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Immunological Investigation of Human Complement Receptor Type II (CR2/CD21) Serum Soluble CD21 in Health and Disease

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Dedicated to:

My Parents & My Teachers

PUBLICATIONS

1. <u>Madhan Masilamani</u>, Hans-Jürgen Apell, Harald Illges
Purification and characterization of soluble CD21 from human plasma by affinity chromatography and density gradient centrifugation, ***J.Immunol.Methods***, 2002, 270(1):11

2. <u>Madhan Masilamani</u>, Elizabeth von Seydlitz, Martin Bastmeyer, Harald Illges
T cell activation induced by cross-linking CD3 and CD28 receptors leads to silencing of Epstein-Barr virus/C3d receptor (CR2/CD21) gene and protein expression, ***Immunobiolog***y, 2002, 206:528-536

3. <u>Madhan Masilamani</u>, Johannes von Kempis and Harald Illges
Shedding of soluble CD21 is specifically impaired in rheumatoid arthritis *(submitted)*

4. <u>Madhan Masilamani</u>, Daniela Kassahn, Stefan Mikkat, Michael Glocker and Harald Illges
Shedding of CD21 from lymphocyte surface depends on serine- and metalloproteases and is inducible by mitogen (PMA) *(submitted)*

5. Solomon S, <u>Masilamani M</u>, Mohanty S. Schwab J.E, Boneberg E.M, Illges H
Generation of pannus-like-3-dimensional tissue *in vitro* from single cell suspensions of synovial fluid cells from arthritis patients. *(submitted-revised)*

ABSTRACTS AND CONFERENCES

1. Poster presentation in 33rd Annual Meeting of the German Society of Immunology Sep 2002, Marburg, Germany.
<u>M. Masilamani</u> and H. Illges**,** Isolation, characterization and quantification of soluble CD21 from human plasma: Is plasma sCD21 an indicator of autoimmune reaction in vivo?, ***Immunobiology*** 2002, 206(1-3):228

2. Poster presentation in Joint Annual Meeting of the German Society of Immunology and the Dutch Society of Immunology, Nov 2001, Duesseldorf, Germany.
<u>M. Masilamani</u>, E.von Seydlitz and H. Illges, T cell expressed CD21: Downregulation during activation by anti-CD3, anti-CD28 and IL2, ***Immunobiology*** 2001, 203(1-3):78

The results of publications 1-4 are presented in this dissertation.

ABBREVIATIONS

Ab, mAb	antibody, monoclonal antibody	ELISA	enzyme-linked immunosorbent assay
Ag	antigen	EtBr	ethidium bromide
APC	antigen present cell	EtOH	ethanol
ATP	adenosine-triphosphate	F(ab´)$_2$	divalent antigen-binding fragment
°C	degree celcius	FACS	fluoresence activated cell sorting
cDNA	complementary DNA		
CD21, sCD21	cluster of differentiation-21, soluble CD21	FCS	fetal calf serum
		FITC	fluorescein isothiocyanate
CO$_2$	carbondioxide	g, mg, µg, ng	gram, milligram, microgram, nanogram
CRI, CRII	complement receptor type I, type II		
		h	hour
CTL	cytotoxic T lymphocyte	HIV	human immunodeficiency virus
CVID	combined variable immune deficiency		
		IC	immune complex
Da, kDa	dalton, kilodalton (molecular weight)	IFN	interferon
		IgG	immunoglobulin G
DMEM	dulbecco´s modified eagle medium	IgM	immunoglobulin M
		IP	immunoprecipitation
DMSO	dimethylsulfoxide	IL	interleukin
DNA	deoxy ribonucleic acid	i.v.	intravenous
EBV	Epstein-Barr Virus	kbp	kilo basepair
EDTA	ethylenediamine-tetraacetic acid	l, ml, µl	liter, milliliter, microliter
e.g.	for example	LPS	lipopolysachharide

m, cm, µm,	meter, centimeter, micrometer	RT-PCR	reverse transcriptase-polymerase chain reaction
M, mM, µM	molar, millimolar, micromolar		
MeOH	methanol	PMN	polymorphonuclear cells
MHC	major histocompatibility complex	r	recombinant
		RA	rheumatoid arthritis
min	minute	RBC	red blood cells
n	number in study or group	RNA	ribonucleic acid
NK	natural killer (cell)	RPM	rounds per minute
NP-40	nonidet P-40	RT	room temperature
O.D	optical density	SDS	sodium dodecyl sulfate
PBL	peripheral blood lymphocytes	SDS-PAGE	SDS-polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cells		
		sec	second
PBS	phosphate buffered saline	SLE	systemic lupus erythematosus
PCR	polymerase chain reaction	S value	Svedberg value
PE	phycoerythrin	TBE	Tris borate EDTA buffer
PHA	phytohemagglutinin	TBS	Tris buffered saline
PKC	protein kinase C	TCR	T cell receptor
PMA	phorbol 12-myristate 13-acetate	Th	T helper (cell)
		TNF	tumor necrosis factor
PWM	pokeweed mitogen	TNF-R	tumor necrosis factor receptor
r	recombinant	V	volt
RPMI	tissue culture medium (Rosewell Park Memorial Institute)	vs.	versus
		WBC	white blood cell

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FIGURES AND TABLES

CHAPTER 1

INTRODUCTION

1.1 Overview of the Human Immune System

The immune system is a highly evolved complicated network conferring the host protection against a vast array of pathogens. The term „immune system" refers to the enormously complex interaction of many types of cells (T-cells, B-cells, NK cells, helper cells, suppressor cells, macrophages, etc.), cell products (many lymphokines, cytokines and cytotoxins), other substances (chemicals, hormones), and physical agents (X-rays, ultraviolet light).

The immune system is broadly classified into two major functional arms namely, the innate immune system and adaptive immune system. Each arm of immunity has its own set of specificities, advantages and drawbacks. Innate immunity provides the first line of defence against invading pathogens. The innate immune response is fast and provides immediate protection against pathogens, but lacks specificity. The adaptive immunity is highly specialized and specific against a particular pathogen or antigen, but there is a delay in the onset of response. A protective adaptive immune response requires a previous encounter with the pathogen/antigen to confer the specificity. There is mounting evidence that both the arms of immunity are highly inter-connected and an efficient immune response against a particular pathogen or antigen requires both the arms of immunity (Paul, 1993).

A variety of organs and cell types are involved in conferring immunity to the host (Fig 1.1). The main organs of the immune system are the lymphoid organs, broadly classified into primary and secondary lymphoid organs. The primary lymphoid organ is the bone marrow which produces a variety of cell types found in the circulation. The secondary lymphoid organs are the spleen, lymph nodes, lymphatic system and thymus. Cells produced by the primary lymphoid organ, the bone marrow circulate in blood and are required to either pass through or rest in the secondary lymphoid organs to be able to mount an appropriate immune response.

All blood cells are derived from pluripotent stem cells, which live mainly in the bone marrow, via a process called hematopoiesis.

The stem cells produce hemocytoblasts and differentiate into the precursors of all the

Figure 1.1 Organs of the Immune system

other types of blood cells. Hemocytoblasts mature into three types of blood cells: erythrocytes (red blood cells or RBCs), leukocytes (white blood cells or WBCs), and thrombocytes (platelets). The leukocytes are further subdivided into granulocytes (containing large granules in the cytoplasm) and agranulocytes (without granules). The granulocytes consist of neutrophils, eosinophils, and basophils. The agranulocytes are the lymphocytes (consisting of B and T cells) and monocytes. Lymphocytes either circulate in the blood and lymphoid systems or rest in the lymphoid organs.

The hematopoeitic system can also be classified as lymphoid and myeloid cells (Fig 1.2). The lymphoid cells comprises B, T and NK lymphocytes while the myeloid cells consists of erythrocytes, thrombocytes, monocytes, macrophages, dendritic cells, mast cells etc. Dendritic cells, macrophages and NK cells contribute most to innate immunity as do lymphocytes for adaptive immunity.
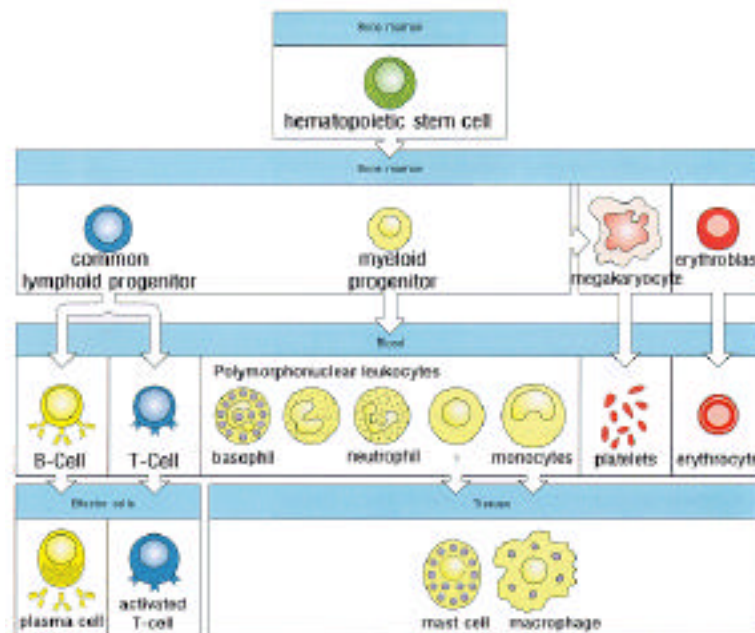
Figure 1.2 The Hematopoietic System (from Janeway, Immunobiology, 1999)

B lymphocytes originate from bone marrow and provide humoral immunity by secreting antibodies that are highly specific for a particular antigen. T lymphocytes mature in the thymus and are involved in cell mediated immunity by destroying the pathogen infected cells or in humoral immunity by secreting soluble mediators called cytokines that help in activation and differentiation of B lymphocytes (Fig 1.3). The interplay of both the cell types is required for an optimal immune response. T lymphocytes are broadly classified as helper T cells and cytotoxic T cells based on the expression of CD4 and CD8 coreceptors on the surface. Helper T cells are further classified into Th1 and Th2 cells which help in cell mediated or humoral immunity respectively depending on the nature of their cytokine secretion.
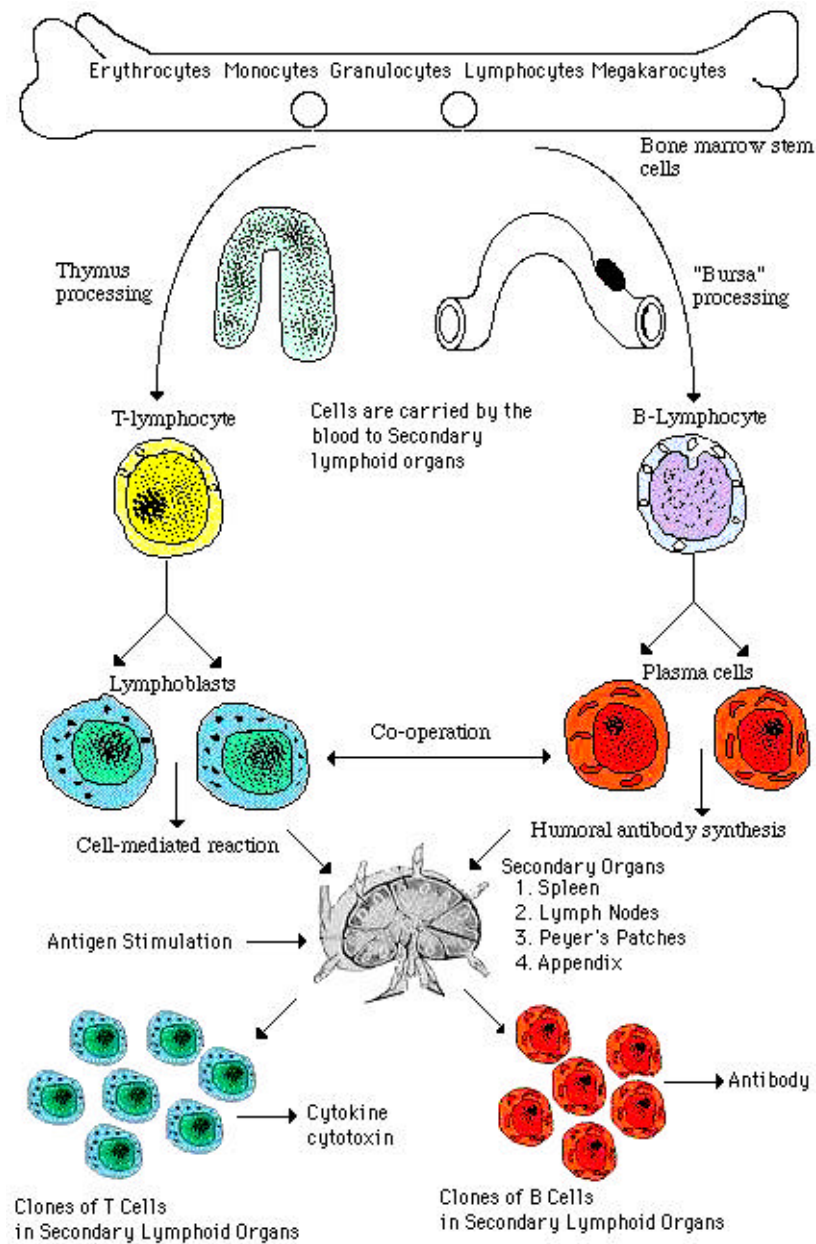
Figure 1.3 Lymphocyte Differentiation

1.1.1 Innate Immunity

The innate immune system is largely nonspecific. It is genetically based and passed on to offsprings. The major components of the innate immune system are the surface barriers, lysozyme, pH, complement system and the cells of myeloid lineage.

The physical barriers such as skin, sticky mucus of respiratory and gastrointestinal tracts provides the host protection against invading pathogens. Saliva, tears, nasal secretions, and perspiration contain lysozyme, an enzyme that destroys Gram positive bacterial cell walls causing cell lysis. Acidic pH (< 7.0) of skin secretions inhibits bacterial growth. Hair follicles secrete sebum that contains lactic acid and fatty acids both of which inhibit the growth of some pathogenic bacteria and fungi. Hydrochloric acid (pH 0.9-3.0) and protein-digesting enzymes in the gastro intestinal tract kills many pathogens.

The cells involved in innate immunity are Natural Killer cells, neutrophils, macrophages, dendritic cells, eosinophils and basophils. Natural Killer (NK) cells are large granular lymphocytes that attach to the glycoproteins on the surfaces of cancer cells and virus-infected cells and kill them. Polymorphonuclear neutrophils are phagocytes that are nondividing, short-lived (1–4 day lifespan), and have a segmented nucleus. They constitute 50–75% of all leukocytes. The neutrophils provide the major defense against pyogenic (pus-forming) bacteria and are the first on the scene to fight infection. They are followed by the wandering macrophages about three to four hours later. Circulating monocytes upon entering the site of injury or inflammation are converted into macrophages whose major function is to engulf and destroy the bacteria and virus infected cells. Release of cytokines by activated macrophages leads to inflammation. Eosinophils are attracted to cells coated with complement fragments, where they release major basic protein (MBP), cationic protein, perforins, and oxygen

metabolites, all of which work together to create holes in cells and helminths (worms). About 13% of the WBCs are eosinophils. Their lifespan is about 8–12 days. Neutrophils, eosinophils, and macrophages are all phagocytes. Dendritic cells are covered with a maze of membranous processes that look like nerve cell dendrites. Most of them are highly efficient antigen presenting cells. There are four basic types: Langerhans cells, interstitial dendritic cells, interdigitating dendritic cells, and circulating dendritic cells. These dendritic cells are distinct from follicular dendritic cells which present antigens and immune complexes (IC) to B cells in secondary lymphoid organs.

Cells of the innate immune system bind to antigen/pathogen using pathogen-associated molecular patterns (PAMP) or pattern-recognition receptors for e.g. Toll receptor family (TLR1, 2, etc) CpG motif receptors, CD14 etc. (Uthaisangsook et al., 2002). Over the course of human development these receptors PAMPs have evolved via natural selection to be specific to certain characteristics of broad classes of infectious organisms. There are several hundred of these receptors and they recognize patterns of bacterial lipopolysaccharide, peptidoglycan, bacterial DNA, dsRNA, and other substances. Clearly, they are set to target both Gram-negative and Gram-positive bacteria as well as yeast and fungi.

The complement system is a highly evolved major enzyme-triggered plasma system. It coats microbes with molecules that make them more susceptible to engulfment by phagocytes (opsonization), and kills the infected cells by making pores into the membrane. The complement system is discussed in detail in the section 1.2.

1.1.2 Adaptive or Acquired Immunity

Parts of the immune system are changeable and can adapt to better attack the invading antigen. There are two fundamental adaptive mechanisms: A) Humoral immunity and B) Cell mediated

immunity. B cells originate and differentiate in bone marrow, are the important mediators of humoral immunity and produce antibodies. T cells are non-antibody-producing lymphocytes which are also produced in the bone marrow but differentiate in the thymus and constitute the basis of cell-mediated immunity.

A) Humoral Immunity

An immunocompetent but as yet immature resting B-lymphocyte is stimulated to further differentiate when an antigen binds to its surface receptors and there is a T helper cell nearby with the same antigenic specificity (to release cytokines). This sensitizes or primes the B cell and undergoes clonal selection and proliferation. B cells may either differentiate into plasma cells secreting highly specific antibodies at a rate of as many as 2000 molecules per second or may become long-lived memory cells.

Antibodies, also called immunoglobulins (Igs), constitute the γ *globulin* part of the plasma proteins. The antibodies inactivate antigens by, (a) complement fixation, (b) neutralization (c) agglutination and (d) precipitation. The constituents of gamma globulin are: IgG-76%, IgA-15%, IgM-8%, IgD-1%, and IgE-0.002%. IgG is the only antibody that can cross the placental barrier to the fetus and it is responsible for the 3 to 6 month immune protection of newborns that is conferred by the mother. IgM is the dominant antibody produced in primary immune responses, while IgG dominates in secondary immune responses. IgG is normally found in circulation as monomeric forms while IgM is pentameric. IgA and IgD may also be found as dimers. Each antibody has two functional arms, namely, antibody binding region (F´ab) and constant region (Fc). The antibody recognizes the epitope (not the entire antigen) through Fab region. The Fc region is responsible for effector functions, i.e., the end to which immune cells can attach through Fc receptors.

B) Cell Mediated Immunity

Macrophages engulf antigens, process them internally, then display antigenic peptides on their surface together with MHC. This „antigen presentation" sensitizes the T cells to recognize these antigens. T cells undergo two selection processes in thymus. First a *positive* selection process weeds out only those T cells with the correct set of receptors that can recognize the MHC molecules responsible for self-recognition. Then a *negative* selection process begins whereby only T cells that can recognize MHC molecules complexed with foreign peptides are allowed to pass out of the thymus.

Cytotoxic or killer T cells (CD8+) functions by releasing lymphotoxins, which cause cell lysis. Helper T cells (CD4+) secrete lymphokines or cytokines that stimulate cytotoxic T cells and B cells to grow and divide, attract neutrophils, and enhance the ability of macrophages to engulf and destroy microbes. Suppressor T cells inhibit the responses of B cells and other T cells to antigens by secreting some inhibitory factors. Memory T cells are programmed to recognize and respond to a pathogen once it has invaded and been repelled.

1.2 The Complement System

The complement system serves as a recognition and effector arm of humoral immunity. Intitially, the complement system was described as a heat labile activity in serum that augments opsonization of bacteria by antibodies and subsequent lysis. Now it is very well established that in addition to „complementing" other immune defenses, especially the humoral immune system, the complement by itself functions as an independent immune network (Carroll, 1998; Carroll and Prodeus, 1998). The importance of complement in defense against pathogenic bacteria is indicated by the serious infectious complications associated with genetic deficiencies of key complement proteins (Ross and Densen, 1984).

The role of complement in innate immunity are a) pathogen recognition, b) inflammation, c) opsonophagocytosis, and d) direct killing of microbes. It also serves the adaptive immune system through recognition of immunoglobulin complexes and regulation of immune responses engaging complement receptor type 2 (CR2, CD21) (section 1.3), and immunomodulation by anaphylotoxins.

The complement system consists of a set of about 30 proteins designed to destroy invading microbes. The activation of the complement system is induced by proteolytic mechanisms triggering a cascade of reactions resulting in activation of other complement proteins. Certain proteolytic products promote inflammatory response, while others facilitate phagocytosis. The terminal components of the complement system destroy bacteria by forming a membrane attack complex creating pores in the bacterial membrane. Because of the highly destructive capability of the complement system, nearly half of the complement proteins serves in regulation. Certain activated complement proteins binds to any nearby surface, immaterial of self or nonself. The further activation of complement proteins is kept in check by regulators present on all host cell surfaces exposed to sera such as decay accelarating factor (DAF/CD55) while the complement cascade triggers a series of proteolytic events and final destruction of invading „nonself" pathogens. Thus the specificity of the complement system to distinguish „self" and „nonself" is highly controlled.

The initial components of the complement system are often serine proteases that activate the next component of the cascade. The later components are membrane-binding proteins which directly insert into the membrane of invading microbe. The regulators of complement activation members are structurally, functionally and genetically related proteins designed to protect the host from inadverdant damage by complement activation. The effector function of complement can be activated through three pathways (Fig 1.4): a) The classical pathway

activated by immune complexes, b) the alternative pathway intiated by binding of complement component to the pathogen surface and c) the mannan-binding lectin pathway, initiated by binding of a serum lectin, the mannan-binding lectin, to mannose-containing carbohydrates bacteria or viruses. Regardless of which route of activation initiates the complement system, the end results are envisaged in three forms. First, to tag the pathogens surface by deposition of complement components thereby marking them (opsonization) as non self leading for phagocytosis. Second, formation of membrane attack complex (MAC), a series of protein interactions that leads to lysis of the pathogen and third, activation of inflammatory responses through by-products of complement activation called as anaphylatoxins.
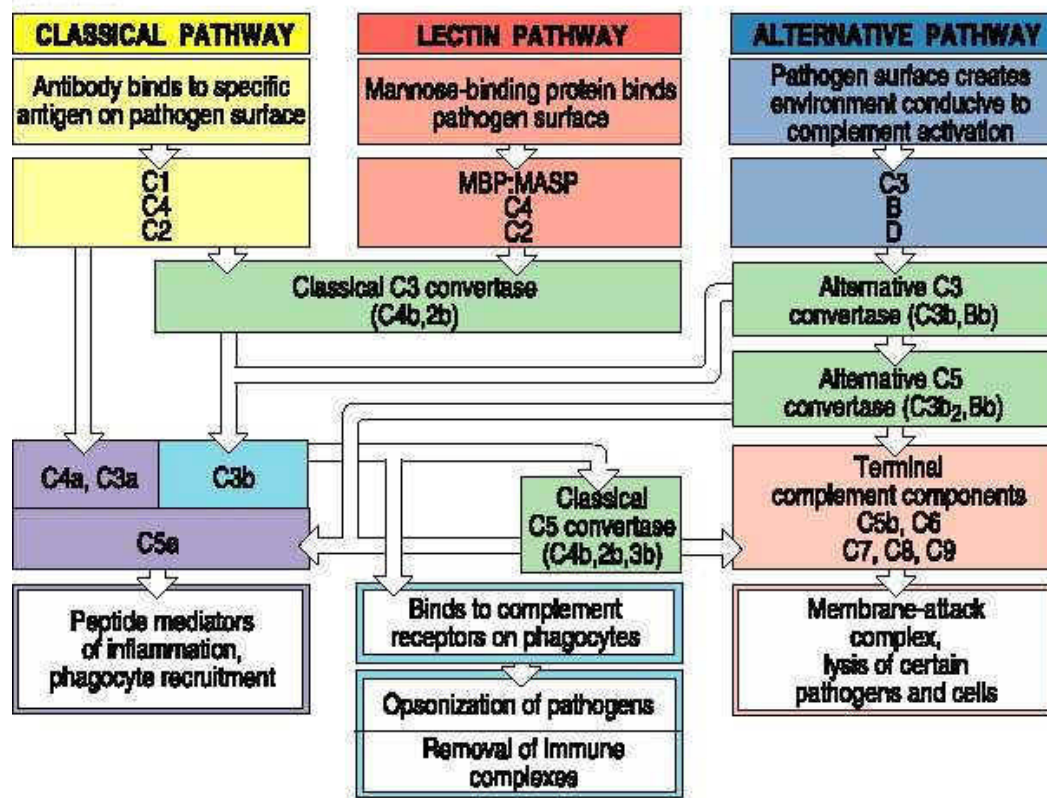
Figure 1.4 The Complement Pathways

1.2.1 The Complement Nomenclature

The components of classical and alternative pathways are designated by the letter C followed by a number. The complement components were numbered in the order of their discovery rather than the sequence of reactions (Fig 1.5). The products of the cleavage reactions are designated by added lower-case letter, the smaller fragment being designated „a" and the larger form „b". Activated complement components are often designated by a horizontal line for example $\overline{C2b}$.
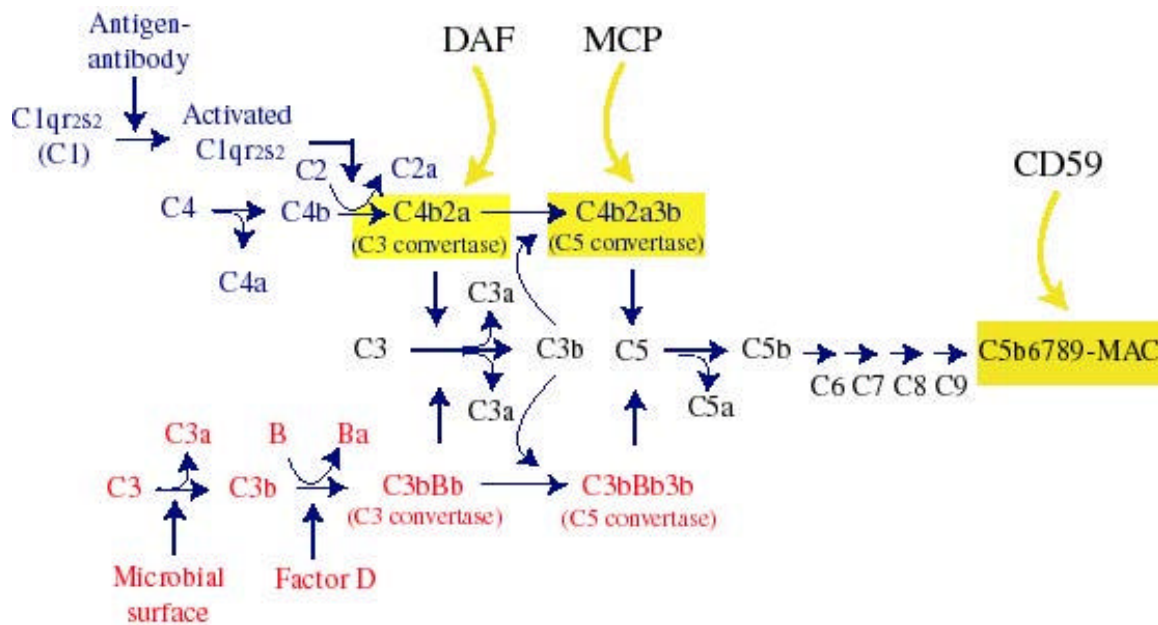
Figure 1.5 Complement Activation (from Kuby, Immunology, 1997)

1.2.2 The Classical Complement Pathway

The first component of classical pathway is C1, which is a multichain complex consisting of the C1q, C1r and C1s proteins. Binding of two globular heads of the C1q molecule to antigen bound antibodies or IC activates C1r, which cleaves and activates the serine protease C1s. Activated C1s cleaves C4 into C4a and C4b, exposing a reactive group on C4b which covalently

binds to the microbial surface. C4b then binds to C2 making it susceptible to cleavage by C1s. The resulting C4b2a complex is an active protease called C3 convertase, which cleaves molecules of C3 to produce C3a and C3b. C3a, the smaller fragment of C3, is a powerful inflammatory mediator. The cell bound C3 convertase deposits a large number of C3b molecules on the pathogen surface. One C4b2a can cleave up to 1000 molecules of C3 to C3b which binds to the pathogen surface. The next step in the cascade is the generation of C5 convertase by binding of C3b to C4b2a to yield C4b2a3b. This complex binds C5 and cleaving of C5 by protease activiy of C2b intiates generation of the membrane attack complex.

1.2.3 The Alternative Complement Pathway

The alternative pathway is activated by covalent binding of C3b to pathogen surface. The source of C3b to alternative pathway is the spontaneous activation of C3 called as „tickover", a phenomenon due to the unstable highly reactive thioester group. The second step of the alternative pathway is binding of C3b to factor B, making it susceptible to cleavage by the plasma protease factor D yielding Ba and Bb. The active protease Bb remains bound to C3b to make the complex C3bBb which is the C3 convertase of the alternative pathway of complement activation. The component C3bBb is an exact structural and functional homologue of C4b2b of classical pathway. Some of the bound C3b binds to preexisting C3 convertase, yielding C3b2Bb, the alternative pathway C5 convertase. C3b2Bb cleaves C5 into C5b initiating the generation of membrane attack complex.

1.2.4 The Membrane Attack Complex

Both classical and alternative pathway converge at the level of C5 convertase to initiate the formation of membrane attack complex. The end result is a pore in the lipid bilayer membrane destroying the proton gradient, thereby killing the microbe. C5b binds to C6 and the C5b6

complex then binds to C7 leading to a conformational change exposing hydrophobic site on C7. The hydrophobic domain of C7 inserts into the lipid bilayer. Similar hydrophobic sites are exposed in later components C8 and C9 when they are bound to the complex. The C8alpha-gamma subunit induces polymerization of 10-16 molecules of C9 into the the annular or ring structure called the membrane-attack complex.

1.2.5 Complement Receptors and Complement Regulatory Proteins

The regulatory functions of complement are envisaged by specific recognition of pathogen bound C3 fragments by complement receptors. The end effect depends of the type of the receptor and the cell. Five types of complement receptors are known to bind complement components, namely, CR1, CR2, CR3, CR4 and C1q receptor.

Complement receptors CR1 (CD35) and CR3 (CD11b/18) on phagocytes bind pathogens opsonized with complement fragments C3b and iC3b respectively. CR1 on erythrocytes clear immune complexes from circulation. CR2 (CD21) plays an important role in B cell signalling as B cell coreceptor. Attachment of C3 to antigens and subsequent binding to complement receptors CR1 and CR2 in addition enhances the antigen uptake by B cells, modulates downstream events such as endosomal targeting of antigen and processing and binding of peptides to major histocompatibility complex (MHC) class I molecules (Cherukuri et al., 2001a; Nielsen and Leslie, 2002; Nielsen et al., 2002). The structure and function of CR2/CD21 is discussed in detail in section 1.3. CR3 and CR4 (CD11c/18) binds to iC3b and promotes phagocytosis. C1q receptor also promotes phagocytosis by binding immune complexes.

The regulatory proteins of complement activation (RCA) are a set of proteins that play an important role in keeping complement activation in check. All these proteins are encoded at a

single chromosomal location on chromosome 1 and contain a number of short consensus repeats (SCRs). The RCA proteins C4b-binding-protein (C4bBP), CR1, and membrane cofactor protein (MCP) prevent assembly of the C3 convertase in the classical complement pathway. Factor H binding to C3b prevents its association with factor B in the alternative pathway. Decay-accelarating factor (DAF) acts on both the pathways causing dissociation of assembled C3 convertase. At the level of the MAC, a serum protein called S protein can bind to C5b67 preventing insertion of C5b67 into the cell membrane. Homologous restriction factor (HRF) and membrane inhibitor of reactive lysis (MIRL) present on the membrane of many cell types, protect cells from nonspecific complement-mediated lysis by binding to C8, preventing assembly and insertion of MAC. Both the proteins display homologous restriction, since the inhibition occurs only if the complement components are from the same species as the target cells (self-nonself recognition) (Kuby, 1997).

