INTRAVENOUS IMMUNOGLOBULIN REGULATION OF T CELL ACTIVATION THROUGH CD45

By

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

Of

MASTER OF SCIENCE

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To my husband

for everything

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LIST OF ABBREVIATIONS

Ab	antibody
AchR	nicotinic acetylcholine receptor
ADCC	antibody-dependent cell mediated cytotoxicity
AECA	anti-endothelial cell antibody
Ag	antigen
Btk	Bruton tyrosine kinase
C2GnT	core 2 β-1, 6-N-acetylglucosaminytransferase
CDR1	complementarity determining region 1
CIDP	chronic inflammatory demyelinating polyneuropathy
CNS	central nervous system
EAE	experimental autoimmune encephalomyelitis
ECL	enhanced chemiluminescence
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein-isothiocyanate-conjugated
GII	glucosidase II
α-galactosyl	Gal-α1-3Galβ1-4GlcNAc-R
GM-CSF	granulocyte mocrophage colony-stimulating factors
GVHD	graft-versus-host disease

HBSS	Hanks balanced salt solution
HLA	human leukocyte antigen
HRP	horseradish peroxidase
^{131}I-dUrd	^{131}I-deoxyuridine
IFN	interferon
IL-1ra	IL-1 receptor antogonist
ITAMs	immunoreceptor tyrosine-based activation motifs
ITP	immune thrombocytopenia
i.v	intravenous
IVIg	intravenous immunoglobulin
IVIgM	intravenous immunoglobulin IgM
Jaks	Janus kinases
KD	Kawasaki's disease
KO	knock-out
LCA	leukocyte common antigen
LPAP	lymphocyte phosphatase-associated phosphoprotein
LPS	lipopolysaccharide
MAC	membrane attack complex
MBP	mannose-binding protein
MCF	mean channel fluorescence
MG	myasthenia gravis
Mgat-5	$\beta_{1,6}$ N-acetylglucosaminyltransferase

M. leprae	mycobacterium leprae
MLR	mixed lymphocyte reaction
M.tb	*mycobacterium tuberculosis*
MS	multiple scerlosis
m.w.	molecular weight
Nab	natural antibody
NB	neuroblastoma
NK	natural killer
NKT	natural killer T
NM	neuraminidase
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PC	pneumococcus
PC5	phycoerythrin-cyanin 5.1
PE	phycoerythrin
PGL-1	phenolic glycolipid-1
PHA	phytohemagglutinin
PMA	phorbol myristate acetate
PNA	peanut agglutinin
PPD	purified protein derivatives of *Mycobacterium*
P_{td}	probability using the paired student's t test
P_{ti}	probability using the independent student's t test

PTPase	phosphotyrosine phosphatase
QR	qantum red
RAG-2$^{-/-}$	recombinase-activating gene-2
RGD	Arg-Gly-Asp
s.c.	subcutaneous
SCID	severe combined immune deficiency
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SFKs	src family kinases
SLE	systemic lupus erythematosus
STATs	signal transducers and activators of transcription
TNBP	tri-n-butyl phosphate
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TPA	12-O-tetradecanoyl-phorbol-13-acetate
VSV	vesicular stomatitis virus
xid	X-linked immunodeficiency

ABSTRACT

Extensive evidence supported a role for natural antibody (NAb) surveillance of developing tumors. Recent observations in a murine system showed T lymphomas treated with the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) to generate variants and then repeatedly selected for high NAb-binding, exhibited a reduced tumorigenicity. Analysis of surface molecule expression on these high NAb-binding cells revealed a consistent increase in binding monoclonal antibodies against activation-associated molecules including CD25 the IL-2R α chain and CD45RA, transiently increased molecules after T cell activation. This data suggested that NAb may also regulate T cell activation. Intravenous immunoglobulin (IVIg) which includes NAb has been shown to provide a benefit against several inflammatory and autoimmune diseases, which were characterized by increased T cell activation. Considering the observations in murine models, we hypothesized that direct down-regulation of T cell activation might contribute to the therapeutic effects of IVIg. To identify the cell surface targets of IVIg on human T cells, we generated model T cells through TPA treatment of Jurkat T leukemia cells and repeated selection four times for high serum IgG plus IgM binding or three times for high human IgG binding. The variants obtained, J4.1 and JIg3.1, exhibited stable increases in binding of IVIg at 10 mg/ml amounting to 67% and 79%, respectively. Moreover, IVIg at 10 mg/ml inhibited J4.1 and JIg3.1 cell growth in vitro by 17% and 26% respectively compared to the untreated cells, while parental Jurkat growth was enhanced by 15% under the same conditions. Additionally, in vivo ^{131}I-dUrd-labeled cell elimination experiments

in mice with severe combined immune deficiency (SCID) showed that JIg3.1 was more sensitive to IVIg than the parental Jurkat cells. Consistent with the increased IVIg binding, flow cytometry analysis of surface molecule expression showed that the two selected cells bound more mAbs against T cell activation-associated markers which increased 1 to 2 days after activation, including CD25, CD134, HLA-class I and class II and high molecule weight (m.w.) CD45 isoforms CD45RA and CD45RB. However, the anti-CD45RO mAb binding was unchanged, while the very early activation marker CD69 which increases within 6 hours and the very late activation marker CD95 were both decreased. Consistent with the changes in detection of CD45 exon products, western blotting of whole cell lysates confirmed that J4.1 and JIg3.1 exhibited markedly more high m.w. CD45 isoforms. Neuraminidase treatment increased the binding by IVIg, anti-CD45RA and anti-pan CD45 mAbs to the Jurkat lines further revealing that IVIg preferentially binds to asialo forms of the high m.w. CD45 isoforms. More specifically, IVIg inhibition of anti-CD45RA and anti-pan CD45 binding by 33% and 35% respectively, further suggested that IVIg bound to high m.w. isoforms of CD45. Together with the observation that IVIg could bind 76% of normal human peripheral blood T cells in vitro at 4 °C the data suggest a new mechanism for the benefit which IVIg provides against various autoimmune diseases and inflammatory disorders through the regulation of T cells. IVIg binding to T cells, especially high m.w. CD45 isoform-expressing pathological T cells, 1 to 2 days after activation, with subsequent reductions in cell growth by CD45-mediated cell apoptosis might contribute to the therapeutic effects of IVIg.

CHAPTER I

INTRODUCTION

LITERATURE REVIEW

I. 1 Natural antibody (NAb)

I. 1.1. NAb - an important component of innate immunity

I. 1.1.1 Innate immunity

The immune system has traditionally been divided into innate and adaptive parts, each with different functions and roles. Compared with adaptive immunity, which is characterized by recent evolution and a more specific immune response, the phylogenetically ancient innate immune system can provide protection from a wide range of pathogens mediated by its polyspecific activity (reviewed in Kimbrell and Beutler, 2001). Innate immunity has been increasingly considered to play a much more important and fundamental role in host defense based on the following facts. 1. Innate immunity is activated immediately and fights infections from the moment of first contact; it acts effectively and rapidly without the demand of previous exposure to a pathogen. 2. To a certain extent, the innate immune system is necessary in directing and assisting the acquired immune response. The susceptibility to infection of patients who have inadequate granulocytes suggested that in the absence of an innate immune system, adaptive immunity could not provide an effective protection. 3. From the view of evolution, the innate immune response developed even before the separation of vertebrates and invertebrates, and so far most multicellular organisms only depend on innate immunity. Taken together the innate immune response exhibits

its vital and exclusive role in the first line of defense against invading pathogens (reviewed in Medzhitov and Janeway 2000).

The components of the innate immune system are the basis of the unique function of innate immunity. They are generally grouped as cellular and humoral mediators including natural killer (NK) cells, natural killer T (NKT) cells, macrophages, γδ T cells, dendritic cells, mast cells, basophils and neutrophils, natural antibodies (NAbs), complement, interferon (IFN) and tumor necrosis factor (TNF) (Shi et al., 2001; Medzhitov and Janeway 1998). All of these individual effectors of the innate immune system contribute to exert the important and exclusive functions of innate immunity. They provide a first line of defense against infection, regulate and instruct the adaptive immune response, defend against early tumor development, and maintain the homeostatic state by clearing dead cells.

I. 1.2 Properties of NAb

Early in this century it was recognized that certain immunoglobulins in the circulation of normal humans and animals can react with a variety of endogenous and exogenous antigens (Ag) (Casali 1996; Turma 1991; Avrameas et al., 1991; Michael et al., 1969 and Boyden et al., 1965). These immunoglobulins were called natural antibodies, defined as antibodies in the serum of healthy individuals generated in the absence of deliberate immunization or intentional exposure to foreign antigens (Medzhitov and Janeway 2000b and Boyden et al., 1965). Compared to antigen-

induced antibodies, NAbs showed many differences in their composition, generation, function and manner of binding. Unlike antigen-induced antibodies which are mainly IgG, NAb consists of a high proportion of IgM along with IgG and traces of IgA (Ochsenbein et al., 2000 and Avrameas et al., 1991). NAb were found to be produced by a subset of B cells, Ly-1$^+$ B cells in mice (Hayakawa et al., 1984). The equivalent NAb-producing cells in humans, CD5$^+$ B cells, were distinguished from "conventional" B cells (Burastero et al., 1989 and Gadol et al., 1986). CD5$^+$ B cells are the predominant lymphocytes in the neonatal B cell repertoire. But CD5$^+$ B cells are not exclusively the only NAb producing cells, some CD5$^-$ B cells also have this capacity (Kasaian et al., 1992). These NAb generating cells have been proposed to undergo a positive selection in an early stage of B cell ontogeny (reviewed in Stollar et al., 1997). Antigens contributing to the early ontogenic selection are mainly endogenous antigens (Portnoi et al., 1986), and it was proposed that exogenous antigens existing in food may be involved as well. Recently a group of studies revealed that exposure to environmental antigens was not necessary for the emergence of NAb-producing cell precursors (Casali et al., 1996) based on the following experiments. A quantitative immunoblotting technique was used to assess the repertoires of natural self-reactive antibody in serum samples coming from the same healthy individuals over a 25-year interval. The results demonstrated that the repertoires of natural IgM and IgG remained conserved during aging as judged by binding to the extracts of histologically normal human kidney, lung, stomach and thymus used as sources of self-antigens and extracts of non-self antigen,

Pseudomonas aeruginosa. Thus, the repertoire stability of NAb may suggest that the NAb-producing B cells were positively selected for self-antigens early in development, and the subsequent exposure to exogenous antigens would not change the repertoire pattern of NAb (Stahl et al., 2001). The comparison of the pattern of NAbs between antigen-exposed and antigen-free mice showed similar results (Bos et al., 1989). Other studies similarly demonstrated that exogenous antigens were not necessary for the generation of the natural IgM repertoires, while external antigenic stimulation may account for the different serum IgG levels between pathogen-free mice, germ-free mice and antigen-free mice, which contained different foreign antigen loads (Haury et al., 1997). The observation that all antigen bands in the immunoblotting assay detected by natural IgG are the same bands as detected by natural IgM, led to the proposal that lack of external antigen stimulation affected the natural IgG level by reducing the stimuli-mediated Ig class switch (Lacroix-Desmazes et al., 1995). Although most repertoire selection of natural IgM antibody is the result of specific endogenous stimulation (reviewed in Coutinho et al., 1995), genetic differences seem to influence NAb titers. For instance, in the analysis of the role of natural antibodies in spontaneous resistance against vesicular stomatitis virus (VSV) in vitro, the titers of the NAb ranged from 1:8 to 1:32 in different inbred mouse strains (Gobet et al., 1988). NAbs are encoded by germline variable (V) genes without extensive somatic mutations (Casali et al., 1996). As a result of the lack of terminal deoxynucleotidyl transferase activity in precursor B cells during early ontogeny

(Feanay et al., 1990 and Gu et al., 1990), the repertoire of NAb is more restricted than that produced by conventional B cells.

The monoclonal antibody hybridoma technique allowed the generation of large panels of monoclonal autoantibody-secreting cells, which facilitate a precise study of the characteristics of the ligands of natural autoantibodies. These studies demonstrated that NAbs were highly polyreactive (Logtenberg 1990). A single monoclonal NAb has the capacity of binding more than one apparently structurally unrelated self and /or non-self antigens (Seigneurin et al., 1988 and Dighiero et al., 1983). Several studies showed that at least 20% of all immunoglobulins in mouse or normal human serum can be autoreactive based on immunoabsorption experiments using a panel of self-antigens, including actin, tubulin, histone, myosin, keratin, DNA, myoglobulin, cholesterol, and phosphatidic acid (Martini et al., 1989; Avrameas et al., 1988 and Shoenfeld et al., 1987). This suggests that polyreactivity and autoreactivity are two markedly characteristic properties of NAbs. NAbs have a wide range of binding affinities from 5×10^{-3} to 5×10^{-11}M (Diaw et al., 1997 and Adib-Conque et al., 1993).

I. 1.3. Functions of NAb

Several functions have been proposed for NAb (Lacroix-Desmazes et al., 1998). They can bind to pathogens and thus play an essential role in the first defense against infection. Their ability to remove tumor cells, altered molecules and senescent cells

contributes to homeostatic regulation and anti-tumor effects. In addition, a number of NAbs found in immune globulin which bind soluble and membrane-associated self molecules may be pertinent to the effects of immune regulation (Hurez et al., 1994; Vassiler et al., 1993; Marchalonis et al., 1992 and Rossi et al., 1989).

I. 1.3.1 Resistance against infection

The ability of NAb to bind to pathogens was considered as a consequence of poly-reactivity or cross-reactivity between self and foreign epitopes (Lacroix-Desmazes et al., 1998). The action of NAb in resistance against infections has been well documented in both animal and human systems. Briles et al. (1981a and 1982) used a mouse strain carrying an X-linked immunodeficiency (xid), an inability to produce normal IgM and IgG3 plus a lack of $CD5^+$ B cells, to assess natural resistance against *S. pneumoniae*. Their results showed that the immunodeficient xid mice were much more susceptible to intravenous infection than the control normal strain. Moreover, resistance to *S. pneumonia* was fully restored by reconstitution with normal serum IgG3 or anti-phosphocholine NAb (Briles et al., 1981b). NAb also demonstrated an important role in clearing pathogens. Recombinase-activating gene-2 ($RAG\text{-}2^{-/-}$) deficient mice with no serum Ig production, were highly sensitive to sub-lethal endotoxin-lipopolysaccharide (LPS) from *S. typhimurium* compared to normal mice. Passive transfer of fresh pooled serum from non-immune wild-type mice significantly enhanced survival of $RAG\text{-}2^{-/-}$ mice from endotoxin shock. This enhancing effect was consistent with the binding ability of NAb to LPS in vitro (Reid et al., 1997). A

similar result has been shown in another mouse strain bearing the Bruton tyrosine kinase (Btk) deficiency (Reid et al., 1997). In addition, NAb can contribute to the defense against infection by controlling early viral and bacterial distribution (Ochsenbein et al., 1999). The role of NAb in preventing pathogen distribution was detected by comparing the viral or bacterial titers in peripheral organs and secondary lymphoid organs. The titers of intravenously introduced bacteria or virus in the antibody-free mice were 10 to 100 times higher in peripheral organs including the kidney and brain than in antibody-competent mice. When compared to the antibody-competent mice, the titers in a secondary lymphoid organ, the spleen was 10 to 100 times lower. This indicated that NAb might enhance antigen-trapping in secondary lymphoid organs to prevent pathogen dissemination.

In humans, NAb is also considered a very important first barrier against infection. Naturally occurring cross-reactive antibodies to *mycobacterium tuberculosis* (M.tb) have been reported to exist in human serum (Bardana et al., 1973). The NAb in the lung mediated complement deposition on the M.tb bacilli and facilitated bacterium uptake by macrophages (Ferguson et al., 2000). Using nonimmune serum demonstrated that NAb was essential in anti-*mycobacterium leprae* (M. leprae) defense by contributing to C3 fixation to the surface of M. leprae and subsequently enhancing phagocytosis by human mononuclear phagocytes. C3 fixation and C1q binding to the major surface glycolipid of M. leprae, phenolic glycolipid-1 (PGL-1), were shown to be strictly NAb dependent (Schlesinger et al., 1994). Natural anti-leishmania antibodies in normal human serum bound leishmania promastigotes and

activated the classical complement pathway. This classical pathway was abolished when NAb was depleted, while natural anti-IgM addition restored C3 binding (Dominguez et al., 2002 and 1999). Most patients with HIV infection were characterized by overexpression of GM2 ganglioside on infected cells which was induced by HIV-1 virus (Okada et al., 1998 and Wu et al., 1996). Some normal individuals were observed to have the antibody (Ab) against GM2 without deliberate immunization (Tai et al., 1985 and Richard et al., 1980). The NAb against GM2 present in normal people showed potent cytolytic activity on HIV-infected cells via complement activation (Wu et al., 1999). Furthermore, when the same group analyzed the $CD4^+$ cell count and the HIV-RNA load in the HIV-infected patients they found that the anti-GM2 IgM NAb titer showed a positive correlation with the $CD4^+$ cell count, but a negative correlation with the HIV-RNA load (Wu et al., 2000). This may suggest that anti-GM2 mAb could help lower the plateau level of the HIV-RNA load and it may be related to the prognosis of the patients. High levels of NAb specific for a carbohydrate moiety, Gal alpha 1-3Gal beta 1-4GlcNAc-R (anti-alpha-galactosyl Ab) in human serum exhibited significant protection against retroviral infection as a result of anti-Gal NAb recognizing the Gal alpha 1-3Gal beta 1-4GlcNAc-R epitope expressed on the retrovival envelope, and triggering the classical pathway-mediated virolysis (Welsh et al., 1998 and Rother et al., 1995).

I. 1.3.2. Anti-tumor effects

The idea that NAb plays a role in tumor defense can be inferred from the increased binding of NAb to oncogenic ras-transformed cells. The widely accepted current theory for neoplastic transformation of a cell is that a tumor develops from a gradual accumulation of several genetic defects, finally ending with increasing aggression and uncontrolled growth properties (Fearon et al., 1990). Early in oncogenesis, altered gene products and oncogenes can be expressed on the surface of the transformed cells, which are considered crucial in initiating the first line of defense against neoplasia. The ras oncogene is the gene found in many tumors as well as oncogenic myc, src and p53. Tough and Chow (1991) demonstrated that the introduction of V-H-ras into murine cells generated transformed cells which exhibited increased binding of NAb. This suggested that NAb has the capacity to monitor incipient changes in cell transformation.

Several studies showed the importance of NAb in tumor resistance and the relationship between tumor resistance and NAb binding. In BALB/Mo mice, the resistance to tumor induced by the Moloney murine sarcoma virus was associated with the level of spontanously existing NAb which reacted with this sarcoma virus, in assays before virus exposure (Zanovello et al., 1984 and D'Andrea et al., 1981). In our laboratory, numerous studies have been performed to investigate the role of NAb in tumor resistance. Chow et al. (1981) revealed a direct relationship between the serum NAb level and tumor resistance. Comparing the relationship between tumorigenicity and NAb levels in old and young mice, old mice with higher levels of

anti-tumor NAb than young mice showed lower tumor frequencies after subcutaneous (s.c.) injection of NK-sensitive or NK-resistant syngeneic lymphomas (Chow et al., 1981). Xid-bearing B cell deficient mice which have lower serum NAb levels exhibited a higher tumor incidence than normal control mice after inoculation with a syngeneic radiation-induced T cell leukemia (Bennet and Chow 1991 and Chow and Bennet, 1989). Passive intravenous injection of NAb from syngeneic mice into the xid deficient strain significantly reduced their susceptibility to a subsequent tumor challenge (Chow et al., 1995). Moreover, the capacity of the tumor NAb binding, exhibited a positive correlation with the anti-tumor effect. Chow et al. (1986 and 1997) inoculated the NK-resistant T lymphoma cell line L5178Y-F9 subcutaneously into syngeneic mice, then retrieved the tumors which grew out in vivo. These cells selected through growth in vivo exhibited increased tumorigenicity and decreased NAb binding compared to the starting cell line (Zhang and Chow 1997 and Brown et al., 1986). Similar results were obtained using the ras-transformed 10T1/2 fibroblast clone I3T2.1, a variant which was selected from a threshold s.c. inoculum of high-NAb-binding ras transformants in syngeneic C3H/HeN mice (Tough et al., 1995). The tumor cells selected in vivo also showed high liver metastasis potential and reduced sensitivities to complement-mediated NAb lysis in vitro (Chow et al., 1984 and 1983) and to hypotonic lysis as well (Brown et al., 1986 and Chow et al., 1984 and 1983). On the other hand, when parental L5178Y-F9 were selected for high natural IgM antibody binding through fluorescence-activated cell sorting (FACS), the selected variants showed high-NAb binding properties and low tumorigenicity (Tough and

Chow, 1988). All of the data from the murine systems strongly suggested that NAb plays an essential role in tumor resistance.

Although there are limitations with the human system regarding in vivo studies, abundant evidence of the anti-tumor activity of NAb has been obtained by using different techniques. Hybridoma technology was used by Bohn et al., (1994) to detect whether the B cells existing in healthy people have the capacity to produce NAb against tumor cells. The antibodies produced by hybridomas generated from B cells derived from tumor-free individuals were screened for reactivity to a large variety of tumor cells. The results demonstrated that the generated NAb-producing hybridomas can produce NAb that indeed react to antigens on tumor cells. The role of NAb against human neuroblastoma (NB) is well documented. NB is the most common extracranial solid neoplasm in infancy, which is characterized by a high spontaneous regression rate (Brodeur et al., 1986 and Beckwith 1963). It was found that natural IgM antibody in gestational sera had cytotoxic activity for human NB cells (Bolande 1990). A few years later, Ollert et al., (1996a) screened 94 normal human sera and showed about one-third of the analysed sera contained cytotoxic natural IgM antibody which could specifically kill 40%-95% of the NB cells. But sera from NB patients with active disease revealed the absence of the anti-IgM antibody. These observations suggested a role of anti-NB NAb in the defense against human NB and that the presence of NAb in the sera of patients may contribute to the phenomenon of spontaneous regression.

The relationship between NAb and tumor resistance has also been revealed by the comparative analysis of serum levels of anti-ganglioside NAb in patients with soft tissue sarcoma and normal people. The anti-tumor function of NAb against MUC1 has been revealed recently. MUC1 is a heavily glycosylated large glycoprotein, which showed increasing expression in a variety of adenocarcinomas such as carcinoma of the lung, ovary and colon (Hiraga 1998 and Ho 1993). An enzyme-linked immunosorbent assay (ELISA) was used to detect the presence of anti-MUC1 NAb in 60 healthy individuals and 30 patients with lung carcinoma. The anti-MUC1 NAb levels in the patients were significantly lower than in normal people. Interestingly, the degree of increase in NAb concentrations strongly correlated with a good outcome of the patients (Hirasawa et al., 2000). Collectively, the evidence suggests that the presence of NAb in the circulation provides an essential barrier in anti-tumor defense.

I. 1.3.3 Cell regulation by NAb

One remarkable characteristic of NAb is its polyreactivity in the sense that it can not only bind to different antigens (Avrameas 1991), but can also recognize a number of self molecules and antibodies in the same person. The immunoregulatory effects of NAb are largely dependent on their ability to interact with soluble and membrane-associated self molecules, as well as idiotypes of other complementary antibodies. The proportion of NAb that is specific for a number of functional molecules of the immune system is believed to be involved in its immunoregulatory effects. The interactions between these NAbs and their target molecules is thought to be directly

relevant to the immunoregulatory function of NAb. In certain autoantibody-mediated autoimmune diseases, the ability of NAb to bind the idiotype of autoantibodies ensures NAb neutralization of the autoantibodies. This may down-regulate the synthesis of autoantibodies by B cells which express the relevant idiotypes (Vassilev 1999 and Dietrich and Kazatchkine, 1990). About 80 percent of patients with the autoimmune disease myasthenia gravis (MG) showed an increased level of autoantibodies against the nicotinic acetylcholine receptor (AchR). The naturally produced anti-idiotypic antibody against disease-associated IgG autoantibodies present in pooled human IgM from normal individuals suppressed anti-thyroglobulin and anti-DNA autoantibody activity in vitro (Hurez 1997). In an vivo experiment, the SCID mouse models of MG were treated with intravenous immunoglobulin IgM (IVIgM) or an equimolar amount of human serum albumin as a control. IVIgM exerted an immunoregulatory effect by significantly decreasing the level of production of anti-AchR autoantibody production, which alleviated the disease (Vassilev 1999). Natural anti-endothelial cell antibody (AECA) present in healthy subjects showed the ability to inhibit endothelial cell secretion of pre-inflammatory factors including thromboxane A2 endothelin and metalloproteinase-9, and inhibited the endothelial cell pro-inflammatory response to TNF-α. Thus, AECA contributed anti-inflammatory effects through regulation of endothelial cells (Ronda et al., 1999).

In previous studies by our lab, murine L5178Y-F9 lymphoma cells were treated with TPA to generate variants and then selected for high NAb binding by cell sorting (Tough and Chow 1988). Consistent with the increased NAb binding, these cells

exhibited an elevated expression of the IL-2R α chain, a characteristic T cell activation marker (Chow et al., 1999). Additionally CD45RA, a marker increased during the first 1 to 2 days of cellular activation (Deans et al., 1992) was also over-expressed on the model cells. Together with the knowledge that TPA treatment rapidly increased CD45RA expression in human T cells (Yamada et al., 1990), the data suggests that the selected cells may exhibit the phenotype of a transitory early stage of T cell activation. Further support for the idea that NAb regulates activated cells came from the observation that the murine C3H 10T1/2 fibroblast which overexpressed PKC, another marker for activated cells introduced by transfection, showed an increase in NAb binding (Wang and Chow 1999). As well, v-H-ras transformed 10T1/2 fibroblasts, which are also considered to be activated cells, exhibited increased NAb binding. Moreover, purified NAb reduced the growth of ras-transformed 10T1/2 cells in vitro with a decreased total cell number and a high proportion of cells in the G0/G1 phase (Wang and Chow, 2000). Thus, it appeared most likely that NAb acted on early activated cells and regulated their cell growth.

