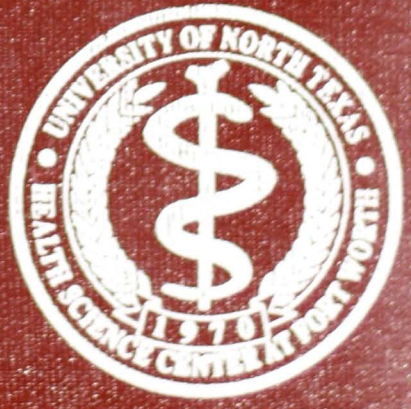

UNIVERSITY OF NORTH TEXAS
HEALTH SCIENCE CENTER AT FORT WORTH
1970

UNTHSC - FW
MØ3AYQ

HECKMAN
BINDERY, INC
AA 015217 J 17 00
12/1/2005

LEWIS LIBRARY
UNT Health Science Center
3500 Camp Bowie Blvd.
Ft. Worth, Texas 76107-2699

ABSTRACT

The purpose of this study was to investigate the roles of CS1 on human lymphocytes. The molecular and functional characterization of CS1 receptor in the natural killer (NK) cells and B lymphocytes was investigated. CS1 (CRACC, novel Ly9) is a novel member of the CD2 family receptor expressed on natural killer (NK), T cells, activated B cells and dendritic cells. To examine the existence of isoform of CS1, library from NK cells was screened based on wild type of CS1 (CS1-L). A splice variant form of CS1 (CS1-S), which lacks immunoreceptor tyrosine-based switch motifs (ITSMs) in cytoplasmic domain, was identified. To demonstrate the function of CS1 on human NK cells, transfectants that stably express each isoform were generated. CS1-L was able to mediate redirect cytotoxicity of P815 target cells as well as intracellular calcium influx in the presence of monoclonal antibody against CS1 suggesting that CS1-L is an activating receptor. CS1-S showed no effect on the cytolytic function and calcium influx suggesting that CS1-L and CS1-S may differentially regulate human NK cell functions.

Although CS1 was also cloned from cDNA library of human B-lymphocytes as well as of NK cells, very little is known regarding its biology on human B-lymphocytes. Here I investigated the expressions and functions of CS1 in human B cells. Human B cells expresses only CS1-L isoform and the levels of CS1 expression are upregulated after activation *in vitro*. Importantly, monoclonal antibody of CS1 (1G10 mAb) strongly

enhances proliferation of both freshly isolated and activated B cells. The enhanced proliferation effects of CS1 were most prominent on B cells activated by anti-CD40 mAbs and/or IL-4. Human cytokine microarray results indicated CS1 enhanced mRNA transcripts of fms-line tyrosine kinase 3 ligand, lymphotoxin A, tumor necrosis factor, and IL-14 which are related with mostly growth promoting activity. These results suggest that autorine cytokines might be the mediators for the function of CS1 on B cell in which it can induce proliferation of activated B cells. This study suggests that CS1 plays important role in human NK cells and B-lymphocytes.

CHARACTERIZATION OF A NOVEL RECEPTOR CS1 IN HUMAN LYMPHOCYTES; STUDIES IN NATURAL KILLER CELLS AND B-LYMPHOCYTES

Jae Kyung Lee, M.S.

APPROVED:

Major Professor, Porunelloor Mathew, Ph.D.

Committee Member, Ming-Chi Wu, Ph.D.

Committee Member, Hriday Das, Ph.D.

Committee Member, Myoung H. Kim, Ph.D.

University Member, S. Dan Dimitrijevich, Ph.D.

Chair, Department of Biomedical Sciences, Thomas Yorio, Ph.D.

Dean, Graduate School of Biomedical Sciences, Thomas Yorio, Ph.D.

CHARACTERIZATION OF A NOVEL RECEPTOR CS1 IN HUMAN LYMPHOCYTES; STUDIES IN NATURAL KILLER CELLS AND B-LYMPHOCYTES

DISSERTATION

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas

Health Science Center at Fort Worth

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

Jae Kyung Lee, M.S.

Fort Worth, Texas

June 2005

COPYRIGHT NOTICE

Permission to use copyright material granted for:

Lee JK, Boles KS, Mathew PA Molecular and functional characterization of a CS1 (CRACC) splice variant expressed in human NK cells that does not contain immunoreceptor tyrosine-based switch motifs. Eur. J. Immunology, 2004 Oct 34; 10: 2791-9

Conditions of permission include proper citation, and that this thesis is not offered for resale.

Permission confirmed by:

Bettina Loycke

Copyright & Licensing Manager

Wiley-VCH Verlag GmbH & Co KG

Boschstr. 12

69469 Weinheim

Germany

Phone: 0049 6201 606 280

Fax: 0049 6201 606 332

Email: rights@wiley-vch.de

ACKNOWLEDGEMENTS

I want to give special gratitude to my mentor, Dr. Porunelloor Mathew for giving great guidance with endless support. You are my ideal role model not only as a scientist but also as a person. You were always there whenever I needed your help. I am realizing how lucky I am to join and have a chance to work for you. I also would like to thank my graduate committee members, Drs. Wu, Das, Kim and Dimitrijevich for their guidance and enthusiasm on my project. Your advice and comment made my dissertation stronger.

I would like to thank Drs. Kitson and Brunson for your help early on in my graduate education. A special thanks to Dr. Myoung Kim for your support and encouragement during my graduate training. I want to give special thanks to Victoria Rudick, a former graduate advisor. You were there whenever I had trouble and I truly appreciate your support and encouragement. I also appreciate for the support of the staffs in the molecular biology and immunology department and graduate school office.

I would like to thank the members of the lab both past and present. You have been great accompanying for me. You have given enjoyable help, great friendship and support. Thanks to Drs. Chuang, Boles and Kumareasan for giving me inspiration of early work. I truly enjoyed while I was in the lab and appreciate all of you to give such a great environment. I also give a special thank to Dr. Sun to kindly share her experiences and technique.

My accomplishments would not have been possible without the contribution of my family. A special thanks to my husband, Jaegwon Chung for your untiring and endless sacrifice for my work and being my closest friend, advisor and love. I truly

appreciate my dad, mom, and sisters for their unselfish support and consistent prey for my work and me.

TABLES OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

CHAPTER I

INTRODUCTION

THE IMMUNE SYSTEM

The immune system is a defense system to protect body from invading pathogenic microorganisms and tumor cells. It is remarkably effective to generate an numerous different cells and molecules that are able to specifically recognize and eliminate enormous variety of foreign invaders as well as abnormal cells. The immune system is divided into two types: Innate and adaptive immunity. Innate immunity provides the first line of defense against infection during the critical period right after the exposure to a pathogen and it is not specific to any one pathogen. The adaptive immune system provides more versatile ways of defense increasing protection against pathogen. It is capable of recognition and selectively eliminating specific and wide repertoires of foreign invaders. In addition, the great advantage of adaptive immunity is 'immunological memory'. Exposure to the same antigen later results in a memory response and the second challenge leads to more rapid and stronger response than the first (1). Since there is a delay of several days before initial adaptive immune response take a place, the innate immune response has a critical role in controlling infection during early period. The activation of innate immune responses produces signals that stimulate and direct subsequent adaptive immune responses. In return the adaptive immune system produces signals and components that stimulate and increase the effectiveness of innate responses (2-4).

HUMAN LYMPHOCYTES

Both innate and adaptive immune responses depend upon the activities of white blood cells, or leukocytes. Leukocytes consist of two groups; the granulocytes and the agranulocytes. The granulocytes include neutrophils, eosinophils and basophil and the agranulocytes include lymphocytes, macrophage and monocytes. Innate immunity involves granulocytes and macrophage and adaptive immune responses mainly depend upon lymphocytes, especially T and B-lymphocytes.

There are three types of lymphocytes: B, T lymphocytes and natural killer (NK) cells. The lymphocytes rise from the bone marrow and circulate in the blood and lymphatic systems. B-lymphocytes develop in the bone marrow and via a process that involves rearrangement and expression of Immunoglobulin (Ig) genes, produce an antigen-specific receptor, which is first manifested in the immature stage. Then immature B cells, called naïve B cell, enter the bloodstream and migrate to the peripheral lymphoid organ and differentiate into plasma cells that secrete antibodies when they encounter foreign antigen. T lymphocytes originate in the bone marrow and they migrate to the thymus to undergo maturation. T cells are composed of cytotoxic T cells that kill cells infected with viruses and helper T cells that activate other cells such as B cells and macrophages. B-lymphocytes bear the B-cell antigen receptor (BCR) that is a membrane –bound form of the antibody and T lymphocytes possess the T-cell antigen receptor (TCR). Both BCRs and TCRs are highly diverse receptors, which are assembled by re-arrangement in the genomic DNA thus, allowing them to recognize wide variety of

antigens (5, 6). Thus, an individual lymphocytes is equipped with receptors that recognizes only one particular antigen.

T cells recognize their targets by detecting peptide fragments derived from these foreign proteins and bound to specialized cell-surface molecules on the infected host cells, phagocytic cells, or B cells. The molecules that display peptide antigen to T cells are membrane glycoprotein encoded in a cluster of genes bearing the major histocompatibility complex (MHC) (6). There are two classes of MHC molecule, MHC class I and MHC class II. The TCR of cytotoxic T cells associates with cell-surface co-receptor CD8 (CD8 T cells) and recognize MHC class I molecules and that of helper T cells associates with co-receptor CD4 (CD4 T cells) recognize peptide bound to MHC class II molecules (7). MHC class I molecules are expressed almost all nucleated cells and deliver peptides originating in the cytosol to the cell surface, and are thus able to display fragments of viral protein on the surface (8). MHC class I molecules bearing viral peptides are recognized by CD8 T cells, which then kill the infected cell. In contrast, MHC class II molecules are found on antigen presenting cells (APCs) such as B cells, macrophage and dendritic cells and deliver peptide originating in vesicular system to the cell surface (9). This way, those display peptides derived from pathogens living in macrophage vesicles or internalized by phagocytic cells and B cells. CD4 T cells recognizing peptide bound to MHC class II molecules on APCs activates macrophage to destroy the pathogen, stimulate B cells to produce antibody (10). CD4 and CD8 are the main co-receptors to regulate T-cell activation, however they are not the only factors, there are co-stimulatory molecules such as CD28 and inducible co-stimulator (ICOS)

(11). CD28 binds B7-1 (CD80) and B7-2 (CD86), expressed on APCs, and interactions of these molecules are essential for TCR-induced proliferation and cytokine production by naïve T cells, as well as for helper T cell polarization. ICOS binds ICOS ligand (ICOS-L), which is expressed on APCs and non-haemopoietic cells this interaction is also necessary for Th-cell polarization (12). CD28 and ICOS are though to function by modifying the TCR-driven signals. There are other receptors, which regulate T-cell activation, including cytokine receptors, TNF-related receptors, chemokine receptors and integrins. However, these receptors do not seem to function by regulating TCR-triggering signal, rather they couple to independent signaling pathways. The signaling lymphocyte activation molecule (SLAM) family is another group of receptors, which can also modulate T-cell activation. Studies have been shown that these receptors are able to affect TCR signaling (13-15). CD2 family receptors can also regulate the functions of several other immune cell types, including NK cells, B cells and dendritic cells (DCs). More details regarding CD2 family receptors will be discussed later.

NK cells are a third lineage of lymphoid cells that lack antigen-specific receptors and are part of the innate immune system. Genes for NK cell receptor are encoded in the germ-line (16). Although NK cells have features that belong to innate immunity. They can also modulate the adaptive immune response (17-19). This study will focus on the NK cells and B-lymphocytes.

NATURAL KILLER CELLS

NK cells are bone marrow derived lymphocytes that function as a key player in innate immunity by recognizing viral, bacterial and parasitic infections and neoplastic target cells (20). They comprise up to 5-10 % peripheral blood lymphocytes and are found in periphery tissues including the liver, peritoneal cavity and placenta (20, 21). Natural killer cells were originally described on the basis of their natural capacity to kill certain tumor cells *in vitro* and depletion of NK cells *in vivo* leads to enhanced tumor formation in several mouse tumor models (22). NK cells also play a role in bone marrow transplantation, pregnancy and in the regulation of immune response and hematopoiesis (21).

Although NK cells are classified as lymphocytes, they contain large granule in the cytoplasm thus morphologically also named as large granular lymphocytes. Phenotype of NK cells is characterized by the absence of conventional receptors for antigen, *i.e.* surface immunoglobulin (sIg) or T cell receptor (TCR) and displays a CD3- CD16+ phenotype. The major effector functions of NK cells are cytotoxicity and cytokine release. Cytokines including IFN-γ, TNF-α, granulocyte-macrophage-CSF as well as matrix metalloproteinases (MMPs) produced by NK cells can modulate other lymphocyte functions (23-26). There are two ways of displaying cytotoxicity of NK cells; natural cytytoxicity and antibody dependent cell mediated cytotoxicity (ADCC). CD16 is one of the earliest characterized NK cell receptors, and it is the receptor for Fc of IgG type IIIA (CD16, FcγRIII). NK cells recognize IgG antibody-coated target cells through CD16 and mediate ADCC (27).

Although NK cells have an important role in innate immune system, there are quite a few evidences that NK cells can modulate the regulation of adaptive immunity. Cross talk between NK cells and dendritic cells (DCs) are well studied and thought to be important in both NK and T cell responses. When freshly isolated NK cells cultured with DCs, NK cells proliferate, acquire cytotoxic activity and produce IFN-γ. In most cases, NK cell activation requires direct contact with DCs (28-30). NK cells promoted adaptive immune responses through their production of type 1 and type 2 cytokines or chemokines and secretion of these factors by activated NK cells influences the differentiation of B and T lymphocytes (31). NK cells can also regulate the function of CD8 T cells. Evidence showed that the depletion of NK cells from peripheral blood mononuclear cells (PBMCs) of healthy tuberculin reactors reduced the frequency of Mycobacterium tuberculosis-responsive CD8 T cells and decreased their capacity to lyse M. tuberculosis-infected monocytes (17).

There are studies showing that NK cells also modulate B lymphocytes by increasing Ig secretions (32). More detail, it has been demonstrated that NK cells regulate B cell function via CD40-CD40 ligand interactions (33). In this study, human NK cells can induce autologous resting B cells to activated B cells, this process requires cell-cell interaction. Recent study demonstrated that NK cells could induce germline transcripts (Igamma2a) necessary for switch recombination to IgG2a (34). In this way, immune system is not regulated by two separate independent systems; innate and adaptive immunity operates in cooperative and interdependent ways.

NK cells kill target cells by either of two major mechanisms that require direct contact between NK cells and target cells. One mechanism is performed by the formation of pores in target cell membranes by secreted molecules of lymphocyte origin, such as perforin and granzymes. Those are secreted by exocytosis and together induce apoptosis of the target cell (21). While this event occurs, intracellular calcium influx is necessary to activate signaling pathway to successfully secrete these molecules (35). The second mechanism initiated by receptor-mediated triggering of apoptosis-inducing target cell surface molecules. This mechanism involves the engagement of death receptors such as Fas/CD95 on target cells by their ligands including FasL and TRAIL on NK cells, resulting in classical caspase-dependent apoptosis (36-38). These two mechanisms are not mutually exclusive and are probably used by different types of effector cells or by the same effector cells at different stages of differentiation. Moreover, the function of NK cells is regulated by engagement of NK cells receptors and ligands on the target cells.

NK CELL RECEPTORS

The effector function of NK cells is controlled by interactions involving specific NK cell receptors and their cognate ligands, either on target cells, or other cells of the immune system. NK cell receptors do not require gene rearrangement machinery that assembles T and B cell receptor genes (39). In fact, studies in mice with disrupted RAG-1 or RAG-2 genes indicated that NK cells could undergo normal development and express receptors capable of mediating natural cytotoxicity (40, 41). Nevertheless, NK cells have an ability to discriminate target cells. NK cells kills target cells that lack surface

expression of MHC class I molecules (42) and are also able to reject MHC-different bone marrow grafts, especially in cases that the donor graft lacks MHC molecules of the host (43). These studies led Kärre and colleagues to propose the 'missing self' hypothesis, in which NK cells attack target cells that do not express normal level of autologous class I molecules on the surfaces (44). This hypothesis suggested the existence of receptors recognizing MHC class I molecules and deliver signal that modulate NK cell function.

There are two distinct families of receptors are responsible for the regulation of NK cell activity. Those include the immunoglobulin-like NK receptors (KIR, LILR, p75/AIRM1, IRp60, NCRs, 2B4/CD244, NTB-A, DNAM1/CD226 and LAIR) and the C-type lectin like NK receptors (CD94/NKG2, NKG2D, NKp80, NKRP1 and the rodents Ly49 receptors) (45-49). These receptors are again characterized by either inhibitory or activating properties and are involved in the fine regulation of NK cell function.

NK cell inhibitory receptors include the human killer-cell immunoglobulin-like receptors (KIRs), the rodent Ly49 receptors and the human and rodent CD94-NKG2A heterodimer molecules (48, 50-52). The inhibitory receptors prevent killing of normal cells and limit the production by NK cells of inflammatory cytokines such as IFN-γ, GM-CSF and TNF-α. All receptor families exhibit the capacity to discriminate between different allelic class Ia isoforms. KIRs belong to the immunoglobulin superfamily and specifically recognized MHC class I alleles, including groups of HLA-A, HLA-B, and HLA-C (53, 54). These inhibitory receptors contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic domains, which recruit intracellular tyrosine phophatases that mediate the inhibition of the NK cells. The ITIMs becomes

tyrosine phosphorylated upon ligand binding on their receptors by an SRC-family tyrosine kinase, which then recruits and activates SH2-domain-containing protein tyrosine phosphatase (SHP)-1, SHP-2 (55) and SH2-domain containing inositol polyphosphate 5' phosphatase (SHIP1) (56). These are responsible for the inhibition of various NK cell-mediated effector functions (57).

The activating receptors can initiate NK cell activation and target cell lysis, provided that NK cells are not turned off by ligation of MHC class I-specific inhibitory receptors, as it occurs when they interact with MHC class I-negative target cells. These triggering surface molecules which mediate NK cell cytotoxicity and their surface expressions are mostly restricted to NK cells are referred to as natural cytotoxicity receptors (NCRs) (58, 59). NKp46, NKp30 and NKp44 are the receptor group which belongs to NCRs and involves in NK cell triggering in the process of natural cytotoxicity but do not recognize HLA-class I molecules (60, 61). NCRs play a major role in the lysis of most tumor cell lines as revealed by monoclonal antibody-mediated receptor blocking experiments. Activating receptors transduce signals through the association with molecules, such as CD3ζ, FcεRIγ and DAP12, which contain in their cytoplasmic region immunoreceptor tyrosine activation motifs (ITAMs) that upon phosphorylation transduce activation via $p72^{syk}$ and ZAP70 cytoplasmic protein tyrosine kinase (PTK). So far, cellular ligands for NCRs have not been identified yet.

NKG2D another receptor which triggers NK-mediated cytolysis through the associated DAP10 polypeptides upon tyrosine phosphorylation, recruit phophatidylinositol 3-kinase (PI3-kinase) (62). Ligands for NKG2D identified include

the stress-inducible molecules MHC class I chain-related A chain (MICA) and B chain (MICB) (63, 64) and the cell surface protein human cytomegalovirus UL-16 binding proteins (ULBP) 1-4 (65, 66). Recent studies showed that NK cells can reject tumor cells that express ligands for the activating NK receptor NKG2D, despite the expression of MHC class I molecules by the tumor cells. (67, 68) This was a greatly meaningful finding because the engagement of NKG2D provides 'dominant' activating signals to the NK cells.

As a member of CD2 receptor family, 2B4 and CS1 are also expressed in NK cells. Cross-linking of murine (m) 2B4 was shown to trigger NK cell-mediated cytotoxicity, IFN-γ and IL-2 secretion and granule exocytosis (69). CS1 has also been shown that it is a self-ligand and increases NK cytotoxicity (70). More detail about 2B4 and CS1 will be discussed in later.

Earlier 'missing-self' model is not correlated as many activating receptors on NK cell have been identified. In some cases, NK cells kills MHC I positive tumor cells. Also there are receptors which can recognize MHC class I molecules but still act as activating receptors (71). Thus, the newly modified model to explain NK cell–target cell discrimination is that NK cell activation is regulated by specific receptors that upon interaction with their respective ligands, may send stimulating or inhibitory signals (21, 48, 58, 72).

B LYMPHOCYTES

The B-lymphocytes has an essential role in host defense and maintains a large complement of cell-surface receptors that regulate its many immunological functions. Major functions of B lymphoctyes are antibody production and antigen presentation. B-lymphocytes develop in bone marrow and mature B cells leave the bone marrow expressing membrane bound immunoglobulin. They may proliferate and differentiate into antibody-secreting plasma cells when stimulated by antigen, and undergo programmed cell death in the absence of stimulation (73). When B cell activation is initiated after recognition of specific antigen by cell surface Ig, which in naïve, unprimed B cells is of IgM or IgD isotype, activated B cell proliferates and differentiates to give rise to antibody-secreting plasma cells or memory cells (74-76).

Cytokines and membrane bound costimulatory molecules are required for subsequent proliferation, Ig isotype switching and differentiation of activated B cells. Cytokines produced by helper T (Th or CD4+) lymphocytes such as IL-4, IL-5 and IL-13 (Th2-type) are involved in providing help for B cells, or IL-2 and IFN-γ (Th1-type) mediate delayed-type hypersensitivity reactions and activate macrophages (77). Monocytes also can regulate B cells proliferation and differentiation through release of cytokines, such as IL-6, IL-8 and IL-10 (78, 79). There are also autocrine cytokines including IL-1, IL-6, IL-10, TNF-α and lymphotoxin produced by activated B cells and they enhance proliferation and differentiation of cells (80-84).

The CD40 molecule is well studied as a membrane bound costimulatory molecule of B lymphocyte activation. It is a member of tumor necrosis factor (TNF) receptor (TNFR) superfamily, however it does not contain death domain in the cytoplasmic region (85). The interactions between CD40 on B cells with CD40 ligand (CD154) on activated CD4+ T cells (86) was shown to be required for proliferation and differentiation of both human and murine B cells (87-89). CD40 ligand (CD154) is a 34-39 kDa type II integral membrane protein expressed on activated but not resting T cells (90), activated B cells (91), and activated platelets (92, 93). The importance of CD40/CD154 interaction had been pointed out by studies using CD40/CD154 knockout mice (94, 95). Engagement of CD40-CD154 leads to B cell clonal expansion, germinal center formation, isotype switching, affinity maturation, and generation of long-lived plasma cells (96).

In human, the study of B lymphocyte activation via CD40 is limited to *in vitro* models. So far, the impact of CD40 stimulation on B lymphoctyes from peripheral blood or tonsils has been using different tools to stimulate CD40: cells transfected with CD154, anti-CD40 Abs (97-100), or soluble CD154 (101) in the presence of IL-4 (102, 103). Banchereau *et al* showed that cross-linking of the B cell CD40 antigen results in major cellular activation further modulated by cytokine. In particular, IL-4 and IL-13 permit establishment of long-term factor-dependent B cell lines, as well as isotype switch towards the production of IgE and IgG4. Addition of IL-10 to CD40-activated B cells results in limited proliferation and remarkable differentiation into plasma cells (104). It has also shown that human resting B cells can enter a state of sustained proliferation upon CD40 stimulation. Additional incubation with IL-4 enhanced the production of IgM and

IgG by B cells and induced them to produce IgE and interestingly IFN-γ enhanced this effect (105).

It had been reported that CD40-activated chronic lymphocytic leukemia (CLL) B-cells induced autocrine production of TNF-α and IFN-γ (106). In their study, TNF-α and IFN-γ had potential role in sensitizing B-cells to fludarabine treatment. Another example of autocrine production of cytokine from human B cells is that, in the presence of IL-4, engagement of CD40/CD154 or anti-CD40 mAb stimulation not only results in B-cell proliferation and induction of IgE synthesis (107) but also increases production of the cytokine lymphotoxin-α (LTA) (108). The study showed that recombinant LTA induces proliferation of human B cells and enhances CD40/IL-4 mediated B cell proliferation and IgE synthesis in a dose-dependent manner and addition of anti-LTA Ab partially inhibited those effects (108). Their study suggested endogenously produced LTA by B cells play a potentially significant role during B-cell proliferation and IgE synthesis.

Signaling lymphocyte activation molecule (SLAM, CD150) is one of the CD2 family receptor and it has soluble and membrane-bound isoforms. It had been shown that SLAM can induce proliferation and Ig synthesis by activated human B lymphocytes (109). CS1, belongs CD150 subfamily receptors, is expressed on activated B cells as well as NK cells. However the function of CS1 on human B cells has not been studied, yet. Details about SLAM and CS1 receptor will be discussed in below.

THE CD2 FAMILY RECEPTORS

The CD2 family of receptors is part of the immunoglobulin superfamily (IgSF) and plays a major role in lymphocyte functions. These receptors are expressed on various hematopoietic cell types and regulate lymphocyte activation and immune responses (13, 110). The CD2 family includes CD2, CD48, CD58, CD84, SLAM (CD150), 2B4 (CD244), Ly-9 (CD299), CS1 (CRACC, 19A), BLAME and NTB-A (Ly-108) (111). Among the CD2 family receptors, 2B4, SLAM, CD84, NTB-A, Ly-9 and CS1 are subgrouped as CD150 family (111). All the members of the CD150 subfamily possess a similar structural organization including an extracellular domains with either two or four Ig-like domains, a single trasmembrane segment and an intracytoplasmic domain with tyrosine-based motifs. They have two or more unique tyrosine-based motifs with the consensus amino acid sequence, TxYxxV/I, where x is any amino acid, called immunoreceptor tyrosine-based switch motifs (ITSMs) (112). ITSMs recruit intracellular kinases, phosphatases, and adapter proteins in response to ligation by their ligands (113).

SLAM, a member of CD150 subfamily, is a glycosylated transmembrane protein with a relative mass of 70 kDa expressed on T cells, a proportion of B cells, dendritic cells, macrophages and activated NK cells (114-116). Originally, SLAM has been cloned and characterized that is involved in T-cell activation and engagement of SLAM triggered T cell proliferation and induced IFN-γ production (117-119). SLAM can also augment T-cell cytotoxicity and CD95-mediated apoptosis, and also regulation of proliferation and differentiation of B cells (109, 120, 121). Moreover, a number of morbilliviruses, including the measles virus, use CD150 as a receptor for cellular entry

(122, 123). SLAM has several isoforms. Among them, soluble and membrane-bound forms of SLAM could induce proliferation and Ig synthesis by activated human B lymphocytes (109). In their study, SLAM acts not only during the initial phase of B cell activation but also during the expansion of pre-activated B cells. In addition, sSLAM enhances production of IgM, IgG, and IgA by B cells activated by anti-CD40 mAbs. SLAM is a high affinity self-ligand and signaling through homophilic SLAM-SLAM binding during B-B and B-T cell interactions may enhance the expansion and differentiation of activated B cells.

2B4 was originally identified in mice by Dr. Mathew in 1993 as a surface receptor and it is a novel member of the CD2 subset of the immunoglobulin superfamily (124). 2B4 is expressed constitutively on the surfaces of NK cells (124). CD48 has been identified as the natural ligand of 2B4 in humans (125, 126). Engagement of 2B4 induces IFN-γ secretion and enhances non-major histocompatibility complex (MHC)-restricted killing by NK cells (69, 124, 127, 128). The extracellular domain of 2B4 contains one constant (C)-like domain and one Variable (V)-like domain. The cytoplasmic domain contains at least two ITSMs, which provide SAP binding motifs (124, 129). In mice, two isoform of 2B4 had been identified, 2B4-L with a long cytoplasmic domain and 2B4-S with a short cytoplasmic domain. Study showed that rat NK cell line (RNK-16) transfected with each isoform suggested that 2B4-S functions as an activating receptor while 2B4-L is inhibitory (130). Study showed that 2B4-S transfected RNK-16 cells give activating signal in NK cells which lead to kill target cells and 2B4-L transfected RNK-16 cells send inhibitory signal which lead to inhibit killing activity of NK cells. There are

also two isoforms identified in human; h2B4-A and h2B4-B. Difference comes from the junction of V and C domain of extracellular region (131) but the functional difference of the each isoforms has not been find out. Activation of 2B4 on human NK cells modulates cytotoxicity, IFN-γ secretion and invasiveness (26, 126, 132). The ITSMs in the cytoplasmic domain of 2B4 recruit various signaling molecules containing SH2 domain including SHP-1 and SHP-2 (133, 134). The ITSM is also involved in the association of small SH2-containing adapter protein 1A (SH2D1A), also called Duncan's disease SH2-protein (DSHP) or SLAM-associated protein (SAP) and regulates signaling through this receptor (135, 136). In X-linked lymphoproliferative disease, NK cells cannot be activated via surface 2B4 (112, 133, 134, 137). Defective signaling via 2B4 and SLAM may contribute to the pathogenesis of XLP due to mutations in SAP.

CS1 (19A, CRACC or novel Ly-9) has two extracellular Ig-like domains, thus belonging to a novel member of the CD2 subset of IgSF (138-140). It is expressed on the CD8+ cytotoxic T lymphocytes, activated B lymphocytes, NK cells and mature dentritic cells (138, 139). The CS1 gene is located on the long arm of the human chromosome 1 and localized at 1q23-24, in between CD48 and Ly9 and has the highest homology to CD84, SLAM, and 2B4 with 47, 44, 40 % similarity (138). It has been shown that CS1 is a self ligand and homophilic interaction of CS1 regulates NK cell cytolytic activity (70). The cytoplasmic domain of CS1 contains two of ITSM motifs observed in other CD150 subfamily members (138, 139). The ITSM motifs provide a docking sites to recruit small SH2-containing adapter proteins, including SH2D1A/SAP and EWS-activated transcript 2 (EAT-2) (141). The ITSM motifs bind directly to the SAP as well as to Src family

kinases, such as Fyn, FynT, Lyn and to the p85 regulatory subunit of PI3K (14, 121, 135, 136, 142, 143). It has been shown that SAP binds to the phosphorylated cytoplasmic tail of human CS1 only when fyn is presented (144). However, there is a report that CS1 mediates NK cell activation through extracellular signal-regulated kinase (Erk)-mediated pathway in a SAP-independent manner (139). Therefore, the mechanism of CS1 signaling respect to SAP association needs to be clear.

X-LINKED LYMPHOPROLIFERATIVE DISEASE (XLP)

X-linked lymphoproliferative disease (XLP) is a severe inherited immune deficiency characterized by abnormal immune deficiency response to Epstein-Barr Virus (EBV). The primary cause of death is hepatic necrosis and bone marrow failure. The extensive tissue destruction of the liver and bone marrow appears to stem from an uncontrolled cytotoxic T-cell response (145). The B-lymphocytes of XLP males do not appear to be resistant to T cell-mediated immunity. XLP-derived EBV-transformed B cells resemble normal lymphoblstoid cell lines (LCLs) with respect to induction of EBV-specific cytotoxic T cells, the ability to present antigens, and the susceptibility to MHC-restricted, CTL-mediated lysis. Thus the failure to eliminate EBV-transformed B cells in XLP does not seemed to be caused by a B cell-specific defect (146). Variable defects in both NK and T cells have been reported (147). The *SAP (SH2D1A)* gene that encodes the SLAM-associated protein (SAP) is deleted or mutated in patients with the XLP (148). Some of CD2 family receptors that have ITSMs have defective function without SAP. NK cells derived from SAP-deficient individuals are no longer activated through 2B4

(134). SAP was also essential for the signal transduction of other CD2 family receptors, such as SLAM/CD150, CD84, and Ly-9, which are differentially expressed on cytotoxic lymphocytes, Th cells, B cells and myeloid cells (14, 134, 149). The finding a defect in SAP as the genetic basis for X-linked lymphoproliferative disease (XLP) implicated a vital role for this molecule in immune regulation (135, 148). The lack of functions of all these receptors in SAP-deficient individuals results in a complex deficit of NK, T, and B cell responses, which leads to uncontrolled EBV infections and to the XLP ultimately.

