FDC-SP is a Novel Chemotactic Peptide for B Cells Activated by T cell Dependent Signals

By

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A Thesis Submitted to the Faculty of Graduate Studies in Partial Fulfillment of the Requirements for the Degree of

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Department of Immunology
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ABBREVIATIONS

AA	**amino acid**
Ab	**antibody**
Ag	**antigen**
AMV	**Avian Myeloblastosis Virus**
Bam32	**The B lymphocyte adaptor**
BCR	**B cell receptor**
BLC	**B-lymphocyte chemokine**
BLR-1	**Burkitt's lymphoma receptor-1**
bp	**base pair**
2-ME	**2 mercaptoethanol**
BSA	**Bovine serum albumin**
CD	**Cluster of differentiation**
cDNA	**Complementary deoxyribonucleic acid**
CDR	**Complementarity determining region**
Cpm	**Counts per minute**
CR	**Complement receptor**
C-terminal	**Carboxyl terminus**
Da	**dalton**
DCAL-1	**Dendritic Cell Associated C-type Lectin-1**
dNTPs	**Deoxynucleotide triphosphates**
E. coli	**Escherichia coli**

EDTA	**Ethylene diamine tetra acetic acid**
EST	**Expressed sequence tags**
EtOH	**ethanol**
FACS	**Fluorescence-activated cell sorting**
FcR	**Receptor for Fc portion**
FCS	**Fetal calf serum.**
FDC	**Follicular dendritic cell**
FDC-SP	**Follicular dendritic cell secreted protein**
FITC	**Fluorescein isothiocyanate**
FLIP	**FLICE-Inhibitory Protein**
FHT	**Follicular helper T cell**
g	**gram**
GALT	**Gut associated lymphoid tissue**
GC	**Germinal center**
GST	**Glutathione S-transferase**
HEV	**High endothelial venule**
hr	**hour**
Ig	**immunoglobulin**
IL	**interleukin**
IPTG	**isopropyl-beta-D-thiogalactopyranoside**
Kda	**Kilodalton**
LB medium	**Luria-Bertani Medium**
LPS	**Lipopolysaccharide**

MALT	**Mucosa associated lymphoid tissue**
MACS	**Magnetic cell sorting**
Mb	**megabase**
mg	**milligram**
MHC	**Major Histocompatibility Complex**
Min	**minute**
ml	**milliliter**
ng	**nanogram**
N-terminus	**amino terminus**
NP-40	**nonidet-p40**
OD	**Optical Density**
PBMC	**Peripheral blood mononuclear cells.**
PBS	**Phosphate buffered saline.**
PCR	**polymerase chain reaction**
PE	**Phycoerythrin**
PI	**PH value for the isoelectric point**
PT	**Pertussis toxin**
RGS	**regulator of G-protein signalling**
RNA	**Ribonucleic Acid**
RNase	**ribonuclease**
rpm	**revolutions per minute**
SDS	**sodium dodecyl sulfate**
SDS-PAGE	**sodium dodecyl sulfate polyacrylamide gel electrophoresis**

RT-PCR	**Reverse Transcriptase-Polymerase Chain Reaction**
SAC	**Staphylococcus aureus Cowan**
Sec	**second**
TBE	**Tris-Borate-EDTA buffer.**
TCR	**The T-cell receptor**
TNF	**Tumor Necrosis Factor**
U	**Unit**
UCi	**microcurie**
ug	**microgram**
ul	**microliter**

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ABSTRACT

B cells undergo affinity maturation, Ig class switching and apoptosis in a specialized microenvironment within lymphoid tissues called the germinal center (GC). These events are believed to be largely regulated by direct surface interaction between B cell and follicular dendritic cell(FDC), a specialized antigen presenting cell for B cells reside in GC, and by soluble mediators produced by FDC. FDC has strong ability to trap and present antigen to B cells. Primary human or mouse FDC cell lines can directly stimulate survival, proliferation, immunoglobulin secretion and expression of co-stimulatory molecules in vitro. However, the exact molecular basis for FDC-B cell interaction remains poorly defined. Here I characterized FDC-SP, a novel candidate molecule for regulation of the germinal center reaction. FDC-SP (follicular dendritic cell secreted protein) gene was cloned from enriched human follicular dendritic cell(FDC) using a subtractive hybridization strategy. FDC-SP encodes an 86 aa peptide with a leader secretion sequence and was previously found to be highly expressed in GC light zone. RT-PCR shows that FDC-SP expression is significantly upregulated in a FDC like cell line, or in mouse spleen cells after stimulation with TNF, but not LPS or SAC. Human PBMC can be induced to express FDC-SP by LPS or SAC, but not CD40 + IL4 or TNF. In contrast, primary B cell do not express FDC-SP whether or not stimulated. FDC-SP can bind to the surface of B cell population in PBMC and mouse spleen cells pretreated with anti-CD40 and this binding can be blocked by monoclonal antibody against FDC-SP. FDC-SP can weakly stimulate B cell proliferation together with Anti-CD40. My

studies also show that FDC-SP is chemotactic specifically for B cells activated by anti-CD-40. This chemotactic effect can be blocked by pretreatment with the inhibitor of G protein coupled receptor signaling, Pertussis toxin, suggesting that FDC-SP exerts its function through binding to this kind of receptor. FDC-SP chemotaxis is also markedly inhibited by further BCR engagement, suggesting that FDC-SP receptor level is down regulated. FDC-SP works synergistically with BLC, another chemotactic factor produced by FDC. I conclude that FDC-SP is produced by FDC during GC formation and play a role in regulating B cell proliferation and positioning through binding to a novel CD40-inducible, G protein coupled receptor.

INTRODUCTION

Adaptive immunity can be divided into two arms according to the main effector components involved: one is called cell mediated immunity, and the other is humoral immunity. Cell mediated immunity is characterized by activation of phagocytic and cytotoxic cells such as macrophage, CD8 T cell and other immune cells involved in killing and clearing foreign invasion. On the other hand, antibodies produced by B cells, together with complement systems are the main mediators of humoral immunity. These two arms of adaptive immunity are not completely separated from each other, rather, they have influence on each other and are functionally interweaved, for example, in antibody dependent cellular cytotoxity (ADCC). However, one aspect may have a dominant role in one infection. T helper cells participate in both cell mediated immunity and humoral immunity, regulating which part dominate in the immune response (HayGlass, 2001).

Humoral immunity.

To activate B cells to produce antibody, there are two kinds of antigen. One is T cell independent antigen which is usually multi-valent or inherently mytogenic, and can activate B cells in a multiclonal manner to produce antibody (Andersson et al., 1977). However, B cells need the help from T helper cells to be activated and produce antibody against most antigen(Parker, 1993). B cells internalize and process antigens and present them to T cells in the form of small peptide bound to MHC II molecule on B cell surface(Lanzavecchia, 1990). The cognate T cell can recognize these form of antigen through specific TCR on their surface(Germain, 1994). T cell is not only needed for the initial activation for B cells, but they also play very important role in subsequent process

of affinity maturation, Ig class switching, apoptosis, and differentiation. T cell exert its influence on these aspects through costimulatory molecules such as CD40 ligand, and secreted cytokine (Croft and Swain, 1991; Lane et al., 1992; Swain et al., 1991; Yan et al., 2000).

B cells can produce different isotypes of antibody such as IgA, IgM, IgD, IgE and IgG through a process called Ig class switching. All these antibody can have the same specificity for antigen binding but differ in their antibody constant region, which confer them different roles in clearing the antigen(Edelman, 1991; Stavnezer, 1996). Antigens bound to antibodies forms immune complex. This complex is efficiently cleared by phagocytic cells (opsonization). Antibody coating of cells facilitates their killing by other cells which recognize immune complex by FcR (ADCC)(Lanier et al., 1986; Lanier and Phillips, 1992). Antibody binding to antigen can activate the complement system. Activated complement can bind to (opsonize) the antigen, allowing binding to complement receptors and facilitate the clearing of antigen by effector cells. End product of activated complement fragment can also directly bind and form permeable pores on certain kind of pathogen and thus lyse them. Some complement fragment can serve as chemoattractant to recruit effector cells to the site of infection(Frank and Fries, 1991). Many cell types play effector roles in humoral immunity, such as monocyte/macrophages, neutrophils, eosinophils basophils, mast cells and NK cells. Humoral immunity is important in clearing extracellular infection and blocking cell to cell transmission by neutralization(Janeway, 2001; Paul, 1999).

Disorders occur when the humoral immune response is inappropriate, excessive, or lacking. For example allergies often involve a humoral immune response to a substance that, in the majority of people, the body perceives as harmless. Transplant rejection involves the destruction of transplanted tissues or organs and can be partially mediated by antibodies (Charlton et al., 1994). Blood transfusion reaction is a complication of blood administration which occurs when there exists recipient antibodies against donor blood group antigen. Autoimmune disorder such as systemic lupus erythematsus, rheumatoid arthritis) occur when the immune system acts to produce antibody against normal body tissues(Tan, 1991). Other immunopathogenesis where humoral immune response may play a role are anaphylaxis, serum sickness and graft versus host disease. So, it is very important to fully understand the process of induction of humoral immunity in order to efficiently control and prevent these related diseases.

Structure and circulation of secondary lymphoid tissue:

Considering the large number of different antigens to be encountered, the vast surface and locations of body needing surveillance and the involvement many cell types of immune cells, coordinating each immune response in a correct spatial and temporal manner is a difficult task. However, the distribution of secondary lymphoid tissues all over the body together with the constant re-circulation of blood and lymph through these organs bringing in immune cells and antigens and sending out effector cells and mediators, provides a very good solution. Secondary lymphoid tissues include lymph nodes, spleen, mucosa associated lymphoid tissues(MALT) and gut associated lymphoid tissues(GALT). The general structure and function are similar but there are some

important differences between these tissues. The spleen functions to provide surveillance of systematic blood-born antigens, so it has no lymphatic circulation. In contrast, lymph nodes are more important in local infections through draining lymph. MALTs locate directly under mucosa to survey these locations. The distinct structure and organization of secondary lymphoid tissues greatly facilitate the initiation of immune response.

The structure of a lymph node is illustrated in figure 1. Lymph nodes are enclosed in a connective capsule. Afferent lymphatic vessels open immediately under the capsule, forming a layer of marginal sinus. Under the marginal capsule, the concentrically arranged outer layer is called the cortex, which is mostly composed of B lymphocytes (B cell zone). B lymphocytes always aggregate spherically around follicular dendritic cells (FDC) to form primary lymphoid follicles. The paracortex lies beneath the cortex in the central area, which is mainly composed of T lymphocytes and professional antigen presenting cells(T cell zone). The inner part of the lymph node is called the medulla, which is mainly composed of T and B lymphocytes, macrophages, and plasma cells. The flow of lymph in the capillary lymphatic vessels collect tissue fluids, which enter lymph node through afferent lymphatic. Lymph from the afferent lymphatic drains continuously through the cortex and paracortex by way of the cortical sinus, passing into the medullary sinus, leaving the lymph node in efferent lymphatic and finally enters blood circulation through thoracic duct. Lymph or blood percolate constantly through these structures, bringing in antigens and immune complexes from the site of infection. Lymphocytes re-circulate between blood and secondary lymphoid tissues in a regulated manner. They enter lymph node through post capillary high endothelial venules (HEVs), percolate

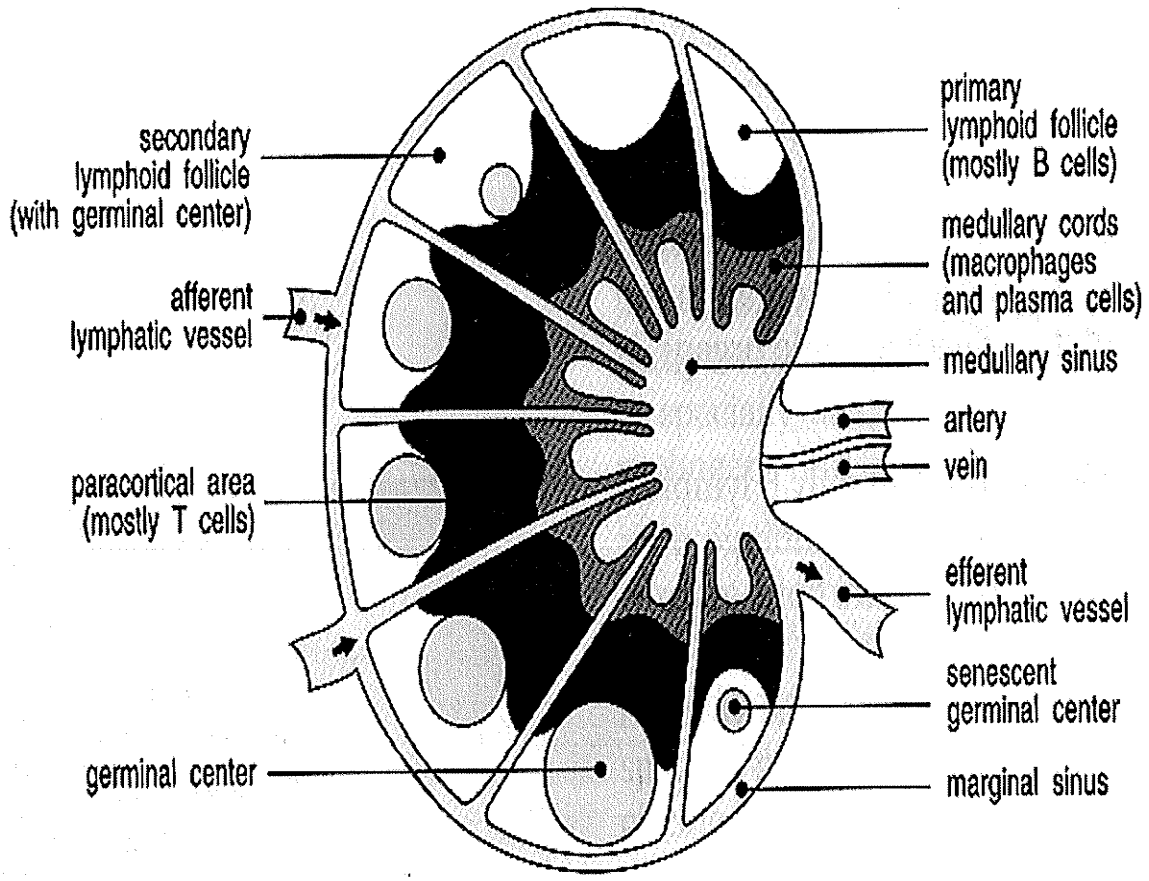


Fig. 1 The structure of lymph node adapted from Janeway, C. A. (2001). Immunobiology : the immune system in health and disease, 5th edn (New York, Garland Publ.).

through lymph nodes and then, reenter blood through the efferent lymphatics, surveying for cognate antigens in these sites. At anytime, there is a large amount of T and B cells residing in these tissues. They occupy "T cell zone" and "B cell zone" respectively.

B cell survival and activation: Initiation of humoral immune response

Primary follicles are important in maintaining B cell homeostasis. This structure provides limited "niches" for B cell to compete for access, thus acquiring surviving signals(Goodnow and Cyster, 1997). In the case of foreign infection, the antigen and the dendritic cells that endocytosed and processed the antigen are brought to the nearest lymph node from site of infection. These dendritic cells present the MHC-bound antigen fragments to specific T cells through TCR, delivering an activation signal with the help of other surface costimulatory molecules(Clark and Ledbetter, 1994; Schlienger et al., 2000; Thery and Amigorena, 2001). When antigen-specific B cells encounter the antigen, they will internalize, process, and present them to activated cognate helper T cells through the MHC II complex(Lanzavecchia, 1990). After engagement with the B cell, the pre-activated T cell will deliver a strong activation signal through binding of CD40 ligand on its surface to CD 40 on B cell surface(Ishida et al., 1995; Kawabe et al., 1994; Laman et al., 1996). Cytokines such as IL-4 produced by helper T cell also play an important role in this activation process(Clark and Ledbetter, 1994; Kishimoto, 1985; Paul and Ohara, 1987). Importantly, the duration of CD40 stimulation and T cell cytokine profile have decisive influence on Ig class switching of B cells(Aversa et al., 1994; Cerutti et al., 1998; Lebman and Coffman, 1988; Toellner et al., 1998). The activated antigen-specific B cell will then migrate into lymphoid follicles, and begin proliferating

rapidly to form germinal center. The combination of signals delivered to B cell ensures the antibody response is confined to the appropriate class, magnitude and location(Gieni et al., 1993; Gieni et al., 1996; HayGlass et al., 2000). Dysregulation of B cell activation can lead to pathological conditions including immuno-deficiency(Minegishi et al., 1999; Tsukada et al., 1993), auto-immune diseases such as systemic lupus erythematsus (Martin et al., 1999; Rieux-Laucat et al., 1995), leukemia(Witte, 1988) or lymphomas(Jungnickel et al., 2000), and allergic diseases(HayGlass, 1995).

Follicular helper T (FHT)

When T cells are activated by antigen-presenting cells, they will undergo proliferation and differentiation and give rise to effector T cells. These effector T cells either migrating to places of inflammation or to the B cell follicles to participate in the formation of germinal centers(Austrup et al., 1997). A fraction of activated T cells develop into memory cells, allowing a fast and effective immune response once re-challenged with the same antigen(Dutton et al., 1998). Based on the expression of the chemokine homing receptor CCR7, memory cells can divided into two group: $CCR7^-$ effector memory T cells (T_{EM}) which will migrate to inflamed tissue, and $CCR7^+$ central memory T cells (T_{CM}) with the potential to home to lymphoid organs(Sallusto et al., 1999).

More recently, a third type of $CXCR5^+$ Th cells has been reported. These cells represent a novel sub-population of Th cells that localize to B cell follicles and GCs to provide the support that B cells need during their differentiation program within germinal center in order to efficiently produce Igs(Breitfeld et al., 2000). These cells are designated here as

follicular B helper T cells (FHT) $CXCR5^+$ T cells are very inefficient in the production of cytokines but potently induce antibody production during co-culture with B cells. CXCR5(+) T cells do not respond to other chemokines present in secondary lymphoid tissues, including secondary lymphoid tissue chemokine (SLC), EBV-induced molecule 1 ligand chemokine (ELC), and stromal cell-derived factor 1 (SDF-1). Instead, they respond to B-lymphocyte chemokine (BLC). They are localized in the mantle and light zone of germinal centers of B cell follicles. FHT cell express high level of activation and costimulatory markers, including CD40 ligand (CD40L), CD69, HLA-DR, and inducible costimulator (ICOS), a recently identified co-stimulatory molecule of the CD28 family(Schaerli et al., 2000). When compared with CD4(+)CD45RO(+)CXCR5(-) cells, CD4(+)CD45RO(+)CXCR5(+) tonsillar T cells efficiently support the production of immunoglobulin (Ig)A and IgG(Kim et al., 2001).

Regulation of B lymphocytes by stromal cells and FDC.

It has long been appreciated that B cell activation and humoral immunity require signals derived from other leukocytes such as T lymphocytes. More recently, it was realized that B lymphocyte development, survival and activation also critically depends upon interaction with specialized non-hematopoietic stromal cells of mesenchymal origin. In bone marrow, B cell progenitors interact closely with stromal cells to support their differentiation(Jacobsen and Osmond, 1990). By using both contact dependent (membrane bound) and soluble growth factors, stromal cells stimulate survival, proliferation and differentiation of B cell precursors(Cumano et al., 1991; Kincade, 1991). In the secondary lymphoid organs, mature B cells interact with morphologically

similar cells known as follicular dendritic cells (FDC)(Cyster et al., 2000; Liu et al., 1996; Tew et al., 1997).