1.3 Complement Receptor Type II (CR2/CD21) – A Review

The complement receptor type II (CD21) belongs to the super gene family of regulators of complement activation. CD21 is the functional receptor for C3d fragments on immune complexes and the Epstein-Barr virus (EBV) envelope protein gp350 (Ahearn and Fearon, 1989). This 140 kDa glycoprotein consists of 15 or 16 extra-cellular short consensus repeats (SCR) of 60-70 amino acids each and a short cytoplasmic part of 34 amino acids (Fig 1.5) (Iida et al., 1983; Weis et al., 1984). CD21 is expressed on mature B-lymphocytes and B cell lines,
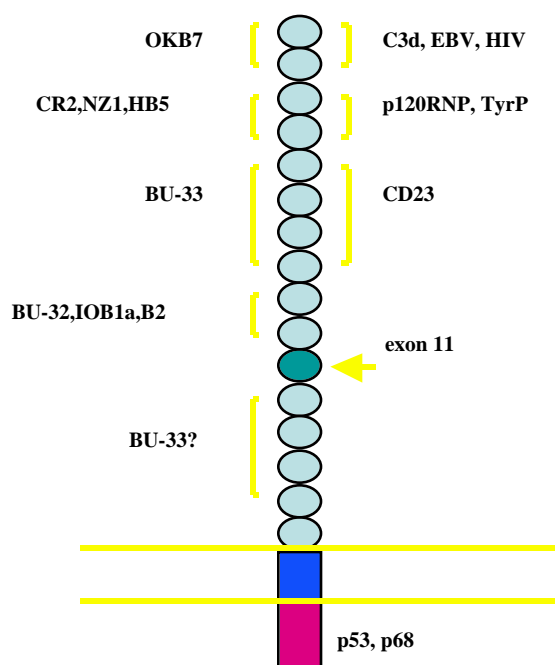
Figure 1.5 Structural Features of CD21

The short consensus repeats (SCR) of CD21 are represented as ovals. The exon 11, present as a splice variant in the long form of CD21 is indicated by an arrow. The ligands binding to their respective SCR are shown on the right and the antibodies against respective epitopes are shown on the left.

but not on early pre and pro B cell and late developmental stages (Tedder et al., 1984). In addition, it is also expressed on peripheral blood and thymic T cells (Fischer et al., 1991), T cell lines (Larcher et al., 1990; Delibrias et al., 1992) and a number of other cell types (Carroll, 1998; Fearon, 2000).

Functionally, CD21 on B cells and follicular dendritic cells (FDC) is implicated in the recognition and binding of immune complexes while the function in T cells and all other cell types is not known. In T cells, the expression of CD21 is developmentally regulated as double negative thymocytes express membrane bound CD21 (Fischer et al., 1999). Ligation of CD21 results in various signals that are critical for normal B cell responses (Fearon and Carroll, 2000). Crosslinking CD21 with C3d or certain anti CD21 antibodies in the presence of T cell factors leads to B cell proliferation and differentiation (Nemerow et al., 1985; Wilson et al., 1985). Crosslinking CD21 with membrane IgM promotes T cell-independent proliferation (Carter et al., 1988; Fingeroth et al., 1989). On mature B cells, CD21 forms a non-covalent signal transduction complex in the plasma membrane together with the CD81, Leu-13 and the pan-B cell antigen CD19. This complex amplifies the signal transmitted through the B cell receptor by specific antigen and thereby reduces the threshold of antigen necessary to initiate cell proliferation (Dempsey et al., 1996; Cherukuri et al., 2001b). The mechanism involved appears to be synergism between the IL-4R and BCR-CD21 signaling pathways in promoting the progression of resting B cells past an early G1 checkpoint (Mongini and Inman, 2001). In addition, CD21 plays a key role in determining B cell survival by limiting apoptosis induced through ligation of membrane IgM (Kozono et al., 1995) and through accumulation of Bcl-2 (Roberts and Snow, 1999).

Alternatively, CD21 participate in the generation of a normal immune response by internalizing and directing C3-bound Ag into the class II processing pathway of B cells (Lanzavecchia et al., 1988; Cherukuri et al., 2001a). CD21 is also shown to have a direct influence on B cell-T cell signal exchange by simultaneous up-regulation of CD80 and CD86 on murine splenic B cells (Kozono et al., 1998). The other functions of CD21 reported in literature though not yet clear are in development and maintenance of B1 cells (Ahearn et al.,

1996). In human pro- and pre-B cells the expression of the CD21 gene is silenced by methylation of a CpG island in its promoter. Expression in mature B cells is accompanied by the loss of CpG-methylation (Schwab and Illges, 2001a; Schwab and Illges, 2001b). C3 deposition on B cells may enhance their interaction with CD21 on FDC and vice versa. CD21 in FDC plays a very important role in rescuing antigen-activated B cells from apoptosis (Liu et al., 1989; Lindhout et al., 1993), promotion of somatic hypermutation (Apel and Berek, 1990; Nie et al., 1997) and class switch (Croix et al., 1996; Wu et al., 1996).

CD21 is also found in a soluble form (sCD21) generated by shedding from lymphocytes in culture and in human plasma (Myones and Ross, 1987; Ling et al., 1991; Huemer et al., 1993). Ling et al (Ling et al., 1991) have purified a 72 kDa form of sCD21 from lymphoblastoid cell lines by affinity chromatography on sepharose-coupled BU34, BU33 and BU36 mAbs followed by DEAE ion exchange chromatography. Later, by metabolically labeling the LICR-LON-Hmy cell line with S^{35}, they could isolate several proteins of molecular range from 30-130 kDa. In addition to a range of proteins isolated from tissue culture supernatants, cell-associated CD21 from cell lysates was detected as a 120-140 kDa molecule and was reduced to 115 kDa upon treatment with endoglycosidase (Ling and Brown, 1992). sCD21 affinity-purified from human serum with THB5 and BU32 mAbs showed a 135 kDa and a 90kDa protein. Moreover, sCD21 circulates as a complex with cleavage fragments of C3 and a trimeric form of soluble CD23 (sCD23). CD21 isolated from human serum showed a smear of 135-190 kDa under non-reducing conditions (Fremeaux-Bacchi et al., 1998b).

1.4 Origin of Proposals

1.4.1 CD21 and T Cell Activation

Previously it was shown that purified peripheral blood B and T lymphocytes from healthy volunteers as well as transformed B and T cells have similar amounts of CD21 mRNA, but differ in surface expression of CD21 glycoprotein (Illges et al., 1997; Braun et al., 1998). Illges et al have shown a reduced expression of CD21 in synovial, possibly activated B and T lymphocytes of patients suffering from various forms of arthritis (Illges et al., 2000). Similarly B cells down regulate CD21 upon differentiation into the blast stage (Tedder et al., 1984). Therefore it would be interesting to know whether a downregulation of CD21 expression would occur during T cell activation. For that purpose, T cells from peripheral blood were activated by cross-linking CD3 and CD28 in the presence of IL-2 to proliferate and differentiate into blasts, as evidenced by its increase in cell size.

Real-time Taqman PCR analysis revealed that CD21 mRNA levels were reduced within 24 to 48 hours of activation and remained so for 7-8 days. The protein expression was investigated by immunofluorescence/confocal microscopy in different cell types. While CD21 protein levels were high and easily detectable by FACS in B cells, T cell expressed protein could only be detected by highly sensitive immunofluorescence/confocal microscopy showing a further reduction upon activation. Its behavior during differentiation of T cells implies that CD21 in peripheral blood T cells performs functions which, however, have yet to be defined.

1.4.2 CD21 Isolation, Characterization and Quantification

There are reports of clinical correlation of sCD21 in plasma with B cell chronic lymphocytic leukemia (B-CLL), common variable immunodeficiency (CVID) and Brutons´s X-lined agammaglobulinemia (Lowe et al., 1989; Ling et al., 1991). Elevated levels of sCD21 were

found in patients with EBV associated malignancies (Huemer et al., 1993). A yet unknown protease is thought to be responsible for shedding of CD21 from the membrane. In order to biochemically define the recognition site of the protease, and to design sensitive assays such as ELISA and to perform other biophysical studies which require higher degree of purity, it is important to purify sCD21 from human serum to homogeneity. Affinity chromatography using antibodies to different epitopes of CD21 co-purifies other proteins as expected. Upon high-speed density gradient centrifugation, pure sCD21 could be isolated with a molecular weight of about 126 kDa. The sedimentation coefficient of the protein and other biophysical parameters were determined. Its mobility in reducing/non-reducing SDS-PAGE and Western blotting compared to cellular CD21 isolated from B and T cell lines was investigated.

As sCD21 could potentially bind to its ligands in plasma, the amount of sCD21 in serum could be a modulator of immunity (Fremeaux-Bacchi et al., 1999). Using purified sCD21 as standard, a specific ELISA was established for the quantification of sCD21 in serum. To study the possible role of sCD21 in immunologically relevant clinical conditions, serum sCD21 levels were estimated in 235 healthy individuals, 209 rheumatoid arthritis patients, 41 CVID patients, 29 pregnant women and in 9 neonates. Attempts to correlate the sCD21 levels to the clinical conditions were made.

1.4.3 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a systemic chronic inflammatory disease, which predominantly affects the joints. The symptoms include pain, swelling, stiffness and loss of function of the joints. The disease often affects the wrist joints and the finger joints closest to the hand and generally occurs in a symmetrical pattern involving both the limbs. It can also affect other parts of the body besides the joints with symptoms of fatigue, occasional fever and malaise.

In RA, the autoimmune response develops against self-antigens inside the joint capsule and causes severe inflammation, called synovitis, resulting in warmth, redness, swelling etc. During the inflammation process, the cells of the synovial membrane are activated and start to grow and divide into the joint cleft. As the disease progresses, the activated synovial cells begin to invade and destroy the cartilage and bone within the joint. The surrounding muscles, ligaments and tendons that support and stabilize the joint become weak and unable to function normally. In addition, many patients develop rheumatoid nodules under the skin that often form in the vicinity the joints and may suffer from anemia, neck pain and dry eyes and mouth.

The etiology of RA is still unknown. A number of genetic, environmental and hormonal factors are involved in the disease. In the extremely complex pathophysiology, innate immunity plays an important role (Corr and Firestein, 2002). Diagnosis is largely based on symptoms related to synovial inflammation and further supported by the presence of rheumatoid factor (RF) and radiological signs of joint damage (Arnett et al., 1988). Only RF has been established as a marker useful for molecular diagnosis while a variety of other serum parameters, e.g. acute phase proteins such as C-reactive protein, fibrinogen etc., only indicate general disease activity. About 65% of all people with RA test positive for RF. Some others who do test positive never develop the disease. Other common tests include erythrocyte sedimentation rate (ESR), white blood cell count and blood tests for anemia. Markers of increased connective tissue turnover are elevated in RA. In addition, certain soluble membrane-bound proteins such as soluble IL2 receptor, soluble CD4, IL6, alpha-1-antitrypsin and complement activation products are elevated in RA (Harris, 1997). Treatment is generally symptomatic aimed to reduce pain and improve the patient´s sense of well-being and ability to function. In addition to medications, other treatments include surgery, joint replacement, tendon reconstruction and synovectomy. Recently anti-TNF- therapy has been approved for use as a treatment for severe RA. Other approaches under research are development of safe and effective inhibitors of mitogen-

activated protein kinase (MAPK) and nuclear factor- B (NF- B) pathways, gene therapy and interaction of stem cells and their growth factors to engineer tissue repair (Feldmann, 2001).

1.4.4 Common Variable Immunodeficiency

Common Variable Immuno Deficiency (CVID) is a heterogenous group of humoral immunodeficiencies of unknown etiology. CVID is a disorder characterized by low levels of serum immunoglobulins and an increased susceptibility to infections. The exact cause of the low levels of serum immunoglobulins is usually not known. It is relatively common form of immunodeficiency, hence the word „common". The degree and type of deficiency of serum immunoglobulins and the clinical course also varies from patient to patient, hence the word „variable". In some patients there is a decrease in both IgG and IgA; in others, all three major types (IgG , IgA and IgM) of immunoglobulins may be decreased. The clinical signs and symptoms also vary from severe to mild. Frequent and unusual infections may occur during eary childhood, adolescence or adult life. In the majority of patients, however, the diagnosis is not made until the 3rd or 4th decade of life. The causes of CVID are largely unknown. Most patients appear to have normal number of B-lymphocytes, but they fail to undergo normal maturation into plasma cells. Other patients lack the helper T-lymphocytes necessary for a normal antibody response. A third group of patients have excessive numbers of cytotoxic T-lymphocytes (Cunningham-Rundles, 2001).

Bryant *et al* have functionally classified the disease according to their capacity to produce IgM, IgA and IgG *in vitro* upon stimulation with *Staphylococcus aureus Cowan* (SAC) plus IL-2 or anti-IgM plus IL-2 (Bryant et al., 1990). PBLs of patients in group A fail to produce any Ig isotype in vitro, while group B patients produce IgM only and group C patients are indistinguishable from healthy controls in producing normal amounts of all isotypes in vitro despite low serum Ig levels in vivo. Another approach to classify the disease has been suggested

by Warnatz K et al (Warnatz et al., 2002), using flow cytometric quantification of class-switched memory and immature B cells in the peripheral blood of patients. Group I patients according to this classification show a reduction of $CD27^+IgM^-IgD^-$ memory B cells below 0.4% while this B cell population exceeded 0.5% in group II patients.

1.4.5 Pregnancy

The suppression of the maternal systemic immune response during pregnancy is only partly understood. The foetus can be regarded as an allograft which would under other circumstances induce rejection. This rejection is prevented by several mechanisms, including production of immunosuppressive factors both at the trophoblast-endometrium interface and the foetus. In some cases, these factors may diffuse into the general circulation to produce a generalized, usually mild, immunosuppression.

Normal pregnancies were characterised by decreased levels of B cells ($CD19^+$), NK cells ($CD56^+$), reduced expression of the IL-2 receptor on lymphocytes ($CD25^+$) as well as HLA-DR+ on T cells (CD3+) and increased level of inactivated $CD4^+CD45RA^+$ T cells. Addition of mitogens to cell cultures in either autologous or AB serum culture media demonstrated the existence of serum and cell mediated suppressor activity. The lymphoproliferative response to mitogens was reduced during normal pregnancies, possibly mediated by PGE2 and the presence of T lymphocytes with suppressor function. Circulating IFN- and IL-4 secreting cells are increased during normal pregnancies. The prevalence of anticardiolipin antibodies (aCL, IgG, ELISA), antinuclear antibodies (ANA, Indirect immunofluorescence) and rheumatoid factor (RF, agglutination test) in a normal pregnant population was low.

1.4.6 Mechanism of CD21 Shedding

Many proteins are shed from the cell surface by endoproteolytic activity associated with the plasma membrane. The large portions of the shed surface molecules are called ectodomains. Usually the ectodomains are not ligand bound and represent the soluble form of the active membrane protein in the extracellular mileau. The ectodomain shedding results in downmodulation of cell surface concentration leading to inhibition of ligand interaction (Rovida et al., 2001), transformation of membrane proteins into soluble competitors for their own ligands (Subramanian et al., 1997) and generation of a transmembrane/cytoplasmic portion of the molecule, which can have signalling functions different from those of the full-length molecule uncontrolled by ligand (Vecchi and Carpenter, 1997).

Altered soluble receptor expression is associated with human disease (Heaney and Golde, 1998). Soluble receptor concentrations in serum are increased in many disease conditions and some are used for therapeutic purposes, for e.g. soluble IL-2 receptor (Kamihira et al., 1994), sCD23 (Beguin et al., 1993), sCD27 (Kersten et al., 1996), sCD30 (Pizzolo et al., 1990), sTNF receptor (Kalinkovich et al., 1992), sIL-6 receptor (Ohtani et al., 1995). The majority of cell surface proteins are resistant to proteolytic release. Therefore, shedding has to be a highly-regulated process, catalysed by specialized proteases (Arribas et al., 1996).

The source and mechanism of CD21 shedding from cell surface was investigated using various protease inhibitors, ELISA and cytometric analyses. The shedding of CD21 from PBL and Raji B cell lines was inhibited by metalloprotease inhibitor, EDTA and serine protease inhibitor, alpha-1-antitrypsin. Activation of Raji B cells with PMA and calcium ionophore induced shedding of sCD21 and is paralleled by a decrease in membrane associated CD21.

CHAPTER 2

AIMS OF THE STUDY

This project was undertaken to understand the biology and function of CD21 molecule in general. The study was conducted in three parts.

The first part of the study investigates the relative gene expression of CD21 in T cells during physiological activation. The goal is to explore the possible functions of the glycoprotein in peripheral blood T cells and also to address the controversy over expression of CD21 in cells of T cell lineage. Peripheral blood T cells were isolated, activated and the expression of CD21 was studied both at the mRNA and protein expression levels.

The second part is devoted to purification and biochemical/biophysical characterization of the soluble form of CD21 shed by the lymphocytes into plasma. Previous attempts to isolate the protein in pure form resulted in co-purification of other proteins and proteolytic products of CD21 were likely generated during the purification process. A combination of affinity chromatography and density gradient centrifugation techniques were employed to achieve pure sCD21 in its native form.

The third aspect of the study was to quantify and establish the absolute amounts of sCD21 in human plasma and the relevance of the quantification in terms of health and disease. A large number of human serum samples of various disorders and health conditions were analysed for this purpose. And finally, experiments concerning the signal transduction events responsible for CD21 shedding were performed to understand the underlying mechanisms.

CHAPTER 3

MATERIALS AND METHODS

3.1 Cells

The mature human B cell lines, Raji (Klein et al., 1975) Dakiki (Steinitz and Klein, 1980), Nalm 6 (Korsmeyer et al., 1983), BJAB (Clements et al., 1975), and T cell lines Molt-4 (Minowada et al., 1972) were grown in Iscove´s DMEM medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS, 1000U/ml penicillin, and 100μg/ml streptomycin at 37°C in 7.5% CO_2. The human T cell line Jurkat (Gillis and Watson, 1980) was grown in RPMI medium (Invitrogen, Karlsruhe, Germany) supplemented with 5% FCS, 1000U/ml penicillin, and 100μg/ml streptomycin. Monoclonal anti-CD21 antibody clones BU32 (IgG1), BU33 (IgG1), BU35 (IgG2b) (Ling and Brown, 1992) and THB5 (Weis et al., 1984) were grown in serum-free hybridoma medium (Invitrogen) at 37°C in 7.5% CO_2. Peripheral blood B and T cells were isolated from fresh blood collected from healthy volunteers.

3.2 Human Plasma and Sera

Human plasma/sera were collected from healthy blood donors, (provided by Dr. Thomas Hartung, Department of Pharmacology, University of Konstanz). Patient sera were kindly provided by Drs. Hartmut H. Peter and Michael Schlesier, Universitätsklinikum Freiburg, Dr. Johannes von Kempis, Kantonspital St Gallen, and Dr. U. Brunner, Konstanz.

3.3 Antibodies and Reagents

Unless otherwise specified, general laboratory chemicals were purchased from standard chemical companies like Sigma, Roth, Promega, Serva, Merck, Fluka, Amersham etc.

The following Abs and reagents were purchased from the indicated companies:

anti-CD3 (UCHT1), anti-CD28 (15E8), anti-CD21 (BU32), and Goat anti-mouse IgG-F(ab´)$_2$	Dianova (Hamburg, Germany)
Goat anti-mouse Alexa488 Goat anti-mouse Cy3	Molecular Probes (Eugene, Oregon, USA)
Human IL-2	Biotest (Dreieich, Germany)
anti-CD4-PE and anti-CD8-FITC	Caltag (Hamburg, Germany)
anti-CD19-PE	DAKO (Glostrup, Denmark)
anti-CD21-FITC	Coulter (Cedex, France)
Density gradient markers	Sigma (Taufkirchen, Germany)
Protein-G sepharose	AP Biotech (Freiburg, Germany)

3.4 Lymphocyte Isolation and Sorting

3.4.1 Principles of Magnetic Activated Cell Sorting

For Magnetic Activated Cell Sorting (MACS) (Fig 3.1), cells are magnetically labeled with antibody conjugate to microbeads (for eg. anti-CD19-microbeads, Miltenyi Biotech, Gladbach, Germany) and separated on a column placed in the magnetic field of a MACS separator (Fig 3.2). The magnetically labeled CD19$^+$ cells were retained in the column while the unlabeled CD19$^-$ cells run through. The unlabeled cells were depleted of CD19$^+$ cells. After removal of the column from the magnetic field, the magnetically retained CD19$^+$ cells were eluted as positively selected cell fraction.

Lymphocytes were purified by ficoll density gradient centrifugation of fresh human blood. B and T cells were enriched by magnetic sorting as previously described (Braun et al., 1998). Briefly, 10^7 PBMCs were suspended in 80µl of MACS buffer (5mM EDTA/PBS, 0.5% FCS). For fewer cells the same volume is used. 20µl of MACS CD19 antibody or CD3
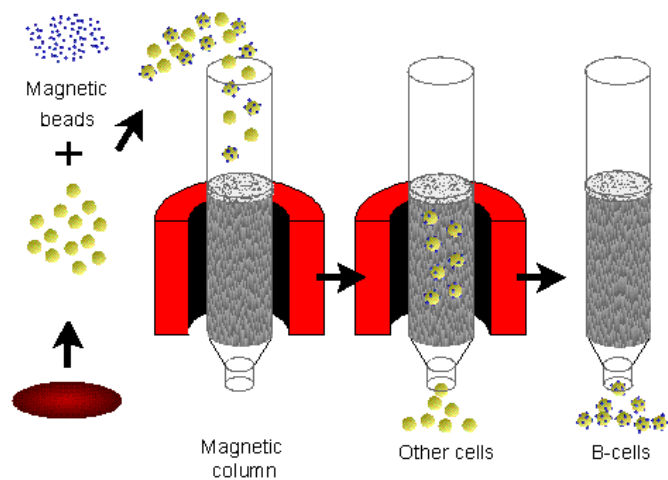
Figure 3.1 Magnetic Activated Cell Sorting

antibody was added for pure B and T cells respectively per 10^7 cells, mixed well and incubated for 15-20 minutes at 4° C. For fewer cells, the same volume is used. Cells were washed by adding 10-20 times MACS buffer, centrifuged at 300g for 10 min, the supernatant removed completely and the cell pellet re-suspended in MACS buffer (500µl buffer for 10^8 cells). The

suspended cells were added on to a previously washed MS Separation column placed on a MiniMACS separation unit (Fig 3.2).

The cell suspension was allowed to run through the column and the effluent collected as negative fraction. The column was washed 3✕ with 500 µl buffer and the total effluent collected as negative fraction. The column was removed from the separator magnet, placed on a new collection tube. The positive fraction was collected by adding 1ml of buffer to the reservoir column and firmly flushing out cells using the plunger supplied.

Figure 3.2 MACS Columns and Separation Units

The relative purity of the cells was determined by cytometric analysis using anti-CD4-PE/anti-CD8-FITC as markers for T cells and anti-CD19-PE/anti-CD21-FITC as markers for B cells. Cells were stained in microtiter plates and loaded into a FACScan (Becton Dickinson, Heidelberg, Germany) using a self constructed loader as previously described (Illges, 1999).

3.4.2 Principles of Flow Cytometry / Fluorescence Activated Cell Sorting

Flow cytometry is a method for quantitating components or structural features of cells primarily by optical means (Fig 3.3). Although it makes measurements on one cell at a time, it can process up to 100,000 cells in a second. Since different cell types can be distinguished by quantitating structural features, flow cytometry can be used to count cells of different types in a mixture. The cells may be alive or fixed at the time of measurement, but must be in monodisperse (single cell) suspension. They are passed single-file through laser beams by continuous flow of a fine stream of the suspension. Each cell scatters some of the laser light,

and also emits fluorescent light excited by the laser. The cytometer typically measures several parameters simultaneously for each cell: the low angle forward scatter intensity, approximately proportional to cell diameter (FSH); the orthogonal (90 degree) scatter intensity (SSH), approximately proportional to the quantity of granular structures within the cell; and the fluorescence intensities at several wavelengths (FL1, FL2, FL3). Light scatter is commonly used to exclude dead cells, cell aggregates, and cell debris from the fluorescence data. Furthermore, It is sufficient to distinguish lymphocytes and monocytes from granulocytes in blood leukocyte samples.

Fluorescence intensities are typically measured at several different wavelengths simultaneously for each cell. Fluorescent probes are used to report the quantities of specific components of the cells. Fluorescent antibodies are often used to

Figure 3.3 Fluorescence Activated Cell Sorting

report the densities of specific surface receptors, and to distinguish subpopulations of differentiated cell types, including cells expressing a transgene. Intracellular components can also be reported by fluorescent probes, including total DNA per cell (allowing cell cycle analysis), newly synthesized DNA, specific nucleotide sequences in DNA or mRNA, filamentous actin, and any structure for which an antibody is available. Flow cytometry can also monitor rapid changes in intracellular free calcium, membrane potential, pH, or free fatty acids.

3.5 T Cell Activation

1×10^6 sorted T cells per ml were cultured in Iscove´s DMEM medium containing 20 units of human IL-2 in tissue culture wells coated with mouse anti-human-CD3 (200 ng/ml/well) and mouse anti-human-CD28 (200 ng/ml/well), precoated with goat anti-mouse-IgG-F(ab´)$_2$ (2.5µg/500µl/well). The cells were incubated at 37°C and 7.5% CO_2. Cells were removed from culture every alternate day, lysed and total RNA was isolated. Cytometric analysis was done on activated T cells once again to confirm activation, indicated by increase in forward and side scatter before RNA isolation.

3.6 RNA Isolation, cDNA Synthesis, RT-PCR and TaqMan PCR

Isolation of RNA was performed using RNAzol B according to manufacturer´s protocol (Wak-Chemie Medical GmbH, Bad Soden, Germany) (Chomczynski and Sacchi, 1987). Briefly, cells were lysed in RNAzol B, a monophase solution containing both phenol and guanidium-isothiocyanate. The addition of bromochloropropane or chloroform and subsequent centrifugation efficiently removes DNA and proteins from an aqueous phase containing the RNA. Undegraded RNA is obtained by isopropanol precipitation, washing of the RNA pellet, and solubilization in an appropriate solution. RNA was isolated from PBLs, sorted B and T cells, T blasts, and Raji B cells. The quality of RNA was checked by running on 0.8% agarose gel containing 2.5µg/ml EtBr and visualized under UV light.

The first strand cDNA synthesis was carried out using oligo dT primers, and SuperScript reverse transcriptase (MBI Fermentas, St.Leon-Rot, Germany). The reverse transcription was carried out at 42° for 50 min. RT-PCR was performed with primers KN127/128 amplifying the region between exons 9-12b of CD21 and KN45/46, the GAPDH control. The sequence of the primers used were :

KN127, 5´ ACC ATC CGT TGT ACA AGC AAT G 3´;

KN128, 5´ CAG TCA ACT GGT ACC CAT CTT G 3´;

KN45, 5´ CCA CCC ATG GCA AAT TCC ATG GCA 3´;

KN46, 5´ TCT AGA CGG CAG GTC AGG TCC ACC 3´.

The following PCR conditions were used: 95°C 2min (hot start), 95°C 1 min, 58°C 30 sec, 72°C 1 min- 35 cycles, 72°C 10 min.

3.6.1 Principles of TaqMan PCR

Qualitative PCR is a well established and straight-forward technology, but quantification of specific nucleic acids present in a sample is a demanding task. Accurate quantification is hampered by a number of variations that may occur during sample preparation, storage or the course of the reaction. Even minor variations in reaction conditions are greatly magnified by the exponential nature of the PCR amplification. These variations may partly be overcome by normalising the amount of PCR products of the specific template with respect to an internal reference template.

Briefly, TaqMan PCR is based on the 5'-3' exonuclease activity of the Taq DNA polymerase, which results in cleavage of fluorescent dye-labelled probes during PCR. The intensity of fluorescence is then measured by a Sequence Detection System (SDS) (Fig 3.4). The TaqMan probe is located between the two PCR primers and has a melting temperature 10°C higher than that of the primers. The binding of the TaqMan probe prior to the primers is crucial because

without the probe, PCR products would be formed without generation of fluorescence signal and thus without being detected. The TaqMan probe has two fluorescent tags attached to it. One is a reporter dye, such as 6-carboxyfluorescein (FAM), which has its emission spectra quenched due to the spatial proximity of a second fluorescent dye, 6-carboxy-tetramethyl-rhodamine

Polymerization

Two fluorescent dyes, a reporter (R) and a quencher (Q), are attached to the probes used with the TaqMan PCR Reagent Kit. The 3' end of the probe is blocked, so it is not extended during the PCR reaction

Strand Displacement

When both dyes are attached to the probe, reporter dye emission is quenched. During each extension cycle, the probe is displaced at the 5' end by the Taq DNA polymerase

Cleavage

Taq DNA polymerase then cleaves the reporter dye from the probe via its 5'-3' exonuclease

Polymerization Completed

Once separated from the quencher, the reporter dye emits its characteristic fluorescence which can then be measured by ABI PRISM 7700. The amount of fluorescence measured is proportional to the amount of PCR product made.

Figure 3.4 Principles of Real-Time TaqMan PCR

(TAMRA). Degradation of the TaqMan probe, by the exonuclease activity of Taq DNA polymerase, frees the reporter dye from the quenching activity of TAMRA and thus the fluorescent activity increases with an increase in cleavage of the probe, which is proportional to the amount of PCR product formed.

The ABI Prism 7700 is a laser-coupled spectrophotometer which monitors the 96-well microtitre plate, 8 times per minute. Most of the data are stored in a true real-time determination and at the end of 40 cycles all the data for quantitative analysis are stored in a SDS file. The amplification plot reflects the generation of the reporter dye during amplification and is directly related to the formation of PCR products. The intersection between the amplification plot and the threshold, where the threshold is defined as 10 times the standard deviation of the background fluorescence intensity and is measured between cycle 3 and 15, is known as the cycle threshold, or CT value. The CT value is directly related to the amount of PCR product and therefore related to the initial amount of target DNA present in the PCR reaction.

A positive TaqMan result is reflected by increasing the fluorescent intensity of the reporter dye, FAM, and by decreasing the fluorescent intensity of the second fluorescent tag, TAMRA. Other fluorescent components present in this procedure are ROX, which is mixed in the PCR buffer to a constant concentration and therefore may be used to normalise fluorescent signals when subtle differences in the volume of the PCR reaction mix occur. Background fluorescence is produced by the plastic of the 96-well plate as well as the optic devices of the detection unit. The quantitative real-time TaqMan PCR technique has several advantages over the classical quantitative PCR system. The use of fluorescent dye-labelled probes increases the sensitivity of the system by at least 7 orders of magnitude and gives rise to a linear relationship between copy number and CT values. In addition, the liquid hybridization assay adds further specificity to the system, comparable to hybridization techniques using blotted PCR products. The elimination of post-amplification steps increases reliability and reproducibility of the assay. A major factor responsible for the accuracy of the kinetic PCR method is the determination of the CT value within the logarithmic phase of the amplification reaction, instead of the endpoint

determination used by conventional systems. The Sequence Detection Software (SDS) calculates the CT value when amplification of PCR products is first detected, in other words at the beginning of the exponential phase of amplification, when accumulation of inhibitory PCR products is unlikely to occur. This system offers great potential for automation. Standardisation is achieved by using specific software for primer-probe design. The default settings of the Primer Express Software (Applied Biosystems, Foster City, CA), designs oligonucleotide triplets (two primers and a matching TaqMan probe) that can all be amplified with the same protocol and an universal mastermix.