The immunoregulatory activity of NAb was also exhibited in its ability to inhibit the autologous mixed lymphocyte reaction (MLR). Pretreatment of phytohemagglutinin(PHA)-activated T lymphocytes with normal human serum resulted in a significant inhibition of the MLR. The inhibition activity was depended on the IgG fraction of the normal serum and mediated by the $F(ab')_2$ portion of IgG (Wolf-Levin et al., 1993). These findings are consistent with the idea that natural IgG and IgM antibodies have the capacity to regulate the immune system.

I.1.4 Mechanisms involved in the biological functions of NAb

Several mechanisms have been proposed for different functions of NAb. The anti-infection properties of NAb have been shown to be directly mediated by neutralization of pathogens in the circulation and indirectly by complement-mediated lysis or formation of Ag-Ab complexes to facilitate efficient elimination in the spleen. Natural antibodies against pneumococcus (PC) in normal serum can protect xid mice from PC infection through neutralization of the intravenously injected pneumococcus (Briles et al., 1981). Results from Reid et al., (1997) suggested that NAb mediated neutralization and clearance of LPS were critical in the protection of antibody-deficient mice from endotoxin shock after the mice were reconstituted with pooled sera from normal mice. In some cases, NAb can enhance its antibacterial and antiviral defense by activation of the complement cascade and formation of the lytic complex (C5-C9). For example, in the defense against infection by *Neisseria gonorrhoeae*, *N. meningitides* and *Salmonella typhimurium*, elimination of these pathogens depended mainly on NAb triggering the formation of the lytic complex (reviewed by Figueroa et al., 1991). In retrovirus infections the direct binding of the complement component C1q to the viral envelope protein P15E lead to classical pathway-mediated virolysis in human serum. If the NAb specific to the carbohydrate epitope on the retroviral envelope has been depleted or blocked, the retrovirus in human serum was not killed (Rother et al., 1995). NAb and antigen complex formation contributes to anti-infection defense by facilitating Ag efficiently being trapped in lymphoid organs, such

as the spleen and being eliminated there. This prevents further hematogenic spread (Baumgarth et al., 2000).

Although a number of observations revealed the involvement of NAb as the first line of defense against tumors, the knowledge of the mechanisms of NAb-mediated elimination of the tumor cells in vivo is still limited. The proposed mechanisms may be complement-dependent cytolysis, antibody-dependent cell mediated cytotoxicity (ADCC) mediated by cytotoxic T cells, NK cells or macrophages and a recently discussed mechanism, NAb-induced apoptosis of tumor cells. Some of these mechanisms have been intensively studied through in vitro experiments. The binding of natural IgM antibodies to the neuroblastoma cell surface has been found to sensitize the tumor cells to the lytic action of complement (Bolande et al., 1990a and b). This NAb mediated complement-dependent neuroblastoma lysis was further confirmed by studies in nude rats bearing human neuroblastoma. The uptake of IgM and massive perivascular complement activation simultaneously happened in the tumors 24 hours after a single intravenous injection of natural IgM antibodies. Repeated injection caused significant growth arrest (Ollert et al., 1997a and b). A very interesting phenomenon was observed by the Bohn group when they exposed human tumors versus normal human T lymphocytes to NAb. Both tumor cells and polyclonally stimulated $CD3^+$ T cells bound NAb, but the unstimulated T lymphocytes did not. In addition, MHC class-I expression was enhanced on the surface of the NAb binding tumor cells. The increased MHC class-I expression makes the tumor cells susceptible to T-cell-mediated tumor cell killing (Bohn et al., 1994).

Very recently several pieces of evidence showed that the induction of apoptosis by NAb may represent a molecular mechanism involved in anti-tumor defense, especially on human neuroblastoma. Natural IgM antibodies from normal individuals which specifically recognized a m.w. 260,000 antigen (NB-p260) on the surface of human neuroblastoma cells, were shown to induce apoptosis of different NB cell lines. After treatment with NAb, the human NB tumor cells showed morphological and biochemical changes typical of apoptosis including membrane blebbing, chromatin condensation and DNA and nuclear fragmation. Both the binding of NAb to NB cells and the induction of apoptosis could be abolished when NB cells were treated with mouse IgG against NB-P260, an identified target of natural IgM antibody (David et al., 2001 and 1999).

The mechanisms related to the immunoregulatory effects of NAb are believed to be largely dependent on V region-mediated idiotypic-anti-idiotypic network interactions (Ochsenbein et al., 2000). A subset of anti-idiotypic antibodies present in NAb recognize functional membrane molecules on immunocompetent cells, and this is the basis of their immunoregulatory properties. At least three mechanisms are proposed for the anti-idiotypic antibody mediated immunoregulation.

First, they can bind to self-reactive autoantibodies by forming an idiotype-anti-idiotype dimer, facilitating the clearance of autoantibodies through Fc receptors in the reticuloendothelial system. This prevents autoantibodies from interacting with self-antigens and the subsequent harmful immune response against self-tissue (Abdou et al., 1981).

Second, NAb may exert its immunoregulatory effects through FcRs on several immunocompetent cells. For instance, the idiotype-anti-idiotype dimers csn bind to B cell FcRs or cross-link BCRs with FcRs on B cells. The B cell function would be inhibited and this would result in a decrease in autoantibody production (reviewed by Anderson et al., 1989). FcRs are heterogeneous groups of receptors which generally can be grouped as stimulatory and inhibitory receptors (Dearon, 1997). Both activating and inhibitory signals can be transduced through Fc following ligation. Crosslinking of stimulating FcRs can initiate a cascade of signaling events including tyrosine phosphorylation of Src (Agarwal et al., 1993) and Lck (Azzoni et al., 1992) which finally mediate cell activation. However, co-ligating of inhibitory FcRs can transduce an inhibitory signal by recruiting SHIP to the immunoreceptor tyrosine-based inhibitory motif (ITIM) (Muta et al., 1994).

Third, anti-idiotypic antibodies bind to T cells which are pivotal in immune responses and thus regulate T cell function. The binding between NAb and its target molecules can exert immunoregulatory effects by enhancing or suppressing the immune responses related to the particular idiotype (reviewed by Varela and Coutinho, 1989). For instance, NAb specific for the complementarity determining region 1(CDR1) public idiotype of the αβ TCR was present in all human sera tested (Marchalonis et al., 1994 and 1992). The immunoregulatory function of TCR-specific NAb has been found by binding this NAb to an ovalbumin-specific T cell line which caused subsequent inhibition of Ag-driven IL-2 production. (Robey et al., 2002). Another component of NAb, IgM has also been demonstrated to exert an

immunoregulatory function through idiotype/anti-idiotype interactions. Pooled normal human IgM can suppress the activity of anti-thyroglubulin and anti-DNA autoantibodies purified from autoimmune patients. In addition, pooled normal human IgM showed the ability to protect F1 (Kewis × Brown-Norway) rats against experimental autoimmune uveitis induced by immunization with the soluble retinal S antigen (Hurez et al., 1997). Moreover, it has long been noted that NAb may preferentially bind to carbohydrate determinants (Sela et al., 1975). In humans, about 1% of circulating IgG NAb has a narrow specificity against the carbohydrate epitopy Gal-α1-3Galβ1-4GlcNAc-R (α-galactosyl epitope). Unlike non-primate mammals with large amounts of the α-galactosyl epitope, humans generally do not express this epitope or express it at a very low level (Galili et al., 1993). Anti-Band 3 IgG NAb has been found to initiate antibody-dependent phagocytic removal of senescent cells from the circulation (Lutz et al., 1987). Additionally, this function was dependent on the binding of anti-band 3 NAb to poly-N-acetyllactosaminyl saccharide chains on band 3 protein of erythrocyte membranes (Beppu et al., 1996). NAb has also been shown to react with a network of idiotypic determinants of CD4, CD5, MHC class-I and the RGD motif of integrins and a number of cytokines by using pooled human IgG (Hurez et al., 1993; Vassilev et al., 1993 and Atlas et al., 1993), which will be reviewed in section I.2.

I.1.5. CD45 – a novel possible target involved in NAb modulation

A long term study has been done in our lab on the regulatory function of NAb on tumor growth and the possible target molecules involved. Early studies showed that low NAb binding was associated with high tumorigenicity (Chow et al., 1981). The $LYNAb^+$ murine T lymphoma cells were repeatedly selected for high NAb binding through cell sorting following tumor promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA) treatment of the parental L5178Y-F9 cells to generate variants (Tough and Chow 1988). The selected high NAb binding phenotype exhibited an inverse correlation with tumor incidence in a threshold s.c. tumor inoculation assay of tumorigenicity (Zhang and Chow 1997). Comparative analysis of surface molecule expression revealed that the selected high NAb-binding cells exhibited increased binding of mAb against CD45RA a high molecular weight isoform of the leukocyte common antigen (LCA), CD45 which is important for TCR signaling (reviewed in Alexander, 2000). Moreover, flow cytometry analysis demonstrated that the patterns of CD45 isoform expression were different between the parental L5178Y-F9, which was $CD45^{high}RA^{low}RB^{high}RC^{low}$, and the high NAb-binding, low tumorigenic $LYNAb^+$ which was $CD45^{low}RA^{high}RB^{-}RC^{high}$. Together with the observation that tumor progression in vivo was associated with reductions in binding of anti-CD45 mAb and NAb, this provided the first evidence that NAb may bind to a CD45RA epitope. Subsequently, experiments provided direct evidence of NAb binding to CD45. A comparison experiment of NAb binding to CD45 negative YAC-N1 cells, CD45RABC transfected CD45 negative cells YAC-33, and wild-type YAC-WT cells

showed NAb binding was higher in YAC-WT and YAC-33 cells versus the $CD45^-$ YAC-N1 cells. A correlation between NAb binding and high molecular weight CD45 expression observed through immunoblotting suggested NAb binding to high MW CD45. Moreover, purified natural IgG inhibited anti-CD45RA binding by 29%, while only slightly inhibiting anti-pan CD45 and anti-CD45RB by 2.8% and 5.8% respectively. In addition, mixtures of purified CD45RB/RO or CD45RABC with natural IgG, competitively inhibited the natural IgG binding to high NAb-binding cells (Zhang and Chow 2000). More specifically, NAb acid-eluted from $LYNAb^+$ cells incubated in normal syngeneic serum, demonstrated significant direct binding to purified CD45RABC and less to CD45RO/RB in an ELISA assay (Zhang et al., 2000). Collectively, all these data strongly argued that high m.w. CD45 isoforms are likely targets of NAb.

I.2 Intravenous immunoglobulin (IVIg)

I.2.1 IVIg for therapeutic use

Intravenous immunoglobulin (IVIg) is a blood product prepared from the serum of between 1000 and 30,000 normal donors per batch and is primarily used as gamma globulin for therapeutic intravenous injection (Mouthon et al., 1996; Dwyer et al., 1992; Berkman et al, 1990 and Eibl et al., 1989). IVIg was first introduced in the 1950's as a replacement for patients with congenital antibody deficiencies. In addition, it has been over 20 years since IVIg was first shown to be effective in the

treatment of patients with autoimmune thrombocytopenic purpura in 1981 (Imbach et al., 1981b). Over the past two decades, IVIg has been established to be an effective treatment of a number of autoimmune and systemic inflammatory disorders. The beneficial effects of IVIg against a wide spectrum of autoimmune diseases and inflammatory disorders were shown to be dependent on the variety of biological functions associated with the components present in IVIg.

All commercial preparations of immune globulin contain primarily intact IgG molecules with a distribution of IgG subclasses similar to that of normal serum, and a small concentration of dimers (Lemm et al., 2002). The half-life of infused IVIg in immunocompetent persons is three weeks in the case of IgG1, IgG2 and IgG4, while in terms of IgG3 the half-life is only one week (Mouthon et al., 1996). IVIg also contains various minor components. These include traces of IgA and trace amounts of soluble material bearing CD4, CD8, HLA (human leukocyte antigen)-class I and HLA-class II. In addition, antibodies against lymphocyte surface determinants, such as CD4, CD5 and HLA molecules have been detected in IVIg (Lam et al., 1993 and Blasczyk et al., 1993). It has been demonstrated that IVIg contains low titers of anti-A, anti-B, anti-C and anti-E blood group antibodies, but no reports on these antibodies are associated with hemolysis (Gaines et al., 2000). Several groups also showed that IVIg contains antibodies against cytokines including IL-1α, IL-6, IL-8, granulocyte macrophage colony-stimulating factors (GM-CSF) and INF-α (Bendtzen 1998; Andersson et al., 1996; Amran et al., 1994 and Shimozato et al., 1991).

Moreover, different IVIg preparations are varied in sugar content, sodium content and pH, which determine the osmolality, stability and the aggregation ability of individual IVIg preparations. Various sugars were added to IVIg preparations as a stabilizer to prevent aggregation. Sodium content was the major contributor to osmolality and varies widely in the range of concentration from trace amounts to 0.9% from different preparations. Since high doses of IVIg (0.4-2gm/kg) are needed to achieve a clinical benefit, all components present in IVIg may effect the biological and therapeutic action of IVIg to various extents.

I.2.2 IVIg and autoimmune diseases

After the Imbach (1981 a and b) group made an inspiring observation that the infusion of IVIg in children with immune thrombocytopenia (ITP) produced a marked improvement in platelet counts, IVIg has been widely used in the treatment of various autoimmune and inflammatory diseases. Increasing clinical and experimental evidence suggests that IVIg preparations provide a safe and efficacious treatment for over 20 different autoimmune, systemic inflammatory or immune-mediated neurological disorders (Otten et al., 1996). For example, IVIg is currently a mainstay for such disorders such as Guillain-Barre syndrome, multiple scerlosis (MS), myasthenia gravis (MG), chronic inflammatory demyelinating polyneuropathy (CIDP) and graft-versus-host disease (GVHD). ITP was the first autoimmune disease in which IVIg was used as an effective treatment modality. It is well known as an immune-mediated bleeding disorder characterized by prematurely destroyed platelets

which were opsonized by increased levels of abnormal autoantibodies (Semple et al., 1995). As for the pathogenic mechanisms of ITP, accumulated evidence showed that T cells played a major role in the onset of ITP. In ITP patients, a consistent finding was increased numbers of $CD3^+$ HLA^+ T lymphocytes, which may suggest the abnormal activation of T cells in vivo (Bouchonnet et al., 1994). Additionally, platelet-induced peripheral blood mononuclear cell proliferation has been seen in ITP patients (Semple et al., 1995 and 1991). Moreover, T lymphocytes of patients with ITP secreted significantly high levels of IL-2 and other proinflammatory cytokines. These data suggested that the pathogenesis of ITP involved abnormal T cell activation and secretion of proinflammatory cytokines that acted as a primary stimulus for the development of anti-platelet autoantibody production (Semple et al., 1996 and 1991).

Kawasaki disease (KD) is another autoimmune disease in which the efficacy of IVIg has been proven in clinical trials. KD is an acute febrile illness that affects infants and young children. This disease carries a characteristic immunoregulatory T cell imbalance (Leung et al., 1982). Patients with KD have an obvious T cell lymphocytopenia with much more pronounced decrease of helper T cells than suppressor T cells. However, among the reduced total lymphocyte population, the proportion of $CD4^+$ helper T cells that express the HLA-DR activation markers was increased (Leung et al., 1987). In addition, T cells from KD patients exhibited an increased capacity to produce cytokines that drove B cell differentiation and produced abnormal antibodies against self-vascular endothelial cells (Leung et al., 1989). As a consequence, KD patients all developed severe systemic vasculitis. Infusion of high-

dose IVIg in the early stage of disease onset dramatically prevented the occurrence of vasculitis, especially coronary disease (Newburger et al., 1986 and Furusho et al., 1984).

Experimental autoimmune encephalomyelitis (EAE) is traditionally considered to be a T-cell-mediated inflammatory disease of the central nervous system (CNS) and serves as an animal model for some aspects of multiple sclerosis (Gold et al., 2000 and Zamvil et al., 1990). When IVIg was administrated through intra-peritoneal injection, the protective ability of IVIg against EAE was found to be associated with T cell unresponsiveness to the immunizing antigens (Pashov et al., 1998).

It is widely accepted that the pathogenics of autoimmune diseases are very complicated. It may involve various aspects of the immune system not just the T cells. So, the proposed mechanisms that contribute to IVIg effects on autoimmune and inflammatory diseases may also involve more aspects of the immune system. But the successful IVIg treatment of several T-cell-mediated diseases in both human and animal models indicated that IVIg had the potential to reduce the abnormal aspects of the immune system caused by the immune-pivotal T cells.

I.2.3. Multiple mechanisms of IVIg action

IVIg has been shown to be an effective and safe treatment for a variety of autoimmune and systemic inflammatory diseases. IVIg may exert its therapeutic effects at several levels of the immune network based on in vivo or in vitro studies on certain diseases in which IVIg is definitely or likely effective. Some of the

mechanisms have been well explored in vivo and in vitro, but some still remain hypothetical.

Fc receptor blockade

The blockade of Fc receptors on macrophages was considered to underlie the mechanism of IVIg in ITP. The increasing platelet count was thought caused by Fc blockade, which prevented removal of antibody-sensitized platelets by the reticuloendothelial system. This led to prolonged survival of autoantibody-coated platelets (reviewed by Bussel 1989 and 1986). Several pieces of evidence support this interpretation. Treatment of ITP patients with the purified Fc γ fragment prepared from Cohn fraction II γ-globulin showed a similar effect to that of IVIg on the improvement of the platelet count (Debré et al., 1993). Infused red cells coated with anti-RhD antibodies survived longer than usual if they were infused after the administration of IVIg (Fehr et al., 1982). In vivo, IVIg can decrease the clearance of anti-D coated autologous erythrocytes (Kimberly et al., 1984). The mechanism of IVIg blockade of Fc receptors on phagocytic cells better explained the very quick changes in platelet count after administration of IVIg, but it could not provide a rationale for the long-term benefits produced by IVIg in several diseases (review in Vassilev and Kazatchkine, 1997 and Mouthon et al., 1996).

Modulation of complement uptake on target cells

IVIg also exerted its therapeutic function through interference with complement uptake on the target cells. The early studies using the guinea pig model of Forssman shock showed that high-dose IVIg prevented death through inhibition of complement

fragment C3 and C4 uptake and activation of endothelial cells. The postulated mechanism involves high levels of IgG in the IVIg hindering the binding of active C3 and C4 to the target cell (Basta et al., 1987). In patients with dermatomyositis which is a complement-mediated endomysial vasculitis, IVIg was thought to act through inhibition of complement activation at the level of C3b. The muscle biopsy specimens from those patients who had been treated with IVIg showed a disappearance of C3b. This limited the available C3b for further incorporation into C5 to form C5 convertase, and prevented the formation of membrane attack complex (MAC) on endomysial capillaries. This prevented the destruction of endothelial cells, muscle ischemia, inflammation and ultimately muscle atrophy (Basta et al. 1996 and Dalakas et al., 1993). In addition, IVIg could accelerate the decay of the C3b into an inactive form and reduce the pathological effects caused by abnormally activated complement (Welch et al., 1995). Again, similar to the function mediated by FcR blockade, the mechanism of interfering with complement uptake was largely dependent on the life span of IVIg, but it could not explain the long-term effects produced by IVIg in several inflammatory and autoimmune diseases.

Modulation of cytokines

Accumulated reports from patients receiving IVIg and in vitro experiments have shown that IVIg can modulate cytokine production and/or release. In an in vitro study, IVIg exhibited the capability to reduce IL-1 production in macrophages (Iwata et al., 1987), causing the dose-dependent decrease of IL-6 production by monocytes, but not affecting TNF-α production (Andersson et al., 1990). Later, other groups observed a

discrepancy in the effect of IVIg on TNF-α. A suppressive effect on TNF-α production was induced by IVIg on lipopolysaccharide-stimulated rabbit macrophages (Shimozato et al., 1991). Assessment of cytokine production at the single cell level in the presence of IVIg showed that significantly less IL-2, IL-10, IFN-γ and TNF-β were produced by anti-CD3 stimulated T cells (Andersson et al., 1996). In addition, IVIg decreased the production of IL-2 and IL-4 which subsequently inhibited mitogen and antigen stimulated T cell proliferation (Amran et al., 1994). IVIg present in the culture of purified human monocytes showed selectively increased gene transcription and extracellular release of the IL-1 receptor antogonist (IL-1ra) (Ruiz-de-Souza et al., 1995 and Poutsiakaet al. 1991). Data from in vitro studies suggested that IVIg behaved as an anti-inflammatory agent as it did not induce the production of the pro-inflammatory cytokines IL-1 and TNF-α, instead production of IL-1ra, the natural antagonist of IL-1 was increased. Compared to in vitro studies, in vivo studies were much more difficult to interpret as the effects of multiple cytokines might be reduced or inhibited by IVIg and the different cytokines might react among each other or interact with cells producing cytokines and antagonists. However, indirect evidence from patients receiving IVIg treatment showed that IVIg exerted different effects depending on the status of the immune system of the patients (Mouthon et al., 1996). In hypogammaglobulinaemic patients, a rapid and significant rise in TNF-α, IL-6 and IL-8 in plasma was observed after infusion of IVIg (Aukrust et al., 1994). While in HIV-infected patients who had high circulating levels of TNF-α, IVIg administration resulted in a decrease in both plasma TNF-α levels and the production of TNF in

LPS-stimulated PBMC supernatants (Aukrust et al., 1995). IVIg could decrease the abnormally elevated TNF-α and IL-6 levels in patients suffering with KS (Leung et al. 1996 and 1992).

Restoration of the idiotype network

In addition, other nonexclusive mechanisms have been postulated to contribute to the broad function of IVIg, such as anti-idiotypic antibodies in IVIg restoration of the idiotype/anti-idiotype network. Being a preparation derived from a large pool of normal human donors, the IgG in IVIg may contain antibodies with a wide range of idiotypic and anti-idiotypic specificities (Roux et al., 1990). The first evidence regarding the presence of anti-idiotypic antibodies in IVIg was demonstrated by the fall of the anti-factor VIII titer in a patient with anti-factor VIII autoimmune disease following IVIg treatment (Sultan et al., 1984). Further study showed purified $F(ab')_2$ fragments could block antibody/antigen binding and interrupted the function of the pathogenic antibody against factor VIII (Sultan et al., 1987). IVIg preparations have been shown to contain antibodies against the Arg-Gly-Asp (RGD) sequence of integrins and the $F(ab')_2$ fragments specific for RGD can be purified from IVIg by chromatography. Anti-RGD $F(ab')_2$ antibody can block the aggregation of platelets induced by adenosine diphosphate, the binding of activated B cells to fibronectin and the adhesion of platelets to Von Willebrand factor. This antibody directed to the RGD motif was proposed to exert an immunoregulatory effect and contribute to anti-inflammatory therapy (Vassilev et al., 1999). Studies by Kazatchkin's group found that $F(ab')_2$ fragments prepared from IVIg could neutralize or bind to more

autoantibodies, such as anti-thyroglobulin, anti-DNA, anti-GM_1 ganglioside, anti-AhR, the nicotinic acetylcholine receptor (Kaveri et al., 1997; Kazatchkine et al., 1994 and Dietrich et al., 1990). This idiotypic-anti-idiotypic interaction has been found to be essential for IVIg regulatory effects. Taken together, the data provide evidence for several mechanisms proposed to contribute to the various actions of IVIg.

I.2.4. IVIg modulation of T cell function

Accumulating observations regarding the success of IVIg in treating a number of T cell-mediated autoimmune and inflammatory diseases has provided strong evidence that IVIg was likely involved in the regulation of T cell function. The studies of several groups demonstrated the comprehensive effects of IVIg on the immune system-pivotal cell, the T lymphocyte.

In commercial IVIg preparations there are a number of minor components including solubilized lymphocyte surface membrane molecules and specific autoantibodies to lymphocyte surface molecules. These components might contribute to IVIg effects by exerting important immunoregulatory activities. Several studies has demonstrated that commercial IVIg contains solubilized CD4, CD8, HLA class I and HLA class II molecules (Lam et al., 1993 and Blasczyk et al., 1993). These molecules could exert a therapeutic effect by blocking the interaction between HLA class II and CD4 or between HLA class I and CD8, resulting in a competitive immunosuppression of T cell mediated responses. In addition, the presence of autoantibodies against CD5,

CD4 and HLA determinants were reported in IVIg preparations and revealed their important modulatory effects. The antibodies recognizing a conserved region of the α_1 helix of HLA class I molecules, could be isolated by affinity chromatography, and exhibited the capacity to inhibit class I restricted $CD8^+$-mediated cytotoxicity of influenza virus specific T cells (Kaveri et al., 1996). The presence of anti-CD4 antibodies could be isolated from IVIg bound to $CD4^+$ T cells by affinity chromatography. Furthermore the binding of anti-CD4 antibodies present in IVIg could inhibited lymphocyte proliferation in a mixed lymphocyte culture (Hurez et al., 1994). In addition, Vassilev reported that IVIg contained antibodies against CD5 molecules and the interaction of the antibodies with CD5 on the majority of mature T and a subpopulation of B cells, might contribute to the therapeutic effects by modulating T cell function through CD5 and regulation the expression of B cell subsets expressing CD5 (Vassilev et al., 1993). Several groups have demonstrated that IVIg had anti-proliferative effects on T cells stimulated with mitogens, anti-CD3 antibodies, tetanus toxoid antigen, and phorbol esters (Amran et al., 1994; Schaik et al., 1992 and Delfraissy et al., 1985). These observations suggested that IVIg may exert some of its homeostatic functions through interference with the expression of genes and/or functions of the gene products involved in cell growth and death of lymphocytes. The inhibitory effect acts in a dose-dependent fashion (Amran et al., 1994) and can be reversed by removal of IVIg through washing (Leung et al, 1995). Moreover, Ameran (1994) also showed that this suppression was reversible by exogenous IL-2. Thus, the inhibitory effect was considered as the result of IVIg

interference with cytokine-mediated T cell proliferation. Another study demonstrated that the anti-staphylococcal toxin antibodies present in IVIg may be the major contributor to the inhibitory effect of IVIg on antigen-mediated proliferation of T cells (Takei et al., 1993).