The exact cellular origin of FDC has been the subject of some controversy. There has been report that monoclonal antibody against FDC surface can recognize potential follicular dendritic cell precursors in the blood and bone marrow, suggesting a hematopoietic origin(Haley et al., 1995). However, current evidence largely favors the idea that FDCs are a special type of non-hemopoietic, stromal cell of mensenchymal origin. Firstly, FDC share several markers with fibroblasts(Lindhout and de Groot, 1995). Secondly, the formation of germinal center is not only restricted to the secondary lymphoid organs. Ectopic FDCs were found at a number of other sites such as thymus(Christensson et al., 1988), blood vessel walls(Houtkamp et al., 2001), synovial tissue(Randen et al., 1995), salivary glands(Aziz et al., 1997) and skin(Rijlaarsdam and Willemze, 1994) under condition of chronic inflammation. Thirdly, although most primary FDC-tumors arise from lymphoid tissues, a number of them has also been found to arise from liver, bile duct, pancreas and so on(Han et al., 2000; Shek et al., 1998). This suggest that conditions required for malignant transformation or chronic inflammation can shift the development of local mensenchymal cells towards the FDC phenotype. Finally, in vitro studies with synovial fibroblast cell line from patients with rheumatoid arthritis showed that some of these cell lines have typical FDC functions, such as binding to GC B cell and switching off apoptosis(Lindhout et al., 1999).

FDC may be seen as antigen presenting cells (APC) for B cells, but they are different from conventional APCs that present antigen to T lymphocytes. They do not internalize and process antigens, and do not present antigenic peptides on MHC class II molecules, because they don't express MHC II on their surface(Denzer et al., 2000). Instead, FDCs are characterized by having long protruding dendrites and strong ability to trap immune complex on their surface. They can hold antigen for months or even years for B cells to recognize(Nossal et al., 1968; Szakal and Hanna, 1968). FDCs express high levels of FcgammaRIIB and low level of Fc epsilon RII, the receptors for antibody Fc proportion(Maeda et al., 1992; Rao et al., 2002). It also expresses very high level of complement receptor CR2/CD21. Both of these types of receptors facilitate trapping of immune complex(Liu et al., 1997; Tew et al., 1997). FDC are thought to represent the central antigen presenting cell for B lymphocytes that drives affinity maturation of antibody response and the generation of memory B cells(Klaus, 1978; Tew et al., 1990; Tew et al., 1979).

Besides presenting antigen to B cells, FDC can support B cell survival and proliferation(Liu et al., 1996). The binding of specific antigen to the BCR of B cells to antigen trapped on FDC surface can deliver important survival signals to B cell(Batista and Neuberger, 2000). This is most likely caused by reduced Fas and Fas-ligand and elevated expression of anti-apoptic Bcl-2 (Smith et al., 2000; Tsunoda et al., 2000). There have also been reports that FDC also express surface molecules that can promote B cell proliferation(Li et al., 2000). FDC can support B cell proliferation, Ig secretion, and expression of co-stimulatory molecules in vitro(Clark et al., 1995; Grouard et al.,

1995; Tew et al., 1997). During germinal center formation, there is a drastic phenotypic change of FDC.

FDCs are also an important source of B cell chemoattractants. FDC expresses B cell chemokines BLC to attract B cell to come close to their dendrites. BLC binds BLR1/CXCR5 as receptor and is so far found the strongest chemoattractant for B cells (Gunn et al., 1998). BLC is playing an important role in secondary lymphoid tissue structural organization(Ansel et al., 2000; Gunn et al., 1998). Deficiency in CXC chemokine receptor 5 (CXCR5) severely affects the development of Peyer's patches(Honda et al., 2001). BLC expression can be induced by lymphotoxins expressed on activated B cell surface(Endres et al., 1999; Fu et al., 1998; Le Hir et al., 1995). Also, BLC can induce lymphotoxin expression on B cells(Luther et al., 2000). Ectopic expression of BLC in pancreatic islets can cause B cell recruitment and lymphotoxin-dependent lymphoid neogenesis(Luther et al., 2000). So there is bi-directional communication between FDC and B cell. Cultured FDCs produced MCP-1, and this production was enhanced by tumor necrosis factor(Husson et al., 2001). This suggest FDC may also be and important source of other chemokines.

Affinity maturation:

The antigen affinity of surface BCR of newly activated B cell clones is often not so high. However, B cell has developed a fine tuning mechanism called affinity maturation. Affinity maturation is facilitated by means of somatic hyper-mutation. With each round of division, B cells acquire point mutations at its BCR CDR region(Wilson et al., 1998),

and a few of them may have higher affinity for the specific antigen and get better chance of survival. There has been abundant evidence that most of the affinity maturation process happens within germinal center. Generation of memory B cells and production of high-affinity antibody is temporally associated with the formation of GC(Kelsoe, 1996; Rajewsky, 1996). B cells with somatically mutated Ig genes were originally identified within GC (Kelsoe, 1996). Competition with previously formed Ab for Ag sequestered in ICs on FDCs is thought to drive affinity maturation of B cells and memory cell development. (MacLennan and Gray, 1986). In GCs high-affinity mutants take over GCs very soon after they appear; the replacement rate is as high as 4 per day(Radmacher et al., 1998). They will have more competence for binding to antigen presented at FDC surface(Berek et al., 1991; Wang et al., 2000). Prolonged availability of antigen is required for somatic mutation and affinity maturation, and FDC or adjuvant facilitate such processes by slowly releasing antigens (Wang et al., 2000). Encounter of soluble antigen by germinal center B cells during an ongoing response can cause B cells to undergo apoptosis(Pulendran et al., 1995; Shokat and Goodnow, 1995). However, tethered antigen on FDC surface can lead to synapse between B cell and FDC(Batista et al., 2001). B-cell antigen receptor accumulates at the synapse, segregated from the CD45 co-receptor which is excluded from the synapse, and there is a corresponding polarization of cytoplasmic effectors in the B cell. B-cell antigen receptor mediates the gathering of antigen into the synapse and its subsequent acquisition, thereby potentiating antigen processing and presentation to T cells with high efficacy(Batista et al., 2001). The physical nature of the antigen extraction process may itself provide discrimination of higher affinity interactions, as the resistance of a chemical interaction to applied force is

related to its affinity. Clathrin and dynamin, proteins involved in the formation of endocytic vesicles, apply significant forces to membrane receptors. This force may effectively test the affinity (in the range 10^6–10^{10} M^{-1}) of the antibody-antigen interaction in the B synapse and provide the necessary feedback for affinity maturation(Batista and Neuberger, 2000).

The B cells with less affinity BCR produced during this process have less affinity to antigens. They will fail to compete for antigen and undergo apoptosis because lack of continued expression of the anti-apoptosis factors cFLIP and Bcl-2, which can be upregulated by CD40 and BCR signaling(Hennino et al., 2001; Tarlinton and Smith, 2000). After many rounds of cell division and mutation, a few B cell clones that has best fit BCR will finally differentiate into antibody producing plasma cells. They will migrate into bone marrow and produce antibodies in large quantity(Kelsoe, 1996; Tarlinton and Smith, 2000).

The dynamic structure and composition of germinal center

Follicles that become filled with activated B lymphocytes during an immune response are referred to as secondary follicles or germinal centers and have a characteristic organized structure that has been well defined by immuno-histology(Liu et al., 1996; MacLennan, 1994; Szakal et al., 1989). Activated B cells undergo a first phase proliferation at the border of T cell and B cell zone, producing a "primary foci" of proliferating B cells which produce some initial low affinity antibodies. The immune complex formed between circulating primary antibody and foreign antigen can be trapped on FDC surface

and is presented to competing B cells that are undergoing affinity maturation later. A small number of B cells from the primary focus will migrate from the T zone into primary follicles. There they will proliferate rapidly; pushing away the non-specific B cells and forming a densely packed area called dark zone. These cells are called centroblasts. They have greatly reduced surface Ig level(Jacob and Kelsoe, 1992; Jacob et al., 1993). Some of these centroblasts will reduce their rate of division, up regulate surface Ig, and migrate into the network of an area of FDC. There they compete for the immune complex on FDC and thus acquire surviving signal, these cells are called centrocytes(Han et al., 1997). Centrocytes that have acquired the antigen, will again enter dividing cycle accompanied by somatic hyper-mutation. They will later again compete for antigens which can deliver surviving and anti-apoptosis signal through BCR engagement. So after many round of competing and mutation, finally the best-fit antibody-producing cell will be produced. These cells will simultaneously undergo Ig class switching and will differentiate into either antibody producing B cells or memory B cells after leaving GC.

Decision making in survival and apoptosis:

In homeostatic conditions, B cells must compete for limited niches within FDC networks for survival signal(Cyster et al., 1994). After first activation by antigen plus helper T cells, B cell undergo proliferation cycles in the GC, however these cells are predisposed to death, they will die if they don't receive further supporting signals(Lebecque et al., 1997; Martinez-Valdez et al., 1996). Mutating GC B cells must acquire antigen from FDC to support their survival(Choe et al., 2000; Kim et al., 1995). However the signal

through the antigen receptor is not enough, interaction with T cell specific for the same antigen is also needed. Auto-reactive B cells will be deleted because of lack of T cell help, since there is no cognate auto-reactive T cell clones existing in the peripheral, they are already deleted during development. Only those B cells which are both non-self reactive and antigen specific are selected by helper T cells(Guzman-Rojas et al., 2002), These selected B cell will go back to undergo further rounds of mutation and selection. CD40 and BCR engagements are critical conditions for B cell survival(Grouard et al., 1995; Schilizzi et al., 1997), although other receptors also likely play roles. The mechanism by which B cells are predisposed to apoptosis is that after initial activation through the BCR, B cells express high level of Fas and Fas ligand that could kill itself, but in the mean time they also express high level of cFLIP, which inhibits apoptotic pathway(Hennino et al., 2001). cFLIP can be diluted after a few rounds of division, so cells must receive further signal from CD40 or BCR to upregulate the expression of this molecule. FDC itself can also induce expression of Fas to kill B cells(Tsunoda et al., 2000; van Eijk et al., 2001).

Migration and chemotaxis route of B and T cell during antibody producing process

B cells re-circulate between secondary lymphoid tissue and blood. During a foreign infection, antigen-specific cells will significantly change their migration property to interact with other types of cells such as T cell and FDC to facilitate the antibody producing process. Dysregulation of these migration process may also lead to pathological conditions such as allergy(Campbell et al., 2002; HayGlass et al., 2000). To achieve these interactions in a timely and spatially coordinated manner, B cells work

through changing their responsiveness to chemokines secreted by other cells. B cells can change the expression level of surface receptors for chemokine(Campbell et al., 2001; Reif et al., 2002) or of intra-cellular signaling molecules. For example, RGS proteins can interact with intracellular domain of a G-protein coupled receptor and regulate their function in B cell migration (Reif and Cyster, 2000). Chemokine receptor level can be regulated by many signaling events, such as IgM, integrin or CD40 engagement (Hargreaves et al., 2001; Reif et al., 2002; Takagi et al., 2002). T cells and FDCs, which are the important sources of chemo-attractant within secondary lymphoid tissues, often change their chemokine expression profile in different situation(Campbell and HayGlass, 2000). The direction of B cell chemotaxis movement is decided by 3 factor: ligand availability, receptor expression and intracelluar signaling molecules (Cyster, 1999). The current model of B cell migration suggest that upon first encounter of antigen, B cell will express higher level of T cell zone chemokine receptors such as CCR7, while down regulating B zone chemokine receptor such as CXCR5(Reif et al., 2002). This facilitates their migration to T cell zone. In the mean time, upon activation by antigen presenting cells, T cell will express higher level of B cell zone chemokine receptor CXCR5, so that it will migrate towards B cell zone(Ansel et al., 1999; Breitfeld et al., 2000). Cognate T and B cell usually meet near the border of T zone and B zone(Garside et al., 1998; Reif et al., 2002). After activation of B cells by T helper cells, B cell will go back to lymphoid follicles to interact with FDCs to form germinal center. The current model for germinal center response is that B cells then cycle between dark zone and light zone, however the signals regulating the migration within GCs are not known. Some of the T helper cells

will also migrate into germinal center. They mostly reside at the peripheral of germinal center and are called follicular helper T cells(Breitfeld et al., 2000; Kim et al., 2001).

Isolation of FDC associated genes

FDC are large cells with fragile long dendrites, often tightly associated with clusters of B cells. This makes it difficult to obtain sufficient amount of pure FDC. To overcome these difficulties, several groups have attempted to obtain long term proliferating FDC lines (Clark et al., 1995; Clark et al., 1992; Kim et al., 1994; Kim et al., 1995). However, these cell lines often do not fully match the phenotype of primary FDC and the FDC-like phenotype tends to be lost with time in culture(Clark et al., 1992; Kim et al., 1994). Thus, studies were undertaken to isolate genes expressed by primary human FDC isolated from human tonsils. This resulted in the discovery of several novel genes, including FDC-SP, the subject of my studies(Marshall et al., 2002a).

Primary studies on FDC-SP

Human FDC-SP is an 86 amino acid (AA) protein. There is a 16 AA leader secretion peptide on it's N-terminus. Immediately after the leader peptide is a region of charged amino acids, followed by a proline rich C terminal region. Searches in the human protein data base showed no homology in terms of AA sequence, functional domains or structural motifs. Most of the C terminal region is of low complexity, and contains a high proportion of proline residues (Fig. 2). A genome blast search was conducted to determine the chromosomal location of the FDC-SP gene. It was found that the FDC-SP gene is located on chromosome 4q13, 3.6 Mb away from a cluster of CXC chemokines

MKKVLLLITAILAVAVGFP*VSQDQER*
*EKRSISDSDE***LASGFFVFPYPYPFRPL**
PPIPFPRFPW**F**RRN**FPIPIP**ES**AP**TT**P**
LPSEK

Fig. 2 Complete amino acid sequence of hFDC-SP. leader secretion signal peptide is underlined, charged region is in italics. Non polar residues are in bolded letters The predicted peptide has a PI(isoelectric point) of 7.3, similar to that of most of small chemokines.

including BLC, IL-8, MIP-2β and NAP-II. It also borders a group of secreted salivary proline-rich proteins PBII, P-B and BPLP. The FDC –SP gene spans a region of 10 Kb, consisting of 5 exons correspond to: 5' untranslated region (exon1); leader secretion signal (exon 2); N terminal charged region (exon 3); the remainder of coding sequence (exon 4); and the 3' untranslated region (exon 5) (Fig. 3). Based on human FDC-SP sequence, we searched the mouse EST and genome database and identified a putative murine counterpart. Murine FDC-SP shows 45% amino acid identity and 54% amino acid similarity with human FDC-SP (Fig 4). The charged N terminal sequence showed the highest degree of conservation between the two FDC-SP counterparts, supporting a functional importance of this region. The proline rich region is conserved in terms of proline content (21.4% overall Vs 18.8% for human). However, the primary amino acid sequence of this region is only modestly conserved. The distribution of the exons of murine FDC-SP is virtually identical to it's human counterpart, further supporting a close evolutionary relationship.

FDC-SP could be detected in both lysates and supernatants from cultures of COS cells transfected with expression constructs encoding FDC-SP fused to a myc epitope tag. This evidence further support that FDC-SP is a secreted protein. Tagged FDC-SP could also be detected on the surface of B lymphoma line BJAB cells transfected with the same construct, which suggest FDC-SP may be specifically binding to to these cells. Interestingly, substitution of certain charged residues with glycine did not affect secretion of FDC-SP, but abolished binding of FDC-SP to BJAB cells. Northern blot analyses showed that FDC-SP is highly expressed in tonsils (a mucosa-associated lymphoid

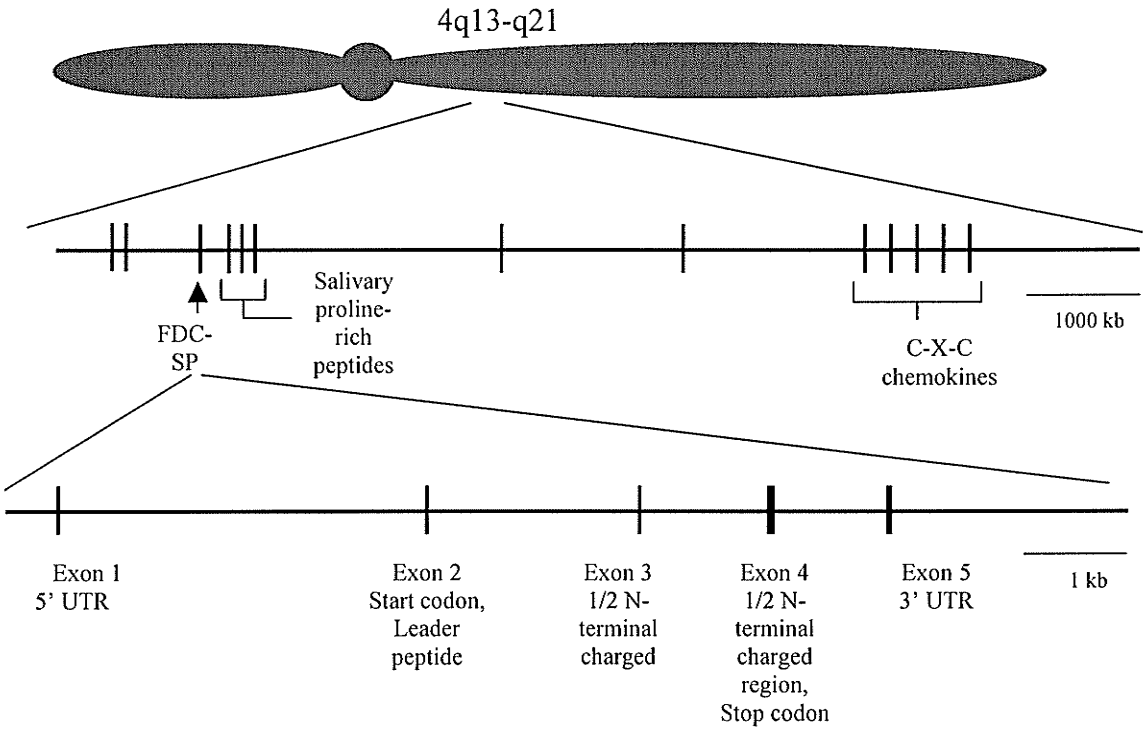


Fig. 3 Chromosomal location and structure of human FDC-SP gene. Alignment of the cDNA sequence with the public draft human genome sequence was done on Pubmed Genome Blast. Note that FDC-SP gene borders a group of secreted salivary proline-rich proteins abd is 3.6 Mb away from the cluster of CXC chemokines.