3.6.2 Relative Quantification of CD21 mRNA by TaqMan PCR

RT-PCR is the technique of choice for analysing extremely low abundance mRNA derived from cells or tissues. The sensitivity of Taqman PCR is a principal advantage over other techniques, such as Northern blots or RNase protection assay. A two-step CD21 RT-PCR was performed with TaqMan MultiScribe reverse transcription reagents (PE Applied Biosystems, Weiterstadt, Germany) using 200 ng RNA in a total volume of 10μl containing random hexamers. The reverse transcription reaction was carried out at 25°C 10 min, 48°C 45 min and 95°C 5 min. 18S rRNA was used to quantitate cDNA contents using the TaqMan 18S rRNA endogenous control reagents. Quantification of CD21 mRNA was done using the ABI PRISM 7700 sequence detection system (PE Applied Biosystems, Weiterstadt, Germany). The primers were designed using Primer Express software between CD21-exon boundaries 1 and 2 (forward primer) and in exon 3 (reverse primer). The following primers and probe sequences were used:

forward primer (TM-1): 5´- GCC GAC ACG ACT ACC AAC CT -3´

reverse primer (TM-2): 5´-TGT GAT GTC CAT TGT GGA TCA TAG -3´

6´-carboxy-flourescein (FAM)-labeled probe: FAM-5´ TGT AAG TGT TTT CCC TCT CGA GTG TCC AGC -3´

Sequence of the amplicon: 5´-GCC GAC ACG ACT ACC AAC CTG TGT AAG TGT TTT CCC TCT CGA GTG TCC AGC ACT TCC TAT GAT CCA CAA TGG ACA TCA CA-3´

PCR conditions: 2 min 50°C; 10 min 95°C; 40 cycles; 15 s 95°C, 1 min 60°C

Real-time PCR was performed in triplicates with 1µl of RT products equivalent of 20 ng total RNA, 200 nM primers each and 100 nM TaqMan probe using the TaqMan universal PCR master mix with AmpliTaq Gold DNA polymerase. Relative quantification was done using the standard curve method. Dilutions of cDNA sample from 1:10 to $1:10^6$ prepared from Raji total RNA were used to construct standard curves for CD21 (FAM) and 18S rRNA (VIC) amplifications. The target amount (CD21) is divided by the endogenous reference amount (18S rRNA) to obtain a normalized target value. Each of the normalized target values is divided by the calibrator normalized target value (T0) to generate the relative expression levels.

3.7 Immunofluorescent Staining and Microscopy

Immunofluoresence staining was performed using anti-CD21 mAb. Briefly, around 1×10^5 cells were immobilized on poly-L-lysine coated slides, fixed in methanol at –20°C for 5 min, treated with anti-CD21 mAb (clone BU32, 0.2µg/ml in PBS containing 1% BSA) for 1h at RT. After three washes in PBS (5 min each) cells were incubated with goat anti-mouse Cy3 (2 µg/ml) or goat anti-mouse Alexa488 (2 µg/ml) for 1h at RT. After three washes in PBS, cells were embedded in Mowiol containing n-propylgallate as an antifading agent. Control preparations were treated in the same way but omitting the primary antibody. Preparations were either observed at a fluorescence microscope (Axioplan, Zeiss) equipped with a digital camera (Kodak 1400E) or at a confocal laser scanning microscope (LSM 510, Zeiss) using a high aperture lens (Plan Apochromat 100✕/1.3 oil, Zeiss).

To quantify fluorescence intensities, 12 bit images were taken with a digital camera under identical exposure times for each cell. Intensities for at least 10 cells from each cell type all from the same batch were measured using MetaMorph software (Visitron Systems, Puchheim, Germany) and the mean values calculated. Since background intensities (without primary antibodies) varied between the different cell types, mean values of background intensities were subtracted from CD21 intensities to normalize CD21 expression. Average grey level intensities were then plotted as percentage of CD21 fluorescence of Raji cells set as 100%.

3.8 Affinity Chromatography

Antibodies were affinity-purified using Protein-G sepharose columns, dialyzed in PBS and coupled to NHS-sepharose beads at concentration of 2mg mAb per ml of column-material according to the manufacturer´s instructions (AP Biotech, Freiburg, Germany). The beads were washed with 100mM Tris; 0.9% NaCl; 0.05% Tween 20 (TBST).

200 ml of human plasma was filtered through a filter paper, centrifuged at 40,000 rpm in a SW40 swing out rotor (Beckman Instruments, Fullerton, USA) for 30 min at 4°C. The top lipid layer and the pellet were discarded. The cleared plasma was incubated with the antibody-coupled beads at 4°C overnight on a roller. The beads were loaded into a column, washed with 10 column volumes of TBST (washing buffer-1) and another 10 column volumes of 20mM sodium-citrate buffer pH 6.4 (washing buffer-2). The column was eluted in 500 µl fractions with high salt, low pH elution buffer (100mM sodium citrate, pH 3.0) and the eluate neutralized with 200 µl of 1M Tris pH 8.5 (neutralizing buffer). The optical density was measured at 280 nm and samples of high optical density were pooled. The samples were analyzed by SDS-PAGE and Western blotting. The yield at this stage was around 1 mg total protein.

3.9 Density Gradient Centrifugation

A semi-discontinuous density gradient of 5% to 50 % glycerol was prepared. Briefly, different concentrations of glycerol were prepared in 50mM HEPES, 5mM NaCl, pH 7.0. One ml of each glycerol solution was placed carefully one above the other with a broad tip-pipette starting from 50% glycerol at the bottom. Starting from a 50% layer the next layers were diluted by a factor of 0.8 till the 5 % layer. Around 500µl of the affinity purified sCD21was added onto the top of the gradient and centrifuged at 40,000 rpm in a SW40 swing out rotor (Beckman Instruments) for 24 hours. This allows fine-separation of the proteins from the affinity column purification. A similar gradient with density gradient marker proteins was run simultaneously to calculate the sedimentation coefficient of the sample. After centrifugation, the bottom of the tube was punctured with a needle and 500µl fractions were collected. 10µl of each fraction was analyzed in SDS-PAGE and Western blot. The total yield of pure sCD21 was around 75-100 µg.

3.10 Partial Specific Volume and Frictional Ratio

The partial specific volume of sCD21 was calculated from the sequence of the extra cellular portion of CD21 (Acc.No. PL0009) (Weis et al., 1986) using the method of Lee and Timasheff (Lee and Timasheff, 1974). Constants for carbohydrate moieties were taken from Gibbons (Gibbons, 1966). The frictional ratio was calculated from the diffusion coefficient, molecular weight and partial specific volume (Bloomfield et al., 1967; Ackers, 1975).

3.11 Immunoprecipitation

Immunoprecipitation of CD21 was carried out using mAb BU32. Briefly, anti-CD21 mAb, BU32 was immobilized on Protein-G-coupled to magnetic beads (Dynal, Hamburg). The cell lysate or the sample was incubated with BU32-Protein-G-Dynabeads for 30 min on a rotor, washed once with 1% NP40-TBS (pH 7.4) and twice with 0.1% NP40-TBS using magnetic particle concentrator (Dynal). The beads were boiled for 5 min at 95°C in SDS-PAGE sample buffer and submitted for electrophoresis and western-blotting analyses.

3.12 SDS-PAGE and Western Blot

The protein samples were run in a 10% polyacrylamide gel under reducing and non-reducing conditions (Laemmli, 1970), either stained with silvernitrate or electrophoretically transferred to nitrocellulose (Towbin et al., 1992). The membranes were blocked with 5% milk powder, washed with TBST and probed with monoclonal anti-CD21 antibody BU32. After three washes the protein band was visualized with goat anti-mouse IgG coupled to horseradish peroxidase followed by Super signal chemiluminescent substrate (Perbio Science, Bonn, Germany).

3.13 Quantification of Soluble CD21 by ELISA

A sandwich ELISA was performed to quantitate sCD21 levels in human serum/plasma (Fig 3.5). The monoclonal antibodies THB5 and biotinylated BU32 were used as capture antibody and revealing antibody respectively. Titration was performed with purified sCD21 and a standard curve was plotted.

Briefly, THB5 was coated onto an ELISA plate (TPP, Trassadingen, Switzerland) at a concentration of 5µg/ml in coating buffer (0.1M Na_2HPO_4/NaH_2PO_4, pH 9.0) for 12-15 hrs at 4° C. After two washes with PBS containing 0.1% Tween 20, the plate was blocked with 1% milk powder in PBST for 2 hours. The serum/plasma samples at appropriate dilutions were
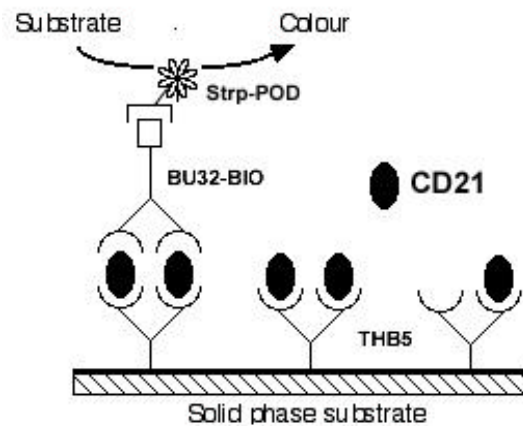
Figure 3.5 Soluble CD21- Sandwich ELISA

added to the plates along with the standard. The plate was incubated at 4°C for 12-15 hrs, washed twice with PBST and then incubated for 2 hrs with BU32-biotin. After two washes, streptavidin coupled to horseradish peroxidase was added as secondary antibody and H_2O_2 /o-phenylenediamine as substrate and colouring agent respectively. The enzyme reaction was quantified by taking O.D at 495 nm in an ELISA reader (Anthos Microsystem, Krefeld, Germany) and sCD21 concentration were calculated extrapolating from the standard graph.

3.14 Statistics

Statistical calculations and graphical illustrations were performed using Instat/Prism software. Kruskal Wallis test for nonparametric analysis of variance (ANOVA) and Mann-Whitney test to obtain nonparametric two-tail P value were performed.

3.14.1 P Value

Having collected the data from several samples and means, it is possible that the populations have the same mean and the difference observed is a coincidence of random sampling. There is no way we can ever be sure whether the difference observed reflects a true difference or a coincidence of random sampling. All we can do is to calculate the probabilities. P value answers this question: If the populations really did have the same mean, what is the probability of observing such a large difference (or larger) between sample means in an experiment of this size? The P value is a probability with a value ranging from zero to one. If the P value is small, we can conclude that the difference is quite unlikely to be caused by random sampling. Instead, the populations have different means. The lower the P value is, the higher is the significance of the observed differences.

3.14.2 Mann-Whitney Test

The Mann-Whitney test, also called the rank sum test, is a nonparametric test that compares two unpaired groups. To perform the Mann-Whitney test, Prism software first ranks all the values from low to high, paying no attention to which group each value belongs. If two values are the same, then they both get the average of the two ranks for which they tie. The smallest number gets a rank of 1. The largest number gets a rank of N, where N is the total number of values in the two groups. Prism then sums the ranks in each group, and reports the two sums. If the sums of the ranks are very different, the P value will be small.

The P value answers this question: If the populations really have the same median, what is the chance that random sampling would result in a sum of ranks as far apart (or more so) as observed in this experiment?

If the samples are small, and there are no ties, Prism calculates an exact P value. If the samples are large, or if there are ties, it approximates the P value from a Gaussian approximation. Here, the term Gaussian has to do with the distribution of sum of ranks, and does not imply that your data need to follow a Gaussian distribution. The approximation is quite accurate with large samples, and is standard (used by all statistics programs).

If the P value is small, the idea that the difference is a coincidence can be rejected and conclude instead that the populations have different medians. If the P value is large, the data do not give any reason to conclude that the overall medians differ. If the sample number is low, the Mann-Whitney test has little power. In fact, if the total sample size is seven or less, the Mann-Whitney test will always give a P value greater than 0.05 no matter how much the groups differ.

3.14.3 Kruskal-Wallis Test

The Kruskal-Wallis test is a nonparametric test that compares three or more unpaired groups. To perform the Kruskal-Wallis test, Prism software first ranks all the values from low to high, disregarding which group each value belongs. If two values are the same, then they both get the average of the two ranks for which they tie. The smallest number gets a rank of 1. The largest number gets a rank of N, where N is the total number of values in all the groups. Prism then sums the ranks in each group, and reports the sums. If the sums of the ranks are very different, the P value will be small. The discrepancies among the rank sums are combined to create a single value called the Kruskal-Wallis statistic. A larger Kruskal-Wallis statistic corresponds to a larger discrepancy among rank sums.

The P value answers this question: If the populations really have the same median, what is the chance that random sampling would result in sums of ranks as far apart (or more so) as observed

in this experiment? More precisely, if the null hypothesis is true then what is the chance of obtaining a Kruskal-Wallis statistic as high (or higher) as observed in this experiment. If the samples are small and no two values are identical (no ties), Prism calculates an exact P value. If the samples are large or if there are ties, it approximates the P value from the chi-square distribution. The approximation is quite accurate with large samples.

3.14.4 Dunn's Post Test

Dunn's post test compares the difference in the sum of ranks between two columns with the expected average difference (based on the number of groups and their size). For each pair of columns, Prism reports the P value as >0.05, <0.05, <0.01 or < 0.001. The calculation of the P value takes into account the number of comparisons made. If the null hypothesis is true (all data are sampled from populations with identical distributions, so all differences between groups are due to random sampling), then there is a 5% chance that at least one of the post tests will have $P<0.05$. The 5% chance does not apply to each comparison but rather to the entire family of comparisons.

CHAPTER 4

RESULTS

4.1 CD21 mRNA is Downregulated During In Vitro T cell Activation

4.1.1 T Cell Activation

T cells were enriched from peripheral blood by magnetic cell sorting and analysed for purity by flow cytometry (Miltenyi et al., 1990; Braun et al., 1998). T cells (10^6 / ml) were activated with anti-CD3 and anti-CD28 monoclonal antibodies and IL-2. The activation was monitored by flow cytometry showing an increased forward and side scatter of activated T cells (day 5) (T blasts) compared to peripheral blood T cells (Fig 4.1).

A   **B**
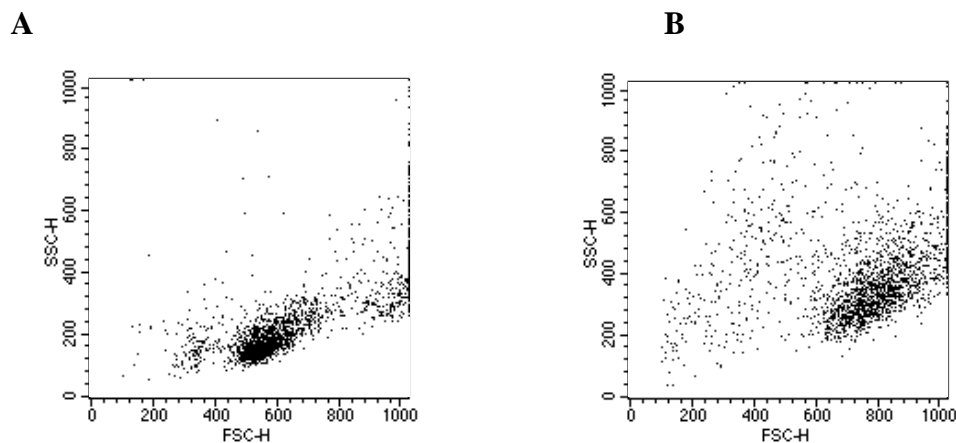
Figure 4.1 Confirmation of T Cell Activation by Flow Cytometry

Freshly sorted peripheral blood T cells (A) and anti-CD3, anti-CD28 and IL-2 activated T blasts (B) were analysed by flow cytometry. Shift in forward and side scatter in T blasts indicates the increase in cell size and granularity respectively, confirming activation.

4.1.2 T Cell Activation Leads to Reduction of CD21 mRNA

RNA was isolated from PBLs, sorted B and T cells, T blasts, and Raji B cells. The quality of RNA was checked by running on 0.8% agarose gel containing 2.5µg/ml EtBr and visualized under UV light (Fig 4.2A). cDNA synthesis and RT-PCR were performed with primers KN127/128 (Fig 4.2B) along with GAPDH controls (Fig 4.2C) and the PCR products were run on 0.8% agarose gel. The primers KN127/128 amplified both the splice variants of CD21 (+/– exon 11). All the cells tested showed the presence of both the splice variants. Raji cells were found to contain relatively maximum amounts of PCR products while it was gradually reduced
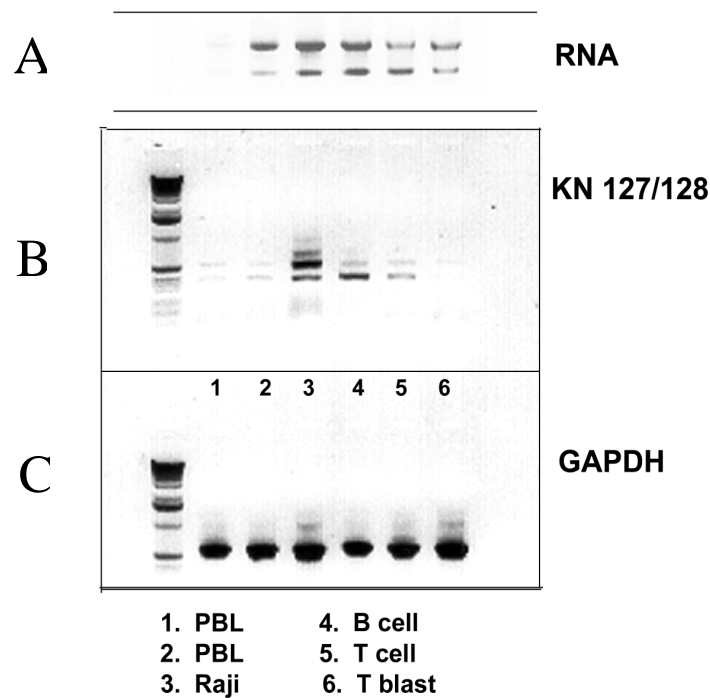
Figure 4.2 T Cells Express CD21
A) Quality of RNA from PBL, Raji cells, B cells, T cells and T blast (lanes 2-4 respectively). B) CD21 RT-PCR was performed in the cells and the PCR products were run on 0.8% agarose gel. C) GAPDH control amplifications

in B cells, T cells and T blast. The difference in intensity of the bands in T cells and T blasts clearly shows a decrease in mRNA levels of CD21 in activated T cells.

To quantify the absolute amounts of CD21 RNA, Taqman PCR was employed to amplify CD21 mRNA from B cells, T cells and T blasts. TaqMan PCR was performed with mRNA from freshly isolated peripheral blood T cells as well as from T blasts at different time-points after activation. Raji B cells served as positive control. The relative amounts of mRNA were determined by the standard curve method taking CD21 levels in unstimulated T cells as 100%. The mRNA levels were normalized with 18S rRNA as endogenous controls. Dilutions of
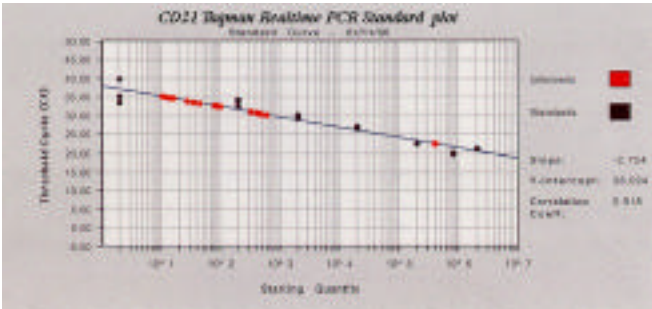
Figure 4.3 Taqman PCR Standard Plot
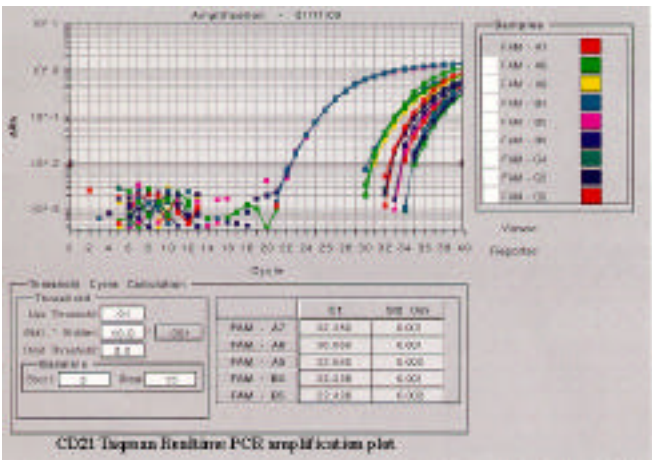
Figure 4.4 CD21 Taqman PCR Amplification Plot

cDNA sample from 1:10 to $1:10^6$ prepared from Raji total RNA are used to construct standard curves for CD21 (Fig 4.3) and 18S rRNA amplifications (not shown). Figure 4.4 shows the amplification plot of CD21 PCR amplification (FAM). A drastic decrease of CD21 mRNA levels were found within 2 days after activation (Fig 4.5). The levels of CD21 transcripts remained low for more than two weeks (data not shown).
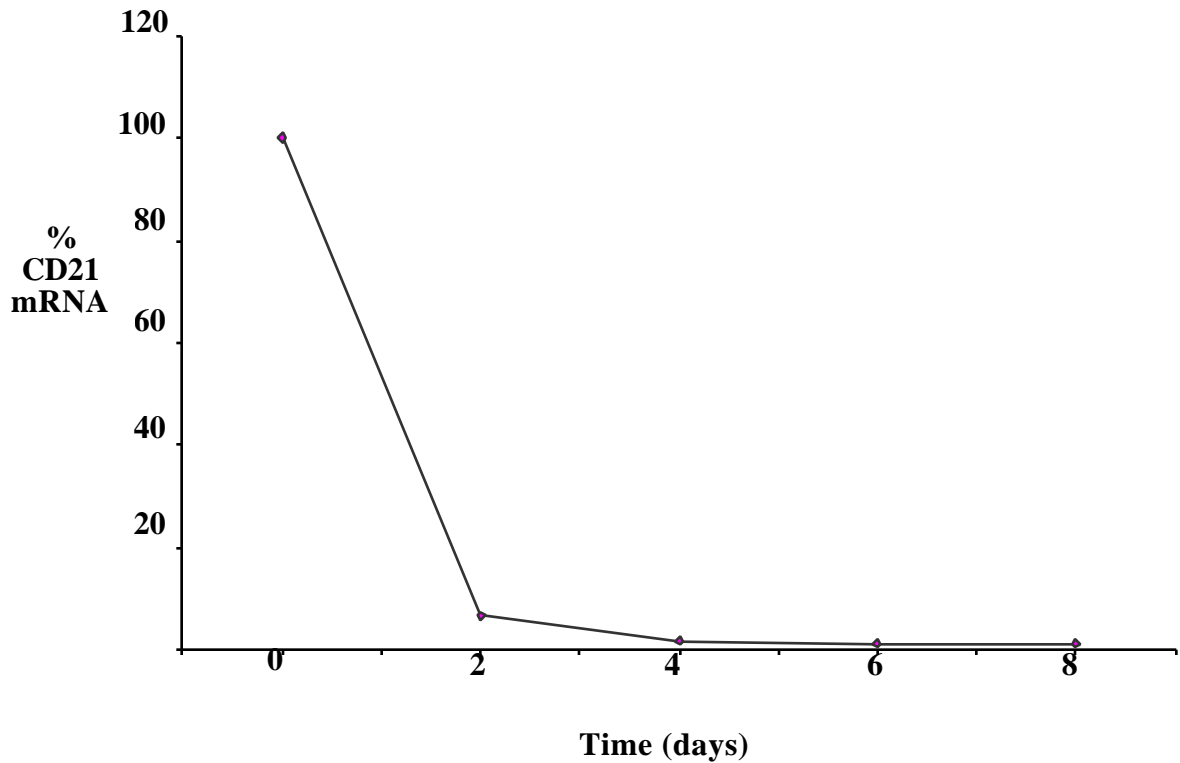
Figure 4.5 CD21 is Downregulated During T cell Activation

T-cells were activated with anti-CD3 and anti-CD28 monoclonal antibodies and IL-2. mRNA was isolated on alternate days. Dilutions of cDNA samples from 1:10 to $1:10^6$ prepared from Raji total RNA are used to construct standard curves for the CD21 and 18S rRNA amplifications. Plotted is the percentage of CD21 mRNA against time, taking the mRNA levels in unstimulated T cells as 100%. Each point represents the data from 3 measurements.

4.1.3 Downregulation of CD21 Protein Expression During T Cell Activation

The expression of CD21 protein during T cell activation was investigated by fluorescent staining technique. Methanol-fixed cells were stained with BU32 antibody and analysed by confocal/fluorescence microscopy. As expected, Raji cells and primary B cells stained brightly for CD21 while cells of T lineage gave weaker signals (Fig 4.6).
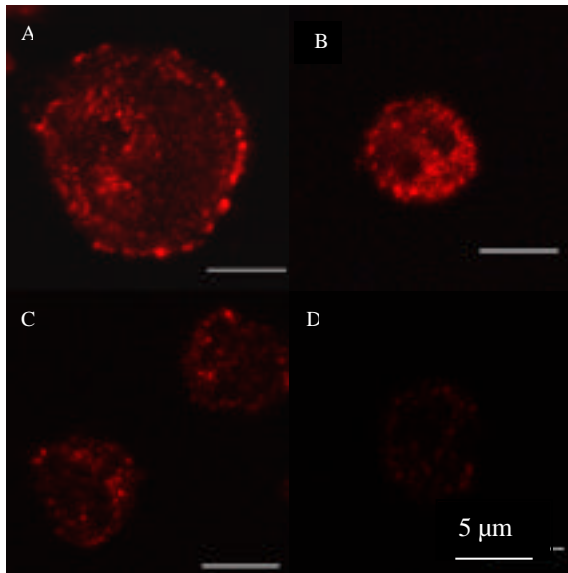
Figure 4.6 CD21 Protein was Detectable by Fluorescent Staining and Confocal Microscopy

Cells were immobilized on poly-L-lysine coated slides, fixed, stained with anti CD21 mAb (BU32) and goat anti-mouse Cy3 secondary antibody, analysed in a laser-scanning microscope. (A) Raji cells (B) B cells (C) T cells and (D) T blasts (day 5). Single confocal sections revealed a punctate staining pattern close to the cell membrane and a less intense staining in the cytoplasm in Raji cells and primary B cells. T cells and T blasts gave weaker signals.

In addition, single confocal sections of CD21 stained cells revealed a punctate staining pattern close to the cell membrane and a less intense staining in the cytoplasm (Fig 4.6). To quantify CD21 staining, the fluorescence intensity of at least 10 cells per slide was measured using a digital camera and metamorph software. Relative staining intensities were normalized for background levels and plotted as average intensities taking Raji cells as 100% (Fig 4.7). Primary B cells, freshly sorted from peripheral blood, contained about 25% of the CD21 protein found in Raji B cells. T cells, isolated from peripheral blood, showed about 17% CD21 protein expression. As expected from the real time PCR analysis, T blasts, activated for 5 days, expressed only 1-2% of CD21. The results show that CD21 protein is expressed in resting, but not in stimulated T cells, and this is paralleled by data obtained through RT-PCR and Taqman PCR.
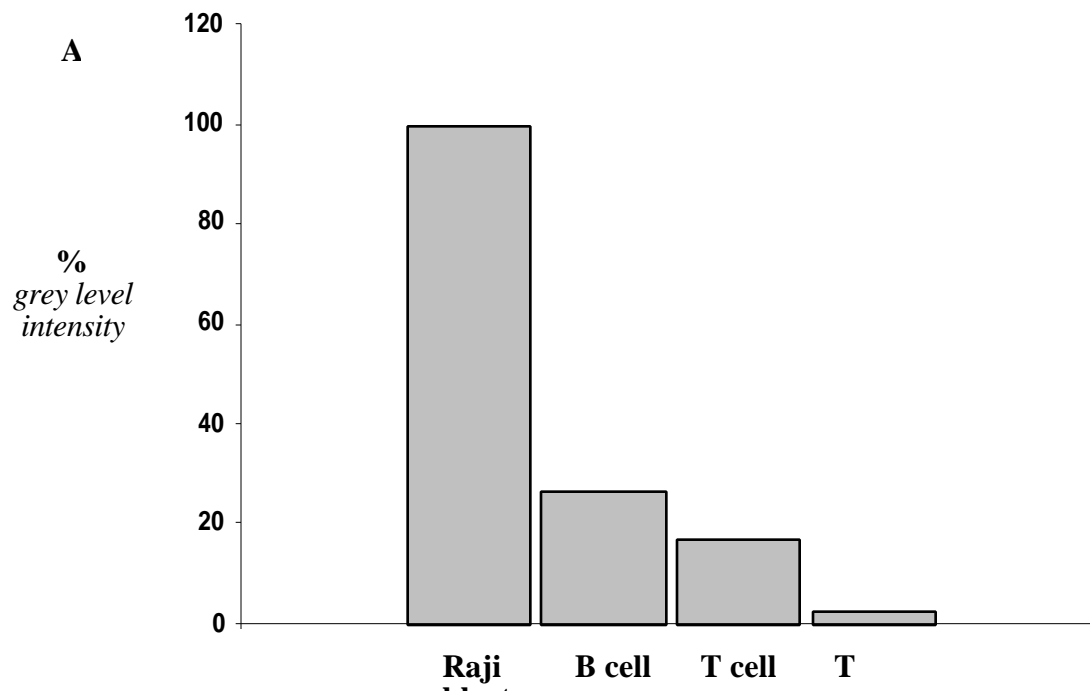
B	Raji	B cell	T cell	T blast
CD21 average grey level intensity	1279±222	487±64	395±67	272±57
Control average grey level intensity	489±126	276±46	262±36	251±27

Figure 4.7 CD21 Relative Quantitation by Microscopy

The fluorescence intensity of B and T cells labeled with mAb BU32 was measured using a digital camera and metamorph software. (A) Relative staining intensities were normalized for background levels and plotted as average intensities taking Raji cells as 100%. (B) Average grey level intensities of fluorescence of BU32 labeled cell types and controls.

4.2 Purification and Characterization of Soluble CD21

4.2.1 Affinity Purification of Soluble CD21 From Human Plasma

Clear pooled human plasma was subjected to affinity chromatography with anti-CD21 antibodies coupled to sepharose as illustrated in the scheme of purification.