Recent studies provided evidences to demonstrate that IVIg has the ability to modulate apoptosis and the cell cycle of T cells. Prasad et al. (1998) found that IVIg could induce apoptosis in human leukemic lymphocytes as well as normal tonsillar B cells. This effect was at least partially dependent on anti-CD95 antibodies present within the IVIg preparations. In contrast, IVIg showed an inhibitory effect on T-cell-mediated, Fas-induced keratinocyte apoptosis in atopic dermatitis (Viard et al., 1998). Later, another study revealed that IVIg suppressed the proliferation of antigen-specific T cells without inducing apoptosis and affected the mRNA expression of bcl-2, an anti-apoptotic molecule (Aktas et al., 2001). In addition, IVIg exhibited a dual effect on peripheral mononuclear cells (PBMC). IVIg induced apoptosis on activated Ki-67, a proliferation marker, and CD95-positive PBMC, while the apoptotic incidence was low in small, non-activated cells that expressed P21/WAF-1, a molecule suggestive of G1 arrest. The long-term survival was associated with upregulation of Bcl-2 expression after the cell was exposured to IVIg (Ekberg et al., 2001). Furthermore, addition of IVIg to the culture lead to an increase in cells accumulating in the G0/G1 phase (Schaik et al., 1992). Taken together, these reports suggested that IVIg could cause cells to arrest at the G0/G1 phase through upregulation of P21/WAF-1 expression inhibiting cells from entering S-phase. When

exposed to IVIg, the final fate for individual cells of the immune system may depend on their current stage in the cell cycle.

I.3 CD45

I.3.1 Structure and isoforms

CD45 is the leukocyte common antigen previously termed L-CA, B-220, T200 and LY-5. It is expressed on all hematopoietic cells except for mature erythrocytes. CD45 is one of the most abundant cell surface glycoproteins occupying up to 10% of the lymphocyte surface (Mattew et al., 1989). There are multiple CD45 isoforms due to alternative splicing of three variably expressed exons 4, 5 and 6 (designated A, B and C) in the extracellular domain. Different isoforms of CD45 are expressed in cell type-specific patterns on functional subpopulation of lymphocytes. The largest isoform RABC includes all three exons, while the smallest one RO lacks all three exons (Trowbridge et al., 1994). The three exons encode multiple sites of O-linked glycosylation that can be variably modified by sialic acid. The different isoforms vary in size, shape and negative charge of the extracellular domain with molecular weights from 180k to 220k. The remaining extracellular domain contains a cysteine-rich region including three fibronectin type III motifs (Matthew et al., 1989). The large cytoplasmic domain has 700 amino acids and contains two tandemly joined phosphotyrosine phosphatase (PTPase) domains D1 and D2. Only the membrane-proximal domain D1 has phosphatase activity (Desai et al., 1994). The function of D2

is unclear. It may relate to structural stability and optimal functional activity of CD45 (Felbery et al., 2000 and 1998).

CD45 has been well known as a heavily glycosylated transmembrane protein with tyrosine phosphatase activity, but only recently, the carbohydrate moiety of CD45 isoforms has attracted considerable interest because it has been shown to be important for special cellular interactions and functions (Uemura et al., 1996; Perillo et al., 1995 and Powell et al., 1993). The heavy glycosylation of CD45 results from N-glycosylation throughout the extracellular domain and O-glycosylation of the products of the variably expressed exons. The tetra- and tri-antennary complex-type sugar chains and α-2, 6-linked sialic acid residues are major N-glycoconjugates of CD45 (Sato et al., 1993). The O-glycoconjugated chains contain mainly core 1 and 2 oligosaccharides and the distribution of the sialylated core 2 oligosaccharides is different among CD45 isoforms. CD45RA has twice as many O-linked sugar chains with core 2 structure as CD45RO (Furukawa et al., 1998). In addition, the glycosylation pattern of CD45 was not only due to the different usage of exons 4-6, but also related to the cell type, development stage and activation state of cells, which may suggest the differential distribution of the carbohydrate could be important in specific cell functions (Thomas et al., 1989). For example, the α-2, 6-linked sialic acid residues of N-linked sugars on CD45 chains on T cells were recognized by CD22, a sialic acid-binding lectin expressed on B cells, and may be involved in cell adhesion (Powell et al., 1993, and Sgroi et al., 1993). The recent study by Ostergaard's group showed that the glycosylation pattern of the CD45 extracellular

domain changes as T cells mature (Baldwin et al., 2001). The changes in the cell surface carbohydrate on CD45 result in changes in the association between glucosidase II (GII), mannose-binding protein (MBP) and CD45, which may affect the development of T cells via the binding of mannose on CD45 on thymocytes to lectins on stromal cells. Moreover, the finding that CD45 modulated galectin-1-induced T cell apopotosis dependent on the expression of core 2 structures on O-glycans on CD45 (Nguyen et al., 2001) and that high m.w CD45 isoforms have more core 2 structures on O-glycans than low m.w CD45RO (Furukawa et al., 1998), suggest different expression of CD45 isoforms might affect the susceptibility of cells to galectin-1 induced apoptosis.

I. 3.2 Protein tyrosine phosphatase (PTPase) activity of CD45

I. 3.2.1 The role as positive regulator

As the first identified transmembrane PTPase, CD45 has been extensively studied and widely accepted as an important controller in positively regulating antigen-receptor signaling via dephosphorylation of Src kinase (Trowbridge et al., 1994; Tonks et al., 1988 and Thomas et al., 1989). In both T and B cells, the enzymatic activity of CD45 is crucial for receptor-induced development, activation, proliferation and cytokine production (Byth et al., 1996; Donovan et al., 1993 and Kishihara et al., 1993). During T cell activation the earliest event after ligation of T cell receptors by antigen is phosphorylation of the Src family protein kinase Lck and Fyn. Src family

kinases (SFKs) subsequently phosphorylate the immunoreceptor tyrosine-based activation motif (ITAMs) present in the ξ and CD3ε, δ and γ subunits of the TCR. Then doubly phosphorylated ITAMs recruit and consequently activate downstream substrates which facilitate T cell activation. SFKs are primary substrates for CD45. CD45 dephosphorylation of the negative regulatory tyrosine residue at the COOH-terminal end of $p56^{lck}$, $p59^{fyn}$ and other Src family kinases is responsible for the initiation of immune responses (Li et al., 2001). SKFs have two major sites for tyrosine phosphorylation and SFK activity is controlled by the phosphorylation of two key tyrosine residues: one is at the COOH-terminal used for negative regulation, the other is within the kinase domain (Latour et al., 2001 and Alexander et al., 2000). The regulatory phosphotyrosine residue at the COOH-terminal end is associated with its own SH2 domain in the inactive form (Eck et al., 1994 and Cooper et al., 1988). The intramolecular interaction blocks the substrate binding site and keeps the kinase inactive. Dephosphorylation of the negative regulatory residues, such as Tyr-505 in $P56^{lck}$ and Tyr-531 in $p59^{fyn}$ can activate these kinases (Mustelin et al., 1990). Studies of CD45-mutant cell lines and CD45-null mice provided more evidence for the positive regulatory role of CD45. Most CD45-deficient cell lines and $CD45^{-/-}$ thymocytes showed Lck and Fyn are hyperphosphorylated at their negative regulatory tyrosine which resulted in complete abortion of TCR intracellular signaling (Seavitt et al., 1999; Stone et al., 1997; Cahir Mcfarland et al., 1993 and Koretzky et al., 1990). In addition, expression of a constitutively active form of Lck as a result of mutation in

tyrosine 505 rescues the block in T cell development in $CD45^{-/-}$ mice (Seavitt et al., 1999 and Pingel et al., 1999).

I. 3.2.2 CD45-mediated negative regulation

Very resent observations showed that the average activity of Src family kinases was paradoxically increased in $CD45^{-/-}$ cell lines, despite hyperphosphorylation of the negative regulatory tyrosine suggesting that CD45 had potential negative regulatory activity for Src family kinases (Ashwell et al., 1999 and D'Oro et al., 1999). The studies of the Ashwell and Baker groups revealed that the structural basis for the CD45 negative regulatory role was dephosphorylation of the positively regulating Tyr-394 residue in PTKase $P56^{lck}$ (Baker et al., 2000 and Ashwell et al., 1999). By dephosphorylating the tyrosine residue located within the kinase domain, CD45 down-regulated Src-family kinases associated with integrin-mediated adhesion, namely the Src family kinases Hck and Lyn (Roach et al., 1997). The paradoxical function of CD45 as both a positive and a negative regulator of Src family kinases has been reconciled in a model of CD45 function which is dependent on CD45 exclusion from or inclusion in signaling complexes. For example, CD45 is known to be specifically included in adhesion sites (Roach et al., 1997). Thus, CD45 functions at adhesion sites to dephosphorylate the autophosphorylation site and attenuate Src-family kinase activation. CD45 serves as a positive regulator of antigen-receptor signaling because it is excluded from interacting with Src kinases at sites of engaged receptors and unable to dephosphorylate the autophosphorylation site within the

cluster. Since CD45 has previously dephosphorylated the inhibitory site, CD45 is a positive regulator and the final effect is activating the Src kinases upon antigen receptor clustering (Thomas et al., 1999). The exclusion of CD45 with the large extracellular domain from interacting with the smaller size antigen receptor at sites of antigen engagement prevent negative regulation by CD45 on antigen-receptor signaling (Thomas et al., 1994).

In addition to Src family kinases, CD45 also negatively regulates cytokine and interferon (IFN) receptor activation by dephosphorylating Janus kinases (Jaks) (Yamada et al., 2002; Irie-Sasaki et al., 2001 and Penninger et al., 2001). Although the exact sites of Jak dephosphorylation by CD45 have not been identified, CD45 directly dephosphorylated and decreased Jak activity of all four Jak family members including Jak1, Jak2, Jak3 and Tyk2 in vitro (Irie-Sasaki et al., 2001). Jaks function as positive regulators in cytokine and chemokine responses by phosphorylating the signal transducers and activators of transcription (STATs) family of transcription factors. As a consequence, CD45 might negatively regulate the antivirus response because interleukin-3 mediated myelopoiesis and erythropoiesis increased in $CD45^{-/-}$ mice accompanied with hyperphosphorylation of Jak2 and STATs 3 and 5 (Irie-Sasaki et al., 2001). Several studies also demonstrated a critical role for CD45 in the negative regulation of cytokine receptor driven cell growth. Ratei et al. (1998) reported that about 10% of patients with acute lymphoblastic leukemia have lost the expression of CD45. Expression of CD45 was found frequently lost in patients with Hodgkins lymphoma (Ozdemirli et al., 1996) and multiple myelomas (Ishikawei et

al., 2000). Mice with a CD45-null background exhibited a high tendency to develop leukemia (Baker et al., 2000). In contrast, overexpression of CD45 in growth factor-dependent cell lines frequently caused growth arrest and/or cell death.

I.3.3 Regulation of CD45 phosphatase activity

Studies on the mechanisms through which the PTPase activity of CD45 was regulated have been the focus of intensive effort for more than a decade. Although progress was slow, several possible mechanisms have been illuminated such as ligand binding to CD45, dimerization of CD45, access of CD45 to substrate and molecular interactions with other cell surface molecules. These kinds of interactions lead to a redistribution of CD45 on the cell surface, covalent modifications and the action of specific intracellular inhibitors.

I. 3.3.1 CD45 ligands

Although the transmembrane Ig superfamily protein CD22 β was first identified as a potential ligand for CD45RO on human T cells (Stamenkovic et al., 1991), subsequent work demonstrated that CD22, a cell surface lectin, can recognized α_2-6 sialic acid-containing carbohydrate structures displayed by several surface molecules other than CD45RO (Sgroi et al., 1993; Powell et al. 1993 and Engel et al., 1993). Very recently the endogenous lectin galectin-1 has been reported to bind to

glycosylated CD45 molucules. Perillo et al. (1995) found that galectin-1 which is expressed on stromal cells in thymus and lymph nodes, could induce apoptosis of activated T cells through CD45. Galectin-1 induced, CD45 mediated T cell apoptosis was highly dependent on the expression of core 2 on CD45 O-glycans (Nguyen et al., 2001). Additionally, galectin-1 proteins showed the ability to bind to N-acetylactosamine residues on CD45 molecules (Symons et al., 2000). Galectin action in negative regulation of T cell activation and autoimmunity was proposed to result from the organization of their cell surface glycoprotein ligands through formation of a galectin-glycoprotein lattice which may restrict TCR recruitment to the site of antigen presentation. This was demonstrated by studies assessing $\beta_{1,6}$ N-acetylglucosaminyltransferase(Mgat-5)-deficiency. Mgat-5 is an enzyme involved in the N-glycosylation pathway and initiates GlcNAc $\beta_{1,6}$ branching on N-glycans, thereby increasing N-acetylactosamine, the galectin ligand. Mgat-5-deficiency exhibits a low level of galectin ligand N-acetylactosamine, which is not efficient in forming the galectin-glycoprotein lattice to restrict TCR recruitment. Thus, Mgat-5 deficiency exhibited lower T cell activation thresholds by directly enhancing TCR clustering (Demetriou et al., 2001).

I. 3.3.2 CD45 interacting proteins

Another mechanism involved in modifying CD45 function was based on its interactions with other surface proteins. Such interactions may subsequently change

the substrate access and/or phosphatase activity of CD45 (Altin et al., 1997). CD45 was found to physically-associate with multiple cell surface molecules including Thy1, CD2, LFA1, CD4 and the TCR complex or the IFN receptor α chain (Johnson et al., 2001; Petricoin et al., 1997 and Trowbridge et al., 1994). A CD45 noncovalent association of CD45 with the lymphocyte phosphatase-associated phosphoprotein (LPAP) through the transmembrane region was established (Kitamura et al., 1995). LPAP is a 32-KD transmembrane protein expressed in T, B and NK cells and in hemotopoietic progenitors (Schraven et al., 1994). Initial research showed that LPAP-mutant mice have reduced T cell proliferation and impaired interactions between CD45 and p56 ^{lck} (Matsuda et al., 1998). Later experiments exhibited a controversial result (Ding et al., 1999 and Kung et al., 1999). Very recently, Hugo's group used the LPAP knock-out (KO) thymocytes to test the sensitivity of these cells to CD45 mediated-killing induced by anti-CD45 mAb. The results indicated that thymocytes from LPAP KO mice did not show any different susceptibility than thymocytes from wild-type mice to CD45-mediated apoptosis. This suggested that LPAP expression was not related to CD45-dependent cell death (Fortin et al., 2002). What role does the LPAP play in regulating CD45 remains to be determined. By using cells transfected with individual isoforms of CD45, it has been found that CD45RO was preferentially associated with CD4/CD8 and the TCR compared to larger isoforms (Dornan et al., 2002; Leitenberg et al., 1999 and Dianzzani et al., 1990). Herold reported that CD45 interacted with CD100 and the association of CD45 and CD100 was necessary for T cell aggregation (Herold et al., 1996). Immunoprecipition experiments have shown

that CD45 associated with the IFN receptor γ chain. The association between CD45 and the IFNγ chain was crucial for transducing IFNγ- mediated growth inhibitory signals in T cells (Petricoin et al., 1997). The chemical cross-linking studies showed that CD2 also interacted with CD45 (Schraven et al., 1990 and Alterogt et al., 1990).

I. 3.3.3 Isoform-differential dimerization

Dimerization was first proposed as a mechanism to regulate CD45 function in 1993 by Weiss's group. A chimeric molecule containing the extracellular and transmembrane domains of the epidermal growth factor receptor (EGFR) was fused to the cytoplasmic domain of CD45 (EGFR-CD45) and this restored TCR signaling in a CD45-deficient T cell line. This activity was abolished by EGF-induced dimerization (Sieh et al., 1993). Similar results have been observed by studies using antibodies to induce CD45 dimerization or cross-linking (Trowbridge et al 1994). Anti-CD45 antibodies have been shown to inhibit T cell activation when cross-linking CD45 on cell surface (Turka et al., 1992; Kanner et al., 1992 and Nel et al., 1991). Other studies demonstrated that dimerization-induced inhibition was caused by the catalytic site of one molecule being blocked by a wedge structure from the membrane-proximal region of its partner (Bilwes et al., 1996). The negative regulatory effects of CD45 induced by dimerization were also confirmed by the fact that a point mutation causing inactivation of the inhibitory wedge could abolished the negative regulation (Majeti et al., 1998 and Desai et al., 1993).

It is well known that CD45 exists as several isoforms and the various isoforms differ in size, shape, content of glycosylation and negative charge. This can explain the dissimilar inclination of different CD45 isoforms to form dimers. In primary and transfectant T cells, the smallest isoform RO dimerizes more efficiently and rapidly than the larger isoforms. The cytoplasmic domain of CD45 was not essential for the dimerization, while sialation and O-glycosylation present in the ectodomain could hinder the dimerization (Xu, et al., 2002 and Majeti et al. 2000). Highly regulated isoform expression and the abundance of CD45 provide another possible mechanism for regulating CD45 by spontaneous and isoform-differential homodimerization (Xu et al., 2002). The finding that a small population of CD45 migrated as dimers after chemical cross-linking in a murine T cell line YAC 1 may raise the possibility that exogeneous protein may modulate CD45 function through ligand mediated dimerization or cross-linking (Takeda et al., 1992).

I. 3.4. CD45 mediated T cell apoptosis

Previously, apoptosis has been extensively studied in lymphocytes related to mechanisms involving Ag receptors (Smith et al., 1989; MacDonald et al., 1990 and Hasbold et al., 1990) or the cell surface protein Fas (CD95) (Nagata et al., 1995 and Grispe et al., 1994). Recently, several studies connected cell apoptosis with the most abundant cell surface glycoprotein, CD45 (Lesage et al., 1997; Klaus et al., 1996 and Perillo et al., 1995). Cross-linking CD45 with the Ag receptor, CD3 or even CD45-

ligation alone can induce apoptosis in both T and B cell lines. CD45–induced apoptotic cells displayed morphologic changes typical of apoptotic cells including the following: nuclear condensation and membrane blebbing, but, unlike CD95-mediated apoptosis, no DNA cleavage causing mono- or oliogonucleosome-size fragments occurred (Lesage et al., 1997 and Klaus et al., 1996). Interestingly, CD45-mediated cell apoptosis exhibited isoform specificity mAbs specific for the CD45RA or CD45RO isoforms can effectively cause T cell death by themselves. A combination of anti-CD45RA and anti-CD45RO can enhance induction of apoptosis. Anti-pan CD45 showed the most effective function of induction of T cell death (Klaus et al., 1996). In addition, activated peripheral T cells stimulated by PMA exhibited more sensitivity to monocyte-mediated T cell apoptosis. This increased susceptibility to apoptosis did not happen in a CD45-deficient Jurkat variant. However, these cells could acquire the sensitivity to cell death after transfection with cDNA encoding CD45RA. This indicted that CD45RA was involved in the T cell apoptosis (Wu et al., 1996).

Another piece of evidence regarding CD45 function in T cell apoptosis was obtained from the studies of galectin-mediated T cell apoptosis. Galectin-1, a proposed natural ligand of CD45 (Fouillit et al., 2000), has a broad tissue distribution (Ahmed et al., 1996 and Baum et al., 1995) and has been shown to play a role in the immune system by mediating apoptosis of activated T cells. The apoptotic effect of galectin-1 was shown to be dependent on expression of CD45 on the T cells. Furthermore, galectin-1 induced apoptosis was also blocked by an anti-CD45

monoclonal antibody (Perillo et al., 1995). Together these data suggested that galectin-1 induced apoptosis was mediated by CD45 glycoproteins (Perillo et al 1995). The binding of galectin-1 and CD45 is crucial in the induction of apoptosis in activated T cells. Interestingly, the binding of galectin to CD45 was found to be carbohydrate dependent and could be completely blocked by pre-incubation of galectin-1 with 20mM lactose (Symons et al., 1999). Consistent with this, Perillo's group demonstrated that the glycosylation status of CD45 was impotant in galectin-1 induced T cell apoptosis. Although both N- and O-glycans on CD45 contained the preferential poly-lactosamine sequence for galectin-1 recognition, inhibition of N-glycan synthesis by treating T cells with swainsonine decreased binding of galectin to T cells and reduced galectin-1-induced cell death. While benzyl-α-GalNAc treatment which inhibits O-glycan elongation also decreased galectin-1 binding, it increased galectin-1 induced cell death. These data suggested that N-glycans are directly involved in galectin-1 induced apoptosis, yet, O-glycan may modulate the effect of galectin-1 by masking N-glycans (Perillo et al., 1995). Very recent studies demonstrated that addition of core 2 O-glycans to CD45 appeared to be specifically required for CD45 mediated galectin-1 induced T cell apoptosis. $CD45^+$ BW5147 T cells lacking the core 2 β-1, 6-N-acetylglucosaminytransferase (C2GnT), an enzyme creating the core 2 O-glycans, were resistant to galectin-1 death. When $CD45^+$ T cells bound to galectin-1 on murine thymic stromal cells, only C2GnT positive T cells underwent apoptosis. In addition, C2GnT expression appeared to increase the susceptibility to apoptosis by facilitating the clustering of CD45 on the T cell surface

after binding to galectin-1 (Nguyen et al. 2001). The clustering of CD45 was considered as one characteristic of the cells undergoing apoptosis after galectin-1 binding (Pace et al., 1999). Moreover, the susceptibility of T cells to galectin-1 induced apoptosis showed a cell cycle-dependent difference: the proliferating cells including S, G_2 or M phase cells were most susceptible to CD45 involved galectin-mediated apoptosis (Perillo et al., 1997).

I. 3.5 CD45: associated diseases and target of therapy

The pivotal role that CD45 plays in hematopoitic cells of the immune system attracted intensive studies on the relationship of CD45 with diseases and its possible role as a therapeutic target. Alterations in CD45 have been reported associated with infantile cholestasis, malnutrition, systemic lupus erythematosus (SLE) (Huck et al., 2001; Blasini et al., 1998 and Takeuchi et al., 1997), rheumatoid arthritis, hematologic malignancies and Alzheimer's disease (Tan et al., 2002). But whether these changes were the causes of the diseases or were results of the diseases was not known. In some diseases, the effect of targeting CD45 for therapeutic purpose has been observed. Lazarovits and Zhang reported that using antibodies that recognized certain CD45 extracellular epitopes can induce long-term engraftment of allogeneic and xenogeneic heart and kidney transplants (Zhang et al., 2000 and Lazarovits et al. 1996 a, b). Additionally, anti-CD45 antibodies successfully prevented allograft rejection of islets in recipient diabetic mice (Auersvald et al., 1997). In Alzheimer's disease which is

characterized by upregulation of CD45 expression on microglia cells, cross-linking CD45 suppressed CD40-ligand-induced microgial activation (Tan et al., 2000). Anti-CD45 antibodies induced anti-leukemic effects when they were used both in unconjugated form or conjugated with radioactive iodine (Nemecek et al., 2002). Thus, the ^{131}I-labeled anti-CD45 antibodies were used as a treatment in bone marrow transplantation with the purpose of reducing the leukemia relapse rate. The studies of Chow suggested that the anti-tumorigenesis of natural antibodies might be mediated by targeting CD45 (Zhang and Chow, 1997). In addition, the MB23G2 monoclonal antibodies against CD45RB showed immunosuppressive activity and have been used as an efficient treatment in preclinical autoimmunity models (Minami et al., 1991). Also MB23G2 treatment can upregulate the expression of CTLA-4 which may contribute to inducing long-term allograft survival (Rothstein et al., 2000).

The accumulating findings that alterations in CD45 associated with a variety of immune disorders and the initial effective therapeutic results obtained from targeting CD45 will encourage more extensive studies on CD45 as a prospective treatment in the future.

PART II. OBJECTIVES

Activation of T cells contributes to inflammation and is involved in many human and animal autoimmune diseases (Ballow et al., 1997 and Mouthon et al., 1996). There is a substantial body of evidence showing that IVIg treatment provides a benefit against a number of immune disorders, including Kawasaki Diseases, ITP and GBS which are all characterized by an increase in the frequency of activated T cells. Additionally, the number of activated T cells decreased after IVIg treatment. However, little is known about the mechanisms of IVIg action or the possible targets of IVIg on the surface of activated T cells. We hypothesized that IVIg, the same as NAb, likely exerts its function on early activated T cells by binding to high asialo-carbohydrate expressing molecules, including CD45. IVIg may achieve its therapeutic benefit by negatively regulating T cells in an early transition phase of activation by preventing their full activation or inducing apoptosis. The aim of this study was to identify the surface targets of IVIg on T cells and to investigate the biological effects of IVIg on T cells. As our model we chose the human T cell leukemia line Jurkat, which is often used as a human T lymphocyte cell model (Uzzo et al., 1999). Jurkat cells express CD4 glycoproteins and they release large amounts of IL-2 when they are fully activated. In this way, Jurkat cells resemble Th1 lymphocytes, which play a key role in initiation and progression of inflammatory and autoimmune diseases (Nindl et al., 2002). These make Jurkat an appropriate cell line to assess IVIg effects on T cell functions. Several approaches were taken. First, it was necessary to generate and select high IVIg-binding human T cell variants which served as models to identify the surface

structures associated with increased IVIg–binding. Following a procedure similar to that used in the murine system, model human T cells were generated by treating the human T cell leukemia line, Jurkat with TPA and then selecting for high serum IgG plus IgM binding or high IVIg binding. The biological function of IVIg was assessed on cells grown in vitro and in vivo. Alterations in the expression of activation-associated cell surface molecules were assessed through flow cytometry using specific mAbs, and changes in CD45 isoform expression were examined through western blotting. As well, direct IVIg binding to CD45 epitopes was investigated through IVIg inhibition of cell binding by anti-CD45 mAbs. Finally, the role of cell surface sialic acid on binding by IVIg and CD45 mAbs was examined by comparing neuraminidase treated and untreated cells.

CHAPTER II. MATERIALS AND METHODS

II.1 Animals

Male immune deficient Fox Chase SCID mice were purchased from Charles River Laboratories (St. Constant, Quebec, Canada), and maintained at the University of Manitoba Vivarium, Winnipeg, Manitoba. Mice used for experiments were five to eight weeks old.