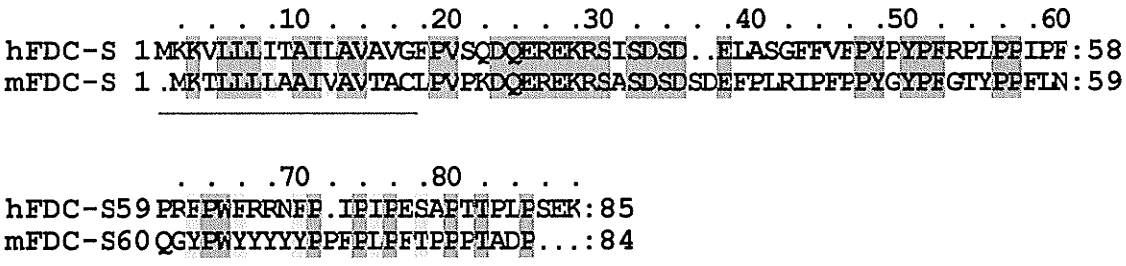


Fig. 4 Sequence alignment of human and mouse FDC-proteins. The secretion signal is underlined. Note that the charged section its highly conserved between human and mouse.

tissue), lymph nodes and trachea. FDC-SP expressed in lymph node and tonsils are likely produced by FDC and activated leukocytes. However, the cells producing FDC-SP in the trachea is unknown. FDC-SP is significantly expressed in prostate, colon, stomach and thyroid, but this expression is much lower compare to that in tonsil or lymph nodes. FDC-SP is not expressed in more than 10 other tissue examined, such as heart, brain, liver or skeletal muscle. Notably, FDC-SP is expressed at very low level or absent in other lymphoid tissues such as spleen, peripheral blood leukocytes and bone marrow. This reveals that FDC-SP has a restricted tissue distribution. FDC-SP expression in a number of cell lines including B lymphocytes, T lymphocytes, myeloid cells, epithelial cells was also examined using FDC isolated from tonsils as a positive control. It was found that FDC-SP is not expressed in any of the cell lines, except the strong expression by primary FDC. These data suggested that FDC-SP expression in lymphoid tissues may be primarily due to expression by FDC.

In an effort to further characterize the expression of FDC-SP within lymphoid tissues, in situ hybridization analysis on mRNA levels in human tonsil sections was carried out. It was found that FDC-SP is mostly expressed in germinal centers, but not in the adjacent T cell area. This further prove FDC-SP is not expressed by T lymphocytes and myeloid and lymphoid-lineage dendritic cells residing in these areas. Particularly, the highest density of signal is observed in the central portion of the germinal centers, namely, the light zone of germinal center which is mainly composed of a dense network of FDC and proliferating B cells. There is much less FDC-SP expression in the dark zone which contains closely packed B lymphocytes. In all germinal centers, the follicular mantle,

which is primarily composed of B lymphocytes not participating in the ongoing immune response, also showed a much less intensity of FDC-SP expression. These data further prove that FDC-SP is expressed by FDC, but not B lymphocytes with which they interact. FDC-SP expression was also detected in tonsil crypts in these analysis. Tonsil crypts are deep invaginations of the epithelia which open into the oral cavity and often filled with inflammatory cells in tonsilitis. Because of the complexity of celluar infiltration components in these sites, its hard to decide what cell types are expressing FDC-SP. However, this expression observed through several layers of infiltrated cells, suggest that expressions of FDC-SP in these sites are not simply from activated epithelial cells and may be produced by infiltrated leukocytes.

Hypothesis and goals

Based on the unique expression pattern of FDC-SP within lymphoid tissues, particularly, its high expression within germinal center light zone, I hypothesize that FDC-SP is produced by follicular dendritic cell and may play a role during ongoing antibody response. I will first set out to definitively prove that FDC-SP is produced by FDC, and to find out what other cell types may produce it and how expression is regulated during an immune response. Secondly, since FDC-SP is expressed in a secreted form within germinal center light zone, I hypothesize that it may exert its function through binding to a surface receptor on target cells. Since B cell is the predominant cell type besides FDC within germinal center, it is most likely that B cell may serve as a target cell. However T cell and other cells should also be tested. Thirdly, once I can prove FDC-SP binds to target cells, I will try to define what is the biological consequence after binding of FDC-

SP to its target cells. Germinal center B cell undergo drastic changes such as proliferation, apoptosis, isotype switching, differentiation. It is very interesting to investigate whether FDC-SP is playing any role in these aspects.

MATERIAL AND METHODS

1, Preparation of total cell RNA

Total RNA was prepared according the established protocol in our lab. Briefly, stimulated or un-stimulated cells were counted and 2 million of them were transferred into 1.5 ml eppendorff tube followed by spin for 10 sec, at 4 °C, 3000 g. The supernatant were then carefully discarded. Cell pellets were re-suspended with remaining supernatant. Four hundred ul Trizol reagent (Gibco) were added to the cell suspension and were mixed well by pipetting. Eighty ul of Chloroform/Isoamyl Alcohol was added to the mixture. The mixture were well mixed by shaking the tubes vigorously for 30 sec. To remove proteins and DNA, the tubes were spun 12500 g,at 4°C, for 15 min. The upper phase were then transferred to a new eppendorff tube. One ul(20ug) of glycogen was added to help the precipitation of RNA. One volume of (~110 ul) of isopropanol was added to the supernatant and mixed well by inverting the tube for a few times. All samples were then spun for 15 sec, at 12500 g, 4 °C to precipitate RNA. The supernatant were carefully discarded and the pellet was washed once with 100 ul of cold 70% EtOH. After aspirating the EtOH wash, the pellets were air dried for 1 hr at room temperature and dissolved in 50 ul of H_2O (Rnase free). One ul of Rnasin (2 U/ul) was added to the tube. The prepared RNA was either used freshly or stored at – 80 °C

2, Reverse transcription

RT reaction Mix was prepared as follows to a total volume of 20 ul per sample on ice:

a) 4 ul of 5 ×AMV RT Buffer

b) 1ul of 2.5mM dNTP

c) 1 ul of Rnasin (2 U/ul) (Promega)

d) 0.5 ul of Random Primer (Promega)

e) 1 ul of AMV RT (10 U/ul) (Promega)

f) 12.5 ul of RNA sample

This mix was then incubated in the GeneAmp Thermocycler (Applied biosciences) at 37 °C for 1 hour, then heated to 95 °C for 5 sec, and was used freshly for PCR or frozen at –80°C

3, RT-PCR

PCR mix was prepared as following on ice:

10× PCR buffer	5 ul
2.5 mM dNTP	3 ul
50 mM MgSO4	1.5 ul
Primer 1(10 uM)	2ul
Primer 2(10 uM)	2 ul
TaQ polymerase	0.5 ul (Invitrogen)
cDNA	1 ul

The PCR reaction was carried out on thermocycler with the following condition:

For human FDC-SP: 94C 2min, 1 cycle; 59°C 30sec, 72°C 30sec, 95°C 15sec, 30 cycles

For mouse FDC-SP: 94°C 2min, 1 cycle; 58°C 30sec, 72°C 30sec, 94C 30sec, 30 cycles

The primers sequences used for these reactions are:

MFR: GTA GCC TTG ATT TA

MFR2: TTC AAG GAA GTG GTT CAT ATT CAA

MFR3:CCA CGG GTA GCC TTG ATT TA

MFL: GAG CAGAGT GGA GAG TTT CAG AAC A

MFL2: CAG CTA GAA GGA GCA GAG TGG

MFL3: GGAGCAGAGTGGAGAGTTTCA

HFL: CTG ACT GAA ACG TTT GAG ATG A

HFL2: CAG CGT CAG AGA GAA AGA ACT GAC TG

HFR: TAC TTT TCG CTA GGA AGG GGA GTT G

The resulting PCR products were loaded together with 100 bp ladder marker on 1% agrose gel containing ethidium bromide, ran for 1hr and were visualized under ultraviolet light.

4, Generation of FDC-SP-GST fusion protein expression vector

4. A, Expression vector construction

PCR reaction was carried out using the primers designed for amplification and cloning of mouse and human FDC-SP. Each pair of primer contains Bam HI and XhoI restriction enzyme site on its 5 and 3 prime respectively. Plasmids containing mouse or human cDNA were used as template. The PCR products and PGEX-5X-2 vector were digested with Bam HI and XhoI. Shrimp alkline phosphatase was also added to the vector digestion mix. Digested fragments were run on agrose gel and recovered using a QiaQuick Column(Qiagen). The recovered PCR fragment and vector were then ligated together overnight at 10 °C using T4 DNA ligase(Promega), transformed into E. coli,

and plated on ampicillin plate. Colonies were picked, cultured and plasmids from these culture were prepared using Qiagen kit. Clones with correct insertion were screened by digestion the plasmids with Bam HI and XhoI. All the positive clones were further confirmed by sequencing.

Primers used for amplifying FDC-SP cDNA (the restriction enzyme sites are underlined):

MFL GST: CGC GGA TCC CCG TGC CTA AGG AC AGG AA

MFR GST: CCG CTC GAG TTA AGG ATC TGC AGT TGG AG

HFL GST: CGC GGA TCC CAG TCT CTC AAG ACC AGG A

HFR GST CCG CTC GAG TTA CTT TTC GCT AGG AAG GGG A

4. B, Recombinant protein expression

Recombinant GST-FDC-SP fusion protein was induced to be expressed in E. coli and purified by absorption to glutathione beads followed by elution with free glutathione. Briefly, a single colony was seeded in 50 ml LB, containing 100ug/ml Ampicillin, shaken at 350 rpm at 37 °C overnight. All the 50 ml culture was then transferred next day to a flask containing pre-warmed 500 ml LB(100ug/ml Amp), shaken at 300 rpm/min for about 1.5 hr, until OD600≈0.8. IPTG of a final concentration of 0.2 mM was added to the culture.

Cells were then incubated at 30 °C for 3 hr in a shaker. Cells then were harvested by spin at 3000 g for 10min and pellet was re-suspended in 10 ml lysis buffer(1× PBS, 1% Triton X 100, 0.05% cocktail protease inhibitor (Sigma). Harvested cells were frozen

and thaw at –80 °C once followed by Sonication 5× on ice, 10 sec each time. Cell lysates were spun 13000 g 10 min and filtered with a 0.45 filter. Fifty ul of sample was collected. To absorb GST fusion proteins, 1 ml Glutathion Sepharose 4B beads was prepared by washing 3× with PBS. Filtered supernatant was applied to bead and shaken gently at 4 °C for 1 hr. Beads were then spin at 200 g and the supernatant was removed. Beads were packed into a mini column (Sigma) and washed 3 times with 5 ml PBS. GST fusion protein was eluted with 5 mM glutathione in ten 1ml fractions collected in 1.5 ml Eppendorff tubes. Elution fractions containing significant amount of protein were combined together, dialyzed at 4 °C in 3 changes of 1000 ml pre-chilled PBS and stirred with magnetic bar. Finally,1 ml allocations were made, quantified on a spectrometer and frozen at –80 °C.

4. C, Factor Xa cleavage of GST fusion protein

The purified product was examined on a mini-SDS PAGE gel before and after Factor Xa cleavage. One hundred ul (0.3 ug/ul) of GST-FDC-SP was mixed with 10 ul of Factor Xa and the mixture was then incubated at room temperature overnight. The digested fusion protein was examined again on a mini-SDS PAGE gel. Gels were stained with Coomassie Blue.

5, Establishment of L cell clones stably expressing myc-tagged FDC-SP

5. A, construction of expression vector

The following primers were used for amplifying and sub-cloning the mouse FDC-SP gene. Each primer contains a Kpn I or Xho I site respectively:

mousemycl: CC GGT ACC ATG AAA ACT CTT CTC CT

mousemycr: CCG CTC GAG AGG ATC TGC AGT TGG AG

The PCR product was digested with KpnI/XhoI and inserted in-frame into the respective cloning site in PcDNA3.1/Myc-His(+)A (Invitrogen). The constructed vector was amplified in E. coli and purified using Qiagen Plasmid Maxi kit and further verified by sequencing. A vector for expressing human FDC-SP was constructed using similar methods.

5. B, Transfection of L cell line

Expression vectors were linearized by digestion with Sca I. The following solutions were prepared in advance:

Solution A: 20.5 ul(15ug) MFDC-SP PcDNA3.1 + 500 ul DMEM

Solution B: 20ul LF2000(lipofectamine) + 500ul DMEM

Solution A and B was mixed, incubated at room temperature for 5min, then added to L cell culture which was grown to 70% confluent. Transfected cell was then cultured at 37 °C overnight. Medium was changed after 18 hr with DMEM containing 10% FCS and 1% Penicillin-Streptomycin.

5. C, screening of high expression clones by limiting dilution

To select the cells expressing transfected genes, a concentration of 2mg/ml of G418 was added to the culture at day 2. Medium was changed once on day 6 and fresh G418 was added. After two weeks, the grown-up cell population was harvested and cell numbers are counted. Cells were then seeded at 0.3 cells/per well (=0.2 ml per well of a 1.5

cell/ml dilution) in 96 well and grown for 2 weeks until colonies are visible by eye. 24 clones for each transfection were transferred to 24 well plates. Cells were grown up until there was enough culture volume for RT-PCR analysis of FDC-SP expression.

6, Preparation of mouse spleen cells

Method A: 4 wk C57 BL/6 male mouse was sacrificed by cervical dislocation, whole spleen was taken and disrupted with a frosted glass tissue grinder in cold PBS containing 2% fetal calf serum. The cell suspension was then passed through a nylon mesh and washed for 2 times with PBS containing 2% FCS. Harvested spleen cells were cultured in Opti-MEM containing 2-ME and 10% FCS.

Method B: whole mouse spleen was cut into small pieces and digested with collagenase (200 U/ml, Sigma) for 1 hr at 37 °C. The digested spleen tissue was gently pipetted up and down a few times to release cells. The released cells were then collected by passing through a nylon mesh and washed twice with PBS, followed by culturing in Opti-MEM containing 2-ME and 10% FCS.

7, Mouse B cell purification

Mouse B cells were prepared by centrifugation of mouse spleen cell in Lympholyte M (Cedarlane Laboratories) followed by MACS negative selection according manufacturer's protocol. Briefly, cells from each mouse spleen prepared by method A were re-suspended in 10 ml PBS, supplemented with 0.5% BSA, and then 5 ml of cells were applied carefully on top of 5 ml Lympholyte M with a Pasteur pipette and

centrifuged at room temperature for 20 min at 1200 g. The yellowish lymphocyte cell layer lie at the inter-phase was carefully collected and spun at 1200 g for 5 minutes. Cells were re-suspended and washed 2 times with PBS, 0.5% BSA. To label the cells, cells were re-suspended in 0.9 ml of PBS, 0.5% BSA. 100 ul of MACS CD43 Microbeads(Miltenyi Biotech) was added, mixed well and incubated for 15 minutes at 10 °C. A CS column (Cedarlane) was prepared by filling and washing the column with 15 ml PBS, 0.5% BSA, then the column was put in a magnetic field. All of the cell suspension was applied in 2 ml buffer on top of the depletion column. The CD43 negative cells (B cells) passed through the column and were collected. The column was further rinsed with 15 ml buffer from top, effluent was collected as negative fraction. Positive cells retained in the column were also collected by taking the column out of magnetic field and eluting with 15 ml of PBS containing 0.5% BSA.

8, FACS analysis of FDC-SP binding

Two and a half ml stable L cell transfectant or the parental L cell line was seeded at 1 million/ml in 6 well plate, grown overnight until it's 80% confluent. Cell lines or primary cells were added to L cell culture at a concentration of 1 million/ ml, grown overnight. Suspension cells were carefully harvested from co-cultures using a Pasteur pipette. Cells were then stained for detection of myc-tagged protein on the cell surface using 1ug/ml FITC-labeled anti-myc Ab(Invitrogen) or 1ug/ml FITC-labeled Mopc21 iso-type control Ab(BD PharMingen). Stained cells were analyzed on a FACSCalibur instrument(BD Biosciences, Mountain View, CA). In some experiments, cells were also labeled with

biotinylated anti-CD19 (BD PharMingen) followed by staining with 0.5ug/ml Streptavidin-PE (Jackson ImmunoResearch Laboratories, West Grove, PA).

9, Proliferation assay

One hundred ul (1million/ml) of L cell and L cell stable transfectant was seeded in each 96 well in the presence of 20 ug/ml mitomycin C. Cells were washed with DMEM after 6 hr and 0.1 ml of B cell populations at 5 million/ml were added to the well and cultured at 37 °C. The cell culture was pulsed with 1 μCi/well ^{3}H Thymidine for 6 hr on day 3 and cells were harvested on a 96 well harvester (Tomtec). ^{3}H radio activity was measured on scintillation counting machine according to manufacturer's protocol.

10, Transwell assay and cell counting

Chemotaxis assay is carried out using Corning-costar 5um trans-well plates. Briefly, purified B cells are washed 3 times with migration buffer(DMEM containing 0.5% BSA) and re-suspended at 10 million/ml in the same buffer. Six hundred ul migration buffer were then added to the lower chamber plus different dose of FDC-SP or control BLC. Then 0.1 of ml cells were added to the upper chamber and the transwell plate was incubated at 37 °C for 3 hr. In some cases, FDC-SP or BLC was added to both lower and upper chamber as controls for chemokinesis. Cells in both the lower and upper chamber are carefully collected, diluted to an equal volume of 0.6 ml and counted on a FACSCalibur instrument(BD Biosciences, Mountain View, CA). Briefly, each sample were run through the instrument with a high flow rate for 2 minutes and the number of cells counted in that time was recorded. The percentage of migration was calculated by

the following formula: Percentage of migration = lower chamber cell number/(lower chamber cell number + upper chamber cell number) × 100%.

11, Statistical analysis

Statistical analyses of the data were performed using Student's *t* test. A value of $p<0.05$ was considered statistically significant.

RESULTS

1, Regulation of FDC-SP expression

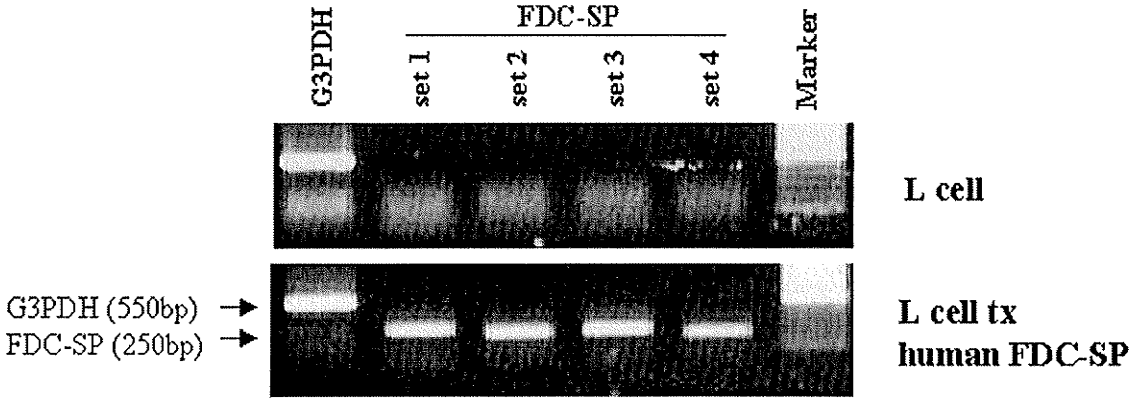
FDC-SP was cloned from enriched human FDC cells, and previous work from our lab showed that it is highly expressed in the germinal center light zone(Marshall et al., 2002a), which is mainly composed of FDC and proliferating B cells. While these results suggest that FDC-SP is produced by FDC in these sites, it is also expressed in infiltrated tonsil crypts(Marshall et al., 2002a), suggesting that FDC-SP may also be produced by other cell types. The overall high expression in lymphoid tissue suggests that FDC-SP may be mainly functionally involved and expressed in immune system during immune response.

1. A, Establishment of RT-PCR assay to test FDC-SP expression

In order to study the expression pattern of FDC-SP in both human and mouse systems by RT-PCR, the primers and reaction conditions first needed to be optimized. I designed 3 pairs of mouse FDC-SP primers and 2 pairs of human FDC-SP primers and tested these primers in different combinations and reaction conditions.

I found that the combination of MFL3 and MFR (252 bp) for mouse and HFL2 and HFR (250 bp) for human have the best amplification results respectively (Fig. 5). So I carried out the following expression study by using these primers for RT-PCR.

A.



B.