Scheme of Soluble CD21 Purification

Human plasma

↓

Centrifuge at 40000 RPM
20 min, at 4°C

↓

Discard top lipid layer and the pellet

↓

Incubate plasma with sulfolink agarose beads coupled to BU32, BU35, THB5
at 4°C for 12hrs

↓

Transfer beads into a column; wash

↓

Elute with high salt-low pH buffer

↓

Glycerol density gradient centrifugation

↓

Analyse samples by immunoblot and
Mass Spectrometry
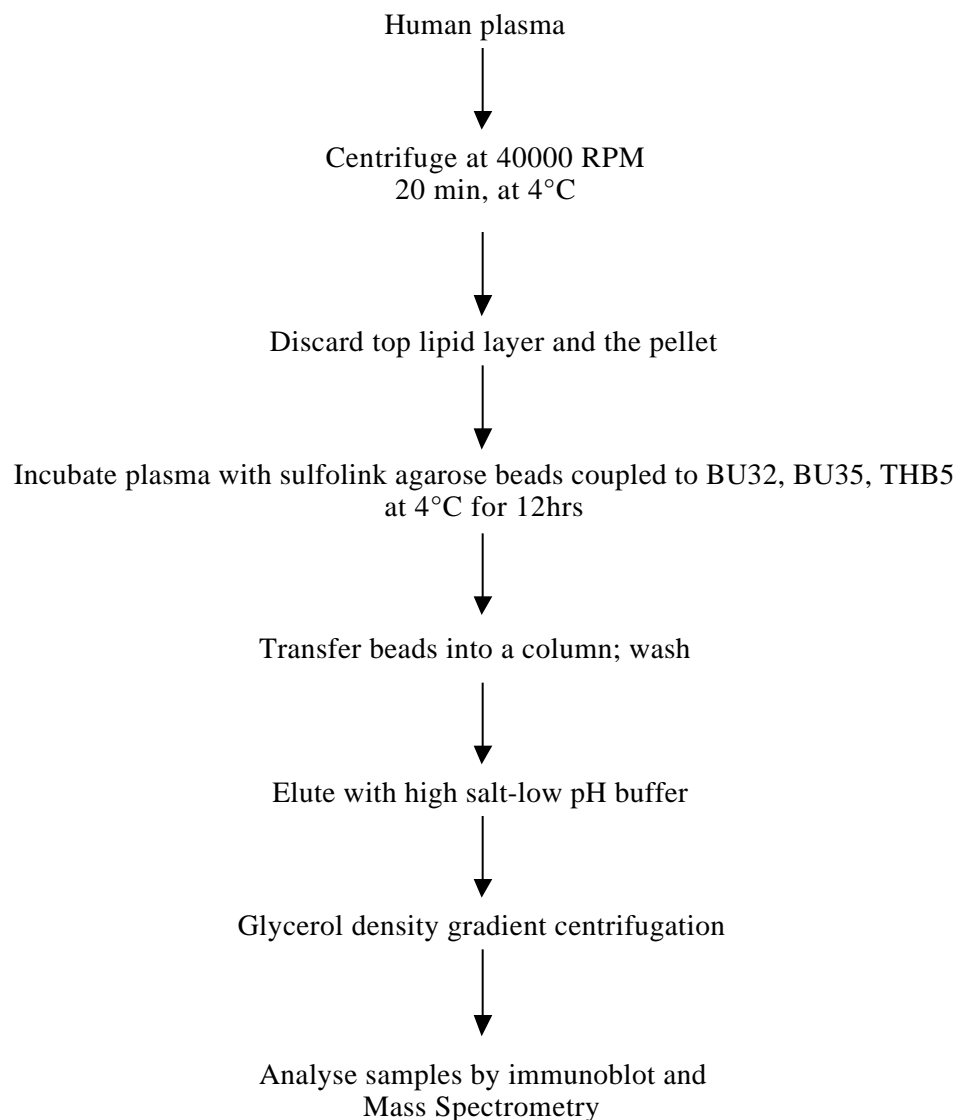
The antibodies used and its corresponding epitopes in CD21 were: THB5: SCR 3-4, BU32: SCR

8-9, BU35: SCR 5-6 (Ling et al., 1994). The eluate was subjected to a 10% gradient SDS-PAGE

under reducing conditions (Fig 4.8A). A band of about 126 kDa was seen upon transferring to

nitrocellulose and probing with anti-CD21 antibody clone BU32 (data not shown). However,

silver staining revealed several additional bands.
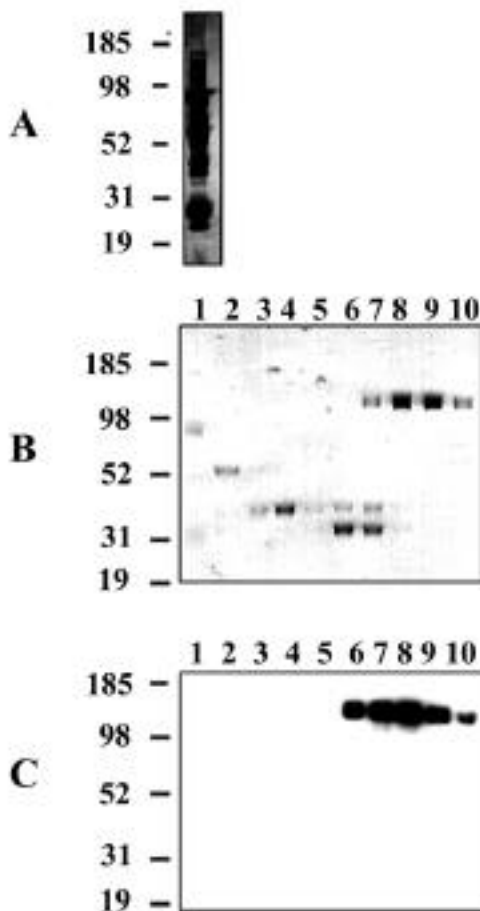
Figure 4.8 Purity of Soluble CD21

A. *10 µl of eluate from affinity chromatography was subjected to 10% polyacrylamide SDS PAGE under reducing conditions and stained with silvernitrate.*

B. *Alternate fractions from density gradient centrifugation were run on a reducing 10% polyacrylamide gel and stained with silvernitrate. Lane 1 to 10, fractions 2 to 20 respectively.*

C. *Western blot of density gradient samples as in B, probed with anti-CD21, mAb BU32. Lanes 6-10; fractions 12, 14, 16, 18, 20 respectively. Pure CD21 protein localizes in fractions 16-18.*

In order to separate sCD21 from co-purified or contaminating proteins, density gradient

centrifugation of the eluate was performed on a semi-discontinuous glycerol gradient. This

method resolves the proteins according to its buoyant density and shape. Fractions were

collected from the bottom of the tube, subjected to two parallel SDS-PAGEs and either silver stained (Fig 4.8B) or blotted and probed with BU32 (Fig 4.8C). sCD21 protein localized itself in fractions 16-18 in the density gradient and ran at 126 kDa. No other band was detected in those fractions by silver staining. An aliquot of the protein was subjected to mass spectrometry-peptide analysis confirming the protein as CD21 (data not shown).

4.2.2 Determination of Sedimentation Coefficient of Soluble CD21

In order to investigate the biophysical nature of the protein, the sedimentation coefficient of sCD21 in serum was determined using density gradient markers of known Svedberg value (S). Apoferritin (17.6 S), -Amylase (8.9 S) and Bovine serum albumin (4.2 S) were used as markers and subjected to similar density gradients and ran parallel along with sCD21. The fractions from marker gradients were subjected to dot blotting, stained with coomassie-brilliant blue-R-250 and the mean fraction numbers in which the proteins localized were determined. The S value of sCD21 was determined by plotting S values upon fraction numbers of the markers and extrapolating the graph for sCD21 (Fig 4.9). The S value of sCD21 was found to be 5.2 S.

The rate at which the sedimentation boundary moves during centrifugation is a measure of sedimentation coefficient of the protein. The sedimentation coefficient depends on the molecular weight (larger proteins sediment faster) and also on molecular shape. Unfolded proteins or one with highly elongated shapes will experience more hydrodynamic friction, and thus will have smaller sedimentation coefficients than a folded, globular protein of the same molecular weight. For a protein of 126 KDa, the S value of 5.2 S is relatively low and indicates that the protein is not a sphere but an elongated molecule.
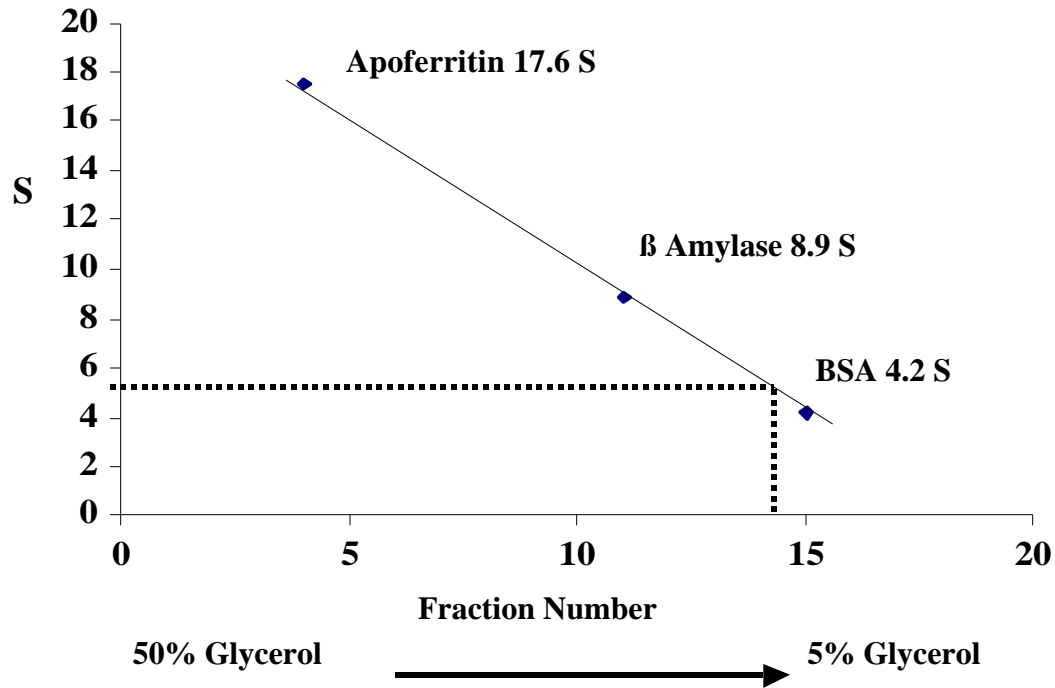
Figure 4.9 Sedimentation Coefficient of Soluble CD21

The sedimentation coefficient of sCD21 was calculated by plotting a standard graph with marker proteins with known S values(X axis) upon fraction number(Y axis). The values shown here are representative of three independent runs. Soluble CD21 localizes in fractions 13-15 in this experiment and correspond to an S-value of 5.2

4.2.3 Hydrodynamic Properties of Soluble CD21

The physical properties of sCD21 were calculated from the sequence assuming that the extra-cellular portion is shed using Einstein relation, Svedberg equation, and Stoke´s law (Ackers, 1975). The partial specific volume of CD21 protein was calculated by the method of Lee and Timashef (Lee and Timasheff, 1974). The partial specific volume indicates how big is a protein molecule. In the absence of special structures like coiled-coil -helices, proteins tend to fold

into compact globular domains. The protein is densely packed in these domains with no holes or water cavities in the interior. The partial specific volume of an average globular protein is 0.73 cm^3/g.

Full-length CD21 is a large protein of molecular weight 145 KDa and is extensively glycosylated. The percentage of glycosylation was calculated from the difference in molecular weight in SDS-PAGE (126 KDa) compared to the predicted molecular weight of the apoprotein (110 KDa). About 12% of the weight of the glycoprotein is contributed by the sugar moieties. The partial specific volume of the apoprotein was calculated from the sequence and was found to be 0.709 cm^3/g. The partial specific volume of the protein was corrected for carbohydrates on the basis of the average partial specific volumes of common sugar moieties (Gibbons, 1966).

- Apparent molecular weight of sCD21 (by PAGE) = 126000

- Molecular weight of apoprotein (calculated from sequence) = 110473

- Percentage of Carbohydrate = 12 %

- Percentage of Protein = 88 %

- Partial specific volume of apoprotein = 0.709 cm^3/g

- Average Partial Specific volume of carbohydrates = 0.6196 cm^3/g

- Final Partial Specific Volume = (0.12 $\times$ 0.6196) + (0.88 $\times$ 0.709) = 0.698 cm^3/g

- Approximate Partial specific volume = 0.7 cm^3/g

The partial specific volume of sCD21 was found to be within in the limits of 0.6950-0.7049 when the percentage of sugar varies between 12±3 % and hence the value was rounded to the second decimal. Since the mobility of the protein is also influenced by the structure, we presume the exact carbohydrate percentage to be within a range of 12±3 %. The frictional coefficient (**F**) and the frictional ratio (**F/F$_0$**) were calculated using Bloomfield equation (Bloomfield et al., 1967).

Bloomfield equation: $s_{20,w} = M (1–\bar{v}\rho) / N_o F$

Frictional Coefficient $F = M (1–\bar{v}\rho) / N_o s_{20,w}$

- Molecular weight (**M**) = 126000 Da (g/mol)

- Partial Specific Volume ($\bar{v}$) = 0.7 cm^3/g

- Density of water (ρ) = 1.0 g/ cm^3

- Avagadro Number (**N_o**) = 6.023 $\times$ 10^{23} mol^{-1}

- Sedimentation Coefficient (**$s_{20,w}$**) = S value $\times$ 10^{-13} = 5.2 $\times$ 10^{-13} s

Frictional Coefficient, **F** = 126 $\times$ 10^3 (1–0.7 $\times$ 1) / (6.023 $\times$ 10^{23}) $\times$ (5.2 $\times$ 10^{-13})

$$= 1.207 \times 10^{-7} \text{ g/s}$$

The frictional coefficient depends on the shape of the protein. A more elongated shape creates more hydrodynamic drag and a larger value of **F.** Also, proteins are hydrated: the layer of `frozen´ water held by hydrogen or ionic bonds, or frozen by the interaction at the hydrophobic surface, increases the effective diameter and hydrodynamic drag of the protein. Thus the factor **F** reflects both the shape and hydration. The hydrodynamic drag is expressed in terms of Stokes Radius **R_s,** which is the radius of a sphere that would have the same frictional coefficient.

Frictional coefficient and Stokes radius are related by the equation

$$\mathbf{F = 6\pi\eta a}$$

where **F** is the frictional coefficient, **a**, the stokes radius and η, viscosity of water (0.01 g/cm.s)

$$\mathbf{a = F / 6\pi\eta}$$

Stokes radius, **a** = 1.207 $\times$ 10^{-7} / 6 $\times$ 3.14 $\times$ 0.001

$$= 6.4 \times 10^{-7} \text{ cm} = 64 \text{ Å}$$

The diffusion coefficient $D_{20,w}$ was calculated using Stokes-Einstein equation (Ackers, 1975).

Stokes-Einstein equation: $D_{20,w} = \kappa\, T/F$

- Boltzmann constant (κ) = 1.38×10^{-16}

- Temperature (**T**, in Kelvin) $20°C = 293$ K

- Frictional Coefficient (**F**) = 1.207×10^{-7} g/s

Diffusion Coefficient, $\mathbf{D_{20,w}} = (1.38 \times 10^{-16}) \times 293 / 1.207 \times 10^{-7}$

$$= 3.35 \times 10^{-7}\ cm^2/s$$

The frictional ratio indicates if a protein is globular or elongated and is calculated from the equation $\mathbf{F / F_o = S / S_{max}}$, where $\mathbf{F_o}$ and $\mathbf{S_{max}}$ are the frictional coefficient and sedimentation coefficient of a smooth, unhydrated sphere (with no shell of bound water) corresponding to the given protein mass. S_{max} is calculated in cgs units (seconds). To express S_{max} in the commonly used Svedberg unit, the value is multiplied by 10^{13}. Since S_{max} assumes the minimum size and most compact shape, and ignores the shell of bound water, the measured S is necessarily always smaller, i.e., real proteins have more drag than this ideal, unhydrated sphere.

The sedimentation coefficient **S** is related to the frictional coefficient **F** and the mass **M** by the following equation:

$$\mathbf{S_{max} = M\,(1{-}\bar{v}\rho) / N_o\,(6\pi\eta R_{min})}$$

- Molecular weight (**M**) = 126000 Da

- Partial Specific Volume ($\bar{v}$) = 0.7 cm^3/g

- Density of water (ρ) = 1.0 g/ cm^3

- Avagadro Number ($\mathbf{N_o}$) = 6.023×10^{23} mol^{-1}

- Viscosity of water (η) = 0.01 g/cm.s

Radius of a sphere of volume $\bar{v}$, $\mathbf{R_{min}}$ is given by the relation

$$\mathbf{R_{min} = 0.066 \times M^{1/3}}$$

$R_{min} = 0.066 \times (126000)^{0.33} = 3.3$ nM

$S_{max} = 126000 \times (1–0.7 \times 1) / 6.023 \times 10^{23} (6 \times 3.14 \times 0.01 \times 3.3)$

$S_{max} = 10.09$

S value calculated from density gradient centrifugation = 5.2

$\mathbf{F / F_o = S / S_{max}} = 10.09/5.2 = 1.94$

The diffusion coefficient of 3.35×10^{-7} (cm^2/s) and the frictional ratio of 1.94 shows that the molecule is elongated and rod shaped. The hydrodynamic properties of sCD21 molecule are given in Table 4.1

Table 4.1 Physical Properties of Soluble CD21

Molecular Weight (apparent, SDS PAGE), (Da)	126000
Molecular Weight of Apoprotein, (Da)	110473
% Carbohydrate	12 %
Partial Specific Volume, (cm^3/g)	0.70
Sedimentation Coefficient, $s_{20,w}$ (S)	5.2
Diffusion Coefficient, $D_{20,w}$ (cm^2/s)	3.35×10^{-7}
Frictional Ratio, F/F_0	1.94
Stokes Radius (Å)	64

4.2.4 Mobility of Soluble and Cellular CD21 During Electrophoresis

In order to further characterize the protein, cellular CD21 was affinity purified from lymphoblastoid cell lines such as the T cell line Molt-4 and B cell line Raji. Both Molt-4 T cells and Raji B cells showed a band of around 140 kDa upon SDS-PAGE/Westernblot with BU32, while the CD21 from plasma was found to be of 126 kDa under reducing conditions. When the same CD21 proteins was run under non-reducing conditions, an increase in mobility to 130 kDa was found for the cellular CD21 and 120 kDa for the plasma derived CD21 (Fig 4.10). No other molecule (e.g. CD23) was associated with CD21 under our purification conditions. This is in contrast to a previous report where purification of CD21 from sera resulted in a smear of 130-190 kDa under non-reducing conditions. The difference to our protocol is that a glycerol density gradient centrifugation step was added after the affinity purification.

Figure 4.10 Mobility of CD21 During Electrophoresis
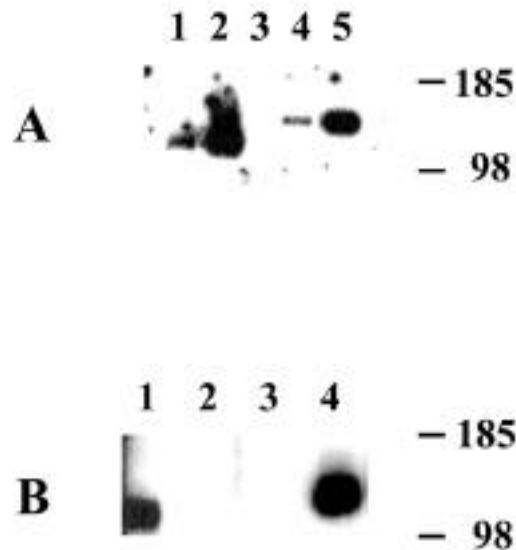
A. Cellular CD21 isolated by affinity chromatography from Molt-4 and Raji cell lines were subjected to 10% polyacrylamide gel electrophoresis, transferred to nitrocellulose and probed with BU32. Lane 1 and Lane 4: Molt-4 CD21 non-reduced and reduced respectively, Lane 2 and Lane 5: Raji CD21 non-reduced and reduced respectively.Lane 3 was loaded only with SDS sample loading buffer to prevent diffusion of reducing agent between the lanes.

B. Westernblot with BU32 of soluble CD21 run under non-reducing (lane 1), and reducing (lane 4) conditions. CD21 runs faster under non-reducing conditions than under reducing conditions. Lane 2 and 3 were loaded only with SDS sample loading buffer.

4.3 Serum Soluble CD21 in Health and Disease

4.3.1 Soluble CD21 ELISA

Serum sCD21 concentrations were determined by sandwich ELISA (Materials and Methods). Monoclonal anti-CD21 antibody clone THB5 directed against SCR 3-4 was used as capture antibody and clone BU32-biotin against exons SCR 8-9 was used as revealing antibody. sCD21 isolated from human plasma by affinity chromatography and density gradient centrifugation was titrated to obtain a standard curve (Fig 4.11). The concentration of sCD21 in appropriately diluted serum was obtained by extrapolating the standard curve. The sensitivity of the assay was found to be <5 ng/ml. The specificity of the assay was controlled using isotype control antibodies for capture and revealing antibodies. ELISA with isotype control antibodies in the place of BU32 and THB5 developed background O.D. values similar to plate control and secondary antibody control.
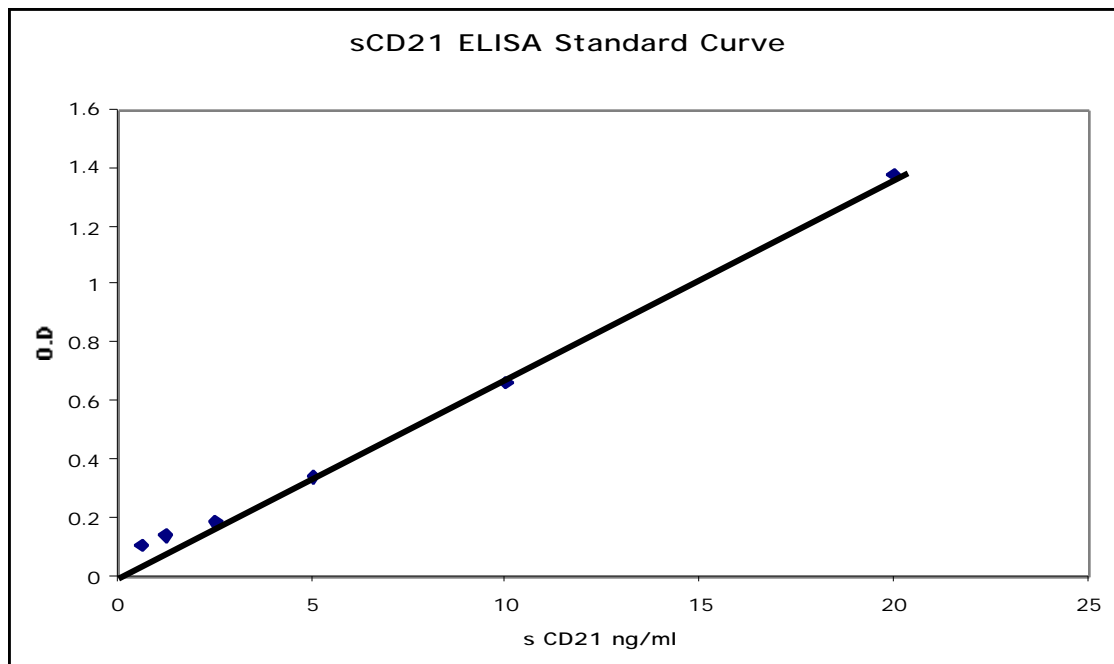
Figure 4.11 Soluble CD21 ELISA Standard Curve

4.3.2 Soluble CD21 Concentration in Healthy Human Sera

Soluble CD21 concentrations were estimated in sera from 235 healthy volunteers of different age. sCD21 level was the highest between 1-20 years and is significantly reduced with age (P value < 0.0001). The normal range of sCD21 in healthy individuals of age between 20-40 years was approximately 100 to 500 ng/ml (with a few outliers) with a median of 292 ng/ml (Fig 4.12). The median values of sCD21 of other age groups were: 1-20 years: 333 ng/ml; 40-60 years: 225 ng/ml; 60-80 years: 209 ng/ml; 80+ years: 211 ng/ml. While the levels of sCD21 were not significantly different between the genders (Fig 4.13), an age dependent decrease was found in both male and female control groups with high significance (P value 0.002 for males and 0.005 for females, data not shown). The sCD21 level range in age groups 20-60 years (n=160) were taken as control values for all the subsequent analyses.
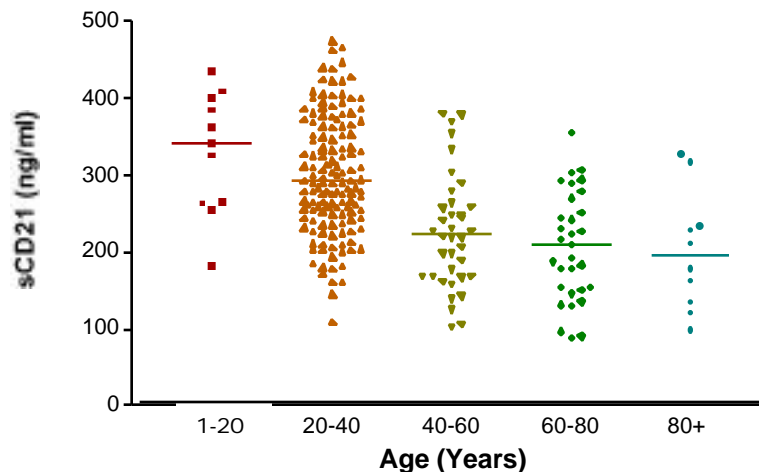
Figure 4.12 Serum Soluble CD21 Concentration Decreases with Age
Serum sCD21 concentrations of 235 healthy volunteers were estimated and the levels were grouped according to age. ■ 1-20 years: n=11; ▲ 20-40 years: n=143; ▼ 40-60 years: n=38; ◆ 60-80 years: n=31; ● 80+ years: n=11. P value <0.0001
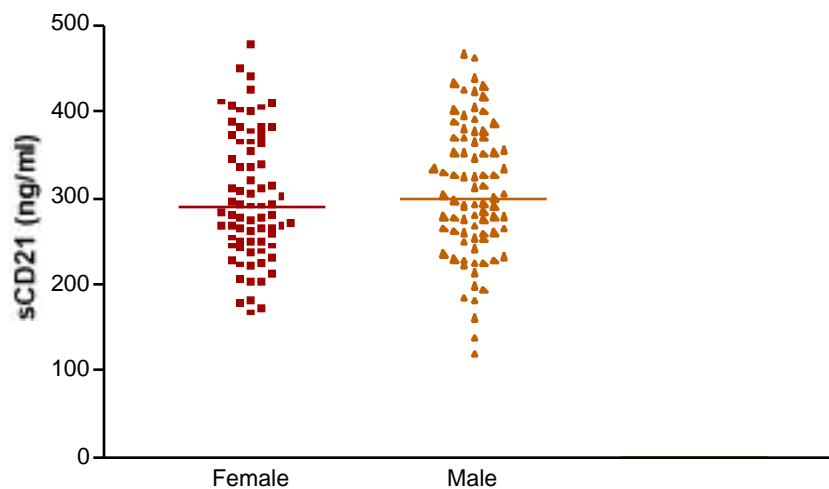
Figure 4.13 Serum Soluble CD21 Concentrations are Similar in Males and Females
Serum sCD21 concentration of 160 healthy volunteers of age 20-60 years were estimated and grouped according to their gender. ■ *Females: n=76,* ▲ *Males: n= 84. P value 0.3014*

Statistics 1: Soluble CD21 Concentrations in Healthy Human Sera, Variance with Age

Kruskal-Wallis Test (Nonparametric ANOVA)
The P value is < 0.0001, considered extremely significant.
Variation among column medians is significantly greater than expected by chance.

The P value is approximate (from chi-square distribution) because at least one column has two or more identical values.

Calculation detail

Group	Number of Points	Sum of Ranks	Mean of Ranks
1-20 Yrs	12	1759.0	146.58
20-40 Yrs	143	19934	139.40
40-60 Yrs	38	3114.5	81.961
60-80 Yrs	31	2075.5	66.952
80+ Yrs	11	847.50	77.045

Kruskal-Wallis Statistic KW = 48.438 (corrected for ties)

Dunn's Multiple Comparisons Test

| | | | Mean | Rank | |
Comparison			Difference		P value
1-20 Yrs vs. 20-40 Yrs			7.188	ns	P>0.05
1-20 Yrs vs. 40-60 Yrs			64.623	*	P<0.05
1-20 Yrs vs. 60-80 Yrs			79.632	**	P<0.01
1-20 Yrs vs. 80+ Yrs			69.538	ns	P>0.05
20-40 Yrs vs. 40-60 Yrs			57.435	***	P<0.001
20-40 Yrs vs. 60-80 Yrs			72.443	***	P<0.001
20-40 Yrs vs. 80+ Yrs			62.350	*	P<0.05
40-60 Yrs vs. 60-80 Yrs			15.009	ns	P>0.05
40-60 Yrs vs. 80+ Yrs			4.915	ns	P>0.05
60-80 Yrs vs. 80+ Yrs			-10.094	ns	P>0.05

Summary of Data

| | Number of | | | |
Group	Points	Median	Minimum	Maximum
1-20 Yrs	12	333.00	135.00	435.00
20-40 Yrs	143	294.00	109.00	623.00
40-60 Yrs	38	225.00	103.00	493.00
60-80 Yrs	31	209.00	88.000	356.00
80+ Yrs	11	211.00	98.000	344.00

Statistics 2: Soluble CD21 Concentrations in Healthy Human Sera, Variance with Gender

Mann-Whitney Test
Do the medians of Female and Male differ significantly?
The two-tailed P value is 0.3014, considered not significant.
The P value is an estimate based on a normal approximation.
The 'exact' method would not be exact, due to tied ranks.

Calculation details
Mann-Whitney U-statistic = 2889.0
U' = 3495.0
Sum of ranks in Female = 5815.0. Sum of ranks in Male = 7065.0.

Summary of Data

Parameter:	Female	Male
Mean:	301.79	314.44
# of points:	76	84
Std deviation:	82.249	86.435
Std error:	9.435	9.431
Minimum:	109.00	121.00
Maximum:	585.00	623.00
Median:	287.50	302.50
Lower 95% CI:	282.97	295.65
Upper 95% CI:	320.61	333.23

4.3.3 Serum Soluble CD21 Concentrations in Rheumatoid Arthritis

Serum samples of 209 confirmed RA patients were tested for sCD21 concentration by ELISA and compared to healthy controls (Fig 4.14). The sCD21 concentrations were found significantly reduced in RA. The serum sCD21 concentration in rheumatoid arthritis ranged approximately from 50 ng/ml to 300 ng/ml with the median of 174 ng/ml.

Figure 4.14 Serum Soluble CD21 Concentration were Significantly Reduced in RA
Serum sCD21 concentrations of 209 RA patients▲, were estimated and compared to healthy controls ■ (n=160). P value <0.0001

<u>***Statistics 3 : Serum Soluble CD21 Concentrations in Rheumatoid Arthritis***</u>

```
Mann-Whitney Test
Do the medians of Control and RA differ significantly?
The two-tailed P value is < 0.0001, considered extremely significant.

The P value is an estimate based on a normal approximation.
The 'exact' method would not be exact, due to tied ranks.

Calculation details
Mann-Whitney U-statistic = 3856.0
U' =  29584
Sum of ranks in Control =  42464.  Sum of ranks in RA =  25801.
```

Summary of Data

Parameter:	Control	RA
Mean:	308.43	182.67
# of points:	160	209
Std deviation:	84.446	81.327
Std error:	6.676	5.625
Minimum:	109.00	36.000
Maximum:	623.00	497.00
Median:	294.00	174.00
Lower 95% CI:	295.35	171.64
Upper 95% CI:	321.52	193.70

In order to make sure that the observed reductions in sCD21 levels were not due to the old age of patients, sCD21 concentrations in RA were compared to age matched healthy controls (Fig 4.15) revealing a highly significant reduction (P value < 0.0001).

Figure 4.15 Serum Soluble CD21 Concentrations were Significantly Reduced in RA, Independent of Age
Serum sCD21 concentrations of RA patients were estimated and compared to age matched healthy controls. ■ 20-40 years, ▼ 40-60 years, ● 60-80 years.
P value< 0.0001

Statistics 4 : Serum Soluble CD21 Concentrations in Rheumatoid Arthritis, Variance with Age

Kruskal-Wallis Test (Nonparametric ANOVA)

The P value is < 0.0001, considered extremely significant.
Variation among column medians is significantly greater than expected
by chance.

