro functional capacity of FDC-SP transgenic B cells. *A*, Normal proliferative response of FDC-SP transgenic (Tg) B cells to various stimuli. Cells were mixed with the medium (Med), LPS (1  $\mu\text{g/ml}$ ), or various doses of anti-IgM ( $\alpha\text{-IgM}$ ) or anti-CD40 ( $\alpha\text{-CD40}$ ) Abs for 72 h. Proliferation was assessed by measuring thymidine uptake and expressed as cpm  $\pm$  SD of two independent experiments. WT, wild type. *B*, FACS dot plot showing the frequency of IgG1 and CD138<sup>+</sup> plasma cells generated after 72 h of activation of B cells with LPS plus IL-4. The numbers show the relative percentage of the cells within the indicated quadrant. *C*, FACS quantitation of the frequency of IgM, IgG1, and IgE after 72 h of activation of B cells with LPS plus IL-4. The data show the relative percentage of each isotype and are representative of two mice per group. *D*, Impaired migration of FDC-SP transgenic B cells. B cells were activated with anti-CD40 plus IL-4 and migration in response to medium or CXCL12 or CXCL13 was assessed. The results are expressed as mean  $\pm$  SD and are representative of four independent experiments. \*,  $p < 0.05$  by Student's *t* test.

CXCL12 and CXCL13 are constitutively produced at low levels by FDC and within GCs (18, 29). We therefore asked whether induced FDC-SP might potentially act in concert with these chemokines by examining the migration of CD40 plus IL-4 prestimulated B cells in response to combinations of FDC-SP and suboptimal doses of CXCL12 or CXCL13. The addition of FDC-SP significantly enhanced migration toward CXCL12 or CXCL13 in this assay (Fig. 9). Strikingly, this enhanced migration response was absent in B cells derived from FDC-SP transgenic mice, consistent with their desensitization to chemotactic stimuli. These results suggest that acute stimulation by FDC-SP could potentially act in concert with CXC chemokines to enhance B cell migration, whereas chronic exposure to high levels of FDC-SP can blunt their migratory responses.

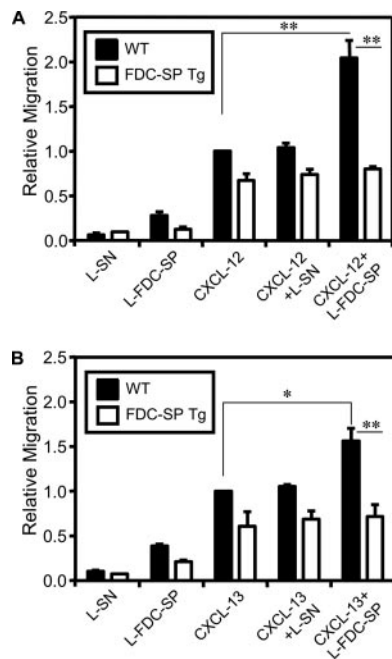


**FIGURE 9.** FDC-SP can enhance chemokine-induced migration. Chemotaxis assays were conducted with CD40 plus IL-4-prestimulated purified splenic B cells by using the indicated combinations of the supernatant from FDC-SP-transfected L cells (L-FDC-SP) or the supernatant from control L cells (L-SN) in combination with suboptimal doses of CXCL12 (A) or CXCL13 (B). Chemokine doses were chosen to give 5–10% migration (20 ng/ml for CXCL12 and 50 ng/ml for CXCL13). Data are normalized to the wild-type (WT) B cell migration obtained with chemokine alone and represent the average and SD of three independent experiments. \*,  $p < 0.05$ ; \*\*,  $p < 0.005$  by Student's  $t$  test comparing the indicated groups.

## Discussion

We have previously isolated human FDC-SP as a gene expressed in human tonsillar GCs of unknown function. In this study we have provided the first evidence for an immunomodulatory function of the FDC-SP gene product, implicating this secreted peptide in the regulation of GC responses and B cell migration. FDC-SP contains an N-terminal charged region adjacent to the secretion signal that is highly conserved between human and mouse counterparts and an N-terminal sequence that is less well conserved. In current sequence databases, FDC-SP homologues are only readily identifiable in human, chimpanzee, mouse, and rat genomes. This apparently recent evolutionary origin of FDC-SP is consistent with unique function(s) in mammalian host defense and with the recent development of the follicular GC reaction in secondary lymphoid tissues (30).

In human studies, the expression of FDC-SP in resting FDC-like cell lines can be induced by exposure to TNF but not LPS, whereas its expression in peripheral blood cells is induced by LPS but not TNF (21). Our present study showed that FDC-SP expression in mouse splenocytes prepared by physical disruption using a glass homogenizer was similar to that observed in human peripheral blood in that it is efficiently induced by LPS, but not TNF. Together, these data suggest that a non-FDC cell subset present in blood and spleen could be induced to express FDC-SP by microbial products. In contrast, FDC-SP induction by CD40 or TNF is most efficient in spleen cells prepared by the gentler method of enzyme digestion. Because enzymatic digestion better preserves the viability of fragile FDCs (31), we conclude that the TNF-inducible expression of FDC-SP under these conditions is likely due to expression by mouse splenic FDCs. As in humans, no expression of FDC-SP was observed in mouse B cells under any circumstances, but its expression after anti-CD40 stimula-

tion appears to be indirectly dependent on B cells. CD40-activated B cells have been reported to express a high level of surface TNF family molecules and to have the ability to induce phenotypic changes of FDC, which is characteristic during GC formation (32). Thus, we postulate that CD40 activation of B cells may allow them to express TNF family cytokines and perhaps other molecules that may, in turn, induce FDC-SP expression by FDCs.