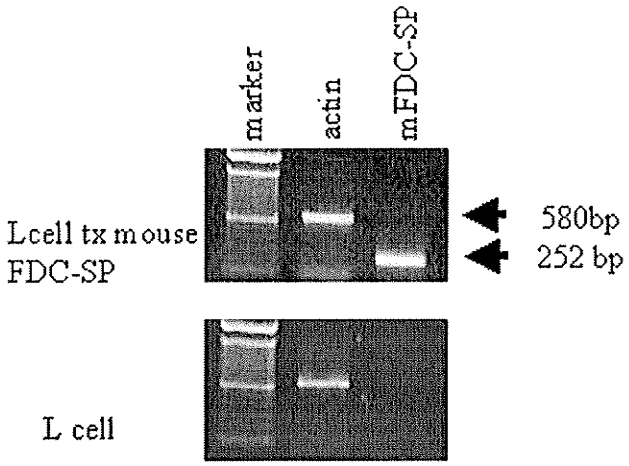


Fig. 5 Establishment of RT-PCR assay. RT-PCR was carried out by using different combinations of primers for human or mouse FDC-SP. RNA for these reactions were prepared from L cell line stably transfected with mouse and human FDC-SP or untransfected L cell as a negative control. **A.** Amplification for human FDC-SP. Note that the third set of primers get relatively better amplification. **B.** Mouse FDC-SP amplification. The primer set used for this amplification is MFL3 and MFR.

1. B, Expression in human system

I first examined the expression of FDC-SP in human PBMC by RT-PCR, after extracting RNA from these cultures (Fig. 6). I found that fresh or cultured PBMC without any stimulation do not produce FDC-SP. Anti-CD40 plus IL-4, which is a B cell dependent signal does not upregulate FDC-SP expression. TNF treatment slightly upregulates FDC-SP expression. In contrast, LPS and SAC can significantly upregulate FDC-SP expression after 24hr treatment, and the expression is sustained after 72hr (Fig. 6).

1. C, Expression in mouse system

Next I checked FDC-SP expression in the mouse system. I prepared mouse spleen cells using two different methods. In first method, the mouse spleens were cut into small pieces followed by digestion with collagenase, in an effort to preserve fragile FDCs that may be harmed by conventional tissue disruption methods. In the second method, mouse spleens were disrupted by using a glass tissue grinder. The results show that the expression pattern of FDC-SP from physically disrupted tissue is similar to human PBMC. Treatment with B cell activation signal does not induce FDC-SP expression, whereas TNF can slightly induce expression of FDC-SP (Fig 7). LPS is still a strong inducer of FDC-SP expression in this case. However, the expression pattern in the case of cells prepared using the digestion method is quite different. Fresh or unstimulated preparations still do not produce FDC-SP. Anti-CD40 plus IL-4 can upregulate FDC-SP expression 48 hr after stimulation at a modest level. LPS can also upregulate its

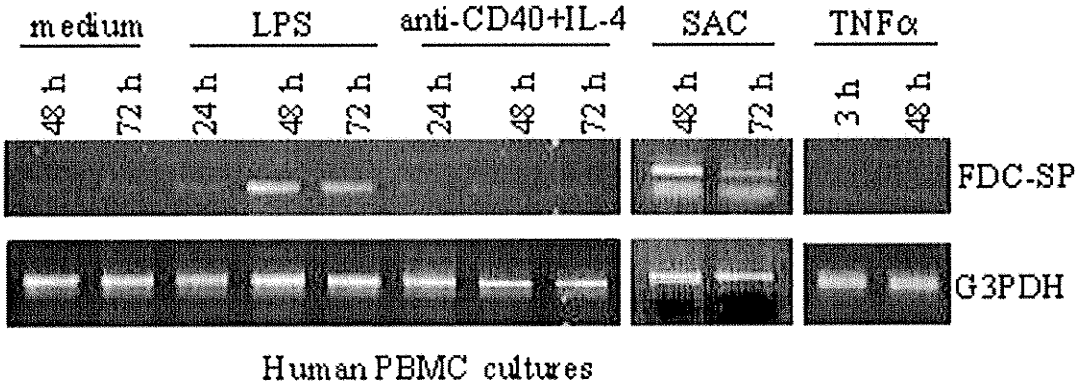


Fig. 6 Human PBMC can be induced to express FDC-SP by LPS or SAC, but not CD40 + IL4 or TNF. PBMC was cultured at 1 million/ml either in medium alone or the presence of following different stimuli: 100ng/ml LPS, 2ug/ml anti-CD40 plus 2ng/ml IL-4, 1/25,000 dilution of Staphylococcus aureus Cowan strain 1, or 10 ng/ml TNF-a, cells were harvested at indicated time and counted, RNA was extracted using Trizol and carried out RT-PCR.

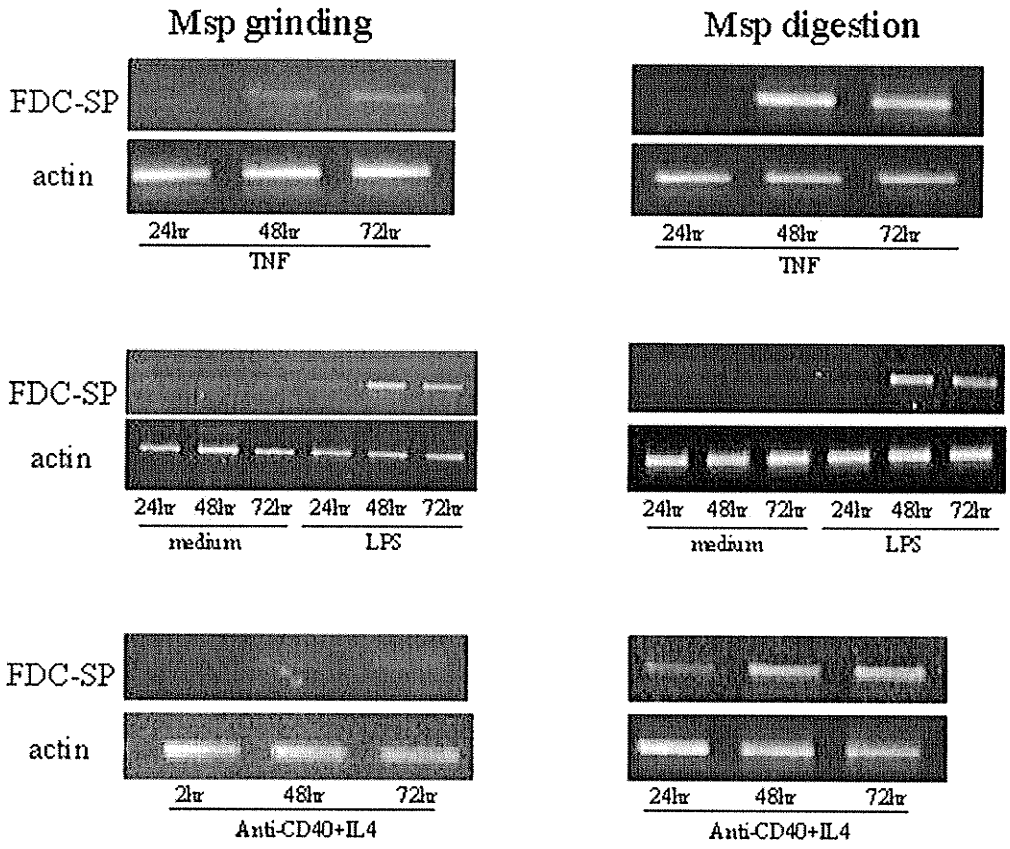


Fig. 7 Mouse spleen cells express FDC-SP in response to different stimuli in a cell preparation protocol dependent manner. Mouse spleen cells were prepared by either disrupting spleen using a glass grinder or digesting with collagenase. Harvested cells were cultured in OPTI-MEM containing 10% FCS, 1%PS, 0.05% 2-ME in the presence of different stimuli. Cells were harvested at different time point to extract RNA and carry out RT-PCR. Note that cells from two different preparation showed similar patterns of FDC-SP expression in response to LPS stimulation. In contrast, they respond quite differently to anti-CD40 plus IL-4 or TNF stimulation. There is no expression of FDC-SP in response to anti-CD40 plus IL-4 stimulation and less (8 fold less by density) expression in response to TNF in the cells prepared by grinding. The cells prepared by enzyme digestion expressed FDC-SP in response to all of the three stimuli, but much more significantly after TNF treatment.

expression. Most strikingly, FDC-SP is significantly upregulated 24 hr after TNF stimulation.(Fig. 7)

To confirm that B cell activation signals do not upregulate FDC-SP expression in B cell itself, I purified B cells from mouse spleen by centrifuge through lympholyte M followed by MACS negative selection. Non-B cells were also collected for analysis. The results show that under none of the treatment condition do B cells produce any FDC-SP. We conclude that FDC-SP is not produced by B cells, but activated B cells may be able to induce FDC-SP expression by non-B cell subsets, such as FDC. (Fig. 8)

My finding that PBMC and physically disrupted mouse spleen cells can be induced to produce FDC-SP by LPS stimulation, together with our previous result showing FDC-SP expression in infiltrated tonsil crypts, suggest that beside follicular dendritic cell, there may be other types of cells that produce FDC-SP upon stimulation. To find out what other cells may produce FDC-SP, I used a series of different mouse cell lines to test their expression. I found that, similar to previous result, B cell, T cell and fibroblasts do not produce FDC-SP. Interestingly, a macrophage cell line produces low level of FDC-SP. (Fig. 9)

Finally I tested FDC-SP expression in two FDC like cell line, FDC-1 and HK cells. We found that both of the cell lines produce basal level of FDC-SP. The expression can be drastically upregulated 3 hours after treating them with TNF, However LPS has no or very little effect on FDC-SP expression of these two cell lines (Fig. 10)

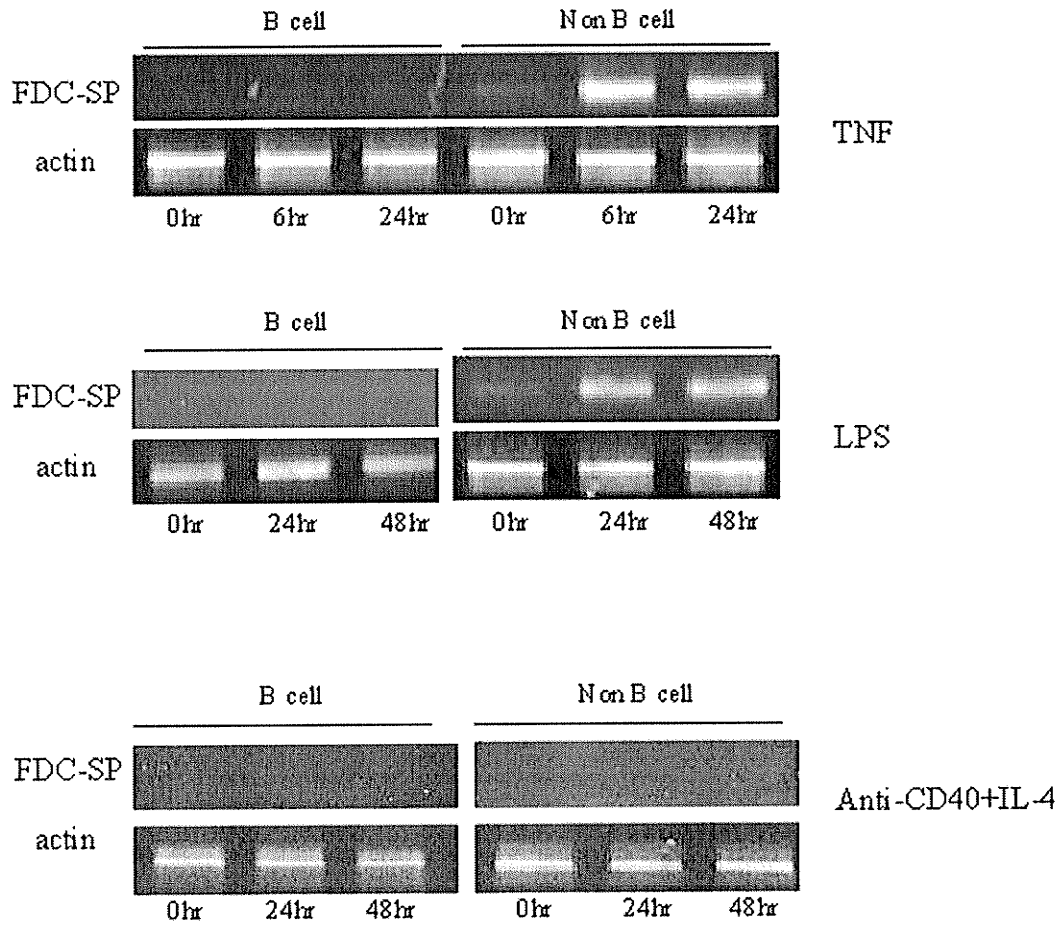


Fig. 8 FDC-SP is produced by non B cell population in mouse spleen. B cells were prepared by grinding the mouse spleen followed by lympholyte M centrifugation and MACS. Non B cell population was also harvested after MACS. Cells were used freshly or treated with different stimuli and harvested at indicated time points. RNA was extracted and RT-PCR was carried out. Note that under none of the conditions do B cells produce FDC-SP. Non B cell population responded to TNF or LPS stimulation but not Anti-CD40+IL-4.

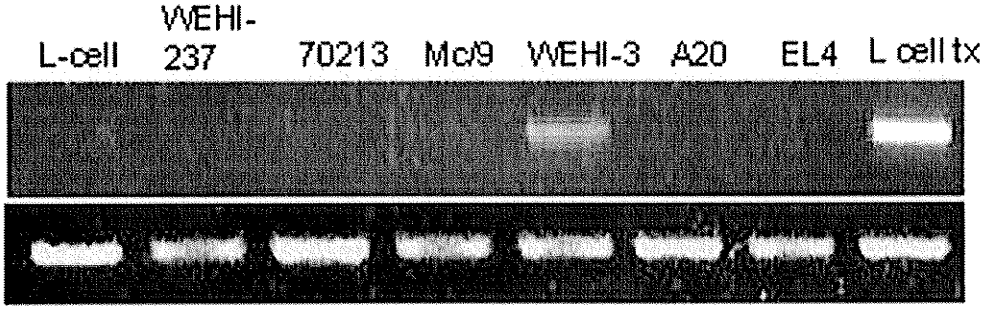


Fig. 9 FDC-SP expression in different mouse cell line. RNA was extracted from different cell line and RT-PCR was carried out to detect FDC-SP expression. Stable L cell FDC-SP transfectant was used as a positive control. Note that WEHI-3 cell line expresses low level (12 fold less than positive control) of FDC-SP compare to stable L cell transfectant. L cell: fibroblast; WEHI-231: B cell line; 70213: pre B cell line; Mc/9: mast cell line; WEHI-3: macrophage cell line; A20: b cell line; EL4: B cell line; L cell tx: stable L cell transfectant.

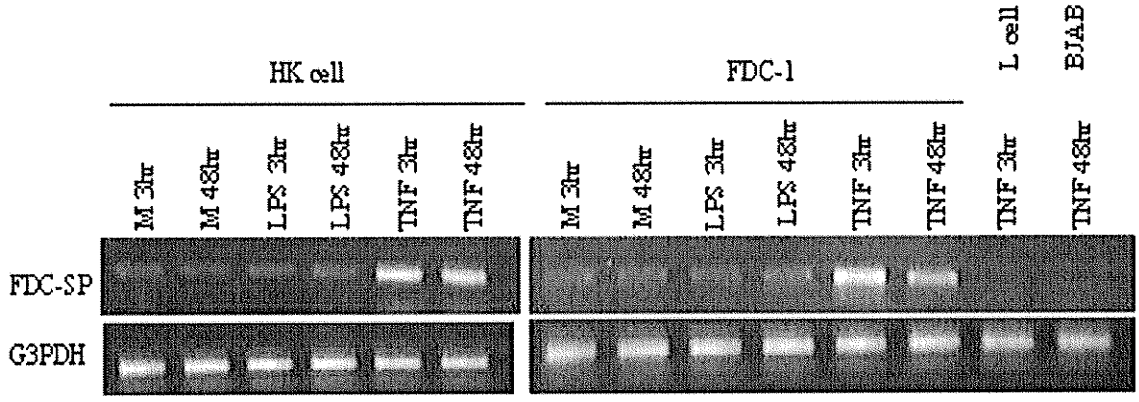


Fig. 10 FDC-SP is expressed by FDC like cell lines under different condition. FDC like cell lines, B lymphoma and fibroblast cell line were treated with 10ng/ml TNF or 100ng/ml LPS and harvested at different time point. FDC-SP expression was examined by RT-PCR. Note that FDC-SP expression is drastically upregulated in FDC like cell lines after 3 hr of TNF treatment.

2, Generation of reagents for functional characterization of FDC-SP

The next step of my study on FDC-SP is to define its physiological function. To fulfill this task, a sufficient amount of FDC-SP had to be obtained. I decided to generate two forms of recombinant FDC-SP: GST fusion protein, and myc-tagged FDC-SP expressed by stably transfected L cell clones. The former could be purified in large quantity from bacterial culture and the later has a lower production but because it is produced in mammalian cell, it should be more correctly processed after translation and thus resemble the natural FDC-SP better.

2. A, Generation of recombinant FDC-SP

To generate GST-FDC-SP fusion protein, I first amplified FDC-SP cDNA by PCR, using primers containing restriction enzyme recognition sequences. Two restriction enzymes BamHI and XhoI were included into the two ends of the primers. The amplified fragments can be subsequently cloned into PGEX-5X-2 vector via Bam HI and XhoI on the multicloning site immediately after GST coding sequence. The resulting vector contains a Factor Xa recognition sequence immediately before FDC-SP (Fig.11) which can be used to release free FDC-SP by digesting the fusion protein with Factor Xa. The expression vector was transformed into E. coli. The host cell was inducted with IPTG to express the fusion protein. The expressed protein was absorbed to glutathione beads and eluted out with glutathion. Eluted protein were visualized on SDS-PAGE gel (Fig. 12)

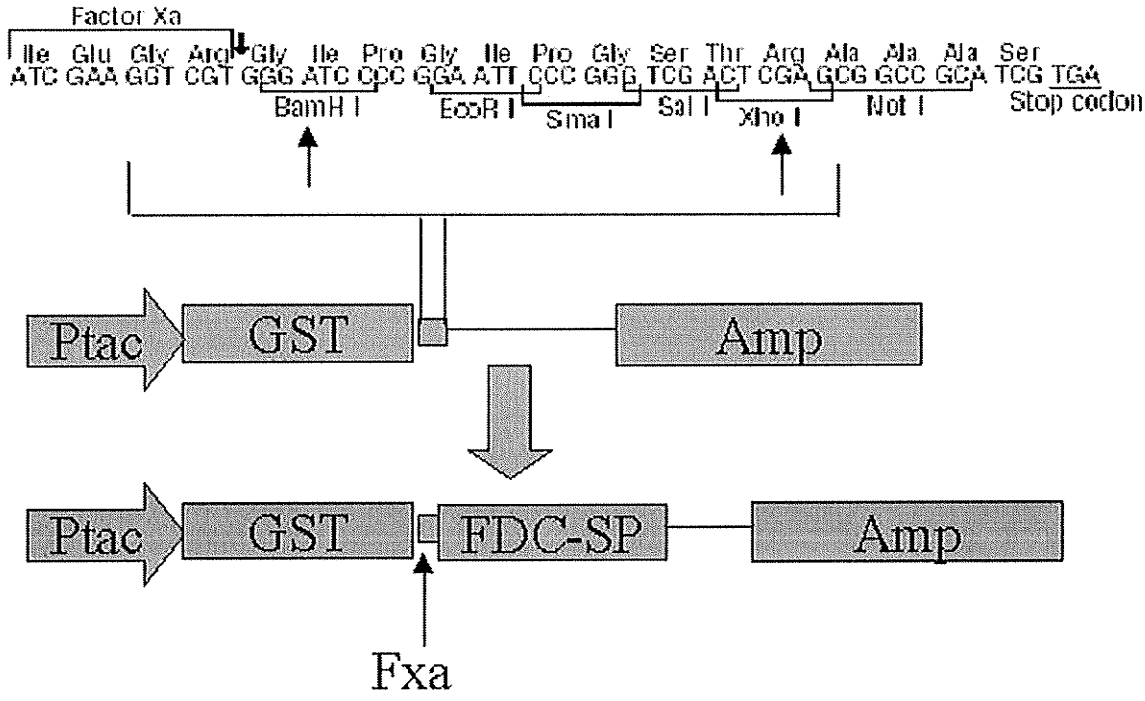


Fig. 11 Construction of GST-FDC-SP fusion protein expression vector.