SLAM-Association Protein (SAP, SH2D1A) has been cloned as a SLAM-association protein independently as a XLP defective gene (114). SAP encodes a 15-kd single SH2 domain that can function as a natural inhibitor of signal transduction events initiated by the cell surface receptor SLAM (114). The cytoplasmic domain of CS1 contains two tyrosine motifs which is present in 2B4, SLAM and CD84 and Ly-9 (117, 124, 135, 143, 150). With exception of SLAM, SAP binding requires the phosphorylation of the tyrosine within the motif (143). SAP associates with SLAM in the absence of tyrosine phosphorylation. SAP binding blocks the recruitment of the tyrosine phosphatase SHP2 to the phosphorylated ITSM of SLAM. In previous study using a three-hybrid system in yeast and COS cell cotransfection, it has been shown that SAP was able to bind to the ITSM of human CS1 (144). In the other hand, although the CS1 receptor contain ITSM motif, CS1 mediated activation of NK cell cytotoxicity is independent of a functional SAP as revealed by studies on NK cells from XLP patients (139). Thus, regarding to the dependency of SAP on the function of CS1 is still debating.

OBJECTIVES

CS1 is expressed on wide range of immune cells including NK cells which belong to innate immunity and CD8 T cells, activated B cells and dendritic cells which belong to adaptive immunity. This study was performed to investigate functions of CS1 on human lymphocytes including NK and B cells. This work composes of two parts. The first part of this work was to demonstrate expression of isoforms of CS1 and evaluate their immunological functions on NK cells. To evaluate the function of each isoform, stable transfectants that express each isoform were generated using a rat NK cell line (RNK-16). The second part was to elucidate the expression and the functions of CS1 on human B lymphoctyes. Monoclonal antibody against CS1 (1G10 mAb) has been used for this study. CS1 enhanced proliferation of fresh isolated as well as activated human B cells. CS1 also induced increases of mRNA expressions of autocrine cytokines from the CD40/IL-4-activated human B cells. Significance of these finding is discussed in chapter VI.

CHAPTER II

MOLECULAR CLONING OF A CS1 (CRACC) SPLICE VARIANT IN HUMAN NK CELLS

SUMMARY

CS1 (CRACC, novel Ly9 or 19A) is a novel member of the CD2 family expressed on natural killer (NK), T and stimulated B cells. Although the cytoplasmic domain of CS1 contains immunoreceptor tyrosine-based switch motifs (ITSM) which enables to recruite SLAM-associated protein (SAP/SH2D1A), it activates NK cells in the absence of a functional SAP. It has been shown that CS1 is a self-ligand and homophilic interaction of CS1 regulates NK cell cytolytic activity. Here I have identified a novel splice variant of CS1 (CS1-S), which lacks ITSM motifs in the cytoplasmic domain. Human NK cells express mRNAs for both wild type CS1 (CS1-L) and CS1-S and their expression level remained steady upon various stimulations.

INTRODUCTION

The CD2 family of receptors is part of the immunoglobulin superfamily (IgSF) and plays a major role in lymphocyte functions. These receptors are expressed on various hematopoietic cell types and regulate lymphocyte activation and immune responses (13, 110). The CD2 family includes CD2, CD48, CD58, CD84, CD150 (SLAM), CD244 (2B4), CD299 (Ly-9), CS1 (CRACC, 19A24), BLAME and NTB-A (111). Particularly, the cytoplasmic domains of 2B4, SLAM, CD84, NTB-A, Ly-9 and CS1 contain two or more unique tyrosine-based motifs with the consensus amino acid sequence, TxYxxV/I, where x is any amino acid, called immunoreceptor tyrosine-based switch motifs (ITSM motifs) (112). Recently these receptors which have ITSM motifs are subgrouped as signaling lymphocyte activation molecule CD150 subfamily (111). They recruit intracellular kinases, phosphatases, and adapter proteins in response to ligation by their ligands (113). Among the CD150 subfamily, 2B4 and SLAM are well-studied receptors in natural killer (NK) cells and T cells respectively, which are involved in cellular activation such as cytokine production, cytotoxicity, and invasiveness (26, 109, 124, 129, 151). Both 2B4 and SLAM exist in different isoforms resulting from differential splicing of hnRNAs.

The murine 2B4 gene consists of at least nine exons with one exon dedicated to the leader sequence, V-like, C2-like, and transmembrane domains, and cytoplasmic domains. Variable usage of exons results in the two forms of 2B4 in the mouse; 2B4-L and 2B4-S (152). Four exons encode the cytoplasmic domain of 2B4-L which contains four tyrosine motifs. 2B4-S is identical to the 5' end of 2B4-L, differing only at the 3'

end in a portion of the cytoplasmic domain. The human 2B4 (h2B4) was homologous to the m2B4-L form based on sequence comparison. Second transcript of 2B4 (h2B4-B) was identified from a library constructed from NK-cell line. It has additional 15 nucleotides inserted at the junction between the V-like and C2 –like domains (153). The difference between h2B4-A and h2B4-B arises from differential splicing of exon3, which contains an internal splice site and results in additional five amino acids in 2B4-B. Since the difference is in the extracellular domains of these two receptors, they might mediate differential ligand interaction or affinity. SLAM also has several splice variants, a membrane bound form (mSLAM), a secreted form (sSLAM) lacking 30 amino acids encompassing the entire 22-aa transmembrane region, a cytoplasmic form (cSLAM) lacking the leader sequence, and a variant membrane form (vmSLAM) with a truncated cytoplasmic tail (109, 117). The detail functions of each isoform still need to be clear.

CS1 (CRACC, novel Ly-9) is a novel member of the CD150 subfamily that activates NK cell-mediated cytotoxicity (70, 138-140, 144). The CS1 gene structure could be exploited from a contiguous gene sequence produced by the human genome project (Genbank accession number AL121958). Boles et al. identified that CS1 is approximately 13-14 kb as estimated by Southern analysis (138) The gene that encodes the CS1 is located on the long arm of human chromosome 1. CS1 follows the same exon arrangement, with three exons coding for the extracellular domains, a single exon for the cytoplasmic domain. The cytoplasmic domain of CS1 contains two of ITSM motifs observed in other CD150 subfamily members. The ITSM motifs provide docking sites to recruit small SH2-containing adapter proteins, including SH2D1A/SAP and EAT-2 (141).

The finding a defect in SAP as the genetic basis for X-linked lymphoproliferative disease (XLP) implicated a vital role for this molecule in immune regulation (135, 148). Here I identified splice variant form of CS1 on human NK cells. Nucleotide sequences and predicted amino acid sequences indicated that the splice variant form of CS1 is identical with its wild type CS1 on extracellular domain and transmembrane domain. The difference comes from cytoplasmic domain due to the elimination of exon 5, which leads to lack of tyrosine motifs. That suggests that CS1 isoforms regulate the ability of CS1 by sending different type of signaling.

MATERIALS AND METHODS

cDNA library screening and sequence analysis

We searched the expressed sequence tag (ETS) database at Genebank (http; //www.ncbi.nlm.nih.gov) with the TblastN program versus a consensus sequence of human members of the CD2 subset. Several overlapping clones were identified and oligonucleotide primers (Table 2.1) were designed to amplify a 363-bp fragment within the cytoplasmic domain. cDNA from a NK cell library constructed in λ phage by Dr. J. Houchins (R&D Systems, Minneapolis, Minn. and kindly provided by Dr. A. Brooks, NIH, Bethesda, Md.) was used as template. PCR cycle conditions were 94°C for 30s, 50°C annealing temperature for 30s and a 72 °C extension for 45s repeated for 30 cycles using Taq DNA polymerase from Gibco-BRL (Grand Island, N.Y.) at 2 mM $MgCl_2$. The same library was screened with the resulting PCR fragment labeled with α^{32}P-dCTP. Approximately 5×10^5 clones were screened for three rounds and positive phage DNA was isolated. All clones were sequenced by automated sequencing (Automated Sequencing Facility, UT Southwestern Medical Center). The cDNA insert from phage DNA was PCR amplified using SP6 and T7 primer sites located on the pGEM vector. PCR products were cloned into the pCIneo (Promega, Madison, MI) vector with the T cloning kit (Invitrogen, Carlsland, CA, USA). Clones were sequenced by DNA sequencing at Seqwright company (Houston, TX).

Human NK cell isolation

Isolation of NK cells from PBMC was performed by depletion of non-NK cells. Mononuclear cells from peripheral blood (PBMC) was obtained by density gradient centrifugation over ficoll paque (Sigma Chem. Co., St. Louis, MO). To deplete T cells, B cells, myeloid cells from PBMC, these cells were magnetically labeled using a cocktail of hapten-conjugated CD3, CD14, CD19, CD36 and anti-IgE antibodies. Cells were labeled with hapten antibody cocktail and FcR blocking reagent. After removing the unbound Abs by washing, the cells were incubated with anti-hapten microbead (Miltenyi Biotec, Auburn, CA) and then magnetically labeled cells were depleted by retaining them on a magnetic column (MACS; Miltenyi Biotec, Auburn, CA). Purity of NK cell was evaluated by labeling cells with PE conjugated anti-CD56 mAb (not shown here).

Reverse Transcriptase (RT)-PCR

Total RNA was isolated with the RNAstat 60 reagent according to the manufacturer's protocol (Teltest Inc, Friendswood, Tex.) and first-strand cDNA was synthesized from 5 μg of total RNA using superscript II (Invitrogen) reverse transcriptase and random primers in a volume of 20ul. PCR was performed using 2 μl of cDNA mixture, under standard conditions for 35 cycles. PCR was performed using primers (Table 2.1) encoding cytoplasmic domain. Oligonucleotides were custom synthesized by integrated DNA Technologies (Coraville, IA). Following amplification, portions of the PCR reactions were electrophoresed through a 1.2 % agarose gel.

RESULTS

cDNA library screening

Previously, wild type CS1 (CS1-L) has been cloned and identified it as a novel member of CD2 family (138). Bouchon *et al* also identified CS1 (CRACC) as a cellular receptor that activated NK cell-mediated cytotoxicity by a SAP-independent pathway (139). cDNA form a NK-cell library constructed in λ phage by Dr. J. Houchins was successfully used as template. The cDNA clones from library screening for CS1 wild type isoform were used for PCR reactions and primers were designed from the partial sequencing of cytoplasmic domain of CS1 to recognize the size difference of splice variant. (The sequence of each primer is shown in Table 2.1). PCR products of two clones, C5D1A and C11C1A showed smaller sizes than the wild type CS1 (Fig 2.1). The C11C1A insert from phage DNA was PCR amplified using SP6 and T7 primer sites located on the pGEM vector (Fig 2.2). Full sequence of C11C1A was subclone into a pCIneo mammalian expression vector. Before it was sent to sequencing, subcloning was confirmed by restriction enzyme digestion (Fig 2.3).

Characterization of a splice variant form of CS1 cDNA clone

The sequence identified another clone, C11C1A (CS1-S), with a cDNA insert of 1097 base pairs containing an open reading frame encoding a polypeptide of 296 amino acid residues (GenBank accession number BC027867) (Fig. 2.4). Comparison of the nucleotide sequence of CS1-S with the CS1-L showed that CS1-S is 104 base pairs shorter than CS1-L. Comparison of the predicted proteins encoded by CS1-L and CS1-S

(Fig. 2.5) revealed that 256 amino acids of the N-terminal sequence of CS1-S, which contain the extracellular domain, transmembrane domain and a few of the cytoplasmic domain region are identical to CS1-L. The remainder of the C-terminal sequences, corresponding to part of the cytoplasmic region, is different and lacks ITSM motifs which are on the cytoplasmic domain of CS1-L (Fig 2.6). This suggests that each isoform could result in transmitting different signals upon interaction with ligand.

Genomic structure of CS1

Gene structure of CS1 has been revealed by a contiguous gene sequence provided by the human genome clone RP11-404F10 (Genbank accession number AL121985). A Blast search using the *CS1* cDNA versus the human genomic databases at GenBank identified a 196-kb contiguous sequence containing the *CS1* gene located on the long arm of Chromosome 1 at 1q23-24. Analysis of the *CS1* gene sequence revealed a size of 13 kb. The CS1 gene contains seven exons; the first exon encodes the 5' untranslated and leader sequence, the second exon for the V domain, the third exon for the C2 domain, the fourth for the transmembrane region, and the last three exons code for the cytoplasmic domain. The short variant of CS1 (CS1-S) is due to the elimination of exon 5, which leads to a frame shift immediately after the transmembrane domain (Fig 2.7).

Expression of the CS1-L and CS1-S isoforms

The expression of CS1 transcripts in human NK cells isolated from PBMC and in various cell lines was analyzed by RT-PCR. Expression level of CS1-S as well as CS1-L mRNA

remained steady with various stimulations (Fig. 2.8A). CS1-L was predominantly expressed in NK92, a human NK cell line, but very low level of CS1-S. Other cell lines such as Jurkat, DB, and HL-60 did not express CS1-S, but showed very low level of expressions of CS1-L transcript (Fig. 2.8B). Previously monoclonal antibodies against the extracellular domain of CS1 were generated by Dr. Mathew's group and one of the clones, 2C7 was selected for its specificity against CS1 (70). Here I selected another hybridoma clone, 1G10 which showed higher affinity to CS1-L and CS1-S.

Table 2.1 Primers used for PCR of CS1 transcript

Primer	Sequence
CS1 727F	5' GTCTCTTTGTACTGGGGCTATTTC 3'
CS1 934R	5' TTTTCCATCTTTTTCGGTATTT 3'
SP6	5' ATTTAGGTGACACTATAG 3'
T7	5' TAATACGACTCACTATAGGG 3'

Figure 2.1 PCR analysis of cDNA library clones. The cDNA clones from library screening for CS1 wild type isoform were used for PCR reactions and primers (CS1 727F and CS1 934R) were designed for the partial sequencing of cytoplasmic domain of CS1 to recognize the size difference of splice variant (The sequence of each primer is shown in Table 2.1). C5D1A (lane 9) and C11C1A (lane19) clones were selected for further processes. Following amplification, portions of the PCR reactions were electrophoresed through a 1.2% agarose gel. Each lane represents cDNA library clones. M represents 100 base pair DNA ladder.

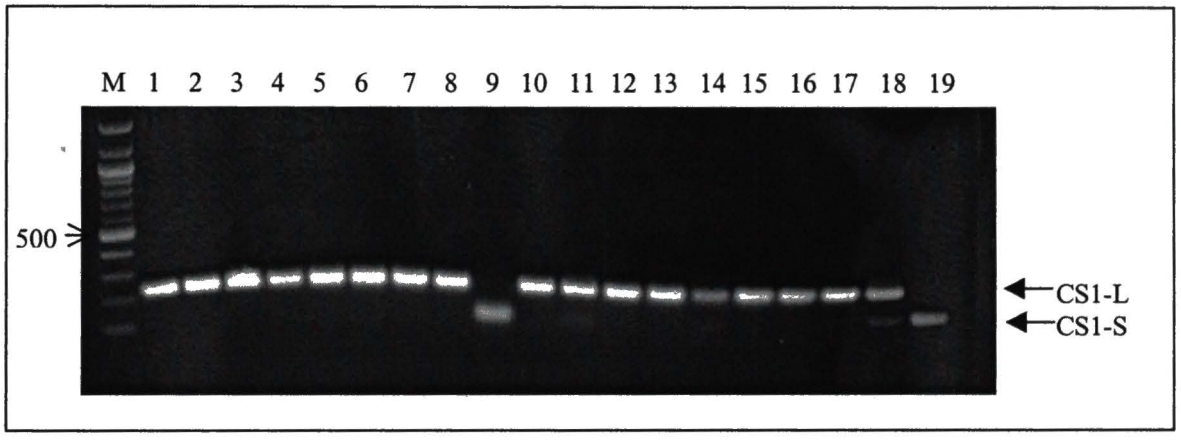

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
500
CS1-L
CS1-S

Figure 2.2 PCR analysis using C11C1A clone. M. 1-kilo basepair DNA ladder.1. PCR product of C11C1A clone using CS1 primers (CS1 727F and CS1 934R) 2. Negative control (Primers only) lane 3. PCR product of C11C1A clone using SP6 and T7 primer. Following amplification, portions of the PCR reactions were electrophoresed through a 1.2% agarose gel.

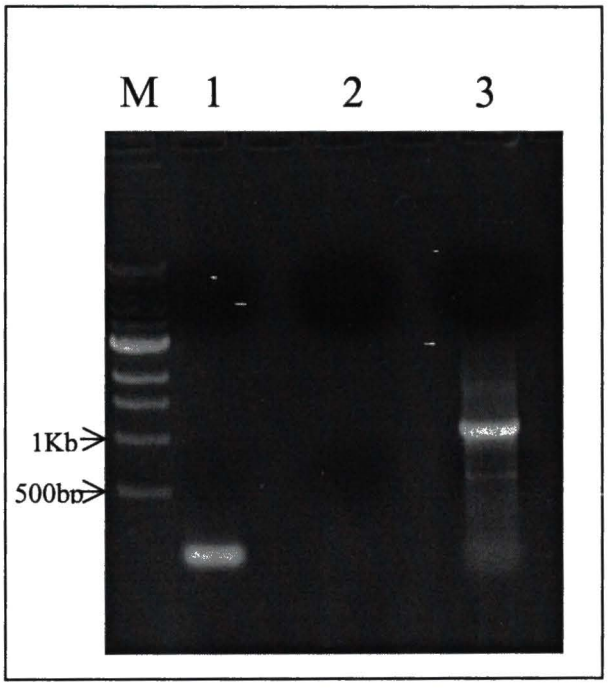

M
1
2
3
1Kb
500bp

Figure 2.3 Restriction enzyme digestion of C11C1A-pCI neo construct. C11C1A has been subcloned into pCIneo mammalian expression vector and digested with restriction enzymes XhoI and EcoRI. M represents 1 kilobase DNA ladder. Lane 1 and 2 represent clones for digestion. Lane 2 showed corresponding size insert in the vector and was selected for sequencing.

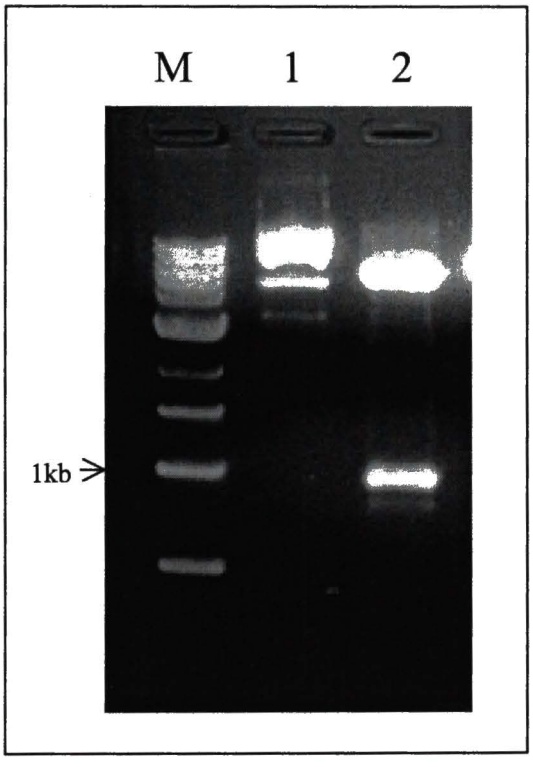
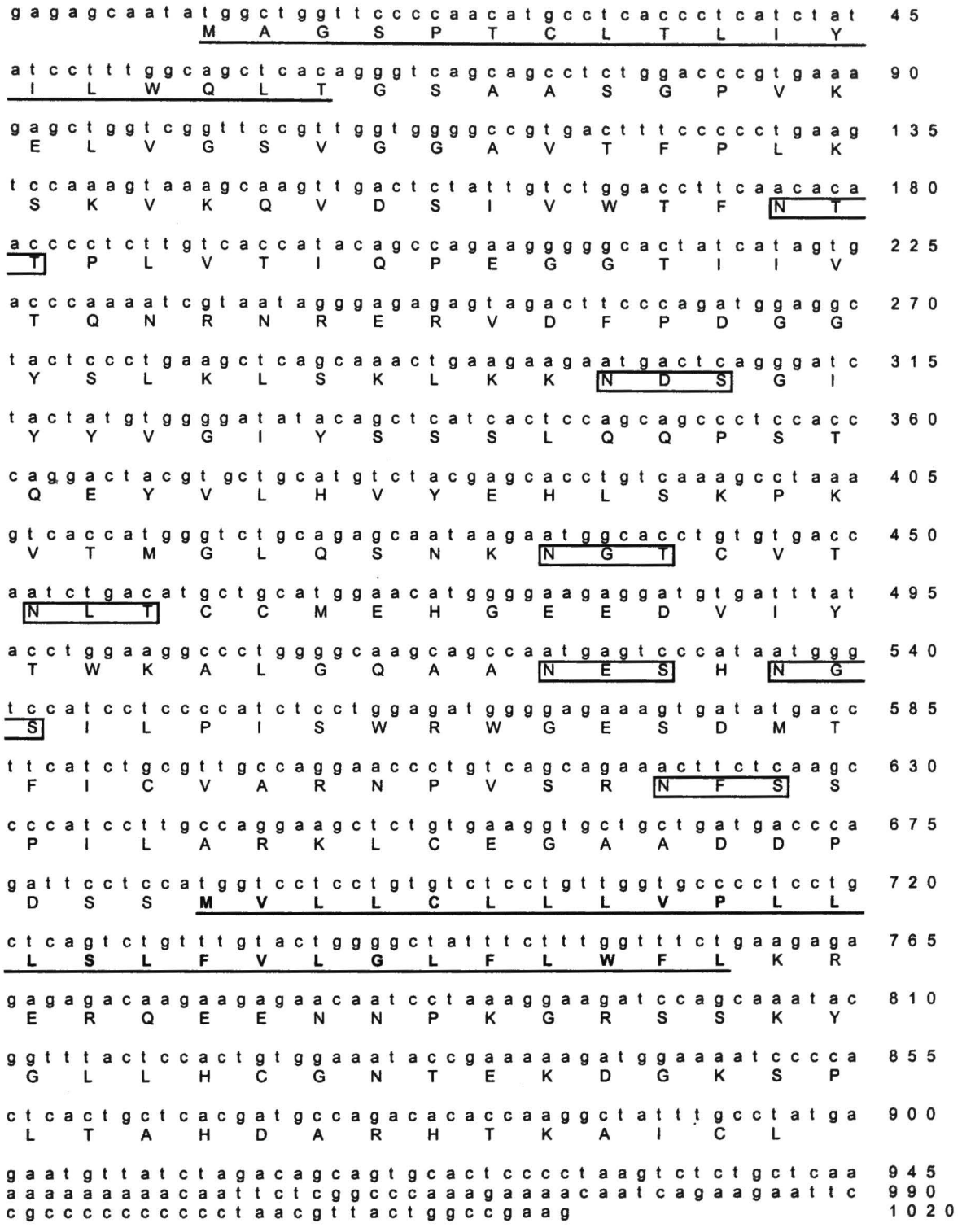

M
1
2
1kb

FIGURE 2.4 Nucleotide sequence of CS1-S cDNA and the predicted amino acid sequence (Genbank accession number BC027867). The signal peptide is underlined. The transmembrane domain is underlined and in bold. Seven potential N-glycosylation sites are boxed.



gagagcaatatggctggttccccaacatgcctcaccctcatctat 45
M A G S P T C L T L I Y

atcctttggcagctcacagggtcagcagcctctggacccgtgaaa 90
I L W Q L T G S A A S G P V K

gagctggtcggttccgttggtggggccgtgactttccccctgaag 135
E L V G S V G G A V T F P L K

tccaaagtaaagcaagttgactctattgtctggaccttcaacaca 180
S K V K Q V D S I V W T F N T

acccctcttgtcaccatacagccagaaggggcactatcatagtg 225
T P L V T I Q P E G G T I I V

acccaaaatcgtaatagggagagagtagacttcccagatggaggc 270
T Q N R N R E R V D F P D G G

tactccctgaagctcagcaaactgaagaagaatgactcagggatc 315
Y S L K L S K L K K N D S G I

tactatgtggggatatacagctcatcactccagcagccctccacc 360
Y Y V G I Y S S S L Q Q P S T

caggactacgtgctgcatgtctacgagcacctgtcaaagcctaaa 405
Q E Y V L H V Y E H L S K P K

gtcaccatgggtctgcagagcaataagaatggcacctgtgtgacc 450
V T M G L Q S N K N G T C V T

aatctgacatgctgcatggaacatggggaagaggatgtgatttat 495
N L T C C M E H G E E D V I Y

acctggaaggccctggggcaagcagccaatgagtcccataatggg 540
T W K A L G Q A A N E S H N G

tccatcctccccatctcctggagatggggagaaaagtgatatgacc 585
S I L P I S W R W G E S D M T

ttcatctgcgttgccaggaaccctgtcagcagaaacttctcaagc 630
F I C V A R N P V S R N F S S

cccatccttgccaggaagctctgtgaaggtgctgctgatgaccca 675
P I L A R K L C E G A A D D P

gattcctccatggtcctcctgtgtctcctgttggtgcccctcctg 720
D S S M V L L C L L L V P L L

ctcagtctgtttgtactggggctatttctttggtttctgaagaga 765
L S L F V L G L F L W F L K R

gagagacaagaagagaacaatcctaaaggaagatccagcaaatac 810
E R Q E E N N P K G R S S K Y

ggtttactccactgtggaaataccgaaaaaagatggaaaatcccca 855
G L L H C G N T E K D G K S P

ctcactgctcacgatgccagacacaccaaggctatttgcctatga 900
L T A H D A R H T K A I C L

gaatgttatctagacagcagtgcactccccctaagtctctgctcaa 945
aaaaaaaaacaattctcggcccaaagaaaacaatcagaagaattc 990
cgccccccccccctaacgttactggccgaag 1020

FIGURE 2.5 Comparison of predicted amino acid sequences of CS1-S and CS1-L (GenBank accession number AF291815). Transmembrane domains are blue colored. The consensus tyrosine sequences in the intracellular domain are pink colored and boxed. The difference of the CS1-L and CS1-S on the C-terminal sequences, corresponding to part of the cytoplasmic region, is underlined.

```
CS1-S  MAGSPTCLTLIYILWQLTGSAASGPVKELVGSVGGAVTFPLKSKV 45
CS1-L  MAGSPTCLTLIYILWQLTGSAASGPVKELVGSVGGAVTFPLKSKV 45

CS1-S  KQVDSIVWTFNTTPLVTIQPEGGTIIVTQNRNRERVDFPDGGYSL 90
CS1-L  KQVDSIVWTFNTTPLVTIQPEGGTIIVTQNRNRERVDFPDGGYSL 90

CS1-S  KLSKLKKNDSGIYYVGIYSSSLQQPSTQEYVLHVYEHLSKPKVTM 135
CS1-L  KLSKLKKNDSGIYYVGIYSSSLQQPSTQEYVLHVYEHLSKPKVTM 135

CS1-S  GLQSNKNGTCVTNLTCCMEHGEEDVIYTWKALGQAANESHNGSIL 180
CS1-L  GLQSNKNGTCVTNLTCCMEHGEEDVIYTWKALGQAANESHNGSIL 180

CS1-S  PISWRWGESDMTFICVARNPVSRNFSSPILARKLCEGAADDPDSS 225
CS1-L  PISWRWGESDMTFICVARNPVSRNFSSPILARKLCEGAADDPDSS 225

CS1-S  MVLLCLLLVPLLLSLFVLGLFLWFLKRERQEENNPKGRSSKYGLL 270
CS1-L  MVLLCLLLVPLLLSLFVLGLFLWFLKRERQEEYIEEKKRVDICRE 270

CS1-S  HCGNTEKDGKSPLTAHDARHTKAICL..................... 296
CS1-L  TPNICPHSGENTEYDTIPHTNRTILKEDPANTVYSTVEIPKKMEN 315

CS1-S  ..................
CS1-L  PHSLLTMPDTPRLFAYENVI                          335
```

FIGURE 2.6 Schematic representations of CS1-L and CS1-S. V represents Ig-V-like domain, C2 represents Ig-C2-like domain, Y1 and Y2 represent immunoreceptor tyrosine-based switch motif (TxYxxV/I).

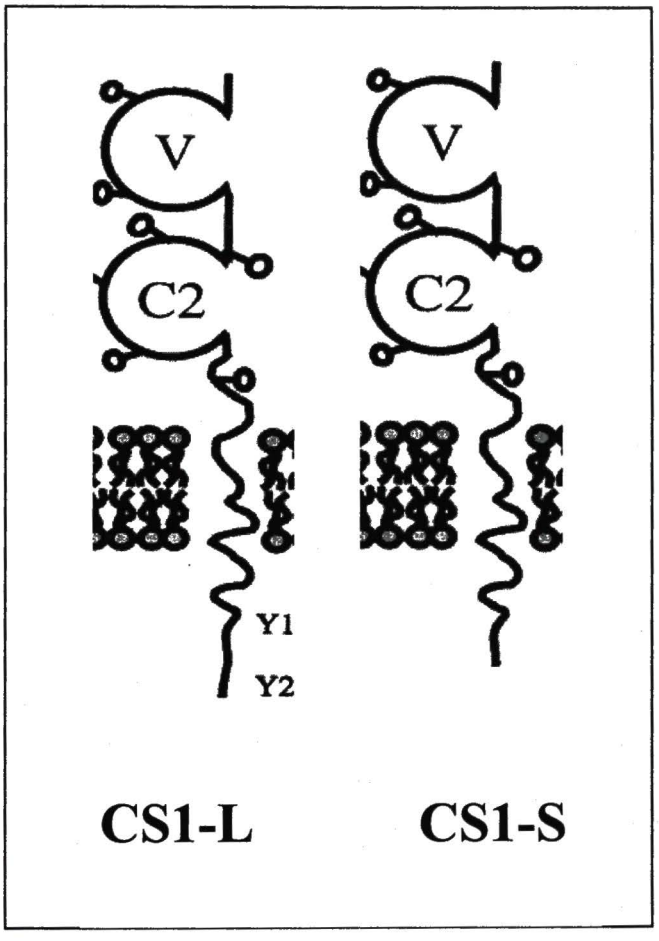

V
C2
Y1
Y2
V
C2
CS1-L
CS1-S

FIGURE 2.7 Schematic of the CS1 gene structure and alternative splicing. Exons are represented by rectangles. Protein domains of the splice variants are indicated by L, V, C2, TM, and Cyto and refer to leader sequence, Ig V-like domain, Ig C2-like domain, transmembrane domain, and cytoplasmic domain, respectively. Y1 and Y2 represent tyrosine-containing motifs.