The P value is approximate (from chi-square distribution) because
at least one column has two or more identical values.

Calculation detail

Group	Number of Points	Sum of Ranks	Mean of Ranks
Control 20-40	149	48641	326.45
RA 20-40	30	5487.5	182.92
Control 40-60	38	8724.5	229.59
RA 40-60	95	13015	136.99
Control 60-80	31	6172.0	199.10
RA 60-80	95	14102	148.44

Kruskal-Wallis Statistic KW = 180.21 (corrected for ties)

Dunn´s Multiple Comparisons Test

Comparison	Mean Rank Difference		P value
Control 20-40 vs. RA 20-40	143.53	***	P<0.001
Control 20-40 vs. Control 40-60	96.854	***	P<0.001
Control 20-40 vs. RA 40-60	189.45	***	P<0.001
Control 20-40 vs. Control 60-80	127.35	***	P<0.001
Control 20-40 vs. RA 60-80	178.00	***	P<0.001
RA 20-40 vs. Control 40-60	-46.675	ns	P>0.05
RA 20-40 vs. RA 40-60	45.922	ns	P>0.05
RA 20-40 vs. Control 60-80	-16.180	ns	P>0.05
RA 20-40 vs. RA 60-80	34.475	ns	P>0.05
Control 40-60 vs. RA 40-60	92.597	**	P<0.01
Control 40-60 vs. Control 60-80	30.495	ns	P>0.05
Control 40-60 vs. RA 60-80	81.150	*	P<0.05
RA 40-60 vs. Control 60-80	-62.102	ns	P>0.05
RA 40-60 vs. RA 60-80	-11.447	ns	P>0.05
Control 60-80 vs. RA 60-80	50.655	ns	P>0.05

Summary of Data

Group	Number of Points	Median	Minimum	Maximum
Control 20-40	149	294.00	109.00	623.00

```
        RA 20-40      30       192.00      107.00        519.00
   Control 40-60      38       225.00      103.00        493.00
        RA 40-60      95       159.00      36.000        497.00
   Control 60-80      31       209.00      88.000        356.00
        RA 60-80      95       174.00      84.000
```

Unlike sCD21 concentrations of controls, there was no statistically significant reduction with age of sCD21 levels in RA (P value 0.0732, data not shown). Therefore the reduction of serum sCD21 is associated with the disease itself, which not only leads to lower levels of sCD21 but also eliminates differences between age groups. In the next set of measurements, levels of serum sCD21 in RA was compared to sera from related connective tissue disorders and chronic inflammatory diseases such as reactive arthritis, borreliosis, collagenosis, scleroderma, systemic sclerosis etc. (Fig 4.16).
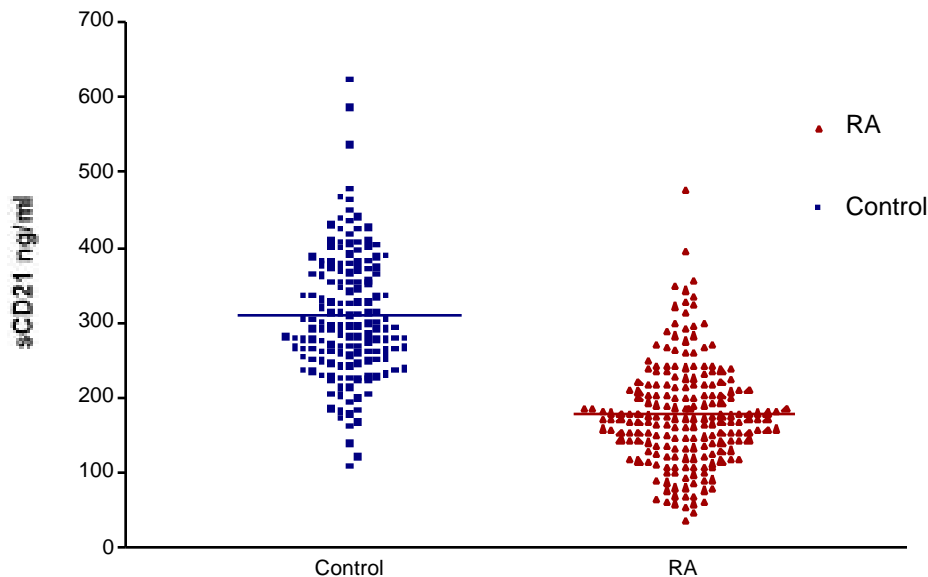

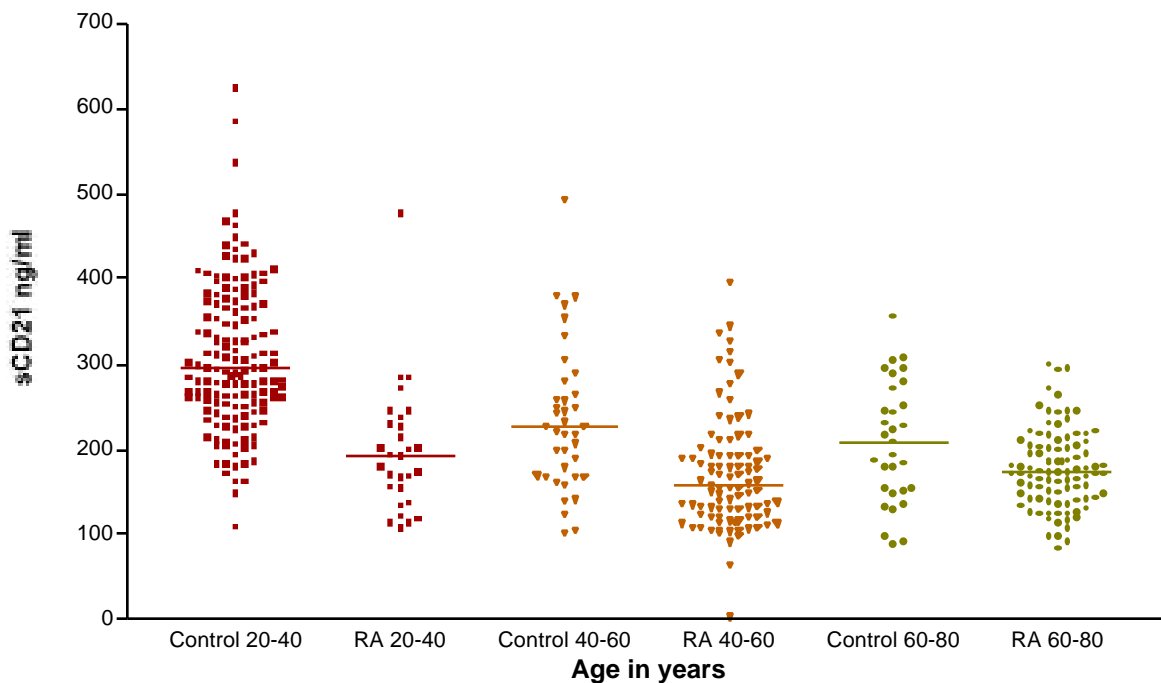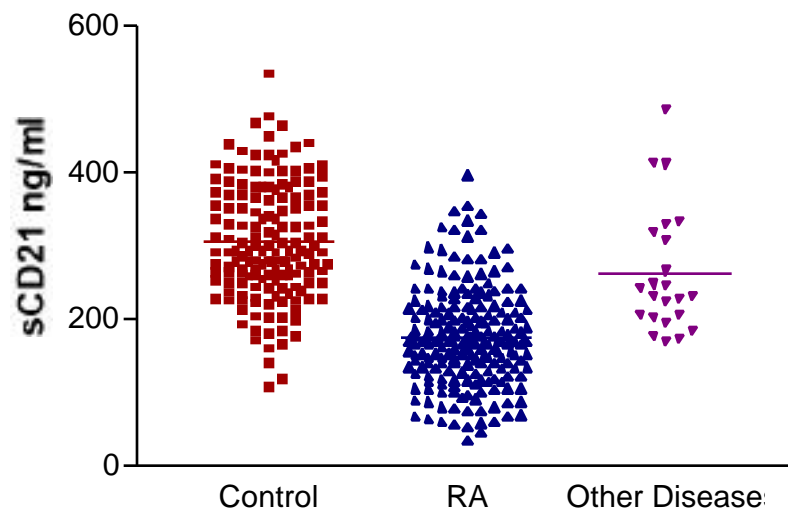*Figure 4.16 Serum Soluble CD21 Concentrations Were Specifically Reduced in RA*

Serum sCD21 concentrations of RA patients (▲ n=209) were estimated and compared to healthy controls (■ n=160) and other related disorders (▼ n=17). The P value between control and RA and between control and other related disorders group were P<0.0001 and P > 0.05 respectively.

The sCD21 concentrations ranged from 171 ng/ml to 414 ng/ml with a median of 247 ng/ml. Statistical analysis using nonparametric ANOVA revealed an extremely significant P value between control and RA (P < 0.001) while there was no significance between control and other related disorders group (P > 0.05). Therefore among the disorders studied, reduced sCD21 concentrations appear to be RA-specific.

Statistics 5 : Serum Soluble CD21 Concentrations in Rheumatoid Arthritis, Variance with Other Related Disorders

Kruskal-Wallis Test (Nonparametric ANOVA)

The P value is < 0.0001, considered extremely significant.
Variation among column medians is significantly greater than expected by chance.

The P value is approximate (from chi-square distribution) because at least one column has two or more identical values.

Calculation detail

Group	Number of Points	Sum of Ranks	Mean of Ranks
Control	160	44241	276.50
RA	209	26420	126.41
Other	17	4030.5	237.09

Kruskal-Wallis Statistic KW = 166.72 (corrected for ties)

Dunn's Multiple Comparisons Test

Comparison	Mean Rank Difference	P value	
Control vs. RA	150.09	***	P<0.001
Control vs. Other	39.415	ns	P>0.05
RA vs. Other	-110.68	***	P<0.001

Summary of Data

Group	Number of Points	Median	Minimum	Maximum
Control	160	294.00	109.00	623.00
RA	209	174.00	36.000	707.00
Other	17	247.00	171.00	414.00

Rheumatoid factor (RF) is one of the diagnostic markers of RA (Arnett et al., 1988). Therefore it would be interesting to know if the presence of rheumatoid factor correlates somehow to sCD21 levels. However, comparison of sCD21 concentrations of RF$^+$ and RF$^-$ patients revealed no significant differences between the two groups (P value 0.6336) (Fig 4.17).
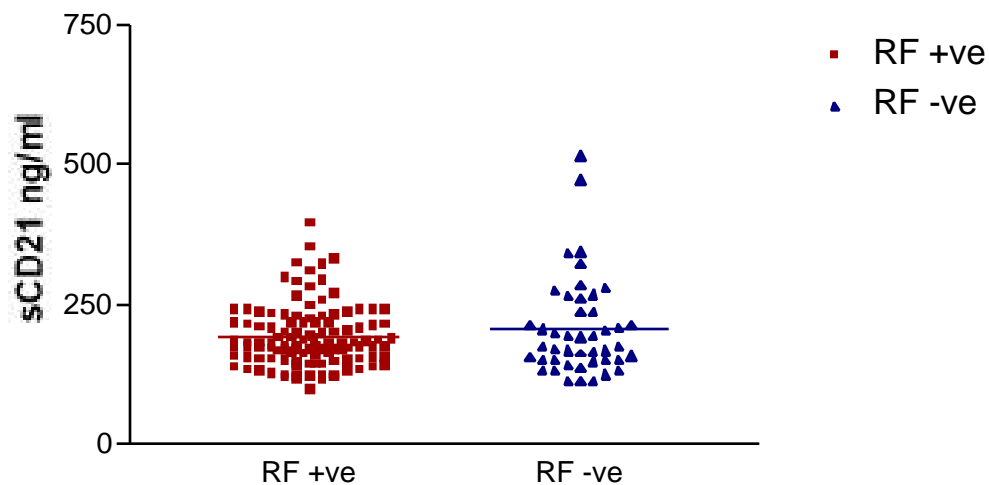
Figure 4.17 Serum Soluble CD21 Concentrations Were Not Associated with RF

Serum sCD21 concentrations of RF$^+$ RA patients (■, n=113) were compared to RF$^-$ RA patients (▲, n=46). P Value = 0.6336

Statistics 6: Serum Soluble CD21 concentrations in rheumatoid arthritis, variance with RF$^+$ and RF$^-$

```
Mann-Whitney Test
Do the medians of RF +ve and RF -ve differ significantly?

The two-tailed P value is 0.6336, considered not significant.
The P value is an estimate based on a normal approximation.
The 'exact' method would not be exact, due to tied ranks.


Calculation details
Mann-Whitney U-statistic = 2473.0
U' = 2725.0
Sum of ranks in RF +ve = 8914.0.  Sum of ranks in RF -ve = 3806.0.

Summary of Data
```

Parameter:	RF +ve	RF -ve
Mean:	194.31	220.98
# of points:	113	46
Std deviation:	55.587	113.10
Std error:	5.229	16.676
Minimum:	101.00	116.00
Maximum:	397.00	707.00
Median:	181.00	187.50
Lower 95% CI:	183.94	187.36
Upper 95% CI:	204.68	254.59

4.3.4 Rheumatoid Arthritis-Lymphocytes Shed Similar Amounts of CD21 as Healthy Controls

Since rheumatoid arthritis is characterized by autoimmune reactions, lymphocyte proliferation and complement activation, it is possible that the reduction in sCD21 could be due to defective shedding of CD21 from lymphocytes. To test this hypothesis, peripheral blood lymphocytes were isolated from two healthy individuals and two RA patients and sorted B and T lymphocytes for tissue culture. The purity of sorted lymphocytes was tested by cytometric analysis (Fig 4.18A & B).
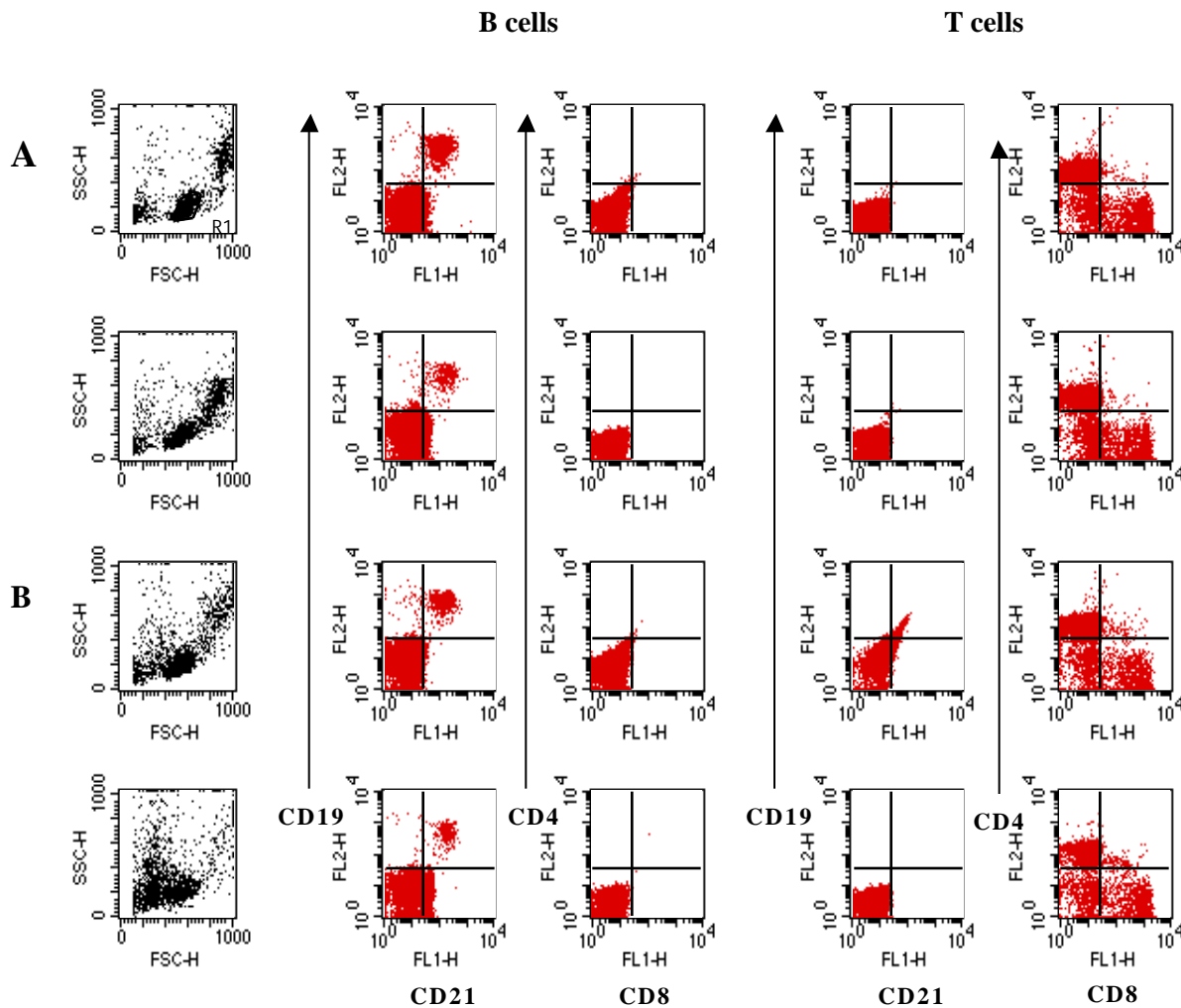
Figure 4.18 Sorting of Lymphocytes from Healthy Controls and RA Patients

*PBLs from two healthy controls **(A)** and two RA patients **(B)** were obtained under sterile conditions. B and T lymphocytes were sorted negatively by magnetic activated cell sorting using CD3 and CD19 magnetic beads respectively and cultured for 36 hours. The purity of separation from each sample of control B and T cells (A, upper and lower panels) and RA-B and T cells (B, upper and lower panels) was determined by FACS analysis. Soluble CD21 concentrations were estimated in the cell culture supernatants.*

Supernatants were collected after 36 hours of incubation and tested for sCD21 by ELISA (Fig 4.19). B cells from both control and RA patients shed similar amounts of CD21 into the supernatant. T cells from both the control and RA patients shed very little or undetectable amounts of CD21 molecules. So I conclude that the shedding of the protein by peripheral B cells is normal in rheumatoid arthritis and the reduced levels observed could be due to either faster degradation or usage of sCD21 in RA plasma.
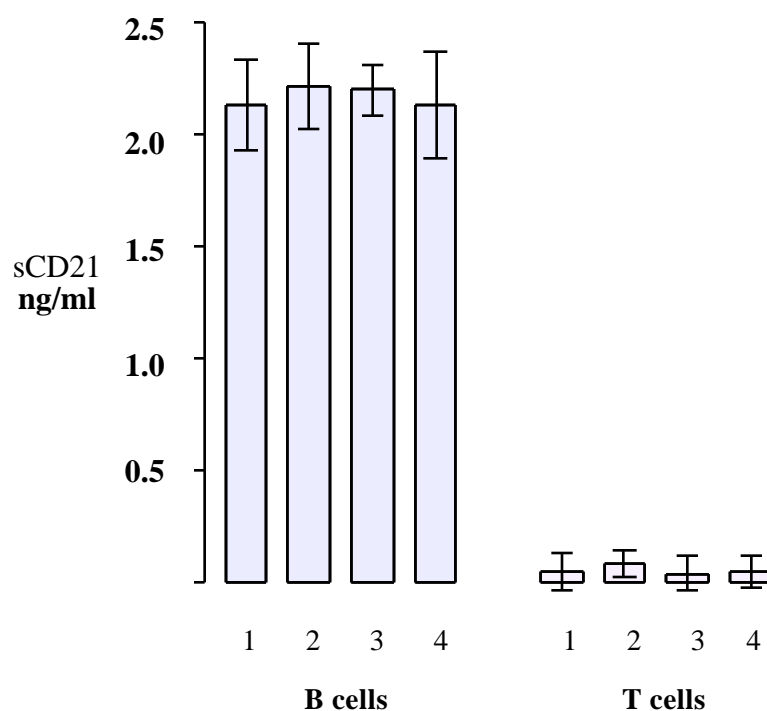
Figure 4.19 Rheumatoid Arthritis- Lymphocytes Shed Similar Amounts of CD21 As Controls

PBLs from two healthy controls (1 and 2) and two RA patients(3 and 4) were obtained under sterile conditions. B and T lymphocytes were sorted negatively by magnetic activated cell sorting using CD3 and CD19 magnetic beads respectively and cultured for 36 hours. Plotted in the graph are the amounts of CD21 shed from B and T cells from controls and RA patients.

4.3.5 Serum Soluble CD21 Concentrations in Common Variable Immunodeficiency

Serum samples from 41 patients with CVID were tested for sCD21 by ELISA. Huge variation of sCD21 levels was found in these assays (Fig 4.20). The CD21 levels I CVID patients ranged from 43 ng/ml to 961 ng/ml. Statistical analysis using Mann-Whitney test showed no significance with a P value 0.7796.
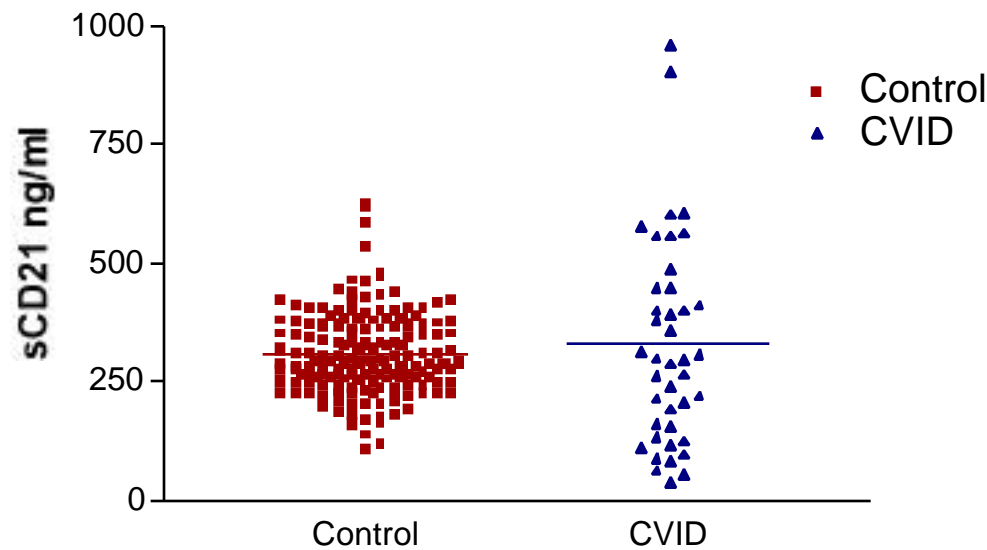
Figure 4.20 Serum Soluble CD21 Concentrations in CVID
Serum sCD21 concentrations of CVID patients ▲ (n=41) were estimated and compared to healthy controls ■ (n=160). P value = 0.7796

Statistics 7: Serum Soluble CD21 Concentrations in CVID

```
Mann-Whitney Test
Do the medians of Control and CVID differ significantly?

The two-tailed P value is 0.7796, considered not significant.
The P value is an estimate based on a normal approximation.
The 'exact' method would not be exact, due to tied ranks.

Calculation details
Mann-Whitney U-statistic = 3186.5
U' = 3373.5
Sum of ranks in Control =  16254.  Sum of ranks in CVID = 4047.5.

Summary of Data
```

Parameter:	Control	CVID

Mean:	308.43	329.51
# of points:	160	41
Std deviation:	84.446	217.44
Std error:	6.676	33.958
Minimum:	109.00	43.000
Maximum:	623.00	961.00
Median:	294.00	299.00
Lower 95% CI:	295.35	260.88
Upper 95% CI:	321.52	398.14

The sCD21 levels were then compared with different subtypes of CVID classification (Fig 4.21). Kruskal-Wallis Test for nonparametric ANOVA was performed to determine the statistical relevance of the data. While the P value of the test is 0.0041 and is considered significant, there was no significance when compared among the CVID subgroups. Also huge variations were seen in every sub groups tested.
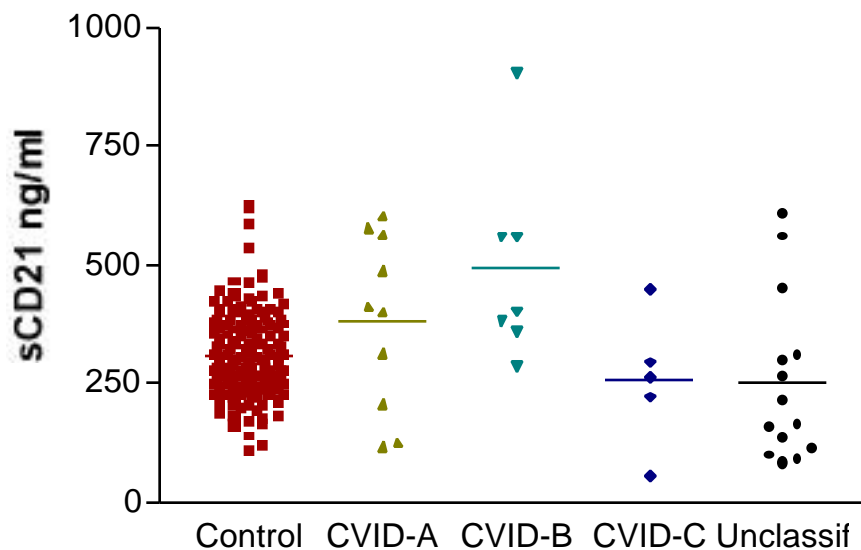
Figure 4.21 Serum Soluble CD21 Concentrations in CVID Subtypes

Serum sCD21 concentrations of patients of CVID subtypes, CVID-A (▲, n=10), CVID-B (▼, n=7), CVID-C (◆, n=5) and Unclassified (●, n=14) were estimated and compared to healthy controls (■, n=160). The P value = 0.0041

Statistics 8: Serum Soluble CD21 Concentrations in CVID, Variance with Subtypes

Kruskal-Wallis Test (Nonparametric ANOVA)

The P value is 0.0041, considered very significant.
Variation among column medians is significantly greater than expected
by chance.

The P value is approximate (from chi-square distribution) because
at least one column has two or more identical values.

Calculation detail

Group	Number of Points	Sum of Ranks	Mean of Ranks
Control	160	15637	97.731
CVID-A	10	1249.0	124.90
CVID-B	7	1110.0	158.57
CVID-C	5	381.50	76.300
CVID-Unknown	14	928.50	66.321

Kruskal-Wallis Statistic KW = 15.318 (corrected for ties)

Dunn's Multiple Comparisons Test

Comparison	Mean Rank Difference		P value
Control vs. CVID-A	-27.169	ns	P>0.05
Control vs. CVID-B	-60.840	ns	P>0.05
Control vs. CVID-C	21.431	ns	P>0.05
Control vs. CVID-Unknown	31.410	ns	P>0.05
CVID-A vs. CVID-B	-33.671	ns	P>0.05
CVID-A vs. CVID-C	48.600	ns	P>0.05
CVID-A vs. CVID-Unknown	58.579	ns	P>0.05
CVID-B vs. CVID-C	82.271	ns	P>0.05
CVID-B vs. CVID-Unknown	92.250	**	P<0.01
CVID-C vs. CVID-Unknown	9.979	ns	P>0.05

Summary of Data

Group	Number of Points	Median	Minimum	Maximum
Control	160	294.00	109.00	623.00
CVID-A	10	409.00	119.00	605.00
CVID-B	7	402.00	290.00	907.00
CVID-C	5	266.00	56.000	452.00
CVID-Unknown	14	191.00	84.000	607.00

Since CVID patients are low in immunoglobulins in plasma, they were given *i.v* injections of human immunoglobulins isolated from pooled plasma (Sandoglobulin) to compensate the reduction. It is possible that the sandoglobulin in serum of CVID patients could influence or contribute to sCD21 levels. So the presence of sCD21 was tested in sandoglobulin samples. 10 mg/ml solution of sandoglobulin gave a signal equivalent of 491 ng/ml sCD21 by ELISA. If a patient receives about 6 g of sandoglobulin as a single dose, taking plasma volume into consideration, the contribution of sandoglobulin to serum sCD21 pool is at the maximum 100 ng/ml. Hence, the variations in sCD21 levels in CVID could be in part due to the sandoglobulin injections received by the patients.

In order to verify the presence of CD21 molecule in sandoglobulin preparations, immunoprecipitation with BU32 antibody was performed in a concentrated solution of sandoglobulin and the precipitate was tested for CD21 by westernblot (Fig 4.22). No band was detected in the molecular range equivalent to CD21 in the precipitate. Therefore, though sandoglobulin did give a positive signal in ELISA, it is not convincing to report that sandoglobulin contains trace CD21 molecules. It is unclear at this moment why there is huge variation of sCD21 in CVID patients.
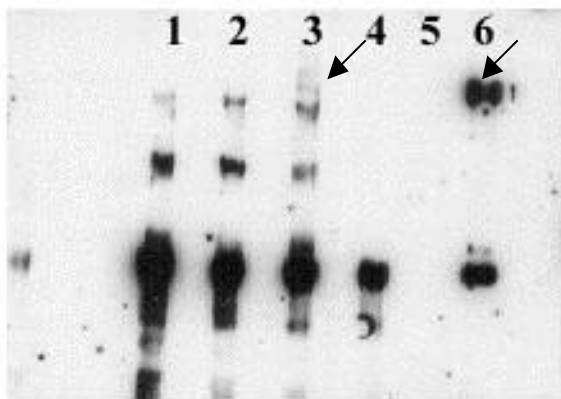
Figure 4.22 Sandoglobulin Did Not Contain CD21

Immunoprecipitation and westernblot analysis were performed from a concentrated solution of sandoglobulin with isotype control IgG (lane 1), BU32 (lane 2). Also included in the gel are control IP from plasma with BU32 (lane 3), Raji supernatant with BU32 (lane 4) and sCD21 positive control (lane 6). The sCD21 band is indicated by an arrow.

4.3.6 Serum Soluble CD21 Concentrations During Pregnancy and in Neonates

Pregnant women are physiologically immuno-compromised. It would be interesting to know if sCD21 levels are associated with the immuno-compromised conditions of pregnancy. To this end, sera from 29 pregnant women were analysed for sCD21 and compared to sCD21 levels in control females (Fig 4.23). The sCD21 levels were significantly reduced (P value <0.0001) in pregnant women. The levels ranged from 90 ng/ml to 260 ng/ml.
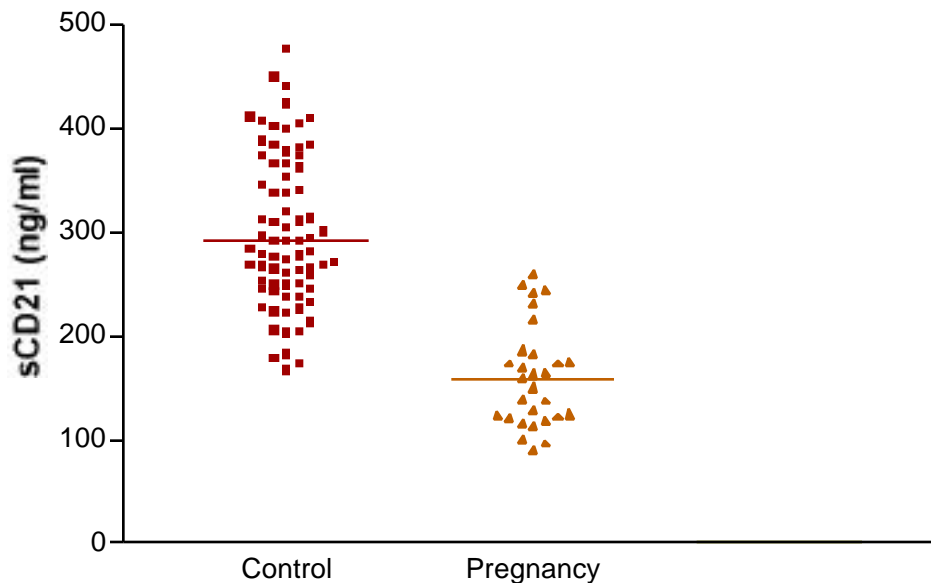
Figure 4.23 Serum Soluble CD21 Concentrations were Reduced During Pregnancy

Serum sCD21 concentrations of 29 pregnant women (▲) were estimated and compared to healthy controls (■, n=160). P value <0.0001

Statistics 9 : Serum Soluble CD21 Concentrations in Pregnant women

Mann-Whitney Test
  Do the medians of Control and Pregnancy differ significantly?