II.2 Cell lines and tissue culture

Jurkat human T lymphoma cells were kindly provided by Dr. John Wilkins, Department of Immunology, University of Manitoba. The Jurkat cells and variants generated from it were maintained in RPMI 1640 medium (Life Technologies, Rockville, MD) containing 10% fetal bovine serum (Life Technologies, Inc. Grand Island, NY) supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin (Life Technologies). Fresh vials of all three cell lines were taken out from liquid nitrogen very 3-5 months to ensure the cell phenotype stability.

II.2.1 Selection of high human serum IgG- plus IgM-binding cells

Jurkat cells were grown in 100 ng/ml tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Calbiochem-Novabiochem Corporation, La Jolla, CA) in RPMI 1640 medium containing 10% fetal bovine serum (FBS) (Life Technologies) to generate variants. The concentration of TPA chosen for this procedure was previously shown to be the minimum amount required for generating

stable and distinguishable variants in our previous murine T cell models (Chow et al., 1984 and 1987). After the Jurkat parental cells had been grown in the presence of TPA for at least one week, 5×10^6 TPA-treated cells were washed once in 10 ml cold RPMI 1640 medium containing 5% FBS. Pellets were resuspended in 1ml human serum diluted 1/3 with HEPES (Life Technologies) buffered 5% FBS-RPMI 1640 and incubated for 30 mins at 4°C. Following an additional wash with 25 ml of the same medium, cell pellets were incubated for 10 min at RT with 0.5 ml of a combination of anti-human IgG and anti-human IgM BioMag magnetic beads (Perseptive Biosystems, Inc. Framingham, MA) in 4.5 ml RPMI medium. The highest binding cells were separated using a magnet for 15 min at RT. After one wash with 5 ml of the same RPMI 1640 media, the cells were resuspended and grown in 10% RPMI 1640 medium containing 100 ng/ml TPA until the next selection. Four successive cycles of TPA treatment followed by the same selection process yielded the final selected cell line, J4.1. After the last selection, the cells were returned to and maintained in standard culture. The first and second selections were performed with sera pooled from 4 normal donors, while the third and fourth selections were done using the pooled sera from 13 normal donors.

II.2.2 Selection of high IVIg-binding cells

Before selection, Jurkat cells were treated with 100 ng/ml TPA for at least one week. Following two washes with 25 ml of cold RPMI 1640 medium containing 1% FBS, and 10 mM HEPES buffer, the pellet of 7×10^6 Jurkat cells were resuspended in 1 ml of IVIg at 7.5 mg/ml IVIg and incubated on ice for 1 hr. After one wash with 25

ml of the same medium, the cell pellet was incubated for 25 mins on ice with 0.35 ml of a 1/15 dilution of FITC-labeled goat anti-human IgG (Sigma Aldrich). Following an additional wash with 25 ml of the same medium, the cell pellet was resuspended in 2 ml of 2% FBS-RPMI 1640 medium. In preparation of high speed cell sorting, the cell suspension was passed through a sterile 40 micron nylon cell stainer (Falcon #35-2340), transferred into a 5 ml 12×75 mm tube (Falcon #35-2054) and the tube mounted in the sample compartment of the cell sorter. The sample tube was surrounded with wet ice during the sorting procedure. An EPICS Altra fluorescence-activated cell sorter (Beckman Coulter, Canada Inc., Mississauga, Canada) was configured for high speed cell sorting using ISOTON II as the sheath fluid. The sample line was treated with 75% ethanol for 15 minutes prior to the introduction of the cell sample. Sort criteria were defined to include single viable cells as identifies by forward angle light scatter and side scatter characteristics. Cellular fluorescence was quantitated and displayed using linear amplification and the most fluorescent 5% of the cells (top 5 percentile) were collected by sorting into 5 ml culture tubes containing 2 ml of HEPES-buffered RPMI medium. Three cycles of TPA treatment followed by sorting yielded the JIg3.1 cells, selected for high IVIg binding.

II.3 IVIg and human serum

Gamimune ® N, 5%, a kind gift of the Bayer (Bayer Corporation, Elkhart, USA) was pooled normal IgG obtained from the plasma of healthy donors and prepared for therapeutic intravenous injection. When reconstituted, Gamimune ® N, 5%, contains

approximately 50 mg/ml IgG, 100 mg/ml maltose, 0.3-0.4% tri-n-butyl phosphate (TNBP) and 0.2-0.3% NaCl. The IVIg was dialyzed to remove the sugar, four times using 1000 ml, 550 ml, 600 ml and 1100 ml of serum-free PBS or dye-free RPMI 1640 at 4° for 24 hours. Stock solutions of approximately 50 mg/ml of IVIg were prepared in the serum-free phosphate-buffered saline (PBS) or RPMI 1640 without phenol red. The protein concentrations of the dialyzed IVIg were quantified using a Bio-Rad DC protein assay kit according to the instructions (Bio-Rad Laboratories, Hercules, CA). IVIg dialyzed in PBS was used throughout the study except for the in vitro cell growth inhibition assay in which IVIg dialyzed in RPMI 1640 was used.

Human serum used for selecting the J4.1 variant and human serum binding assays was collected from 13 healthy donors by The Canadian Blood Service, Winnipeg Center. It was prepared from left-over plasma after the clot was removed. Before use, the plasma was recalcified and centrifuged at room temperature for 10 min. The serum was subsequently isolated and stored at -70 °C.

II.4 Antibodies

Mouse anti-human monoclonal antibodies (mAbs) for flow cytometry included the following: HI100 anti-CD45RA ($IgG_{2b,\kappa}$), M-A251 anti-CD25 (IgG_1), Mik-β2 anti-CD122 ($IgG_{2a,\kappa}$), DX2 anti-CD95 ($IgG_{1,\kappa}$), NOK-1 anti-CD95L (IgG_1), R-phycoerythrin (PE)-conjugated mAbs: FN anti-CD69 (IgG_1), ACT35 anti-CD134 (IgG_1), G46-2.6 anti-HLA-A,B,C (IgG_1) and 9-49 anti-HLA-DRDPDQ (IgG_{2a}) (PharMingen, San Diego, CA). Bra-11 anti-CD45RB ($IgG_{1,\kappa}$), UCHL1 anti-CD45RO

($IgG_{2a,}\kappa$), Bra-55 anti-pan CD45 (IgG_1), Quantum Red (QR)-conjugated mAb UCHT-1 anti-CD3 (IgG_1), fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG (specific for the whole molecule), IgG (specific for the γ chain), IgM (specific for the μ-chain) and FITC-goat anti-human IgG (specific for the whole molecule) were obtained from Sigma Aldrich. Phycoerythrin-cyanin 5.1 (PC5)-conjugated UCHT1 anti-CD3 (IgG_1) was purched from Beckman Coulter (Canada INC., Mississauga, Canada). Isotype controls, MOPC141 mouse IgG2b, MOPC21 mouse IgG1, UPC10 mouse IgG2a, QR-conjugated mouse IgG1 and FITC-conjugated goat IgG (fraction from normal serum) were obtained from Sigma Aldrich. Additionally a PE-conjugated MOPC21 mouse IgG_1 was obtained from PharMingen. Antibodies used in western blotting included mouse anti-human Bra-55 anti-pan CD45 (IgG_1) and horseradish peroxidase-labeled goat anti-mouse IgG (The Jackson Laboratory, West Grove, Pennsylvania).

II.5 Fluorescence-detected IVIg and human serum binding

Flow cytometry was used to assay IVIg and human serum binding to the parental Jurkat cell line and the high binding variant cell lines J4.1 and JIg3.1 according to Tough's procedure (Tough and Chow, 1991) with minor modification. Aliquots of $3x10^5$ cells were washed in 1ml of cold RPMI 1640 containing 1% FBS, 10 mM HEPES buffer and 0.1% sodium azide (Sigma Aldrich). Cell pellets were resuspended in 50 μl of IVIg diluted in the RPMI wash medium. Control samples were resuspended in wash medium only. After incubation on ice for 1 h, both test and

control samples were washed once in 4 ml of cold wash medium and then stained by incubating the cells for 20 mins at 4 °C in 100 µl of FITC anti-human IgG diluted 1/13 in RPMI wash medium. The cells were washed once with 3 ml cold RPMI wash medium and fixed with 600 µl of 2% paraformaldehyde (Sigma Aldrich) in PBS at pH 7.3 for at least 10 min at 4°C.

For human serum binding, aliquots of $3x10^5$ cells were incubated for 1 h at 4°C in 50 µl of a ¼ or ½ dilution of pooled whole human serum. After one wash, the cells were further incubated for 30 min at 4°C to 100 µl aliquots of a 1/10 dilution of FITC-labeled anti-human IgG (specific for the whole molecule), a 1/10 dilution of FITC anti-human IgM (specific for the µ-chain) or a combination of a 1/20 dilution of FITC anti-human IgG and a 1/20 dilution of FITC anti-human IgM. This was done to quantify human IgG, IgM, and IgG- plus IgM-binding ability, respectively. The cells were then washed once with 3ml cold wash medium and fixed in 600 µl of 2% paraformaldehyde in PBS at pH 7.3 for at least 10 min at 4°C.

Data were collected on 5000 viable cells satisfying light scatter gating criteria using an EPICS Altra fluorescence-activated cell sorter (Beckman Coulter Canada INC) or a FACSCalibur (Becton Dickinson, San Jose CA). Cellular fluorescence was quantified and displayed on a linear basis in the parameter histograms as shown in Figure I. The levels of IVIg or human serum binding were reported in terms of the mean channel fluorescence (MCF) after the control MCF was subtracted.

II.6 Fluorescence-detected monoclonal antibody binding

Aliquots of 3×10^5 cells of the Jurkat parental, J4.1 and JIg3.1 lines were assayed simultaneously to compare cell surface marker expression. Following one wash with cold RPMI 1640 wash medium as before and centrifugation at 500g for 10 min at 4°C, the cells were resuspended in 50 ul aliquots of saturating levels of purified mAbs at 0.6-114 μg/ml, including the following: mouse anti-human CD45RA (IgG_{2b}), anti-CD45RB (IgG_1,κ), anti-CD45RO (IgG_{2a}), anti-pan CD45 (IgG_1), anti-CD95 (IgG_1,κ), anti-CD95L (IgG_1,κ) and anti-CD122 (IgG2a,κ) or the same concentration of control monoclonals. Both the detecting and isotype control antibodies were diluted in the same RPMI wash medium and incubated with the cells for 1 hour at 4°C. After this incubation the cells were washed once with 2 ml cold RPMI wash medium and then stained with 100 μl of a 1/20 dilution of FITC-goat anti-mouse IgG (specfic for the whole molecule) for 20 min at 4°C. Following a single wash with 2 ml of cold RPMI wash medium, the cells were fixed in 600 μl of 2% paraformaldehyde at 4°C for at least 10 min before being analyzed by flow cytometry. Binding of fluorescence conjugated monoclonal antibodies was carried out similarly using the following antibodies including PE-conjugated anti-human HLA-ABC, anti-human HLADRDPDQ, anti-CD25, anti-CD69 and anti-CD134, PC_5-conjugated anti-CD3, and individual isotype controls. The cells were incubated for 1 hour at 4°C, except that anti-CD25 and anti-HLADRDPDQ were incubated for only 40 min at 4°C. After this incubation, 2 ml of cold RPMI wash medium was added and the cells were

centrifuged at 500 g for 10 min at 4°C. The cells were fixed in 500 µl of 2% paraformaldehyde for at least 10 min at 4°C. The flow cytometry data were collected on 5000 viable cells using a FACSCalibur and the binding was reported after subtraction of the MCF for the isotype control.

II.7 IVIg inhibition of cell growth in vitro

To find minimal nutrient conditions for Jurkat cell lines to grow in the 96-well flat-bottom cell culture plates (ICN Biomedicals, Urora, OH), JIg3.1 were initially cultured in different FBS concentrations to assess the cell growth. JIg3.1 cells were washed once at RT in 16 ml of 3% FBS/RPMI 1640 medium at 500 g for 10 min. Cell pellets were resuspendent and set at 1.5×10^5/ml in 200 µl of RPMI 1640 medium containing the following different concentrations of FBS: 3%, 5%, 7%, 9% and 10%. After 3 days of growth, the cells were stained with trypan blue and living cells were counted using a microscope. From this primary experiment, 3% FBS/RPMI 1640 medium was chosen as the culture medium used for cell culture in 96-well flat-bottom plates since cell growth was about 90% of control cultures using 10% RPMI.

In the assay of IVIg inhibition of cell growth in vitro, Jurkat, J4.1 and JIg3.1 were washed once at RT in 16 ml of 3% FBS/RPMI 1640 medium at 500 g for 10 min. Aliquots of 3×10^4 cells were resuspendent in 200 µl of 3% FBS/RPMI 1640 medium containing IVIg at final concentrations of 2.5, 5, and 10 mg/ml or 3% FBS/RPMI 1640 medium as control. The cells were cultured for 3 days in 96-well flat-bottom plates at 37°C in a humidified incubator with 5% CO_2. Then, the cells were harvested

and stained with trypan blue. After counting the living cells, the total cell growth was calculated by subtracting the total number of starting cells from the total final number of cells. The impact of IVIg at each concentration was expressed as a percentage of the total cell growth of control cells which grew in 3% FBS/RPMI medium.

II.8 ^{131}I-deoxyuridine (^{131}I-dUrd) cell labeling and in vivo elimination assay

Cell labeling with ^{131}I-dUrd was performed as described previously (Chow et al., 1983). Briefly, cell growth was initiated with aliquots of 1.2×10^6 Jurkat or JIg3.1 cells at 3.0×10^5/ml in RPMI medium containing 10% FBS and then ^{131}I-dUrd was added at a final concentration of 4.25 μCi/ml. The ^{131}I-dUrd incorporation was allowed to proceed for 24 hrs at 37°C in a humidified incubator with 5% CO_2. Then the cells were washed three times with Hanks balanced salt solution (HBSS) (Life Technologies, Chagrin Falls, Ohio) at RT. At least 2 hrs before challenge with ^{131}I-dUrd-labeled cells, a group of four mice were each pretreated with a single i.v. injection of 200 μl of 50 mg/ml, non-dialyzed IVIg or 200 μl of PBS as control. An aliquot of 1×10^5 ^{131}I-dUrd-labeled cells in 200 μl was injected i.v. into each of four IVIg-treated or PBS-treated mice for each cell line. As time passes, the injected radio-labeled cells will be lysed and the radiolabel eliminated in the urine. Then, the mice were whole-body counted in a Beckman 8,000 gamma counter (Irvine, CA) at time 0, at 2 h, 4 h, 12 h and 18 h after the cell inoculation. The amount of radioactive label

remaining in the mice at each time point was expressed as a percentage of the whole body counts at time 0.

II.9 IVIg binding to peripheral blood T lymphocytes

Using a sterile needle, 5 ml of peripheral blood was drawn from healthy donors into sterile pyrogen-free vacuum blood collection tubes (Becton Dickinson, Franklin Lake, NJ) using EDTA as an anticoagulant. Blood was centrifuged at 500 g for 10 min at RT to separate the whole blood into three components, the buffy coat, cells and serum. Then, the buffy coat was transferred and resuspended in 5 ml of RPMI 1640 wash medium containing 1% FBS and 1% HEPES buffer. The mixture of buffy coat and wash medium were carefully laid on top of 5 ml of Histopaque-1077 (Sigma Aldrich). Then, the peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation at 700 g for 30 min at RT. The PBMCs were washed in 16 ml cold RPMI wash medium by centrifugation at 500g for 10 min at 4°C. The cell pellet was resuspended at a concentration of 5×10^7/ml. Aliquots of 20 µl of this cell suspension were incubated at 4°C for 1 hour with 20 µl of IVIg at 10 mg/ml or RPMI wash medium and shaken 3 times during the incubation. The cells were then washed with 4 ml of cold RPMI wash medium and centrifuged at 500 g for 10 min at 4°C. The cell pellets previously incubated with IVIg or RPMI medium were resuspended in 75 µl detecting mAbs at 4 µg/ml of PC_5-conjugated anti-CD3 plus 145 µg/ml of FITC-conjugated goat anti-human IgG (specific for the γ chain). Isotype

controls were prepared for each IVIg treated and for untreated samples by substituting FITC-conjugated goat IgG for FITC-conjugated goat IgG anti-human IgG. Following a 35 min incubation in the dark at 4°C, cells were washed with 3 ml of cold RPMI wash medium and fixed overnight with 600 µl of 2% paraformaldehyde in PBS at 4°C. All samples were analyzed by an EPICS Altra fluorescence-activated cell sorter. Data were collected on 2000-5000 cells defined as lymphocytes by light scatter measurement.

II.10 Western blotting for CD45

Aliquots of 10^7 Jurkat, J4.1 and JIg3.1 cells were harvested and treated simultaneously in the following procedure. The cells were washed with 20 ml of cold PBS and resuspended with 0.5 ml RIPA buffer containing 1M Tris/HCl pH 7.4, 1.5M NaCl, 1% Nonidet P-40, 1.0% deoxycholate, 0.1M PMSF and 1 tablet/10 ml Roche Complete mini tablets (Riche, Diagnostics GmbH, Mannaheim, Germany). In this cell lysis procedure, the cells were set on ice for 15 min and sonicated for 10 seconds. The lysates were then centrifuged at 17,000 g for 7 mins and the supernatant lysate was removed from insoluble debris including nuclei. The protein concentrations of the lysates were quantified using a Bio-Rad DC protein assay kit according to the instructions (Bio-Rad Laboratories, Hercules, CA). Then a 50 µg and a 100 µg aliquot of the lysate solution was removed and boiled in 4 × SDS sample loading buffer for 5 min. The high range molecular weight (m.w.) standards (Bio-Rad laboratories, Inc., Hercules, CA) were boiled for only 2 min as longer boiling of the standards caused a

smear to be observed on the western blot. Then the samples were loaded onto a 6 % SDS-PAGE gel (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). After the SDS-PAGE had been performed at RT using 200v for 45 min, the gel was transferred to a PVDF membrane (Roche Diagnostices Corporation, Indianapolis, IN), in a cold transfer buffer containing 192 mM glycine, 25 mM Tris base and 20% methanol using 100V for 1.5 hours at 4°C. To block the portions of membrane without protein bound to it, the PVDF membrane was incubated with TBS/5% nonfat dried milk overnight at 4°C. The membrane was incubated with a 1:100 dilution of mouse anti-human Bra-55 anti-CD45 in 5%milk-TBS and 0.1% Tween-20 (TBS-MT) on a shaker at RT for 1 hour. After 3 repetitions of 15 ml TBS-T washes for 10 min, the membrane was incubated on a rotator at RT for 1 hour in a 1:800 dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Jackson Laboratory), and then washed 3 times. Meanwhile, the portion of the membrane containing the high MW standards was maintained in the 5% milk-TBS blocking solution, and then was washed three times. The membrane was then incubated with 10 ml of a 1:500 dilution of Strepavidin-HRP (Bio-Rad laboratory) in TBST without milk for 60 min at RT on a rotator and was washed extensively in TBST solution. The protein present on the membrane was detected by using the enhanced chemiluminescence (ECL) system (Amersham pharmacia biotech, Buckinghamshire, England). The membrane was incubated in a mixture of 2 ml of western blotting detection reagent A and 2 ml of reagent B for 1 min and was then placed inside a clear plastic sheet protector and exposed to photographic film to detect the light emission. Detection of actin served as

a control for the equal protein content of samples. The PVDF membrane was stripped at 50 °C for 40 mins in stripping solution containing 2% SDS and 0.7% β-mercaptoethanol in TBS and subsequently incubated with 5% milk-TBS overnight at 4°C. Following 2 washes with 15 ml TBS-T washes for 30 mins, the membrane was incubated in a 1:100 dilution of anti-actin (Sigma Aldrich) in TBS-MT on a shaker at RT for 40 mins. After one 15 ml TBS-T wash for 10 mins, the membrane was incubated with a 1:500 dilution of HRP-conjugated goat anti-rabbit IgG (Life Technologies) in TBS-MT on a rotator at RT for 1 hour. Detection of actin was performed by using the ECL chemiluminescence system.

II.11 IVIg inhibition of cells surface binding by anti-CD45 mAbs

Use of mAb concentrations which were optimum for binding revealed little or no inhibition by IVIg. The standard mAb binding method was used to perform a careful titration of the binding of the monoclonal antibodies against CD45RA, CD45RB, CD45RO and pan-CD45. After the binding curves were completed ranging from the minimal binding to the plateau, we chose the concentrations of mAb for inhibition experiments which showed 80% of optimal binding under the standard conditions. JIg3.1 was the primary model cell used for this inhibition study, except that J4.1 was used for anti-CD45RB inhibition because J4.1 has the highest anti-CD45RB binding of all three cell lines. Aliquots of 3×10^5 cells were washed once with RPMI 1640 medium containing 1% FBS, 1% HEPES buffer and 0.1% NaN_3. Cell pellets were resuspended in 25 µl of IVIg at 10 mg/ml diluted in RPMI wash medium or wash

medium without IVIg as control. After incubating for 1 hour at 4 °C, 5 µl of anti-CD45RA, anti-CD45RB, anti-CD45RO and anti-pan CD45 or isotype control mAbs were added to make final concentrations at 80% of saturating concentrations and the incubation was continued for 15 min at 4°C. Then, 3 ml of cold RPMI wash medium was added to all tubes. To ensure that the resulting supernatant would have the same amount of IVIg, 25 µl of IVIg at 10 mg/ml was added to the tubes without IVIg present. Thus, the differences observed between the IVIg treated/untreated cells were not due to IVIg affecting the detection of the antibodies. After washing with 4 ml cold RPMI washing medium, a 100 µl aliquot of a 1/18 dilution of FITC-goat anti-mouse IgG (specific for whole molecule, absorbed with human serum) was added. After incubation for 20 min at 4°C, the cells were washed with 2 ml of cold RPMI wash medium. Then, 500 µl of 2% paraformaldehyde was added to fix the cells. IVIg inhibition of binding by each of the CD45 isoforms was expressed as a percentage of CD45 mAb binding to cells not treated with IVIg.

II.12 Neuraminidase (NM) treatment of Jurkat cells

For all the lectin, IVIg and anti-CD45 mAb binding assays, Jurkat parental cells were treated with neuraminidase Type V (NM) (Sigma Aldrich) simultaneously in the same way. Aliquots of 3.5×10^6 cells were washed once with 50 ml HBSS at RT. Cell pellets were resuspended in 300 µl of HBSS/NaOH at pH 9.3 plus 12 µl of NM at 100 IUs/ml or 312 µl of HBSS/NaOH as the control. This incubation took place at 37°C in

a humidified incubator with 5% CO_2 for 1 hour with shaking every 20 min. The cells were washed with 4 ml cold 1640 RPMI wash medium. Aliquots of 3×10^5 NM treated or control non-treated cells were incubated with FITC-conjugated peanut agglutinin (PNA) (Sigma Aldrich) for 30 min at 4°C. After one wash with 3 ml RPMI 1640 wash medium, the lectin binding was analyzed by flow cytometry using a FACSCalibur. Assessment of the binding of IVIg and mAbs against CD45RA, RB, RO and pan-CD45 followed our standard protocol as outlined previously.

II.13 Statistical Analysis

The paired Student's t test (td) and the independent Student's t test (ti) were used to determine whether the differences between the sets of data collected in this study were significant. A probability $P > 0.05$ was not considered significant (NS).

CHAPTER III. RESULTS

III.1. Human serum IgG plus IgM binding to parental Jurkat, J4.1 and JIg3.1 cells

To examine the effect of IVIg on human T cells, we selected two Jurkat variant cell lines, J4.1 and JIg3.1. These were selected on the basis of high binding of human serum IgG plus IgM and for binding of human IgG in IVIg, respectively. These cell lines served as human T cell models and were compared to the parental Jurkat cell line.

The immunoglobulin binding abilities of the Jurkat, J4.1 and JIg3.1 cell lines were assessed by flow cytometry (Table I). Comparing the binding ability of human serum IgG and IgM between parental and selected variants suggested that both J4.1 and JIg3.1 showed similarly increased capacities to bind human IgG and IgM. Compared to the parental Jurkat cells, J4.1 bound 33% more IgG, and 43% more IgM, and 38% more IgG plus IgM. Similarly, JIg3.1 showed increases of 47%, 27% and 45% respectively for binding of human IgG, IgM and IgG plus IgM. The binding profiles of the two selected cell lines were slightly different. J4.1, which was selected for high IgG plus IgM binding in the context of human serum, showed a smaller increase in IgG binding, but a larger increase in IgM binding than the JIg3.1 which was selected for high IgG binding in the context of IVIg. These differential increases in IgG-binding or IgM-binding seem to reflect the different selection conditions. Additionally, examination of IgG binding in human serum indicated the impact of the

TABLE I BINDING OF SERUM IgS TO Ig-SELECTED JURKAT CELLS

		Mean Channel Fluorescence ± SE [a]				
Expt.#	Serum	Jurkat	J4.1	% Increase	JIg3.1	% Increase
1. [b]	IgG	151.1±9.8	201.0±17.7**	33	222.2±13.8**	47
	IgM	33.8±6.7	48.5±8.6**	43	43.0±9.0**	27
2. [c]	IgG+IgM	99.9±13.9	137.6±15.1*	38	144.8±12.8*	45

[a] All experiments were performed using a 1/4 dilution of pooled serum from 13 normal donors.The results were calculated after subtraction of the MCF for the controls. Increases were calculated as a percentage of the Ig binding to Jurkat.
[b] Four experiments were performed.
[c] Three experiments were performed in parallel with [b] experiments.
* $P_{td} < 0.04$ versus Jurkat * *$P_{td} < 0.03$ versus Jurkat.

presence of IgM on IgG binding. This would be important for the action of IVIg in vivo.

III.2 IVIg binding to parental Jurkat, J4.1 and JIg3.1 cells

When Jurkat, J4.1 and JIg3.1 cell lines were incubated at 4 °C with the following concentrations of IVIg: 4 or 5 mg/ml, 10 mg/ml and 12.5 mg/ml, J4.1 and JIg3.1 cells showed higher binding than the parental cells at all concentrations of IVIg (Table II, Figure I). J4.1 cells exhibited increases in the MCF of 31%, 67% and 40% respectively for binding of IVIg at 4 or 5 mg/ml, 10 mg/ml and 12.5 mg/ml of IVIg with statistical significance achieved for the two lower concentrations. Increases in JIg3.1 binding amounted to 20%, 79% and 50% respectively, for binding of IVIg at 4 or 5 mg/ml, 10 mg/ml and 12.5 mg/ml, with significant differences in binding achieved at the two higher concentrations.