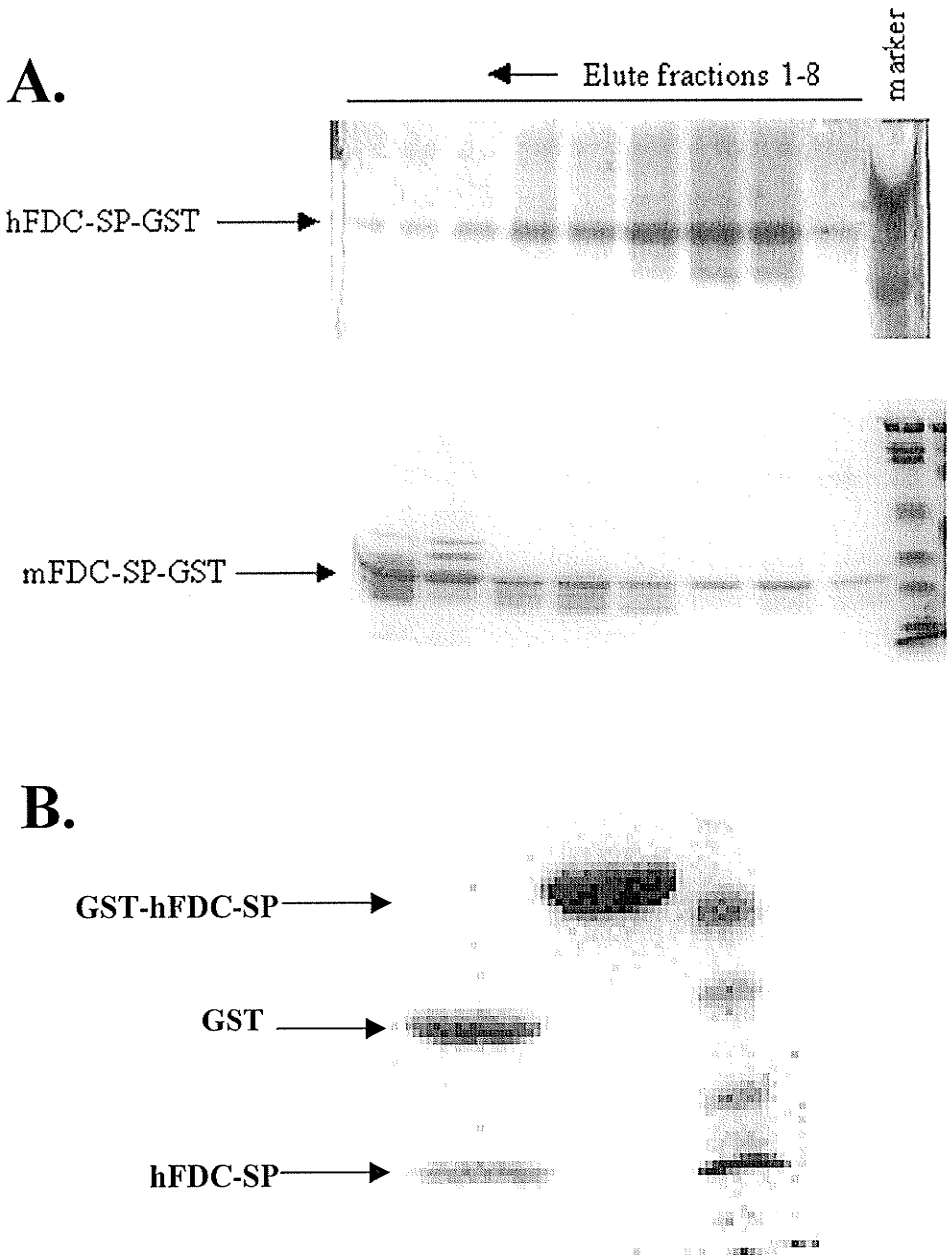


Fig. 12 SDS-PAGE analysis of purified human and mouse FDC-SP-GST fusion protein. Samples were mixed with an equal volume of 2× denaturing loading buffer, boiled for 5 Min, ran on a 4% stacking and 12% separation gel at 100 V, 4.5 hr. Gels were then stained with Coomassie Blue to visualize proteins. A. Sequential elution fractions. B. Factor Xa cleaved sample.

2. B, L cell clones were established to stably express myc-tagged FDC-SP

Although FDC-SP GST fusion protein can be acquired in large quantity and can be used for functional analysis, it first needs to be cleaved out to release free FDC-SP. This make it difficult to detect its binding to cell surface. In addition, proteins expressed in mammalian cells may have been properly glycosylated and thus may have better physiological function. So I decided to establish L cell clones expressing myc-tagged FDC-SP as another source of FDC-SP. As showed in figure below, FDC-SP was cloned into expression vector of PcDNA3.1 (Fig 13), under the control of a strong mammalian promoter Pcmv. FDC-SP is inserted right before a myc epitope and a 6× his epitope so that the expressed protein can be easily detected by antibodies against these two C-terminal epitopes which are commercially available. Linearized expression vectors were transfected into L cell, a mouse fibroblast cell line and stably integrated clones were selected using G418. Clones were checked for FDC-SP expression using RT-PCR, and I found 4 clones of both mouse and human that expressed high level of FDC-SP mRNA (Fig.14).

3, FDC-SP binds to activated B cell surface

As has been reported previously, FDC-SP is a secreted protein. It has a highly restricted and inducible expression pattern. All these prompted me to hypothesize that this secreted protein may function through binding to target cells through a surface receptor in its anatomical location of production. The main cell types involved in the germinal center reaction are FDCs, T cells, B cells and macrophages. Since FDCs are known to play a very important role in regulating B cell function through both surface molecules and

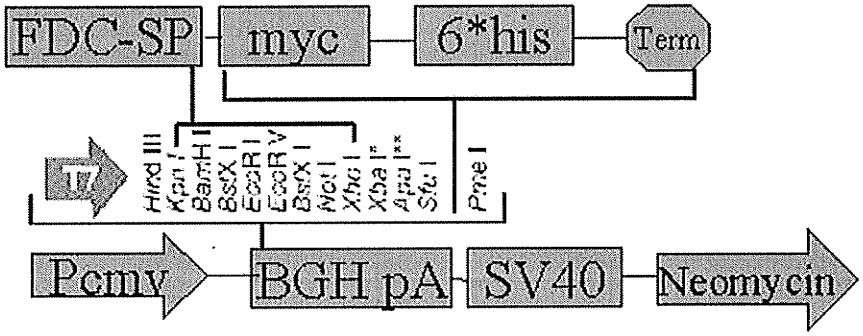


Fig. 13 Construction of mammalian vector expressing myc-tagged FDC-SP

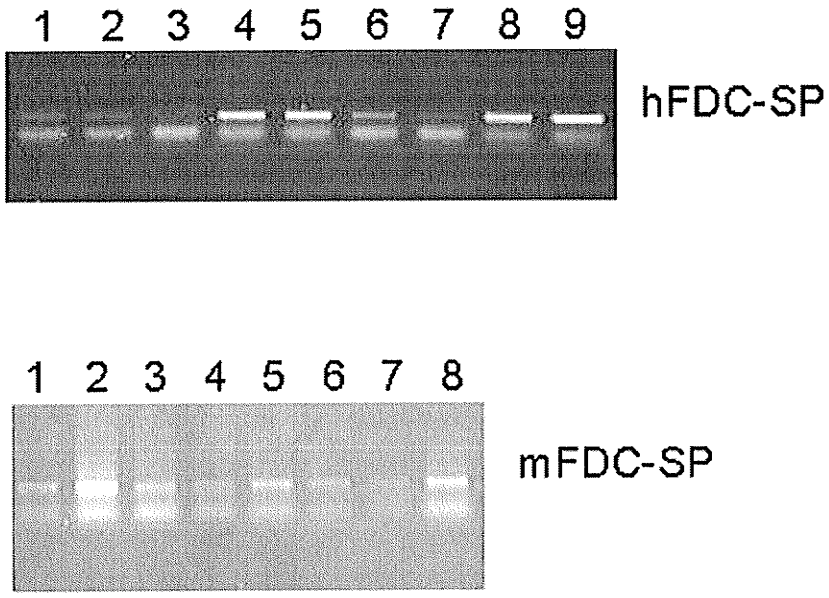


Fig. 14 Detection of mouse and human FDC-SP expression in stable L cell transfectants. L cell clones grown from single cell was grow up in DMEM containing 9% FCS, 1% PS. RNA was extracted from 2 million cells and RT-PCR was carried out. Note that FDC-SP expression level is different among different clones.

soluble mediators during germinal center formation(Hollmann and Gerdes, 1999; Tew et al., 1997), B cells are a likely target of FDC-SP binding. I decided to first test whether FDC-SP can bind to B cells or T cells by using two cell lines BJAB and Jurkat, which are B cell and T cell lymphomas respectively. To do this, I used stable L cell transfectants expressing myc-tagged human or mouse FDC-SP as source of FDC-SP. I used a co-culture system by making use of the adherent nature of L cell line. Target cells were added to L cell culture after it has grown to confluent. After 18 hrs of co-culture, the non-adherent target cells are carefully removed and FDC-SP binding is detected by FACS analysis after cells are stained with FITC-labeled anti-myc antibody. In accordance to our hypothesis, I found that BJAB cell showed significant ability to bind FDC-SP, whereas the Jurkat cell line shows no signs of binding (Fig. 15).

Next I decided to use primary cells to further prove this binding. To our surprise, fresh or un-stimulated cultured human PBMC showed no significant binding to FDC-SP. Since B cells in germinal center are activated blasts, I decided to test the binding again after stimulating these cells with anti-CD40, which is a critical activating signal delivered to B cell from T cells and is critical for germinal center initiation(Kawabe et al., 1994; Koopman et al., 1997; Xu et al., 1994) A sub-population of cells of about 30 percent showed binding to FDC-SP after this stimulation. Double color staining with anti-myc and anti-CD19, which is a B cell marker (Pesando et al., 1989; Stamenkovic and Seed, 1988), showed clearly that the population that binds to FDC-SP are B cells (Fig.16). This result was confirmed in mouse system (Fig. 17).

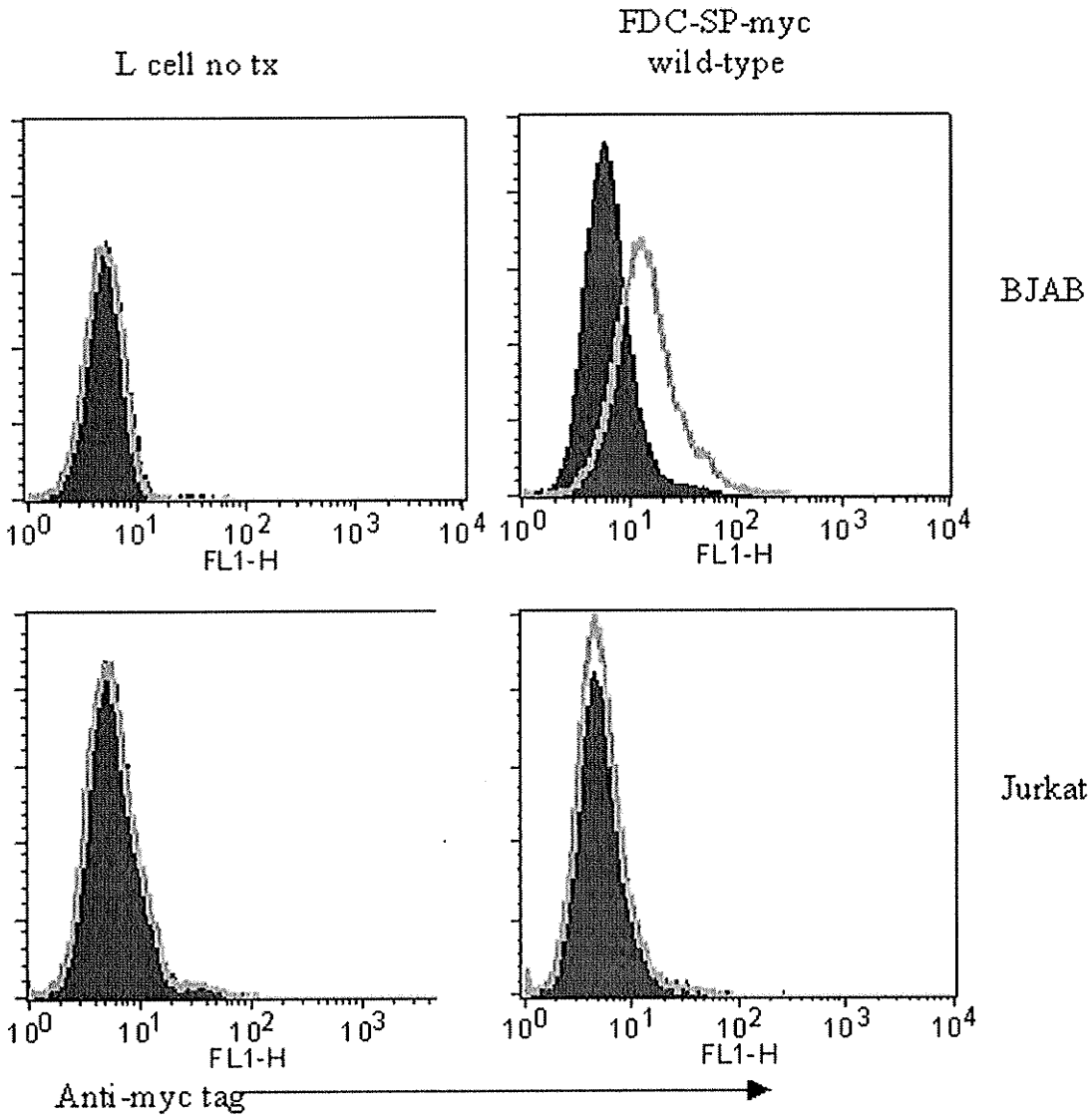


Fig. 15 FDC-SP binds to the surface of B, but not T lymphoma cells. B or T lymphoma cells were co-cultured with mouse fibroblasts (L cells) stably transfected with myc-tagged human FDC-SP proteins. Non-adherent cells were harvested after 18 hours of co-culture and analyzed for myc-tagged protein at the cell surface by staining with FITC-labeled anti-myc mAb Green traces indicate anti-myc staining, while blue filled traces represent staining with the isotype control mAb. Residual L cells were excluded from the analysis by side scatter gating. Note that BJAB cells, but not Jurkat cells, show binding to FDC-SP. Each figure is representative of 3 experiments.

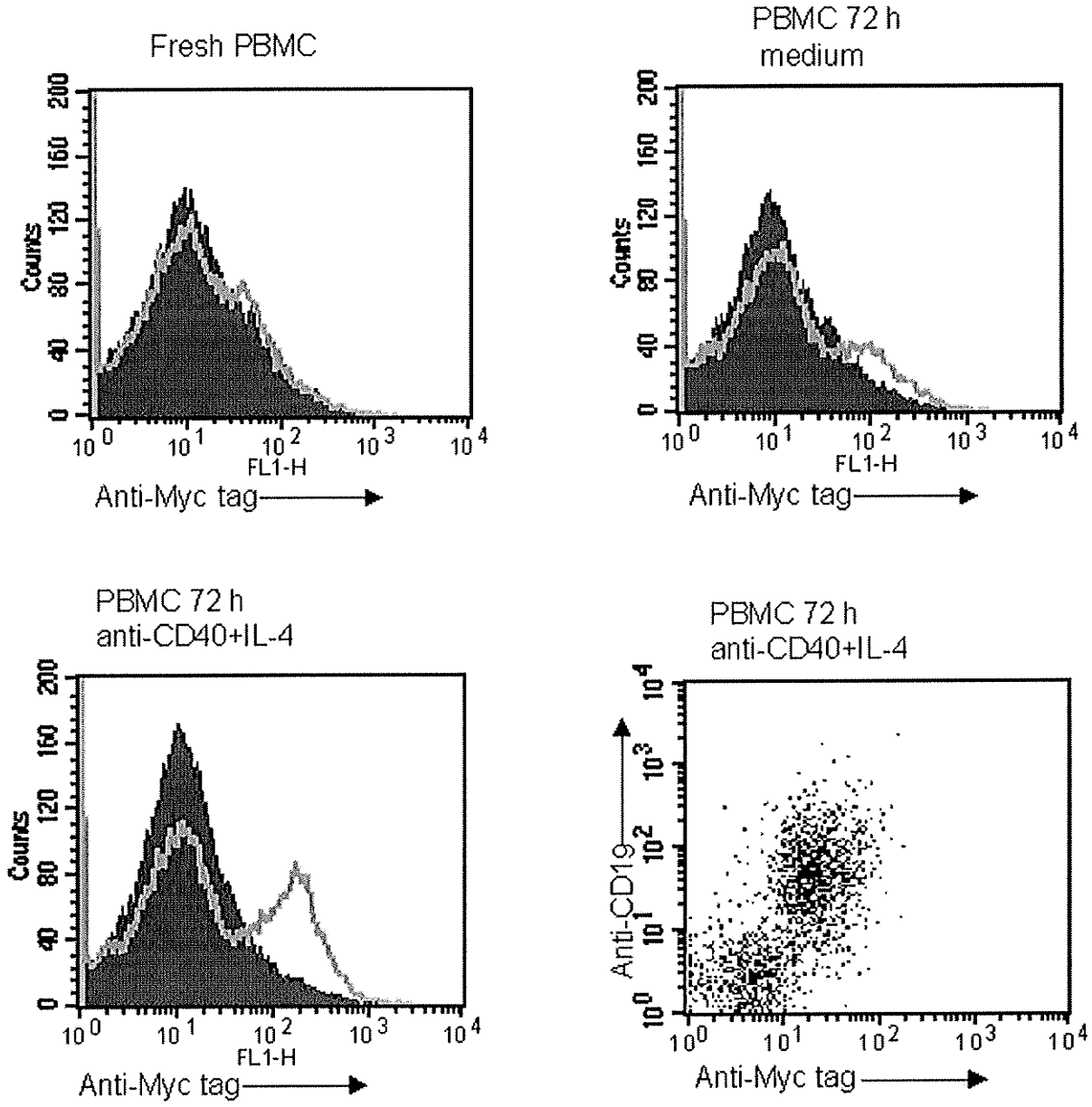


Fig. 16 FDC-SP binding to peripheral blood B cells is enhanced by T-dependent activation signals. Human PBMC were cultured for 3 days in medium or with anti-CD40+IL4 and then co-cultured with FDC-SP-myc L cell transfectants for 18 hours prior to analysis. **A.** Cells were stained with FITC-anti-myc or isotype control and analyzed by flow cytometry. **B.** After 3 days of culture with anti-CD40+IL-4, PBMC cells were stained with FITC-anti-myc and PE-anti-CD19.