The number and size of GCs formed postimmunization with a T-dependent Ag were substantially decreased in FDC-SP transgenic mice. The GCs that do form in these mice appear to be positioned normally at the interface between T zones and B zones, unlike those formed in CXCR5-deficient mice (33); however, they fail to form dense PNA-bright cores of proliferating GC B cells, suggesting that FDC-SP may also affect the development of GCs after their initiation. In contrast, the formation of FDC networks and the generation of T cell responses appear grossly normal in FDC-SP transgenic mice. These results demonstrate a specific immunomodulatory activity of FDC-SP impinging upon GC responses; although further work will be required to define the precise mechanism for the effect, our current results are consistent with the hypothesis that FDC-SP can act directly upon B cells. This hypothesis is supported by results showing that FDC-SP can bind directly to B cells (Ref. 21 and data not shown). The *in vitro* function of B cells that develop in FDC-SP transgenic mice seems relatively normal in terms of proliferation, Ig production, and plasma cell differentiation. However, prolonged exposure to high levels of FDC-SP in these mice causes B cells to become partially refractory to chemotaxis *in vitro*, suggesting that FDC-SP may act by modulating B cell migration. The impaired chemotaxis responses of FDC-SP transgenic B cells may be the result of mechanisms such as heterologous desensitization or regulation of RGS protein expression (34–36).

Deficits in the T-dependent primary IgG2a and IgE Ab responses were observed in FDC-SP transgenic mice, providing evidence that this molecule can potentially regulate specific aspects of humoral immunity. The decreased levels of IgG2a and IgE Abs in primary responses may be related to the reduced GC response, because these structures can support isotype switching and generation of memory B cells (reviewed in Ref. 2); however, their exact roles and importance are still controversial (37). M. K. Jenkins's group found that Ag-specific, IgG2a-switched cells undergo rapid expansion in the GCs (38); thus, the decreased expansion of IgG2a-switched cells within the FDC-SP transgenic GC, rather than impaired switching *per se*, may explain the reduced IgG2a titers seen in our model. The selective reduction in type II T-independent IgG3 production provides further evidence that FDC-SP can regulate humoral immunity. In the spleen, at least, B cell proliferation in response to NP-Ficoll occurs mainly outside of follicles (39); thus, this result suggests that FDC-SP may also regulate extrafollicular B cell responses.

In both mice and humans the FDC-SP gene is located near a gene cluster encoding proline-rich salivary peptides of largely unknown function that is, in turn, adjacent to a cluster of CXC chemokine genes. Although human FDC-SP contains a number of prolines in its C-terminal half, mouse FDC-SP has fewer prolines, and the overall m.w., amino acid composition, and charge of FDC-SP are more similar to those of chemokines than to those of the proline-rich salivary peptides. CXCL13, which is located in the adjacent chemokine gene cluster, is constitutively expressed in lymphoid tissues and serves homeostatic roles in lymphoid structure organization (40) in contrast to FDC-SP, whose expression appears to be more restricted and dynamically regulated by inflammatory stimuli. *In vitro*, FDC-SP can promote the migration of activated B cells to a greater extent than naive B cells, suggesting that FDC-SP may functionally target activated B cells.

FDC-SP-induced chemotaxis appears to represent a typical directional migration involving G protein signaling because it is lost after pertussis toxin treatment or in the absence of a concentration gradient. The charged or polar residues near the N terminus of FDC-SP may be critical for FDC-SP chemotactic activity, because FDC-SP tagged at its N terminus was more efficient after removal of the tag, which may spatially hinder FDC-SP function. Because several posttranslationally modified forms of FDC-SP can be produced, perhaps through glycosylation and/or protease cleavage (Ref. 21) and data not shown), it will be important to further define the bioactive form of FDC-SP. The migration of activated B cells toward FDC-SP consistently plateaus at a lower level than that of CXC chemokines, leading us to hypothesize that it may function in concert with other chemotactic factors present in GCs such as CXCL12 and CXCL13. Given the relatively weak chemotactic activity of FDC-SP alone, it is striking that it can markedly enhance chemotaxis toward strong chemoattractants such as CXCL12 and CXCL13, even when the latter are used at arguably superphysiological concentrations in vitro. FDC-SP seems to work through a directional chemotactic mechanism to cooperate with chemokines, because the preincubation of B cells with FDC-SP did not enhance their migration toward chemokines (data not shown). Elucidation of the mechanism for FDC-SP-induced chemotaxis will require identification of the receptor mediating the biological activities demonstrated here, an important goal for future studies.

Our study provides the first evidence for a functional role of FDC-SP in humoral immunity, consistent with our discovery of FDC-SP in human GCs. The lack of other overt phenotypes in FDC-SP transgenic mice suggest that this secreted peptide mainly targets immune processes, making it an attractive immunomodulator candidate.

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## Disclosures

The authors have no financial conflict of interest.

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