In initial experiments, the level of binding of the three cell lines to IVIg at higher concentrations from 2.5 to 30 mg/ml were analyzed (Table III and IV). The data showed that for IVIg concentrations in the range of 2.5 to 10 mg/ml, Jurkat and J4.1 cell lines exhibited a dose-dependent increase in binding to IVIg and JIg3.1 tended to increased binding. However, when the concentration of IVIg was over 10 mg/ml, variable binding of IVIg was observed for all three cell lines. All cell lines showed a tendency toward decreased binding at concentrations around 12.5 mg/ml of IVIg and increases at the higher concentrations of 20 mg/ml and 30 mg/ml. We proposed that

TABLE II BINDING OF IVIg TO Ig-SELECTED JURKAT CELLS

Expt.#	IVIG Concentration	Mean Channel Fluorescence ± SE [a]				
		Jurkat	J4.1	% Increase	JIg3.1	% Increase
1. [b]	4or5 mg/ml	125.6±27.6	165.5±22.9*	31	150.6±28.6	20
2. [c]	10 mg/ml	100.6±20.4	167.6±16.4**	67	179.8±23.3**	79
	12.5 mg/ml	56.5±12.7	79.2±13.4	40	84.7±20.7*	50

[a] The results were calculated after subtraction of the MCF for the controls. Increases were calculated as a percentage of IVIg binding to Jurkat.
[b] Nine experiments were performed.
[c] Nine experiments were performed in parallel.
* $P_{td} < 0.05$ versus Jurkat. ** $P_{td} < 0.003$ versus Jurkat.

FIGURE I BINDING OF 10 MG/ML IVIg TO Ig-SELECTED JURKAT CELLS

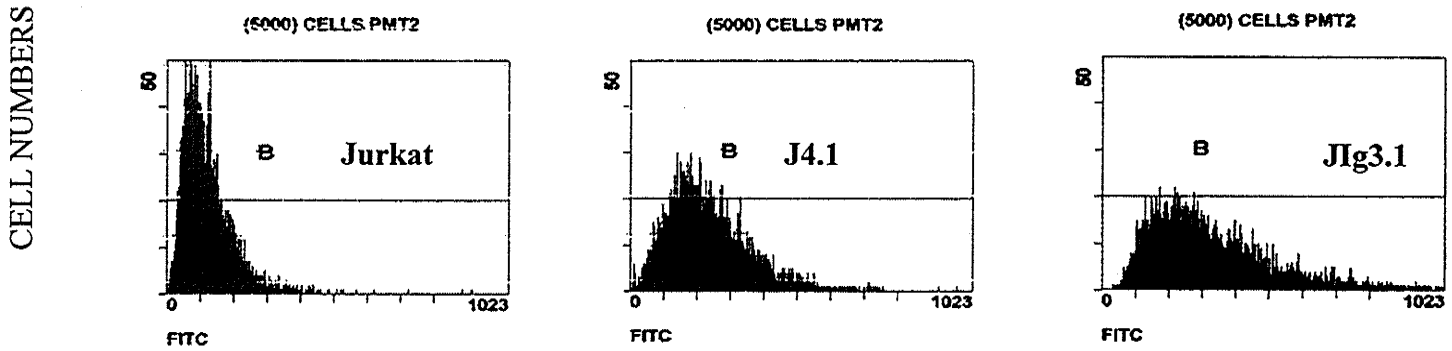


Fig.I. The selected high human serum IgG plus IgM and high IVIg binding variants J4.1 and JIg3.1 respectively, exhibited markedly more IVIg binding than the parental Jurkat.

TABLE III BINDING OF IVIg AT DIFFERENT CONCENTRATIONS TO JURKAT AND J4.1

EXP.#	Cells	Mean Channel Fluorescence± SE						
		IVIg Concentration (mg/ml)						
		2.5	5	10	12.5	15	20	30
1 [a]	Jurkat	25.3±9.3	44.3±8.8	127.3±31.3	81.5±16.2	136.9±22.7	189.9±19.6	260.8±54.2
	J4.1	27.4±12.5	60.0±21.0	118.9±25.3	66.2±18.9	108.7±13.0	142.9±19.1	133.8±42.0

[a] Five experiments were performed.

TABLE IV BINDING OF IVIg AT DIFFERENT CONCENTRATIONS TO JIg3.1

EXP.#	Cells	Mean Channel Fluorescence± SE					
		IVIg Concentration (mg/ml)					
		6	8	10	12	16	20
1 [a]	JIg3.1	21.9±11.5	30.1±16.1	31.6±8.6	50.1±8.3	81.1±18.3	150.1±38.0

[a] Four experiments were performed.

this phenomenon may occur as follows: at low concentrations ranging from 2.5 to 10 mg/ml, as the amount of IVIg increased, the binding to the cell surface increased correspondingly. Up to probably 12.5 mg/ml in this study, the surface bound IVIg achieved a high level of binding which was unstable resulting in a loss of bound Ig with or without the surface molecules which IVIg targeted. This may result in the exposure of the new surface targets or unmasking of several previously bound target molecules. The free IVIg available in the supernatant can bind to the cells producing another increase. Considering the results of these preliminary experiments, we chose the concentrations of IVIg ranging from 2.5 mg/ml to 10 mg/ml to use in the later experiments.

III.3. IVIg inhibition of cell growth in vitro

Several early studies demonstrated that IVIg has antiproliferative effects on T cells (Schaik. et al., 1992; Delfraissye et al., 1985 and Amran et al., 1994). To determine whether IVIg had a similar effect on our selected T cell models and if there was any difference between the IVIg sensitivity of the cell lines, the three cell lines Jurkat, J4.1 and JIg3.1 were tested in parallel.

To choose the conditions for testing IVIg-inhibition of cell growth in 96-well flat bottom tissue culture plate, JIg3.1 cells were set at $1.5x10^5$/ml in 200 ul RPMI 1640 medium containing the following different concentrations of FBS, 3%, 5%, 7%, 9% and 10%, the last concentration being our standard concentration for cell culture.

TABLE V GROWTH OF JIg3.1 IN RPMI MEDIUM CONTAINING DIFFERENT CONCENTRATIONS OF FBS

FBS in Medium	3%	5%	7%	9%	10%
Final Cell Number ($\times 10^4$)	20.3±2.1*	21.3±2.3	22.4±2.4	22.8±2.4	22.5±2.7
Final Cell Concentration ($\times 10^5$)	11.1±1.3 *	11.7±1.3	12.2±1.3	12.4±1.3	12.3±1.4
Percentage change in Growth [a]	−11.3	−6.2	−0.5	1.5	—

[a] The changes in cell growth in different concentrations of FBS were calculated by comparing the final cell number minus the starting number for test cells and for cells grown in standard 10% FBS medium.

* $P_{td} < 0.05$ versus the cells grown in 10% FBS RPMI medium.

After 3 days of growth, the cells were stained with trypan blue and the living cells were counted. The data (Table V) revealed that the cells growing in RPMI 1640 medium containing 3% FBS caused a reduction of about 10% in the total cell number compared with growth in 10% FBS RPMI medium. However, even the cells that were cultured in 3% FBS-RPMI 1640 medium, achieved a final cell concentration of $11.2x10^5$/ml which was much higher than our normal final cell concentration of about $6.0x10^5$/ml after 3 days growth from $1.5x10^5$/ml in RPMI 1640 medium containing 10% FBS, our standard culture conditions. This might indicate that when cells were cultured in 96-well plates in as small a volume as 200 μl, the standard 10% FBS RPMI medium provided more nutrition than the cells actually required. Since much more evaporation occurred as the result of a more broad surface exposure area relative to the volume. We chose the RPMI 1640 medium containing 3% FBS as the culture medium for examining the impact of IVIg on cells growing in 96-well plates because the cells grew very well but just slightly less than optimally.

The presence of 2.5 mg/ml, 5 mg/ml and 10 mg/ml IVIg in cell culture for 3 days enhanced parental Jurkat cell growth compared with untreated cells by 15%, 19% and 15% respectively. In contrast, the same treatment reduced cell growth of J4.1 by 3%, 13% and 17% respectively, while JIg3.1 growth was reduced by 12%, 21% and 26% respectively (Table VI, Figure II). The significant inhibition of IVIg on J4.1 and JIg3.1 cell growth exhibited a dose-dependent pattern, 10mg/ml IVIg showed the highest rate of inhibition which is consistent with the observation that the amount of IVIg binding was higher at this concentration (Table IV). Furthermore, consistent

TABLE VI IVIg INHIBITION OF Ig-SELECTED JURKAT VARIANT GROWTH IN VITRO

Cells	Total Cell Growth (Cell Number × 10^4)						
	IVIg 0 mg/ml	IVIg 2.5 mg/ml	% Growth Inhibition	IVIg 5 mg/ml	% Growth Inhibition	IVIg 10 mg/ml	% Growth Inhibition
Jurkat	20.6±1.6	23.1±1.8	−15	23.9±2.0	−19	23.1±2.4	−15
J4.1	15.0±2.0	14.3±1.6**	3	12.8±1.2**	13	12.3±1.1*	17
JIg3.1	20.4±1.2	18.0±1.1**	12	16.1±1.1**	21	15.0±1.1*	26

[a] Five experiments were performed. Total cell growth was calculated as final cell number minus starting number. Growth of the cells in 3% FBS/RPMI medium was considered to be 100%. The effect of IVIg was compared to this control.
** $P_{td} < 0.01$ versus Jurkat * $P_{td} < 0.05$ versus Jurkat.

FIGURE II IVIg INHIBITION OF Ig-SELECTED JURKAT VARIANT GROWTH IN VITRO

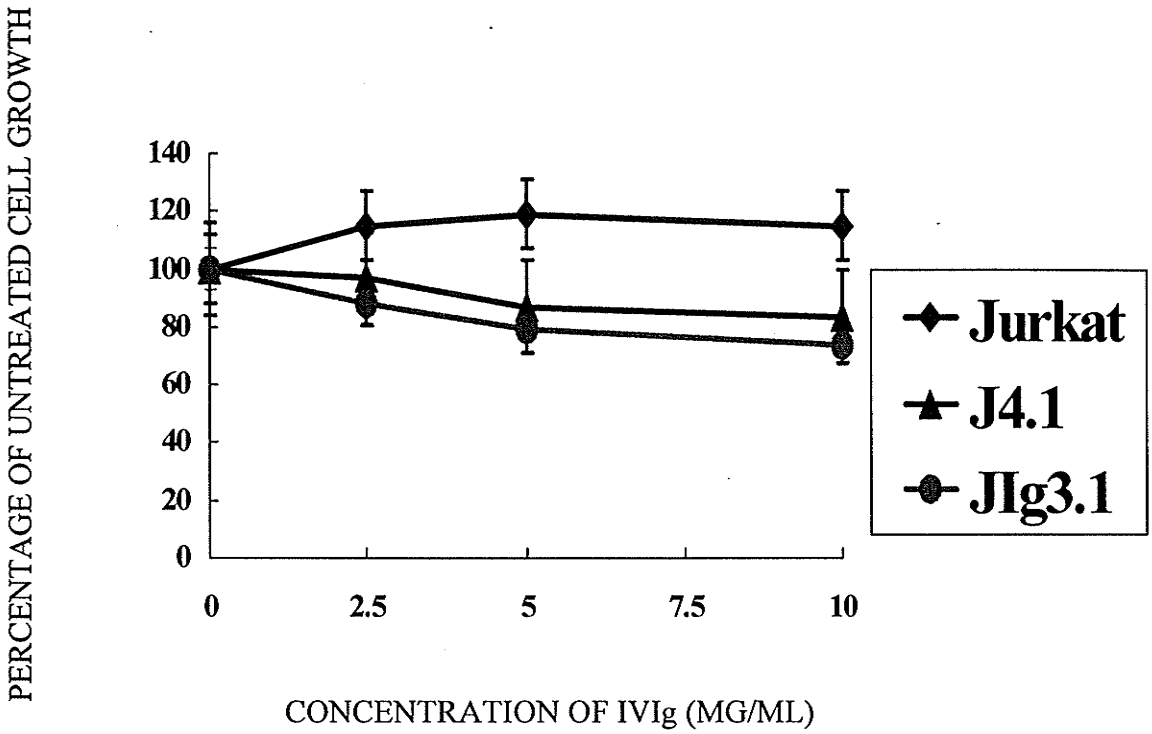


Fig.II. IVIg showed different effects on the growth of Jurkat parental and the selected high IVIg-binding J4.1 and JIg3.1 cells. IVIg significantly inhibited the cell growth of J4.1 and JIg3.1 in a dose-dependent fashion. In contrast, the cell growth of the parental Jurkat was increased with IVIg present in the same concentration range.

with the fact that JIg3.1 could bind the most IVIg among the three cell lines, the cell growth of JIg3.1 was inhibited by IVIg to a greater extent than was J4.1. In contrast, stimulatory effects were observed on the growth of the parental Jurkat cell, the lowest IVIg binding cell line among the three models. Thus, the high IVIg-binding cells were more sensitive to IVIg growth control in vitro, indicating that the inhibitory effects of IVIg correlated positively with the ability of the cells to bind IVIg.

III.4. Impact of IVIg on cell elimination in vivo

The impact of IVIg on cell elimination of high IVIg-binding JIg3.1 and parental Jurkat cells was assessed in immune deficient SCID mice by comparing the elimination of cells from mice pretreated with IVIg versus mice treated with PBS. Within 24 hours after i.v. injection of ^{131}I-dUrd-labelled Jurkat or JIg3.1 cells, whole body gamma counting revealed that mice pretreated with i.v. IVIg showed a similar trend toward increased elimination of both Jurkat and JIg3.1 cells compared to control mice pretreated with PBS (Table VII and Figure III a and b). In groups of mice challenged with JIg3.1, mice pretreated with IVIg demonstrated a statistically significant faster elimination of JIg3.1 cells within 2 hours with less retention of JIg3.1 at 72% compared to 94.7% retained in the PBS treated control mice. However, IVIg pretreatment lead to only a slight decrease in ^{131}I-dUrd-labelled Jurkat cell retained at 84.4% compared to that of control mice at 90.6%. This data suggests that

TABLE VII ELIMINATION OF ^{131}I-dUrd-LABELED JURKAT AND JIg3.1 CELLS IN VIVO

Pretreatment	Cells	Mean Percentage Retained Radioactivity ± SE [a]				
		0	2 h	4 h	12 h	18 h
PBS	JIg3.1	100	94.7±1.8	57.0±6.7	28.1±1.4	16.7±1.0
IVIg	JIg3.1	100	71.8±4.5 *	55.4±5.5	22.5±3.0	17.7±2.3
PBS	Jurkat	100	90.6±3.3	80.2±3.3	34.7±2.9	25.5±2.2
IVIg	Jurkat	100	84.4± 4.3	72.4±4.0	33.1±3.5	21.0±1.2

[a] Male immune deficient SCID mice, 4-6 weeks old were employed in two complete experiments each with 4 mice in each group. Aliquots of 1.5×10^5 ^{131}I-dUrd-labelled cells were injected i.v. and whole body retained radioactivity assayed at the indicated time points was expressed as a percentage of cpm at time 0 immediately after the cell injection.

* $P_{ti} < 0.02$ compared with control mice pretreated with PBS.

FIGURE III a IMPACT OF IVIg ON ELIMINATION OF IV JIg3.1

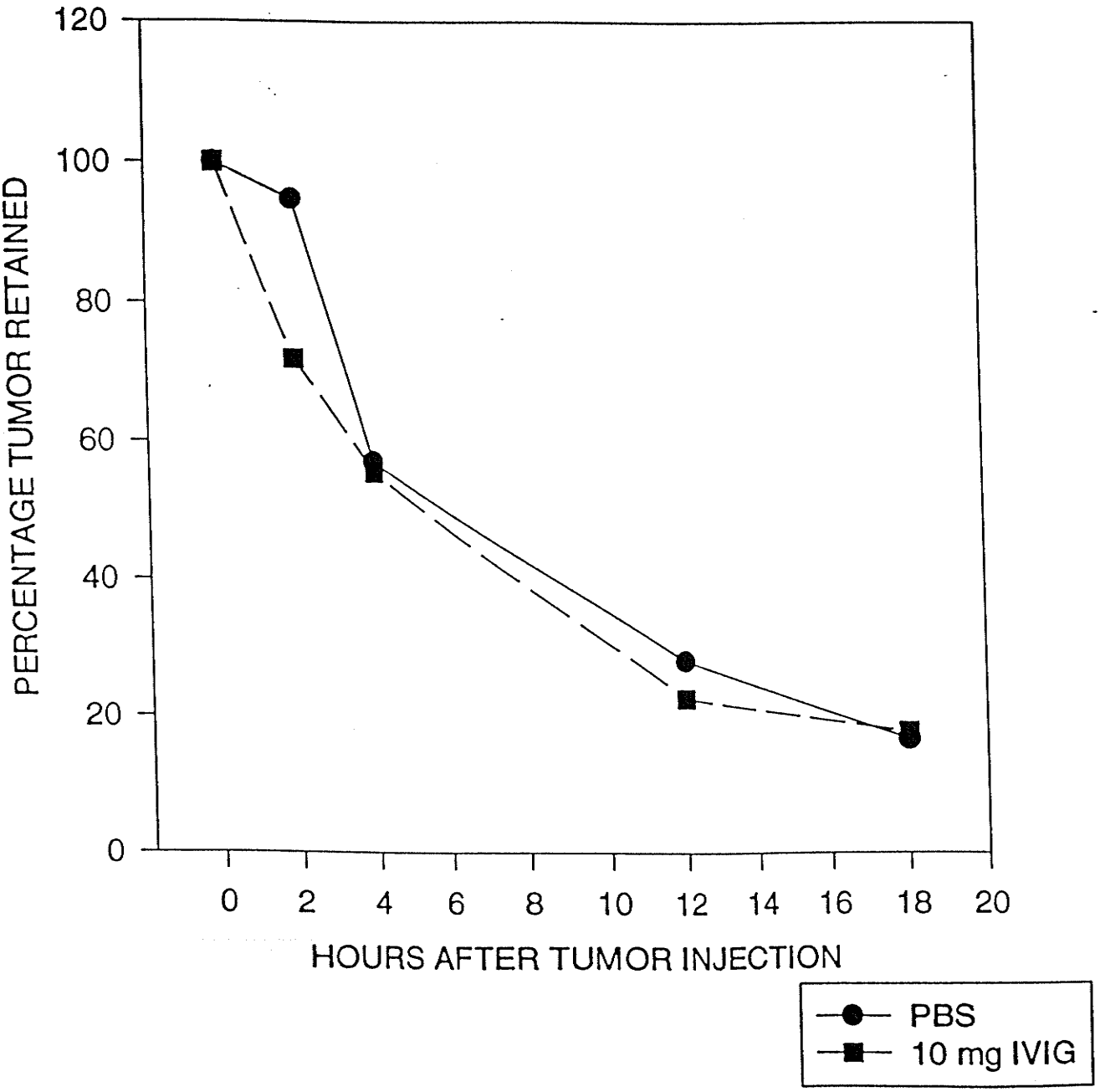
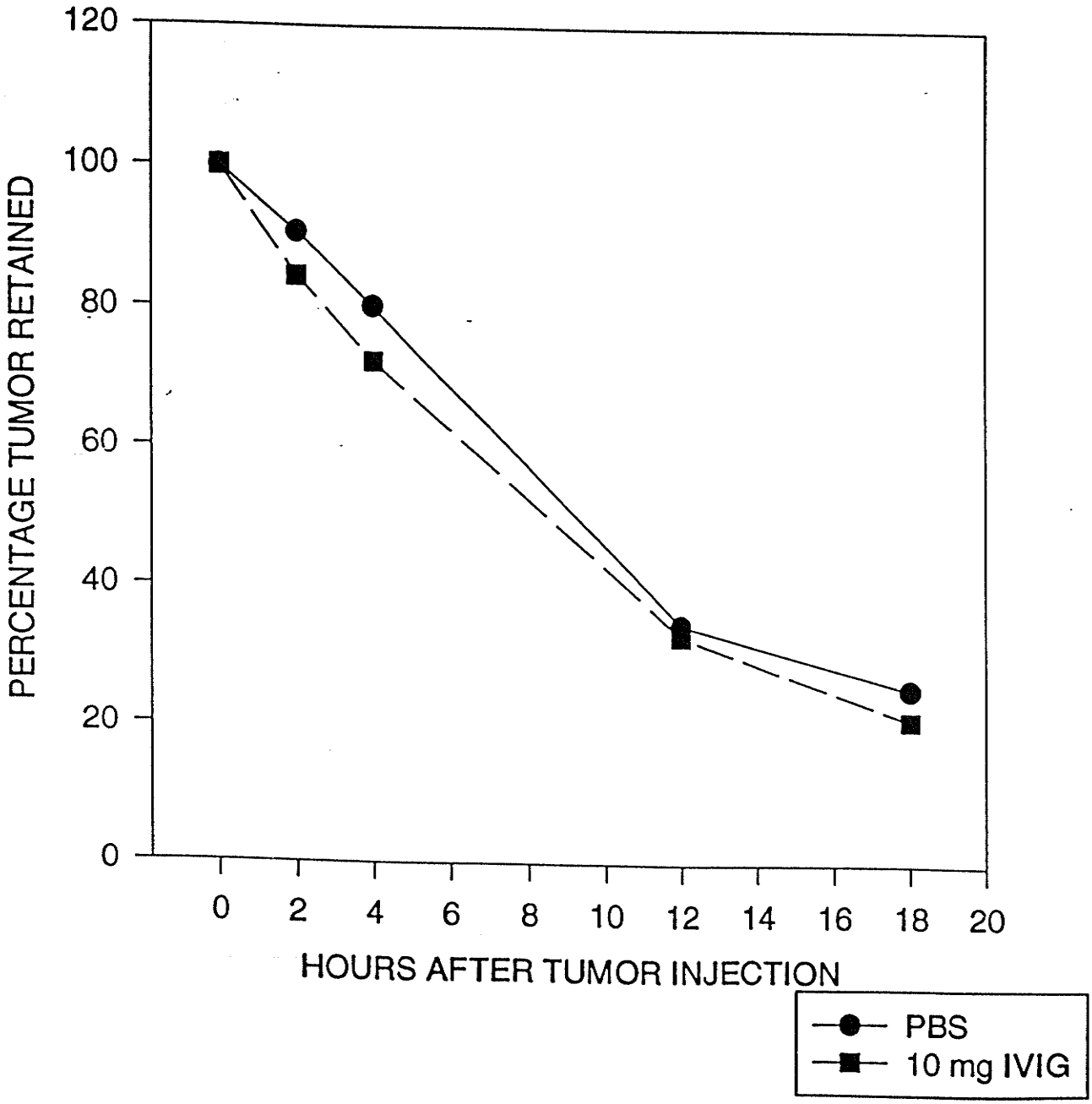


FIGURE III b IMPACT OF IVIg ON ELIMINATION OF IV JURKAT



the impact of IVIg on JIg3.1 elimination in vivo was greater than on the parental Jurkat elimination which indicated IVIg may be important for the quick elimination of rapidly proliferation target cells.

III.5. High IVIg-binding variant expression of cell surface markers

Having shown that the two selected J4.1 and JIg3.1 cells exhibited an increased ability to bind IVIg at several concentrations, these two stable, high IVIg-binding variants were used as human T cell models to investigate the possible T cell surface targets of IVIg which would be increased consistent with their IVIg binding. Previous results from our lab using murine systems showed that high NAb binding was consistent with the increased expression of T cell activation-associated molecules including CD25, CD45RA and CD45RC (Zhang and Chow, 1997). The high m.w. CD45RA is a transient marker of T cell activation (Deans et al., 1992) and CD45RC is another marker increased in expression on both $CD45RC^{+}$ and RC^{-} T cells upon T cell activation (Nishimura et al., 1992). Thus, to identify the possible cell surface molecules involved in the effects of IVIg on human T cells, we focused on surveying the expression of several T cell activation markers on the parental and selected cells. It was hypothesized that surface markers which were more highly expressed on the selected high IVIg binding cell phenotypes most likely would be the targets recognized by IVIg. Accompanying the increased IVIg binding by J4.1 and JIg3.1, the expression of CD25 (IL-2R α chain) and CD134 which are T cell activation markers that appear about 1 day post activation (Muller-Suur et al., 2000 and Stuber

and Strober, 1996) were increased (Table VIII). As well, the expression of HLA-class I and HLA-class II, two T cell activation-related molecules, were increased. Among the above increased activated T cell markers, there was not much binding of anti-HLA class II mAb 9-49 to the selected high IVIg-binding cells. It might that the anti-HLA-DRDPDQ mAb 9-49 we used in our assay did not function very well. Alternatively, the low binding of anti-HLA class II to Jurkat parental and the two selected high IVIg-binding variants might be the result of a lack of HLA class II expression on Jurkat cells (Saifuddin et al., 2000).