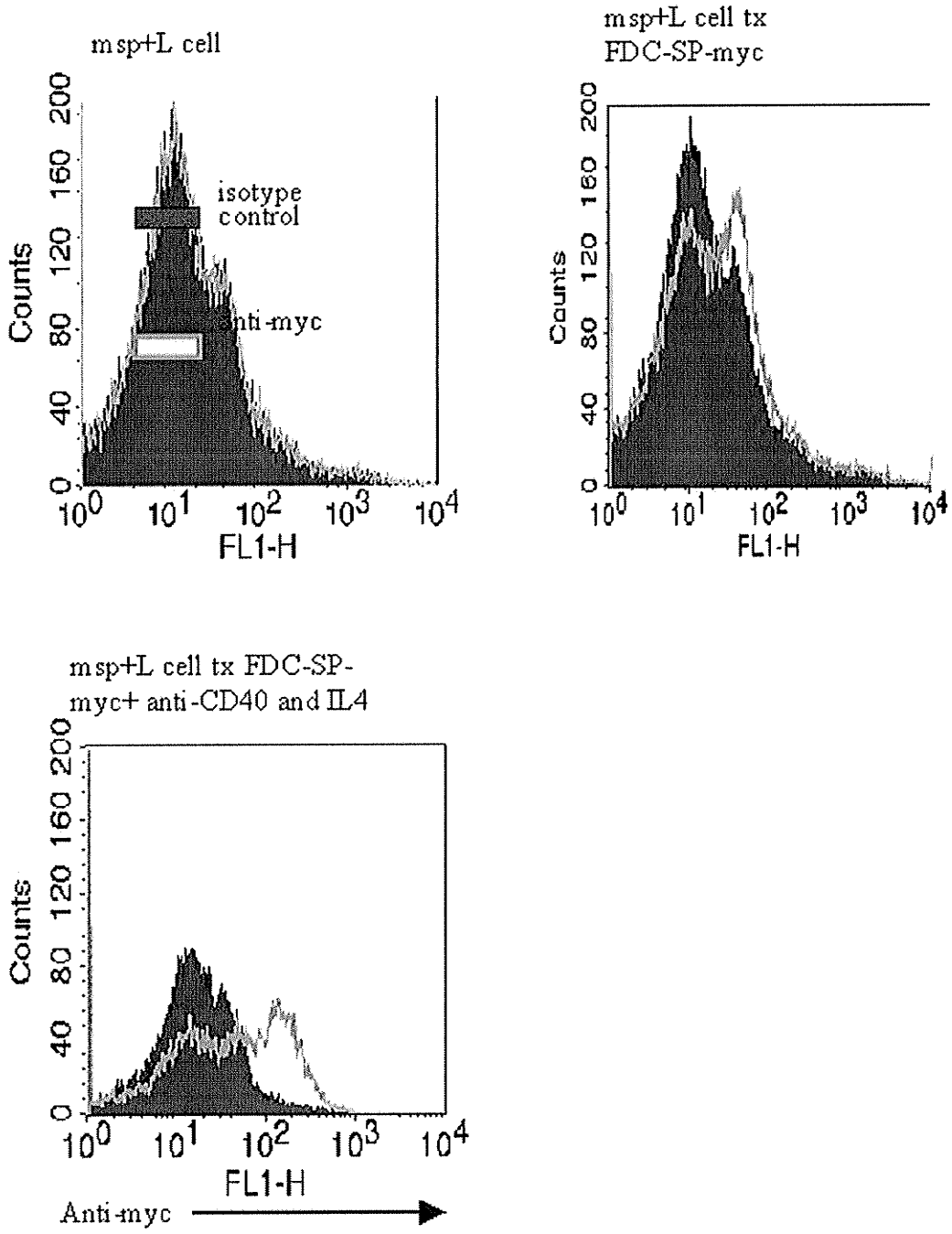


Fig. 17 FDC-SP binding to mouse spleen cells is also enhanced by CD40+IL-4 activation. Mouse spleen cells were cultured and stimulated as above before cells were stained with FITC-anti-myc or isotype control and analyzed by flow cytometry. These experiments has been repeated for 3 times.

I further tested this binding using purified B cells and showed similar binding pattern. (Fig. 18).

4, Effect of FDC-SP on B cell proliferation

To investigate the functional role of FDC-SP, I first checked its possible influence on B cell proliferation, which is a prominent feature for B cells in germinal center. To do this, I used the established co-culture method in mouse system. Purified mouse spleen B cells were co-cultured with L cell or stable L cell transfectant with or without the presence of anti-CD40. Proliferation was assessed by a standard 3H-thymidine uptake assay. In a series pre-experiments, I find that the most significant proliferation of B cells appeared at day 3 and thereafter. The incorporation of 3H-thymidine has reached a plateau 6 hours after it was added into the culture. So I used these conditions as standard in subsequent assays. I found that in the presence of FDC-SP, B cells proliferate reproducibly better than the group without FDC-SP. Although the difference is not very large, it is consistently around 15% in over 8 experiments and it is statistically significant ($P<0.05$). FDC-SP alone does not appear to promote proliferation, but has a synergistic effect with anti-CD40. Particularly, the effect of FDC-SP on B cell proliferation is more pronounced when anti-CD40 is removed after 2 days of stimulation, and proliferation is measured on day 3-4 in the continued presence of FDC-SP. (fig 19). A very similar proliferation pattern is acquired with total mouse spleen cell.

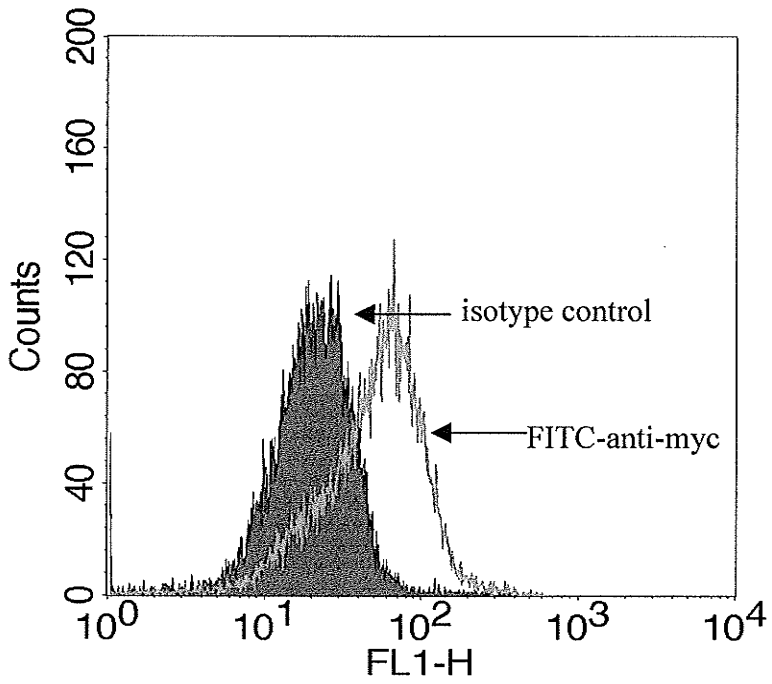


Fig. 18 FDC-SP binds to purified B cells Purified B cells were cultured for 3 days in medium or with anti-CD40+IL4 and then co-cultured with FDC-SP-myc L cell transfectants for 18 hours prior to analysis.

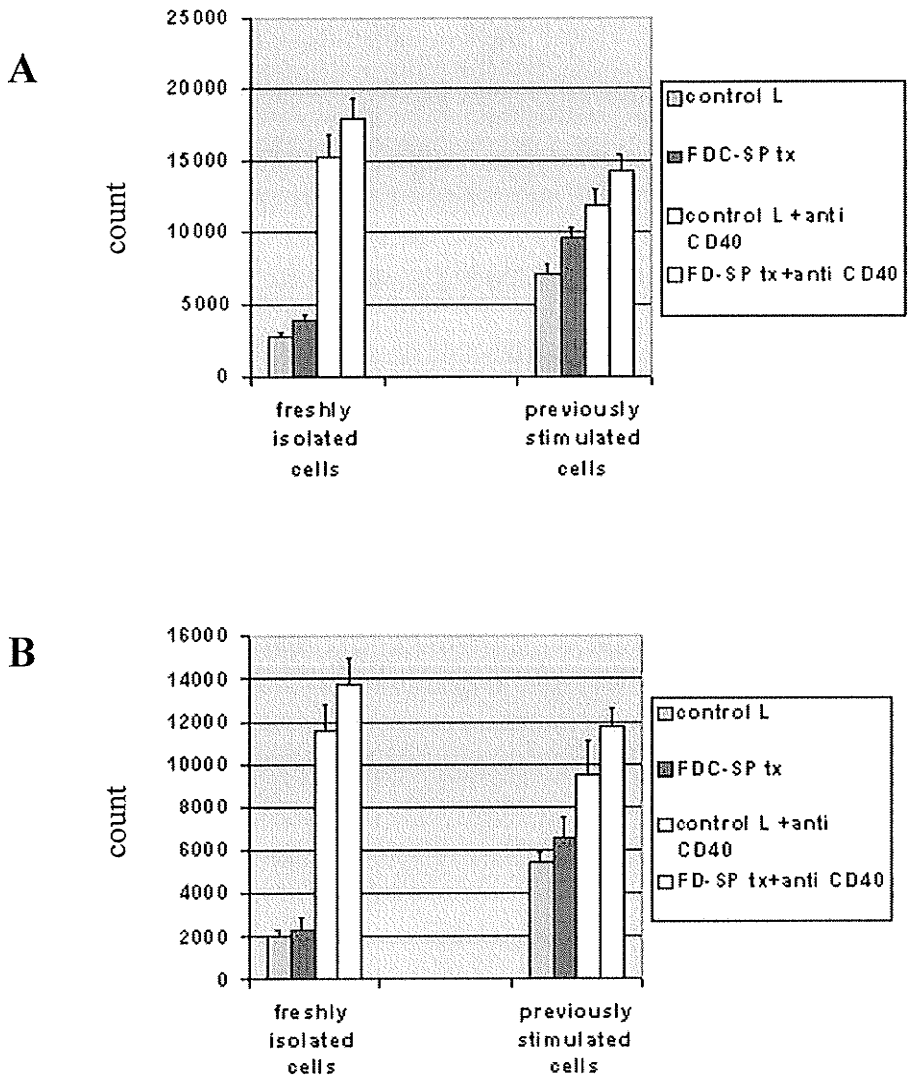


Fig. 19 FDC-SP stimulate B cell proliferation synergistically with Anti-CD40. Purified B cells (A) or whole spleen cells (B) were co-cultured with control or FDC-SP transfected L cell clones with or without anti-CD40 antibody, pulsed with H^3-thymidine at day 2 for 6 hours. Cells were harvested and analyzed on a scintillation counter. Previously stimulated group: purified B cell or spleen cells were pretreated with anti-CD40 antibody for 2 day, washed, then co-cultured with L cell clones for a further 2 days with or with out anti-CD40 before pulse with H^3-thymidine. Each figure is representative of 8 experiments.

5, Chemotactic activity of FDC-SP

The PI (PH at isoelectric point) of FDC-SP is 7.3. Its secreted peptide composes of 70 AA and contains a quite polar N terminal. These properties are quite similar to that of chemokines like IL-8 and BLC, although it does not contain signature cysteine residues. The FDC-SP gene is located 3.6 Mb away from this cluster of chemokines which represents a relatively close linkage on chromosome 4q13. These properties lead me to raise the hypothesis that FDC-SP may also have similar chemotactic effect on its target cells. To test this hypothesis, I used an established transwell system to detect chemotactic movements of B cells. In these experiments, chemotactic factors were placed in the lower chamber of a transwell, which is separated from a upper chamber by a 5 um pore size filter. B cells were added to upper chamber and the percentage of migrated cells from upper chamber into lower chamber was counted after different time of incubation. I find migration reached its peak after 3 hours of incubation, which is then used as standard incubation time for subsequent chemotaxis assays. BLC was used as a positive control, because BLC is so far found the strongest chemokine to attract B cell to lymphoid follicles. I used both recombinant FDC-SP-GST fusion protein and the L cell transfectant co-culture system in the chemotaxis assay to check if FDC-SP has any chemotactic effect. B cells were purified from mouse spleen and used freshly or after stimulation with anti-CD40. FDC-SP GST fusion protein was pre-treated with Factor Xa to release free FDC-SP.

5. A, Establishment of FDC-SP chemotaxis

Using both GST fusion protein and L cell transfectants as sources of FDC-SP, FDC-SP showed repeatedly a measurable chemotactic effect. Recombinant control GST fusion protein prepared by the same procedure did not cause significant migration, proving that the purification of the recombinant protein is free of other microbial products that may affect chemotaxis. The chemotaxis effect is comparable between the two system when the GST fusion protein dose is 500ng/ml. Particularly, in accordance with the binding pattern of FDC-SP to B cells, I find that B cells migrate towards FDC-SP much more significantly after they are pre-treated for 2 days with anti-CD40 plus IL-4. In these same experiments, B cells showed very strong migration towards BLC, typically 2-3 times higher than that for FDC-SP, the difference is also statistically significant ($p<0.05$). It seems that there are similar levels of B cell migration towards BLC whether or not pre-treated with anti-CD40plus IL-4 (Fig. 20).

In order to better define the chemotactic property of FDC-SP for B cells, I carried out a dose response study for both recombinant GST-FDC-SP and BLC. I find that FDC-SP showed a minimal chemotactic effect at a dose of 50ng, and the effect reaches a plateau at 1000ng/ml dosage. By the mean time, the effect of BLC reaches its plateau at a dose of 100ng/ml (Fig . 21). This dose effective dose range difference may be most likely caused by protein quantification differences between self-prepared recombinant protein and commercially acquired chemokine.

5. B, Factor Xa digested GST-FDC-SP has stronger chemotactic effect than intact fusion protein

FDC-SP is composed of a region of charged polar N terminal, a proline rich, low complexity middle region and a shorter C terminal region. Alignment of mouse and human counterpart showed almost identical N terminal region. This unique feature of structures suggest that the N terminal portion could serve as a binding motif when it comes into contact with its receptor. FDC-SP is fused through its N terminal to the GST part in the fusion protein which could spatially hinder the interaction between its N terminal and receptor. To test this, I compared the chemotactic properties of intact fusion protein and the Factor Xa cleaved protein, in which free FDC-SP is released. I find that as expected, uncleaved intact protein has much less ability to attract B cells compare to cleaved fusion protein.(Fig. 22) Also this result confirms the specificity of the chemotactic response and rules out any effect of contaminant proteins in the GST-FDC-SP preparation.

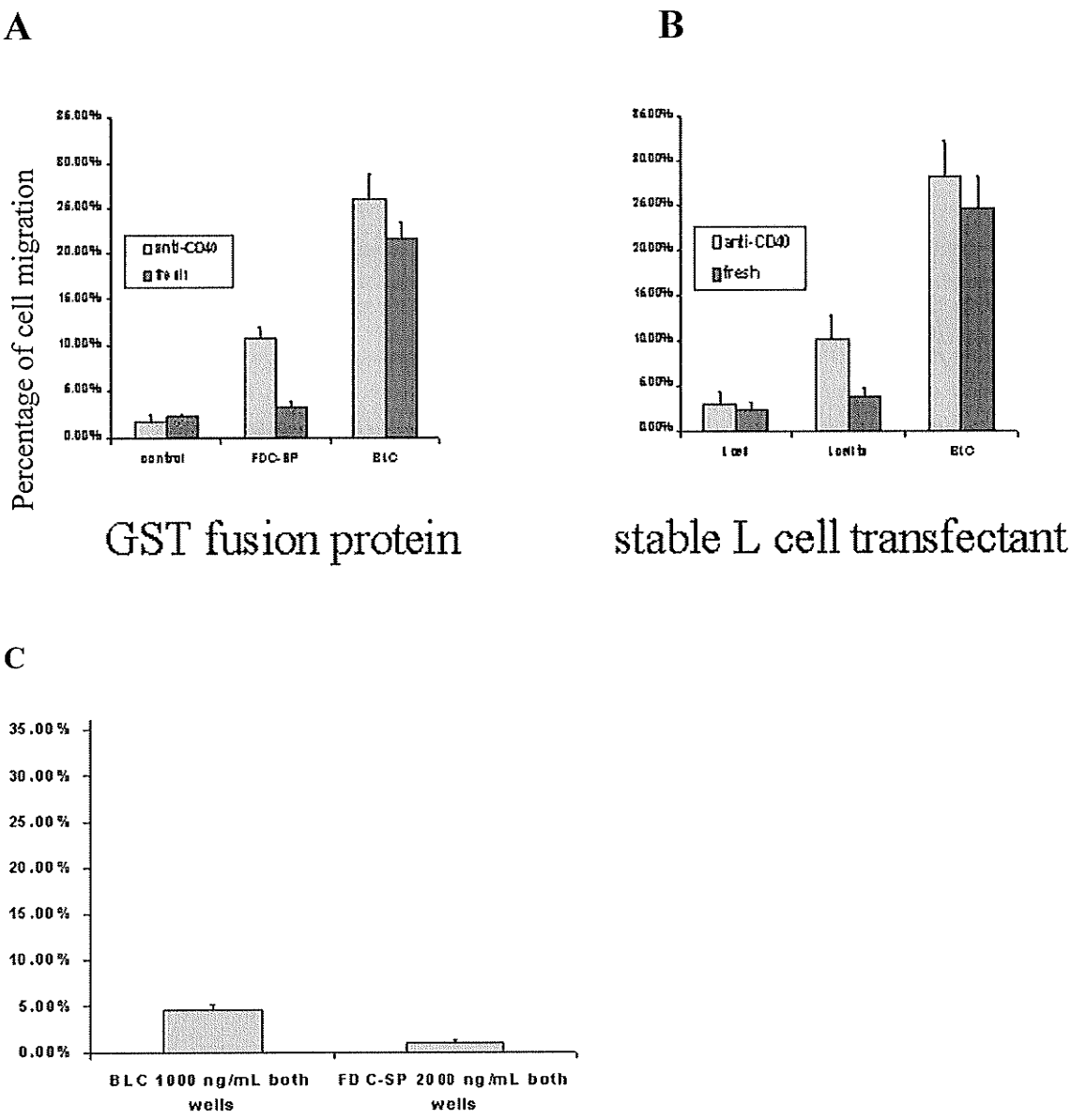
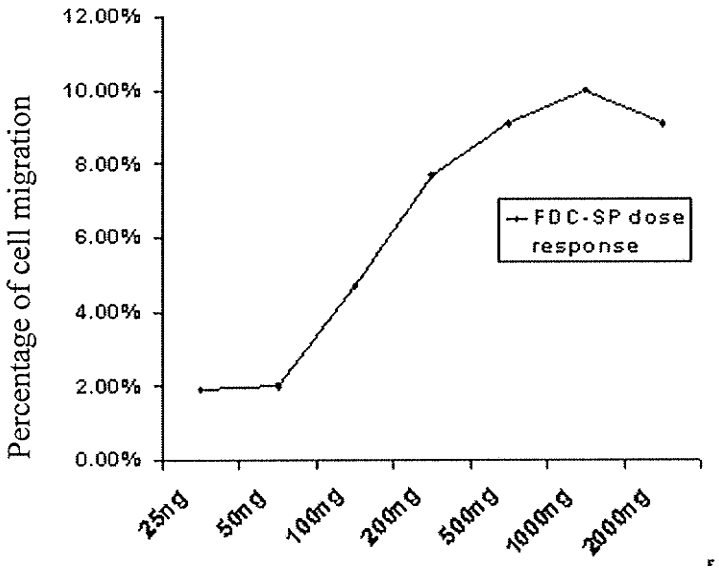


Fig. 20 FDC-SP is a chemoattractant for activated B cell. Purified B cells were used for chemotaxis assay either directly ex vivo or cultured for 2 days with 2ug/ml anti-CD40 plus 2ng/ml IL-4. RPMI 1640 containing 0.5% BSA was used as migration buffer. Migrated cells were counted by flow cytometry. **A,** Chemotactic effect of GST-FDC-SP fusion protein. The fusion proteins were treated with Factor Xa before assay to release free FDC-SP and the control protein. **B,** Chemotactic effect of supernatant from stable L cell transfectants. **C,** control, BLC or FDC-SP were added to both lower and upper chamber. L cell lines were grown in lower chamber of transwell plates to 80% confluent, washed 3 times with migration buffer, then cultured in migration buffer for another hours before carrying out chemotaxis assay. L cell tx: stable L cell transfectant expressing FDC-SP. These experiments has been repeated for 3 times.