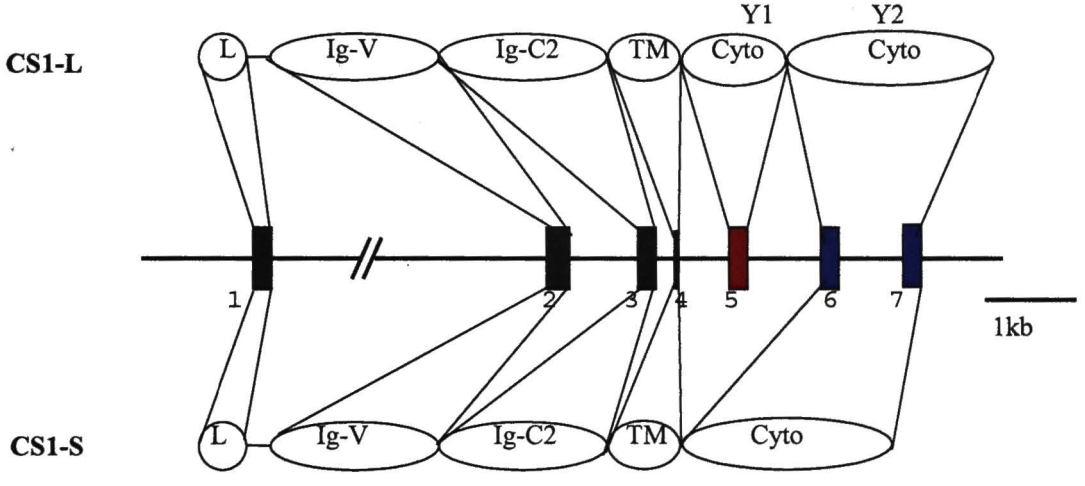
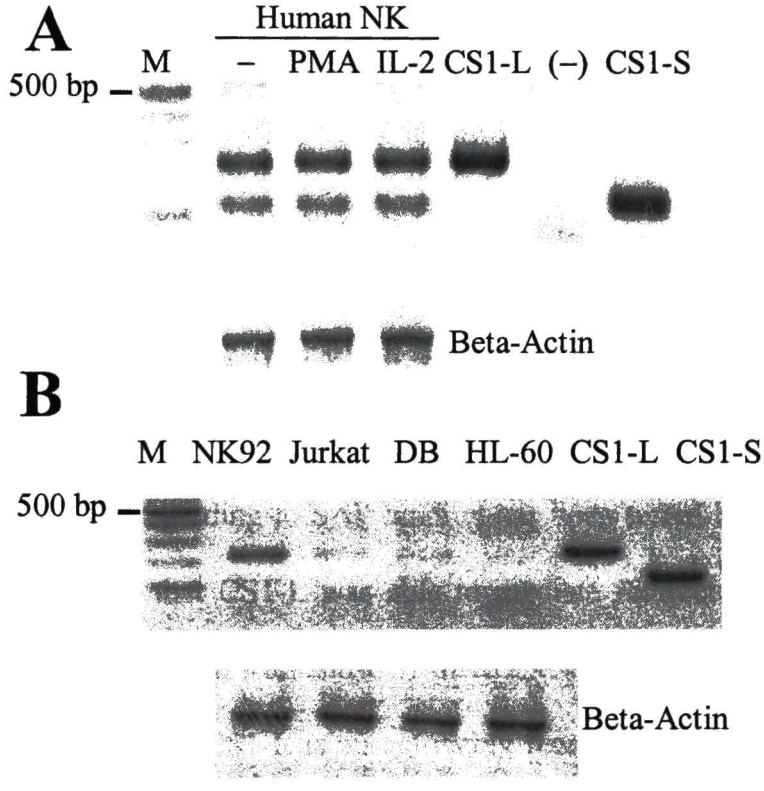

CS1-L
L
Ig-V
Ig-C2
TM
Y1
Cyto
Y2
Cyto
1
2
3
4
5
6
7
1kb
CS1-S
L
Ig-V
Ig-C2
TM
Cyto

FIGURE 2.8 mRNA expression levels of CS1-L and CS1-S in human NK cells with various stimulation and in different cell lines. (A) Freshly isolated human NK cells from a healthy donor were stimulated with PMA (50μg/ml) and IL-2 (1000U/ml). (B) mRNA from NK92, Jurkat, DB, HL-60 tumor cells were isolated and used for RT-PCR cDNA synthesis. cDNA clones corresponding to CS1-L and CS1-S were used as positive controls. The RT-PCR products were run on a 1. 2 % agarose gel and stained with ethidium bromide. PCR amplification of beta-actin from the same cDNA is shown at the bottom. Molecular mass standards are indicated on the left.


A
Human NK
M
–
PMA
IL-2
CS1-L
(–)
CS1-S
500 bp
Beta-Actin
B
M
NK92
Jurkat
DB
HL-60
CS1-L
CS1-S
500 bp
Beta-Actin

CHAPTER III

FUNCTIONAL ANALYSIS OF CS1 ISOFORMS IN HUMAN NK CELLS

SUMMARY

Two isoforms of CS1 have identified on human NK cells. Newly found splice variant form of CS1 lacks ITSMs in its cytoplasmic domain and human NK cells constitutively express both isoforms. This study was to determine the functions of each isoform. For this purpose, cDNAs for CS1-L and CS1-S were transfected into the rat NK cell line RNK-16. Functions of each isoform were investigated by performing redirected cytotoxicity assays and calcium flux experiments using these transfectants. Also signal transduction of CS1 regarding to SAP binding has been tested by immunoprecipitation. The results indicated that CS1-L was able to mediate redirected cytotoxicity of P815 target cells upon stimulation with anti-CS1 mAb and a rise in intracellular calcium within RNK-16, suggesting that CS1-L is an activating receptor. However, CS1-S showed no effects. Interestingly, SAP only associated with unstimulated CS1-L and dissociated upon pervanadate treatment. These results indicate that CS1-L and CS1-S may differentially regulate human NK cell functions.

INTRODUCTION

NK cells have a number of cell surface molecules that modulate NK cell recognition and activation (46) and NK cell functions are regulated by a delicate balance between negative and positive signals transmitted through these receptors (21, 48, 154, 155). Members of the CD2 family receptors play a major role in lymphocyte functions and do not recognize MHC molecules (150).

It has been shown that murine 2B4 has two splice variants, 2B4-L and 2B4-S as a result of alternative splicing (152). Functional study to determine difference of isoforms in which 2B4-L and 2B4-S were transfected into a rat leukemia cell line RNK-16. 2B4-S was able to mediate redirected lyses of P815 tumor targets suggested that 2B4-S represents activating receptor while 2B4-L expression led to an inhibition of redirect lysis of P815 target (130). In their study, 2B4-L also inhibited lysis of YAC-1 tumor targets and removal of the tyrosine motifs abrogated the inhibitory function of 2B4-L. In addition, 2B4-L associated with SHP-2 which is adaptor protein recruited by other inhibitory receptors as well. These results support potentially different functions for the each isoform.

SLAM also has several splice variants; a membrane bound form (mSLAM), a soluble or secreted form (sSLAM), a cytoplasmic form (cSLAM), and a variant membrane form (vmSLAM) with a truncated cytoplasmic tail (109). Functions of each variant are not clearly identified, however mSLAM and sSLAM were previously shown to have potent growth promoting effects on B cells and sSLAM enhanced production of

immunoglobulins (109). In addition to B cell and T cell activation SLAM acts as a cellular receptor for measles virus (117, 122). These studies support our hypothesis that the splice variants have distinct functions in immune system.

CS1 is expressed on NK cells, CD8+ T cells, activated B cells and mature dendritic cells (138, 139). The cytoplasmic domain of CS1 contains two tyrosine motifs (TxYxxI/V) which are SAP binding motifs (138). The presence of two tyrosine motifs suggests that it associates with SAP and regulates immune responses. From the study in chapter II, I identified new splice variant form of CS1 (CS1-S). Comparison of the predicted proteins encoded by CS1 (CS1-L) and CS1-S revealed that 256 amino acids of the N-terminal sequence of CS1-S, which contain the extracellular domain, transmembrane domain and a few of the cytoplasmic domain region are identical to CS1-L. The remainder of the C-terminal sequences, corresponding to part of the cytoplasmic region, is different and lacks ITSM motifs which are on the cytoplasmic domain of CS1-L. This suggests that each isoform could result in transmitting different signals upon interaction with ligand.

CS1 is a self-ligand and homophilic interaction of CS1 regulates NK cell cytolytic activity (70).It has been shown that SAP binds to the phosphorylated cytoplasmic tail of human CS1 only when fyn is presented (144). However, there is a report that the CS1 triggers NK-cell mediated cytotoxicity through a extracellular signal-regulated kinase (Erk)-mediated pathway in a SAP-independent manner (139). Therefore, the mechanism of CS1 signaling respect to SAP association needs to be clear. So far the functional studies of CS1 in human NK cells were the cytotoxicity related with XLP and signal

transduction including association of CS1 and SAP which is still conflicting. Thus, in this study I performed experiments to investigate the role of CS1 in human NK cells and the different function of CS1 isoforms by performing cytotoxicity assay, calcium influx experiment and ELISA for cytokine secretion. Immunoprecipitation was also performed to determine whether SAP could associate with CS1.

MATERIALS AND METHODS

Cells and Tissue culture

RNK-16 cells, a spontaneous NK cell leukemic cell line from F344 rat, were grown in RPMI 1640 medium supplemented with 10 % fetal bovine serum (Hyclone, Logan, UT), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 10 mM nonessential amino acids) and 55μM 2-mercaptoethanol. P815 cells, a murine mastocytoma, were used as target cells and also cultured in complete RPMI 1640 medium. Various RNK16 transfectants were grown in complete RPMI 1640 medium supplemented with 0.5 mg/ml G418. Cells were maintained at 37°C in a humidified 5 % CO_2/95 % air incubator. All products related to tissue culture were purchased from Invitrogen (Carlsbad, CA).

Vector constructs and transfections

cDNAs corresponding to each of the CS1-L and CS1-S were subcloned into the *XhoI & SalI* and *EcoRI & SalI* sites of the expression vector pEMCV.SRα, respectively (a kind gift from Dr. John D. Schatzle, UT Southwestern Medical Center, Dallas). Constructs were confirmed by sequencing before transfections. Electroporations were performed using 10 μg of *ClaI* linearized plasmids that had been purified by two rounds of CsCl centrifugation.

Flow cytometry

Production of anti-CS1 monoclonal antibody was described previously (70). Monoclonal antibody against CS1, 1G10, was used for labeling CS1. The 1×10^6 of cells were incubated with 1G10 mAb and washed with cold PBS containing 3 % BSA. Cells were then incubated with FITC-conjugated anti-mouse secondary antibodies for 30 min at 4 °C. The Ig isotype control (22B5) was run in parallel. Cell populations were analyzed on a FACSscan (Becton Dikinson). Dead cells were analyzed out on the basis of their light scatter properties.

Cytotoxicity assay

Target cells were labeled by incubating 1×10^6 cells with 2 MBq of $Na_2{}^{51}CrO_4$ (NEN Research Products, Boston, MA) for 90 min at 37 °C under 5 % CO_2 in air. The target cells (100 μl) were incubated with effector cell suspension (100 μl) under various conditions. The mAb used were anti-CS1 (1G10), anti-NKR P1 (10/78) (Pharmingen, San Diego, CA) and Ig isotype control (22B5). After incubation for 4 hr at 37°C under 5 % CO_2 in air, the cells were pelleted at 250 *g* for 5 min, 100 μl of the supernatants were removed and their radioactivity was measured. The percentage of specific lysis was calculated by the following equation: $(a–b/c–b) \times 100$, where *a* is the radioactivity of the supernatant of target cells mixed with effector cells, *b* is that in the supernatant of target cells incubated alone, and *c* is that in the supernatant after lysis of target cells with 1 % Nonident P-40.

Calcium flux assay

Calcium flux assay was performed according to the protocol by Prasanna *et al.*(156). Briefly, in order to measure intracellular calcium mobilization, RNK-16 cells expressing CS1-L or CS1-S were seeded on coverslips and loaded with 3 μM of Fura-2 dye (Molecular Probes, Eugene, OR) in a modified Krebs-Ringer buffer solution (KRB, in millimoles: 115 NaCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 24 $NaHCO_3$, 5 KCl, 5 glucose, and 25 HEPES, pH 7.4) for 30 minutes at 37°C. Then, anti-CS1 mAb or anti-NKRP1A (10/78) mAb was used to induce receptor cross-linking and cross-linking goat anti-mouse Ig (18 μg/ml) (Pharmingen, San Diego, CA) was added. Fura-2 fluorescence from these cells was monitored at 37°C by the ratio technique (excitation at 340 and 380 nm, emission at 500 nm) under a Nikon Diaphot microscope using Metafluor software (Universal Imaging, West Chester, PA). Calibrations were performed in vivo, and conditions of high $[Ca^{2+}]_i$ were achieved by adding the Ca^{2+} ionophore 4-Bromo-A23187 (1–3 μM; Calbiochem, San Diego, CA), whereas conditions of low $[Ca^{2+}]_i$ were obtained by adding EGTA (4–5 mM). Statistical significance of $[Ca^{2+}]_i$ between control and treatments was determined either parametrically by Student's *t*-test at $P < 0.05$.

Immunoprecipitation and western blotting

Approximately 1×10^8 cells were treated with pervanadate 1 mM for 20 min at 37 °C. Cells were then lysed with 1 % dodecylmatoside, 10 mM Tris pH 7.4, 150 mM NaCl, 100 μg/ml PMSF, and protease inhibitor mixture (Sigma-Aldrich, St. Louis, MO) for 30

min on ice. The lysate were precleared with the addition of protein G/A plus agarose bead and rotated for 1 h at 4 °C. The precleared supernatant were incubated with anti-CS1 (1G10) mAb. The immune complex was then precipitated by the incubation with protein G/A plus agarose bead for 2 h at 4 °C. Isotype control IgG was used as negative control. Immunoprecipitates were separated by 8-10 % SDS-PAGE. Membranes were probed with anti-SAP and SHP-1 (Santa cruz Biotechnology, Santa cruz, CA), anti-SHIP1 and SHP-2 (BD Biosciences, San Jose, CA). Western blots were performed according to the manufacturer's chemiluminiscence detection system instructions (Kirkegaad & Perry Laboratories, Gaithersburg, MD)

Statistical analysis

Statistical analysis was done using Student's t-test for two samples with equal variance, A p value of 0.05 or less was considered significant.

RESULTS

Subcloning CS1-S and CS1-L into pEMCV vector

RNK-16 cells, a spontaneous NK cell leukemic cell line from F344 rat, were selected for the stable transfection of CS1 isoforms. For the effective transfection of this cell line, pEMCV vector were used. The pEMCV.SRα vector (Fig 3.1) was generously presented by Dr. John D. Schatzle and used for transfection. cDNAs corresponding to each of the CS1-L and CS1-S were subcloned into the *XhoI & SalI* and *EcoRI & SalI* sites of the pEMCV.SRα mammalian expression vector respectively (Fig. 3.2). Constructs were confirmed by sequencing before transfections.

Stable expression of CS1 clones in RNK-16 cells

Since the extracellular domains of CS1-L and CS1-S are the same, expressions of each isoform was confirmed by RT-PCR using primers that amplified only cytoplasmic domain. The two potential tyrosine phosphorylation sites, similar to those found in ITSM motifs of other SLAM family receptors, located at amino acids 284 and 304 in the cytoplasmic region of CS1-L are shown schematically in fig. 3.3A. RT-PCR and immunoblotting results confirmed the expression of each isoform in transfectants (Fig. 3.3B, Fig 3.4A). Surface expression of CS1-L and CS1-S on representative selected transfectants was analyzed by flow cytometry (Fig. 3.4B). I used the 1G10 mAb to analyze surface expression levels of CS1-L as well as CS1-S. Clones expressing similar levels were selected for further studies.

1G10 mAb increased cell cytotoxicity and intracellular calcium flux of CS1-L transfectants but not of CS1-S transfectants.

CS1-L has been previously shown to activate NK cell-mediated cytotoxicity (70, 139). We performed redirected cytotoxicity assay using RNK-16 transfectants to examine the function of each isoforms separately. We used murine mastocytoma, P815 which express the Fc receptor on the surface (FcγR+) as target cells (Fig 3.5). For the positive control of the experiments, it has been shown that cross-linking of anti-NKR P1 mAb 3.2.3. or 10/78 mediated redirected lysis by RNK-16 cells and induce a calcium flux in RNK-16 cells (157-159). Therefore, we used 10/78 mAb as the positive control in cytotoxicity assays and calcium assays (Fig. 3.6B). CS1-L transfected RNK-16 cells were able to mediate redirected lysis of the target P815 in the presence of the 1G10 mAb. However, CS1-S transfectants were not able to mediate redirected lysis of P815 cells (Fig. 3.6A).

While NK cells kill the target, calcium influx is essential process to exert granzyme secretion as well as signal transduction to activate cytotoxicity. Thus, I also performed intracelluar calcium flux experiments using CS1-L or CS1-S RNK-16 transfectants to confirm the functional data obtained in the redirected lysis assay. As shown in Fig. 3.7A, cross-linking of NKR-P1 using 10/78 mAb on CS1-L RNK-16 or CS1-S RNK-16 transfectants resulted in a calcium flux as measured by fura-2 over time (Fig. 3.7A) which were provided as positive controls of each isoform transfectants. Only cross-linking of CS1 using 1G10 mAb on CS1-L RNK-16 transfectants resulted in a significant calcium flux, while cross-linking of CS1 on CS1-S RNK-16 transfectants did

not show any discernible calcium mobilization (Fig. 3.7B). These results supported previous redirected lysis data and lead us to conclude that only CS1-L is an activating receptor and that CS1-indued NK cell cytotoxicity in the previous studies were mediated by CS1-L isoform not by CS1-S isoform.

SAP was associated only with CS1-L isoform.

Since CS1-L has two ITSM motifs in cytoplasmic domain, I investigated whether CS1 associates with SAP or any other SH2-containing phosphatases which have been shown to bind with other members of the SLAM family. We performed immunoprecipitation using 1G10 mAb with lysate of transfectants which express each isoform. Cells were treated with or without sodium pervanadate for stimulation. Immunoprecipitation after pervanadate treatment failed to show an association of CS1-L neither with the SAP nor with other molecules such as SHIP1 (Fig. 3.8), SHP-1 or SHP-2 (data not shown). Interestingly, an association between CS1-L and the SAP was observed particularly in unstimulated condition. No association of CS1-S with SAP was observed. Therefore, only CS1-L is able to associate with SAP constitutively without any stimulation and binding was dissociated after pervanadate treatment (Fig. 3.8).

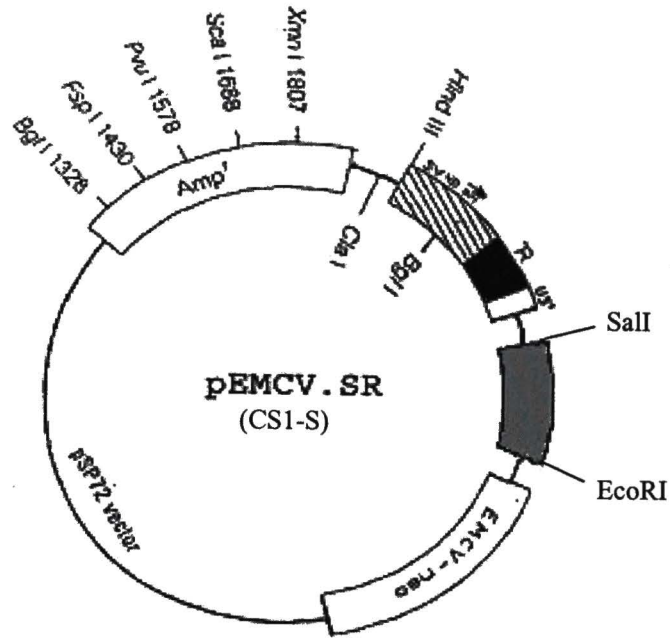
1G10 mAb was not able to induce IFN-γ secretion.

2B4 that is another CD150 subfamily receptors has both functions in NK cells including cytotoxicity activity and IFN-γ secretion. IFN-γ production is a major function of NK cells in response to NK –target cell contact (21). So, I tested whether CS1 could

induce IFN-γ production or not. Although CS1 has been shown that it increase the cytolytic activity of NK 92 cells against P815 tumor target cells, it did not induce IFN-γ production regardless of the presence of K562 tumor target cells (Fig 3.9). Thus these results indicate that CS1 receptor is involved only in the function of NK cell cytotoxicity but not of cytokine secretion.

Figure 3.1 Map of pEMCV.SRα mammalian expression vectors. A, CS1-S insert (1097 bp) was subcloned into EcoRI & SalI enzyme sites of pEMCV.SRα vector. B, CS1-L insert (1201 bp) sucloned into SmaI & XhoI sites of pEMCV.SRα vector.

A


Xmn I 1807
Sca I 1688
Pvu I 1578
Fsp I 1430
Bgl I 1328
Hind III
Amp'
Cla I
Bgl I
R
U5'
SalI
pEMCV.SR
(CS1-S)
pSP72 vector
EMCV-neo
EcoRI

B

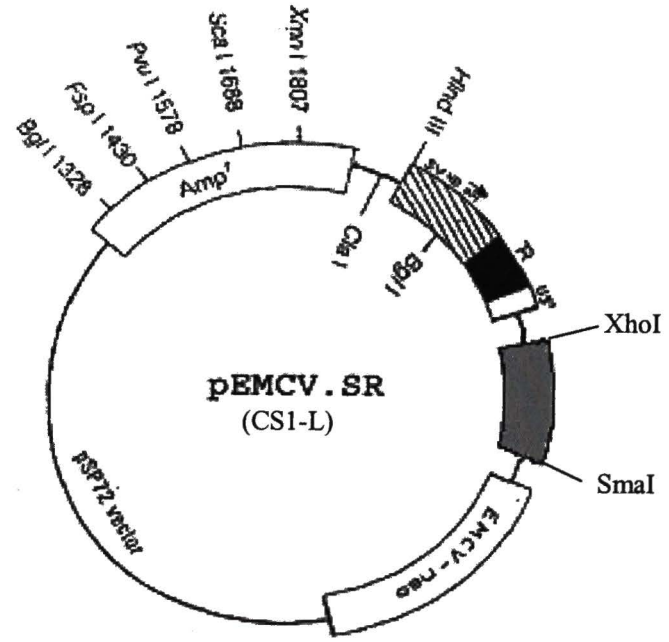

Xmn I 1807
Sca I 1688
Pvu I 1578
Fsp I 1430
Bgl I 1328
Hind III
Amp'
Cla I
Bgl I
R
U5'
XhoI
pEMCV.SR
(CS1-L)
pSP72 vector
EMCV-neo
SmaI

Figure 3.2 Restriction exzyme digestion of CS1-pEMCV.SR construct. A, CS1-S-pEMCV.SR construct was digested with EcoRI & SalI. B, CS1-L pEMCV.SR construct was digested with SmaI & XhoI. M represents 1 kb DNA ladder.

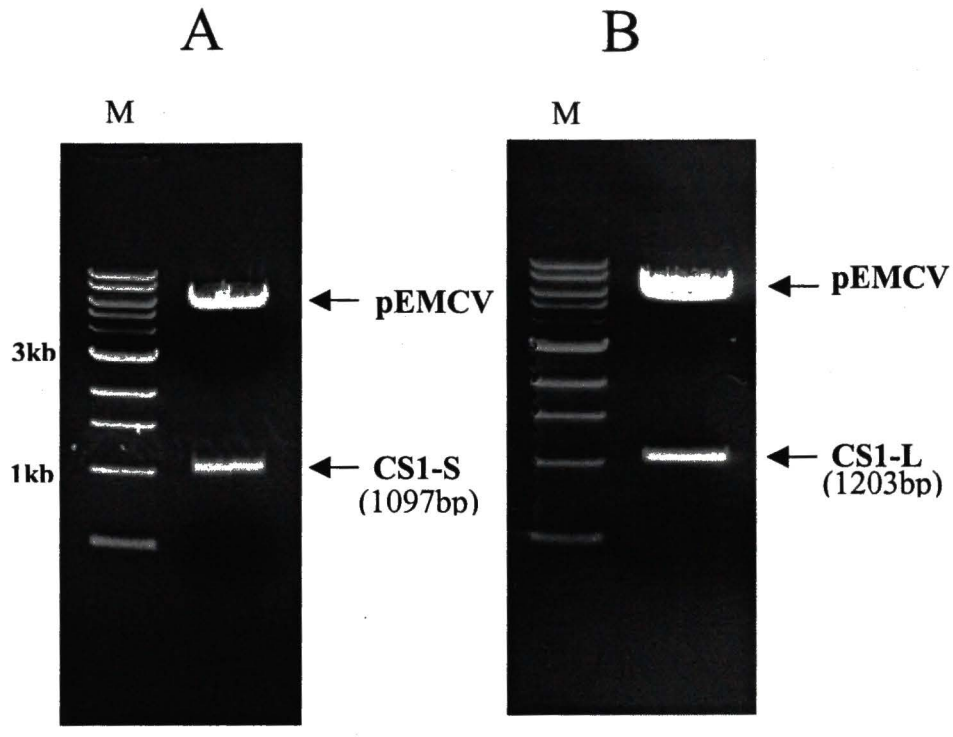
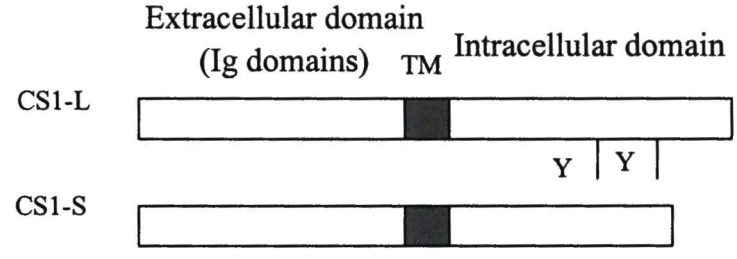

A
B
M
M
3kb
1kb
pEMCV
CS1-S
(1097bp)
pEMCV
CS1-L
(1203bp)

FIGURE 3.3 Expression of CS1-L and CS1-S in RNK-16 transfectants. RNK-16 cells were stably transfected with CS1-L or CS1-S expression vector. (A) Diagram of the protein structure (CS1-L or CS1-S). TM indicates transmembrane domain and the potential tyrosine phosphorylation sites are represented by (Y). (B) mRNA expressions of each transfectant (CS1-L or CS1-S) and untransfected RNK-16 (empty) were confirmed by RT-PCR.

A


Extracellular domain
(Ig domains)
TM
Intracellular domain
CS1-L
Y
Y
CS1-S

B

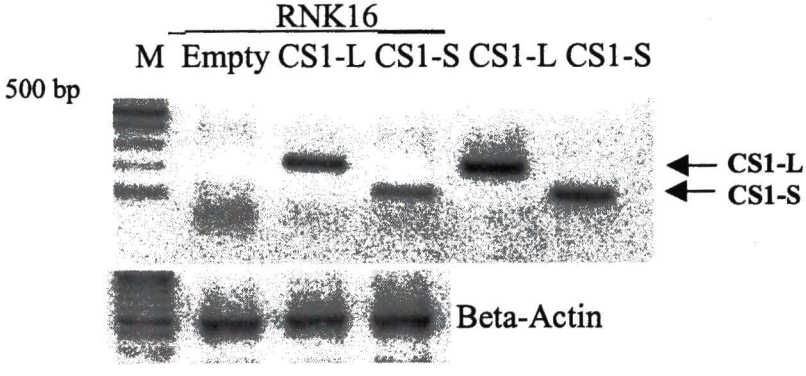

RNK16
M Empty CS1-L CS1-S CS1-L CS1-S
500 bp
CS1-L
CS1-S
Beta-Actin

FIGURE 3.4 Protein expression and surface expression of CS1-L and CS1-S in RNK-16 transfectants. RNK-16 cells were stably transfected with CS1-L or CS1-S expression vectors. (A) Protein expression was detected by western blot using anti-CS1 mAb (1G10). (B) Surface expression levels were measured by FACS analysis by labeling transfectants with mAb 1G10. RNK16 transfectants labelled stained with a control mAb (22B5, open) or 1G10 mAb (closed).

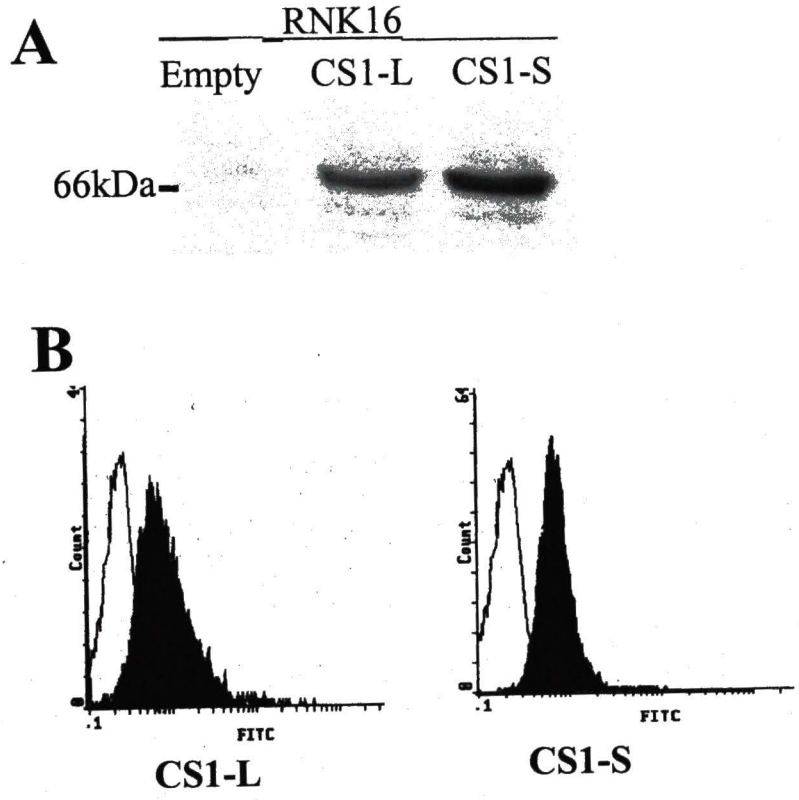

A
RNK16
Empty
CS1-L
CS1-S
66kDa
B
Count
FITC
CS1-L
Count
FITC
CS1-S

FIGURE 3.5 Model of the redirected lysis. RNK16 cells transfected with each CS1 expression construct (CS1-L or CS1-S) were used as effector cells in a standard chromium release assay against the murine FcR+ target P815.