In contrast, both J4.1 and JIg3.1 showed a decrease in the binding of anti-CD3 mAb UCHT-1, which is considered to be a pan-reactive T cell marker. The detection of a very early activation marker CD69, a surface marker that is highly expressed about 6 hours after activation, was decreased on both J4.1 and JIg3.1 cell surfaces. In addition, the apoptosis receptor Fas, a very much later activation marker of T cells were decreased (Table VIII). In general, Jurkat cells are considered as Fas-expressing T cells and have been used as targets for killing by cells with FasL (Shiraki et al., 1997; Strand et al., 1996 and O'Connell et al., 1996). Thus, Jurkat may not express FasL consistent with our inability to detect FasL on any of the three cell lines. CD122 on the IL-2R β chain is another component required to form the high affinity human IL-2 receptor in addition to IL-2R α (Reem et al., 1992). Unlike IL-2R α which is expressed only on activated T cells, the IL-2R β chain is expressed constitutively on a subpopulation of resting T cells (Gunes et al., 1993). However, the anti-CD122 antibody we employed in this study did not exibit a good binding to any of the three

TABLE VIII BINDING OF MAbS AGAINST NORMAL AND ACTIVATED T-CELL MARKERS TO Ig-SELECTED JURKAT CELLS

MAbs	Specificity	Dil'n/ Conc'n	Mean Channel Fluorescence ± SE[a] Jurkat	J4.1	% Increase	JIg3.1	% Increase
UCHT-1	CD3	1/10	202.9±9.2	59.8±2.3 *	-71	175.2±11.4	-14
MOPC21(IgG_1)	Isotype control	1/10	17.6±l3.6	21.0±4.6	(6)	16.0±3.6	(6)
G46-2.6	HLA-A,B,C	2.4 μg/ml	149.3±7.9	205.2±12.3 *	37	246.1±15.7	68
MOPC21(IgG_1)	Isotype control	2.4 μg/ml	5.0±0.38	7.5±0.43	(3)	4.8±0.35	(3)
9-49	HLA-DRDPDQ	5.6 μg/ml	47.6±5.4	63.9±10.9	154	50.3±8.3	44
U7.27(IgG_{2a})	Isotype control	5.6 μg/ml	45.2±6.5	57.8±10.0	(3)	45.9±5.7	(3)
M-A251	CD25(IL-2Rα)	5.0 μg/ml	62.2±3.1	73.9±7.1	89	68.9±3.5	68
MOPC21(IgG_1)	Isotype control	5.0 μg/ml	53.4±3.0	57.2±4.0	(5)	54.1±4.2	(5)
Mik-β2	CD122(IL2Rβ)	50 μg/ml	53.1±5.4	55.0±5.3	-160	52.1±5.9	-155
UPC-10($IgG_{2a}κ$)	Isotype control	50 μg/ml	49.1±5.4	57.4±6.1	(5)	54.5±8.1	(5)
FN50	CD69	0.6 μg/ml	180.8±4.8	119.7±12.4 *	-47	74.5±7.6	-70
MOPC21(IgG_1)	Isotype control	0.6 μg/ml	38.0±2.1	43.9±2.3	(4)	32.0±1.3	(4)
ACT35	CD134	2.0 μg/ml	110.8±7.2	185.5±11.4 *	113	129.0±12.0 *	40
MOPC21(IgG_1)	Isotype control	2.0 μg/ml	47.1±1.5	49.7±3.0	(4)	39.6±2.8	(4)
DX2	FAS	2.0 μg/ml	434.1±1.5	372.3±16.5	-14	239.5±9.7	-45
MOPC21(IgG_1)	Isptype control	2.0 μg/ml	66.8±5.2	69.1±7.5	(3)	54.8±3.6	(3)
NOK-1	FASL	1,2,5,10 μg/ml	66.4±0.3	80.0±0.7	0	53.8±2.1	0
		0μg/ml	66.9	78.7	(1)	52.1	(1)

[a]Numbers of experiment are shown in parentheses. Increases were calculated after subtraction of the MCF for the isotype control. Changes in expression were presented as the percentage of each mAb binding to Jurkat. * P_{td} <0.05 versus Jurkat.

cell lines. It might be caused by the Mik-β2 anti-CD122 antibody not working well. To assess the changes in expression of CD45 isoforms related to the increased IVIg binding of our selected cell lines, we compared the expression of the various CD45 isoforms on the parental and the selected high IVIg-binding variants. The data demonstrated that the increases in IVIg binding of J4.1 and JIg3.1 were accompanied by remarkably increased expression of the higher m.w. CD45 isoforms. CD45RA, marker of naïve T cells which undergoes a transient increase in expression about 1 day after T cell activation, was increased approximately 2 and 4 fold on J4.1 and JIg3.1 respectively (Table IX). Additionally, the binding of anti-CD45RB and anti-pan CD45 mAbs were shown to be increased by 67% and 37% respectively for J4.1 and, in the case of JIg3.1, the increases were 24% and 44% respectively. Interestingly, the binding of mAb against CD45RO, the low m.w. CD45 isoform which characterizes memory T cells was unchanged on J4.1 cells, while there was a slight decrease in the binding of this antibody to JIg3.1 cells. The disparity in the changes in CD45RA and CD45RO expression in the selected high IVIg-binding variants, together with the increased expression of the T cell activation markers unregulated 1 to 2 days after activation including CD25 and CD134 argues that IVIg preferentially binds to and regulates the high density, $CD45RA^{+}$ early stage activation T cells, and is not likely to bind CD45RO memory T cells.

Finally, the decrease in expression of the pan-reactive T cell marker CD3, as well as the decreases in CD69, CD95 and CD122 detection suggested that the observed increases in the expression of early activated T cell markers, CD25, CD45RA and

TABLE IX BINDING OF ANTI-CD45 MABS TO Ig-SELECTED JURKAT CELLS

		Mean Channel Fluorescence ± SE [b]				
MAbs [a]	Specificity	Jurkat	J4.1	% Increase	JIg3.1	% Increase
HI100	CD45RA	34.7±9.1	82.2±20.1*	172	133.6±28.5*	374
MOPC141(IgG_{2b})	Isotype Control	8.3±2.9	10.3±3.4		8.4±2.9	
BRA-11	CD45RB	61.5±13.8	100.1±27.2*	67	74.8±17.5	24
MOPC 21(IgG_1)	Isotype Control	7.1±2.4	9.1±3.0		7.5±2.5	
UCHL1	CD45RO	38.9±8.9	42.6±12.1	0	35.7±10.2	0
UPC 10(IgG_{2a})	Isotype Control	7.7±2.6	10.1±3.3		7.3±2.5	
BRA-55	Pan CD45	202.7±39.4	278.1±49.1*	37	281.4±52.6	44
MOPC 21(IgG1)	Isotype Control	7.1±2.5	9.7±3.2		7.4±2.6	

[a] All experiments were performed five times in two step assays. Increases were calculated after subtraction of the MCF for the isotype control and compared to that of Jurkat. * $P_{td} < 0.05$ versus Jurkat.

CD134 were not simply the result of selection for cells larger than parental cells.

III.6. High IVIg-binding variant expression of CD45 isoforms

The flow cytometry data indicated that the two selected high IVIg-binding variants J4.1 and JIg3.1 bound more of the mAbs against high m.w. CD45 isoforms. To confirm the different changes in CD45 isoform expression between parental and the selected cell lines, we used whole cell lysates of Jurkat, J4.1 and JIg3.1 cells for western blot analysis. The lysates were fractionated by SDS-PAGE and the proteins were transferred to PVDF membrane. Immunoblotting with anti-pan CD45 mAb Bra-55 which recognized all isoforms of CD45 revealed clear differences in the CD45 isoform profiles of these three cell lines consistent with the flow cytometry data. Studies of others have demonstrated that the Jurkat cell line expressed a markedly low level of the largest isoforms which contain exon A (McKenney et al., 1995). In agreement with that, our data showed the parental Jurkat cell line expressed predominantly low molecular weight CD45 isoforms around 180-190 KD; while both the selected J4.1 and JIg3.1 expressed a new band at the range from 200 to 220 KD, which represented markedly more of the higher molecular weight isoforms of CD45 (Figure IV). The density of the bands for each cell line showed a loading dose-dependent increase. Considering the flow cytometry data which revealed increased expression of CD45RA and CD45RB determinants in the selected cells, the more predominantly expressed higher m.w band on the selected cell lines may be the result of increased expression of CD45RA and RB. Previous observations that CD45RA and

FIGURE IV WESTERN BLOT DETECTION OF CD45

Immunoblotting of lysates from J, J4.1 and JIg3.1 with anti-pan CD45

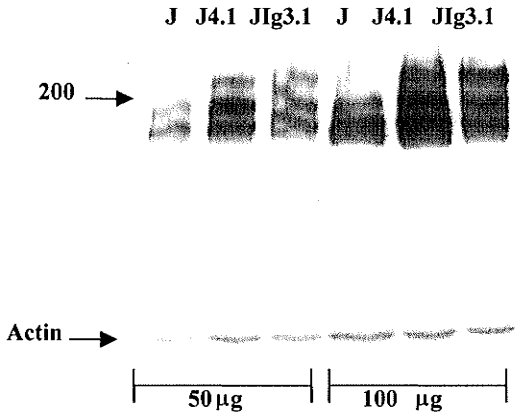


Fig. IV. The comparison of CD45 isoform expression between the Jurkat parental (J) and the selected high IVIg-binding J4.1 and JIg3.1. The result was one of six independent experiments which produced similar observations.
The position of Myosin at 200 kD of molecular-weight standard is indicated on the left side of the figure.
Detection of actin binding served as a control for equal protein loading.

CD45RC were increased in association with more NAb reactivity with selected murine LYNAb$^+$ cells, suggests that CD45RC may also contribute as well in the Jurkat system.

III.7. IVIg inhibition of anti-CD45RA binding to the cell surface

In previous studies, extensive efforts had been made in the lab to identify the NAb-binding surface molecules using several approaches employing immunoprecipitation in the murine T cell models, but we found that all these approaches were not useful because of the low affinity of NAb binding. Even though the flow cytometry results showed good binding to the high binding variants, the procedure of lysing the cell surface may easily break the weak binding between IVIg and the cell surface targets of IVIg. Thus, to assess the specificity of the surface molecules that IVIg recognized, we chose the approach of IVIg inhibition of binding to cells by mAbs against CD45 isoforms. In this approach the cells were first incubated with IVIg for 1 hour to ensure formation of stable binding, and then, the mAbs against CD45RA, CD45RB, CD45RO or pan-CD45 were added. The binding of IVIg to their targets on the cell surface should inhibit the similarly targeted mAbs from recognizing their ligands. The comparison of the inhibition of IVIg among the different mAbs would suggest the most likely cell surface targets that IVIg binds.

In this approach, use of our standard optimal mAb concentrations revealed little or no IVIg inhibition. Thus, it was critical to employ sub-optimal concentrations of the mAbs, and the same percentage of optimal binding was chosen for each mAb to

FIGURE V TITRATION OF MABS OF CD45 ISOFORMS

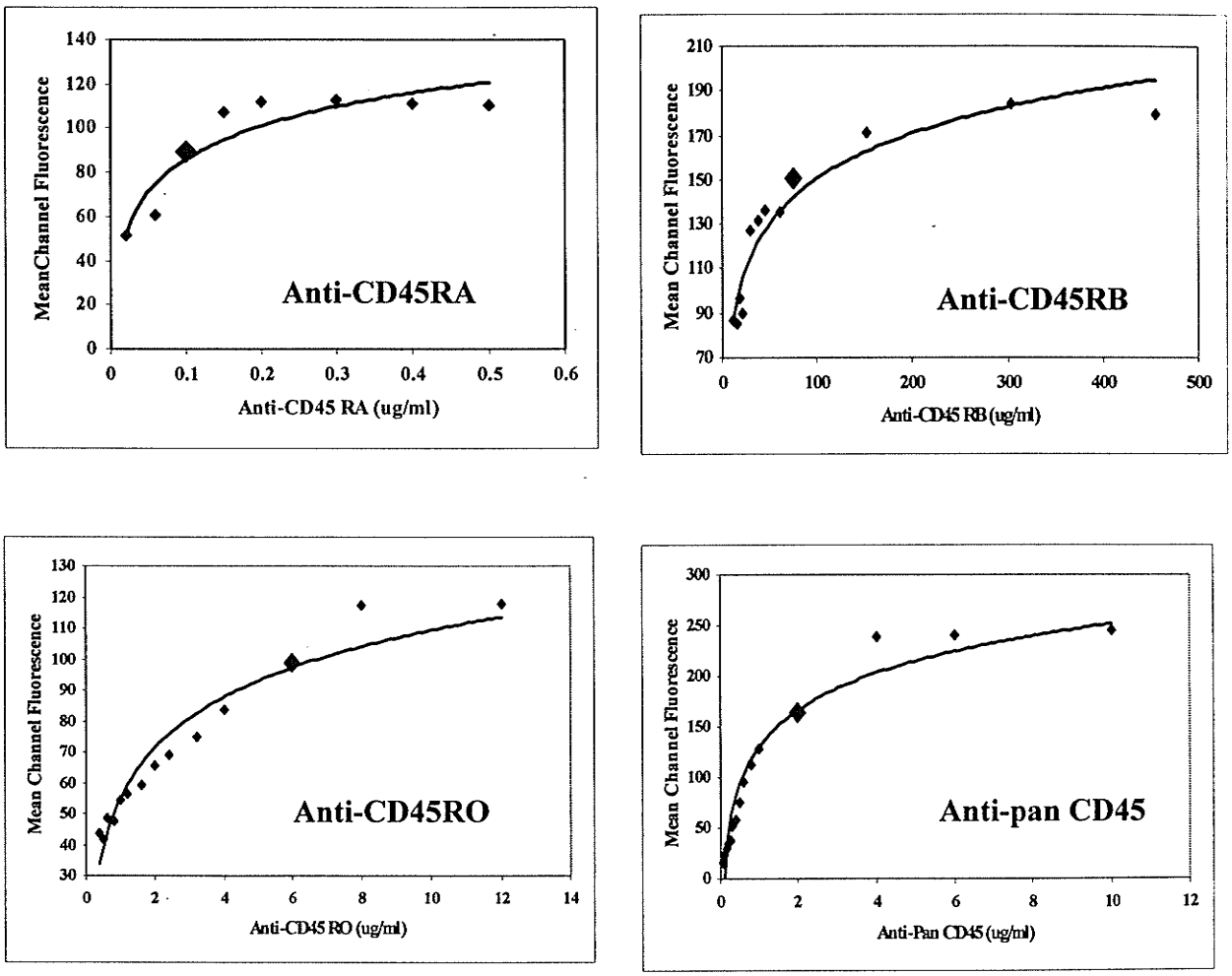


Fig.V The standard mAb binding method with JIg3.1 was used to titrate the binding of the monoclonal anti-CD45RA, -CD45RO and -pan-CD45 antibodies. J4.1 was used to titrate anti-CD45RB. The binding curves ranging from the minimal binding to the plateau were prepared using the Excel program and the concentrations of mAbs that showed 80% of optimal binding at standard conditions were used for the inhibition experiments (♦).

examine IVIg inhibition of binding to their ligands. The titrations of individual mAbs against CD45RA, CD45RB, CD45RO and pan-CD45 were performed using our standard mAb binding method and the final concentrations chosen for the inhibition experiments were 0.1, 76, 6 and 2 μg/ml respectively, which exhibited 80% of optimal binding for each individual mAb (Figure V).

In this assay JIg3.1 were the primary cells used for the inhibition of anti-CD45RA, -CD45RO and -pan-CD45 mAbs because JIg3.1 were the highest binding cells for anti-CD45RA and anti-pan CD45 and binding of anti-CD45RO was similar on all three cell lines. Being the highest anti-CD45RB binding cell line, J4.1 was used for the anti-CD45RB inhibition experiments.

The data showed that IVIg at 10 mg/ml inhibited 33% of HI100 anti-CD45RA mAb binding, slightly inhibited Bra-11 anti-CD45RB mAb binding at a 5% reduction and inhibited 35% of Bra-55 anti-pan CD45 mAb binding (Table X). Interestingly, instead of reducing UCHLI anti-CD45RO binding to CD45RO after incubation of the cells with IVIg, the bind of anti-CD45RO showed a 41% increase when IVIg was present. This was possibly due to a better exposure of the short isoform of CD45RO after IVIg cross-linking and pulling away of the high m.w CD45 isoforms.

III.8. Effects of NM on binding by IVIg and anti-CD45 mAbs

Earlier studies by our lab in the murine system revealed that NAb binding epitopes could be carbohydrates masked by sialic acid (Reese and Chow 1992). Sialic acid was reported to exist on the glycoproteins of almost all cell surfaces (Narayanan et al.,

TABLE X IVIg INHIBITION OF CELL SURFACE BINDING BY ANTI-CD45MABS

Cell	MAbs [b]	Specificity	Mean Channel Fluorescence±SE [a] RPMI Control	IVIg (10 mg/ml)	% Inhibition [c]	P_{td}
JIg3.1	HI100	RA	102.6±15.3	89.0±13.7	33 (3)	<0.02
	MOPC 141(IgG_{2b})		31.3±3.2	33.4±3.6		
J4.1	BRA-11	RB	126.9±4.8	120.7±5.0	5 (6)	NS
	MOPC21($IgG_{1\kappa}$)		7.0±0.6	7.6±0.6		
JIg3.1	UCHL1	RO	73.8±3.2	83.0±4.6	- 41(5)	<0.01
	UPC10(IgG_{2a})		45.3±2.8	43.6±2.5		
JIg3.1	BRA-55	PAN	73.0±6.5	50.5±4.1	35 (6)	<0.01
	MOPC21($IgG_{1\kappa}$)		8.5±0.7	9.3±0.6		

[a] The binding of CD45 mAbs to cells with/without IVIg present were calculated after subtraction of MCF for the isotype control. Inhibition is expressed as a percentage of mAb binding to cells without IVIg present.

[b] MAb concentrations were 0.1-76 ug/ml, 80% of optimal binding at standard conditions.

[c] The number of assays performed is indicated in parenthesis.

NS not significant

1994). Considering the fact that high m.w. CD45 isoforms, the possible surface targets of IVIg are highly glycosylated proteins, we investigated the effect of enzymatic digestion of sialic acid on the cell surface epitopes recognized by IVIg and anti-CD45 mAbs by treating cells with NM to remove the terminal sialic acid (Schlamowitz et al., 1975). Jurkat parental cells were used in these assays because consistent with their lower IVIg binding ability, they may express more sialic acid on the cell surface. Thus, any changes observed when the cells were treated with NM should be more obvious. The efficacy of NM pretreatment was first tested by examining the lectin binding ability of NM treated cells. Flow cytometry assessment of the binding by FITC-conjugated PNA lectin, which is specific for D-Gal, D-Gal/Nac, showed a significant 14.5 fold increase in lectin binding to the neuraminidase treated cells compared to non-treated controls (Table XI). The binding of IVIg to Jurkat cells also showed a significant increase of 135% after the removal of sialic acid by NM treatment, which suggested that IVIg preferentially bound to asialo forms and that sialic acid could mask the epitopes recognized by IVIg. Consistent with the increases in IVIg binding after cells were pretreated by NM, the binding of anti-CD45RA HI100 and anti-pan CD45 BRA-55 on NM treated cells exhibited increases of 11% and 25%, respectively (Table XI). This suggested that, similar to IVIg binding, the epitopes recognized by HI100 and BRA-55 were masked by sialic acid. NM treatment may change the conformation of glycosylated CD45 by removal of highly negatively charged sialic acid. This may facilitate anti-CD45RA, anti-pan CD45 and IVIg binding to the asialo forms of cell surface molecules. On the other

TABLE XI BINDING OF PNA, IVIg AND ANTI-CD45 ISOFORM MABS TO NEURAMINIDASE TREATED JURKAT CELLS

Lectin MAbs	Specificity	Conc. (ug/ml)	Cell Growth (days)	Mean Channel Fluorescence ± SE – NM	Mean Channel Fluorescence ± SE + NM	% Increase	Ptd [a]
FITC-PNA	Gal,GalNAc	10	1 or 2	25.0±1.8	387.8±18.5	1450	<0.01 (9)
IVIg	IgG	5000	1 or 2	152.3±12.8	339.2±41.0	135	<0.02
RPMI 1640	Medium control	0	1 or 2	41.9±3.2	79.9±4.2		(9)
HI100	CD45RA	0.2	1 or 2	97.1±7.4	107.4±7.2	11	<0.08
MOPC141(IgG_{2b})	Isotype control	0.2	1 or 2	36.6±0.4	44.8±1.1		(9)
BRA-11	CD45RB	152	1 or 2	122.4±3.7	9.5±0.5	-99	<0.01
MOPC-21(IgG_1)	Isotype control	152	1 or 2	6.0±0.2	9.0±0.6		(5)
UCHL1	CD45RO	8.0	1 or 2	389.8±17.9	64.4±2.5	-99	<0.01
UPC10(IgG_{2a})	Isotype control	8.0	1 or 2	54.3±1.3	61.8±1.8		(5)
BRA-55	Pan CD45	4.0	1 or 2	455.7±7.2	566.8±7.9	25	<0.01
MOPC 21(IgG_1)	Isotype control	4.0	1 or 2	11.1±0.9	13.4±0.4		(5)

[a] Changes were calculated after subtraction of the MCF for the isotype controls and expressed as a percentage of binding to cells without NM treatment.

[b] Numbers of experiment were shown in parentheses.

hand, NM treatment of Jurkat cells resulted in almost complete elimination of anti-CD45RB BRA-11 and anti-CD45RO UCHL1 binding (Table XI) suggesting that their binding depended largely on the presence of sialic acid, and that removal of negative charges per se, did not necessarily lead to an increase in mAb binding. In agreement with our observations, previous studies by other groups reported that anti-CD45RB and anti-CD45RO mAbs recognized an epitope dependent on sialic acid structure of O-linked sugars on CD45 (Pulido et al., 1990 and 1989). In contrast, CD45RA and pan-CD45 epitopes were resistant to NM digestion, whereas the CD45RB and CD45RO epitopes disappeared (Pulido et al., 1989).

In the initial experiments, the binding data derived from the cells grown for 1 day and 2 days in culture were pooled because we did not expect that there would be any difference between them. When we did the statistical analysis we found that NM treatment caused a different increase in the binding of CD45RA between the cells grown for 1 day and 2 days. However there was no different increase in anti-pan CD45, anti-CD45RB and anti-CD45RO binding between 1 day and 2 days of cell growth. To determine whether the observed difference in anti-CD45RA binding was significant or not, more parallel experiments were performed on cells grown for 1 and 2 days. The data showed (Table XII) that the increases in lectin, and IVIg binding were only slightly different after 1 and 2 days growth. Interestingly, after NM treatment, the increase in CD45RA binding by 26% in the cells grown for 2 days was more significant than the 1% increase exhibited by cells grown for 1 day.

TABLE XII BINDING OF PNA, IVIg AND ANTI-CD45RA TO NEURAMINIDASE TREATED JURKA T CELLS (1 vs 2days) [a]

Lectin MAbs	Specificity	Conc. (ug/ml)	Cell Growth (day)	Mean Channel Fluorescence ± SE [b] – NM	Mean Channel Fluorescence ± SE [b] + NM	% Increase	Ptd
FITC-PNA	Gal,GalNAc	10	1	25.3±3.1	375.8±30.7	1390	<0.01
IVIg	IgG	5000	1	146.5±17.1	285.5±52.9	99	<0.03
RPMI 1640	Medium control	0	1	39.7±3.0	73.0±5.9		
HI100	CD45RA	0.2	1	143.8±9.5	152.2±12.2	1.0	<0.8
MOPC141(IgG_{2b})	Isotype control	0.2	1	36.7±0.6	44.3±1.6		

Lectin MAbs	Specificity	Conc. (ug/ml)	Cell Growth (days)	Mean Channel Fluorescence ± SE [c] – NM	Mean Channel Fluorescence ± SE [c] + NM	% Increase	Ptd
FITC-PNA	Gal,GalNAc	10	2	24.6±1.4	402.8±18.4	1540	<0.01
IVIg	IgG	5000	2	144.7±21.5	297.3±50.4	109	<0.01
RPMI 1640	Medium control	0	2	44.6±6.6	88.5±2.1		
HI100	CD45RA	0.2	2	121.0±9.1	152.3±9.1	26	<0.04
MOPC141(IgG_{2b})	Isotype control	0.2	2	36.5±0.5	45.4±1.5		

[a] Cells grown for 1 day were set at 3.5 $\times 10^5$/ml. Cells grown for 2 days were set at 2.0×10^5/ml. Changes were calculated after subtraction of the MCF for the isotype controls and expressed as a percentage of binding to cells without NM treatment.
[b] Four experiments were performed. [c] Five experiments were performed.

Additionally, the results of the parallel experiments showed the binding of anti-CD45RA at MFC of 121.0 on cells grown for 2 days was less than that of cells grown for 1 day at MCF of 143.8. This difference between 1 and 2 days growth may be caused by differential expression of sialic acid by cells at different stage of growth. It has been well documented that CD45 glycoprotein sialylation is tightly regulated during T cell development and maturation and the process of sialylation is dependent on the activity of related sialyltransferase enzymes (Baum et al., 1996). As thymocytes were maturing, more sialylated forms of CD45 were expressed and mature thymocytes exhibited fully sialylated CD45 (Whiteheart et al., 1990). Considering these observations, we proposed that in our situation, changing fresh medium during the cell culture probably to a certain extent started a new cell growth cycle due to differences in pH, metabolites, nutrition and other growth-related ingredients. This may cause a different activation level of sialyltransferase which results in different expression of sialic acid on CD45RA between cells grown for 1 and 2 days. The cells grown for 2 days may bear more sialic acid, thus, anti-CD45RA binding may be lower and neuraminidase treatment may cause more change in binding of anti-CD45RA compared to cells grown for 1 day which had less sialic acid.