  The two-tailed P value is < 0.0001, considered extremely significant.
  The P value is an estimate based on a normal approximation.
  The 'exact' method would not be exact, due to tied ranks.

  Calculation details
  Mann-Whitney U-statistic = 111.00
  U' = 2064.0
  Sum of ranks in Control = 4914.0. Sum of ranks in Pregnancy = 546.00.

  Summary of Data

Parameter:	Control	Pregnancy
Mean:	304.36	161.52
# of points:	75	29
Std deviation:	79.671	49.278
Std error:	9.200	9.151
Minimum:	167.00	90.000
Maximum:	585.00	260.00
Median:	291.00	159.00
Lower 95% CI:	286.00	142.78
Upper 95% CI:	322.72	180.26

To determine if the sCD21 levels are restored to normal levels after parturition, serum samples were obtained from 10 pregnant women on the day before and after parturition. Chord blood (neonatal blood) was also obtained after delivery. Samples were tested for sCD21 by ELISA (Fig 4.24).
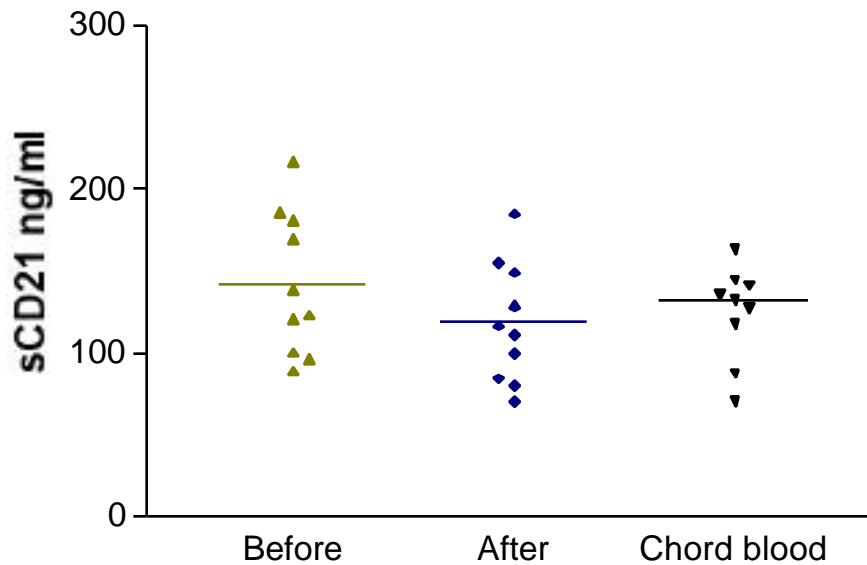
**Figure 4.24 Serum Soluble CD21 Concentrations Did Not Differ Significantly
Shortly Before and After Parturition, and in Neonates**

*Serum sCD21 concentrations of 10 pregnant women were estimated shortly
before parturition,(▲), compared to sCD21 levels after 3 days of parturition (◆)
and in chord (neonatal) blood (▼). P value=0.4139*

Statistics 10 : Serum Soluble CD21 Concentrations in Pregnency, Variance with Neonates, Before and After Partutition

Kruskal-Wallis Test (Nonparametric ANOVA)

*The P value is 0.4139, considered not significant.
Variation among column medians is not significantly greater than
expected
by chance.*

*The P value is approximate (from chi-square distribution) because
at least one column has two or more identical values.*

Calculation detail

Group	Number of Points	Sum of Ranks	Mean of Ranks
Before Delivery	10	176.00	17.600
Chord Blood	9	133.50	14.833
After Delivery	10	125.50	12.550

Kruskal-Wallis Statistic KW = 1.764 (corrected for ties)

Post tests were not calculated because the P value was greater than 0.05.
Summary of Data

Group	Number of Points	Median	Minimum	Maximum
Before Delivery	10	131.50	90.000	218.00
Chord Blood	9	133.00	71.000	164.00
After Delivery	10	114.50	71.000	186.00

A small reduction of sCD21 levels was found in serum obtained after parturition (Fig 4.25 A), but not statistically significant (P value 0.2176). Comparison of sera from mother and neonate also did not show any significant decrease (Fig 4.25 B) (P value 0.4967). It would be interesting to know when the sCD21 levels are restored to normal levels in women after parturition and possible correlation with hormonal changes. More analyses needs to be performed in these lines.

A

B
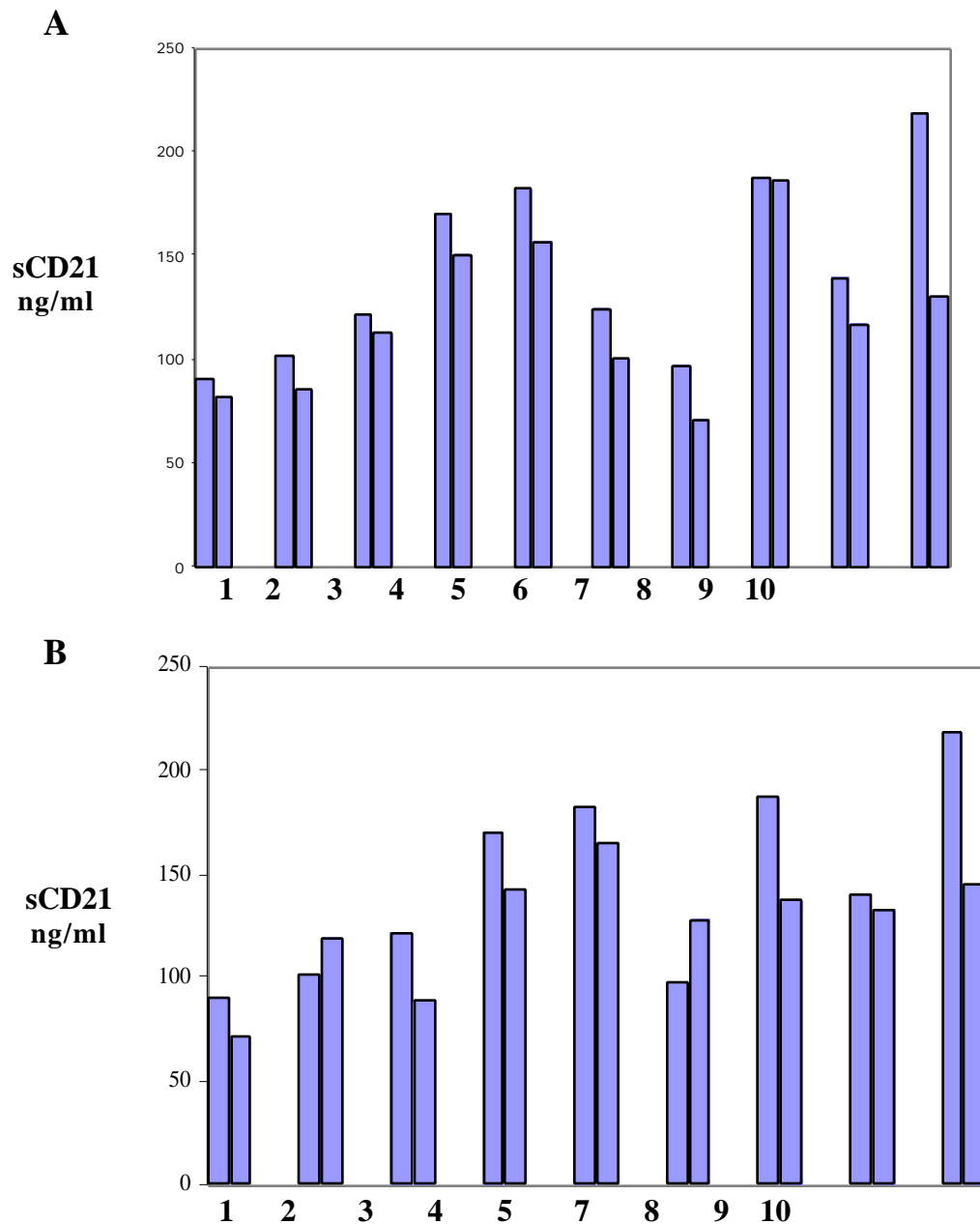
***Figure 4.25 Serum Soluble CD21 Concentrations Did Not Differ Significantly Shortly
Before and After Parturition and Between Mother and Neonate***

*A. Serum sCD21 concentrations of 10 pregnant women were estimated shortly before
and three days after parturition and plotted next to each other.*
*B. Serum sCD21 concentrations of mother and neonates shortly after birth were
estimated and plotted next to each other*

4.4 Mechanism of Soluble CD21 Shedding From Cell Surface

4.4.1 Peripheral Blood B Cells Contribute to Plasma Soluble CD21 Pool

As explained above soluble CD21 occurs at about 300 ng/ml in the blood of healthy person and levels change under pathological conditions. However, no definite data exist onto which primary cell type sheds sCD21. B and T lymphocytes are the primary hematopoeitic cells expressing CD21 in the circulation under non pathological conditions. To investigate the source of sCD21 in plasma, peripheral blood lymphocytes were isolated from healthy individuals, B and T lymphocytes were sorted and taken into culture. Cell supernatants were collected after 36 hours of incubation and tested for sCD21 by ELISA (Fig 4.26). Only B cells were found to shed CD21 into the supernatant. T cells shed very little or no detectable amounts of CD21 molecules under these culture conditions. Thus although T cells out number B cells in the peripheral blood and express CD21 they do not contribute to the sCD21 serum pool.
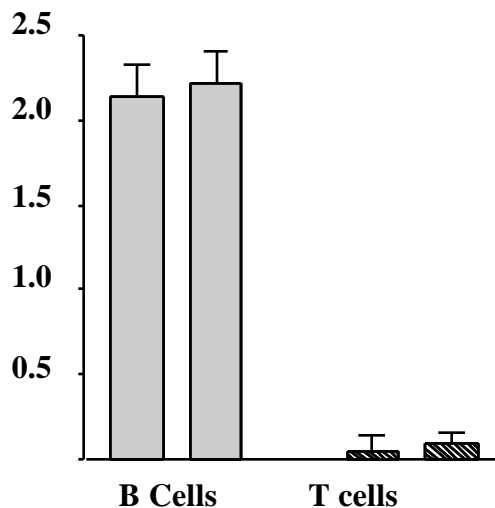
Figure 4.26 Peripheral Blood B Cells, But Not T Cells Shed CD21

Peripheral blood lymphocytes were isolated from fresh human blood and sorted B and T lymphocytes. 5×10^6 cells/ml were cultured in serum free medium for 36 hours, the supernatant was tested for sCD21 concentration. B cells shed about 2-3 ng sCD21 while T cells shed very little or undetectable amounts under the culture conditions.

4.4.2 Inhibition of Serine Proteases and Metalloproteases Prevents CD21 Shedding

In order to explore the nature of the elusive CD21 specific protease responsible for shedding CD21 into circulation, PBLs were cultured in the presence of different protease inhibitors and the supernatants were used for sCD21 estimation. The following classes of inhibitors were used. EDTA to inhibit metalloproteases, Pepstatin A for aspartic proteinases, Leupeptin for cysteine/serine proteases and $_1$-antitrypsin for serine proteases. PBLs were incubated with the protease inhibitors for 36 hours and the supernatants were analysed for sCD21 concentrations. $_1$-antitrypsin and EDTA prevented shedding of CD21 from cell membrane (Fig 4.27A). While Pepstatin did not show any difference compared to the controls, Leupeptin incubation had little effect under the culture conditions. There was no difference in concentrations of membrane associated CD21 between controls and protease inhibitor treated PBLs at 36 hours as determined by flow cytometry (Fig 4.27B).
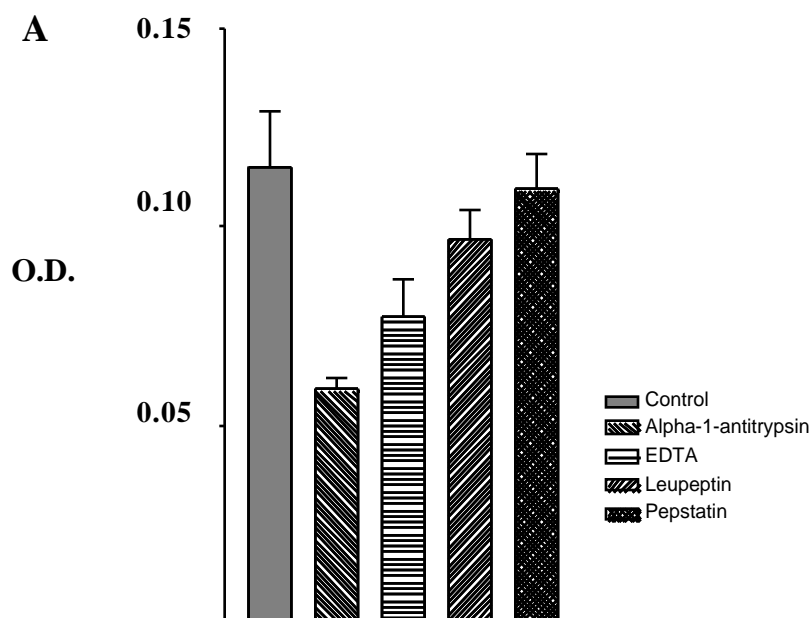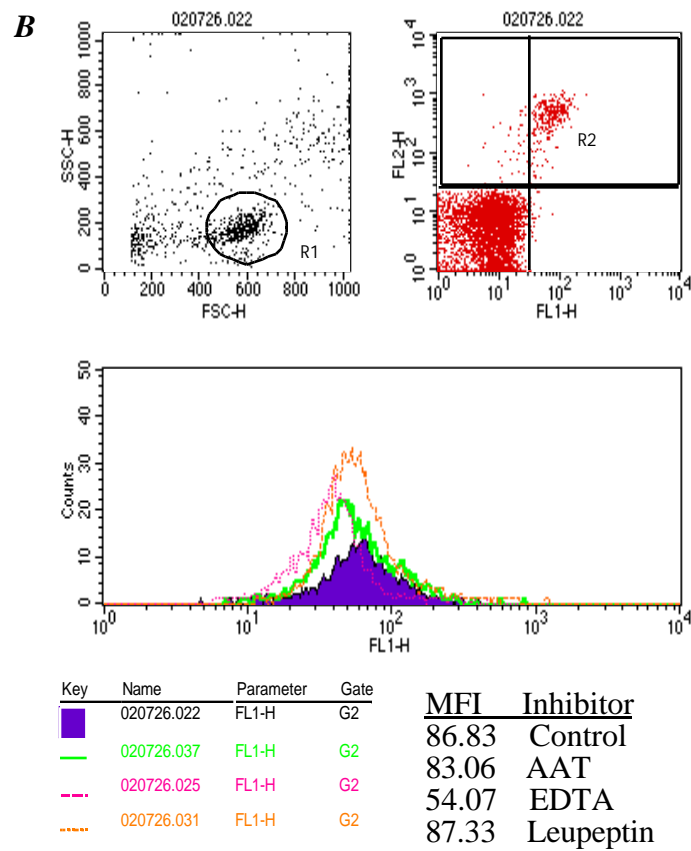
Fig 4.27 Effect of Protease Inhibitors on CD21 Shedding in Peripheral Blood Cells

5×10^6 *peripheral blood lymphocytes were cultured in the presence of various protease inhibitors for 36 hours and*
A. Soluble CD21 concentration in the supernatant was estimated and compared to control without any inhibitor. The inhibitors tested were, α_1-antitrypsin, 1mg/ml; EDTA, 2μM; Leupeptin, 100μM; Pepstatin, 1μM. α_1-antitrypsin had the maximum effect of sCD21 shedding among the inhibitors tested.
B. The cells were stained with CD21-FITC and cytometric analysis was performed to investigate the membrane levels of CD21.

4.4.3 Kinetics of CD21 Shedding

As Raji B cells were found to shed at least 10 times more CD21 than PBLs, Raji B cells was chosen to investigate the kinetics of CD21 shedding. Raji B cells were incubated with protease inhibitors $_1$-antitrypsin, EDTA and Leupeptin (as a negative control) for five days and followed the shedding (Fig 4.28a) and surface expression every 24 hours (Fig 4.28b). While there was no significant difference found in surface expression of CD21 by FACS, alpha-1-antitrypsin and EDTA prevented the shedding of CD21 into the tissue culture medium. The inhibition of CD21 shedding was not complete since the sCD21 concentrations in the supernatant increased with time albeit at a reduced level than in control cells.
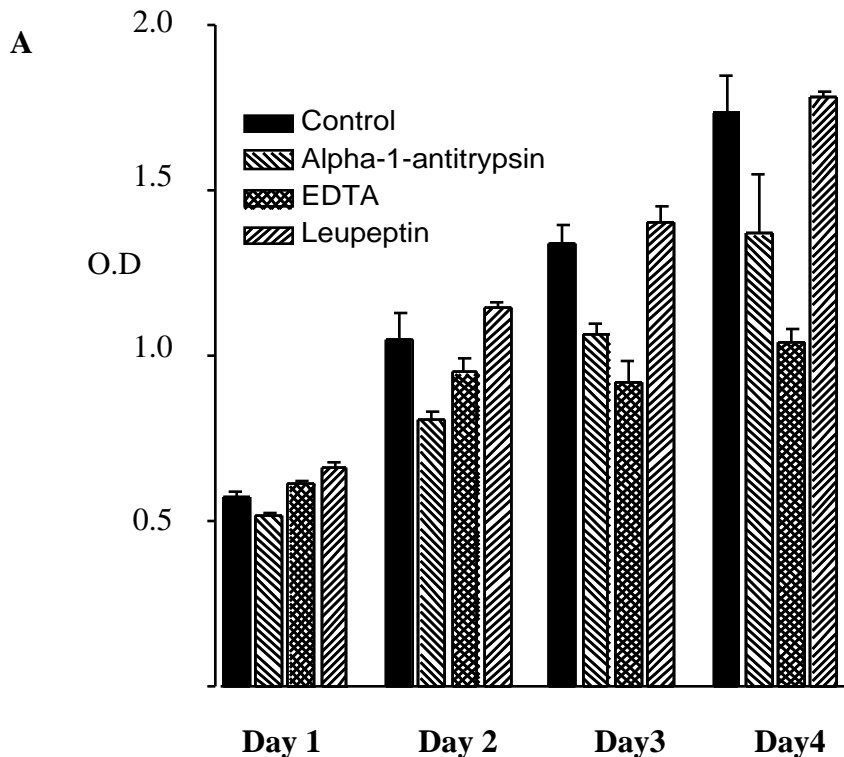
Figure 4.28A Time Dependent Effect of Protease Inhibitors on CD21 Shedding in Raji B Cells

Shedding of CD21 was followed every 24 hours for four days in 10^6 Raji B cells per ml, cultured in the presence of α_1-antitrypsin, 1mg/ml; EDTA, 2μM and Leupeptin, 100μM. Soluble CD21 concentrations estimated by ELISA in tissue culture supernatants

B

Key	Name	Parameter	Gate	MFI Day 1
(purple)	020806.001	FL1-H	G3	28.52
(green)	020806.011	FL1-H	G3	30.15
(magenta dashed)	020806.003	FL1-H	G3	26.98
(blue dotted)	020806.005	FL1-H	G3	30.56
				Day1

Key	Name	Parameter	Gate	Day 2
(purple)	020811.001	FL1-H	G1	20.34
(green)	020811.004	FL1-H	G1	13.85
(magenta dashed)	020811.002	FL1-H	G1	23.07
(blue dotted)	020811.003	FL1-H	G1	20.23

Key	Name	Parameter	Gate	Day 3
(purple)	020812.001	FL1-H	G1	19.42
(green)	020812.004	FL1-H	G1	9.53
(magenta dashed)	020812.002	FL1-H	G1	23.09
(blue dotted)	020812.003	FL1-H	G1	23.70

Key	Name	Parameter	Gate	Day 4
(purple)	020813.001	FL1-H	G1	19.98
(green)	020813.004	FL1-H	G1	12.89
(magenta dashed)	020813.002	FL1-H	G1	25.53
(blue dotted)	020813.003	FL1-H	G1	21.82

Figure 4.28 B. *Membrane CD21 Expression on Raji B Cells Treated with Protease Inhibitors for 24 hours*

■ : Control,   : α_1-antitrypsin, ------: EDTA, ········: Leupeptin

4.4.4 Mitogen (PMA) Activation of B Cells Induces Soluble CD21 Shedding

Many immune mediators act locally rather than systemically. Local concentrations of sCD21 might be higher at places where B or T cells are engaged in a immune reaction, e.g. in the germinal center or sides of local inflammation. Therefore it would be interesting if CD21 shedding is associated with mitogen activation of lymphocytes. Peripheral blood lymphocytes from three healthy individuals were activated with the phorbol ester PMA and Ca^{++} ionophore for 5 hours and sCD21 estimated in the cell culture supernatants. PBLs from all the three donors tested shed higher amounts of sCD21 when activated with PMA (Fig 4.29). To study the role of protease inhibitors during PMA activation, PBLs from three healthy donors were activated with PMA in the presence of alpha-1-antitrypsin for 5 hours. Alpha-1-antitrypsin completely abrogated the PMA induced shedding in all the three samples tested (Fig 4.29). Therefore PMA/calcium ionophore activation directly acts on the activity of the protease.

Similarly, Raji B cells also shed more CD21 into the tissue culture medium when activated with PMA and Ca^{++} ionophore for 5 hours (Fig 4.30A). The shedding was more pronounced during activation in the presence of Ca^{++} ionphore than with PMA alone. Flow cytometric analysis was performed to analyse the surface expression of CD21 during activation in Raji cells. The shedding of sCD21 was paralleled by a significant decrease in membrane CD21 (Fig 4.30B).
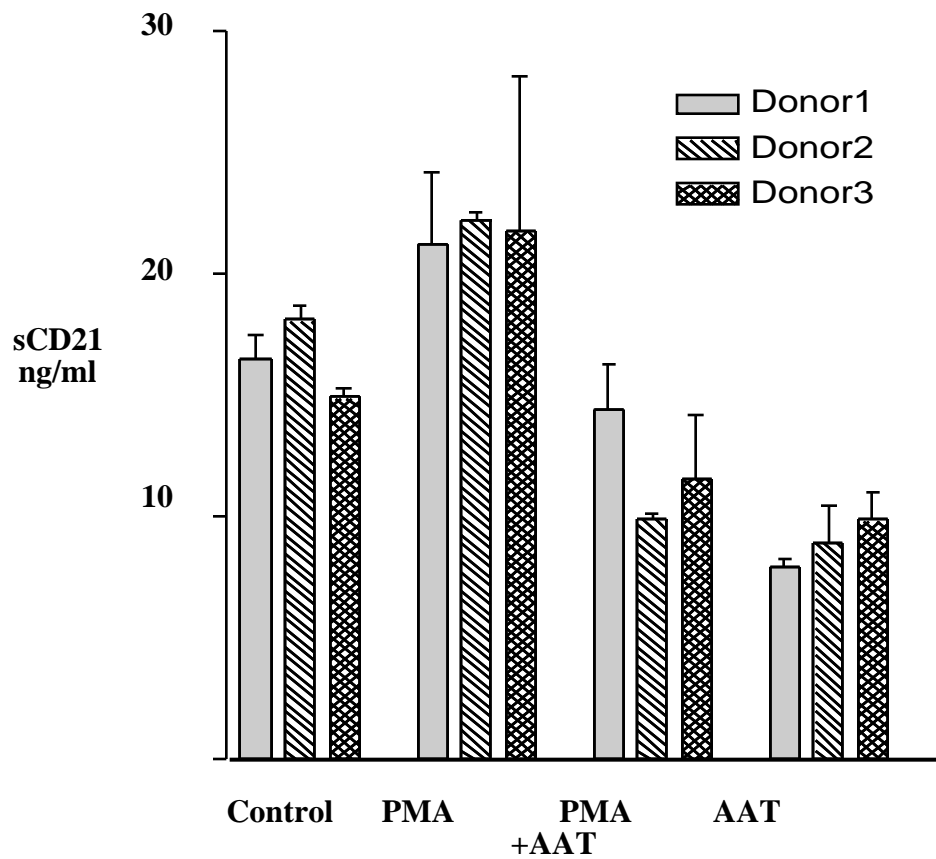
***Figure 4.29** α_1-antitrypsin Inhibits PMA-Induced CD21 Shedding*

5 × 10^6 peripheral blood lymphocytes /100 µl from three healthy donors were activated with PMA in the presence of calcium ionophore with and without α_1-antitrypsin. sCD21 concentrations were estimated in the tissue culture supernatants. Only PMA activation induced sCD21 shedding into the extracellular medium. Lymphocytes from all the three donors tested shed less sCD21 when activated with PMA and calcium ionophore in the presence of α_1-antitrypsin.
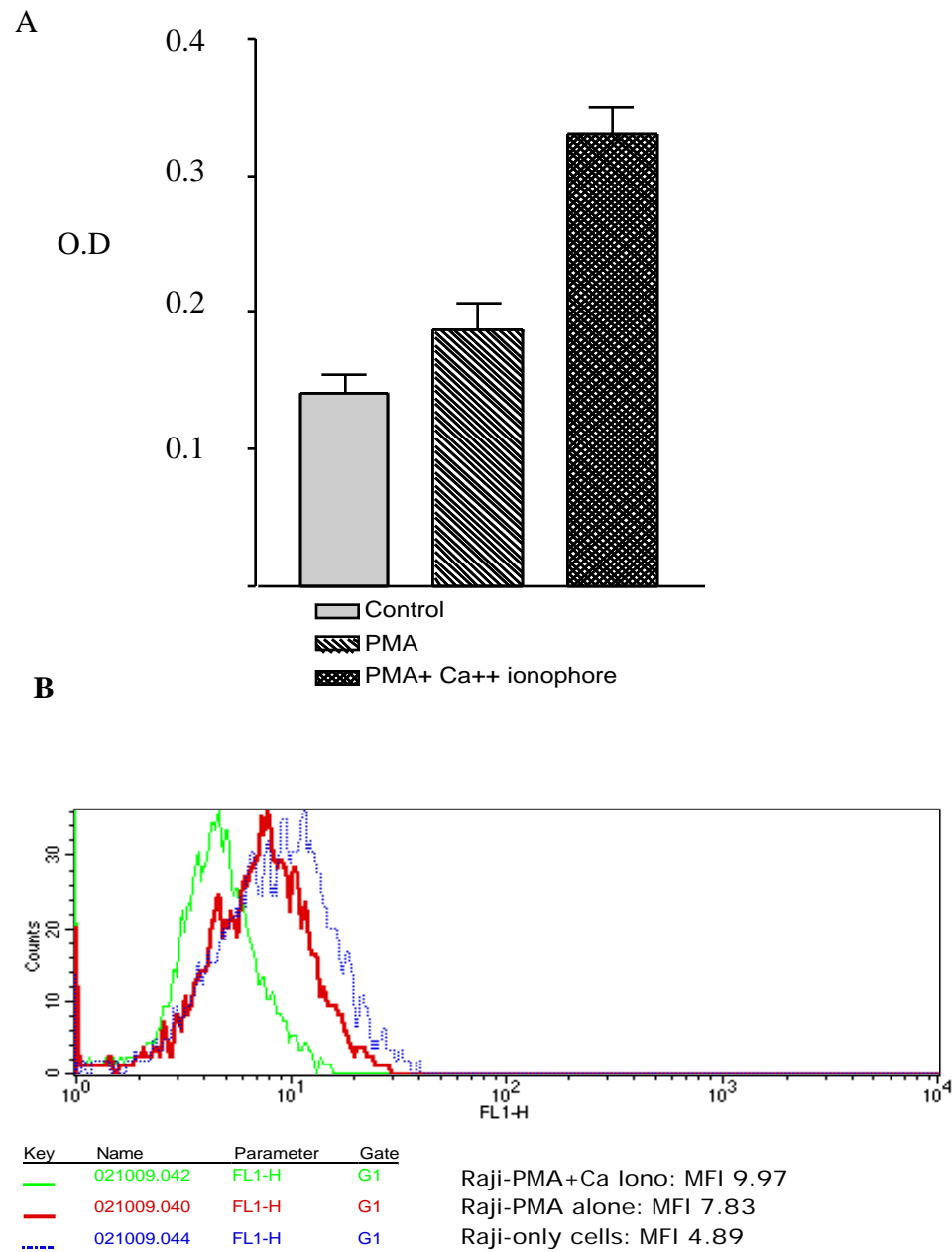
Figure 4.30 PMA Activation Induces CD21 Shedding in Raji B cells

10^6 Raji B cells were activated by PMA with and without calcium ionophore for 5 hours. A) sCD21 concentrations were estimated in the tissue culture supernatant by ELISA and B) the surface expression of CD21 was measured by FACS. —— :PMA + Ca^{++} ionophore activation, —— : Only PMA activation, ········ : Control(only cells)

CHAPTER 5

DISCUSSION

5.1 Regulation of CD21 Expression During T Cell Activation

CD21 plays a major role in B cell activation, proliferation and differentiation (Bohnsack and Cooper, 1988; Hatzfeld et al., 1988; Carter and Fearon, 1989). Peripheral blood B cells show decreased expression of CD21 upon activation with poke-weed-mitogen (PWM) or cross-linking of the B cell receptor by anti-μ treatment (Roome and Reading, 1987). In T-lineage cells, the immature double-negative thymocytes express CD21, which is down regulated at the double-positive/single-positive stage of differentiation (Fischer et al., 1999).

The functional role of CD21 in T cells is not yet known. HTLV-1 infection of a human T cell line resulted in induction of CD21 expression (Schultz et al., 1986). Ligation of CD21 with the mAb OKB7 mediate an increased concentration of Ca_i^{2+}, cap formation and ligand internalization on human HPB-ALL T cell line (Delibrias et al., 1992; Prodinger et al., 1996). The presence of EBV genome is reported in several T cell malignancies which implies that the cells express CD21 because it is needed as receptor for EBV (Watry et al., 1991).

Conflicting reports of CD21 expression in normal T cells are found in the literature. Some reports claim membrane bound CD21 only on a subpopulation of peripheral blood T cells (Fischer et al., 1991). Fischer et al (Fischer et al., 1991) reported that the percentage of CD21 expressing T cells decreased from 20% to 4% and 8% upon activation by PHA and ConA

respectively by cytometric analysis. Sandilands et al (Sandilands et al., 1999) found permeabilized PHA-activated T cells to show higher levels of CD21 by FACS. However, others and we were unable to detect CD21 by surface staining of T cells at all by cytometry (Tedder et al., 1984; Braun et al., 1998) and so is the case in western blot. These contrasting reports led us to investigate CD21 in T cells upon physiological activation.

TaqMan PCR analysis showed that the CD21 mRNA is present in resting T cells, but is down regulated by a factor of 50-100 upon the activation of T lymphocytes within two to three days. Human peripheral blood T lymphocytes were activated using anti-CD3, anti-CD28 and IL-2, which mimics physiological activation of T lymphocytes in vivo. In immunostainings, the difference in T and B cell derived protein (Fig. 4.6) could be the result of the low expression on T cells or the lack of the corresponding epitope for the monoclonal antibody used. The analysis involving fluorescence microscopy showed that all primary T cells express CD21 glycoprotein.