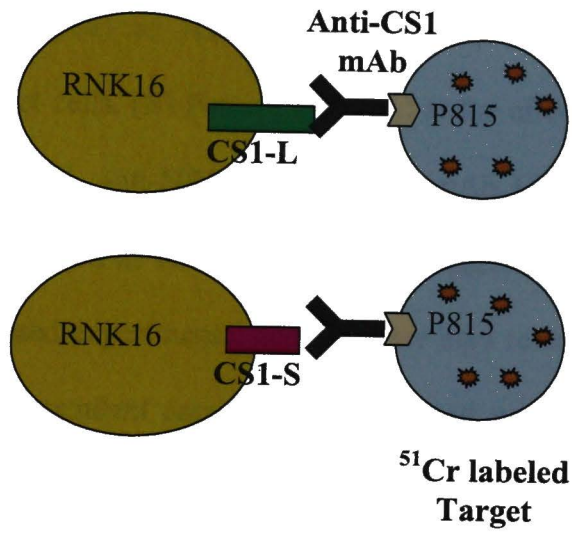

Anti-CS1
mAb
RNK16
P815
CS1-L
RNK16
P815
CS1-S
51Cr labeled
Target

FIGURE 3.6 Only CS1-L transfectants had killing activity against P815 target cells. Redirected lysis of P815 targets by CS1-L or CS1-S transfectants. RNK16 cells transfected with each CS1 expression construct (CS1-L or CS1-S) was used as effector cells in a standard chromium release assay against the murine FcR+ target P815. (A) CS1-L (■,□) or CS1-S (●,○) RNK16 transfectants were incubated with control antibody (22B5) (open) or 1G10 mAb (20ug/ml) (closed) for 40 min prior to the incubation of target cells. (B) Percent lysis (%) at the effector to target ratio of 100 to 1. As a positive control, anti-NKR P1A (10/78) mAb (5ug/ml) was used. Assays were performed triplicates on at least three separate occasions. Each data point is the mean value of the repeated experiments, and the error bars refer to the mean ±S.D. generated from the three independent assays. Student's *t* test was used to calculate *p* values. *, $p<0.01$.

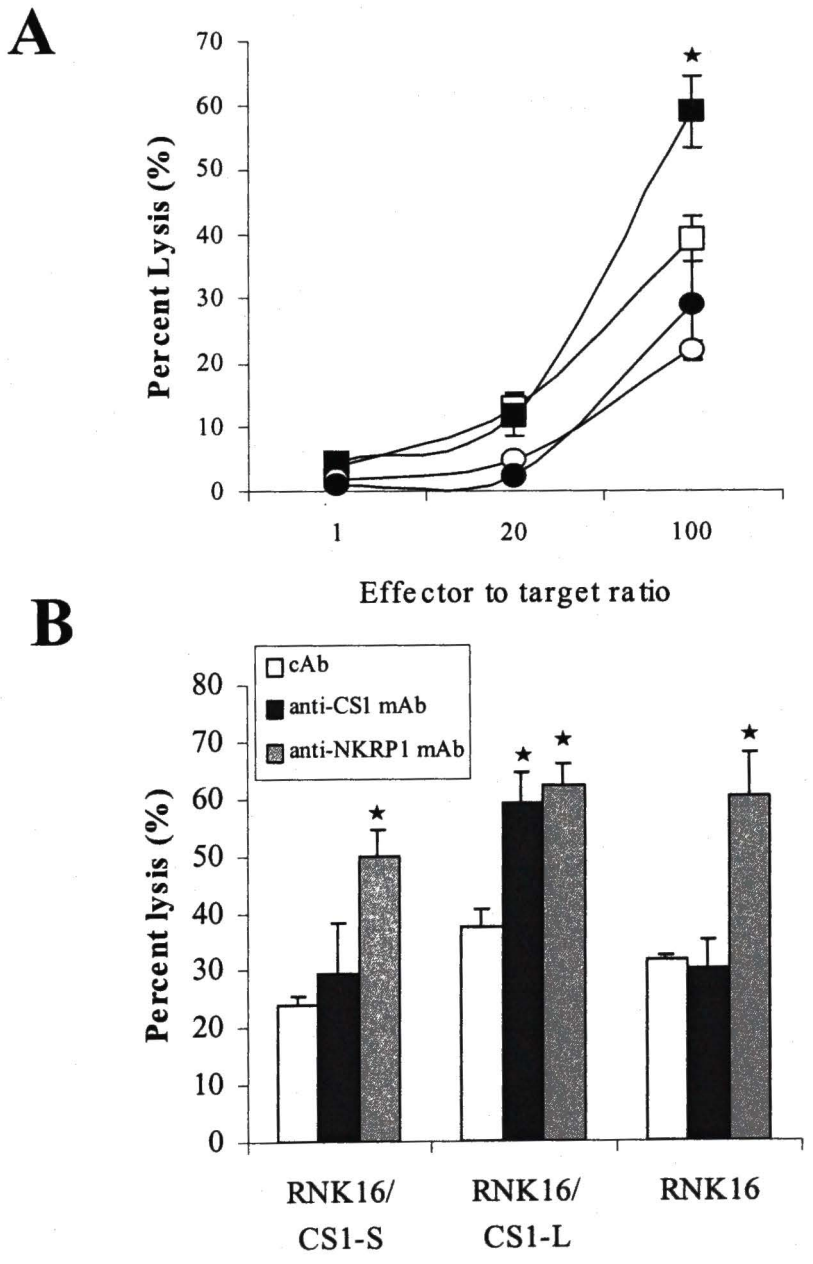
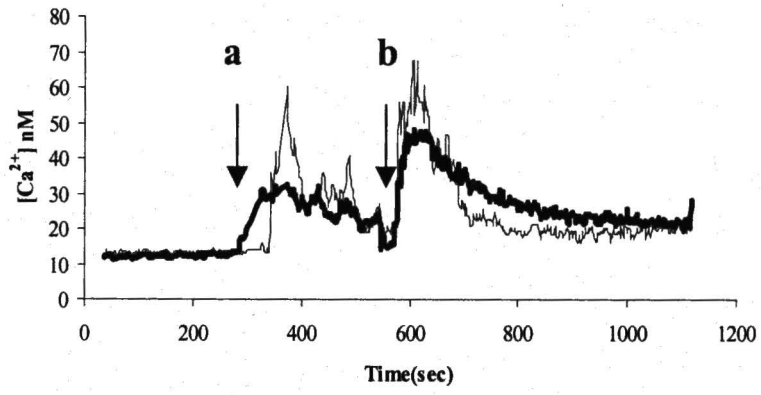

A
Percent Lysis (%)
70
60
50
40
30
20
10
0
1
20
100
Effector to target ratio
B
cAb
anti-CS1 mAb
anti-NKRP1 mAb
Percent lysis (%)
80
70
60
50
40
30
20
10
0
RNK16/ CS1-S
RNK16/ CS1-L
RNK16

FIGURE 3.7 Calcium mobilization determined by the ratio technique fura-2 over time. (A) CS1-L (thick) or CS1-S (thin) RNK-16 transfectants were treated with 5 μg/ml of anti-NKR P1A (10/78) mAb (a) followed by 20 μg/ml of goat anti-mouse Ig (b). (B) CS1-L (□) or CS1-S (○) RNK-16 transfectants were treated with 10 μg/ml of 1G10 mAb (a) and subsequently exposed to 20 μg/ml of goat anti-mouse Ig (b). The data are representative of three separate experiments.

A


80
70
60
50
40
30
20
10
0
[Ca2+] nM
a
b
0
200
400
600
800
1000
1200
Time(sec)

B

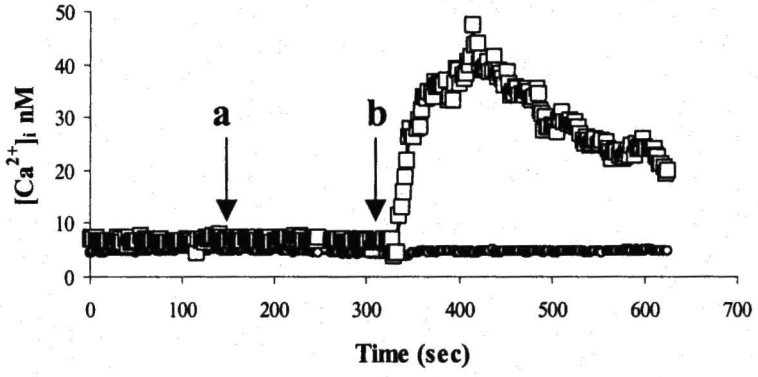

50
40
30
20
10
0
[Ca2+]i nM
a
b
0
100
200
300
400
500
600
700
Time (sec)

FIGURE 3.8 SAP was associated with CS1-L. Interactions between SAP and CS1 were studied by RNK16 cells which were transfected with CS1-L or CS1-S. Pervanadate-treated (+) or untreated (-) transfectants were subjected to immunoprecipitation with 1G10 mAb. The precipitates were separated by 10 % polyacrylamide gels and analyzed by westernblotting with anti-SAP antibody (top) and anti-SHIP1 antibody (bottom). Molecular mass markers are indicated on the left. TL indicates total lysate.

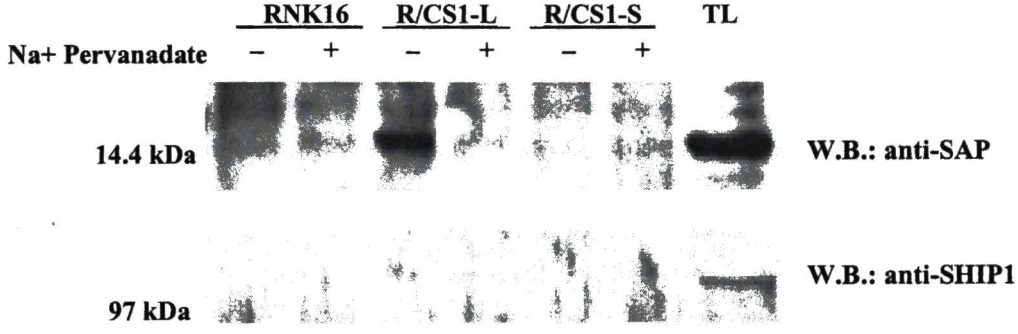
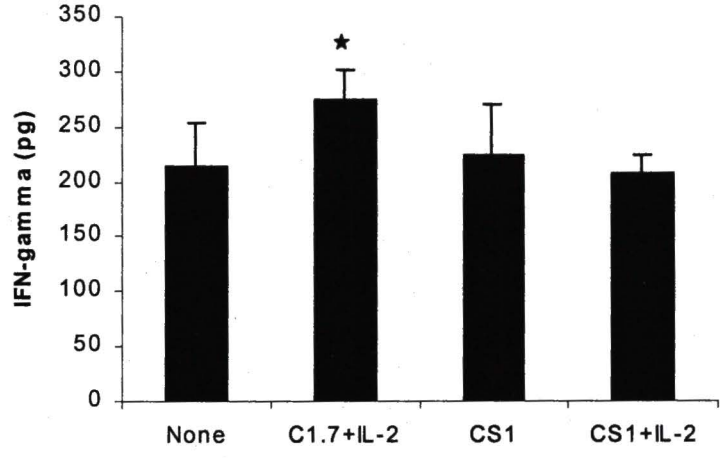
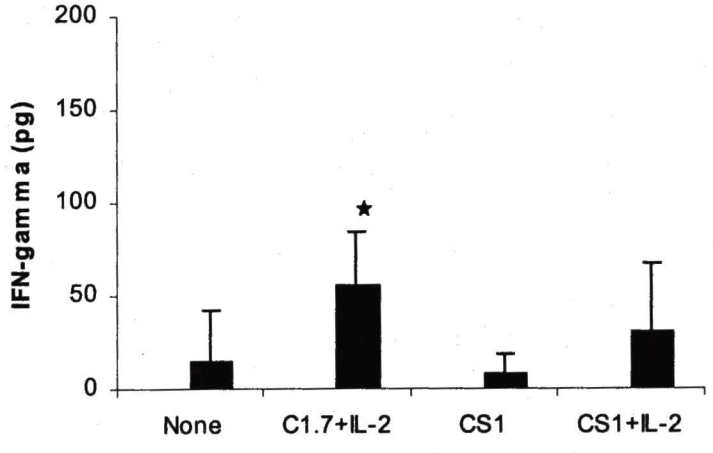

RNK16
R/CS1-L
R/CS1-S
TL
Na+ Pervanadate
– + – + – +
14.4 kDa
W.B.: anti-SAP
97 kDa
W.B.: anti-SHIP1

FIGURE 3.9 CS1 did not induce IFN-γ secretion on NK cells. IFN-γ was studied using NK92 cell line. NK92 cells were incubated with anti-CS1 mAb (30 μg/ml) and/or IL-2 (100 U/ml) for 24h in the presence (A) or absence (B) of K562 as target cells. Anti-2B4 mAb (C1.7) (200 ng/ml) and IL-2 (100 U/ml) were used as a control. IFN-γ was measured by quantitative ELISA as described in materials and methods. Assays were performed quadruplicated and on at least three separate occasions. Each data point is the mean value of the repeated experiments, and the error bars refer to the mean ± S.D. generated from the three independent assays. Student's *t* test was used to calculate *p* values. *, $p<0.05$.

A


350
300
250
200
150
100
50
0
IFN-gamma (pg)
None
C1.7+IL-2
CS1
CS1+IL-2

B


200
150
100
50
0
IFN-gamma (pg)
None
C1.7+IL-2
CS1
CS1+IL-2

CHAPTER IV

EXPRESSION OF CS1 ON FRESHLY ISOLATED AND ACTIVATED HUMAN B LYMPHOCYTES

SUMMARY

It has been shown that CS1 is expressed on activated B cells (139) and that enforced expression of the CS1 gene promoted homotypic B-cell adhesion (140). Since splice variant form of CS1 was identified in human NK cells, the existence of CS1 isoforms was determined by RT-PCR on naïve human B cells as well as CD40-activated B cells. To determine surface expression of CS1, human peripheral blood B cells were negatively isolated and doublestained with anti-CD19 mAb as a B cell marker and anti-CS1 mAb (1G10 mAb). More than 95 % human B cells express CS1 on the surface without any stimulation and upregulated with CD40 stimulation. Various B cell stimulators including anti-CD40 mAb, IL-4, anti-μ Ab and *Staphylococcus aureus* Cowan I (SAC) increased surface expression of CS1 on B cells effectively. Among these, the effect was most prominent especially with anti-CD40 mAb stimulation. This chapter shows the existence isoforms and the expression pattern of CS1 in human B-lymphocytes.

INTRODUCTION

B cell activation is initiated after recognition of specific antigen through cell surface immunoglobulin (Ig), which is of IgM or IgD isotype in naïve, unprimed B cells. Costimulatory molecules and cytokines are required for subsequent proliferation, Ig isotype switching and differentiation of activated B cells. Along with B cell receptor (BCR) engagement, cytokines and ligation of CD40 as a costimulatory stimulation lead to T cell-dependent B cell activation.

CD40 engagement results in B cell proliferation, isotype switching, differentiation to antibody-secreting plasma cells and generation of memory cells (160, 161). Human interleukin–4 (IL-4) represents a cytokine produced by CD4 T cells upon B-T cell interaction. Recombinant (r) IL-4 has pleiotropic effects, it induces proliferation of activated B cells, T cells, thymocytes, and natural killer cell clones (162-164). Indeed, IL-4 acts as a growth and differentiation factor for preactivated B lymphocytes (163, 165) and directly modulates expression of CD23 on both normal and malignant tumor cells (166, 167), a function that is critical for the production of IgE (168). Anti-μ antibody can induce B cell proliferation through activating BCR. Activation with anti-μ triggers a number of protein tyrosine kinases (PTK), including four *src* family members, and Syk which bears homology to the T cell Zap70 (169). Anti-μ cross-linking induced PKC translocation from the cytosolic to the membrane compartment and tyrosine phosphorylation of the p85 and p110 subunits of phosphatidylinositol 3-kinase (P13-kinase)(170). Formaldehyde-fixed *Staphylococcus aureus* Cowan I (SAC), a

staphylococcal strain rich in protein A, is a polyclonal B cell activator that has provided considerable insight into the sequence of events in and factors requisite for normal B cell activation (171). It has been shown that SAC elicited IgM-rheumatoid factor production by human PBMC (172).

It has been shown that CS1 is expressed on activated B cells (139) and enforced expression of the CS1 gene promoted homotypic B-cell adhesion (140). However detailed information about the expression of CS1 on human B cells has not been studied. In this study, I investigated the existence of CS1 isoforms and the expression of CS1 on freshly isolated human B cell or activated B cells with various stimulators including anti-CD40 mAb, IL-4, anti-μ mAb and SAC. Results indicated that CS1 is highly expressed on human B cells and surface expression is upregulated upon B cell stimulations. This effect was mostly prominent on the B cells activated with anti-CD40 mAb and IL-4.

MATERIALS AND METHODS

Reagents

Purified human recombinant (r) IL-4, IL-2, IL-10 and IL-12 were purchased from BD Bioscience. The purified anti-CD40 mAbs and anti-μ mAb were purchased from Beckman coulter and BD Bioscience, respectively. *Staphylococcus aureus* Cowan I cells (SAC) were purchased form Sigma-Aldrich Co. (St. Louis, MO). Anti-CS1 mAb (1G10 mAb) was generated and purified as described earlier (70).

B cell isolation

B cells were obtained by density gradient centrifugation over Histopaque-1077 (Sigma Chem. Co., St. Louis, MO). For depletion of T cells, NK cells, monocytes, granuloytes, dendrtic cells, basophile, platelets and early erythroid cells from PBMC, cells were magnetically labeled using a cocktail of hapten-conjugated CD2, CD4, CD11b, CD16, CD36 and anti-IgE antibodies (Miltenyi Biotec). Cells were labeled for 10 min at 4°C with hapten antibody cocktail and FcR blocking reagent (Miltenyi Biotec). After removing the unbound Abs by washing with cold PBS plus 1% BSA, the cells were incubated with anti-hapten microbead (Miltenyi Biotec) for 15 min at 6°C-12°C and then magnetic labeled cells were depleted by retaining them on a magnetic column (MACS; Miltenyi Biotec)

Culture conditions

Purified B cells were cultured in round-bottomed 96-well plates in 0.2 ml RPMI 1640 medium supplemented with 10 % FBS and incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cultures were performed in triplicate when proliferation of B cells was studied and in quadruplicated when Ig synthesis was studied. Total negatively selected B cells purified by magnetic beads were cultured in 10^5 cells/well.

Reverse Transcriptase (RT)-PCR

Total RNA was isolated with the RNAstat 60 reagent according to the manufacturer's protocol (Teltest Inc, Friendswood, Tex.) and first-strand cDNA was synthesized from 5 μg of total RNA using superscript II (Invitrogen) reverse transcriptase and random primers in a volume of 20ul. PCR was performed using 2 μl of cDNA mixture, under standard conditions for 35 cycles. PCR was performed using primers which were described before (173). Following amplification, portions of the PCR reactions were electrophoresed through a 1.2 % agarose gel.

Flow Cytomery

B cells were cultured as described above and cells were harvested and washed in PBS. Cells were incubated with anti-CS1 mAb followed by PE conjugated mouse IgG. For double staining, FITC conjugated CD19 mAbs were used. FITC- and PE-conjugate mAbs with irrelevant specificity were used as negative controls. A total of 10^4 cells with

light scatter characteristics of lymphocytes of each sample were analyzed using FACScan flow cytometry (Corixa EPICS XL-MCL). The mean fluorescence intensity in the binding assay represents the average of three independent experiments with similar results.

Western blotting

Approximately 2 × 10^6 cells were incubated with or without anti-CD40 mAb (10 ug/ml) for 24 min at 37 °C. Cells were then lysed with 1 % NP-40, 10 mM Tris.Cl pH 7.4, 150 mM NaCl, 100 μg/ml PMSF, and protease inhibitor mixture (Sigma-Aldrich, St. Louis, MO) for 30 min on ice. The lysate was separated by 7.5 % SDS-PAGE. Membranes were probed with 1G10 mAb and anti-actin (Santa cruz Biotechnology, Santa cruz, CA). Western blots were performed according to the manufacturer's chemiluminiscence detection system instructions (Kirkegaad & Perry Laboratories, Gaithersburg, MD).

RESULTS

Only CS1-L express on human B lymphocytes

The expression of CS1 on human B-lymphocytes was studied by RT-PCR. In earlier study, I found that there are two isoforms of CS1 on human NK cells; CS1-L containing two immuno tyrosine switch motif (ITSM) in cytoplasmic domain and CS1-S that does not contain ITSMs (173). Interestingly, RT-PCR result indicates human B cells only expressed long isoform of CS1 (CS-L) while NK cells had both isoforms. The levels of mRNA have not changed upon stimulation with anti-CD40 mAb for 72 hours (Fig. 4.1).

Surface expression of CS1 on human B-lymphocytes.

The surface expression of CS1 on B cells was studied by performing double immunofluorscence using mAbs specific for CD19 and CS1. Freshly isolated human B cells and B cells incubated with anti-CD40 mAb for 24 hours were used. The purity of human B cells from PBMCs was more than 90 % in each experiment. Results showed that surface expression of CS1 on fresh isolated B cells was more than 95 %. Moreover, after cells were stimulated with anti-CD40 mAb for 24 hours, surface expression (Fig. 4. 2) as well as protein expression (Fig. 4. 3) have been upregulated.

CS1 is upregulated on B cells after activation.

I studied the effects of various other activations on CS1 expression on B cells. B cells were negatively selected from PBMC and incubated with B cell stimulators

including anti-CD40 mAb, IL-4, anti-μ mAb and SAC. CS1 expression on B cells was more than 95 %. The surface expressions of CS1 without stimulation have not been changed for 72 hours. However, anti-CD40 mAbs, anti-μ Abs and SAC and IL-4 stimulation increased the levels of the surface expression of CS1 during a culture period of 24 hours and the high expression levels were sustained for up to 72 hours. The most rapid effect was observed in response to anti-CD40 mAbs (Fig 4. 4), which induced high levels of CS1 expression on B cells during a culture period of 6 hours (data not shown). Whereas optimal enhancement of CS1 expression by anti-μ was observed after 24 hours (Fig 4. 6) and IL-4 (Fig 4. 5) effect was observed after 48 hours. Effect of SAC was minimal but there was minor increase of expression (Fig 4. 7). This suggests that CS1 is expressed on B cells constitutively and level is rapidly upregulated after activation of B-lymphocytes.

FIGURE 4.1 mRNA expression of CS1 in human B cells. Fresh isolated human B cells of a healthy donor were cultured in medium for 0h, 72h, and 72h with the stimulation of anti-CD40 mAbs (10μg/ml). RT-PCR was performed using CS1 primers (table 2.1) and products were run on a 1.2 % agarose gel and stained with ethidium bromide. Molecular mass standards are indicated on the left.

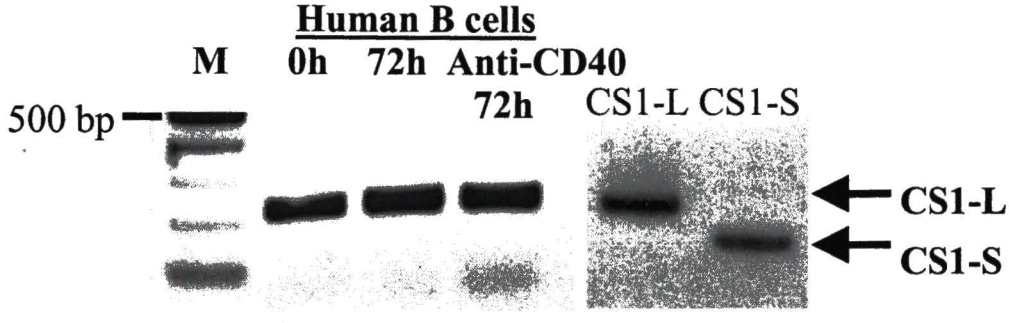

Human B cells
M
0h
72h
Anti-CD40
72h
CS1-L
CS1-S
500 bp
CS1-L
CS1-S

FIGURE 4.2 Surface expression of CS1 on human B cells. Negatively selected human B cells were incubated in medium or activated with anti-CD40 mAbs for 24 hours and stained with FITC-conjugated anti-CD19 mAbs and 1G10 mAbs followed by PE-conjugated secondary Abs.

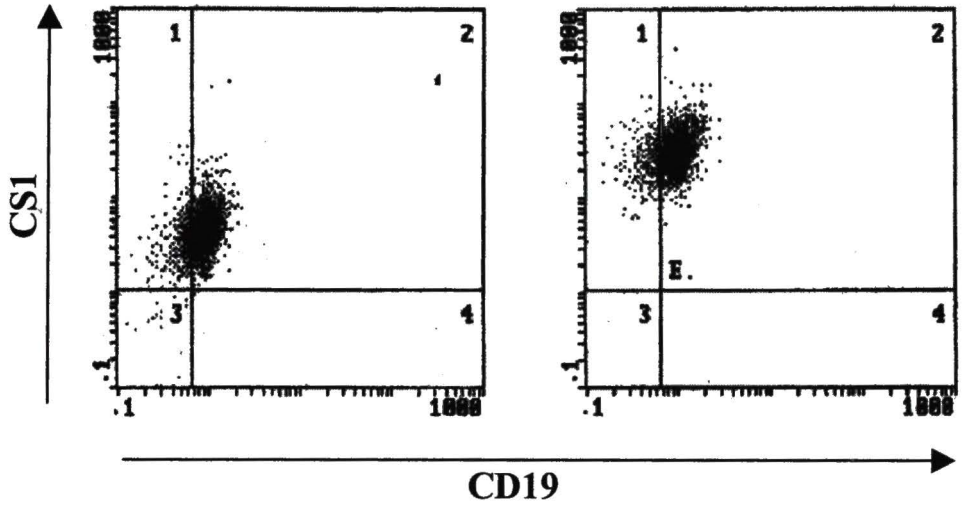

CS1
1
2
3
4
.1
1000
CD19
Medium 0h
Anti-CD40 mAb 24h

FIGURE 4.3 Protein expression of CS1 on human B cells. Negatively selected human B cells were incubated in medium or activated with anti-CD40 mAbs for 24 hours. The lysate were separated by 7.5 % SDS-PAGE. Membranes were probed with 1G10 mAb and anti-actin mAb.

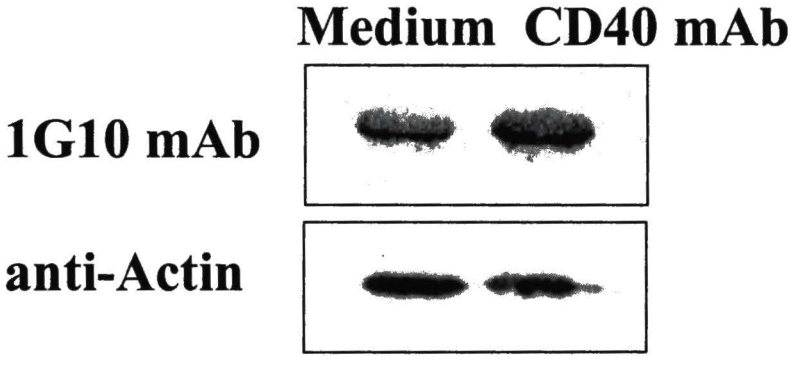

Medium
CD40 mAb
1G10 mAb
anti-Actin

FIGURE 4.4 Effects of anti-CD40 mAb-activated B cells on CS1 expression. Negatively selected human B cells were cultured in medium or anti-CD40 mAb (10 μg/ml) for indicated time points. Thereafter, the cell were harvested, washed and stained with mAb CD19-FITC and anti-CS1 mAb followed by PE-conjugated secondary antibody. Open and filled histograms represent staining with control Ab and mAb 1G10, respectively. CS1 expression on CD19 positive cells was analyzed using FACScan flow cytometer. Numbers in the histogram indicate mean fluorescence intensity (MFI) values. The data are representative of three independent experiments are shown.

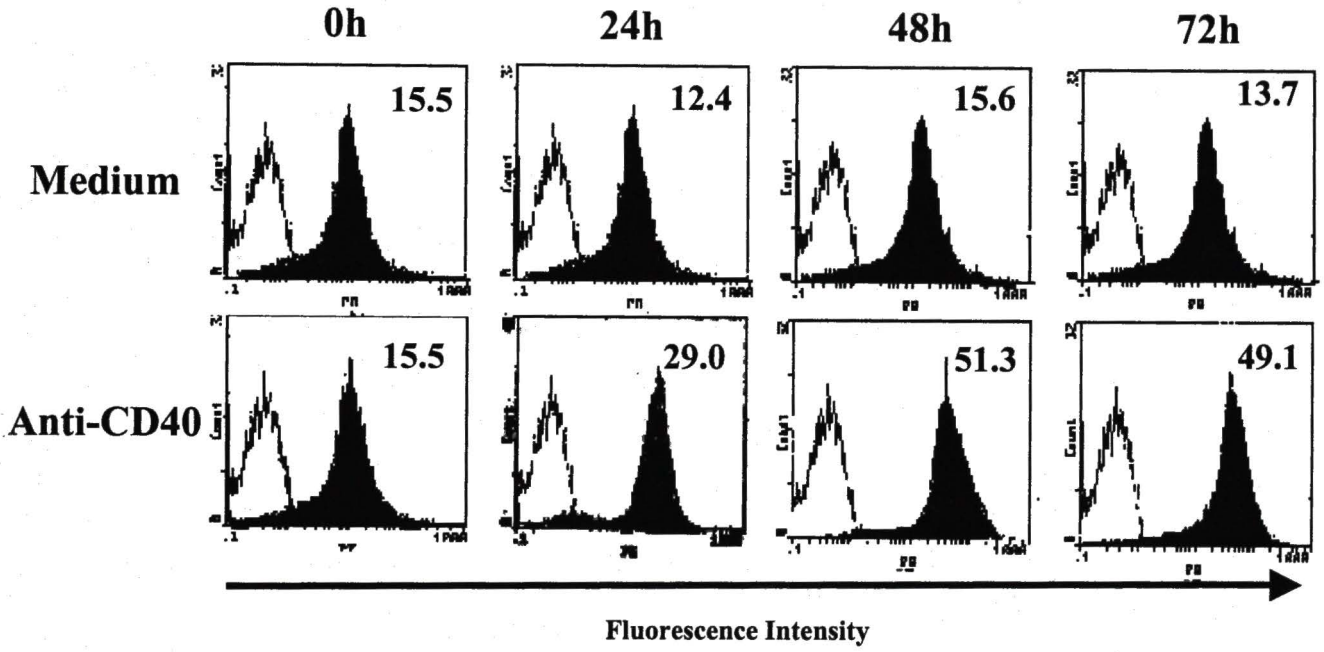

0h
24h
48h
72h
Medium
Anti-CD40
15.5
12.4
15.6
13.7
15.5
29.0
51.3
49.1
Fluorescence Intensity

FIGURE 4.5 Effects of IL-4-activated B cells on CS1 expression. Negatively selected human B cells were cultured in medium or hrIL-4 (5 ng/ml) for indicated time points. Thereafter, the cell were harvested, washed and stained with mAb CD19-FITC and anti-CS1 mAb followed by PE-conjugated secondary antibody. Open and filled histograms represent staining with control Ab and mAb 1G10, respectively. CS1 expression on CD19 positive cells was analyzed using FACScan flow cytometer. Numbers in the histogram indicate mean fluorescence intensity (MFI) values. The data are representative of three experiments is shown.