III.9. IVIg binding to peripheral blood T cells

Evidence of IVIg providing a benefit against the autoimmune disease ITP has been well documented since the early 1980's. More than one mechanism has been

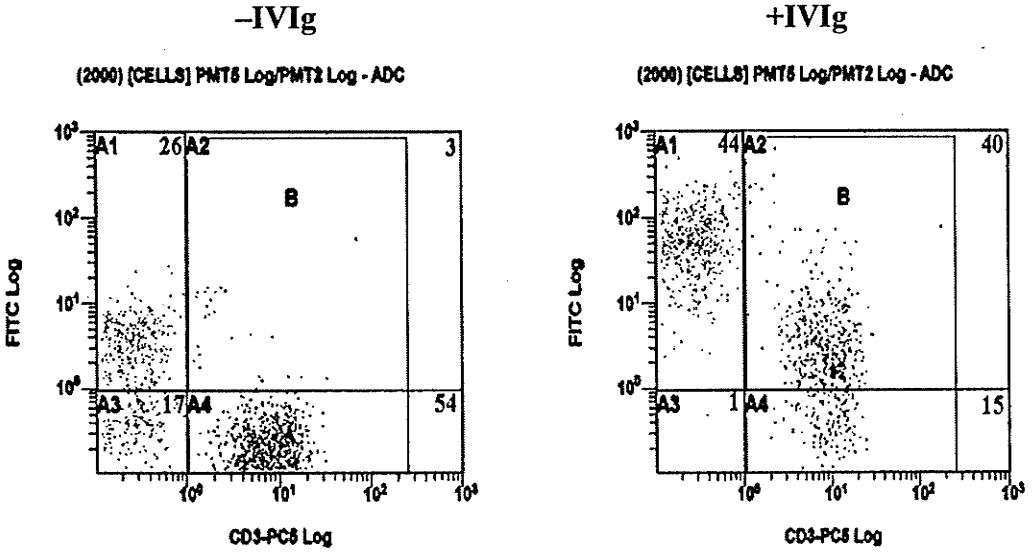
proposed for IVIg action (Ballow et al. 1997 and Mouthon et al., 1996), but how IVIg reduces autoimmune activation in this disease is still unclear. One of the characteristics of ITP patients is an increased number of circulating activated T cells (Garci-Suarez et al., 1993 and Mizutani et al., 1987). Considering our data showed that IVIg preferentially bound to T cells with an early activation phenotype and inhibited cell growth of the higher binding J4.1 and JIg3.1 cells, we assessed the possibility of IVIg affecting the pathogenic activated T cells of ITP patients by comparing the ability of IVIg to bind freshly isolated peripheral blood T cells from ITP patients and normal individuals. We hypothesized that IVIg might bind to more T cells of ITP patients due to high IVIg binding of activated cells and the known increased levels of activated T cells in ITP. To do this we developed a protocol to compare the binding of IVIg by isolated peripheral blood T cells in vitro. A comparison was made between the proportions of these cells with IgG already bound to their surface before isolation. Additionally, to imitate therapeutic exposure to IVIg and stabilize all possible binding, the cells were incubated with 10 mg/ml IVIg at 4°C, which is within the concentration range of circulating IVIg achieved for therapeutic doses of 0.4-2 gm/kg used in clinical treatment (Prasad et al., 1998; Van Schaik et al., 1992 and Kawada et al., 1987). In this protocol, freshly isolated PBMC were stained with PC_5-anti-human CD3 plus FITC-anti-human IgG and analyzed by two color flow cytometry. The results (Table XIII, Figure VI) demonstrated that approximately 6.7% of lymphocytes from normal individuals were CD3 positive as well as anti-human IgG positive, equivalent to 9.6% of the T cells. After exposure to IVIg, the proportion

TABLE XIII IVIg BINDING TO PERIPHERAL T CELLS OF NORMAL INDIVIDUALS AND ONE ITP PATIENT

MAbs	% Ig Positive T Cells (Of Total Lymphocytes) [a]							
	Normal People [b]				ITP Patient [c]			
	-IVIg	Δ	+IVIg	Δ	- IVIg	Δ	+ IVIg	Δ
FITC-goat anti-human IgG + PC_5-anti-CD3	10.7±2.5 (7.4±1.9)	9.6±2.4 (6.7±1.6)	75.7±2.4 (55.3±3.1)	74.7±2.2* (54.6±2.8)	31.3 (19.8)	17.5 (11.1)	93.3 (68.0)	90.1 (65.4)
FITC-goat IgG+PC_5-anti-CD3	1.1±0.2 (0.7±0.2)	---	1.0±0.5 (0.7± 0.3)	---	12.8 (8.1)	---	3.8 (2.6)	---

[a] Numbers indicated in parentheses are the percentage of total lymphocytes which are Ig positive T cells.
[b] Ten experiments were performed.
[c] One experiment was performed.
Δ The numbers were calculated after subtraction of the percentage of positive cells for the controls.
*Ptd < 0.001 versus IVIg untreated cells.

FIGURE VI IgG BINDING TO PERIPHERAL BLOOD T CELLS TREATED WITH OR WITHOUT IVIg



FITC-α -huIgG+ PC_5- α -CD3

Fig.VI showed staining for representative examples of health donor PBMC (n=9). Cells were gated on lymphocytes by FSC and SSC parameters. The A2 quadrant describes $CD3^+IgG^+$ cells. FITC-anti-human IgG detection of IgG binding to peripheral blood T cells treated with IVIg (+ IVIg) contain higher $CD3^+IgG^+$ cells than untreated peripheral blood T cells (-IVIg).

of lymphocytes that were both $CD3^+$ and anti-human IgG^+ exhibited a surprising increase to 54.6%, amounting to about 75% of the total number of T cells. The results from one ITP patient showed a slight increase in these two populations compared with that of the normals. The $CD3^+$ and IgG^+ double positive cells accounted for 11.7% of all lymphocyte or 17.5% of just the T cells, and after IVIg incubation about 65.4% of the lymphocytes were double positive or 90.1% of the T cells. This data demonstrated that IVIg exhibited a great capacity to bind to peripheral T cells from normal people and possibly ITP patients at 4°C, suggesting that IVIg has a great potential to regulate circulating T cells. We wondered whether this surprising increase of number of T cells binding to IVIg was caused by the low temperature of 4°C inducing T cell apoptosis and increasing non-specific binding to IVIg during the preparation process and overnight storage of the sample at 4°C after labeling and fixing. Some studies reported that a temperature of 4°C would not affect the viability of PBMC subsets because the cell viability showed no significant difference between fresh PBMCs and PBMCs stored for 24 h at 4°C (Paxton et al., 1993; Ekong et al., 1993; Ashmore et al., 1989 and Ponzio et al., 1984). On the other hand, a temperature shift of 4°C/1 hr to 37°C/1 hr did induce obvious cell surface morphology changes (Liepins and Bustamante, 1994).

At the physiological temperature of 37°C, IVIg would likely be constantly binding and releasing from cells, IVIg may preferentially regulate the 35% of circulating T cells that are $CD45RA^+$ naïve T cells, in addition to the transiently increased high m.w. CD45RA isoform-bearing and other IVIg target-bearing, early activated T cells.

CHAPTER IV. DISCUSSION

IV.1 Selection for high-IVIg binding cells correlated with susceptibility to IVIg in vitro and in vivo

J4.1 and JIg3.1 were the two human T cell models used in this study. They had been selected for high human serum IgG plus IgM and high IVIg binding respectively, from the human T leukemia cell line Jurkat treated with TPA. Flow cytometry assessment showed that both selected cell lines exhibited increases in IVIg binding at concentrations of IVIg ranging from 4 to 10 mg/ml compared to the starting Jurkat cells (Tables III and IV). This suggested that the selection procedure generated cell models with augmented IVIg binding and could provide an invaluable tool to investigate the role of IVIg in T cell regulation. When these high IVIg binding cells were cultured with different concentrations of IVIg ranging from 2.5 to 10 mg/ml, the cell growth was inhibited by IVIg in a dose-dependent manner. In contrast, IVIg stimulated the cell growth of the parental Jurkat line at the same concentrations (Table VI and Figure II). Our data indicated a correlation between IVIg binding level and sensitivity to IVIg inhibition of cell growth in vitro. Additionally, cells with different levels of IVIg binding ability showed different susceptibilities to IVIg augmentation of in vivo elimination. SCID mice pretreated intravenously with IVIg before injection of JIg3.1 cells showed a statistically significant increase in elimination of JIg3.1 cells within 2 hrs compared to the control mice pretreated with PBS. Similar IVIg pretreatment led to only a slight increase in Jurkat elimination

compared to that in PBS treated mice, suggesting that the impact of IVIg on JIg3.1 was greater than on Jurkat. Further it suggests, that IVIg augmented elimination of certain types of cells in vivo, was related to the level of IVIg binding to these cells. Previous studies in mice have shown similar increases in cell elimination in vivo using serum NAb which contains normal IgM and IgG. A single intravenous dose of CBA/J serum NAb or ammonium precipitated, dialyzed and reconstituted NAb, injected into B-cell deficient CBA/N mice 2 hrs prior to an intravenous challenge with ^{131}I-dUrd-labelled syngeneic T leukemia cells, caused an increased elimination of the cells versus control saline treatment (Chow 1995). In addition, an intravenous injection of IVIg at 0.1gm/kg given 2 hrs before an intravenous injection of L5178Y-F9 T lymphoma cells reduced the subsequent liver metastasis in syngeneic mice (Chow et al., 1992). Daily doses of 0.8 gm/kg IVIg injected i.p. injection showed the capability of ameliorating the progression of experimental autoimmune myocarditis (EAM) in the rat by reducing the proliferative capacity of antigen-reactive lymphocytes (George et al., 2001). Several reports have demonstrated that IVIg administration benefited animal models with autoimmune and inflammatory conditions through inducing a state of antigen-specific anergy of T lymphocytes (Saoudi et al., 1993) or modulating the production of cytokines by monocytes and T cells in vivo (Pashov et al., 1998; Andersson et al., 1994 and 1993). These data provide more evidence to support the idea that IVIg can affect cell growth in vivo.

IVIg modulation of cell growth in vitro has been extensively studied in the past decade. Van Schaik et al. (1992) used twenty autonomously growing cell lines with

various origins to investigate the effects of IVIg on cell growth, including ten neuroblastoma lines, three B-cell hybridomas, five Epstein-Barr virus-transformed human B-cells, the myelomonocytic cell line HL60, the erthroblastoma cell line K562 and the T cell line Molt 4. At higher concentrations ranging from 15 to 50 mg/ml, IVIg showed an inhibitory effect on cell growth for all of the above cell lines. However, at low concentrations ranging from 0.1 to 10 mg/ml, IVIg accelerated the cell growth of approximately half of the cell lines including all of the hematopietic cells, the B cell hybridomas and half of the neuroblastoma cell lines. In addition to autonomously growing cells, IVIg exerted inhibitory effects on lymphocyte proliferation in vitro induced by phytohemagglutinin (PHA) (Klaesson et al 1993; Van Schail et al. 1992 and Kawada et al. 1987), concanavalin A, pokeweed mitogen, staphylococcus aureus protein A (Van Schaik et al., 1992), TPA with ionomycin (Andersson et al., 1993) and anti-CD3 monoclonal antibody (Amran et al., 1994 and Andersson et al., 1993) and in the mixed lymphocyte reaction (Klaesson et al 1993 and Van Schaik et al., 1992). The effects of IVIg concentration on cellular proliferation of lymphocytes activated by various stimuli were similar to those on autonomously growing cells. At concentrations above 20 mg/ml, IVIg completely inhibited PBMC proliferation regardless of how the cells were activated. However, at IVIg concentrations ranging from 1 to 10 mg/ml, PHA- and purified protein derivatives of *Mycobacterium* (PPD)-induced proliferation of PBMC did not show significant inhibition. Anti-CD3 mAb-induced proliferation was inhibited in a dose-dependent manner at concentrations between 1 and 10 mg/ml (Lee et al. 2001). These

data showed that IVIg has varying effects on cell proliferation in the low concentration range from 1 to 10 mg/ml. Collectively, these results indicate several general characteristics of IVIg regarding the effects on cell proliferation. High-doses of IVIg over 15 mg/ml induced inhibition regardless of the origin of the cells and the way the cells were activated. But, IVIg at lower concentrations, ranging from 0.1 to 10 mg/ml, exhibited varying effects on the growth of different cell lines and cells activated by different stimuli.

The inhibitory mechanisms of IVIg on cell proliferation are at present unknown. One study suggested that high-concentrations of IVIg exert a growth inhibitory effect by arresting the cell cycle in G0/G1 stage (Van Schaik et al 1992). In the murine system developed in our lab, NAb similarly reduced the cell growth of ras-transformed 10T1/2 fibroblast cells, with a higher proportion of cells arrested in the G0/G1 phase (Wang and Chow 2000). More recently it has been reported that high dose IVIg induced cell apoptosis in vitro in leukemic lymphocytes as well as in normal tonsillar B cells. This effect was at least partially dependent on anti-CD95 antibodies present within IVIg preparations (Prasad et al. 1998). Although these mechanisms can reasonably explain the inhibitory effects of IVIg exhibited at the high-concentration ranges, the varying effects IVIg exerts on the growth of different cells of different origin or on the same cells activated with different stimuli, can not be explained completely by these mechanisms.

As a blood product prepared from the serum of over 10,000 health donors, IVIg has been found to contain antibodies specific for a broad range of molecules, which

reflects the antibody repertoire of the thousands of donors included in each batch of commercial preparation. Several components present in IVIg have been reported to regulate cell growth including the activities of the antibodies in IVIg. Anti-interferon-γ (IFN-γ) antibodies in IVIg have been shown to inhibit the production of IFN-γ in vitro, and reduce IFN-γ-mediated T cell proliferation (Toungouz et al., 1995). Several groups found that IVIg contained antibodies against TNF (Abe et al 1994 and Boyle et al 1993). This anti-TNF antibody contributed to increases in cell growth as a result of directly blocking the cytotoxic effect of TNF (Abe et al 1994). Additionally IVIg contains antibodies against CD4 (Hurez et al., 1994) and the interaction of IVIg with CD4 on the cell surface suppressed the mixed lymphocyte proliferation. Moreover, several studies have revealed that IVIg can modulate the production of a number of cytokines that function in cell growth and proliferation. For instance, IVIg reduced IL-2 levels in culture supernatants of IVIg-supplemented mixed lymphocyte reactions (Nachbaur et al 1997). The addition of IVIg to anti-CD3-stimulated cultures markedly inhibited the production of T cell derived IL-2, IL-10, TNF-α and IFN-γ (Andersson et al 1996). These results suggest that IVIg affects cell growth via interactions of various components with different effects. These contributing components can be roughly divided into two groups based on their ability to inhibit or augment cell growth. The inhibitory components include anti-IFN-γ and anti-CD4 antibodies. In contrast, anti-INF-α antibodies present in IVIg may account for the ability of IVIg to reduce the production of TNF-α by monocytes or T cells. This may be the major contributor responsible for IVIg augmenting cell growth by protecting cells from

TNF-α-induced cell death (Menezes et al 1997 and Stangel et al 1997). The balance between inhibitory and enhancing antibodies present in IVIg may be one of the factors determining whether the overall effect is an increase or decrease in cell growth.

In our studies, other factors may contribute to the observed increase in cell growth of parental Jurkat leukemia cells aside from the above outlined components present in IVIg. For example, Jurkat leukemia cells have been reported to produce prolactin, an autocrine growth factor for Jurkat cell growth in vitro (Matera et al., 1997 and O' Neal et al., 1992). Additionally, a human T leukemia cell demonstrated the ability to autonomously produce cytokine G-CSF (Matsushita et al., 2000), IL-2 and IL-15 (Kukita et al., 2002), with all serving as autocrine growth factors. These autocrine growth factors and cell growth enhancing components present in IVIg, such as anti-TNF antibodies, could explain the increased cell growth of the parental Jurkat cell line when IVIg was present in the culture. However, at the same low concentration range, IVIg exerted the paradoxically inhibitory effect on high-IVIg binding variants J4.1 and JIg3.1 and half of twenty cells lines used in cell growth experiments by Van Schaik et al. (1994). This suggests that intrinsic differences between different cell lines exist and were probably the major factor to determine whether inhibitory or enhancing effect of IVIg would be exerted on the individual cells.

Our data demonstrated that high IVIg-binding selected variants were more sensitive to IVIg growth control than the lower IVIg-binding parental cells (Table VI). This indicated that the increased ability of the selected cells to bind IVIg was the

underlying cause of the inhibitory growth effects of IVIg. The increased binding of IVIg is logically connected with the increased expression of cell surface targets of IVIg. Thus, it is possible that different cell lines have intrinsic differences in the expression of the cell surface targets of IVIg. Therefore in this model, the level of expression of specific cell surface targets of IVIg is likely the deciding factor in determining the IVIg effect, ie. whether growth inhibition or enhancement will predominate.

Considering both factors, the intrinsic differences in cell surface target expression and the variety of components present in IVIg, we proposed a model to explain the discrepancy in the effects of IVIg on in vitro cell growth at different concentration ranges. When IVIg is present at a low concentration, the cell growth may be primarily affected by relatively major components of IVIg such as anti-TNF and/or prolactin that lead to augmentation of cell growth. In contrast, the components of IVIg with inhibitory effects on cell growth may form a minor proportion. At the low concentrations, whether IVIg can exert inhibitory effects on cell growth may largely depend on the density of the target molecules expressed on the cell surface. If certain types of cells have more targets of IVIg, the limited amount of specific antibodies with the inhibitory effects in IVIg would still bind efficiently to these cells and might subsequently induce an inhibitory signal strong enough to overcome the cell growth enhancing components. Otherwise, at the lower concentrations, the predominant effect of IVIg will be an acceleration of cell growth probably mediated by a reduction of TNF-α caused by the anti-TNF-α antibodies present in IVIg, or in the case of Jurkat

cells, by the autocrine growth factors secreted by the Jurkat leukemia line. As the concentration of IVIg is increased, the minor components will approach a critical mass. At this point the inhibitory effects of IVIg can finally overcome the enhancing effects and the overall result of IVIg treatment on cell growth may switch to inhibition above this critical concentration. In our experiments and those of others, this critical concentration was determined to be approximately 10 mg/ml. Thus, at high concentrations of IVIg ranging from 15 to 50 mg/ml, IVIg exerts the overall inhibitory effects regardless of the different cell types, as a result of the inhibitory minor specific components of functional antibodies present in an overwhelming amount, which ensures the recognition of and binding to all possible targets on the individual cell surfaces.

IV. 2 IVIg regulation of T cells

A body of evidence indicates the potential for IVIg binding and regulating T cells. First, clinical observations suggested IVIg was an effective treatment for several T-cell-mediated diseases such as ITP (Kuhne et al., 1998 and George et al., 1997), multiple sclerosis (Pashov et al., 1998), Kawasaki Syndrome (Meissner et al., 1994) and Guillain-Barre syndrome (Abd-Allah et al., 1997). The benefits of IVIg against these diseases have been found to be associated with reductions in the number of activated T cells (Pashov et al., 1998; Tekgul et al., 1998 and Leung et al., 1989 and 1987). Second, IVIg demonstrated an extensively inhibitory effect on T cell

proliferation in vitro. IVIg exerted anti-proliferation effects on autonomously growing cell lines of various origins (Van Schaik et al., 1992) and peripheral blood lymphocytes activated by PHA, TPA or anti-CD3 mAb (Lee et al., 2001 and Andersson et al., 1993). IVIg also inhibited cell growth in the mixed lymphocyte reaction (Van Schaik et al., 1993 and Klaesson et al., 1993).

The idea that IVIg can modulate T cell functions is further supported by the observations that NAb, one of the major components present in IVIg, has the ability to regulate T cells. Previous experiments using syngeneic murine tumor models including the NK-resistant DBA/2 T-lymphoma L5178Y-F9 showed that NAb provided resistance against small T lymphoma foci in vivo (Chow et al., 1981). A subsequent preliminary assessment of NAb binding to cell surface molecules known to participate in T cell activation, proliferation, differentiation and maturation indicated that anti-transferrin receptor and anti-Thy1.2 monoclonal antibody reactivity did not correlate with NAb binding (Chow et al., 1999). However, the selected high NAb-binding variant LYNAb$^+$ showed an increased expression of the IL-2R α chain, an indicator of activated T cells and increased CD45RA and CD45RC expression (Tough and Chow 1988). CD45RA, a widely accepted marker of naïve T cells showed a marked transient increase in density shortly after T cell activation (Deans et al., 1992). CD45RC is a marker which exhibited an increase in expression on both CD45RC$^+$CD4$^+$ and CD45RC$^-$CD4$^+$ T cells upon stimulation with concanavalin A (Nishimura et al., 1992). Thus, NAb might bind to activated T cells by targeting activation-associated T cell surface molecules including the IL-2R α chain, CD45RA

and CD45RC (Zhang and Chow 1997). This implied that NAb might preferentially bind and regulate activated T cells. Moreover, previous research using murine fibroblasts provided evidence to support the idea that NAb can bind and regulate activated cells other than lymphocytes. V-H-ras transformed C3H 10T1/2 murine fibroblasts that were considered to be activated cells, showed a parallel increase in NAb binding with transformation (Tough and Chow, 1995). Additionally, preneoplastic C3H 10T1/2 cells, which overexpressed an introduced gene for PKC, a key molecule of cell activation, also exhibited increased NAb binding (Wang and Chow, 1999). Constitutive increases in the basal activity of PKC in both the ras transformation and PKC preneoplasia models suggested that PKC upregulated NAb binding structures and that NAb recognized activated cells and cells in the early stages of neoplasia (Chow et al., 1999). Furthermore, other investigators have shown that tumor binding IgM human natural antibodies bound to activated T cells (Bohn et al., 1994). In addition, normal serum IgG bound to autologous PHA-activated T lymphocytes (Wolf-Levin et al., 1993). These observations raised the possibility that IVIg provided a benefit against T cell-mediated immune disorders by down regulating abnormally activated T cell growth. However, little is known about the precise mechanisms or the contribution of the cell surface targets.

IV. 2.1 Increased IVIg binding associated with increased early markers of T cell activation

The increased IVIg binding by J4.1 and JIg3.1 which corresponded with their increased susceptibility to IVIg-mediated cell growth inhibition in vitro and in vivo

suggested that their stable high IVIg binding phenotypes were biologically relevant and therefore could be used as human T cell models to reveal the T cell surface targets of IVIg. From the information obtained from the murine system, the cell surface molecules we chose to assess were those which were involved in T cell activation or were characteristic of the normal T cell surface. Surface expression analysis revealed that consistent with the increased IVIg binding, the selected cells bound more monoclonal antibodies against T cell activation-related molecules including CD45RA, CD45RB, CD25, CD134, HLA-ABC and possibly HLA-DRDPDQ, but less CD69 was bound and no binding of anti-FASL on any of the cells. Other surface molecules such as FAS and CD3, a pan-reactive T-cell marker, were decreased in the selected high IVIg-binding cells.

The increases in CD25 detection accompanying increased IVIg binding were similar to the results obtained from our murine system. CD25 on the IL-2R α chain is expressed upon T cell activation. It was shown to be essential for T cell activation and formation of the high affinity receptor for IL-2 (DukoVich et al., 1987 and Robb et al., 1984). It is normally considered as an indicator of T cell activation and exhibited maximal expression 24 hrs after activation (Muller-Suur et al., 2000 and Stuber and Strober, 1996). Moreover, studies of others showed that T lymphocytes including Jurkat cells exposed to TPA for 24 hrs produced a marked increase in IL-2R α chain mRNAs (Makover et al., 1990).

As a member of the tumor necrosis factor receptor (TNFR) surperfamily, CD134 is specifically expressed on activated T cells with the maximum level at 24 hrs

after stimulation (Van Kooten et al., 1996; Al-Shamkhani et al., 1996 and Gruss et al., 1995). Previous study demonstrated that the protection by IVIg against the alloantigen response in GVHD which exhibited abnormally increased $CD134^+$ alloreactive T cells, was associated with a decrease in the number of $CD134^+$ activated T cells caused by IVIg induced apoptosis (Caccavelli et al., 2001). Our data showed that increases in expression of CD134 correlated with the increased IVIg binding of J4.1 and JIg3.1 further suggesting that IVIg exerted its effects likely by targeting CD134.

Detection of HLA class I molecules was increased on the selected high IVIg binding cell lines (Table VIII). Together with the observation that human colon carcinoma cell lines incubated with natural human IgM antibodies for 48 hrs exhibited an enhanced expression of HLA class I (Bohn et al., 1994), this indicates that IVIg is one of the reagents that induced increases in HLA class I expression and targeting of HLA class I molecules might contribute to the in vivo tumor surveillance function of IVIg against tumors and otherwise activated cells.

CD69, the earliest marker which appears within 2 hrs after T cell activation (Santis et al., 1995) was decreased in the selected high IVIg-binding cells. In addition, the late T cell activation marker CD95, which maximally increased at 72 hrs after T cell activation with TPA (Nalbant et al., 2002), was also decreased in expression on our high IVIg-binding cells. As a member of the TNFR superfamily which can initiate programmed cell death, the decrease in expression of CD95 on our selected cell lines suggested that the FAS pathway might not be the major pathway for explaining IVIg inhibition of cell growth in vitro. However, our data could not rule out the possibility

that in the physiological situation IVIg may regulate activated T cells through Fas-mediated cell apoptosis (Prasad et al., 1998). It is possible that during our selection procedure the Fas positive cells were eliminated upon interaction with IVIg, the remaining selected high IVIg binding cells may be the most Fas negative of the population. The observations that IVIg induced apoptosis on activated PBMCs which were CD95-positive and proliferation marker Ki-67 positive, while the non-activated, G1 arrested cells exhibited less apoptosis, might suggest that IVIg exerts different effects on cells at different stages of the cell cycle (Aktas et al., 2001). Thus, another possible result of our selection procedure might be the elimination of CD95-positive, later phase activated cells, while the major population of the remaining cells were in G0/G1 phase or in an early stage of activation.

The reduction in binding of CD3, the pan-reactive T cell surface marker on the selected cells argued against IVIg binding to CD3. The lack of IVIg binding to CD3 might be due to its relatively short extension from the cell surface causing inaccessibility to IVIg (Pace et al., 1999).

Previous studies from our lab demonstrated that high CD45RA and RC expression accompanied FACS selection of the $LYNAb^{+}$ for high NAb binding on murine T lymphoma cells suggesting that high m.w CD45 isoforms were the targets of NAb (Zhang and Chow 1997). Expanding this study to the human system using IVIg to select the high binding variants, we found that not all CD45 isoforms were increased with the increase in IVIg binding. In addition to the increased binding of the mAb against CD45RA to the selected cell lines, the binding of mAbs against CD45RB and

pan-CD45 showed slight increases, with binding of anti-CD45RO mAb low and unchanged relative to the parental cell line. The correlation between the expression of high m.w CD45RA- and RB-bearing isforms and increased IVIg binding in the human model T cells further confirmed and extended the data from the murine system that the high m.w. CD45 isoforms were the targets of IVIg.