It has been speculated that CD21 in T cells may not have any functional significance because in B cells the signal transduction of this protein requires an association with CD19 protein that is not found in T cells (Prodinger et al., 1996). However Bouillie et al (Bouillie et al., 1999) has recently found that a new signal transduction pathway for CD21 triggering phosphatidylinositol-3-kinase activity in human B lymphocytes independent of CD19. Therefore it would be interesting to explore the functional significance of CD21 in T cells. It is likely that similar mechanisms may take place in T cells also and may have significance during T cell activation and proliferation. Identification of molecular aspects of this phenomenon may throw light on the roles of this glycoprotein in T cell function.

5.2. Isolation And Characterization of Soluble CD21

Several membrane molecules are cleaved from the surface by endogenous cellular enzymes, releasing their soluble fragments in the extra-cellular medium and exerting its physiological function in vivo (Bazil, 1995). Several reports support the functions of sCD21. Recombinant soluble CD21 has been shown to interfere with the attachment of EBV to B cells (Nemerow et al., 1990; Moore et al., 1991). Administration of a soluble CD21-IgG chimera suppressed antibody responses to T cell-dependent antigens in mice (Hebell et al., 1991) suggesting an immuno-modulatory function of sCD21 in vivo. In addition to binding to iC3b, sCD21 complexes with CD23 and inhibits sCD23 induced IgE synthesis by B cells (Fremeaux-Bacchi et al., 1998b). Soluble CD21 induces activation and differentiation of human monocytes through binding to membrane associated CD23, activating nitric oxide synthase (NOS) (Fremeaux-Bacchi et al., 1998a). CD21 also has functions unrelated to complement/EBV binding such as being a receptor for interferon- (Delcayre et al., 1991). Elevated amounts of sCD21 are found in the case of B cell chronic lymphocytic leukemia. The sCD21 levels were related to the surface expression of antigen on the leukemic cells and the number of cells in the blood (Lowe et al., 1989). Although B cells are thought to be a major source of sCD21 in plasma, alternative sources, such as T cells and FDCs may also contribute to the serum pool. Concentration of sCD21 in serum of patients with hypo-gammaglobulinemia were not significantly different from those of normal individuals (Ling et al., 1991). Reduced expression of CD21 in synovial B and T cells in rheumatic patients and in peripheral B cells in sytemic lupus erythematosus leads to a role in autoimmunity (Takahashi et al., 1997; Illges et al., 2000). Therefore, it would be interesting to investigate if changes in serum levels of sCD21 would influence immuno-regulation.

Even though sCD21 is known to exist as a complex with soluble CD23 in circulation (Fremeaux-Bacchi et al., 1998b), density gradient centrifugation separates almost all the proteins that co-purify in affinity chromatography. Though there are several forms of sCD21 reported to be in circulation (Ling and Brown, 1992), our method of density gradient centrifugation purifies a single species of 126 kDa. Since any other form of the protein could not be identified by western blot, I propose that the 126 kDa form of sCD21 is the predominant form in circulation. One cannot rule out the possibility that the lower molecular weight forms of sCD21 reported could be degradation products of full-length CD21. The possible degradation products may not contain the epitopes recognized by the monoclonal antibodies used. It is shown here that the soluble form of CD21 exist as a monomer and is not covalently linked to any other protein. This finding is in accordance with previous reports that the interactions of sCD21 with its binding partners in serum are mainly through lectin interactions with linked oligosaccharides instead of protein-protein interaction (Aubry et al., 1994). The sedimentation coefficient of sCD21 was determined by density gradient centrifugation. The sedimentation coefficient of a protein depends on its molecular weight and shape and gives some clues on the conformation of the protein. The S value of sCD21 is found to be 5.2, which is relatively low for a protein of 126kD. Calculations based on the hydrodynamic properties such as diffusion coefficient $(D_{20,w})$ and frictional ratio (F/F_0) of sCD21 show that sCD21 is a rod shaped molecule. Previous studies on these properties were done by Moore et al using a recombinant form of CD21 comprising the extra-cellular portion expressed in baculovirus expression system (Moore et al., 1989). The variations found in the values to the published hydrodynamic properties may be due to the different glycosylation pattern of the expression system used.

The native CD21 molecule consists of 15 or 16 short consensus repeats (SCRs) repeats and is extensively N-glycosylated. The beads-on-string structure of the molecule may provide a complex secondary structure that retards the movement in a centrifugal field. This structure may also account for anomalous high apparent molecular weight of 320 kDa in gel filtration as

reported by Ling et al (Ling et al., 1991). SDS-PAGE/Westernblot of both cellular and soluble CD21 with BU32 under non-reducing conditions shows an increased mobility of CD21 in contrast to 135-190 kDa smear of serum CD21 as reported by Fremeaux-Bacchi (Fremeaux-Bacchi et al., 1998b). The results presented here (Fig 4.8C) demonstrate that the density gradient purified protein is not associated covalently (at least through intermolecular disulfide bonds) with any other molecule and the resulting faster mobility upon boiling with a reducing agent is because of breakdown of intra-molecular disulfide bonds giving rise to a single polypeptide chain. This finding also emphasizes the fact that the native CD21 in serum may not be in circulation as a complex protein and exist as monomer.

5.3 Serum Soluble CD21 Concentration as a Parameter of Immunoregulation

5.3.1 Serum Soluble CD21 Levels in Rheumatoid Arthritis

The activation of different parts of the complement cascade in RA has been known for a long time. Recent studies showed that molecules of the innate immune system play central roles in the development or progression of autoimmunity in different diseases including RA (Fearon and Carroll, 2000; Ji et al., 2002). One finding among many showed that levels of the CD21 ligand C3d are elevated in RA synovial fluids (Mollnes et al., 1986).

The study presented here demonstrates that the levels of sCD21 in the sera of RA patients were significantly reduced as compared to healthy controls and to patients with other related disorders. The data indicate that this is not due to the age of the patients or related to the presence of rheumatoid factor. The reduction could be due to different reasons. Firstly, increased complement activation products (e.g. C3d) could bind to sCD21 leading to increased clearance from plasma. The lack of significant reduction in other immune complex associated

autoimmune conditions suggests that classical immune complexes might not be involved in this process. However, in RA patients the turnover of C3 (which is about 1 g/L in healthy human sera) might be slightly elevated as compared to other diseases. Comparing the amounts of C3 and sCD21 in plasma, a small percentage of converted C3 would readily be sufficient to "neutralize" sCD21. Secondly, the elevated concentrations of protease inhibitors such as alpha-1-antitrypsin in plasma could prevent shedding of CD21 from the lymphocyte surface by inhibiting the proteases associated with cell membrane (Papiha et al., 1989). Thirdly, the release of sCD21 from cells other than lymphocytes or lymphocytes outside peripheral blood might have been impaired during rheumatoid arthritis.

In RA patients the switched memory B cell pool is enlarged and $CD38^+$ plasma-cell-like B cells are present in higher numbers (Reparon-Schuijt et al., 1998). However, the reduction of sCD21 cannot be accounted to peripheral blood B cells alone because peripheral blood B cells from RA patients were shown to produce the same low amounts as those from healthy individuals. This finding suggests that the majority of sCD21 is produced by other cell types or by B cells in different locations, for example in the spleen.

In conclusion, the reduction of sCD21 serum levels seen in RA underlines the importance of the innate immune system in autoimmune reactions and possibly in the pathogenesis of rheumatoid arthritis. Experiments using tissues from patients as well as healthy donors needs to be done to understand the mechanisms leading to the reduction of sCD21 in the peripheral blood of RA patients.

5.3.2 Serum Soluble CD21 Levels in Combined Variable Immunodeficiency

CVID is a heterogenous disease characterized by defective antibody production (IUIS-Report, 1999). Because of hypogammaglobulinemia, the patients usually have recurrent bacterial infections. In addition, there are a number of unexplained manifestations including inflammatory conditions, autoimmune diseases and lymphomas (Cunningham-Rundles, 2001). Pneumonia, Sinusitis and Otitis are the most common infectious manifestations. Other infections suggestive of severe T cell defects includes *Pneumcystis carinii*, Herpes zoster, candidiasis, *M.avium* and cytomegalovirus enteritis. Development of autoimmune disease is a paradox in CVID. Approximately 20-50% of CVID patients develop autoimmune diseases such as idiopathic thrombocytopenic purpura (ITP), autoimmune hemolytic anemia, rhematoid arthritis, systemic lupus erythematosus, and vasculitis. In the other end of the spectrum, the incidence of lymphoma and cancer is equally high and is the major single cause of death in CVID patients (Cunningham-Rundles, 1989). Most of the lymphomas that develop in CVID patients are of non-Hodgkin´s type of extra nodal origin and B cell in type (Cunningham-Rundles et al., 1987; Cunningham-Rundles et al., 1991; Sander et al., 1992).

The pathogenesis of CVID is equally varied as its manifestations. Though the total number of CVID B cells is often (but not always) normal, there is an increased proportion of phenotypically immature IgG bearing B cells (Fiorilli et al., 1986). Cellular immunity is also poor or non existant with often anergic to recall antigens. The T cell abnormalities include decreased lymphocyte proliferation, a relative lack of CD4+ T cells and a deficiency of antigen-primed T cells in circulation (Hermaszewski and Webster, 1993; Hammarstrom et al., 2000). The deficiency of CD40 and IL2 secretion could play a role in pathogenesis of B cell defects (Aukrust et al., 1997).

I propose that the huge variation of serum sCD21 found in CVID patients is due to the varied manifestations of the syndromes. It is quite likely that patients who develop autoimmune disorders show highly reduced serum sCD21 concentrations and those developing lymphomas, particularly of B cell orgin are the most likely candidates who show elevated levels. The varied immunological manifestation also could play a major role in regulated shedding of sCD21 in CVID patients. It is not clear at this point if sCD21 levels are the cause or the result of these clinical conditions. Analysis of patient data and a thorough investigation of the pathogenesis and disease manifestations of the patients analysed would shed more light in this direction.

5.3.3 Serum Soluble CD21 Levels During Pregnancy

The survival of fetal allograft, for a long time, is thought to be due to suppression of maternal immune system. Recent studies indicate the the fetal-maternal allograft, and several immunological features that arise during pregancy are still paradoxical. Moreover, the very idea of immune-suppression during preganancy by itself is viewed as a misconception (Stirrat, 1994).

However, the maternal immune response undergoes some changes both in humoral and cell mediated immunity during pregnancy. The antibody production tend towards the IgG1 isotype and away from the complement fixing IgG2a isotype particularly for antibodies against fetal allo antigens. Cytokines produced by activated lymphocytes during murine pregnancy tend to favour antibody production over cytotoxic responses (Dudley et al., 1993). Human pregnancy serum is capable of inhibiting IL-2 generation during lymphocyte generation (Nicholas et al., 1984) and hence depress cell mediated immunity during pregnancy. Certain allergic and some immunologic conditions such as RA improve considerably during pregnancy, providing evidence of generalised, but mild immunosuppression (Holland et al., 1984).

Analysis of 29 sera samples during various stages of pregnancy showed a significant reduction in serum sCD21 levels. The observed reduction in sCD21 levels may be due to the generalised immunosuppression and the hormonal changes associated with pregnancy. Further studies needs to be performed along these lines to assertively show the correlation of these findings and pregnancy. The serum sCD21 levels in chord blood was found not to differ much from that of the mother. These low levels may be due to decreased expression of CD21 on B cells compared to adult peripheral blood B cells (Griffioen et al., 1993) and in infant marginal zone B cells (Timens et al., 1989). The reduced CD21 on neonatal B cells could be attributed to unresponsiveness to bacterial capsular polyshaccharides, making them susceptible for infections (Rijkers et al., 1998). The capacity to mount humoral response is not fully developed in the human newborn. In vivo production of antibodies in the first 3 months of age is limited to the IgM class of immunoglobulins. Only IgM-secreting cells are induced when chord blood B cells were activated by polyclonal B cell activators in vitro (Pabst and Kreth, 1980; Holland et al., 1984).

5.4 Mechanism of CD21 Shedding

Peripheral blood B cells shed more sCD21 into tissue culture medium than T cells. B cells shed about 3 ng/ml in 36 hours while sCD21 in normal individuals averages to about 150-300 ng/ml. Assuming that the artificial cell culture conditions were not changing the ability of the lymphocytes to shed sCD21 by one or two orders of magnitude, one has to conclude that peripheral blood B cells and T cells play a minor role in the generation of the sCD21 pool in the blood. However it might well be that B and/or T cells in lymphoid organs are promoted by the environment to shed the larger amounts. The estimated theoretical half life of sCD21 according to N-end rule (Ciechanover and Schwartz, 1989) is 20 hours and is classified as unstable.

Considering the less stability of the protein and the low shedding from peripheral blood B cells, circulating B cells may not be the only source of sCD21.

Inhibition of sCD21 shedding by alpha-1-antitrypsin and EDTA clearly shows an involvement of a serine- and metallo proteases in regulating sCD21 shedding. Alpha-1-antitrypsin has been shown to inhibit shedding of CD23 from lymphocytes co-cultured with activated polymorphonuclear neutrophils (Brignone et al., 2001). It is not clear at this point if the same CD23 sheddase is also involved in sCD21 shedding as well. A number of reports are available on the involvement of metalloproteases in ectodomain shedding of cell membrane associated molecules (Dello and Rovida, 2002). The data suggesting the involvement of both metallo protease and serine protease very well coincides with the earlier observation that certain classes of metallo proteases (e.g. a disintegrin and a metalloprotease, ADAM family) are converted to their active forms in the trans-golgi by furin-type serine endoproteases, during their progress through protein sorting (Steiner et al., 1992).

PMA activation of Raji B cells induces a series of signal transduction events through protein kinase C (Krauss, 1999). The activation is augmented by addition of Ca^{++} ionophore, which makes calcium ions available for the activity of PKC by releasing the intra cellular storage of Ca^{++} from endoplasmic reticulum. Taking into consideration that PMA activation also mimics signal transduction through BCR, it is possible that shedding of sCD21 could be due to BCR signalling. Based on the results shown above, I propose a hypothesis that the shedding of CD21 leads to a local accumulation of the glycoprotein and could be involved in fine regulation of B cell responses. Soluble CD21 represents a fully functional part of the molecule without the trans-membrane and intra cellular portions and potentially could bind to all of its known ligands. The most likely ligands of sCD21 in a local environment such as a lymph node and/or germinal centers are iC3b, C3d and CD23.

On mature B cells CD21 forms a non-covalent signal transduction complex with CD19 and TAPA1 coreceptor complex, reducing the threshold of antigen necessary to initiate cell proliferation (Fearon and Carroll, 2000; Cherukuri et al., 2001b). CD21 on FDC plays an important role in the initiation of germinal center reaction and B cell differentiation against a T dependent antigen by binding immune complexes opsonized with complement fragments such as iC3b and C3d (Dempsey et al., 1996). Ligation of CD23 with CD21 inhibits sCD23 induced IgE synthesis (Fremeaux-Bacchi et al., 1998b). Soluble CD21 also induces activation and differentiation of human monocytes through CD23, activating nitric oxide synthesis (NOS) (Fremeaux-Bacchi et al., 1998a). As CD23 is also present in FDC, sCD21 could potentially regulate FDC activity through CD21.

Therefore, any local increase in sCD21 concentration could competitively inhibit the binding of CD21 ligands and could very well contribute to fine regulation of CD21 dependent responses. Another implication of this effect could be in maturation of B cells during an immune response. There B cell maturation requires a delicate balance between affinity of antigen binding and co-stimulation (Carroll, 2000). CD21 was shown to be essential for B cells to survive the germinal center reaction (Fischer et al., 1998). In a given scenario that sCD21 could block ligand binding and thereby increase the threshold for BCR signalling, a much higher affinity of antigen-antibody interaction would be required to initiate the required B cell response. A B cell entering a germinal center containing IC of the right specificity may initially express a B cell receptor of low affinity. The stimulation of the B cell will then be facilitated by the help of the CD21 coreceptor. During subsequent cell divisions the B cell will undergo affinity maturation thereby enhancing the affinity to the specific antigen. Thereby the threshold for activation via the BCR is lowered and less coreceptor participation is required. During the presence of the B cell in the germinal center sCD21 is produced and blocks potential ligands reducing the availability of coreceptor stimuli. Thus a B cell which produces sCD21 locally decreases coreceptor interactions and may have to compensate that through higher affinity of BCR binding in order to

survive. Therefore sCD21 might not only control B cell survival in the germinal center, but could well be involved in a sophisticated balance of B cell activation resulting in the selection of high affinity B cells.

Yet another facet is the potential role of sCD21 in the development of autoimmune disease. It is more likely that decreased levels of sCD21 facilitate maturation of autoreactive B cells, because of increased availability of C3d on opsonized IC. Along with this, the plasma levels of sCD21 have been found to be reduced in sera of rheumatoid arthritis patients suggesting a possible role in autoimmunity. This finding is yet another indication of requirement of normal levels of sCD21 to maintain tolerance against auto antigens, as proposed by Fearon and Carroll (Fearon and Carroll, 2000).

Soluble CD21 might therefore be a candidate for therapeutic purposes. Studies in model organisms, such as mice have already revealed the importance of CD21 in autoimmune response and the germinal center reaction, but the function of sCD21 still remains less clear. The availability of CD21 deficient mice might be a useful tool to introduce transgeneic CD21 to explore the possible functions discussed above.

CHAPTER 6

SUMMARY

In this study, the expression and regulation of membrane associated human complement receptor type-II (CR2/CD21) and its soluble form were investigated.

The expression of CD21 was analysed in normal and activated T cells. PCR analyses and immunofluorescence/confocal microscopy of peripheral blood T cells and of activated T cells showed reduction in CD21 mRNA and protein expression upon activation. The regulation of CD21 expression may modulate life span or immunological reactivity of T cells and the susceptibility of cells to infection by lymphotropic viruses.

CD21 is released as a soluble form from the cell surface by proteolytic activity known as „Shedding". Soluble CD21 was isolated and purified from human plasma to homogeneity applying affinity chromatography and density gradient centrifugation. Soluble CD21 was found to be a single 126 kDa molecular species. By determining the sedimentation coefficient of sCD21, the hydrodynamic properties such as diffusion coefficient and the frictional coefficient were calculated. These values show that the sCD21 isolated from human plasma is an elongated rod shaped molecule. A specific ELISA was developed using purified sCD21 as a standard for the determination of sCD21 concentration in human sera. Soluble CD21 levels were estimated in serum samples from 235 healthy donors, 209 RA patients, 17 patients with arthritis related disorders, 41 CVID patients, 10 healthy pregnant women and 10 neonates. The normal values of plasma sCD21 in healthy individuals of age between 20-40 years range from 100-500 ng/ml (median 292 ng/ml) with a tendency to decrease with age but did not differ with gender. sCD21

levels were significantly reduced in rheumatoid arthritis (P<0.0001). Soluble CD21 levels in RA ranged from 50-300 ng/ml (median 182 ng/ml), did not differ with age and were independent of rheumatoid factor. Patients with other arthritis-related disorders showed near normal CD21 levels (range: 170-400 ng/ml, median: 247 ng/ml). Peripheral blood B and T lymphocytes were isolated from healthy donors and rheumatoid arthritis patients, cultured and supernatants were analyzed for CD21 shedding. However, sorted B cells of rheumatoid arthritis patients released amounts of CD21 comparable with those of normal controls suggesting that either sCD21 is less shed from other sources or more consumed in rheumatoid arthritis. Huge variation of sCD21 levels were found in CVID patients. The sCD21 levels ranged from 40-960 ng/ml (median: 299 ng/ml). The levels did not differ significantly in various subtypes of CVID. The reason for these variation could be due to heterogeneity of the disease and may also be due to prevalence of autoimmunity and lymphoma in CVID patients. The sCD21 levels in pregnancy were significantly reduced (P<0.0001) and the levels were not restored to normalcy soon after parturition. Also, the sCD21 levels did not differ significantly between mother and the neonate.

The source and mechanism of CD21 shedding was studied in primary lymphocytes and lymphoid cell lines. B cells shed more CD21 than T cells and contribute to plasma sCD21 pool. Mitogen (PMA) activation of lymphocytes induced shedding of CD21 and is paralleled by an decreased in cell-surface CD21 in Raji B cells. The shedding was inhibited by α_1-antitrypsin, a serine protease inhibitor and EDTA, a metalloprotease inhibitor. α_1-antitrypsin also inhibited PMA induced CD21 shedding. These results show that a PMA-inducible protease is responsible for shedding of CD21. Protein kinase C pathway is likely to be involved in the regulated shedding of CD21 as PMA specifically binds to PKC. Further dissection of the signal transduction pathways might lead to identification of novel mechanism leading to regulated shedding of CD21, modulating the immune response.

ZUSAMMENFASSUNG

In dieser Doktorarbeit wurde die Expression und Regulation des humanen membraneständiger Komplementrezeptors Type-II (CR2/CD21) und siner löslichem CD21 form untersucht.

Die Expression von CD21 wurde in normalen und aktivierten T-Zellen analysiert. Mit Hilfe von PCR und Immunofluoreszenz/konfokaler Mikroskopie von peripheren Blut-T-Zellen und aktivierten T-Zellen wurde nach Aktivierung eine Reduktion in der CD21 mRNA und in der Proteinexpression gezeigt. Die Regulierung der CD21-Expression könnte die Lebensdauer oder die immunologische Reaktivität von T-Zellen und die Anfälligkeit für Infektionen durch lymphotrophe Viren regulieren.

Lösliches CD21 wurde durch Affinitätschromatographie und Dichtegradientenzentrifugation aus menschlichem Plasma isoliert und aufgereinigt. Es handelt sich dabei um ein einzelnes 126 kDa großes Molekül. Mit der Bestimmung des Sedimentationskoeffizienten von sCD21, wurden die hydrodynamischen Eigenschaften wie der Diffusionskoeffizient und der Reibungskoeffizient berechnet. Diese Werte zeigen, dass das aus menschlichem Serum isolierte sCD21 ein lang gestrecktes routenförmiges Molekül ist. Es wurde ein spezifischer ELISA entwickelt, in dem gereinigtes sCD21 als Standard für die Berechnung des sCD21 in Humanseren verwendet wurde. Der Gehalt an löslichem CD21 wurde in Seren von 235 gesunden Spendern, 209 RA Patienten, 17 Patienten mit Arthritis verwandten Krankheiten, 41 CVID Patienten, 10 gesunden schwangeren Frauen und 10 Neugeborenen ermittelt. Die normalen sCD21 Werte in gesunden Spendern zwischen 20 und 40 Jahren liegen zwischen 100-500 ng/ml (Median: 292 ng/ml) mit einer Tendenz zur Abnahme mit steigendem Alter aber unabhängig vom Geschlecht. Die sCD21-Werte waren in Patienten mit Rheumatoider Arthritis signifikant geringer als in den Kontrollseren (P<0,0001). Sie lagen zwischen 50-300 ng/ml (Median: 182 ng/ml) und waren unabhängig von Alter und Rheumafaktor. Patienten mit anderen Arthritis-verwandten Krankheiten zeigten nahezu

normale Werte (zwischen 170-400 ng/ml, Median: 247 ng/ml). Periphere Blut-T- und B-Lymphozyten wurden aus gesunden Spendern und Patienten mit Rheumatoider Arthritis isoliert, in Kultur gesetzt und der Überstand auf CD21-Shedding untersucht. Dabei zeigte sich jedoch in Patienten mit Rheumatoider Arthritis ein vergleichbares Shedding von CD21 durch B-Zellen wie bei den gesunden Kontrollen. Dies könnte daran liegen, dass das sCD21-Shedding aus anderen Quellen reduziert ist oder dass mehr sCD21 in RA Patienten verbraucht wird. Es wurde eine breite Variation im sCD21-Gehalt bei CVID Patienten gefunden. Die Werte lagen zwischen 43-961 ng/ml (Median: 299 ng/ml). Die Werte unterschieden sich nicht signifikant zwischen den unterschiedlichen CVID-Subtypen. Der Grund für diese Varianz unter den CVID Patienten könnte die Heterogenität des Krankheitsbildes sein bzw. könnte an dem Vorkommen von Autoimmunität und Lymphomen in CVID Patienten liegen. Der sCD21-Gehalt bei Schwangeren war signifikant reduziert ($P < 0{,}0001$) und erlangte auch kurz nach der Geburt nicht wieder seinen Normalwert. Der sCD21-Gehalt unterschied sich nicht signifikant zwischen Mutter und Neugeborenem.

Die Quelle und der Mechanismus des CD21-Shedding wurde in primären Lymphozyten und lymphoiden Zelllinien untersucht. In B-Zellen existiert ein höheres CD21-Shedding als in T-Zellen, daher tragen diese zum Plasma sCD21-Pool bei. Mitogen (PMA) Aktivierung von Lymphozyten induziert Shedding von CD21 und korreliert mit einer Abnahme des CD21-Gehalts auf der Zelloberfläche in Raji B-Zellen. Das Shedding wurde durch Alpha1-Antitrypsin, ein Serinproteasehemmer und EDTA, ein Metalloproteasehemmer, inhibiert. Alpha1-Antitrypsin inhibierte auch das PMA induzierte CD21-Shedding. Diese Ergebnisse zeigen, dass eine PMA-induzierbare Protease für das Shedding von CD21 verantwortlich ist. Der Proteinkinase C-Reaktionsweg ist wahrscheinlich in dem regulierten Shedding von CD21 involviert. PMA bindet spezifisch an PKC. Eine weitere Untergliederung des Signaltransduktionsweges führt möglicherweise zur Identifizierung von neuen Mechanismen, die zum regulierten Shedding von CD21 führen und somit zur Modulierung der Immunantwort.

CHAPTER 7

BIBLIOGRAPHY

Ackers, G.K. (1975) Molecular sieve methods of analysis. In: H. Neurath, Hill, R., Boeder, C-L. (Ed) The Proteins. Academic Press, Inc., London.

Ahearn, J.M. and Fearon, D.T. (1989) Structure and function of the complement receptors, CR1 (CD35) and CR2 (CD21). Adv Immunol 46, 183-219.

Ahearn, J.M., Fischer, M.B., Croix, D., Goerg, S., Ma, M., Xia, J., Zhou, X., Howard, R.G., Rothstein, T.L. and Carroll, M.C. (1996) Disruption of the Cr2 locus results in a reduction in B-1a cells and in an impaired B cell response to T-dependent antigen. Immunity 4, 251-62.

Apel, M. and Berek, C. (1990) Somatic mutations in antibodies expressed by germinal centre B cells early after primary immunization. Int Immunol 2, 813-9.

Arnett, F.C., Edworthy, S.M., Bloch, D.A., McShane, D.J., Fries, J.F., Cooper, N.S., Healey, L.A., Kaplan, S.R., Liang, M.H., Luthra, H.S. and et al. (1988) The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 31, 315-24.

Arribas, J., Coodly, L., Vollmer, P., Kishimoto, T.K., Rose-John, S. and Massague, J. (1996) Diverse cell surface protein ectodomains are shed by a system sensitive to metalloprotease inhibitors. J Biol Chem 271, 11376-82.

Aubry, J.P., Pochon, S., Gauchat, J.F., Nueda-Marin, A., Holers, V.M., Graber, P., Siegfried, C. and Bonnefoy, J.Y. (1994) CD23 interacts with a new functional extracytoplasmic domain involving N-linked oligosaccharides on CD21. J Immunol 152, 5806-13.

Aukrust, P., Berge, R.K., Muller, F., Ueland, P.M., Svardal, A.M. and Froland, S.S. (1997) Elevated plasma levels of reduced homocysteine in common variable immunodeficiency--a marker of enhanced oxidative stress. Eur J Clin Invest 27, 723-30.

Bazil, V. (1995) Physiological enzymatic cleavage of leukocyte membrane molecules. Immunol Today 16, 135-40.

Beguin, Y., Lampertz, S., De Groote, D., Igot, D., Malaise, M. and Fillet, G. (1993) Soluble CD23 and other receptors (CD4, CD8, CD25, CD71) in serum of patients with chronic lymphocytic leukemia. Leukemia 7, 2019-25.

Bloomfield, V., Dalton, W.O. and Van Holde, K.E. (1967) Frictional coefficients of multisubunit structures. I. Theory. Biopolymers 5, 135-48.

Bohnsack, J.F. and Cooper, N.R. (1988) CR2 ligands modulate human B cell activation. J Immunol 141, 2569-76.

Bouillie, S., Barel, M. and Frade, R. (1999) Signaling through the EBV/C3d receptor (CR2, CD21) in human B lymphocytes: activation of phosphatidylinositol 3-kinase via a CD19-independent pathway. J Immunol 162, 136-43.

Braun, M., Melchers, I., Peter, H.H. and Illges, H. (1998) Human B and T lymphocytes have similar amounts of CD21 mRNA, but differ in surface expression of the CD21 glycoprotein. Int Immunol 10, 1197-202.

Brignone, C., Munoz, O., Batoz, M., Rouquette-Jazdanian, A. and Cousin, J.L. (2001) Proteases produced by activated neutrophils are able to release soluble CD23 fragments endowed with proinflammatory effects. Faseb J 15, 2027-9.

Bryant, A., Calver, N.C., Toubi, E., Webster, A.D. and Farrant, J. (1990) Classification of patients with common variable immunodeficiency by B cell secretion of IgM and IgG in response to anti-IgM and interleukin-2. Clin Immunol Immunopathol 56, 239-48.

Carroll, M.C. (1998) The role of complement and complement receptors in induction and regulation of immunity. Annu Rev Immunol 16, 545-68.

Carroll, M.C. (2000) The role of complement in B cell activation and tolerance. Adv Immunol 74, 61-88.

Carroll, M.C. and Prodeus, A.P. (1998) Linkages of innate and adaptive immunity. Curr Opin Immunol 10, 36-40.

Carter, R.H. and Fearon, D.T. (1989) Polymeric C3dg primes human B lymphocytes for proliferation induced by anti-IgM. J Immunol 143, 1755-60.

Carter, R.H., Spycher, M.O., Ng, Y.C., Hoffman, R. and Fearon, D.T. (1988) Synergistic interaction between complement receptor type 2 and membrane IgM on B lymphocytes. J Immunol 141, 457-63.

Cherukuri, A., Cheng, P.C. and Pierce, S.K. (2001a) The role of the CD19/CD21 complex in B cell processing and presentation of complement-tagged antigens. J Immunol 167, 163-72.

Cherukuri, A., Cheng, P.C., Sohn, H.W. and Pierce, S.K. (2001b) The CD19/CD21 complex functions to prolong B cell antigen receptor signaling from lipid rafts. Immunity 14, 169-79.

Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate- phenol-chloroform extraction. Anal Biochem 162, 156-9.

Ciechanover, A. and Schwartz, A.L. (1989) How are substrates recognized by the ubiquitin-mediated proteolytic system? Trends Biochem Sci 14, 483-8.

Clements, G.B., Klein, G. and Povey, S. (1975) Production by EBV infection of an EBNA-positive subline from an EBNA- negative human lymphoma cell line without detectable EBV DNA. Int J Cancer 16, 125-33.

Corr, M. and Firestein, G.S. (2002) Innate immunity as a hired gun: but is it rheumatoid arthritis? J Exp Med 195, F33-5.

Croix, D.A., Ahearn, J.M., Rosengard, A.M., Han, S., Kelsoe, G., Ma, M. and Carroll, M.C. (1996) Antibody response to a T-dependent antigen requires B cell expression of complement receptors. J Exp Med 183, 1857-64.