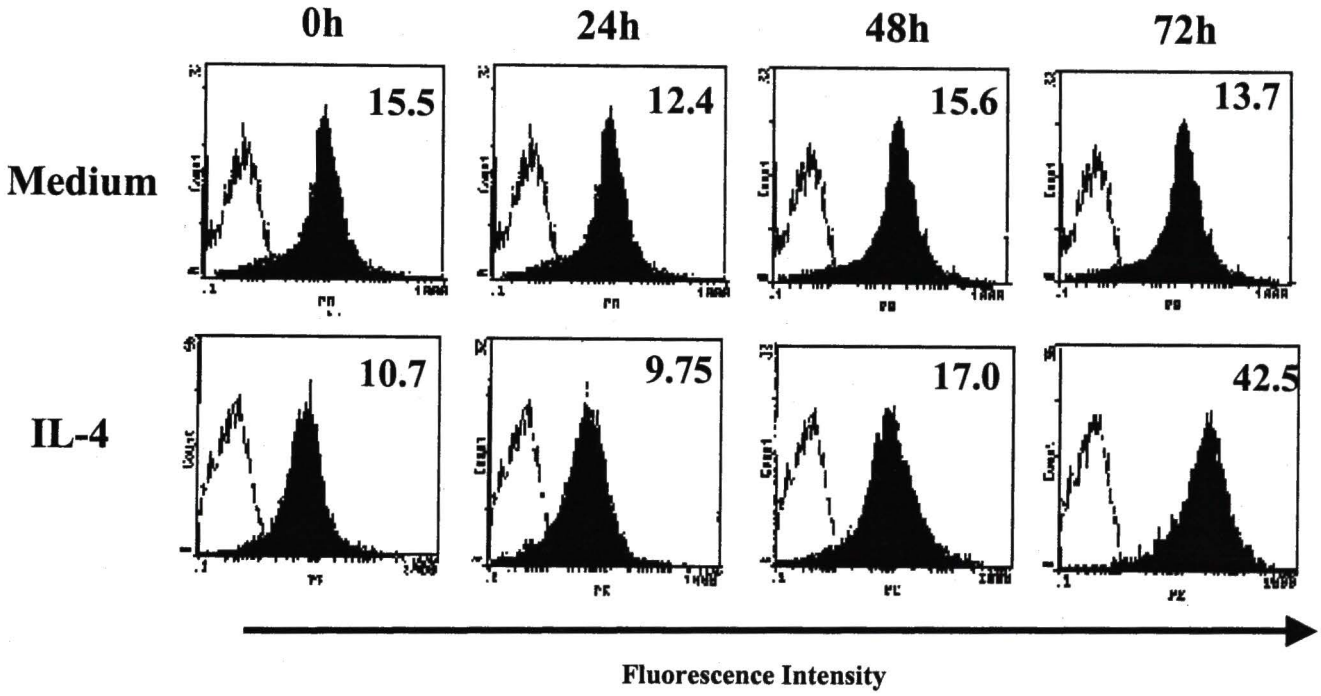

0h
24h
48h
72h
Medium
IL-4
15.5
12.4
15.6
13.7
10.7
9.75
17.0
42.5
Fluorescence Intensity

FIGURE 4.6 Effects of anti-μ mAb-activated B cells on CS1 expression. Negatively selected human B cells were cultured in medium or anti-μ mAb (10 μg/ml) for indicated time points. Thereafter, the cell were harvested, washed and stained with mAb CD19-FITC and anti-CS1 mAb followed by PE-conjugated secondary antibody. Open and filled histograms represent stainings with control Ab and mAb 1G10, respectively. CS1 expression on CD19 positive cells was analyzed using FACScan flow cytometer. Numbers in the histogram indicate mean fluorescence intensity (MFI) values. The data are representative of three experiments is shown.

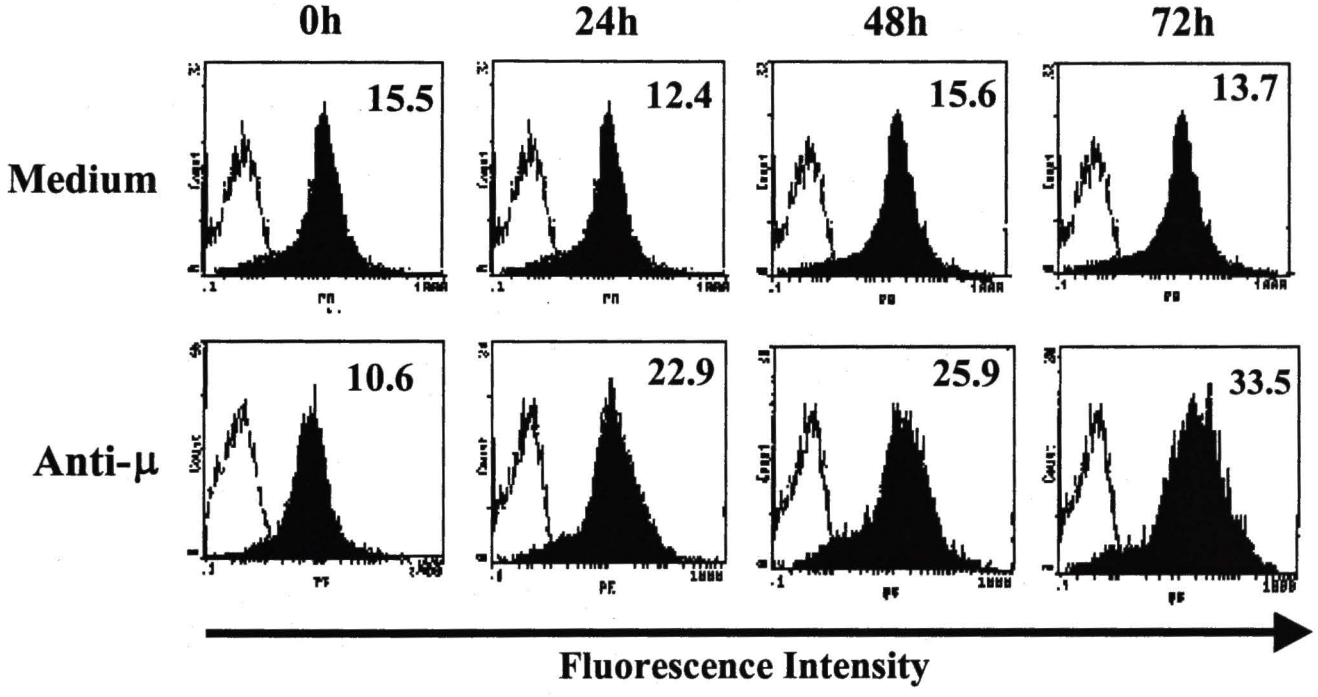
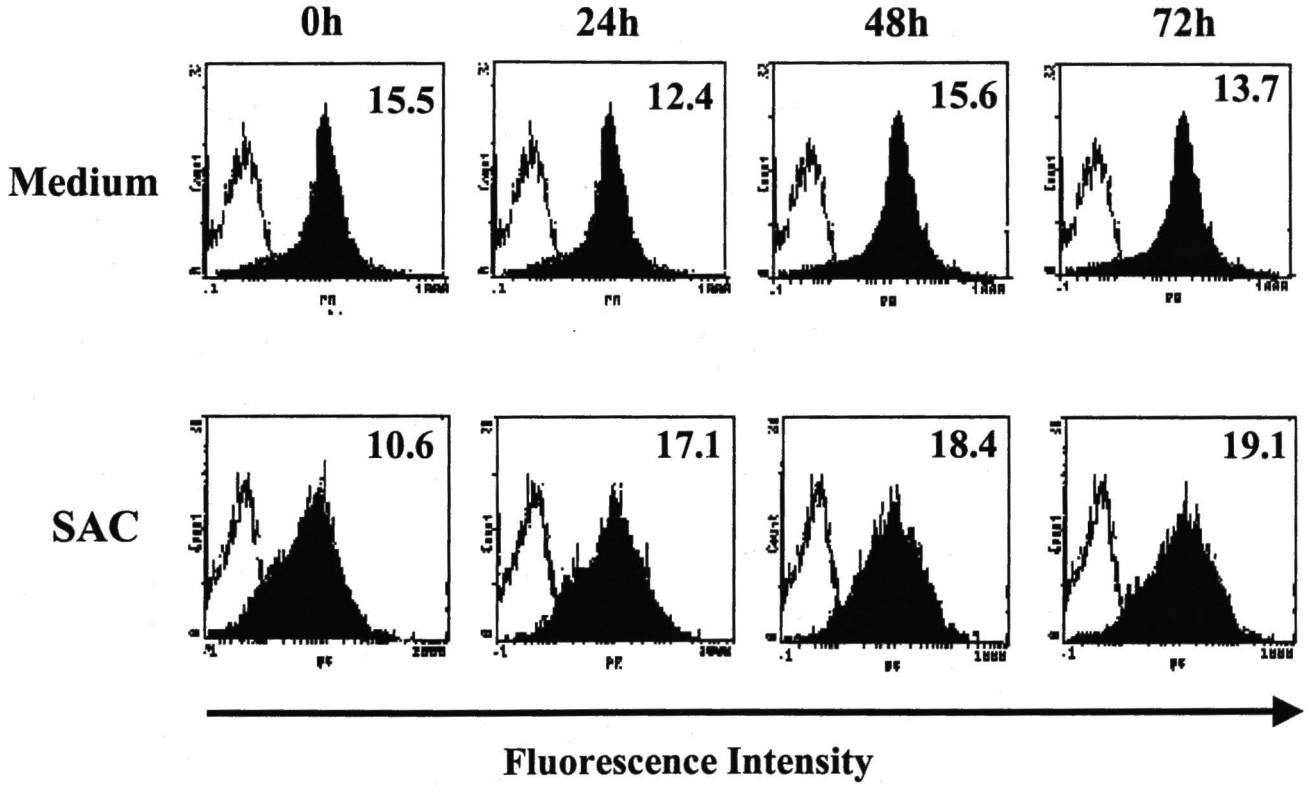

0h
24h
48h
72h
Medium
Anti-μ
15.5
12.4
15.6
13.7
10.6
22.9
25.9
33.5
Fluorescence Intensity

FIGURE 4.7 Effects of SAC-activated B cells on CS1 expression. Negatively selected human B cells were cultured in medium or SAC (0.005%) for indicated time points. Thereafter, the cell were harvested, washed and stained with mAb CD19-FITC and anti-CS1 mAb followed by PE-conjugated secondary antibody. Open and filled histograms represent staining with control Ab and mAb 1G10, respectively. CS1 expression on CD19 positive cells was analyzed using FACScan flow cytometer. Numbers in the histogram indicate mean fluorescence intensity (MFI) values. The data are representative of three experiments is shown.


0h
24h
48h
72h
Medium
SAC
15.5
12.4
15.6
13.7
10.6
17.1
18.4
19.1
Fluorescence Intensity

CHAPTER V

FUNCTIONAL STUDIES OF CS1 IN HUMAN B LYMPHOCYTES

SUMMARY

CS1 is a member of the CD2 subset of the immunoglobulin superfamily of cell surface receptors. Earlier we have been studied expression and function of CS1 on NK cells. Although CS1 was also cloned from cDNA library of human B-lymphocytes as well as of NK cells, very little is known regarding its biology on human B-lymphocytes. Here we investigated the function of CS1 on human B cells. Human B cells expressed only CS1-L isoform and the levels of CS1 expression are upregulated after activation in vitro. Results indicated that anti-CS1 monoclonal antibody strongly enhanced proliferation of both freshly isolated B cells and activated B cells. The enhanced proliferation effects of CS1 were most prominent on B cells activated by anti-CD40 mAbs and/or hrIL-4. Human cytokine microarray results indicated CS1 enhanced mRNA transcripts of flt3 ligand, lymphotoxin A, TNF, and IL-14 which are related with mostly growth promoting activity. These results suggest that autorine cytokines might be the mediators for the function of CS1 on B cell in which it can induce B cell proliferation and also enhance proliferation of activated B cells

INTRODUCTION

The proliferation and differentiation of lymphocytes are regulated by receptors localized on the cell surface. Especially, activation of B cells is initiated by recognition of specific antigen by cell surface Ig, which is pivotal in determining the antigen specificity of the response. Cytokines derived from T helper 2 (Th2) cells such as IL-4, IL-5, and IL-13 and membrane-bound costimulatory molecules expressed by activated CD4+T cells required for subsequent proliferation, Ig production, Ig isotype switching and differentiation of activated B cells (77, 174). Regulation of B cell proliferation and differentiation can be also mediated by monocytes through release of cytokines such as IL-8 and IL-10 (78, 79). B cell co-receptor, CD19, has recently been shown to regulate positive selection and maturation in B lymphopoiesis (175). The CD40 on B cells interacts with CD40L (CD154) on activated CD4+ T cells to provide an essential signal for T-cell-dependent B-cell activation (87-89, 176). One of the CD2 family receptors, signaling lymphocyte activation molecule (SLAM, CD150) could induce proliferation and Ig synthesis by activated human B lymphocytes (109). SLAM has several isoforms, among which soluble, secreted form of SLAM (sSLAM) and membrane-form of SLAM (mSLAM) had B cell growth promoting effects even in the absence of other stimuli and had more potent effect in the presence of polyclonal B cell stimuli and cytokines (109).

CS1 (CRACC, 19A, novel Ly-9) is a novel member of the CD2 family (138-140) and is expressed on the activated B lymphocytes, NK cells, CD8+ cytotoxic T lymphocytes and mature dentritic cells (138, 139). It has been shown that CS1 is a self

ligand and homophilic interaction of CS1 regulates NK cell cytolytic activity (70). The cytoplasmic domain contains two immunoreceptor tyrosine-based switch motifs (ITSMs) observed in some of CD2 family members (138, 139) that provide a docking sites to (141). Additionally, human NK cells express splice variant form of CS1 (CS1-S) which lack the ITSM in the cytoplasmic domain as well as original CS1 (CS1-L). Only CS1 (CS1-L) that contained ITSMs was able to mediate cytotoxicity in human NK cells (173). Even though in earlier study, it had been shown that it is expressed on activated B lymphocytes and enforced expression of CS1 promoted homotypic B-cell adhesion (140), this study showed expression of CS1 on naïve B cell as well and further detailed function on B lymphoctyes has not been studied yet.

In this chapter, I investigated the functions of CS1 on human B-lymphocytes. The data indicated that CS1 could alone induce B cell proliferation. In addition, CS1 also could enhance proliferation of activated B cells, and this effect was prominent in the B cells activated with anti-CD40 mAb or combined with IL-4. Interestingly, cytokine microarray data showed that CS1 increased mRNA transcript of autocrine cytokines which mostly involve in B cell growth effect.

MATERIALS AND METHODS

Reagents

Purified human recombinants (r) IL-4, IL-2, IL-10 and IL-12 were purchased from BD Bioscience. The purified anti-CD40 mAbs and anti-μ mAb were purchased from Beckman Coulter and BD Bioscience. Anti-CS1 mAb (1G10 mAb) was generated and purified as described earlier (70).

B cell isolation

B cells were obtained by density gradient centrifugation over Histopaque-1077 (Sigma Chem. Co., St. Louis, MO). For depletion of T cells, NK cells, monocytes, granuloytes, dendrtic cells, basophile, platelets and early erythroid cells from PBMC, these cells were magnetically labeled using a cocktail of hapten-conjugated CD2, CD4, CD11b, CD16, CD36 and anti-IgE antibodies (Miltenyi Biotec). Cells were labeled for 10 min at 6°C-12°C with hapten antibody cocktail and FcR blocking reagent (Miltenyi Biotec). After removing the unbound Abs by washing with cold PBS plus 1% BSA, the cells were incubated with anti-hapten microbead (Miltenyi Biotec) for 15 min at 6°C-12°C and then magnetically labeled cells were depleted by retaining them on a magnetic column (MACS; Miltenyi Biotec)

Culture conditions

Purified B cells were cultured in round-bottomed 96-well plates in 0.2 ml RPMI 1640 medium supplemented with 10 % FBS and incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cultures were performed in triplicate when proliferation of B cells was studied and in quadruplicated when Ig synthesis was studied. Total negatively selected B cells purified by magnetic beads were cultured in 10^5 cells/well.

Proliferation assay

Purified B cells were cultured in the presence of increasing concentrations of anti-CS1 mAbs. B cells were also activated with anti-μ mAbs (10 μg/ml) or anti-CD40 mAbs (10 μg/ml) in the presence of absence of IL-4 (5 ng.ml), IL-2, IL-10 or IL-12 (100 U/ml) and proliferation was measured by MTS/PMS (Promega, Madison, WI) incorporation during the last 4 h of a 4-day culture. Cultures were performed in triplicate.

Ig analysis ELISA

Quantitation of IgG in cell-free supernatants was performed by an ELISA specific for human IgG (Rockland, PA). 96-well plates were coated with isotype-specific capture Abs at 1μg/well in 0.1 ml of 0.5 M sodium carbonate (pH 9.6) for overnight at RT. The plates were washed five times with a wash solution containing 50 mM of Tris (pH 8.0), 0.1 M of NaCl, and 0.05% Tween 20 and incubated for 30 min at RT with a postcoat

solution (1% BSA in 50 mM of Tris (pH 8.0), 0.15 M of NaCl) to block nonspecific binding. Subsequently, cell-free supernatants and standards were added in duplicate to the plate wells (100 µl/well). Dilution of the standards was made in a blocking solution to avoid binding of serum components to the wells. All plates were incubated for 1h at RT, washed five times with wash solution, and incubated 1 h at RT with 100 µl/well optimal concentration of isotype-specific (anti-human IgG) Abs conjugated with HRP. After washing three times, the enzyme substrate hydrogen peroxide plus 3, 3', 5, 5'-tetramethylbenzidine was added for 20 min at RT in the dark and the reaction was stopped with 50 µl of 2M of H_2SO_4. Plates were read in a microplate reader and isotype concentrations are extrapolated from a reference curve. A revelation program incorporated with the microplate reader calculated the Ig concentration.

Microarray experiments and analysis

Total cellular RNA was isolated using the RNeasy Mini kit (Qiagen) according to the manufacture's instruction. Human common cytokine array was obtained from SuperArray Bioscience Corporation and used according to manufacturer's instructions. Briefly, 2 µg of total RNA was used for generating the biotin-16-UTP (Roche) labeled cRNA. The arrays were exposed to an Alpha Innotech FlourChem™ Image system for image correction. The images were then analyzed using GEArray Expression Analysis Suite (SuperArray Bioscience Corporation). The level of gene expression was determined as the average density and mean had been adjusted to value of 100 and minimum positive value was 10. Background correction was set on minimum value which is a method in

which the lowest average density spot on the array is found and the average across that spot is used as the background correction value. Normalization was performed using GAPDH with comparable results. Comparison of the two arrays was performed using a fold (ratiometric analysis (*x/y*)). Genes showing a fold change of >2.5 were selected for further evaluation.

Quantitative real-time PCR

Total RNA was reverse transcribed to cDNA using Omniscript™ Reverse tracscriptase (Quiagen) and random primers (New England Bio Labs). The RT^2 PCR Primer sets from Superarray (SuperArray Bioscience Corporation) were used for each gene, which were designed to analyze the relative expression of a specific gene based on the sequence from Genebank. Primers were LTA (Cat No. PPH00337A), TNF (Cat No. PPH00341A), IFNG (Cat No. PPH00380A), IL-14 (Cat No. PPH00806A), TNFSF13B (Cat No. PPH01180A) and FLT3LG (Cat No. PPH06324A). RT-PCRs using 2 μg of total RNA were performed under conditions recommended by the manufacturer. PCRs were conducted in the Cepheid SmartCycler® (Cepheid) in a 25-μl reaction mixture containing 12.5 μl of RT^2 Real-Time™ SYBR Green PCR Master Mixture (SuperArray Bioscience Corporation), 1.0 μl of RT^2 PCR primer set, and 11.5 μl of Rnase-free H_2O containing a diluted template cDNA. Samples were preincubated for 15 min at 95°C, them subjected to 40 cycles of amplification at 95°C for 30s for denaturing and at 55°C for 30s for annealing-extension. Data were displayed by SmartCycler® Software 2.0 (Cepheid). The

relative mRNA unit for a given gene measured form a single reverse-transcription reaction was divided by the value obtained for β-actin.

Statistical analysis

Statistical analysis was done using Student's t-test for two samples with equal variance, p value of 0.05 or less was considered significant.

RESULTS

1G10 mAb Induces Proliferation of freshly Isolated B Cells.

In the earlier studies, monoclonal antibody of CS1 (1G10 mAb) has been generated and used to characterize CS1 on human NK cells (173). I tested MTS assay sensitivity with hrIL-4 to decide optimal culture condition to evaluate the effect of CS1 in proliferation on B cells (Fig 5.1). Here, I used our specific antibody for CS1 (1G10 mAb) to study effect of CS1 on proliferation of human B cells. 1G10 mAbs directly induced B cell proliferation of freshly isolated B cells in a dose-dependent manner. Those effects were in the similar range as that induced by IL-2 or lL-4 (Fig. 5.2). Optimal effects by 1G10 mAbs were observed even at concentrations of 2.2 ug/ml. Upon increasing concentrations there was slight decrease of proliferation which was not statistically significant.

Effects of 1G10 mAb on B cell proliferation in the presence of B cell activators.

Anti-CS1 mAbs also enhanced proliferation of B cells cultured in the presence of other B cell growth promoting signals. CS1 stimulation had most synergistic effect on B cell proliferation induced by anti-CD40 mAbs and IL-4 (Fig. 5.3A) and it also enhanced proliferation of B cells cultured in the presence of anti-μ mAbs (Fig. 5.3B). Importantly, proliferation of B cells activated by anti-CD40 mAb alone was enhanced by CS1 stimulation. B cell growth promoting effects was also there when saturating concentrations (100 U/ml) of IL-4 were added to cultures stimulated with anti-CD40 mAbs suggesting that the signaling pathway of CS1 is dependent of that of CD40 or

CD40 combined with IL-4 cytokines. When the cells were stimulated with other cytokines, there was no significant enhancing on the B cell proliferation (Fig. 5.4).

Effects of 1G10 mAb on Ig Synthesis by B Cells.

I studied the effects of CS1 on Ig-production by purified B cells cultured in the presence or absence of anti-CD40 mAbs. Neither anti-CS1 mAbs alone did not induced spontaneous IgG synthesis nor combined with anti CD-40 mAbs did not enhanced IgG synthesis produced by highly purified total B cells, although effect of anti-CD40 mAbs in IgG production was quite obvious as a positive control (Fig. 5.5).

Change of gene expressions of human common cytokines in activated human B-lymphocytes upon 1G10 mAb stimulations.

To predict the mechanism of CS1 to induce B cell proliferation, I performed the cytokine microarray to check any difference in the transcription of autocrine cytokines upon CS1 stimulation. Human common cytokine microarray contained 114 known cytokine genes. Human B cells were negatively selected from PBMCs and stimulated with anti-CD40 mAb and IL-4 for 4 days in the presence or absence of 1G10 mAb. Total RNAs were isolated from each sample and used for generating biotinylated cRNA probe. The results were that 19 cytokine genes (18.3 %) were presented when the B cells were activated with CD40/IL-4. 35 genes (30.8 %) were presented when the activated B cells were additionally stimulated with 1G10 mAb. The data indicated that 12 genes with more than 2.4-fold increased could be differentially expressed between 1G10 mAb stimulated

and unstimulated B cells, of which 7 were highly expressed in 1G10 mAb stimulated human B cells (Table 5.1).

Cytokine expressions in activated human B cells upon 1G10 mAb stimulation

To confirm the differential transcriptional activities of human cytokines between 1G10 mAb stimulated and unstimulated B cells, the mRNA of six cytokines (FLT3LG, IFN-gamma, IL-14, LTA, TNF and TNFSF13B) were measured by real-time quantitative PCR. The mRNA level of LTA was increased with about 3-fold, and the transcripts of FLT3LG, IL-14, and TNF were also showed significant increasing as much as 1.5- to 2.5-fold than that in the 1G10 mAb unstimulated B cells (Fig. 5.7)

FIGURE 5.1 Effect of IL-4 on proliferation of freshly isolated human B cells. Negatively selected human B cells were cultured in the presence of increasing concentration (ng/ml) of IL-4 for 4 days. Proliferation was measured by MTS incorporation during last 4 hour of 4-day culture. Mean ± SD of triplicate cultures that are representative of three experiments is shown.

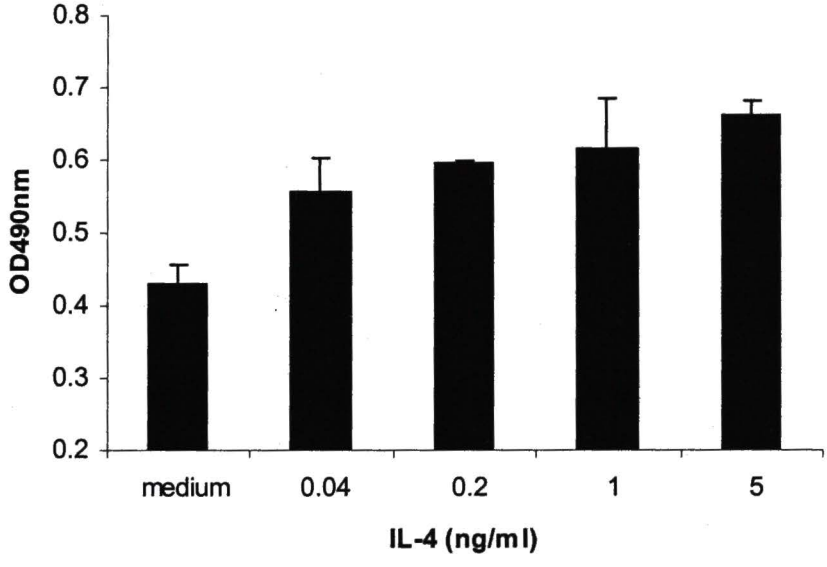

0.8
0.7
0.6
0.5
0.4
0.3
0.2
OD490nm
medium
0.04
0.2
1
5
IL-4 (ng/ml)

FIGURE 5.2 Effect of 1G10 mAb on proliferation of freshly isolated human B cells. Negatively selected human B cells were cultured in the presence of increasing concentration (μg/ml) of 1G10 mAb for 4 days. IL-4 (5 ng/ml) and IL-2 (100U/ml) were treated on B cells as positive controls. Proliferation was measured by MTS incorporation during last 4 hour of 4-day culture. Mean ± SD of triplicate cultures in are representative of three experiments is shown.

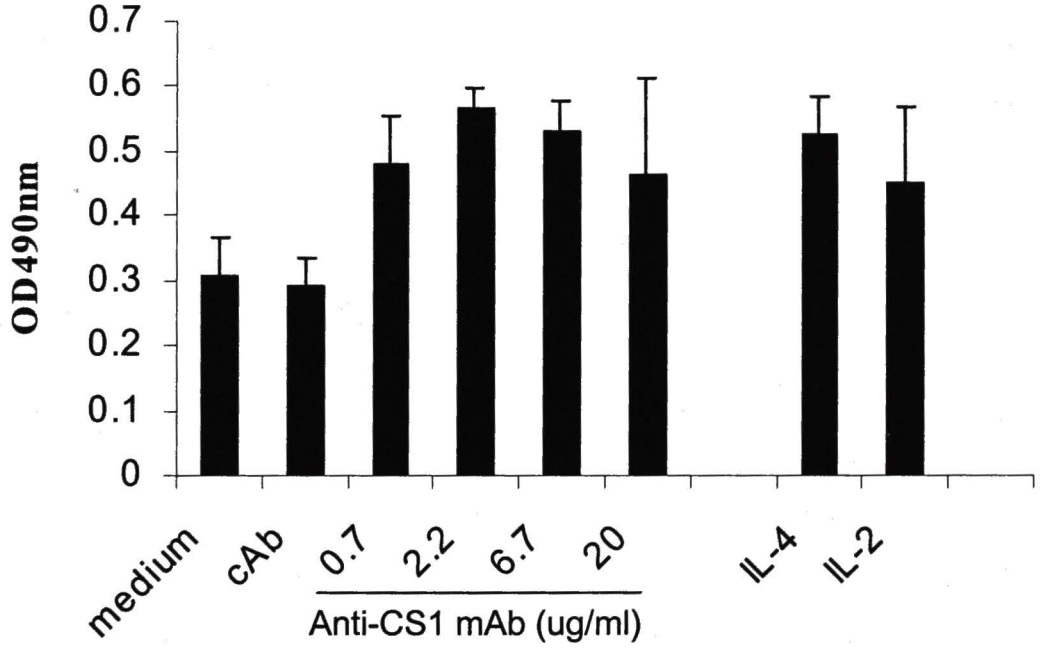
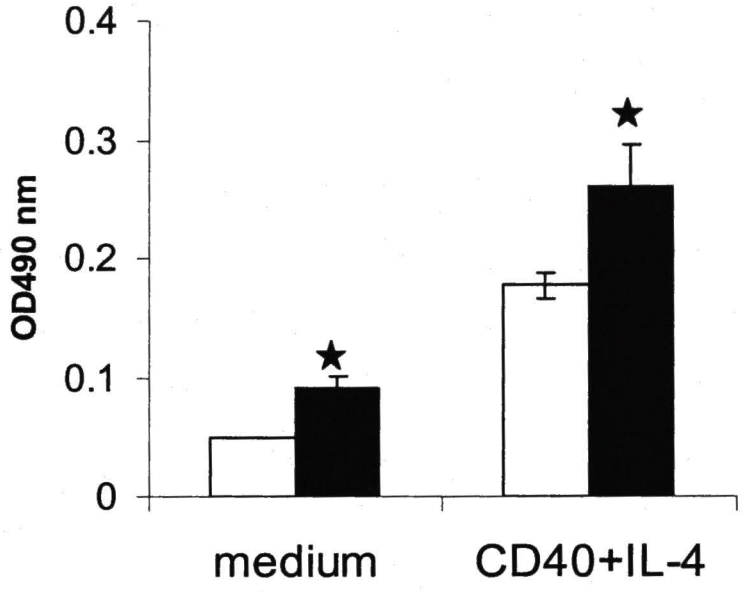

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD490nm
medium
cAb
0.7
2.2
6.7
20
IL-4
IL-2
Anti-CS1 mAb (ug/ml)

FIGURE 5.3 Effects of 1G10 mAb on the proliferation of activated B cells. (A) Negatively selected human B cells were cultured in medium or anti-CD40 mAb (10 μg/ml) and IL-4 (5 ng/ml) in the presence (filled) or absence (open) of 1G10 mAb (20 μg/ml) for 4 days. (B) Freshly isolated human B cells were cultured in medium or anti-μ mAbs (10 μg/ml) in the presence or absence of 1G10 mAb. Proliferation was measured by MTS incorporation during the last 4 h of a 4-d culture. Mean ± SD of triplicate cultures in are representative of three experiments is shown. *, $p < 0.05$