CD45 is the major transmembrane tyrosine phosphatase and is highly expressed on all hematopoietic cells (Thomas 1989). The occupancy of 10% of the cell surface area of T cells established CD45 as an important regulator of T cell functions (Matthew et al., 1989). CD45 exists in at least eight isoforms as a result of alternative RNA splicing among the extracellular exons A, B, C (reviewed in Thomas and Lefrancois 1988). CD45RA, RB, RC are determinants on higher molecular weight species of the leukocyte common antigen and are restricted to isoforms expressing exons 4, 5, 6 respectively. Lack of the expression of the variably expressed exons will yield the low m.w. isoform CD45RO. Alternative splicing and isoform expression are highly conserved and precisely controlled in leukocyte differentiation and activation (Thomas et al., 1989). Naïve T cells predominantly express the large CD45RA isoform, about 24 hrs after activation, the density of CD45RA on the cell surface showed a transient marked increase (Deans et al., 1992). Over the course of 3-5 days after activation, the expression of CD45RA on the cell surface switches to expression of the smallest CD45RO isoform (Birkeland et al., 1989). CD45RB highly expressing T cells are generally considered as naïve T cells and the CD45ROCD45Rb^{lo} phenotype are characterized as memory cells. Thus the popular view is that T cell

activation results in down-regulation of the high m.w. isoforms and concomitant up-regulation of low m.w isoforms (Lee et al., 1990 and Birkeland et al., 1988). However, subsequent studies demonstrated the possibility that cells expressing high and low m.w. isoform can interconvert (Bunce et al., 1997; Michie et al., 1992 and Rothstein et al., 1991). Moreover, CD45RB showed a slight increase within 1 day on activated OVA-specific T cells after OVA stimulation and then the expression of CD45RB went down (Sun et al., 1999). Although, CD45RB showed a slight increase within 1 day after stimulation, the actual increase in CD45RB might have been more than what was detected because recognition by anti-CD45RB mAb employed was dependent on sialic acid decorating CD45RB molecules, but conditions after T cell activation favor expression of asialo forms due to the upregulation of neuraminidase activity (Landolfi et al., 1985).

Increased expression of CD45RC, another high m.w. CD45 isoform also appears closely related to T cell activation. Upon stimulation with Con A, the conversion of $CD45RC^{-}CD4^{+}$ T cell to $CD45RC^{+}$ cells started as early as 6 hrs after stimulation and lasted for 120 hrs. In addition, the expression of CD45RC on the $CD45RC^{+}CD4^{+}$ T cell subset was increased (Nishimura et al., 1992).

CD45RO is widely accepted as a marker of memory T cells. From the aspect of T cell activation, it could be considered as a later activation marker because the shift of high m.w. CD45 to CD45RO occurred 3-5 days after T cell activation (Birkeland et al., 1989). All these data suggested that upon T cell activation there is a trend toward a transient phase of increased expression of high m.w. CD45 followed by a shift to the

low m.w. CD45 isoform and this transiently expressed phenotype may be particularly sensitive to regulation by IVIg. Additionally, the most remarkable disparity in the changes in CD45RA and CD45RO argued for IVIg regulation of high density $CD45RA^+$ early stage activated T cells and possibly to a lesser extent $CD45RA^+$ naïve T cells but not $CD45RO^+$ memory T cells.

Collectively, the comparison between the selected high IVIg binding cells and the parental cells showed parallel changes between increased IVIg binding and increased surface expression of early T cell activation markers. In the process of selection, the length of time for increased expression of the activation marker to occur after cell stimulation may be important. These data suggested that IVIg preferentially bound to the surface activation markers appearing about 1 to 2 days after activation, but not to the very early marker CD69, nor to the later, well expressed marker CD95.

IV. 2.2 IVIg binding to CD45

Although several early activated T cell surface markers showed increased expression consistent with the increased IVIg binding on our selected J4.1 and JIg3.1 cell lines, CD45RA was the most markedly increased molecule among those assessed. Compared to the parental cells J4.1 and JIg3.1 expressed more CD45RA by 172% and 374% respectively. The expression of CD45RB and a pan-CD45 epitope showed slight increases on the selected cells, while the expression of CD45RO, a characteristic marker of memory T cells was unchanged (Table IX). The difference in

CD45 isoform expression between the selected high IVIg-binding cells and parental cells was confirmed by western blotting using whole cell lysates and immunoblotting with anti-pan CD45. The result showed that the two selected high IVIg-binding J4.1 and JIg3.1 lines, expressed markedly more higher molecular weight CD45 isoforms ranging from 200-220KD. Jurkat parental cells expressed predominantly low molecular weight CD45 isforms around 180-190KD and lacked the band of protein above 200KD (Figure IV). Thus, results of both flow cytometry and western blot analysis demonstrated a consistent correspondence between expression of high molecule weight CD45 isoforms and increased IVIg binding on the selected cell lines. The most remarkable increase in CD45RA in association with increased IVIg binding, argued that CD45RA was important in contributing to the increased IVIg binding of the selected cells and it was most likely a major target of IVIg. These data further supported the previous findings in the murine system that the same correlation was seen between increased high m.w isoforms of CD45RA and CD45RC expression versus NAb binding (Zhang and Chow, 1997).

IV. 2.3 Asialo CD45RA, B, C likely targets for IVIg

NAb binding to neuraminidase treated murine L5178Y-F9 cells was always significantly increased compared with untreated controls (Zhang and Chow, 1997). Vibrio cholerae neuraminidase treated human peripheral blood monocytes bound about 30% more human IgM than untreated PBMCs (Haegert, 1979). These data

revealed that immunoglobulin including NAb were carbohydrate reactive particularly with asialo carbohydrates. Our data similarly showed that treating Jurkat cells with neuraminidase prior to IVIg incubation increased IVIg binding by 135% (Table XI). This indicated that IVIg preferentially bound to the asialo forms of surface markers. Neuraminidase treatment of target cells revealed increased binding of anti-CD45RA and CD45RC mAbs in the murine system. Similar to that, neuraminidase treatment Jurkat cells also caused an increase in the anti-CD45RA mAb binding by 11% and anti-pan CD45 binding by 25%, indicating that the epitopes detected by these antibodies may have been masked by sialic acid. Alternatively, neuraminidase treatment may have changed the conformation of highly glycosylated CD45 by removing the negatively charged sialic acid. This might have facilitated the binding by IVIg, anti-CD45RA and anti-pan CD45. However, mAb detection of CD45RO and CD45RB were completely lost, suggesting that sialic acid was required for these two detecting antibodies to bind and that a general decrease in cell surface negative charge may not be the reason for increases in binding by other mAbs.

Several experimental approaches had been used to determine whether high m.w. CD45 might be a target of NAb in the murine system. IgG NAb partially inhibited the binding of anti-CD45RA and anti-CD45RB relative to anti-pan CD45 on $LYNAb^{+}$ cells selected for high NAb-binding. Purified CD45 containing the variably expressed exon products inhibited NAb binding to L5178Y-F9 cells. Tumor-eluted NAb bound directly to purified CD45RABC and less to CD45RO/RB in an ELISA assay and CD45-deficient YAC cell variants exhibited low NAb binding versus wild type and

CD45RABC transfected CD45-deficient variants (Zhang et al., 2000). These observations strongly suggested that NAb directly bound to high m.w CD45 isoforms. Following the information obtained from the murine system, the assessment of IVIg inhibition of the binding of anti-CD45 mAbs against different epitopes on the cells was employed to find direct evidence of IVIg binding to CD45. A similar assay has been used by Kazatchkin's group that found that IVIg recognized the cell surface molecule CD5 using mouse CD5-transfected lymphocytes to ensure enough CD5 molecules were expressed on the cells. Similarly, the selected model human T cells we used showed markedly more expression of high m.w. CD45 isoforms and unchanged CD45RO, which provided a better opportunity to reveal IVIg inhibition of binding to CD45 by individual mAbs. The inhibition assay showed that IVIg inhibited anti-CD45RA HI-100 binding and anti-pan CD45 Bra-55 binding by 33% and 35%, respectively. Anti-CD45RB Bra-11 binding was slightly inhibited by 5%. Interestingly, the anti-CD45RO UCHLI binding exhibited an increase when IVIg was present (Table X).

IVIg partial inhibition of anti-CD45RA binding might be due to IVIg directly binding and/or masking the same, or part of the same epitope which was recognized by anti-CD45RA. Although IVIg only partially inhibited anti-CD45RA and pan-CD45 binding, considering the possibility that the relatively weak IVIg binding may be replaced by higher affinity specific mAb binding and that the replacement was happening continuously, the actual interference in mAb binding by IVIg may be more than what we were able to show here.

The correlation between increased binding of anti-CD45RB and that of IVIg, to the selected high IVIg-binding variants argued that CD45RB might be one of the targets of IVIg. However, the results (Table XI) showing that neuraminidase treatment induced a complete loss in binding of anti-CD45RB mAb Bra-11 indicated that the Bra-11 anti-CD45RB recognized a sialic acid-dependent epitope. It also suggested that the increased expression of the CD45RB epitope detected by Bra-11 mAb on the selected cells was the sialic acid-bearing portion of CD45RB. Considering the observation that IVIg preferentially binds to asialo forms of molecules, we propose that the detected increase in CD45RB with sialic acid decoration might not be the ideal targets of IVIg. Additionally, the contrasting observation of a decrease in sialic acid-dependent binding of anti-CD45RB to cells selected for high NAb binding in the murine system might be due to expression of only the sialylated form of CD45RB on the L5178Y-F9 starting cell line. It is likely that the asialo form of CD45RB also exists on the surface of the selected high IVIg-binding Jurkat cells and can be recognized by IVIg. This possibility could be clarified by using an anti-CD45RB mAb which recognizes the asialo form of CD45RB, but such an antibody is not currently available.

CD45RC is another high MW isoform of CD45 and the asialo form of CD45RC has been found to be another likely target of NAb in the murine system (Zhang and Chow 1997). In our human T cell models, CD45RC is probably another high m.w. CD45 isoform which serves as one of the targets of IVIg but we could not assess this due to the lack of a mAb against any human CD45RC epitope.

As for the increased binding effect of IVIg on anti-CD45RO mAb binding to the cells, we propose that it probably is due to a better exposure of the short CD45RO molecules after IVIg cross-links and pulls away the higher m.w. CD45 isoform molecules. Similar observations have been reported by Hamann et al. (1996). They found that preincubating cells with anti-pan CD45 or anti-CD45RA mAb induced a marked increase in binding of BL-Tsub/2 an antibody recognizing the N-terminal end of CD45 and partially overlapping with CD45RO binding sites. This was proposed to be caused by cross-linking of high m.w. CD45 isoform molecules leading to better exposure and thus increased binding of this CD45RO-like mAb. In our assay, preincubating the cells with IVIg may have caused the same effect of increasing CD45RO exposure as did anti-CD45RA mAb preincubation. Likewise, it suggested that IVIg, similar to anti-CD45RA might have the ability to bind and cross-link CD45RA. This also indirectly supports the hypothesis that CD45RA is a target of IVIg and that IVIg can recognize a similar structure which is recognized by anti-CD45RA.

Together the data suggested that IVIg reacted with T cells at a transient phase 1 to 2 days after activation, which exhibited a phenotype of increased expression of high m.w. CD45 isoforms CD45RA, CD45RB and CD45RC on the cell surfaces. Neuraminidase treatment increased IVIg binding and monosaccharides and pronase significantly inhibited NAb binding (Chow and Reese, in preparation) indicating that IVIg preferentially binds to asialo forms of cell surface N-linked and O-linked carbohydrate epitopes on high IVIg binding cells. Moreover the finding that an

increased neuraminidase activity 24-48 hours after T cell activation (Landolfi et al., 1985) could further facilitate IVIg binding to the T cells at a transient phase 1 to 2 days after activation by producing more of the asialo-form of cell surface targets including high m.w. CD45RA, CD45RB and CD45RC. Thus, these data implicate IVIg in preferential binding to asialo targets expressed on early activated T cells and exerting its effects on these cells.

IV. 3 Possible mechanisms of IVIg cell regulation through CD45

In the present work, we found that IVIg could significantly inhibit the cell growth of two selected high IVIg-binding variants. Together with the observation of the positive correlation between increased IVIg binding and more expression of the likely cell surface targets, high m.w. CD45 isoforms, this suggested that IVIg binding to and modulation of high m.w. CD45 on the target cell surface may contribute to the inhibitory effect of IVIg on cell growth. The possible mechanism underlying CD45-mediating growth inhibition by IVIg might be caused by cell death and/or negative regulation of T cell activation.

IV. 3.1 Dimerization mediated inhibition of CD45 activity

One possible mechanism of IVIg regulation of T cells may be through modulating CD45 dimerization. Previous research suggested that CD45 molecules spontaneously

homodimerize without the aid of a ligand, and this homodimerization can cause CD45 to become inactive by dimerization-induced formation of an inhibitory wedge in the phosphatase domain (Xu and Weiss 2002 and Jiang et al., 2000). However, the different CD45 isoforms homodimerized at different rates because of the size, shape and negative charges present on the various CD45 isoforms. The smallest isoform CD45RO dimerized more efficiently and rapidly than the large isoforms. Mechanistic studies revealed that dimerization was partially impeded by O-glycosylation and sialylation of the different isoforms of CD45. Likely, CD45RO exhibited more efficient spontaneous dimerization as a result of the lack of all three variably expressed exon products A, B, C and their multiple sites of O-linked glycosylation. Moreover, sialidase which catalyzes the removal of sialic acid residues, increased the efficiency of dimerization by the large CD45RABC, further revealing that sialylation plays a role in hindering CD45 isoform dimerization (Xu and Weiss 2002). Based on the above results and the fact that CD45RA and CD45RO are generally found on naïve and memory T cells respectively, a model was proposed for regulating CD45 function. In the situation without any external ligands, there is an equilibrium between CD45 monomers and dimers on the cell surface for all isoforms. This equilibrium is controlled by the isoform expression. When a T cell is naïve, the predominant expression of large CD45RA isoforms would shift the equilibrium to monomers because the O-glycosylation, sialylation and large size causes spatial and electrical barriers to homodimer formation. Thus, on naïve T cells, CD45RA exists primarily as active phosphatase monomers. Upon TCR stimulation, the active phosphatase of

CD45RA can activate Src family protein tyrosine kinases and subsequently stimulate the downstream signaling pathway. After TCR stimulation, there is an early transient increase in the density of CD45RA expression, which provides a favored condition for T cells undergoing further activation. During the next 3-5 days, TCR signaling can induce the expression of CD45RO through PKC and Ras, but the replacement of CD45RA by CD45RO on the cell surface will take several days. The expression of CD45RO on activated T cells shifts the equilibrium toward dimers due to its more efficient homodimerization which renders it less active. Thus the isoform switch acts as the controller to terminate the T cell response through dimerization mediated negative regulation. Collectively, all of the above supports the idea that spontaneous dimerization of CD45 likely contributes to reduced cell activation and cessation of the immune response.

Based on our present studies showing that high m.w. CD45 isoforms were likely targets of IVIg and IVIg can directly bind to CD45RA, a mechanism for regulating high CD45RA function when IVIg is present as a ligand can be proposed as an example of IVIg regulation of other high m.w. CD45 isoforms. Without any external ligand present, CD45RA showed low dimerization efficiency and more phosphatase activity to initiate TCR signaling. However, when IVIg is present, it is possible that IVIg preferentially binds and cross-links asialo-carbohydrate components on the T cell surface facilitating the efficient dimerization of the large CD45RA-bearing isoforms. The increased neuraminidase activity 24-48 hours after T cell activation and a transient high density of CD45RA expression at about the same time would render

these large asialo CD45 isoforms subject to negative regulation by formation of an inhibitory wedge. This probably causes an increase in the activation threshold on early activated T cells as the result of reduced CD45RA PTPase activity. Thus, on early activated T cells, the reduction of total CD45 phosphatase activity caused by IVIg mediated CD45RA dimerization might prevent further T cell activation.

IV. 3.2 CD45 mediated cell apoptosis

Current data provides several pieces of evidence for a role for CD45 function in lymphocyte apoptosis. T cells treated with insoluble anti-CD45 mAb, which could cross-link CD45 on the cell surface, showed a dramatic increase in cells undergoing apoptosis. Cross-linking CD45 mediated cell apoptosis was dose-dependent and increased with higher concentrations of anti-CD45 (Klaus et al., 1996). In addition to T cells, cross-linking of CD45 also can induce apoptosis of B cells (Lesage et al., 1997).

Recently, the role of CD45 in galectin-1 mediated cell apoptosis has been extensively studied. Binding of galectin-1 to CD45 on the T cell surface induced an apoptotic signal to the T cells and caused the cells to undergo apoptosis. Galectin-1, one physiological ligand of CD45, is a member of a family of animal lectins that share structural similarities within the carbohydrate binding domain (Barondes et al., 1994 and Leffler et al., 1986). Several glycoproteins including CD45 have been identified to bind to galectin-1 (Baum et al., 1995). In order for galectin-1 to induce an apoptotic

signal, it was necessary that CD45 be expressed on the T cell surface (Symons et al., 2000). Additionally, it has been shown that galectin-1 induces apoptosis of peripheral T cells that are activated and proliferating (Perillo et al., 1997). Several similarities can be seen regarding the actions of galectin-1 and IVIg, such as carbohydrate reactivity, targeting CD45 and acting on activated or proliferating cells. This suggested that IVIg and galectin-1 might act by similar mechanisms. The inhibitory effect of IVIg on cell growth may occur through induction of apoptosis after IVIg binding to surface CD45.

Although the CD45-mediated signaling pathway of galectin-1 induced apoptosis has not been well documented, cross-linking of CD45 is essential for initiating apoptotic signaling and triggering the galectin-1-mediated apoptosis (Perillo et al., 1995). In addition to galectin-1, Ab cross-linking of CD45 also induced an apoptotic signal in human T cells (Klaus et al., 1996 and Lesage et al., 1996). Other studies found that galectin-1 treatment of Jurkat T cells resulted in decreased phosphate activity of CD45 (Walzel et al., 1999). Moreover, blockade of CD45 PTPase activity by PTPase inhibitors showed an enhancement of galectin-1-induced T cell death (Nguyen et al. 2001). Thus CD45 acts as a negative regulator of T cell apoptosis in the absence of ligand induced sequestration (Byth et al., 1996). These data indicated that sequestration of CD45 by galectin-1-mediated crosslinking and the subsequent decrease in phosphatase activity of CD45 is essential for facilitating T cell apoptosis. Thus, a proposed model of CD45-mediated galectin-1-induced cell apoptosis works as follows: galectin-1-induced CD45 cross-linking and sequestration would functionally

pull CD45 PTPase away from a pro-apoptosis signaling complex, which ensures the apoptosis signal is propagated forward and finally induces apoptosis. Otherwise, CD45 will function as a negative regulator and abort the pro-apoptosis signaling. (Pace et al., 1999).

IV. 3.3 CD45-mediated inhibition of T cell functions

Another possible outcome following IVIg binding to CD45 may be inhibition of T cell activation, which might also contribute to IVIg inhibition of cell growth. Although CD45 is widely accepted as a positive regulator of T cell receptor signaling by constitutive priming of $p56^{lck}$ through the dephosphorylation of the C-terminal negative regulatory phosphotyrosine site, CD45 can also exert negative effects on Src family kinase $p56^{lck}$ by dephosphorylating the positive regulating Tyr-394 residue. Additionally, CD45 has a potential negative regulatory activity on cell processes including cytokine receptor-mediated proliferation, differentiation and anti-viral responses by inhibiting activation of the Jak-STAT pathway (Irie-sasaki et al., 2001). The activity of CD45 as a negative regulator of cytokine receptor-driven cell growth was further confirmed by the following observations. In humans, the tumor cells of more than 10% of patients with acute lymphoblastic leukemia showed a lack of CD45 expression (Ratei et al., 1998). Loss of CD45 expression also frequently occurred on the tumor cells of patients with Hodgkin's lymphoma (Ozdemirli et al., 1996) and multiple myelomas (Ishikawa et al., 2000). Mice expressing nononcogenic amounts of

active lck F505 developed aggressive thymic lymphomas on a CD45-null background (Baker et al., 2000). Additionally, in growth factor dependent cell lines, overexpression of CD45 normally caused growth arrest and/or cell death in vitro (Ogimoto et al., 1994). All of these data suggested that CD45 was necessary to control the cell growth, as well as tumorigenesis. However, the kind of ligand binding to CD45 that can initiate the negative regulation of cell growth through the Jak-STAT pathway is still unknown. Data from our previous research revealed high CD45RA and CD45RC expression on murine LYNAb$^+$ cells selected for high NAb-binding (Zhang and Chow 1997). These high NAb-binding and higher CD45RA and CD45RC expressing cells exhibited low tumorigenicity (Chow et al., 1981 and Tough et al., 1995). Growth of the LYNAb$^+$ from a threshold s.c. inoculum in syngeneic DBA/2 mice yielded more tumorigenic cells which bound less NAb, anti-CD45RA and anti-CD45RC. The observation that these in vivo passaged LYNAb$^+$ variants expressed predominantly low m.w. CD45 isoforms indicated the necessary role of high m.w. CD45 isoforms in the cell growth control (Zhang and Chow 1997). Additionally, in the present studies, the human model T cells selected for high IVIg binding, which exhibited markedly increased expression of higher m.w. CD45RA- and CD45RB-bearing isoforms, were sensitive to cell growth inhibition by IVIg. These results might suggest that consistent with the previously revealed negative regulation by CD45 in controlling cell growth, high m.w. CD45 isoforms bearing CD45RA and CD45RC were crucial in negative regulation of cell growth. However, whether NAb binding to high m.w. CD45 initiated the negative regulatory function by inhibiting the Jak-STAT

pathway is not known. This will be a new area to be investigated to further understand the mechanism of IVIg negative regulating of cell growth.

The data so far suggested that IVIg bound to $CD45RA^+$ early activated T cells and reduced the cell proliferation. This provided important information arguing for IVIg regulation of T cells undergoing activation as a new mechanism for the beneficial therapeutic effects of IVIg in inflammatory and autoimmune diseases. Still the exact mechanisms of CD45 mediated IVIg inhibitory effects on cell growth are unknown. In the future, the assessment of cell cycle arrest or apoptosis on IVIg treated cells will help to understand the mechanism of IVIg-mediated cell growth inhibition. Additionally, future research aim to understand the mechanisms of this regulation could be sought by investigating the substrates of CD45RA activity after IVIg binding and further identify the signaling pathway which will help to better reveal the mechanisms. For example, changes in the activity of different sets of CD45 substrates could be analysed after IVIg binding. If Jak family protein tyrosine showed less phosphorylation after IVIg treatment, this may suggest IVIg effects were mediated through the Jak pathway. Similarly we can compare the dephosphorylation level of Lck kinase at the negative Y505 site or the positive regulation site Y394, to investigate whether CD45 exerts negative or positive effects on substrates upon IVIg binding. Comparison of IVIg effects in CD45-deficient T cell model versus our high IVIg-binding models can be used as another approach to confirm several CD45-dependent effects on IVIg functions, such as cell growth control. Alternatively, considering the observation that galectin-1 binding and redistribution of CD45, CD43

and CD7 was required for triggering apoptosis, we proposed the following possibility of IVIg action. IVIg, similar to galectin-1, may exert its function by inducing co-localization or isolation of CD45RA together with other molecules which are increased on selected cells including other high m.w. CD45 isoforms bearing CD45RB and CD45RC and CD134 and CD25 molecules. In particular, CD134 showed a dramatic reduction following treatment with IVIg or $F(ab')_2$ fragments which likely contributed to IVIg induced apoptosis of CD134 bearing activated T cells (Caccavelli et al., 2001) making it a good candidate for investigating its involvement with CD45RA. IVIg induced interactions between CD45RA and other increased target molecules can be assessed by using confocal microscopy in the future. The identification of other surface markers which are likely involved with CD45RA in IVIg-induced signaling will contribute to revealing the underlying mechanisms. A better understanding of the mechanisms of IVIg regulation may provide opportunities for future enhancement of IVIg treatment.

IV.4 Summary

To identify T cell surface targets of IVIg and to assess the relationship between IVIg binding and the impact on cell functions, we selected the high human serum IgG plus IgM and human IVIg binding variants by cell sorting from the parental Jurkat, human T leukemia cell line. The selected J4.1 and JIg3.1 with stable increases in IVIg binding were more susceptible to growth control by IVIg in vitro. In addition, an in

vivo cell elimination assay showed that ^{131}I-dUrd-labelled JIg3.1 cells injected into SCID mice were more susceptible to clearance by IVIg-pretreatment than Jurkat parental cells. Thus, IVIg had a greater impact on the higher binding cells by inhibiting their cell proliferation in vitro and facilitating their cell elimination in vivo. Cell surface molecule expression analysis demonstrated that, consistent with the increased IVIg binding, J4.1 and JIg3.1 exhibited an increase in expression of T cell activation-associated markers. This included high m.w CD45 isoforms CD45RA, CD45RB, CD134, CD25 and HLA class I and class II. However, the detection of mAbs against CD69, a very early T cell activation marker with the maximum expression in 6 hours, CD95, a later expressed activation marker and CD45RO which is considered another later activation marker and characteristic marker of "memory" T cell were decreased on the selected high binding variants. These data argued that IVIg preferentially binds to T cells at a transient phase of activation, 1 to 2 days after stimulation, but not T cells at very early or late phases of activation, nor memory CD45RO$^+$ T cells. Furthermore, the difference in CD45 isoform expression between the selected cells and Jurkat parental cells was confirmed by western blotting using anti-pan CD45 mAb. Neuraminidase treated cells showed that the parallel increases in anti-CD45RA, anti-pan CD45 and IVIg binding were highly suggestive of IVIg binding to asialo forms of high m.w. CD45 isoforms. Finally, by employing an inhibition assay, we provided direct evidence of IVIg binding to high m.w CD45 isoforms. Considering the fact that CD45 is the key molecule in T cell activation and plays a role in inducing cell apoptosis, the present findings suggest that IVIg may

bind to high m.w. CD45 isoforms expressed during a transient phase of T cell activation and reduce cell proliferation of these susceptible T cells probably by CD45 mediated cell apoptosis. This argues for IVIg regulation of T cells undergoing activation as a new mechanism for the therapeutic effects of IVIg in antoimmune and inflammatory diseases.

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