Cunningham-Rundles, C. (1989) Clinical and immunologic analyses of 103 patients with common variable immunodeficiency. J Clin Immunol 9, 22-33.

Cunningham-Rundles, C. (2001) Common variable immunodeficiency. Curr Allergy Asthma Rep 1, 421-9.

Cunningham-Rundles, C., Lieberman, P., Hellman, G. and Chaganti, R.S. (1991) Non-Hodgkin lymphoma in common variable immunodeficiency. Am J Hematol 37, 69-74.

Cunningham-Rundles, C., Siegal, F.P., Cunningham-Rundles, S. and Lieberman, P. (1987) Incidence of cancer in 98 patients with common varied immunodeficiency. J Clin Immunol 7, 294-9.

Delcayre, A.X., Salas, F., Mathur, S., Kovats, K., Lotz, M. and Lernhardt, W. (1991) Epstein Barr virus/complement C3d receptor is an interferon alpha receptor. Embo J 10, 919-26.

Delibrias, C.C., Fischer, E., Bismuth, G. and Kazatchkine, M.D. (1992) Expression, molecular association, and functions of C3 complement receptors CR1 (CD35) and CR2 (CD21) on the human T cell line HPB-ALL. J Immunol 149, 768-74.

Dello, S.P. and Rovida, E. (2002) Transmodulation of cell surface regulatory molecules via ectodomain shedding. Biol Chem 383, 69-83.

Dempsey, P.W., Allison, M.E., Akkaraju, S., Goodnow, C.C. and Fearon, D.T. (1996) C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. Science 271, 348-50.

Dudley, D.J., Chen, C.L., Mitchell, M.D., Daynes, R.A. and Araneo, B.A. (1993) Adaptive immune responses during murine pregnancy: pregnancy-induced regulation of lymphokine production by activated T lymphocytes. Am J Obstet Gynecol 168, 1155-63.

Fearon, D.T. (2000) Innate immunity--beginning to fulfill its promise? Nat Immunol 1, 102-3.

Fearon, D.T. and Carroll, M.C. (2000) Regulation of B lymphocyte responses to foreign and self-antigens by the CD19/CD21 complex. Annu Rev Immunol 18, 393-422.

Feldmann, M. (2001) Pathogenesis of arthritis: recent research progress. Nat Immunol 2, 771-3.

Fingeroth, J.D., Heath, M.E. and Ambrosino, D.M. (1989) Proliferation of resting B cells is modulated by CR2 and CR1. Immunol Lett 21, 291-301.

Fiorilli, M., Crescenzi, M., Carbonari, M., Tedesco, L., Russo, G., Gaetano, C. and Aiuti, F. (1986) Phenotypically immature IgG-bearing B cells in patients with hypogammaglobulinemia. J Clin Immunol 6, 21-5.

Fischer, E., Delibrias, C. and Kazatchkine, M.D. (1991) Expression of CR2 (the C3dg/EBV receptor, CD21) on normal human peripheral blood T lymphocytes. J Immunol 146, 865-9.

Fischer, E.M., Mouhoub, A., Maillet, F., Fremeaux-Bacchi, V., Krief, C., Gould, H., Berrih-Aknin, S. and Kazatchkine, M.D. (1999) Expression of CD21 is developmentally regulated during thymic maturation of human T lymphocytes. Int Immunol 11, 1841-9.

Fischer, M.B., Goerg, S., Shen, L., Prodeus, A.P., Goodnow, C.C., Kelsoe, G. and Carroll, M.C. (1998) Dependence of germinal center B cells on expression of CD21/CD35 for survival. Science 280, 582-5.

Fremeaux-Bacchi, V., Aubry, J.P., Bonnefoy, J.Y., Kazatchkine, M.D., Kolb, J.P. and Fischer, E.M. (1998a) Soluble CD21 induces activation and differentiation of human monocytes through binding to membrane CD23. Eur J Immunol 28, 4268-74.

Fremeaux-Bacchi, V., Fischer, E., Lecoanet-Henchoz, S., Mani, J.C., Bonnefoy, J.Y. and Kazatchkine, M.D. (1998b) Soluble CD21 (sCD21) forms biologically active complexes with CD23: sCD21 is present in normal plasma as a complex with trimeric CD23 and inhibits soluble CD23-induced IgE synthesis by B cells. Int Immunol 10, 1459-66.

Fremeaux-Bacchi, V., Kolb, J.P., Rakotobe, S., Kazatchkine, M.D. and Fischer, E.M. (1999) Functional properties of soluble CD21. Immunopharmacology 42, 31-7.

Gibbons, R.A. (1966) Physico-chemical methods for the determination of the purity, molecular size and shape of glycoprotiens. In: A. Gottschalk (Ed) Glycoproteins. Elsevier Publishing Company, Amsterdam, p. 31-140.

Gillis, S. and Watson, J. (1980) Biochemical and biological characterization of lymphocyte regulatory molecules. V. Identification of an interleukin 2-producing human leukemia T cell line. J Exp Med 152, 1709-19.

Griffioen, A.W., Franklin, S.W., Zegers, B.J. and Rijkers, G.T. (1993) Expression and functional characteristics of the complement receptor type 2 on adult and neonatal B lymphocytes. Clin Immunol Immunopathol 69, 1-8.

Hammarstrom, L., Vorechovsky, I. and Webster, D. (2000) Selective IgA deficiency (SIgAD) and common variable immunodeficiency (CVID). Clin Exp Immunol 120, 225-31.

Harris, E.D.J. (1997) Rhematoid Arthritis. W.B.Saunders Company, California.

Hatzfeld, A., Fischer, E., Levesque, J.P., Perrin, R., Hatzfeld, J. and Kazatchkine, M.D. (1988) Binding of C3 and C3dg to the CR2 complement receptor induces growth of an Epstein-Barr virus-positive human B cell line. J Immunol 140, 170-5.

Heaney, M.L. and Golde, D.W. (1998) Soluble receptors in human disease. J Leukoc Biol 64, 135-46.

Hebell, T., Ahearn, J.M. and Fearon, D.T. (1991) Suppression of the immune response by a soluble complement receptor of B lymphocytes. Science 254, 102-5.

Hermaszewski, R.A. and Webster, A.D. (1993) Primary hypogammaglobulinaemia: a survey of clinical manifestations and complications. Q J Med 86, 31-42.

Holland, D., Bretscher, P. and Russell, A.S. (1984) Immunologic and inflammatory responses during pregnancy. J Clin Lab Immunol 14, 177-9.

Huemer, H.P., Larcher, C., Prodinger, W.M., Petzer, A.L., Mitterer, M. and Falser, N. (1993) Determination of soluble CD21 as a parameter of B cell activation. Clin Exp Immunol 93, 195-9.

Iida, K., Nadler, L. and Nussenzweig, V. (1983) Identification of the membrane receptor for the complement fragment C3d by means of a monoclonal antibody. J Exp Med 158, 1021-33.

Illges, H. (1999) An automated 96-well-plate loader for FACScan. Anal Cell Pathol 19, 99-103.

Illges, H., Braun, M., Peter, H.H. and Melchers, I. (1997) Analysis of the human CD21 transcription unit reveals differential splicing of exon 11 in mature transcripts and excludes alternative splicing as the mechanism causing solubilization of CD21. Mol Immunol 34, 683-93.

Illges, H., Braun, M., Peter, H.H. and Melchers, I. (2000) Reduced expression of the complement receptor type 2 (CR2, CD21) by synovial fluid B and T lymphocytes. Clin Exp Immunol 122, 270-6.

IUIS-Report. (1999) Primary immunodeficiency diseases. Report of an IUIS Scientific Committee. International Union of Immunological Societies. Clin Exp Immunol 118 Suppl 1, 1-28.

Janeway, Jr. C.A., Travers, P., Walport, M., Capra, J.D., (1999) Immunobiology, The Immune system in Health and Disease. 4th Ed. Cuurent Biology Publications, New York.

Ji, H., Ohmura, K., Mahmood, U., Lee, D.M., Hofhuis, F.M., Boackle, S.A., Takahashi, K., Holers, V.M., Walport, M., Gerard, C., Ezekowitz, A., Carroll, M.C., Brenner, M., Weissleder, R., Verbeek, J.S., Duchatelle, V., Degott, C., Benoist, C. and Mathis, D. (2002) Arthritis critically dependent on innate immune system players. Immunity 16, 157-68.

Kalinkovich, A., Engelmann, H., Harpaz, N., Burstein, R., Barak, V., Kalickman, I., Wallach, D. and Bentwich, Z. (1992) Elevated serum levels of soluble tumour necrosis factor receptors (sTNF-R) in patients with HIV infection. Clin Exp Immunol 89, 351-5.

Kamihira, S., Atogami, S., Sohda, H., Momita, S., Yamada, Y. and Tomonaga, M. (1994) Significance of soluble interleukin-2 receptor levels for evaluation of the progression of adult T-cell leukemia. Cancer 73, 2753-8.

Kersten, M.J., Evers, L.M., Dellemijn, P.L., van den Berg, H., Portegies, P., Hintzen, R.Q., van Lier, R.A., von dem Borne, A.E. and van Oers, R.H. (1996) Elevation of cerebrospinal fluid soluble

CD27 levels in patients with meningeal localization of lymphoid malignancies. Blood 87, 1985-9.

Klein, G., Giovanella, B., Westman, A., Stehlin, J.S. and Mumford, D. (1975) An EBV-genome-negative cell line established from an American Burkitt lymphoma; receptor characteristics. EBV infectibility and permanent conversion into EBV-positive sublines by in vitro infection. Intervirology 5, 319-34.

Korsmeyer, S.J., Arnold, A., Bakhshi, A., Ravetch, J.V., Siebenlist, U., Hieter, P.A., Sharrow, S.O., LeBien, T.W., Kersey, J.H., Poplack, D.G., Leder, P. and Waldmann, T.A. (1983) Immunoglobulin gene rearrangement and cell surface antigen expression in acute lymphocytic leukemias of T cell and B cell precursor origins. J Clin Invest 71, 301-13.

Kozono, Y., Abe, R., Kozono, H., Kelly, R.G., Azuma, T. and Holers, V.M. (1998) Cross-linking CD21/CD35 or CD19 increases both B7-1 and B7-2 expression on murine splenic B cells. J Immunol 160, 1565-72.

Kozono, Y., Duke, R.C., Schleicher, M.S. and Holers, V.M. (1995) Co-ligation of mouse complement receptors 1 and 2 with surface IgM rescues splenic B cells and WEHI-231 cells from anti-surface IgM- induced apoptosis. Eur J Immunol 25, 1013-7.

Krauss, G. (1999) Signal Transduction and Regulation. Wiley-VCH Verlag GmbH, Weinheim.

Kuby, J. (1997) Immunology. W.H.Freeman and Company, New York.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-5.

Lanzavecchia, A., Abrignani, S., Scheidegger, D., Obrist, R., Dorken, B. and Moldenhauer, G. (1988) Antibodies as antigens. The use of mouse monoclonal antibodies to focus human T cells against selected targets. J Exp Med 167, 345-52.

Larcher, C., Schulz, T.F., Hofbauer, J., Hengster, P., Romani, N., Wachter, H. and Dierich, M.P. (1990) Expression of the C3d/EBV receptor and of other cell membrane surface markers is altered upon HIV-1 infection of myeloid, T, and B cells. J Acquir Immune Defic Syndr 3, 103-8.

Lee, J.C. and Timasheff, S.N. (1974) The calculation of partial specific volumes of proteins in guanidine hydrochloride. Arch Biochem Biophys 165, 268-73.

Lindhout, E., Mevissen, M.L., Kwekkeboom, J., Tager, J.M. and de Groot, C. (1993) Direct evidence that human follicular dendritic cells (FDC) rescue germinal centre B cells from death by apoptosis. Clin Exp Immunol 91, 330-6.

Ling, N., Hansel, T., Richardson, P. and Brown, B. (1991) Cellular origins of serum complement receptor type 2 in normal individuals and in hypogammaglobulinaemia. Clin Exp Immunol 84, 16-22.

Ling, N.R. and Brown, B. (1992) Properties of soluble CR2 in human serum. Immunobiology 185, 403-14.

Ling, N.R., Brown, B. and Hardie, D. (1994) Synergy test for recognition of epitopes on soluble proteins; its application in the study of CD21 and CD23 antigens and their respective antibodies. J Immunol Methods 173, 11-7.

Liu, Y.J., Joshua, D.E., Williams, G.T., Smith, C.A., Gordon, J. and MacLennan, I.C. (1989) Mechanism of antigen-driven selection in germinal centres. Nature 342, 929-31.

Lowe, J., Brown, B., Hardie, D., Richardson, P. and Ling, N. (1989) Soluble forms of CD21 and CD23 antigens in the serum in B cell chronic lymphocytic leukaemia. Immunol Lett 20, 103-9.

Miltenyi, S., Muller, W., Weichel, W. and Radbruch, A. (1990) High gradient magnetic cell separation with MACS. Cytometry 11, 231-8 02142-1479, USA.

Minowada, J., Onuma, T. and Moore, G.E. (1972) Rosette-forming human lymphoid cell lines. I. Establishment and evidence for origin of thymus-derived lymphocytes. J Natl Cancer Inst 49, 891-5.

Mollnes, T.E., Lea, T., Mellbye, O.J., Pahle, J., Grand, O. and Harboe, M. (1986) Complement activation in rheumatoid arthritis evaluated by C3dg and the terminal complement complex. Arthritis Rheum 29, 715-21.

Mongini, P.K. and Inman, J.K. (2001) Cytokine dependency of human B cell cycle progression elicited by ligands which coengage BCR and the CD21/CD19/CD81 costimulatory complex. Cell Immunol 207, 127-40.

Moore, M.D., Cannon, M.J., Sewall, A., Finlayson, M., Okimoto, M. and Nemerow, G.R. (1991) Inhibition of Epstein-Barr virus infection in vitro and in vivo by soluble CR2 (CD21) containing two short consensus repeats. J Virol 65, 3559-65.

Moore, M.D., DiScipio, R.G., Cooper, N.R. and Nemerow, G.R. (1989) Hydrodynamic, electron microscopic, and ligand-binding analysis of the Epstein-Barr virus/C3dg receptor (CR2). J Biol Chem 264, 20576-82.

Myones, B.L. and Ross, G.D. (1987) Identification of a spontaneously shed fragment of B cell complement receptor type two (CR2) containing the C3d-binding site. Complement 4, 87-98.

Nemerow, G.R., McNaughton, M.E. and Cooper, N.R. (1985) Monoclonal antibody to the Epstein-Barr virus receptor induces human B lymphocyte activation and differentiation. Trans Assoc Am Physicians 98, 290-300.

Nemerow, G.R., Mullen, J.J., 3rd, Dickson, P.W. and Cooper, N.R. (1990) Soluble recombinant CR2 (CD21) inhibits Epstein-Barr virus infection. J Virol 64, 1348-52.

Nicholas, N.S., Panayi, G.S. and Nouri, A.M. (1984) Human pregnancy serum inhibits interleukin-2 production. Clin Exp Immunol 58, 587-95.

Nie, X., Basu, S. and Cerny, J. (1997) Immunization with immune complex alters the repertoire of antigen- reactive B cells in the germinal centers. Eur J Immunol 27, 3517-25.

Nielsen, C.H. and Leslie, R.G. (2002) Complement's participation in acquired immunity. J Leukoc Biol 72, 249-61.

Nielsen, C.H., Pedersen, M.L., Marquart, H.V., Prodinger, W.M. and Leslie, R.G. (2002) The role of complement receptors type 1 (CR1, CD35) and 2 (CR2, CD21) in promoting C3 fragment deposition and membrane attack complex formation on normal peripheral human B cells. Eur J Immunol 32, 1359-67.

Ohtani, K., Ninomiya, H., Hasegawa, Y., Kobayashi, T., Kojima, H., Nagasawa, T. and Abe, T. (1995) Clinical significance of elevated soluble interleukin-6 receptor levels in the sera of patients with plasma cell dyscrasias. Br J Haematol 91, 116-20.

Pabst, H.F. and Kreth, H.W. (1980) Ontogeny of the immune response as a basis of childhood disease. J Pediatr 97, 519-34.

Papiha, S.S., Pal, B., Walker, D., Mangion, P. and Hossain, M.A. (1989) Alpha 1 antitrypsin (PI) phenotypes in two rheumatic diseases: a reappraisal of the association of PI subtypes in rheumatoid arthritis. Ann Rheum Dis 48, 48-52.

Paul, W.E. (1993) Fundamental Immunology. In: W.E. Paul (Ed). Raven Press.

Pizzolo, G., Vinante, F., Chilosi, M., Dallenbach, F., Josimovic-Alasevic, O., Diamantstein, T. and Stein, H. (1990) Serum levels of soluble CD30 molecule (Ki-1 antigen) in Hodgkin's disease: relationship with disease activity and clinical stage. Br J Haematol 75, 282-4.

Prodinger, W.M., Larcher, C., Schwendinger, M. and Dierich, M.P. (1996) Ligation of the functional domain of complement receptor type 2 (CR2, CD21) is relevant for complex formation in T cell lines. J Immunol 156, 2580-4.

Reparon-Schuijt, C.C., van Esch, W.J., van Kooten, C., Levarht, E.W., Breedveld, F.C. and Verweij, C.L. (1998) Functional analysis of rheumatoid factor-producing B cells from the synovial fluid of rheumatoid arthritis patients. Arthritis Rheum 41, 2211-20.

Rijkers, G.T., Sanders, E.A., Breukels, M.A. and Zegers, B.J. (1998) Infant B cell responses to polysaccharide determinants. Vaccine 16, 1396-400.

Roberts, T. and Snow, E.C. (1999) Cutting edge: recruitment of the CD19/CD21 coreceptor to B cell antigen receptor is required for antigen-mediated expression of Bcl-2 by resting and cycling hen egg lysozyme transgenic B cells. J Immunol 162, 4377-80.

Roome, A.J. and Reading, C.L. (1987) Frequency of B-lymphocyte transformation by Epstein-Barr virus decreases with entry into the cell cycle. Immunology 60, 195-201.

Ross, S.C. and Densen, P. (1984) Complement deficiency states and infection: epidemiology, pathogenesis and consequences of neisserial and other infections in an immune deficiency. Medicine (Baltimore) 63, 243-73.

Rovida, E., Paccagnini, A., Del Rosso, M., Peschon, J. and Dello Sbarba, P. (2001) TNF-alpha-converting enzyme cleaves the macrophage colony-stimulating factor receptor in macrophages undergoing activation. J Immunol 166, 1583-9.

Sander, C.A., Medeiros, L.J., Weiss, L.M., Yano, T., Sneller, M.C. and Jaffe, E.S. (1992) Lymphoproliferative lesions in patients with common variable immunodeficiency syndrome. Am J Surg Pathol 16, 1170-82.

Sandilands, G.P., Perry, M., Wootton, M., Hair, J. and More, I.A. (1999) B-cell antigens within normal and activated human T cells. Immunology 96, 424-33.

Schultz, T.F., A.Petzer, R., Staudere, A., Eigentler and M.P.Dierich. (1986) Expression of CR2 (C3d/EBV-receptor) on HTLV-1 transformed human T-cells. Proceedings of the 6th International Congress of Immunology, National Research Council, Ottawa, Canada, 188.

Schwab, J. and Illges, H. (2001a) Regulation of CD21 expression by DNA methylation and histone deacetylation. Int Immunol 13, 705-10.

Schwab, J. and Illges, H. (2001b) Silencing of CD21 expression in synovial lymphocytes is independent of methylation of the CD21 promoter CpG island. Rheumatol Int 20, 133-7.

Steiner, D.F., Smeekens, S.P., Ohagi, S. and Chan, S.J. (1992) The new enzymology of precursor processing endoproteases. J Biol Chem 267, 23435-8.

Steinitz, M. and Klein, G. (1980) EBV-transformation of surface IgA-positive human lymphocytes. J Immunol 125, 194-6.

Stirrat, G.M. (1994) Pregnancy and immunity. Bmj 308, 1385-6.

Subramanian, S.V., Fitzgerald, M.L. and Bernfield, M. (1997) Regulated shedding of syndecan-1 and -4 ectodomains by thrombin and growth factor receptor activation. J Biol Chem 272, 14713-20.

Takahashi, K., Kozono, Y., Waldschmidt, T.J., Berthiaume, D., Quigg, R.J., Baron, A. and Holers, V.M. (1997) Mouse complement receptors type 1 (CR1;CD35) and type 2 (CR2;CD21): expression on normal B cell subpopulations and decreased levels during the development of autoimmunity in MRL/lpr mice. J Immunol 159, 1557-69.

Tedder, T.F., Clement, L.T. and Cooper, M.D. (1984) Expression of C3d receptors during human B cell differentiation: immunofluorescence analysis with the HB-5 monoclonal antibody. J Immunol 133, 678-83.

Timens, W., Boes, A., Rozeboom-Uiterwijk, T. and Poppema, S. (1989) Immaturity of the human splenic marginal zone in infancy. Possible contribution to the deficient infant immune response. J Immunol 143, 3200-6.

Towbin, H., Staehelin, T. and Gordon, J. (1992) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. 1979. Biotechnology 24, 145-9.

Uthaisangsook, S., Day, N.K., Bahna, S.L., Good, R.A. and Haraguchi, S. (2002) Innate immunity and its role against infections. Ann Allergy Asthma Immunol 88, 253-64; quiz 265-6, 318.

Vecchi, M. and Carpenter, G. (1997) Constitutive proteolysis of the ErbB-4 receptor tyrosine kinase by a unique, sequential mechanism. J Cell Biol 139, 995-1003.

Warnatz, K., Denz, A., Drager, R., Braun, M., Groth, C., Wolff-Vorbeck, G., Eibel, H., Schlesier, M. and Peter, H.H. (2002) Severe deficiency of switched memory B cells (CD27(+)IgM(-)IgD(-)) in subgroups of patients with common variable immunodeficiency: a new approach to classify a heterogeneous disease. Blood 99, 1544-51.

Watry, D., Hedrick, J.A., Siervo, S., Rhodes, G., Lamberti, J.J., Lambris, J.D. and Tsoukas, C.D. (1991) Infection of human thymocytes by Epstein-Barr virus. J Exp Med 173, 971-80.

Weis, J.J., Fearon, D.T., Klickstein, L.B., Wong, W.W., Richards, S.A., de Bruyn Kops, A., Smith, J.A. and Weis, J.H. (1986) Identification of a partial cDNA clone for the C3d/Epstein-Barr virus receptor of human B lymphocytes: homology with the receptor for fragments C3b and C4b of the third and fourth components of complement. Proc Natl Acad Sci U S A 83, 5639-43.

Weis, J.J., Tedder, T.F. and Fearon, D.T. (1984) Identification of a 145,000 Mr membrane protein as the C3d receptor (CR2) of human B lymphocytes. Proc Natl Acad Sci U S A 81, 881-5.

Wilson, B.S., Platt, J.L. and Kay, N.E. (1985) Monoclonal antibodies to the 140,000 mol wt glycoprotein of B lymphocyte membranes (CR2 receptor) initiates proliferation of B cells in vitro. Blood 66, 824-9.

Wu, J., Qin, D., Burton, G.F., Szakal, A.K. and Tew, J.G. (1996) Follicular dendritic cell-derived antigen and accessory activity in initiation of memory IgG responses in vitro. J Immunol 157, 3404-11.

APPENDIX: REAGENTS AND SOLUTIONS

**ALSIEVER´S ANTICOAGULANT
SOLUTION**
For 100 ml,
0.42g NaCl
o.8g Trisodiumcitrate.2H$_2$O
(or 11g Citric acid monohydrate)
2.05g D-Glucose

Adjust pH to 6.1 with 10% citric acid
Filter sterilize (do not autoclave)
Use 1:1 to 1:5 with Blood

CITRATE BUFFERS (for antibody isolation)
100 mM Citric acid,
100 mM Trisodiumcitrate
mix to get pH 3.0 or 6.4
100 mM Citric acid: 2.1g Citric acid in 100 ml
ddH$_2$O
100 mM Trisodiumcitrate: 2.9 g Trisodium
citrate-2-hydrate in 100 ml ddH$_2$O.

Citrate Washing buffer: 20mM Na-Citrate
buffer pH 6.4
Mix 100 mM Citric acid and 100mM
Trisodiumcitrate to get pH 6.0.
Dilute 1=>5 for 20mM

Citrate Elution buffer-100mM Na-Citrate buffer
pH 3.0
Mix 100mM citric acid and 100mM
Trisodiumcitrate to get pH 3.0
100mM Citric acid: 2.1g Citric acid =>100ml
ddH$_2$O
100mM TriSodium Citrate-2-hydrate
2.9 Trisodium citrate-2-hydrate=>100ml ddH$_2$O

COLLOIDAL COOMASIE STAINING
SOLUTION
Stock: (w/v) 2%Phosphoric acid (20g of 85%
phosphoric acid)
(w/v) 10% Ammonium sulphate 100g
0.1%(w/v)CBB-G250(20ml stock, 1g in 20ml
water)
Dissolve in 1L water
Fixing solution: 10% AcOH and 50% EtOH
2h- over night
Staining:
Working Solution: 80ml stock staining
solution+20ml EtOH(or) MeOH
Stain for 8-10 hrs

Wash: Short wash 1X 25% EtOH 15min
Overnight with 25% EtOH

COUPLING BUFFER
(Bicarbonate buffer for coupling to sepharose)

0.2M NaHCO$_3$,O.5M NaCl,pH 8.3
84.01 g NaHCO$_3$
146.1 g NaCl
Adjust pH to 8.3
Make upto 5L

COATING/ BINDING BUFFER (for ELISA)
0.1M Na$_2$HPO4/Na$_2$H$_2$PO4   pH 9.0
0.1M Na$_2$HPO4.2H$_2$O => 1.779g in 100ml
0.1M NaH$_2$PO4.H$_2$O   => 1.379g in 100ml
Mix both to adjust the pH to 9.0

LOADING BUFFER
(For antibody purification)
50mM Tris pH 8.0, 5mM EDTA, 0.5% NP4
25 ml Tris pH 8.0
12.5ml 0.2M EDTA
2.5 ml 10% NP40
make upto 500 ml with ddH$_2$O

For 100 ml:
5ml 1M Tris pH 8.0
2.5 of 0.2M EDTA
0.5 ml 10% NP40
make upto 100ml with ddH$_2$O

SINGLE DETERGENT LYSIS BUFFER
(For cell lysates for Western Blot)
50mM Tris.Cl (ph 8.0); 150 mM NaCl 0.02%
NaN$_3$
100 µg/ml of PMSF; 1µg/mlAprotinin (or) 25%
Protease inhibitor cocktail tablet ;1% Triton-X-
100 (or) NP40
For 50ml
302.75 mg Trisma base
438.3 mg NaCl
Adjust pH to 8.0 with HCl
Add 10 mg sodium azide
500 µl Triton-X-100
Add 25X Protease inhibitor just before use.

NP40 LYSIS BUFFER
(For Immunoprecipitation)
50mM Tris; 150 mM NaCl; 2mM EDTA pH 8.0
1% NP40 for lysis.
0.1% NP40 for wash.

Stock: 5M NaCl, 0.2M EDTA pH 8.0, 1M Tris pH 7.6, NP40 10% W/V

Mix 2.5 ml Tris + 1.5ml NaCl+ 0.5 ml EDTA NP40 in 50ml

Stock:
5M NaCl:14.61g NaCl in 50ml ddH_2O.
1M Tris pH 7.6: 6.055g in 50ml ddH_2O and adjust pH to 7.6 with HCl.
0.2 M EDTA pH 8: 3.72g in 50ml and adjust pH 8.0 with NaOH.

Working solution:
2.5 ml   1M Tris
1.5 ml   5M NaCl
0.5 ml   0.2M EDTA
5 ml   NP40 (10% w/v) or
0.5 ml NP40(10% w/v for 0.1% buffer)

LYSIS BUFFER
(For phosphorylation studies)
50 mM Tris pH 7.6; 150 mM NaCl; 5mM EDTA
50 mM NaF ; 1mM Na_3V04; 30mM $Na_4P_2O_7$
1% NP40 + Protease inhibitors

Stock
1M Tris, 5M NaCl, 0.2M EDTA, 10% NP40, 1M NaF
250 mM $Na_4P_2O_7.10H_2O$
0.5 M Na_3VO_4
For 50ml
1M Tris pH 7.6 - 2.5ml
5M NaCl   -1.5ml
0.2M EDTA   -1.25ml
1M NaF   - 2.5ml
250mM $Na_4P_2O_7.10H_2O$-6ml
0.5M Na_3VO_4 -100µl
10% NP4O   -5ml
Make upto 50ml (Before use add 25X Protease Inhibitor in required amount)

PHOSPHATE BUFFERED SALINE (PBS)
pH 7.4 (10X)
for 500ml,
40g NaCl
1g KCl
7.2g Na_2HPO_4
1.2 g KH_2PO_4
in 500ml (Adjust pH in 1X)

SUBSTRATE BUFFER (For ELISA)
M Citric acid; 0.2M Na_2HPO_4
2.1g Citric acid and 3.56 g Na_2HPO_4
Make up to 100 ml
Adjust pH 6.0 with NaOH (approx 50-53 tablets)

TRIS BUFFERS
(For SDS PAGE and Western Blot)

RESOLVING GEL -BUFFER 1
1M Tris-Cl pH 8.8

STACKING GEL-BUFFER 2
1M Tris-HCl pH6.8

For 500ml
60.55g=> 500ml
Adjust pH to 8.8 for Buffer1
Adjust pH to 6.8 for Buffer2

SDS GEL RUNNING BUFFER
10X :
30g Tris
140 g Glycine
1g SDS
in 1000ml ddH_2O
dil 1 in 10

3X SDS SAMPLE BUFFER
187.5 mM Tris pH 6.7 - 2.27g
6% SDS   -   6g
30% Sucrose   -   30g
in 100 ml
 pH 6.7-6.8
Dissolve Tris and adjust pH with HCl , add SDS and Sucrose
Warm it until sucrose dissolves and make upto 100ml
Add little amount of Bromophenol blue
Add BetaMercapto Ethanol just before use
3X => 15% v/v (150µl=>850µl)
2X =>   10% v/v
1X =>   5% (50 µl=> 950µl)

MES SDS RUNNING BUFFER (20X)
For NuPAGE /Bis-Tris gels
MES 48.8g
(2-(N-Morpholino)-ethane sulfonic acid
Tris base   - 30.3g (1.00M)
SDS   - 5g   (69.3mM)
EDTA   - 1.5g   (20.5 mM)
ddH_2O -250 ml
Dilute   50ml to 1L
1X buffer should be pH 7.3

WESTERN BLOT –TRANSFER BUFFER
20mM Tris ;150mM Glycine; 20% MeOH
10X: 12g Tris
        56g Glycine
in 400ml ddH$_2$O

1X:
80ml 10X buffer
800ml ddH$_2$O
200 ml MeOH

STRIPPING BUFFER
(For Western Blot)
62.5 mM Tris HCl pH 6.7, 2% SDS, 100mM 2-
Mercapto ethanol
757.1mg Tris => adjust pH to 6.7
2g SDS (or) 10ml 20% SDS
Make upto 100ml
Add 2-mercapto ethanol fresh before use
7µl/ml or 70µl/10ml.

For 200ml => 1.514 g Tris
4g SDS or 20 ml 20% SDS

TRIS BUFFERED SALINE (TBS)
100 mM Tris ;0.9% NaCl pH7.5
10X
121.1 g Tris
90 g NaCl
Adjust pH to 7.5 with HCl

TRIS BUFFERED SALINE –TWEEN 20
(TBS –T)
For Western blot wash
60.55 g Tris
45g NaCl pH 7.5=> 5L ddH$_2$O
Add 2.5 ml Tween-20