A


0.4
0.3
0.2
0.1
0
OD490 nm
medium
CD40+IL-4

B

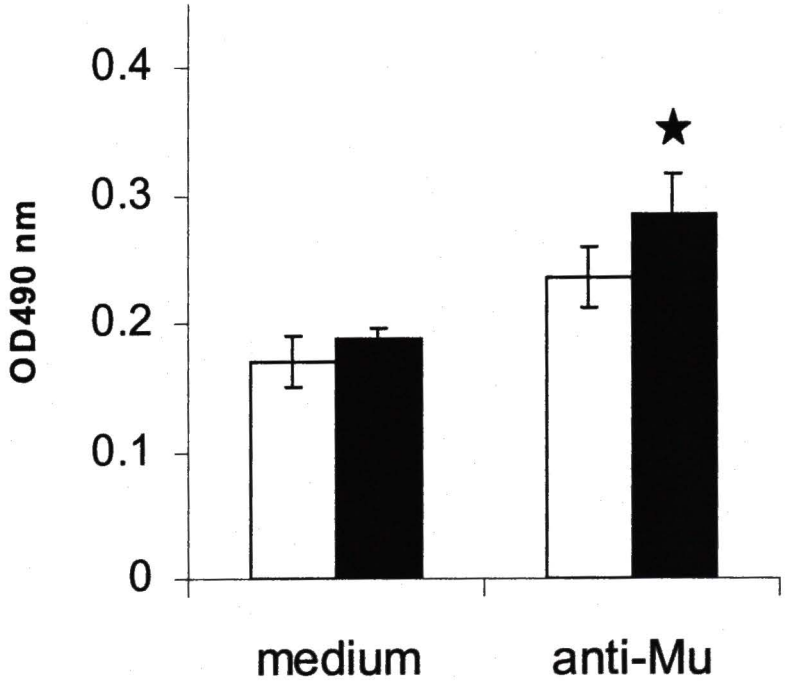

0.4
0.3
0.2
0.1
0
OD490 nm
medium
anti-Mu

FIGURE 5.4 Effects of 1G10 mAb on the proliferation of activated B cells. Negatively selected human B cells were cultured in medium or anti-CD40 mAb (10 μg/ml) in the presence (filled) or absence (open) of 1G10 mAb (20 μg/ml) for 4 days. Human recombinant (hr) IL-2 (100 U/ml), IL-4 (5 ng/ml), IL-10 or IL-12 (100 U/ml) were also used to stimulate B cells. Proliferation was measured by MTS incorporation during the last 4 h of a 4-d culture. Mean ± SD of triplicate cultures in are representative of three experiments is shown. *, $p < 0.05$

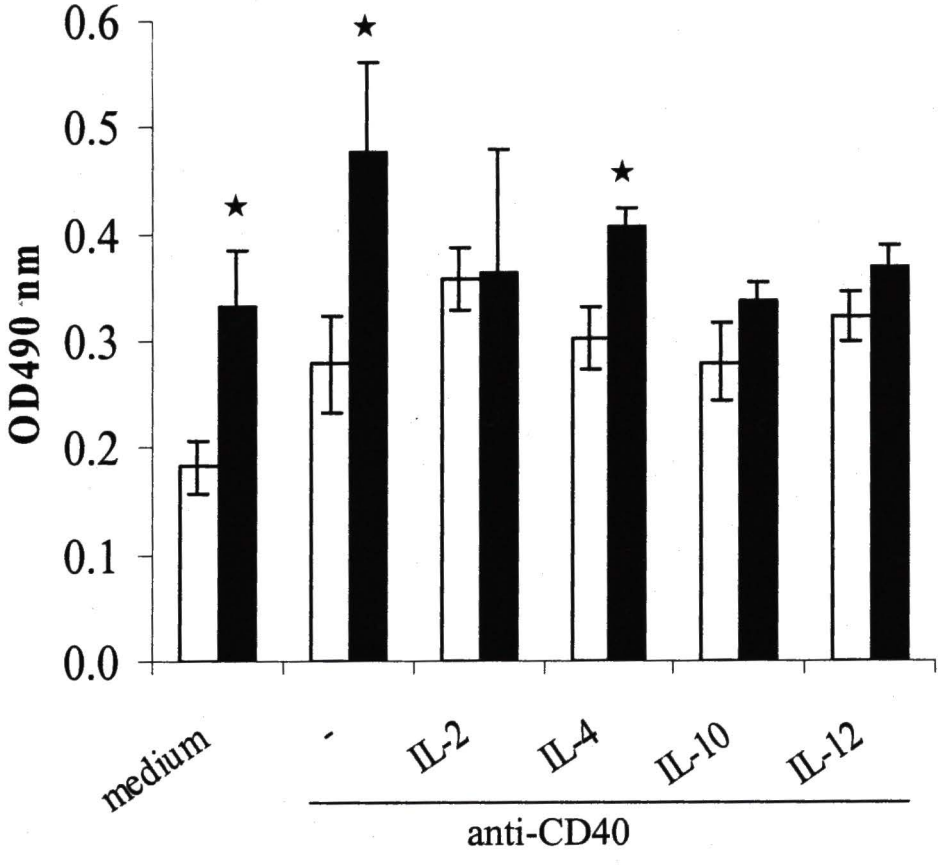

0.6
0.5
0.4
0.3
0.2
0.1
0.0
OD490 nm
medium
-
IL-2
IL-4
IL-10
IL-12
anti-CD40

FIGURE 5.5 Effects of 1G10 mAb on Ig synthesis by purified B cells. Negatively selected total B cells were cultured in the presence or absence of anti-CD40 mAbs (10 μg/ml) and 1G10 mAb (20 μg/ml) for 12 days. Ig levels in the cultured supernatants were measured by ELISA. The data represent mean ± SEM of quadruplicate cultures in four experiments.

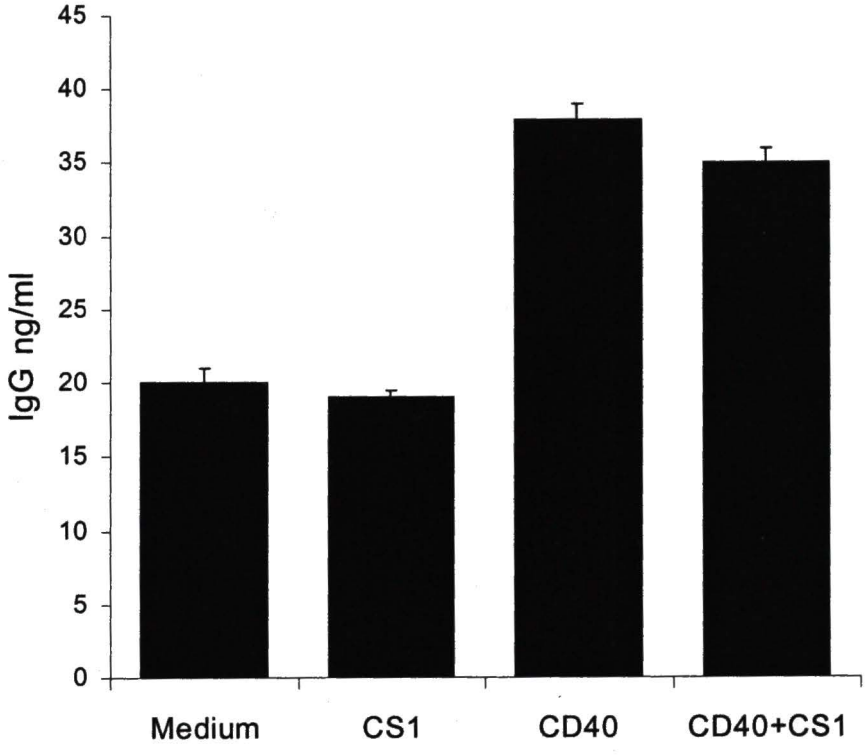

45
40
35
30
25
20
15
10
5
0
IgG ng/ml
Medium
CS1
CD40
CD40+CS1

FIGURE 5.6 Human cytokine microarray on activated human B cells. Negatively isolated human B cells were isolated and cultured in CD40 mAb (10 μg/ml) and IL-4 (5 ng/ml) in the presence (B) or absence (A) of 1G10 mAbs (20 μg/ml) for 4 days. Total RNAs were isolated and biotinylated cRNAs were used as probes and hybridization was performed as described in materials and methods. The arrays were exposed to an Alpha Innotech FlourChem™ Image system for image correction. Normalization was performed using GAPDH with comparable results. Comparison of the two arrays was performed using a fold (ratiometric analysis (x/y)) analysis. Genes showing a fold change of >2.4 were selected for further evaluation.

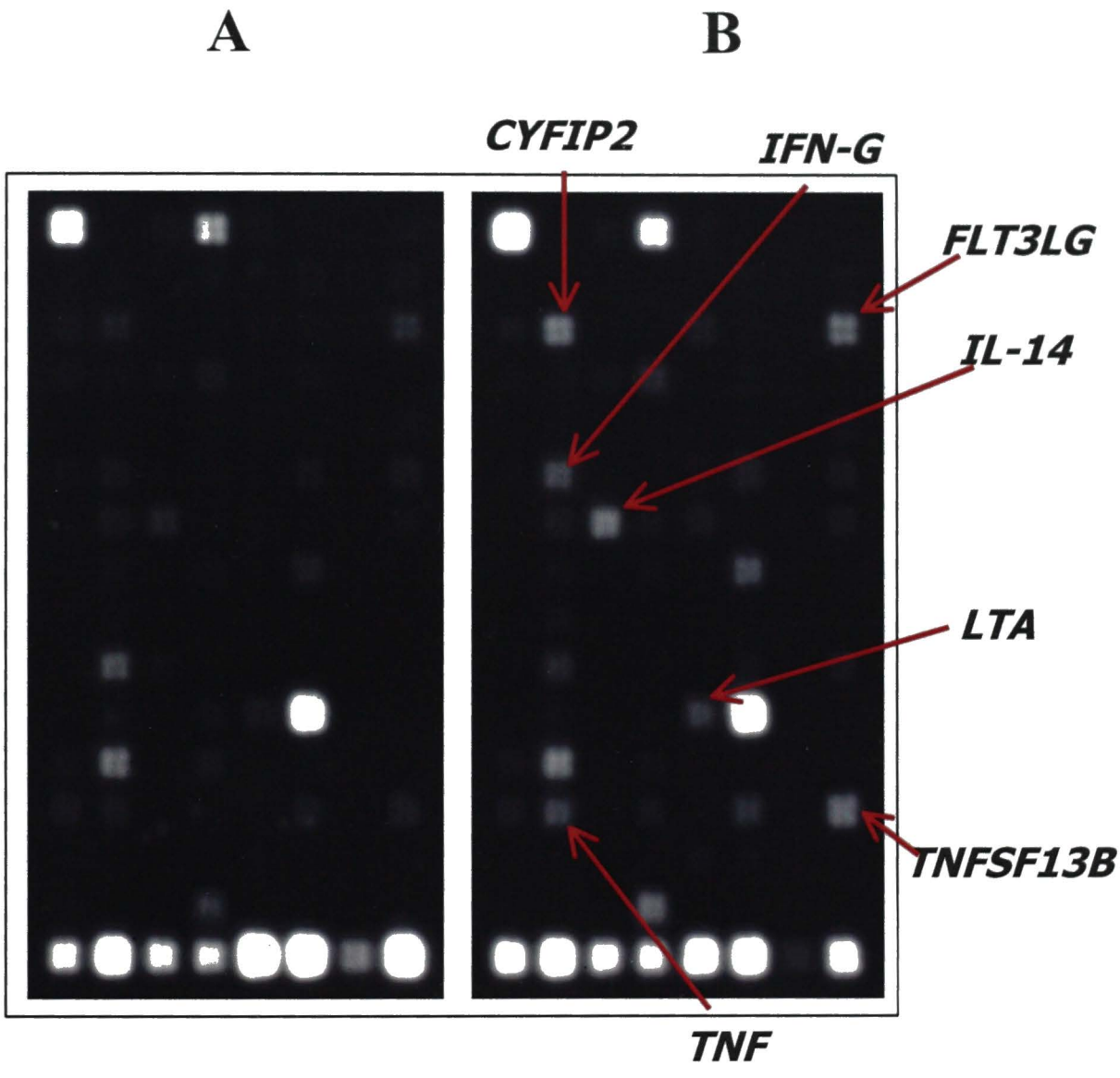
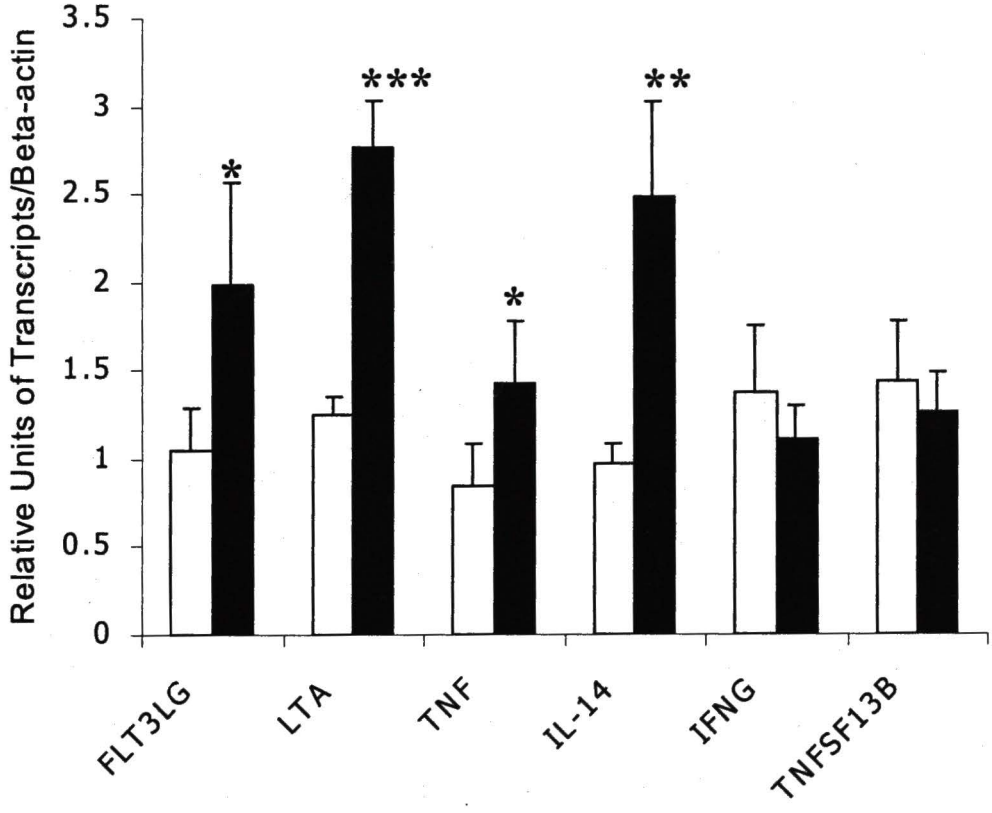

A
B
CYFIP2
IFN-G
FLT3LG
IL-14
LTA
TNFSF13B
TNF

Table 5.1 Upregulated genes induced by CS1 on activated human B cells

Unigene No.	Gene Description	Gene Symbol	CS1	Control	Ratio (CS1/Control)
Hs.519702	Cytoplasmic FMR1 interacting protein 2	CYFIP2	315.68	102.10	3.09
Hs.434053	Family with sequence similarity 3, member C	FAM3C	86.42	31.67	2.73
Hs.428	Fms-related tyrosine kinase 3 ligand	FLT3LG	283.28	112.35	2.52
Hs.856	Interferon, gamma	IFNG	193.63	59.03	3.28
Hs.1121150	Interleukin 14 (Taxilin)	IL-14	302.53	106.53	2.84
Hs.459095	Interleukin 16	IL-16	62.79	59.03	3.34
Hs.512234	Interleukin 6	IL-6	44.24	10.77	4.11
Hs.624	Interleukin 8	IL-8	59.46	13.00	4.57
Hs.36	Lymphotoxin alpha	LTA	152.56	63.66	2.40
Hs.241570	Tumor necrosis factor	TNF	171.64	60.25	2.85
Hs.478275	Tumor necrosis factor, member 10	TNFSF10	60.11	19.12	3.14
Hs.525157	Tumor necrosis factor, member 13B	TNFSF13B	271.99	88.70	3.14

FIGURE 5.7 Higher expression of LTA, TNF, IL-14, and FLT3L genes in CS1-treated CD40/IL-4-activated human B cells. Freshly isolated human B cells were negatively selected from PBMC and activated with CD40 (10μg/ml) and IL-4 (5ng/ml) in the presence or absence of 1G10 mAb for 4 days. Real-time PCR of each transcript was performed. Results are presented as relative units of each transcript compared with β-actin. *, $p < 0.05$, **, $p < 0.01$, *** $p < 0.001$ (Compared with CS1 treated B cells)


Relative Units of Transcripts/Beta-actin
3.5
3
2.5
2
1.5
1
0.5
0
*
***
*
**
FLT3LG
LTA
TNF
IL-14
IFNG
TNFSF13B

CHAPTER VII

DISCUSSION

CS1 is a novel CD150 family receptor identified in human NK cells and B-lymphocytes. It is expressed on NK cells, activated B cells, CD8 T cells, and dendritic cells. Earlier studies have shown that CS1 is a self-ligand and increases NK cell cytolytic activity. In B cells, it increased the homophilic adhesion. However, the detailed immunological functions and biology of CS1 have not been studied, even though it is expressed on wide range of immune cells belonged in both innate and adaptive immunity. First half of this study was to determine the existence of isoforms and the role of CS1 receptor on human NK cells. Second half of the study was to characterize CS1 in human B-lymphocytes.

Characterization of CS1 in human NK cells

In this study, I have cloned a novel splice variant of CS1, CS1-S, that lacks ITSM motifs in the cytoplasmic domain. The existence of CS1 splice variant which differs in the cytoplasmic domain suggested that these isoforms may be involved in sending different signals upon ligand interaction. This study demonstrated that only CS1-L was able to activate NK cell cytotoxicity which means CS1-L transfected RNK-16 cells showed increased killing activity against P815 target cells. Interestingly, there was difference in baseline lysis by the two RNK-16 transfectants. When we performed

redirect lysis assay using untransfected RNK-16 cells, basal lysis of CS1-L transfectants was higher than the basal lysis of untransfected RNK-16 cells as well as CS1-S transfectants. It has been previously shown that CS1 is self-ligand (70). Hence, homophilic interactions between CS1-L on neighboring cells at higher effector cell concentration may lead to an increased basal killing by the CS1-L transfected RNK-16 cells.

Bouchon *et al.* showed anti-CS1 mAb activated lysis of P815 cells by NK92 and also triggered lysis by both SAP-deficient and normal NK cells while the anti-2B4 mAb triggered lysis of P815 only by normal NK cells (139). These results indicate that CS1-mediated NK cell cytotoxicity is independent of SAP binding (139). Nevertheless, Tovar *et al.* showed that SAP binds to the cytoplasmic tail of human CS1-L when the src-related tyrosine kinase, fyn, is presented in transfected COS-7 cells (144). Since CS1-L has been debated with the possibility of binding to SAP and it has two ITSM motifs in its cytoplasmic domain; I examined whether CS1 associates with SAP. Current study demonstrated that CS1-L associated with SAP only in unstimulated condition. This suggests that CS1-L may constitutively bind SAP and SAP dissociates from CS1-L upon stimulation.

SAP plays a significant role in the immune system. Dysfunctional SAP has been identified as the genetic defect in patients with X-linked lymphoproliferative disease (XLP) (EBV) (135, 148). Its mutations also found in patients with B cell non-Hodgkin's lymphoma, some cases of common variable immunodeficiency syndrome and familial hemophoagocytic lymphohistiocytosis (135, 148, 177-179). SAP has recently been

shown to play a crucial role in CD4+ T-cell function for generating lone-term humoral immunity (180).

It has been shown that SAP interacts physically with the SLAM and this interaction serves to block a competing interaction between SLAM and SHP-2 (141). There are other SH2-containing phosphatases which bind to other members of SLAM family receptors such as SH-2 containing protein tyrosine phosphatases (SHP-1, SHP-2) or SH-2 containing inositol phosphatase (SHIP1). Li *et al* showed that SAP binds with comparable affinities to the same sites on receptors, which have ITSM motifs in cytoplasmic domain, as of the SH2 domains of SHP-2 and SHIP1, suggesting that these three proteins may compete against one another in binding to a given receptors (181). But our study did not show the binding with these molecules to CS1-L or CS1-S. This suggests that the mechanism regulating CS1 function may be different than other SLAM family receptors. NK cells from XLP patients which is triggered by anti-CS1 mAb still have the ability to kill target cells (137, 139). In this way, if the cytotoxicity mediated by CS1 did not require SAP, killing activity of NK cells from XLP patients who has nonfunctional SAP might be mediated by CS1 receptors.

Expression of CS1 on human B-lymphocytes

Human peripheral blood B-lymphocytes can be divided into two distinct populations including naïve B lymphocytes (60 %) and memory B lymphocytes (40 %) (182). Following antigen challenge, responding B lymphocytes proliferates and forms germinal centers (183, 184) while interacting with activated T lymphocytes by direct cell-

cell contact and soluble factors (76, 185). This response requires the stimulation of CD40 expression on B lymphocytes by its ligand, CD 154 on activated T lymphocytes (94).

When Murphy *et al.* identified CS1 as a novel immunoglobulin superfamily receptor, 19A, enforced expression of the CS1 gene promoted homotypic cell adhesion in a B-cell-line model (140). Our study showed that CS1 expressed on the surface of most human B cells and level of expression was upregulated by various stimulations especially with anti-CD40 mAbs as early as in 6 hour. B cell activation requires two signals; the first signal required is delivered through its antigen receptor. The second signal is delivered by a helper T cell that recognizes degraded fragments of the antigen as peptides bound to MHC class II molecules on the B-cell surface; the interaction between CD40 ligand (CD40L) on the T cell and CD40 on the B cell contributes an essential part of this second signal. CD40 signaling activates different mediators and pathways including tyrosine kinases (syk and lyn), serine/threonine kinases (c-jun aminoterminal kinase (JNK), p38 mitogen-activated protein kinase (p38 MAPK) and extracellular signal-regulated kinase (ERK)) and phosphatidylinositol 3 (PI-3) kinase (186). These signaling pathways lead to the activation of transcription factors, including nuclear factor-κB (NF-κB), nuclear factor of activated T cells (NF-ATs), and activator protein 1 (AP-1) (187, 188). Through these signaling pathways, CD40 signals can induces proliferation in B cells (86, 188) (103, 189-191), induction of isotype switching (160, 161), differentiation to Ab-secreting plasma cells (105, 192), generation of memory (193) and stimulate up-regulation of cell surface protein such as LFA-1 (194), ICAM-1 (194, 195), CD23 (196), and B7 (197). These studies brought an attention to the effect of CD40 signaling on

upregulating expression of the cell surface proteins which are related with cell-adhesion. In the process of B cell activation, cell-cell interaction is most important step, thus cell-adhesion molecules must be induced or upregulated. Although other stimulations were not as much effective as anti-CD40 mAb, they could also upregulate CS1 expression. Each of the mechanism to regulate CS1 expression might be different. However, it lead to suggest that CS1 might be the one of the cell-adhesion molecules response to B cell activation, which require for the proliferation or further activation of B cells.

Functional studies of CS1 in human B-lymphocytes

There are many studies showing that other members of the CD2 family of cell surface molecules could mediate activation of lymphocytes. Meuer *et al.* showed that CD2 engagement induces T cell proliferation in the absence of T cell receptor signaling (198, 199), and its natural ligand, CD58 (200), on antigen-presenting cells reduces the concentration of antigen required for T cell activation (201). Anti-CD2 mAb also could induce proliferation of CD2 positive human B progenitor cell lines (202, 203). Moreover, blocking of CD2 on the surface of T cells inhibits the capacity of these cells to mediate productive T-B cell interactions (204). It has been reported that mAbs specific for CD58 induce IgE isotype switching and IgE synthesis in a CD40-independent manner in the presence of IL-4 (205) suggesting the stimulatory function of CD58 on B cell isotype switching. Recombinant sSLAM and murine L cells transfected with cDNA encoding mSLAM induced proliferation and IgM, IgG, and IgA production by unfractionated B cells, but only IgM production by sorted $sIgD^+$ B cells was observed, indicating that

SLAM acts as a B cell growth and differentiation promoting molecule, but not as an Ig isotype switch factor (109).

Anti-CS1 mAb (1G10 mAb) alone could increase B cell proliferation and it also enhanced activated B cell proliferation as well. Detailed mechanism of CS1 on B cell proliferation needs to be studied, but I could raise two possible mechanisms: the first mechanism is that CS1 signaling itself could induce B cell proliferation by activating growth related signaling pathways. CS1 contains ITSMs in its cytoplasmic domain and ITSMs can recruit small adaptor molecules including SAP, which is expressed in T and NK cells (116), binds to the ITSMs in the cytoplasmic tail of SLAM (CD150) (135), 2B4 (CD244) (46, 114, 133, 136)), Ly-9 (CD229) and CD84 (143) via its SH2 domain. However SAP is absent in antigen presenting cells (APCs) including B-lymphocytes, instead EAT-2 that encodes a 132 amino acid single SH2 domain protein (206) was identified. Morra *et al.* showed that EAT-2 is the SAP equivalent in B-lymphocytes and macrophages as it binds to CD84, CD150, CD244 and CD229 through its SH2 domain. The structure of a complex of EAT-2 with a phosphotyrosine peptide (pTyr281) derived from the CD150 cytoplasmic tail is very similar to that of SH2D1A with the same peptide. However EAT-2 has high affinity for the pTyr motif in the cytoplasmic tail of CD150, unlike SH2D1A, EAT-2 does not bind to non-phosphorylated CD150 (14). They also have mentioned that preliminary binding experiments using peptides with an amino acid sequence surrounding EAT-2 Tyr127 indicated that the phospholipase-C-γ (PLC-γ) enzyme might bind the EAT-2 tail if phosphorylated. PLC-γ hydrolyzes phosphatidylinositol 4, 5-bisphosphate (PIP_2) to generate two secondary messengers

including inositol 1,4,5-triphophate (IP_3) and diacylglycerol (DAG) (207). DAG activates protein kinase C (PKC) and lead to activate small GTP-binding protein such as Ras and Raf, and mitogen activating protein kinase (MAPK) cascade pathway (208). IP_3 induce intracellular calcium increase and lead to also activate PKC pathway. The specific signaling pathway with the CS1 function on B cells remains to be elucidated. However it is possible that CS1 receptor might transduce the pathways related to B cell growth.

Second mechanism is that CS1 might induce secretion of autocrine cytokines, which are related with B cell growth. Recent studies shows that B cells may regulate immune responses by secreting B cell effector cytokines to immune modulation (209, 210). However, little is known about the factors regulating such cytokine production. Our results suggested that 1G10 mAb can enhance autocrine cytokine production by increasing cytokine mRNA expressions on CD40/IL-4 activated B cells. The mRNA of six cytokines including flt3 (fms-line tyrosine kinase 3) ligand (flt3L), IFN-γ, IL-14, LTA, tumor necrosis factor (TNF) and TNF superfamily factor 13B (TNFSF13B) were selected by the microarray. Real-time quantitative PCR confirmed mRNA level of LTA was increased about 3-fold, and the transcripts of FLT3L, IL-14, and TNF were also showed 1.5- to 2.5- fold increase over unstimulated B cells.

Duddy *et al.* reported that TNF-α, lymphotoxin-α (LTA), and IL-6 were secreted from B cells that are stimulated by sequential BCR and CD40 stimulation. These cytokines could act as autocrine growth and differentiation factors as well as modulators of immune response (211). Li *et al.* demonstrated IFN-γ was secreted by activated B cells in response to IL-12 and anti-IFN-γ antibody completely abrogated B-cell growth factor

activity suggesting B cell proliferative effect of IL-12 may be mediated by autocrine IFN-γ (212).

In the presence of IL-4, engagement of CD40/CD154 or anti-CD40 mAb stimulation not only results in B-cell proliferation and induction of IgE synthesis (107) but also increased production of the cytokine LTA (108). In their study, LTA, also known as TNF-beta-β (TNF-β), can induce proliferation of B cells, enhances CD40/IL-4-stimulated B cells and increase IgE synthesis in normal and atopic donor suggesting that LTA plays a potentially significant role during B-cell proliferation and IgE secretion.

Interleukin-14 (IL-14), called high molecular weight (HMW)-B-cell growth factors (BCGFs), had been identified in non-Hodgkin's lymphoma (NHL)-B. There was constitutive expression of IL-14 by aggressive intermediated lymphomas of the B-cell type NHL-B from the patient. Also the proliferation of lymphoma B cells was increased by IL-14 suggesting that autocrine and paracrine production of IL-14 may play a significant role in the rapid proliferation of aggressive NHL-B (213).

The human TNF-α gene is one of the earliest genes transcribed after stimulation of T and B cells through their antigen receptors. It has been shown that anti-CD40 mAb/IL-4 induced rapid TNF-α gene transcription. B cell proliferation induced by CD40/IL-4 stimulation was inhibited by anti-TNF-α antibody suggesting TNF-α is a required autocrine B-cell growth factor (83).

In the stage of B cell development, interaction between B cell progenitors and stromal cells are quite important. Flt3L is a potent hematopoietic cytokine that affects the growth and differentiation of progenitor and stem cells both in vivo and in vitro (214). It

has been shown that flt3L synergizes with IL-7 in the proliferation of committed B220+proB cells and may contribute to the maintenance of an earlier pro-B cell population suggesting an importance of flt3L in the differentiation and proliferation of early B cell progenitors in vitro (215). Recent study using deficient mice showed that signaling through the cytokine tyrosine kinase receptor flt3L and IL-7R alpha are indispensable for fetal and adult B cell development (216).

It is very interesting that all cytokines that were enhanced by 1G10 mAb stimulation is related with growth promoting activity on B-lymphocytes according to previous literatures. Although the protein levels of cytokine secretion should be confirmed, it is enough to raise hypothesis that CS1 can accelerate proliferation of CD40/IL-4 activated human B cell by giving the prolonged secretions of autocrine cytokines related with B cell growth activity. This way it will be also interesting to investigate which cytokines are responsible for the function of CS1 on enhancing B cell proliferation.

Since 1G10 mAb alone could increase B cell proliferation as well, another question may be raised whether CS1 exert these effect with the same mechanism or different mechanism. This might be reveal new function of CS1 on early B cell development because one of the cytokine enhanced by CS1 was flt3L that plays important role in early B cell development.