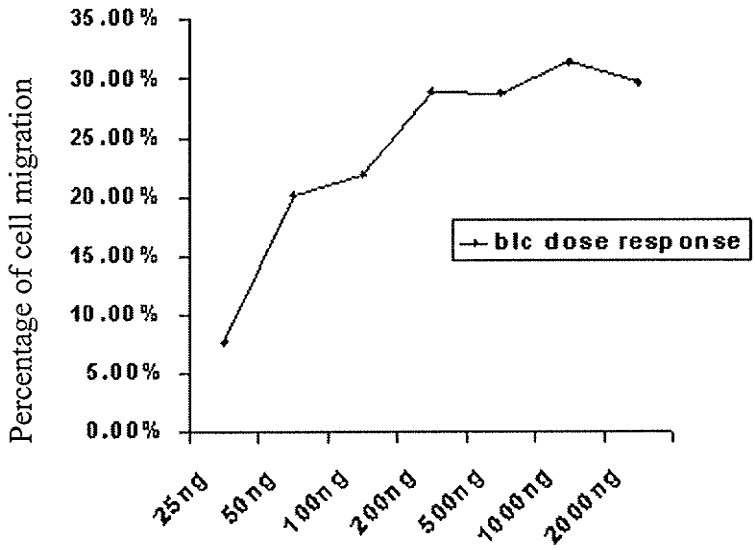


Fig. 21 Dose response of B cell migration for FDC-SP and BLC. Note that in each set of experiment there is about 1 to 2 percent of background migration caused by chemokinesis (based on other experiments) which has not been subtracted from the total migration number

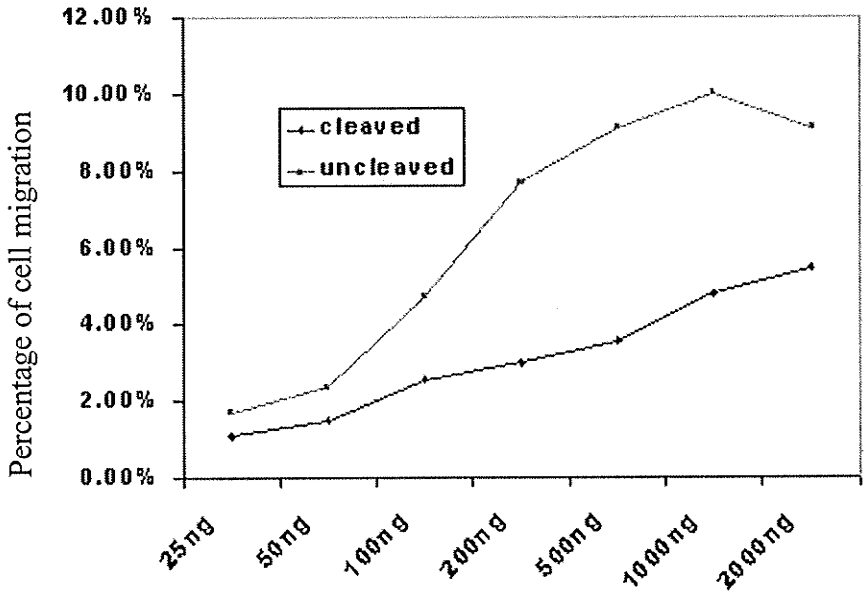


Fig. 22 Factor Xa digested GST-FDC-SP has stronger chemotactic effect than intact fusion protein. Chemotaxis assays were carried using intact or Factor Xa cleaved GST-FDC-SP fusion protein

5. C, FDC-chemotactic effect is blocked by Pertussis toxin

Since most chemotactic factors function through binding to a G protein coupled receptor on their target cells, I decided to test if this is also the case for FDC-SP. The kind of G protein coupled to most chemotactic receptors is Gi protein (Neptune and Bourne, 1997; Neptune et al., 1999). Its activity can be blocked by treating the cells with Pertussis Toxin. Therefore, the stimulated cells were treated with medium containing 1ug/ml of PT for 2 hrs at 37 C before chemotaxis assays were carried out. The results show that the chemotactic effect is totally abolished after PT treatment for both FDC-SP and BLC group, indicating that FDC-SP may also function through a G protein coupled receptor (Fig. 23).

5. D, Regulation of FDC-SP chemotaxis by activation stimuli

The migration properties of lymphocytes are often quite dynamic during an immune response, so that they can interact with different types of cell involved at different stage. It is important to know the kinetics of B cell response to FDC-SP at different stages of activation and how do B cells respond to different stimuli, either alone or in combination. I, therefore, examined in detail the regulation of FDC-SP chemotaxis by different activation stimuli and compared this to BLC.

I first examined B cell migration at different time points after B cells were stimulated with anti-CD40 alone. I find that B cells reach their highest ability to migrate towards FDC-SP after 2 day's of CD40 stimulation and retain this high ability after 96 hrs of stimulation (Fig 24), IL-4 does not seem to be required. There is a 5 to 8 fold increase of

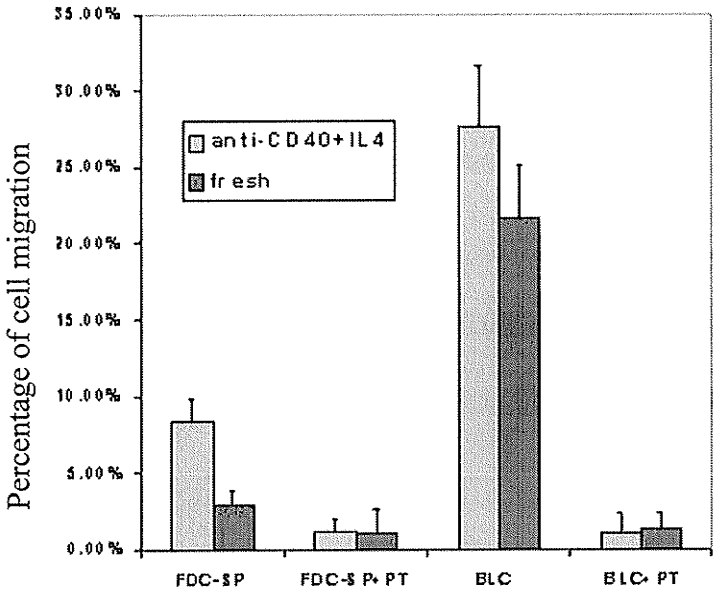


Fig. 23 FDC-SP chemotactic effect can be blocked by treatment of B cells with Pertussis toxin. B cells were either used directly or incubated at 37 °C for 2 hr with the presence of 1ug/ml Pertussis Toxin. B cells were then washed 3 times with migration buffer before transwell assays were carried out. This figure is a representative of 3 experiments.

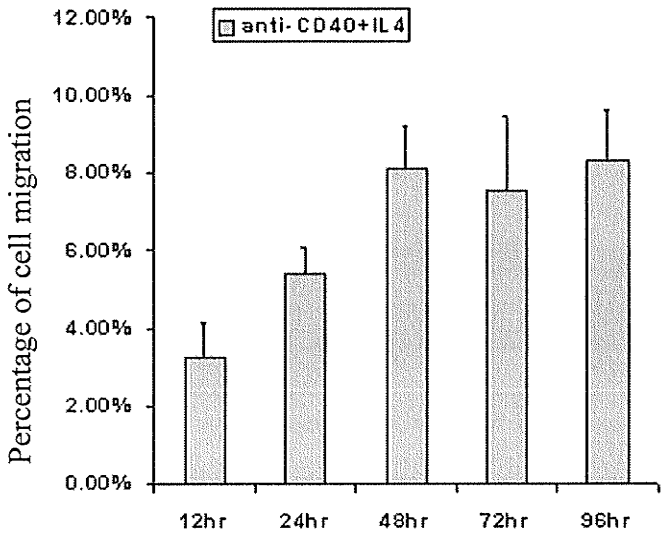


Fig. 24 B cell migration towards FDC-SP after different time of CD40 stimulation

migration towards FDC-SP after CD40 stimulation. In contrast, there is only about 1.2 fold increase in migration towards BLC after CD40 stimulation (Fig. 25)

Next I examined whether B cell change its responsiveness to FDC-SP after further BCR engagement which may naturally occur before, during or after CD40 stimulation from T cells. To test this, B cells were stimulated with anti-CD40 for 2 days followed by three washes with medium. Cells were cultured for another 24 hrs in medium with or without the presence of anti-IgM. I found that cells cultured in medium alone after washing off anti-CD40 still retained most of their ability to migrate towards FDC-SP. However, cells cultured in medium containing anti-IgM lost most of this ability. Interestingly, B cell migration towards BLC was also inhibited after further IgM engagement, but to a much less extent (Fig 26, 27).

To determine how does B cell may respond to FDC-SP under other physiological situations, I treated B cells with different stimuli and carried out chemotaxis assay. I found that neither LPS or IL-4 stimulation has major effect on B cell response towards FDC-SP. As expected from the results above, BCR stimulation does not activate FDC-SP chemotaxis on its own, and can partially inhibit when given in combination with anti-CD40. B cell migration towards BLC didn't change either in these experiments (Fig. 28~29)

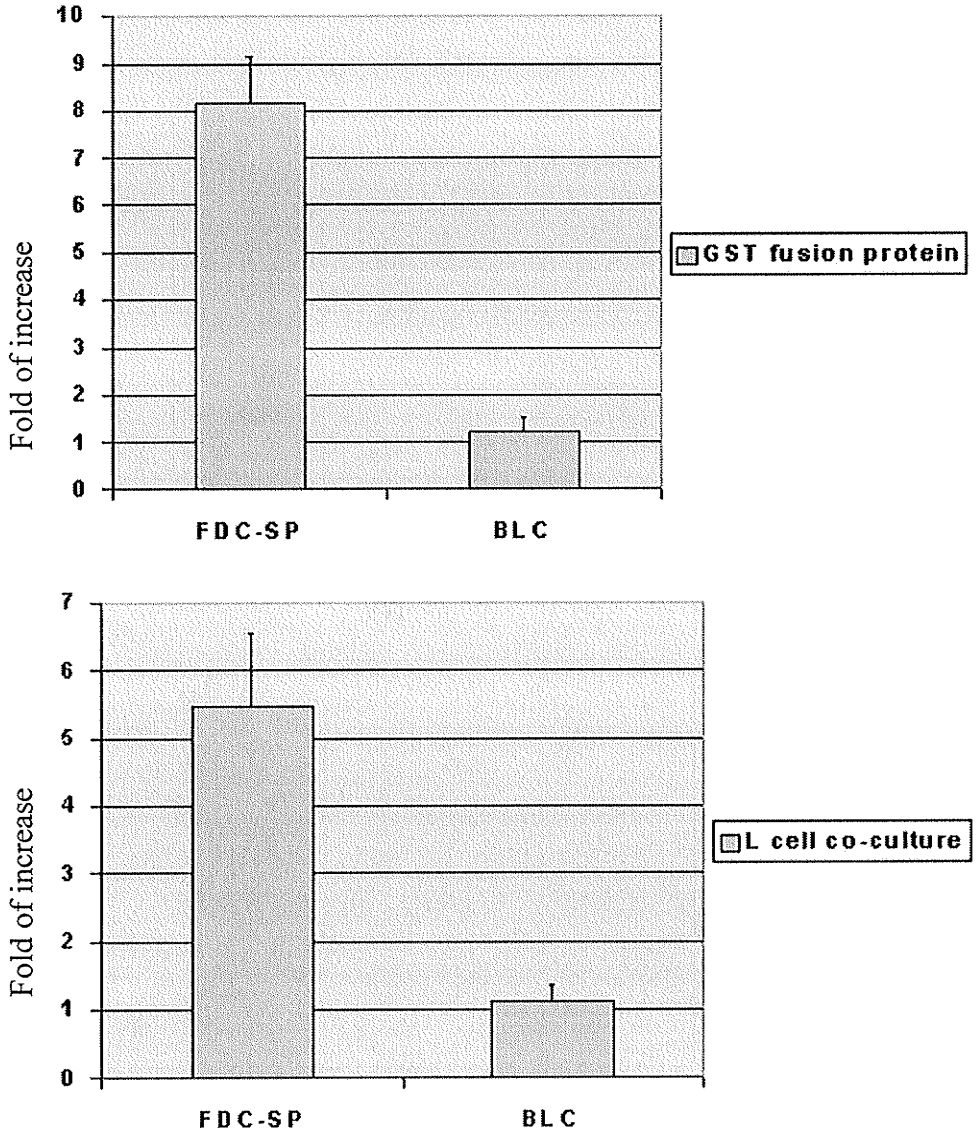


Fig. 25 Fold of increase of B cell migration after anti-CD40 stimulation. The numbers are calculated as a ratio between the migration rate of stimulated and unstimulated B cell preparations after deducting the migration rate of respective control group.

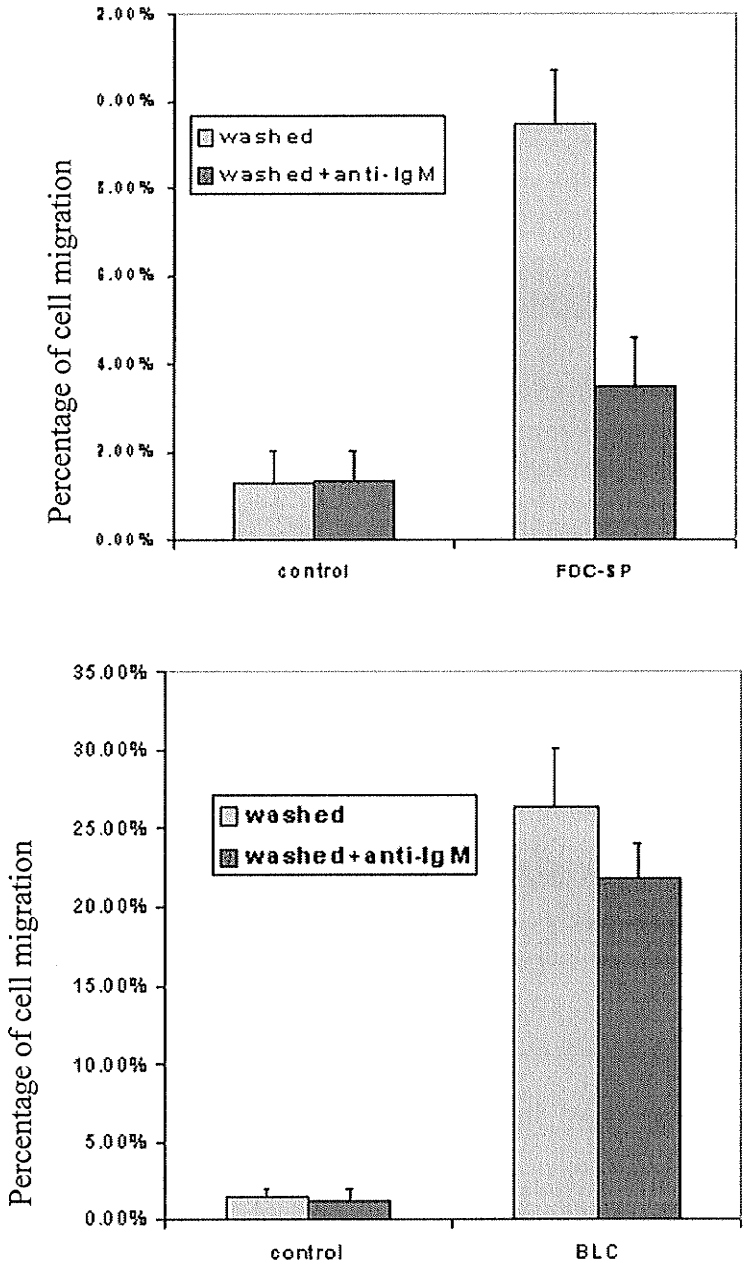


Fig. 26 Further BCR engagement reduce the responsiveness of pre-activated B cell to FDC-SP and BLC. Purified B cells were stimulated for 2 days with 2ug/ml anti-CD40 followed by 3 washes of RPMI 1640 medium. Washed cells are cultured for another 12 hr at 37c with or without 2ug/ml anti-IgM before used for chemotaxis assay A, Chemotaxis assay with FDC-SP. B, chemotaxis assay with BLC.

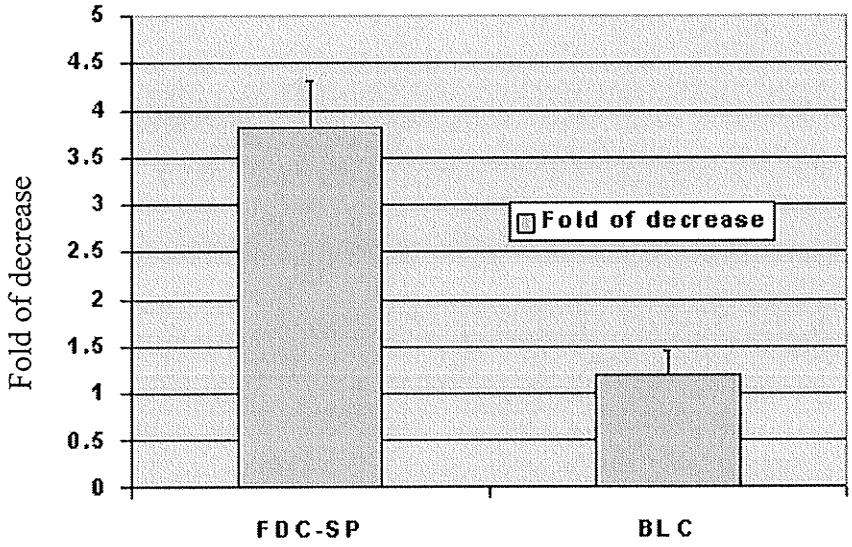


Fig. 27 Fold of decrease in migration after further BCR engagement The numbers are calculated as a ratio between the migration rate of anti-CD40 pre-stimulated B cell preparations with or without further IgM stimulation after deducting the migration rate of respective control groups.