Interaction of NK-B cells

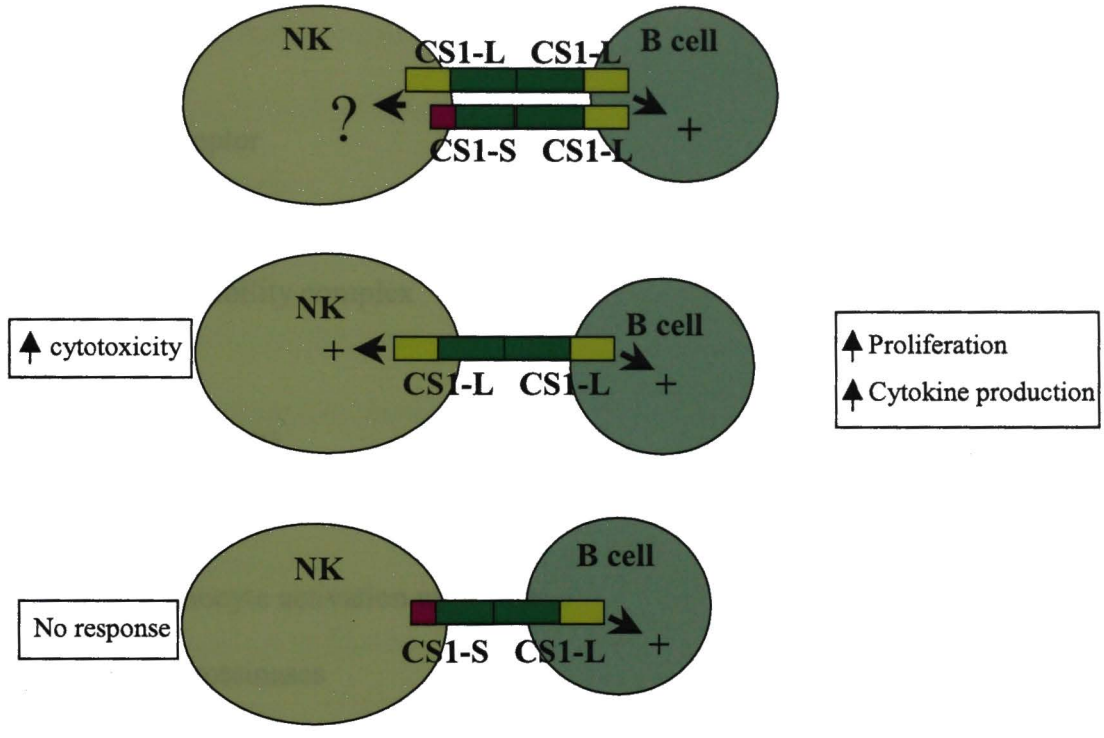
The interaction of NK cells and B-lymphocytes are well reported (217, 218). NK cells have been reported with a variety of other functions in immune response including the regulation of hemopoiesis (217, 218) and regulation of Ig production (219-224) in addition to their cytotoxic function and cytokine secretion. NK cells can regulate B cells by suppressing or enhancing antibody production. These events require cell-cell interaction between NK cells and resting B cells (225). It has been also demonstrated that acitvated human NK cells have the ability to induce resting B cells to secrete immunoglobulin (225). They showed that human NK cells can induce T cell-independent Ab production by a two step process. The first step consists of surface contact between NK cells and resting B cells that enables these B cells to become cytokine responsive. The second step is the secretion of appropriate cytokines by activated NK cells. This way, NK cells are capable of inducing resting B cells to produce IgM and IgG.

When NK cells and B-lymphocytes modulate each other's functions, it requires cell-cell contact and surface receptors on each cells might play important role. I observed that CS1 was expressed on more than 95 % of fresh isolated human B cells. Since CS1 is self-ligand molecule and it expresses on NK cells and B-lymphocytes, CS1 might be one of the key molecules for NK cell-B cell interaction and give a rise such functions on both cells. The finding that only CS1-L acted as an activating receptor implicates the CS1 isoforms may differentially regulate NK and B cell functions. Especially human NK cells express both CS1-L and CS1-S isoform; this led me to speculate three senarios of NK-B cell interactions. One is when NK cell express both CS1-L and CS1-S on the surface, B

cell will be activated in any cases. However, in this case, NK cells may get activating signal because CS1-L function as an activating receptor and CS1-S did not show any function. Second senario is when NK cell expresses CS1-L dominantely, the interaction of CS1 on NK and B cells results in both way. Thus NK cell will be activated and lead to increase cytolytic activity. B cell is also stimulated by CS1 ligation and result in proliferation and cytokine productions. However, when NK cell express CS1-S dominantely, the effect will be only into B lymphocytes which increase proliferation and cytokine production (Fig 6.1).

How the expressions of CS1 isoforms in NK cells are regulated needs to be studied. In addition, CS1 is also found on dendritic cells (DCs) and CD8 T cells. As I mentioned in introduction, there are recent studies showing the importance of the interaction between NK cells and DCs in the aspect of the cross talk of innate and adaptive immune responses. The isoforms and the function of CS1 in DCs and T cells have not been identified, yet. Based on the information in current study, CS1 play an important role in NK cells and B cells. It will be very interesting to demonstrate the function of CS1 on DCs and T cells and investigate effect of the interaction among these cell types through CS1 receptor. These results might lead to novel information to find out the mechanism of immune disease including XLP.

Figure 6.1 Senarios of NK-B cell interactions through CS1. Ligation of CS1-L on NK cell and CS1-L on B cell lead to activate both cells (two-way response), however ligation of CS1-S on NK cell and CL1-L on B cell lead to activate only B cells (one-way response).


NK
CS1-L CS1-L
B cell
?
+
CS1-S CS1-L
NK
B cell
cytotoxicity
+
CS1-L CS1-L
+
Proliferation
Cytokine production
NK
B cell
No response
CS1-S CS1-L
+

LIST OF ABBREVIATIONS

NK, natural killer

Ig, Immunoglobulin

BCR, B-cell antigen receptor

TCR, T-cell antigen receptor

MHC, major histocompatibility complex

IFN, interferon

APCs, antigen presenting cells

ICOS, inducible co-stimulator

SLAM, signaling lymphocyte activation molecule

MMPs, matrix metalloproteinases

ADCC, antibody dependent cell mediated cytotoxicity

DCs, dendritic cells

PBMCs, peripheral blood mononuclear cells

KIRs, killer-cell immunoglobulin-like receptors

ITIMs, immunoreceptor tyrosine-based inhibitory motifs

SHP, SH2-domain-containing protein tyrosine phosphatase

SHIP, SH2-domain containing inositol polyphosphate 5' phosphatase

NCRs, natural cytotoxicity receptors

ITAMs, immunoreceptor tyrosine activation motifs

PI3-kinase, phophatidylinositol 3-kinase

ULBP, cytomegalovirus UL-16 binding proteins

TNF, tumor necrosis factor

LTA, lymphotoxin-α

ITSMs, immunoreceptor tyrosine-based switch motifs

SH2D1A, SH2-containing adapter protein 1A

SAP, SLAM-associated protein

EAT-2, EWS-activated transcript 2

Erk, extracellular signal-regulated kinase

XLP, X-linked lymphoproliferative disease

EBV, Epstein-Barr Virus

LCLs, lymphoblstoid cell lines

mSLAM, membrane bound form SLAM

sSLAM, secreted form SLAM

vmSLAM, variant membrane form SLAM

cSLAM, cytoplasmic form SLAM

IL-4, interleukin–4

PTK, protein tyrosine kinases

NF-κB, nuclear factor-κB

NF-ATs, nuclear factor of activated T cells

AP-1, activator protein 1

PLC-γ, phospholipase-C-γ

PIP_2, phosphatidylinositol 4, 5-bisphosphate

DAG, inositol 1,4,5-triphophate (IP_3) and diacylglycerol

PKC, protein kinase C

MAPK, mitogen activating protein kinase

flt3, fms-line tyrosine kinase 3

BCGFs, high molecular weight (HMW)-B-cell growth factors

REFERENCES

1. Vitetta, E. S., M. T. Berton, C. Burger, M. Kepron, W. T. Lee, and X. M. Yin. 1991. Memory B and T cells. *Annu Rev Immunol 9:193.*

2. Castriconi, R., M. Della Chiesa, and A. Moretta. 2004. Shaping of adaptive immunity by innate interactions. *C R Biol 327:533.*

3. Ehlers, S. 2004. Commentary: adaptive immunity in the absence of innate immune responses? The un-Tolled truth of the silent invaders. *Eur J Immunol 34:1783.*

4. Castellano, G., A. M. Woltman, F. P. Schena, A. Roos, M. R. Daha, and C. van Kooten. 2004. Dendritic cells and complement: at the cross road of innate and adaptive immunity. *Mol Immunol 41:133.*

5. Hozumi, N., and S. Tonegawa. 1976. Evidence for somatic rearrangement of immunoglobulin genes coding for variable and constant regions. *Proc Natl Acad Sci U S A 73:3628.*

6. Davis, M. M., and P. J. Bjorkman. 1988. T-cell antigen receptor genes and T-cell recognition. *Nature 334:395.*

7. Zamoyska, R. 1998. CD4 and CD8: modulators of T-cell receptor recognition of antigen and of immune responses? *Curr Opin Immunol 10:82.*

8. Paulsson, K. M. 2004. Evolutionary and functional perspectives of the major histocompatibility complex class I antigen-processing machinery. *Cell Mol Life Sci 61:2446.*

9. Bryant, P., and H. Ploegh. 2004. Class II MHC peptide loading by the professionals. *Curr Opin Immunol 16:96.*

10. Germain, R. N. 1994. MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. *Cell 76:287.*

11. Sharpe, A. H., and G. J. Freeman. 2002. The B7-CD28 superfamily. *Nat Rev Immunol 2:116.*

12. Arimura, Y., H. Kato, U. Dianzani, T. Okamoto, S. Kamekura, D. Buonfiglio, T. Miyoshi-Akiyama, T. Uchiyama, and J. Yagi. 2002. A co-stimulatory molecule on activated T cells, H4/ICOS, delivers specific signals in T(h) cells and regulates their responses. *Int Immunol 14:555.*

13. Tangye, S. G., J. H. Phillips, and L. L. Lanier. 2000. The CD2-subset of the Ig superfamily of cell surface molecules: receptor-ligand pairs expressed by NK cells and other immune cells. *Semin Immunol 12:149.*

14. Morra, M., D. Howie, M. S. Grande, J. Sayos, N. Wang, C. Wu, P. Engel, and C. Terhorst. 2001. X-linked lymphoproliferative disease: a progressive immunodeficiency. *Annu Rev Immunol 19:657.*

15. Veillette, A. 2002. The SAP family: a new class of adaptor-like molecules that regulates immune cell functions. *Sci STKE 2002:PE8.*

16. Lanier, L. L., A. M. Le, S. Cwirla, N. Federspiel, and J. H. Phillips. 1986. Antigenic, functional, and molecular genetic studies of human natural killer cells and cytotoxic T lymphocytes not restricted by the major histocompatibility complex. *Fed Proc 45:2823.*

17. Vankayalapati, R., P. Klucar, B. Wizel, S. E. Weis, B. Samten, H. Safi, H. Shams, and P. F. Barnes. 2004. NK cells regulate CD8+ T cell effector function in response to an intracellular pathogen. *J Immunol 172:130.*

18. Shi, F. D., H. B. Wang, H. Li, S. Hong, M. Taniguchi, H. Link, L. Van Kaer, and H. G. Ljunggren. 2000. Natural killer cells determine the outcome of B cell-mediated autoimmunity. *Nat Immunol 1:245.*

19. Ferlazzo, G., B. Morandi, A. D'Agostino, R. Meazza, G. Melioli, A. Moretta, and L. Moretta. 2003. The interaction between NK cells and dendritic cells in bacterial infections results in rapid induction of NK cell activation and in the lysis of uninfected dendritic cells. *Eur J Immunol 33:306.*

20. Cerwenka, A., and L. L. Lanier. 2001. Natural killer cells, viruses and cancer. *Nat Rev Immunol 1:41.*

21. Trinchieri, G. 1989. Biology of natural killer cells. *Adv Immunol 47:187.*

22. Smyth, M. J., D. I. Godfrey, and J. A. Trapani. 2001. A fresh look at tumor immunosurveillance and immunotherapy. *Nat Immunol 2:293.*

23. Biron, C. A. 1997. Activation and function of natural killer cell responses during viral infections. *Curr Opin Immunol 9:24.*

24. Naume, B., and T. Espevik. 1994. Immunoregulatory effects of cytokines on natural killer cells. *Scand J Immunol 40:128.*

25. Kitson, R. P., P. M. Appasamy, U. Nannmark, P. Albertsson, M. K. Gabauer, and R. H. Goldfarb. 1998. Matrix metalloproteinases produced by rat IL-2-activated NK cells. *J Immunol 160:4248.*

26. Chuang, S. S., M. H. Kim, L. A. Johnson, P. Albertsson, R. P. Kitson, U. Nannmark, R. H. Goldfarb, and P. A. Mathew. 2000. 2B4 stimulation of YT cells induces natural killer cell cytolytic function and invasiveness. *Immunology 100:378.*

27. Moretta, A., C. Bottino, M. Vitale, D. Pende, R. Biassoni, M. C. Mingari, and L. Moretta. 1996. Receptors for HLA class-I molecules in human natural killer cells. *Annu Rev Immunol 14:619.*

28. Ferlazzo, G., M. L. Tsang, L. Moretta, G. Melioli, R. M. Steinman, and C. Munz. 2002. Human dendritic cells activate resting natural killer (NK) cells and are recognized via the NKp30 receptor by activated NK cells. *J Exp Med 195:343.*

29. Fernandez, N. C., C. Flament, F. Crepineau, E. Angevin, E. Vivier, and L. Zitvogel. 2002. Dendritic cells (DC) promote natural killer (NK) cell functions: dynamics of the human DC/NK cell cross talk. *Eur Cytokine Netw 13:17.*

30. Gerosa, F., B. Baldani-Guerra, C. Nisii, V. Marchesini, G. Carra, and G. Trinchieri. 2002. Reciprocal activating interaction between natural killer cells and dendritic cells. *J Exp Med 195:327.*

31. Zingoni, A., T. Sornasse, B. G. Cocks, Y. Tanaka, A. Santoni, and L. L. Lanier. 2005. NK cell regulation of T cell-mediated responses. *Mol Immunol 42:451.*

32. De Arruda Hinds, L. B., M. S. Alexandre-Moreira, D. Decote-Ricardo, M. P. Nunes, and L. M. Pecanha. 2001. Increased immunoglobulin secretion by B lymphocytes from Trypanosoma cruzi infected mice after B lymphocytes-natural killer cell interaction. *Parasite Immunol 23:581.*

33. Blanca, I. R., E. W. Bere, H. A. Young, and J. R. Ortaldo. 2001. Human B cell activation by autologous NK cells is regulated by CD40-CD40 ligand interaction: role of memory B cells and CD5+ B cells. *J Immunol 167:6132.*

34. Gao, N., T. Dang, W. A. Dunnick, J. T. Collins, B. R. Blazar, and D. Yuan. 2005. Receptors and counterreceptors involved in NK-B cell interactions. *J Immunol 174:4113.*

35. Berke, G. 1994. The binding and lysis of target cells by cytotoxic lymphocytes: molecular and cellular aspects. *Annu Rev Immunol 12:735.*

36. Smyth, M. J., E. Cretney, J. M. Kelly, J. A. Westwood, S. E. Street, H. Yagita, K. Takeda, S. L. van Dommelen, M. A. Degli-Esposti, and Y. Hayakawa. 2005. Activation of NK cell cytotoxicity. *Mol Immunol 42:501.*

37. Yagita, H., K. Takeda, Y. Hayakawa, M. J. Smyth, and K. Okumura. 2004. TRAIL and its receptors as targets for cancer therapy. *Cancer Sci 95:777.*

38. Hayakawa, Y., V. Screpanti, H. Yagita, A. Grandien, H. G. Ljunggren, M. J. Smyth, and B. J. Chambers. 2004. NK cell TRAIL eliminates immature dendritic cells in vivo and limits dendritic cell vaccination efficacy. *J Immunol 172:123.*

39. Murphy, W. J., V. Kumar, and M. Bennett. 1987. Rejection of bone marrow allografts by mice with severe combined immune deficiency (SCID). Evidence

that natural killer cells can mediate the specificity of marrow graft rejection. *J Exp Med 165:1212.*

40. Shinkai, Y., G. Rathbun, K. P. Lam, E. M. Oltz, V. Stewart, M. Mendelsohn, J. Charron, M. Datta, F. Young, A. M. Stall, and et al. 1992. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell 68:855.*

41. Mombaerts, P., J. Iacomini, R. S. Johnson, K. Herrup, S. Tonegawa, and V. E. Papaioannou. 1992. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell 68:869.*

42. Moretta, A., R. Biassoni, C. Bottino, D. Pende, M. Vitale, A. Poggi, M. C. Mingari, and L. Moretta. 1997. Major histocompatibility complex class I-specific receptors on human natural killer and T lymphocytes. *Immunol Rev 155:105.*

43. Bennett, M. 1987. Biology and genetics of hybrid resistance. *Adv Immunol 41:333.*

44. Karre, K., H. G. Ljunggren, G. Piontek, and R. Kiessling. 1986. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature 319:675.*

45. Biassoni, R., C. Cantoni, D. Pende, S. Sivori, S. Parolini, M. Vitale, C. Bottino, and A. Moretta. 2001. Human natural killer cell receptors and co-receptors. *Immunol Rev 181:203.*

46. Lanier, L. L. 1998. NK cell receptors. *Annu Rev Immunol 16:359.*

47. Natarajan, K., N. Dimasi, J. Wang, R. A. Mariuzza, and D. H. Margulies. 2002. Structure and function of natural killer cell receptors: multiple molecular solutions to self, nonself discrimination. *Annu Rev Immunol 20:853.*

48. Long, E. O. 1999. Regulation of immune responses through inhibitory receptors. *Annu Rev Immunol 17:875.*

49. Moretta, A., C. Bottino, M. Vitale, D. Pende, C. Cantoni, M. C. Mingari, R. Biassoni, and L. Moretta. 2001. Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. *Annu Rev Immunol 19:197.*

50. Moretta, L., R. Biassoni, C. Bottino, M. C. Mingari, and A. Moretta. 2000. Human NK-cell receptors. *Immunol Today 21:420.*

51. Yokoyama, W. M., and W. E. Seaman. 1993. The Ly-49 and NKR-P1 gene families encoding lectin-like receptors on natural killer cells: the NK gene complex. *Annu Rev Immunol 11:613.*

52. Lopez-Botet, M., M. Llano, F. Navarro, and T. Bellon. 2000. NK cell recognition of non-classical HLA class I molecules. *Semin Immunol 12:109.*

53. Pende, D., R. Biassoni, C. Cantoni, S. Verdiani, M. Falco, C. di Donato, L. Accame, C. Bottino, A. Moretta, and L. Moretta. 1996. The natural killer cell receptor specific for HLA-A allotypes: a novel member of the p58/p70 family of inhibitory receptors that is characterized by three immunoglobulin-like domains and is expressed as a 140-kD disulphide-linked dimer. *J Exp Med 184:505.*

54. Wagtmann, N., S. Rajagopalan, C. C. Winter, M. Peruzzi, and E. O. Long. 1995. Killer cell inhibitory receptors specific for HLA-C and HLA-B identified by direct binding and by functional transfer. *Immunity 3:801.*

55. Yusa, S., T. L. Catina, and K. S. Campbell. 2004. KIR2DL5 can inhibit human NK cell activation via recruitment of Src homology region 2-containing protein tyrosine phosphatase-2 (SHP-2). *J Immunol 172:7385.*

56. Wang, J. W., J. M. Howson, T. Ghansah, C. Desponts, J. M. Ninos, S. L. May, K. H. Nguyen, N. Toyama-Sorimachi, and W. G. Kerr. 2002. Influence of SHIP on the NK repertoire and allogeneic bone marrow transplantation. *Science 295:2094.*

57. Long, E. O., D. F. Barber, D. N. Burshtyn, M. Faure, M. Peterson, S. Rajagopalan, V. Renard, M. Sandusky, C. C. Stebbins, N. Wagtmann, and C.

Watzl. 2001. Inhibition of natural killer cell activation signals by killer cell immunoglobulin-like receptors (CD158). *Immunol Rev 181:223.*

58. Moretta, A., R. Biassoni, C. Bottino, M. C. Mingari, and L. Moretta. 2000. Natural cytotoxicity receptors that trigger human NK-cell-mediated cytolysis. *Immunol Today 21:228.*

59. Bottino, C., R. Biassoni, R. Millo, L. Moretta, and A. Moretta. 2000. The human natural cytotoxicity receptors (NCR) that induce HLA class I-independent NK cell triggering. *Hum Immunol 61:1.*

60. De Maria, A., R. Biassoni, M. Fogli, M. Rizzi, C. Cantoni, P. Costa, R. Conte, D. Mavilio, B. Ensoli, A. Cafaro, A. Moretta, and L. Moretta. 2001. Identification, molecular cloning and functional characterization of NKp46 and NKp30 natural cytotoxicity receptors in Macaca fascicularis NK cells. *Eur J Immunol 31:3546.*

61. Cantoni, C., C. Bottino, M. Vitale, A. Pessino, R. Augugliaro, A. Malaspina, S. Parolini, L. Moretta, A. Moretta, and R. Biassoni. 1999. NKp44, a triggering receptor involved in tumor cell lysis by activated human natural killer cells, is a novel member of the immunoglobulin superfamily. *J Exp Med 189:787.*

62. Watzl, C. 2003. The NKG2D receptor and its ligands-recognition beyond the "missing self"? *Microbes Infect 5:31.*

63. Bauer, S., V. Groh, J. Wu, A. Steinle, J. H. Phillips, L. L. Lanier, and T. Spies. 1999. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA [see comments]. *Science 285:727.*

64. Groh, V., R. Rhinehart, H. Secrist, S. Bauer, K. H. Grabstein, and T. Spies. 1999. Broad tumor-associated expression and recognition by tumor-derived gamma delta T cells of MICA and MICB. *Proc Natl Acad Sci U S A 96:6879.*

65. Cosman, D., J. Mullberg, C. L. Sutherland, W. Chin, R. Armitage, W. Fanslow, M. Kubin, and N. J. Chalupny. 2001. ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity 14:123.*

66. Jan Chalupny, N., C. L. Sutherland, W. A. Lawrence, A. Rein-Weston, and D. Cosman. 2003. ULBP4 is a novel ligand for human NKG2D. *Biochem Biophys Res Commun 305:129.*

67. Diefenbach, A., E. R. Jensen, A. M. Jamieson, and D. H. Raulet. 2001. Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. *Nature 413:165.*

68. Cerwenka, A., J. L. Baron, and L. L. Lanier. 2001. Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo. *Proc Natl Acad Sci U S A 98:11521.*

69. Garni-Wagner, B. A., A. Purohit, P. A. Mathew, M. Bennett, and V. Kumar. 1993. A novel function-associated molecule related to non-MHC-restricted cytotoxicity mediated by activated natural killer cells and T cells. *J Immunol 151:60.*

70. Kumaresan, P. R., W. C. Lai, S. S. Chuang, M. Bennett, and P. A. Mathew. 2002. CS1, a novel member of the CD2 family, is homophilic and regulates NK cell function. *Mol Immunol 39:1.*

71. Moretta, L., R. Biassoni, C. Bottino, C. Cantoni, D. Pende, M. C. Mingari, and A. Moretta. 2002. Human NK cells and their receptors. *Microbes Infect 4:1539.*

72. Lanier, L. L. 2000. Turning on natural killer cells. *J Exp Med 191:1259.*

73. Tarakhovsky, A. 1997. Antigen receptor signalling in B cells. *Res Immunol 148:457.*

74. Hasler, P., and M. Zouali. 2001. B cell receptor signaling and autoimmunity. *Faseb J 15:2085.*

75. DeFranco, A. L. 1997. The complexity of signaling pathways activated by the BCR. *Curr Opin Immunol 9:296.*

76. Bishop, G. A., and B. S. Hostager. 2001. B lymphocyte activation by contact-mediated interactions with T lymphocytes. *Curr Opin Immunol 13:278.*

77. Mosmann, T. R., and R. L. Coffman. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol 7:145.*

78. Kimata, H., A. Yoshida, C. Ishioka, I. Lindley, and H. Mikawa. 1992. Interleukin 8 (IL-8) selectively inhibits immunoglobulin E production induced by IL-4 in human B cells. *J Exp Med 176:1227.*

79. Rousset, F., E. Garcia, T. Defrance, C. Peronne, N. Vezzio, D. H. Hsu, R. Kastelein, K. W. Moore, and J. Banchereau. 1992. Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes. *Proc Natl Acad Sci U S A 89:1890.*

80. Wakasugi, H., L. Rimsky, Y. Mahe, A. M. Kamel, D. Fradelizi, T. Tursz, and J. Bertoglio. 1987. Epstein-Barr virus-containing B-cell line produces an interleukin 1 that it uses as a growth factor. *Proc Natl Acad Sci U S A 84:804.*

81. Rieckmann, P., F. D'Alessandro, R. P. Nordan, A. S. Fauci, and J. H. Kehrl. 1991. IL-6 and tumor necrosis factor-alpha. Autocrine and paracrine cytokines involved in B cell function. *J Immunol 146:3462.*

82. Burdin, N., C. Peronne, J. Banchereau, and F. Rousset. 1993. Epstein-Barr virus transformation induces B lymphocytes to produce human interleukin 10. *J Exp Med 177:295.*

83. Boussiotis, V. A., L. M. Nadler, J. L. Strominger, and A. E. Goldfeld. 1994. Tumor necrosis factor alpha is an autocrine growth factor for normal human B cells. *Proc Natl Acad Sci U S A 91:7007.*

84. Estrov, Z., R. Kurzrock, E. Pocsik, S. Pathak, H. M. Kantarjian, T. F. Zipf, D. Harris, M. Talpaz, and B. B. Aggarwal. 1993. Lymphotoxin is an autocrine growth factor for Epstein-Barr virus-infected B cell lines. *J Exp Med 177:763.*

85. Smith, C. A., T. Farrah, and R. G. Goodwin. 1994. The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. *Cell 76:959.*

86. Clark, E. A., and J. A. Ledbetter. 1986. Activation of human B cells mediated through two distinct cell surface differentiation antigens, Bp35 and Bp50. *Proc Natl Acad Sci U S A 83:4494.*

87. Armitage, R. J., W. C. Fanslow, L. Strockbine, T. A. Sato, K. N. Clifford, B. M. Macduff, D. M. Anderson, S. D. Gimpel, T. Davis-Smith, C. R. Maliszewski, and et al. 1992. Molecular and biological characterization of a murine ligand for CD40. *Nature 357:80.*

88. Hollenbaugh, D., L. S. Grosmaire, C. D. Kullas, N. J. Chalupny, S. Braesch-Andersen, R. J. Noelle, I. Stamenkovic, J. A. Ledbetter, and A. Aruffo. 1992. The human T cell antigen gp39, a member of the TNF gene family, is a ligand for the

CD40 receptor: expression of a soluble form of gp39 with B cell co-stimulatory activity. *Embo J 11:4313.*

89. Clark, E. A., and J. A. Ledbetter. 1994. How B and T cells talk to each other. *Nature 367:425.*

90. Klaus, G. G., M. S. Choi, E. W. Lam, C. Johnson-Leger, and J. Cliff. 1997. CD40: a pivotal receptor in the determination of life/death decisions in B lymphocytes. *Int Rev Immunol 15:5.*

91. Higuchi, T., Y. Aiba, T. Nomura, J. Matsuda, K. Mochida, M. Suzuki, H. Kikutani, T. Honjo, K. Nishioka, and T. Tsubata. 2002. Cutting Edge: Ectopic expression of CD40 ligand on B cells induces lupus-like autoimmune disease. *J Immunol 168:9.*

92. Henn, V., J. R. Slupsky, M. Grafe, I. Anagnostopoulos, R. Forster, G. Muller-Berghaus, and R. A. Kroczek. 1998. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature 391:591.*

93. Danese, S., C. de la Motte, A. Sturm, J. D. Vogel, G. A. West, S. A. Strong, J. A. Katz, and C. Fiocchi. 2003. Platelets trigger a CD40-dependent inflammatory response in the microvasculature of inflammatory bowel disease patients. *Gastroenterology 124:1249.*

94. van Kooten, C., and J. Banchereau. 2000. CD40-CD40 ligand. *J Leukoc Biol 67:2.*

95. Foy, T. M., A. Aruffo, J. Bajorath, J. E. Buhlmann, and R. J. Noelle. 1996. Immune regulation by CD40 and its ligand GP39. *Annu Rev Immunol 14:591.*

96. Garside, P., E. Ingulli, R. R. Merica, J. G. Johnson, R. J. Noelle, and M. K. Jenkins. 1998. Visualization of specific B and T lymphocyte interactions in the lymph node. *Science 281:96.*

97. Kindler, V., and R. H. Zubler. 1997. Memory, but not naive, peripheral blood B lymphocytes differentiate into Ig-secreting cells after CD40 ligation and costimulation with IL-4 and the differentiation factors IL-2, IL-10, and IL-3. *J Immunol 159:2085.*

98. Galibert, L., I. Durand, J. Banchereau, and F. Rousset. 1994. CD40-activated surface IgD-positive lymphocytes constitute the long term IL-4-dependent proliferating B cell pool. *J Immunol 152:22.*

99. Nagumo, H., K. Agematsu, K. Shinozaki, S. Hokibara, S. Ito, M. Takamoto, T. Nikaido, K. Yasui, Y. Uehara, A. Yachie, and A. Komiyama. 1998. CD27/CD70 interaction augments IgE secretion by promoting the differentiation of memory B cells into plasma cells. *J Immunol 161:6496.*

100. Nagumo, H., K. Agematsu, N. Kobayashi, K. Shinozaki, S. Hokibara, H. Nagase, M. Takamoto, K. Yasui, K. Sugane, and A. Komiyama. 2002. The different process of class switching and somatic hypermutation; a novel analysis by CD27(-) naive B cells. *Blood 99:567.*

101. Werner-Favre, C., F. Bovia, P. Schneider, N. Holler, M. Barnet, V. Kindler, J. Tschopp, and R. H. Zubler. 2001. IgG subclass switch capacity is low in switched and in IgM-only, but high in IgD+IgM+, post-germinal center (CD27+) human B cells. *Eur J Immunol 31:243.*

102. Banchereau, J., and F. Rousset. 1991. Growing human B lymphocytes in the CD40 system. *Nature 353:678.*

103. Banchereau, J., P. de Paoli, A. Valle, E. Garcia, and F. Rousset. 1991. Long-term human B cell lines dependent on interleukin-4 and antibody to CD40. *Science 251:70.*

104. Banchereau, J., F. Briere, Y. J. Liu, and F. Rousset. 1994. Molecular control of B lymphocyte growth and differentiation. *Stem Cells 12:278.*

105. Rousset, F., E. Garcia, and J. Banchereau. 1991. Cytokine-induced proliferation and immunoglobulin production of human B lymphocytes triggered through their CD40 antigen. *J Exp Med 173:705.*

106. de Totero, D., P. L. Tazzari, M. Capaia, M. P. Montera, M. Clavio, E. Balleari, R. Foa, and M. Gobbi. 2003. CD40 triggering enhances fludarabine-induced apoptosis of chronic lymphocytic leukemia B-cells through autocrine release of tumor necrosis factor-alpha and interferon-gama and tumor necrosis factor receptor-I-II upregulation. *Haematologica 88:148.*

107. Jabara, H. H., S. M. Fu, R. S. Geha, and D. Vercelli. 1990. CD40 and IgE: synergism between anti-CD40 monoclonal antibody and interleukin 4 in the induction of IgE synthesis by highly purified human B cells. *J Exp Med 172:1861.*

108. Worm, M., K. Ebermayer, and B. Henz. 1998. Lymphotoxin-alpha is an important autocrine factor for CD40 + interleukin-4-mediated B-cell activation in normal and atopic donors. *Immunology 94:395.*

109. Punnonen, J., B. G. Cocks, J. M. Carballido, B. Bennett, D. Peterson, G. Aversa, and J. E. de Vries. 1997. Soluble and membrane-bound forms of signaling lymphocytic activation molecule (SLAM) induce proliferation and Ig synthesis by activated human B lymphocytes. *J Exp Med 185:993.*