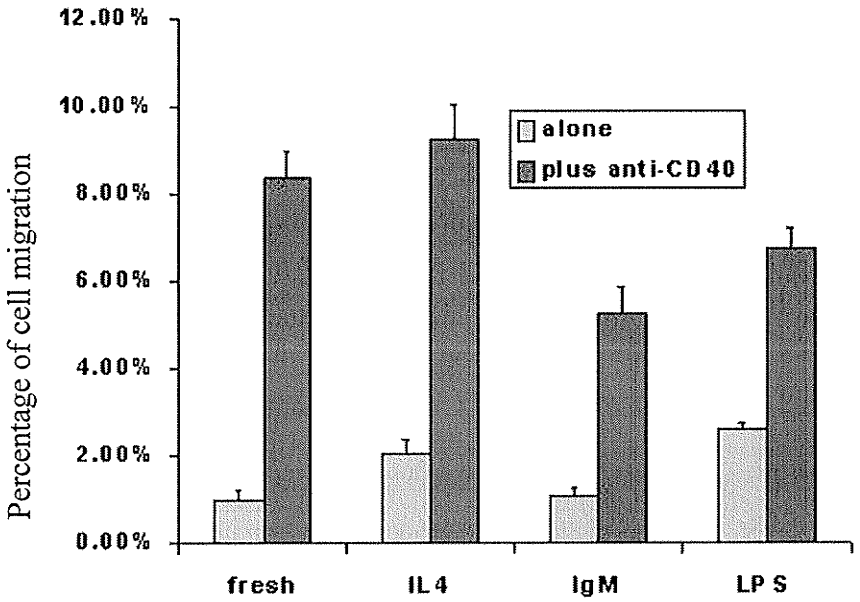


Fig. 28 IL-4, anti-IgM or LPS alone or in combination with anti-CD40 has no significant influence on B cell migration towards FDC-SP. B cells were treated with 2ng/ml IL-4, 2ug/ml anti-IgM or 100ng/ml LPS alone or combined with 2ug/ml anti CD-40 for 2 days before carrying out chemotaxis assay

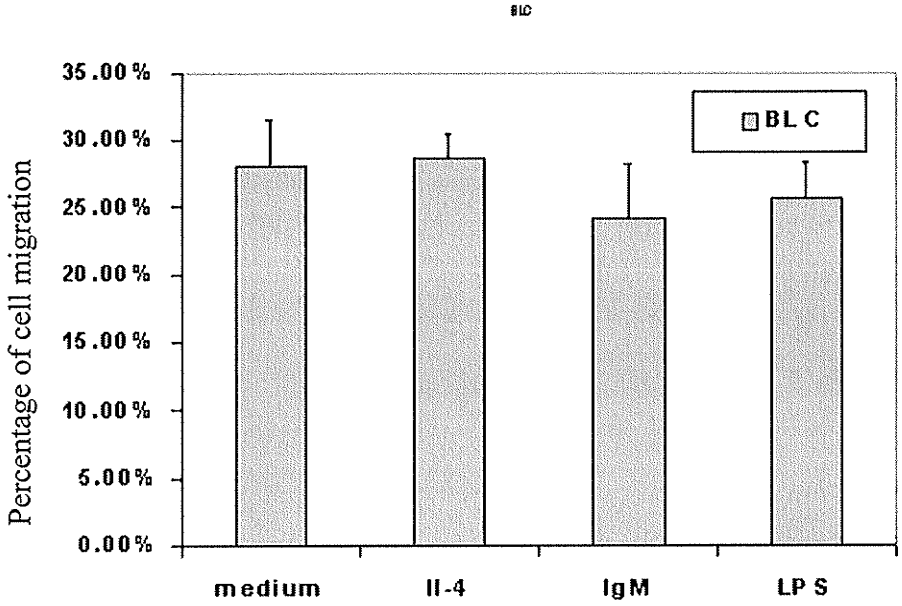


Fig. 29 IL-4, anti-IgM or LPS has no significant influence on B cell migration towards BLC.

5. E, FDC-SP works synergistically with BLC

Since both FDC-SP and BLC are produced by FDC to attract B cells, it is important to determine how these two chemoattractants might coordinate in vivo. To test this, I combined these two proteins together in the chemotaxis assays. I find that without anti-CD40 stimulation, there is no difference between the response to BLC alone or the two together. If B cells were pre-stimulated with anti-CD40, the migration rate of B cell towards a combination of sub-optimal dose of both BLC and FDC-SP is significantly higher than migration towards either of them alone. Especially, there is no significant difference of migration towards BLC alone before or after anti-CD40 stimulation. This migration is also higher than the simple adding up of the migration towards the two chemoattractant separately, and this difference has been tested to be statistically significant. Suggesting that these two factors may synergize to specifically attract B cells pre-activated by T-dependent signal anti-CD40. (Fig. 30). Although there are many reports on the synergism effect of different cytokines, there are very few reports of synergism between chemokines so far, making it difficult to make a comparison to synergistic effect of other chemokines.

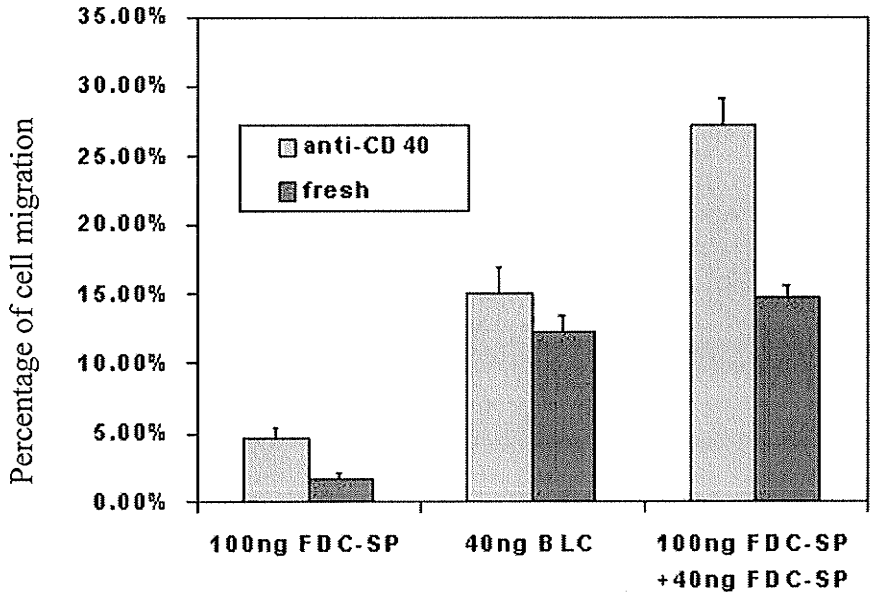


Fig. 30 FDC-SP works synergistically with BLC at sub-optimal dose. Purified B cells were stimulated with 2ug/ml anti-CD40 for 2 days. Chemotaxis assay was carried out using a combination of 100ng/ml FDC-SP and 40 ng/ml BLC in the lower chamber. This figure is representative of 4 experiments.

DISCUSSION

Regulation of FDC-SP expression by FDCs

The fact that TNF can upregulate FDC-SP expression in FDC correlates very well with the fact that CD40 activated B cell can express high level of surface TNF family molecules and induce major phenotypic changes of FDC which is characteristic during germinal center formation(Endres et al., 1999). FDC-SP expression is only upregulated by anti-CD40 stimulation in mouse spleen cells prepared by enzyme digestion method, but is absent from spleen cells from physical disruption method. The difference of the two method is that the former can preserve viable FDCs by not destroying their fragile dendrites. I have shown that both B cell and non-B cell population from mouse spleen cells prepared by physical disruption method does not respond to anti-CD40 stimulation to produce FDC-SP. Since there has been no report of CD40 expression on FDCs, it is unlikely that anti-CD40 antibody could act directly on FDC. From these evidence we can infer that FDC-SP expression in mouse spleen cell prepared by enzyme digestion may be due to firstly activating B cells, which then express TNF family molecules on their surface, which then in turn may induce FDCs to express FDC-SP. To further exclude the possibility of FDCs responding to anti-CD40 stimulation directly, it might be helpful to treat FDC cell lines with anti-CD40 and check FDC-SP expression in the future. I have also shown that FDC cell lines do not respond to LPS stimulation to express FDC-SP, however it might be interesting to test whether there is any change in FDC-SP expression after trapping immune complexes on its surface through their Fc and complement receptor. The overall expression level of FDC-SP is low or not detectable in various unstimulated cells including FDC.

Regulation of FDC-SP expression in other cell types

Although FDC-SP is most obviously expressed by TNF treated FDC and within germinal centers, we can not neglect the evidence that it is also expressed by other cells in PBMC and infiltrated tonsil crypts. Its expression by these cells seems to be regulated in a quite different manner. PBMC and mouse spleen cells prepared by physical disruption does not increase FDC-SP expression in response to TNF, but respond well to microbial products such as LPS and SAC. Compare to the expression of FDC-SP by FDCs after TNF stimulation, this induced expression level is at a relatively lower level. Besides HK and FDC-1 cell lines, a series of cell lines was checked for FDC-SP expression and the only other cell line that was found to produce FDC-SP is a macrophage cell line WEHI-3. This suggest that the expression of FDC-SP in PBMC and homogenized mouse spleen cell may be at least partly by macrophages. However we can not yet exclude that dendritic cell or other cell types may also express FDC-SP.

The correlation between expression and function of FDC-SP

Previous work show that FDC-SP expression is high in trachea, which may contain abundant mucosa associated lymphoid tissue, and in tonsil (Marshall et al., 2002a). But its expression is low in spleen which contains mostly primary follicles. Even the binding to of FDC-SP to B cells is only produced when B cell is activated. This suggests that FDC-SP is not a homeostatic chemoattractant like BLC, ELC and SLC which are important in lymphoid structure organization. But its predominant expression site in germinal center distinguishes itself from other activation-induced chemoattractants.

Whether FDC-SP is playing other role outside germinal center still needs to be determined. It's been reported that germinal center structure can be formed at the inflammation site of rheumatoid arthritis which is usually devoid of lymphoid tissues(Lindhout et al., 1999). Also IgA secreting B cells tends to locate under mucosa, it's not yet known whether the switch to IgA secreting plasma cell happened before the B cell relocation under mucosa or happened in situ after arrival under mucosa(Brandtzaeg et al., 1999). It's interesting to investigate whether FDC-SP is playing a role in any of these aspects. Infiltrated macrophage could be a source of FDC-SP to attract B cells at these sites.

Evolution link between FDC-SP, chemokines and antimicrobial peptides.

Recently there have been reports of antimicrobial effect of chemokines and chemotaxis effect of proline rich antimicrobial peptides (Marshall et al., 2002a; Wu et al., 2003; Yang et al., 2003). Proline rich antimicobial peptides are evolutionarily more ancient than chemokines. It seems there is a close evolutionary link between these two kind proteins. Also, in some case the gene locations of these proteins are close to each other(Durr and Peschel, 2002). FDC-SP seems fall into half way between the these two kind of proteins, it's proline rich, charged on N terminal which resemble a anti-microbial peptide. But it's overall composition and chemical property is more similar to that of a chemokine. More importantly, FDC-SP gene is located right beside a group of proline rich salivary anti-microbial peptides, which in turn located beside a cluster of chemokines, prompting that all these protein maybe evolutionarily linked together. There has been observations in my experiments during purifying recombinant fusion GST-FDC-SP protein that E. coli

expressing both human or mouse FDC-SP grow significantly slower after induction with IPTG and can never reach an OD over 0.9. I have done some preliminary experiments on the anti-microbial effect of FDC-SP using recombinant FDC-SP and E. coli. Although there is no significant antimicrobial effect detected, these experiments need to be optimized and further carry out.

The binding and chemotaxis effect of FDC-SP on B cell

Binding experiments with human PBMC and mouse spleen cells have shown that FDC-SP only binds to B cells activated by CD40 stimulation. This can be readily explained by the reason that, after CD-40 stimulation B cell up regulate their surface expression of FDC-SP receptor. However before we could identify a receptor for FDC-SP and directly measure its surface expression level, we can not exclude other possibility. For example, some other surface molecule may have effect on otherwise consistently expressed FDC-SP receptor conformation and change their affinity for FDC-SP. More over, the binding pattern of FDC-SP to B cell correlates very well with it's ability to induce chemotaxis movement on B cell.

The chemotaxis effect of FDC-SP can be blocked by Pertussis toxin treatment. This suggest that it functions through binding to a G protein coupled receptor on B cell surface. It could be a yet unknown receptor, or known orphan receptor, or simply another known receptor found for chemokines or other chemoattractant. For example antimicrobial peptide defensin is using chemokine receptor for its chemotactic function(Schutte and McCray, 2002). This also supports an evolutionary link between them. This raise another question of how does B cell combine signals from different

chemokine. Since one would imagine there should be always relevant chemoattractant within the microenvironment at any given time. It's a big question for cells regarding how to integrate these signals. Besides a receptor changing strategy, there is report that some chemokines have a competitive advantage in attracting certain cells towards them. Even at a small amount, they can override the effect of another chemokine which present at a high concentration. For some chemokines, the outcome for the migration may be just an additive effect of all the chemokine present in the microenvironment.

The synergistic effect of FDC-SP and BLC is a very interesting phenomenon. The exact mechanism of this synergism is worth further investigation. It could be a cross-talking between the intracellular pathways of the two attractant, so that the effect is amplified or it could happen outside on the cell surface. It could be that the occupancy of both receptor for FDC-SP and BLC better facilitate the homo and heterodimerization for the receptor, since the homo and heterodimerization is also needed for other effective chemokine signaling (Mellado et al., 2001; Rodriguez-Frade et al., 2001). Since chemokines bind promiscuously to their receptors, these two chemoattractant may also bind to the same receptor. Thus, the binding of these two chemoattractants to one receptor at the same time may have better signaling potency. It will be a very interesting job to clone the receptor for FDC-SP to facilitate the investigation into these possibilities.

In my experiments, primary B cells prepared from mouse spleen shows binding and migrate towards FDC-SP after anti-CD40 stimulation. The *in vivo* situation is much more complicated for B cell differentiation within germinal center. Sequential events are

coordinated in different temporal and spatial manner may have different effect on B cell responsiveness to FDC-SP. I have showed that B cell migration towards FDC-SP is abolished after further BCR engagement. But what about BCR engagement before CD40 stimulation? Several phenotypes of B cell including the centroblasts, centrocytes, plasmablasts and memory B cells exist within germinal center. The centroblasts have low level of surface BCR, whereas centrocytes have higher level of surface BCR. With this regard, more refined experiments need to be done with different phenotypes of B cell purified from germinal centers after immunizing the mouse.

Possible role of FDC-SP in germinal center development

My research on FDC-SP showed that FDC-SP is a novel chemoattractant specifically for B cells activated by CD40 ligation. According to the three check point model of B cell development during antibody response (van Eijk et al., 2001), and the gradual nature of somatic mutation needed for affinity maturation, proliferating B cells has to come back and forth between FDC and follicular helper T (FHT) cell frequently to check BCR fitness for antigen. Considering that the germinal center is full of rapid expanding and dying B cell population, one can imagine that selected B cell must need some kind of guidance to fulfill this task in a short time. As CXCR5 expressing FHT cells mostly reside at the outer edge of germinal center light zone(Breitfeld et al., 2000), proliferating centrocytes have to shuttle between FDC and FHT to pass the last two check points. The expression of FDC-SP by FDC seems to help to solve this problem. Compared to BLC, the B cell response to FDC-SP changes more drastically depending upon whether they are stimulated with CD40 ligation or BCR engagement. Thus, the chemotactic effect of FDC-SP is much more dynamic compared to that of BLC. At the same time, BLC and

FDC-SP seems working synergistically on attracting B cell. This gives those B cells selected by cognate T cells an advantage to migrate more quickly towards FDC. After BCR is further engaged with antigen, B cell significantly down regulate its responsiveness to FDC-SP, this will facilitate them to go away from FDC to proliferate, and to interact with T cells again. After many rounds of this kind of shuttling, B cell clones expressing best fit antibody could be eventually selected. In my experiments, I used soluble anti-IgM to engage BCR, however antigens are usually attached on FDC surface and need some physical strength to wrench it down from FDC. It's interesting to check with tethered antigens on the effect of B cell response to FDC-SP. B cell response to BLC also seem to be down regulated after further BCR engagement. Whether B cells extracted Ag are passively pushed towards cognate T cells or they may start to express some T Zone chemokines to migrate to T cell still needs to be checked out.

Pro-proliferation effect of FDC-SP on CD40 activated B cell

Another interesting observation is that FDC-SP have some pro-proliferation effect on CD-40 activated B cells. This effect is not so significant compare to the effect of CD40. However, it is reproducible in at least 8 experiments and is statistically significant. This result is not surprise to us, since there has been report that both chemokines and antimicrobial peptides has been reported to have some pro-proliferation effect on their target cell. The G-protein coupled receptor signaling can lead to the activation of Janus kinase (JAK) kinases, a protein family originally implicated in cytokine signaling. This in turn phosphorylates the chemokine receptors in tyrosine residues and activates signal transducers and trans-activators of transcription (STAT) transcription factors thus leads to

cell proliferation(Rodriguez-Frade et al., 2001). It will be interesting to investigate whether FDC-SP is simply induce anti-apoptosis signal within the target cells. This would give cells attracted close to FDCs more opportunity to interact with and to extract antigens trapped on FDCs before they undergo apoptosis.

Possible signal transduction pathways for FDC-SP

One of the next step of this study would be checking intracelluar signaling event after FDC-SP binding which has largely not been investigated yet. PI3K and PTEN is two important enzymes linked to G protein coupled receptor in mediating chemotaxis movement(Funamoto et al., 2002). The phospholipids can recruit many adapter proteins at the polar side of chemokine binding(Marshall et al., 2002b; Marshall et al., 2000a; Marshall et al., 2000b). This in turn leads to the rapid reorganization of cytoskeletal proteins such as actin to carry out the cell movement. Calcium flux is also important for this process.

The relation between structure and function of FDC-SP

The structure of FDC-SP is characterized by a charged or polar N terminal followed by a proline rich middle region. I did blast searches with Pubmed PSI and Rpsbalst program within existing protein domain database and found nothing homologous to FDC-SP. But clearly these program suggest the middle proline rich portion is of "low complexity" and should be filtered out for the functional domain search. The characteristic N terminal received most of scrutiny in my research. It's composed of about 20 AA, most of them are charged or polar residues, which may favor a better strength of interaction with its ligand. Genomically, it's composed of one intact exon and mostly conserved between

human and mouse counterparts. Typical AA residues at specific position on cytokine has been reported(Boulay et al., 2003). It's also interesting to point out that GST fusion protein of FDC-SP after cleavage with Factor Xa functions much better than the uncleaved protein. This further suggest the GST fusion partner which is fused to the N terminal of FDC-SP is spatially hindering FDC-SP function. It's also possible that without GST part, FDC-SP can reach and enter cells more easily because of smaller size. In contrary, myc-tagged protein, which has the tag on its C terminal functions similarly to those without tag.

SUMMARY:

Molecules of different structure can serve as chemoattractant for different motile cells. These include chemokines, complement fragments, growth factors, lipids, N-formyl peptides and antimicrobial peptides. Chemotactic peptides play a role in many physiological aspects such as leukocyte traffic, th1 and th2 polarization, embryogenesis, neuron development, auto-immune disease, angiogenesis, cancer metastasis(Rossi and Zlotnik, 2000). Most chemotactic factors use G-protein coupled receptors (Arai and Charo, 1996). By overall observation, my results suggest that FDC-SP functions is a novel chemoattractant mainly expressed by FDC, and have a specific role in B cell migration during ongoing antibody response within germinal center.

The dynamic nature of B cell responsiveness to FDC-SP, the regulation pattern and location within germinal center of its expression by FDC and the synergistic chemotactic effect on B cell with BLC has all implicated to us that germinal center reaction is also marked by a coordinated B cell migration and positioning rather than a random one within this micro-environment. This may greatly facilitate B cell interaction with T cell or FDC at different stages of its development, bringing the rapid massive B cell proliferation and apoptosis under tight control. This subsequently may have influence other aspects of B cell function such as somatic hyper-mutation, isotype switching and differentiation. The in vivo study of FDC-SP expression after antigen immunization and the mouse model of transgenic or gene knock out would greatly help this research in the future.

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