110. Davis, S. J., S. Ikemizu, M. K. Wild, and P. A. van der Merwe. 1998. CD2 and the nature of protein interactions mediating cell-cell recognition. *Immunol Rev 163:217.*

111. Sidorenko, S. P., and E. A. Clark. 2003. The dual-function CD150 receptor subfamily: the viral attraction. *Nat Immunol 4:19.*

112. Tangye, S. G., J. H. Phillips, L. L. Lanier, and K. E. Nichols. 2000. Functional requirement for SAP in 2B4-mediated activation of human natural killer cells as revealed by the X-linked lymphoproliferative syndrome. *J Immunol 165:2932.*

113. Latour, S., and A. Veillette. 2001. Proximal protein tyrosine kinases in immunoreceptor signaling. *Curr Opin Immunol 13:299.*

114. Sayos, J., K. B. Nguyen, C. Wu, S. E. Stepp, D. Howie, J. D. Schatzle, V. Kumar, C. A. Biron, and C. Terhorst. 2000. Potential pathways for regulation of NK and T cell responses: differential X-linked lymphoproliferative syndrome gene product SAP interactions with SLAM and 2B4. *Int Immunol 12:1749.*

115. Bleharski, J. R., K. R. Niazi, P. A. Sieling, G. Cheng, and R. L. Modlin. 2001. Signaling lymphocytic activation molecule is expressed on CD40 ligand-activated dendritic cells and directly augments production of inflammatory cytokines. *J Immunol 167:3174.*

116. Nagy, N., C. Cerboni, K. Mattsson, A. Maeda, P. Gogolak, J. Sumegi, A. Lanyi, L. Szekely, E. Carbone, G. Klein, and E. Klein. 2000. SH2D1A and SLAM protein expression in human lymphocytes and derived cell lines. *Int J Cancer 88:439.*

117. Cocks, B. G., C. C. Chang, J. M. Carballido, H. Yssel, J. E. de Vries, and G. Aversa. 1995. A novel receptor involved in T-cell activation. *Nature 376:260.*

118. Aversa, G., J. Carballido, J. Punnonen, C. C. Chang, T. Hauser, B. G. Cocks, and J. E. De Vries. 1997. SLAM and its role in T cell activation and Th cell responses. *Immunol Cell Biol 75:202.*

119. Carballido, J. M., G. Aversa, K. Kaltoft, B. G. Cocks, J. Punnonen, H. Yssel, K. Thestrup-Pedersen, and J. E. de Vries. 1997. Reversal of human allergic T helper 2 responses by engagement of signaling lymphocytic activation molecule. *J Immunol 159:4316.*

120. Henning, G., M. S. Kraft, T. Derfuss, R. Pirzer, G. de Saint-Basile, G. Aversa, B. Fleckenstein, and E. Meinl. 2001. Signaling lymphocytic activation molecule (SLAM) regulates T cellular cytotoxicity. *Eur J Immunol 31:2741.*

121. Mikhalap, S. V., L. M. Shlapatska, A. G. Berdova, C. L. Law, E. A. Clark, and S. P. Sidorenko. 1999. CDw150 associates with src-homology 2-containing inositol phosphatase and modulates CD95-mediated apoptosis. *J Immunol 162:5719.*

122. Tatsuo, H., N. Ono, K. Tanaka, and Y. Yanagi. 2000. SLAM (CDw150) is a cellular receptor for measles virus. *Nature 406:893.*

123. Tatsuo, H., and Y. Yanagi. 2002. The morbillivirus receptor SLAM (CD150). *Microbiol Immunol 46:135.*

124. Mathew, P. A., B. A. Garni-Wagner, K. Land, A. Takashima, E. Stoneman, M. Bennett, and V. Kumar. 1993. Cloning and characterization of the 2B4 gene encoding a molecule associated with non-MHC-restricted killing mediated by activated natural killer cells and T cells. *J Immunol 151:5328.*

125. Kubin, M. Z., D. L. Parshley, W. Din, J. Y. Waugh, T. Davis-Smith, C. A. Smith, B. M. Macduff, R. J. Armitage, W. Chin, L. Cassiano, L. Borges, M. Petersen, G. Trinchieri, and R. G. Goodwin. 1999. Molecular cloning and biological characterization of NK cell activation- inducing ligand, a counterstructure for CD48. *Eur J Immunol 29:3466.*

126. Nakajima, H., M. Cella, H. Langen, A. Friedlein, and M. Colonna. 1999. Activating interactions in human NK cell recognition: the role of 2B4- CD48. *Eur J Immunol 29:1676.*

127. Brown, M. H., K. Boles, P. A. van der Merwe, V. Kumar, P. A. Mathew, and A. N. Barclay. 1998. 2B4, the natural killer and T cell immunoglobulin superfamily surface protein, is a ligand for CD48. *J Exp Med 188:2083.*

128. Peritt, D., D. A. Sesok-Pizzini, R. Schretzenmair, R. R. Macgregor, N. M. Valiante, X. Tu, G. Trinchieri, and M. Kamoun. 1999. C1.7 antigen expression on

CD8+ T cells is activation dependent: increased proportion of C1.7+CD8+ T cells in HIV-1-infected patients with progressing disease. *J Immunol 162:7563.*

129. Boles, K. S., H. Nakajima, M. Colonna, S. S. Chuang, S. E. Stepp, M. Bennett, V. Kumar, and P. A. Mathew. 1999. Molecular characterization of a novel human natural killer cell receptor homologous to mouse 2B4. *Tissue Antigens 54:27.*

130. Schatzle, J. D., S. Sheu, S. E. Stepp, P. A. Mathew, M. Bennett, and V. Kumar. 1999. Characterization of inhibitory and stimulatory forms of the murine natural killer cell receptor 2B4. *Proc Natl Acad Sci U S A 96:3870.*

131. Kumaresan, P. R., and P. A. Mathew. 2000. Structure of the human natural killer cell receptor *2B4* gene and identification of a novel altenative transcript. *Immunogenetics 51:987.*

132. Valiante, N. M., and G. Trinchieri. 1993. Identification of a novel signal transduction surface molecule on human cytotoxic lymphocytes. *J Exp Med 178:1397.*

133. Parolini, S., C. Bottino, M. Falco, R. Augugliaro, S. Giliani, R. Franceschini, H. D. Ochs, H. Wolf, J. Y. Bonnefoy, R. Biassoni, L. Moretta, L. D. Notarangelo, and A. Moretta. 2000. X-linked lymphoproliferative disease. 2B4 molecules displaying inhibitory rather than activating function are responsible for the

inability of natural killer cells to kill Epstein-Barr virus-infected cells. *J Exp Med 192:337.*

134. Nakajima, H., M. Cella, A. Bouchon, H. L. Grierson, J. Lewis, C. S. Duckett, J. I. Cohen, and M. Colonna. 2000. Patients with X-linked lymphoproliferative disease have a defect in 2B4 receptor-mediated NK cell cytotoxicity. *Eur J Immunol 30:3309.*

135. Sayos, J., C. Wu, M. Morra, N. Wang, X. Zhang, D. Allen, S. van Schaik, L. Notarangelo, R. Geha, M. G. Roncarolo, H. Oettgen, J. E. De Vries, G. Aversa, and C. Terhorst. 1998. The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM. *Nature 395:462.*

136. Tangye, S. G., S. Lazetic, E. Woollatt, G. R. Sutherland, L. L. Lanier, and J. H. Phillips. 1999. Cutting edge: human 2B4, an activating NK cell receptor, recruits the protein tyrosine phosphatase SHP-2 and the adaptor signaling protein SAP. *J Immunol 162:6981.*

137. Benoit, L., X. Wang, H. F. Pabst, J. Dutz, and R. Tan. 2000. Defective NK cell activation in X-linked lymphoproliferative disease. *J Immunol 165:3549.*

138. Boles, K. S., and P. A. Mathew. 2001. Molecular cloning of CS1, a novel human natural killer cell receptor belonging to the CD2 subset of the immunoglobulin superfamily. *Immunogenetics 52:302.*

139. Bouchon, A., M. Cella, H. L. Grierson, J. I. Cohen, and M. Colonna. 2001. Activation of NK cell-mediated cytotoxicity by a SAP-independent receptor of the CD2 family. *J Immunol 167:5517.*

140. Murphy, J. J., P. Hobby, J. Vilarino-Varela, B. Bishop, P. Iordanidou, B. J. Sutton, and J. D. Norton. 2002. A novel immunoglobulin superfamily receptor (19A) related to CD2 is expressed on activated lymphocytes and promotes homotypic B-cell adhesion. *Biochem J 361:431.*

141. Morra, M., M. Simarro-Grande, M. Martin, A. S. Chen, A. Lanyi, O. Silander, S. Calpe, J. Davis, T. Pawson, M. J. Eck, J. Sumegi, P. Engel, S. C. Li, and C. Terhorst. 2001. Characterization of SH2D1A missense mutations identified in X-linked lymphoproliferative disease patients. *J Biol Chem 276:36809.*

142. Aoukaty, A., and R. Tan. 2002. Association of the X-linked lymphoproliferative disease gene product SAP/SH2D1A with 2B4, a natural killer cell-activating molecule, is dependent on phosphoinositide 3-kinase. *J Biol Chem 277:13331.*

143. Sayos, J., M. Martin, A. Chen, M. Simarro, D. Howie, M. Morra, P. Engel, and C. Terhorst. 2001. Cell surface receptors Ly-9 and CD84 recruit the X-linked lymphoproliferative disease gene product SAP. *Blood 97:3867.*

144. Tovar, V., J. del Valle, N. Zapater, M. Martin, X. Romero, P. Pizcueta, J. Bosch, C. Terhorst, and P. Engel. 2002. Mouse novel Ly9: a new member of the

expanding CD150 (SLAM) family of leukocyte cell-surface receptors. *Immunogenetics 54:394.*

145. Coffey, A. J., R. A. Brooksbank, O. Brandau, T. Oohashi, G. R. Howell, J. M. Bye, A. P. Cahn, J. Durham, P. Heath, P. Wray, R. Pavitt, J. Wilkinson, M. Leversha, E. Huckle, C. J. Shaw-Smith, A. Dunham, S. Rhodes, V. Schuster, G. Porta, L. Yin, P. Serafini, B. Sylla, M. Zollo, B. Franco, D. R. Bentley, and et al. 1998. Host response to EBV infection in X-linked lymphoproliferative disease results from mutations in an SH2-domain encoding gene. *Nat Genet 20:129.*

146. Jager, M., G. Benninger-Doring, N. Prang, B. S. Sylla, B. Laumbacher, R. Wank, H. Wolf, and F. Schwarzmann. 1998. Epstein-Barr virus-infected B cells of males with the X-linked lymphoproliferative syndrome stimulate and are susceptible to T-cell-mediated lysis. *Int J Cancer 76:694.*

147. Sullivan, J. L., K. S. Byron, F. E. Brewster, S. M. Baker, and H. D. Ochs. 1983. X-linked lymphoproliferative syndrome. Natural history of the immunodeficiency. *J Clin Invest 71:1765.*

148. Coffey, A. J., R. A. Brooksbank, O. Brandau, T. Oohashi, G. R. Howell, J. M. Bye, A. P. Cahn, J. Durham, P. Heath, P. Wray, R. Pavitt, J. Wilkinson, M. Leversha, E. Huckle, C. J. Shaw-Smith, A. Dunham, S. Rhodes, V. Schuster, G. Porta, L. Yin, P. Serafini, B. Sylla, M. Zollo, B. Franco, D. R. Bentley, and et al. 1998. Host response to EBV infection in X-linked lymphoproliferative disease

results from mutations in an SH2-domain encoding gene [see comments]. *Nat Genet 20:129.*

149. Nichols, K. E. 2000. X-linked lymphoproliferative disease: genetics and biochemistry. *Rev Immunogenet 2:256.*

150. Boles, K. S., S. E. Stepp, M. Bennett, V. Kumar, and P. A. Mathew. 2001. 2B4 (CD244) and CS1: novel members of the CD2 subset of the immunoglobulin superfamily molecules expressed on natural killer cells and other leukocytes. *Immunol Rev 181:234.*

151. Aversa, G., C. C. Chang, J. M. Carballido, B. G. Cocks, and J. E. de Vries. 1997. Engagement of the signaling lymphocytic activation molecule (SLAM) on activated T cells results in IL-2-independent, cyclosporin A-sensitive T cell proliferation and IFN-gamma production. *J Immunol 158:4036.*

152. Stepp, S. E., J. D. Schatzle, M. Bennett, V. Kumar, and P. A. Mathew. 1999. Gene structure of the murine NK cell receptor 2B4: presence of two alternatively spliced isoforms with distinct cytoplasmic domains. *Eur J Immunol 29:2392.*

153. Kumaresan, P. R., and P. A. Mathew. 2000. Structure of the human natural killer cell receptor 2B4 gene and identification of a novel alternative transcript. *Immunogenetics 51:987.*

154. Bakker, A. B., J. Wu, J. H. Phillips, and L. L. Lanier. 2000. NK cell activation: distinct stimulatory pathways counterbalancing inhibitory signals. *Hum Immunol 61:18.*

155. Colonna, M., H. Nakajima, and M. Cella. 1999. Inhibitory and activating receptors involved in immune surveillance by human NK and myeloid cells. *J Leukoc Biol 66:718.*

156. Prasanna, G., A. I. Dibas, and T. Yorio. 2000. Cholinergic and adrenergic modulation of the Ca2+ response to endothelin-1 in human ciliary muscle cells. *Invest Ophthalmol Vis Sci 41:1142.*

157. Ryan, J. C., E. C. Niemi, R. D. Goldfien, J. C. Hiserodt, and W. E. Seaman. 1991. NKR-P1, an activating molecule on rat natural killer cells, stimulates phosphoinositide turnover and a rise in intracellular calcium. *J Immunol 147:3244.*

158. Chambers, W. H., N. L. Vujanovic, A. B. DeLeo, M. W. Olszowy, R. B. Herberman, and J. C. Hiserodt. 1989. Monoclonal antibody to a triggering structure expressed on rat natural killer cells and adherent lymphokine-activated killer cells. *J Exp Med 169:1373.*

159. Li, J., B. A. Rabinovich, R. Hurren, J. Shannon, and R. G. Miller. 2003. Expression cloning and function of the rat NK activating and inhibitory receptors NKR-P1A and -P1B. *Int Immunol 15:411.*

160. Clark, L. B., T. M. Foy, and R. J. Noelle. 1996. CD40 and its ligand. *Adv Immunol 63:43.*

161. van Kooten, C., and J. Banchereau. 1997. Functions of CD40 on B cells, dendritic cells and other cells. *Curr Opin Immunol 9:330.*

162. Yokota, T., T. Otsuka, T. Mosmann, J. Banchereau, T. DeFrance, D. Blanchard, J. E. De Vries, F. Lee, and K. Arai. 1986. Isolation and characterization of a human interleukin cDNA clone, homologous to mouse B-cell stimulatory factor 1, that expresses B-cell- and T-cell-stimulating activities. *Proc Natl Acad Sci U S A 83:5894.*

163. Defrance, T., B. Vanbervliet, J. P. Aubry, Y. Takebe, N. Arai, A. Miyajima, T. Yokota, F. Lee, K. Arai, J. E. de Vries, and et al. 1987. B cell growth-promoting activity of recombinant human interleukin 4. *J Immunol 139:1135.*

164. Spits, H., H. Yssel, Y. Takebe, N. Arai, T. Yokota, F. Lee, K. Arai, J. Banchereau, and J. E. de Vries. 1987. Recombinant interleukin 4 promotes the growth of human T cells. *J Immunol 139:1142.*

165. Defrance, T., B. Vanbervliet, J. Pene, and J. Banchereau. 1988. Human recombinant IL-4 induces activated B lymphocytes to produce IgG and IgM. *J Immunol 141:2000.*

166. Defrance, T., J. P. Aubry, F. Rousset, B. Vanbervliet, J. Y. Bonnefoy, N. Arai, Y. Takebe, T. Yokota, F. Lee, K. Arai, and et al. 1987. Human recombinant interleukin 4 induces Fc epsilon receptors (CD23) on normal human B lymphocytes. *J Exp Med 165:1459.*

167. Rousset, F., R. W. Malefijt, B. Slierendregt, J. P. Aubry, J. Y. Bonnefoy, T. Defrance, J. Banchereau, and J. E. de Vries. 1988. Regulation of Fc receptor for IgE (CD23) and class II MHC antigen expression on Burkitt's lymphoma cell lines by human IL-4 and IFN-gamma. *J Immunol 140:2625.*

168. Pene, J., F. Rousset, F. Briere, I. Chretien, J. Y. Bonnefoy, H. Spits, T. Yokota, N. Arai, K. Arai, J. Banchereau, and et al. 1988. IgE production by normal human lymphocytes is induced by interleukin 4 and suppressed by interferons gamma and alpha and prostaglandin E2. *Proc Natl Acad Sci U S A 85:6880.*

169. Nedellec, S., Y. Renaudineau, A. Bordron, C. Berthou, N. Porakishvili, P. M. Lydyard, J. O. Pers, and P. Youinou. 2005. B cell response to surface IgM cross-linking identifies different prognostic groups of B-chronic lymphocytic leukemia patients. *J Immunol 174:3749.*

170. Kuwahara, K., T. Kawai, S. Mitsuyoshi, Y. Matsuo, H. Kikuchi, S. Imajoh-Ohmi, E. Hashimoto, S. Inui, M. D. Cooper, and N. Sakaguchi. 1996. Cross-linking of B cell antigen receptor-related structure of pre-B cell lines induces tyrosine phosphorylation of p85 and p110 subunits and activation of phosphatidylinositol 3-kinase. *Int Immunol 8:1273.*

171. Muraguchi, A., J. H. Kehrl, J. L. Butler, and A. S. Fauci. 1984. Regulation of human B-cell activation, proliferation, and differentiation by soluble factors. *J Clin Immunol 4:337.*

172. Levinson, A. I., L. Tar, C. Carafa, and M. Haidar. 1986. Staphylococcus aureus Cowan I. Potent stimulus of immunoglobulin M rheumatoid factor production. *J Clin Invest 78:612.*

173. Lee, J. K., K. S. Boles, and P. A. Mathew. 2004. Molecular and functional characterization of a CS1 (CRACC) splice variant expressed in human NK cells that does not contain immunoreceptor tyrosine-based switch motifs. *Eur J Immunol 34:2791.*

174. Paul, W. E., and R. A. Seder. 1994. Lymphocyte responses and cytokines. *Cell 76:241.*

175. Diamant, E., Z. Keren, and D. Melamed. 2005. CD19 regulates positive selection and maturation in B lymphopoiesis: lack of CD19 imposes developmental arrest

of immature B cells and consequential stimulation of receptor editing. *Blood 105:3247.*

176. Cocks, B. G., R. de Waal Malefyt, J. P. Galizzi, J. E. de Vries, and G. Aversa. 1993. IL-13 induces proliferation and differentiation of human B cells activated by the CD40 ligand. *Int Immunol 5:657.*

177. Brandau, O., V. Schuster, M. Weiss, H. Hellebrand, F. M. Fink, A. Kreczy, W. Friedrich, B. Strahm, C. Niemeyer, B. H. Belohradsky, and A. Meindl. 1999. Epstein-Barr virus-negative boys with non-Hodgkin lymphoma are mutated in the SH2D1A gene, as are patients with X-linked lymphoproliferative disease (XLP). *Hum Mol Genet 8:2407.*

178. Strahm, B., K. Rittweiler, U. Duffner, O. Brandau, M. Orlowska-Volk, M. A. Karajannis, U. Stadt, M. Tiemann, A. Reiter, M. Brandis, A. Meindl, and C. M. Niemeyer. 2000. Recurrent B-cell non-Hodgkin's lymphoma in two brothers with X-linked lymphoproliferative disease without evidence for Epstein-Barr virus infection. *Br J Haematol 108:377.*

179. Arico, M., S. Imashuku, R. Clementi, S. Hibi, T. Teramura, C. Danesino, D. A. Haber, and K. E. Nichols. 2001. Hemophagocytic lymphohistiocytosis due to germline mutations in SH2D1A, the X-linked lymphoproliferative disease gene. *Blood 97:1131.*

180. Crotty, S., E. N. Kersh, J. Cannons, P. L. Schwartzberg, and R. Ahmed. 2003. SAP is required for generating long-term humoral immunity. *Nature 421:282.*

181. Li, C., C. Iosef, C. Y. Jia, V. K. Han, and S. S. Li. 2003. Dual functional roles for the X-linked lymphoproliferative syndrome gene product SAP/SH2D1A in signaling through the signaling lymphocyte activation molecule (SLAM) family of immune receptors. *J Biol Chem 278:3852.*

182. Klein, U., K. Rajewsky, and R. Kuppers. 1998. Human immunoglobulin (Ig)M+IgD+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells. *J Exp Med 188:1679.*

183. Tarlinton, D. 1998. Germinal centers: form and function. *Curr Opin Immunol 10:245.*

184. Neuberger, M. S., M. R. Ehrenstein, C. Rada, J. Sale, F. D. Batista, G. Williams, and C. Milstein. 2000. Memory in the B-cell compartment: antibody affinity maturation. *Philos Trans R Soc Lond B Biol Sci 355:357.*

185. Lahvis, G. P., and J. Cerny. 1997. Induction of germinal center B cell markers in vitro by activated CD4+ T lymphocytes: the role of CD40 ligand, soluble factors, and B cell antigen receptor cross-linking. *J Immunol 159:1783.*

186. Salmon, R. A., I. N. Foltz, P. R. Young, and J. W. Schrader. 1997. The p38 mitogen-activated protein kinase is activated by ligation of the T or B lymphocyte antigen receptors, Fas or CD40, but suppression of kinase activity does not inhibit apoptosis induced by antigen receptors. *J Immunol 159:5309.*

187. Berberich, I., G. L. Shu, and E. A. Clark. 1994. Cross-linking CD40 on B cells rapidly activates nuclear factor-kappa B. *J Immunol 153:4357.*

188. Francis, D. A., J. G. Karras, X. Y. Ke, R. Sen, and T. L. Rothstein. 1995. Induction of the transcription factors NF-kappa B, AP-1 and NF-AT during B cell stimulation through the CD40 receptor. *Int Immunol 7:151.*

189. Gordon, J., M. J. Millsum, G. R. Guy, and J. A. Ledbetter. 1988. Resting B lymphocytes can be triggered directly through the CDw40 (Bp50) antigen. A comparison with IL-4-mediated signaling. *J Immunol 140:1425.*

190. Paulie, S., A. Rosen, B. Ehlin-Henriksson, S. Braesch-Andersen, E. Jakobson, H. Koho, and P. Perlmann. 1989. The human B lymphocyte and carcinoma antigen, CDw40, is a phosphoprotein involved in growth signal transduction. *J Immunol 142:590.*

191. Valle, A., C. E. Zuber, T. Defrance, O. Djossou, M. De Rie, and J. Banchereau. 1989. Activation of human B lymphocytes through CD40 and interleukin 4. *Eur J Immunol 19:1463.*

192. Nonoyama, S., D. Hollenbaugh, A. Aruffo, J. A. Ledbetter, and H. D. Ochs. 1993. B cell activation via CD40 is required for specific antibody production by antigen-stimulated human B cells. *J Exp Med 178:1097.*

193. Foy, T. M., J. D. Laman, J. A. Ledbetter, A. Aruffo, E. Claassen, and R. J. Noelle. 1994. gp39-CD40 interactions are essential for germinal center formation and the development of B cell memory. *J Exp Med 180:157.*

194. Bjorck, P., and S. Paulie. 1993. Inhibition of LFA-1-dependent human B-cell aggregation induced by CD40 antibodies and interleukin-4 leads to decreased IgE synthesis. *Immunology 78:218.*

195. Barrett, T. B., G. Shu, and E. A. Clark. 1991. CD40 signaling activates CD11a/CD18 (LFA-1)-mediated adhesion in B cells. *J Immunol 146:1722.*

196. Schattner, E. J., K. B. Elkon, D. H. Yoo, J. Tumang, P. H. Krammer, M. K. Crow, and S. M. Friedman. 1995. CD40 ligation induces Apo-1/Fas expression on human B lymphocytes and facilitates apoptosis through the Apo-1/Fas pathway. *J Exp Med 182:1557.*

197. Ranheim, E. A., and T. J. Kipps. 1993. Activated T cells induce expression of B7/BB1 on normal or leukemic B cells through a CD40-dependent signal. *J Exp Med 177:925.*

198. Meuer, S. C., R. E. Hussey, M. Fabbi, D. Fox, O. Acuto, K. A. Fitzgerald, J. C. Hodgdon, J. P. Protentis, S. F. Schlossman, and E. L. Reinherz. 1984. An alternative pathway of T-cell activation: a functional role for the 50 kd T11 sheep erythrocyte receptor protein. *Cell 36:897.*

199. Siliciano, R. F., J. C. Pratt, R. E. Schmidt, J. Ritz, and E. L. Reinherz. 1985. Activation of cytolytic T lymphocyte and natural killer cell function through the T11 sheep erythrocyte binding protein. *Nature 317:428.*

200. Selvaraj, P., M. L. Plunkett, M. Dustin, M. E. Sanders, S. Shaw, and T. A. Springer. 1987. The T lymphocyte glycoprotein CD2 binds the cell surface ligand LFA-3. *Nature 326:400.*

201. Koyasu, S., T. Lawton, D. Novick, M. A. Recny, R. F. Siliciano, B. P. Wallner, and E. L. Reinherz. 1990. Role of interaction of CD2 molecules with lymphocyte function-associated antigen 3 in T-cell recognition of nominal antigen. *Proc Natl Acad Sci U S A 87:2603.*

202. Muraguchi, A., N. Kawamura, A. Hori, Y. Horii, Y. Ichigi, M. Kimoto, and T. Kishimoto. 1992. Expression of the CD2 molecule on human B lymphoid progenitors. *Int Immunol 4:841.*

203. Punnonen, J., and J. E. de Vries. 1993. Characterization of a novel CD2+ human thymic B cell subset. *J Immunol 151:100.*

204. Sen, J., P. Bossu, S. J. Burakoff, and A. K. Abbas. 1992. T cell surface molecules regulating noncognate B lymphocyte activation. Role of CD2 and LFA-1. *J Immunol 148:1037.*

205. Diaz-Sanchez, D., S. Chegini, K. Zhang, and A. Saxon. 1994. CD58 (LFA-3) stimulation provides a signal for human isotype switching and IgE production distinct from CD40. *J Immunol 153:10.*

206. Thompson, A. D., B. S. Braun, A. Arvand, S. D. Stewart, W. A. May, E. Chen, J. Korenberg, and C. Denny. 1996. EAT-2 is a novel SH2 domain containing protein that is up regulated by Ewing's sarcoma EWS/FLI1 fusion gene. *Oncogene 13:2649.*

207. Singer, W. D., H. A. Brown, and P. C. Sternweis. 1997. Regulation of eukaryotic phosphatidylinositol-specific phospholipase C and phospholipase D. *Annu Rev Biochem 66:475.*

208. Porter, A. C., and R. R. Vaillancourt. 1998. Tyrosine kinase receptor-activated signal transduction pathways which lead to oncogenesis. *Oncogene 17:1343.*

209. Pistoia, V. 1997. Production of cytokines by human B cells in health and disease. *Immunol Today 18:343.*

210. Harris, D. P., L. Haynes, P. C. Sayles, D. K. Duso, S. M. Eaton, N. M. Lepak, L. L. Johnson, S. L. Swain, and F. E. Lund. 2000. Reciprocal regulation of polarized cytokine production by effector B and T cells. *Nat Immunol 1:475.*

211. Duddy, M. E., A. Alter, and A. Bar-Or. 2004. Distinct profiles of human B cell effector cytokines: a role in immune regulation? *J Immunol 172:3422.*

212. Li, L., D. Young, S. F. Wolf, and Y. S. Choi. 1996. Interleukin-12 stimulates B cell growth by inducing IFN-gamma. *Cell Immunol 168:133.*

213. Ford, R., A. Tamayo, B. Martin, K. Niu, K. Claypool, F. Cabanillas, and J. Ambrus, Jr. 1995. Identification of B-cell growth factors (interleukin-14; high molecular weight-B-cell growth factors) in effusion fluids from patients with aggressive B-cell lymphomas. *Blood 86:283.*

214. Antonysamy, M. A., and A. W. Thomson. 2000. Flt3 ligand (FL) and its influence on immune reactivity. *Cytokine 12:87.*

215. Ray, R. J., C. J. Paige, C. Furlonger, S. D. Lyman, and R. Rottapel. 1996. Flt3 ligand supports the differentiation of early B cell progenitors in the presence of interleukin-11 and interleukin-7. *Eur J Immunol 26:1504.*

216. Sitnicka, E., C. Brakebusch, I. L. Martensson, M. Svensson, W. W. Agace, M. Sigvardsson, N. Buza-Vidas, D. Bryder, C. M. Cilio, H. Ahlenius, E.

Maraskovsky, J. J. Peschon, and S. E. Jacobsen. 2003. Complementary signaling through flt3 and interleukin-7 receptor alpha is indispensable for fetal and adult B cell genesis. *J Exp Med 198:1495.*

217. Degliantoni, G., M. Murphy, M. Kobayashi, M. K. Francis, B. Perussia, and G. Trinchieri. 1985. Natural killer (NK) cell-derived hematopoietic colony-inhibiting activity and NK cytotoxic factor. Relationship with tumor necrosis factor and synergism with immune interferon. *J Exp Med 162:1512.*

218. Herrmann, F., R. E. Schmidt, J. Ritz, and J. D. Griffin. 1987. In vitro regulation of human hematopoiesis by natural killer cells: analysis at a clonal level. *Blood 69:246.*

219. Tilden, A. B., T. Abo, and C. M. Balch. 1983. Suppressor cell function of human granular lymphocytes identified by the HNK-1 (Leu 7) monoclonal antibody. *J Immunol 130:1171.*

220. Kuwano, K., S. Arai, T. Munakata, Y. Tomita, Y. Yoshitake, and K. Kumagai. 1986. Suppressive effect of human natural killer cells on Epstein-Barr virus-induced immunoglobulin synthesis. *J Immunol 137:1462.*

221. Abo, W., J. D. Gray, A. C. Bakke, and D. A. Horwitz. 1987. Studies on human blood lymphocytes with iC3b (type 3) complement receptors. II. Characterization

of subsets which regulate pokeweed mitogen-induced lymphocyte proliferation and immunoglobulin synthesis. *Clin Exp Immunol 67:544.*

222. Vyakarnam, A., M. K. Brenner, J. E. Reittie, C. H. Houlker, and P. J. Lachmann. 1985. Human clones with natural killer function can activate B cells and secrete B cell differentiation factors. *Eur J Immunol 15:606.*

223. Kimata, H., F. Shanahan, M. Brogan, S. Targan, and A. Saxon. 1987. Modulation of ongoing human immunoglobulin synthesis by natural killer cells. *Cell Immunol 107:74.*

224. Becker, J. C., W. Kolanus, C. Lonnemann, and R. E. Schmidt. 1990. Human natural killer clones enhance in vitro antibody production by tumour necrosis factor alpha and gamma interferon. *Scand J Immunol 32:153.*

225. Gray, J. D., and D. A. Horwitz. 1995. Activated human NK cells can stimulate resting B cells to secrete immunoglobulin. *J Immunol 154:5656.*